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

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Running head: Rhamnolipids toxicity and modulation of cadmium bioavailability

Abstract

In this study the mixture of mono- and di-rhamnolipids produced by *Pseudomonas aeruginosa* DS10-129 was characterized for its toxicity and modulatory effects on Cd availability to different bacteria. Gram-negative naturally bioluminescent *Vibrio fischeri* and recombinant bioluminescent *Pseudomonas fluorescens, Pseudomonas aeruginosa, Escherichia coli* and Gram-positive *Bacillus subtilis* were used as model organisms. Rhamnolipids reduced the bioluminescence of these bacteria in less than a second of exposure even in relatively low concentrations (30-min EC_{50} 45–167 mg l^{-1}) which might be due to the enhancement in permeability of bacterial membranes as confirmed by the hydrophobic 1-N-phenylnaphthylamine dye.

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

The analysis of the concentration of free Cd^{2+} in Cd-spiked soils showed that only a minor fraction (0.13% of the total Cd in soil contaning 1.5 mg of Cd kg^{-1}) was desorbed from soil if rhamnolipids were not added and as maximum two-fold higher Cd desorption was measured in the presence of 40 mg l^{-1} rhamnolipids. However, even this small fraction of desorbed Cd remained not available to Cd-sensing recombinant bacteria. Since the concentrations of rhamnolipids showing effects on bacterial membranes (>10 mg l^{-1} of rhamnolipids) and bioluminescence inhibition (>45 mg l^{-1} of rhamnolipids) did not influence the viability of Gram-negative and Gram-positive test bacteria, the rhamnolipids could be considered harmless also to soil bacteria. Hence, rhamnolipids could be applied for the remediation of polluted areas, provided the range of concentrations will be carefully chosen.

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

Introduction

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

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

Objectives of the study

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

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

Materials and Methods

1. Materials

$CdCl_2$ (>98%) was obtained from Sigma, Tween 80 from Serva, components of growth media were either from LabM or Sigma, L-rhamnose, orcinol and 1-N-phenylnaphthylamine (1-NPN) were from Sigma-Aldrich. Rhamnolipids were purified from the culture broth of *Pseudomonas aeruginosa* DS10-129 as described below. A sandy soil (initial concentration of Cd 0.17 mg kg^{-1}) spiked with $CdCl_2$ (1.5, 15, 150, 1500 or 15 000 mg of Cd kg^{-1}) as previously reported [11] was used for the bioavailability studies. Before spiking, the soil was characterised in a certified laboratory and had the following properties: 10.6% of clay, 10.6% of silt, 72.8% of sand, 5.7% of organic matter; 39 g·kg^{-1} of $CaCO_3$, 3.59 g·kg^{-1} of N, 0.62 g·kg^{-1} of P, 0.17 mg kg^{-1} of Cd; with 2.3 $cmol^{+}kg^{-1}$ of CEC (cation exchange capacity) and pH of 7.3. Deionised water was used throughout the study.

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

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

3. Isolation, purification and characterization of rhamnolipids

Rhamnolipids were isolated from *P. aeruginosa* DS10-129 cell-free supernatant. *P. aeruginosa* was grown on the mineral medium containing (per l water) 20 g of glycerol, 0.7 g of KH_2PO_4, 2 g of Na_2HPO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2$ and 0.001 g of $FeSO_4 \cdot H_2O$ and 1% of Yeast Extract. The presence of the rhamnolipids was verified by thin layer chromatography on silica gel 60 F_{254} according to Matsufuji et al., [21]. Rhamnolipids were extracted from the cell-free supernatant of the 4-day bacterial culture by centrifugation at 8000 g for 20 minutes at 4°C and the subsequent filtration through a sterile filter (pore size 2 µm). The cell-free culture was acidified to pH 2 with 2 M H_2SO_4 and the precipitated rhamnolipids were extracted with an equal volume of 2:1 dichloromethane/methanol (liquid-liquid extraction) [38]. The organic phase was dried with anhydrous Na_2SO_4 to remove excess water and evaporated on a rotary evaporator (Buchi) at 60–70 °C to yield rhamnolipids. The rhamnolipids were dissolved in 0.05 M $NaHCO_3$.

The concentration of rhamnolipids was determined using the orcinol assay [6] by mixing 100 µl of diluted solution of rhamnolipids (purified with liquid-liquid extraction) with 900 µl of freshly prepared 0.19% orcinol solution in 53% H_2SO_4. The mixture was heated at 80^{o}C for 30 min and its absorbance was measured spectrophotometrically at 421 nm. The concentration of rhamnolipids was calculated according to L-rhamnose standard curve (0 to 50 mg l^{-1}) and by multiplying the result with a coefficient of 3.4 obtained from the correlation of pure rhamnolipids/rhamnose [3]. The critical micelle concentration (CMC) was determined by measuring the surface tension of serial dilutions of the rhamnolipids [17]. Fourier Transform Infrared spectrophotometer (FTIR) Perkin Elmer 100 series was used to determine the molecular structure of the rhamnolipids. The cell-free supernatant was acidified to pH 2 by adding drops of 2M Sulphuric acid to precipitate the rhamnolipids. The precipitated rhamnolipids were extracted with an equal volume of 2:1 dichloromethane/methanol. The organic phase was dried with anhydrous Sodium Sulphate (Na_2SO_4) and evaporated on a rotary evaporator (Buchi, Rota vapour R-200

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

4. Luminescent bacterial strains for toxicity and bioavailability assays

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

5. Analysis of toxicity and Cd bioavailability

Luminescent bacterial strains were either rehydrated from lyophilised culture (*Vibrio fischeri*) obtained from Aboatox, Turku, Finland or cultivated. *V. fischeri* was reconstituted in heavy metal MOPS medium (HMM medium) supplemented with 2% NaCl at room temperature for 1 h. The HMM medium contained (per l of deionised / distilled? water): 8.4 g of MOPS buffer, 0.4 g of glucose, 0.22 g of glycerol-2-phosphate, 3.7 g of KCl, 0.54 g of NH_4Cl, 0.06g of $MgSO_4$, 0.162 mg of $FeCl_3$ ~~[23]~~. All recombinant bacteria were cultivated freshly by growing the cultures overnight in

3 ml of LB medium ~~[44]~~ supplemented with appropriate antibiotics as in Table 1. The overnight culture was diluted 1:50 with 10–50 ml of HMM medium, grown until OD_{600} of 0.3 and then diluted to OD_{600} of ~0.1 prior to test.

5.1. Toxicity testing

To measure toxicity $CdCl_2$ or $CdCl_2$-rhamnolipids mixtures were analyzed by measuring the inhibition of bioluminescence of constitutively luminescent bacterial strains (Table 1). In addition, *Pseudomonas aeruginosa* DS10-129(pDNcadRPcadAlux) was used to assess the inhibitory effect of rhamnolipids. The effect of rhamnolipids on viability of bacteria was evaluated by plating the treated bacteria on solidified growth medium (see below). Dilutions of $CdCl_2$ (0.1 – 100 mg l^{-1} as final concentrations), rhamnolipids (5 – 200 mg l^{-1} as final concentrations) or Cd-rhamnolipids mixture (the final concentration of rhamnolipids was 50 mg l^{-1} or a concentration reducing the light output of bacteria by 20%, indicated below) were prepared in HMM medium or HMM medium supplemented with 2% NaCl (in case of *V. fischeri*). For toxicity assessment, luminescence inhibition assay was performed in 96-well microplates essentially as in Mortimer et al. [23]. Briefly, 100 µl of the diluted test compound(s) was pipetted into 96-well microplate and 100 µl of the bacterial suspension was automatically dispensed into the wells. Bacteria were incubated at 20°C (*V. fischeri*) or 30°C (recombinant luminescent bacteria) and the luminescence was continuously recorded during the first 30 seconds of exposure and once after 30 minutes of incubation using Fluoroskan Ascent FL plate luminometer (ThermoLabsystems). Inhibition of bacterial bioluminescence by the tested compounds/mixtures was calculated as percentage of the unaffected control (HMM medium or HMM supplemented with 2% NaCl, respectively*).* 30-s and 30- min EC_{50} and EC_{20} values (the concentration of chemical which reduces the light output of bacteria by 50 or 20% after the respective exposure times) were calculated by linear regression from dose-response curves of the studied compounds. Measurements were performed in three independent assays.

Viability of bacteria was assessed after their exposure to 100 mg l^{-1} of rhamnolipids by plating the bacteria onto LB agar plates containing appropriate antibiotics. Plates were incubated for 24 h at 30°C after which colony forming units (CFU) were 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]. $CdCl_2$ 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 $CdCl_2$ dilutions. Rhamnolipids
229 were added to $CdCl_2$ 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^{o}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 ($CdCl_2$, $CdCl_2$-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-phenylnaphthylamine (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

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

7. Analysis of free Cd^{2+} with ion-selective electrode

A Cd-selective electrode (Thermo Orion 96-48 and Thermo Orion 4-star meter; ThermoOrion) was used to measure the free Cd^{2+} in the aqueous environment and in soil-water suspensions. Before use, the electrode was polished with alumina strips. The inner filling solution of the electrode was replaced at least weekly. $CdCl_2$ solutions at final concentrations of 11.2-1120 mg l^{-1} were prepared in deionised / distilled? water; 1:10 water suspensions of Cd-spiked soils were rotated for 24 h prior to the test. Rhamnolipids were added to these solutions at final concentrations of 10, 20 and 40 mg l^{-1} and incubated at 30^{o}C for 2 hours. The concentrations of free Cd^{2+} were calculated by comparing the results from different test conditions with the response of the electrode to $CdCl_2$ solutions in distilled / deionised? water.

8. Analysis of Cd desorption from soil

The effect of rhamnolipids on desorption of Cd in Cd-spiked soil was analyzed by Atomic Absorption Spectroscopy (Shimadzu, Kyoto, Japan). Rhamnolipids are added at final concentrations of 10, 20 and 40 mg l^{-1} to 1:10 water suspensions of Cd-spiked soil, incubated for 2 hours and centrifuged at 13,000×g for 5 minutes. The resulting soil-water extracts were acidified with 1% HNO_3 and the concentration of Cd in the extracts was analysed.

9. Analysis of rhamnolipids sorption to soil

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

Results

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

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

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

The presence of rhamnolipids in the *P. aeruginosa* DS10-129 culture broth was confirmed by thin layer chromatography where two anise aldehyde positive spots (Rf 0.32 and Rf 0.52, corresponding to di- and mono-rhamnolipids) were detected. The concentration of rhamnolipids extracted from the bacterial culture broth was 174 mg l^{-1} and the critical micelle concentration (CMC) of the rhamnolipids was 22 mg l^{-1}. FTIR spectroscopy was used to determine the molecular structure of the rhamnolipids (Figure 2). Strong and broad bands of the hydroxyl group (-OH) free stretch due to hydrogen bonding were observed in the region A (3368 cm^{-1}). The presence of carboxylic acid functional group in the molecule was confirmed by the bending of the hydroxyl (O-H) of medium intensity bands in the region D (1455-1380cm^{-1}). The aliphatic bonds CH_3, CH_2 and C-H stretching with strong bands are shown in regions B and D (2925-2856 and 1455-1380 cm^{-1}). The carbonyl (C=O) stretching was found in the region C (1737cm^{-1}) with strong intensity bands. Two other strong peaks between 1300 cm^{-1} and 1033 cm^{-1} in region E due to C-O stretch are characteristic of an ester functional group. The peaks in the range of 1121–1033 cm^{-1} were also

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

3. Toxicity of rhamnolipids to bacteria

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

4. Effect of rhamnolipids on toxicity, bioavailability and concentration of free Cd^{2+} in aqueous environment

4.1. The effect of rhamnolipids on Cd toxicity

Acute effects of $CdCl_2$ solutions on the bioluminescence of four different bacterial strains were analysed. In contrast to the rhamnolipids, whose effects on bacterial bioluminescence occurred during the first seconds of exposure (Figure 3), the toxic effect of Cd on bacteria was observed only after 30 minutes of exposure. The 30-min EC_{50} of $CdCl_2$ solutions were (mg Cd l^{-1}): 0.16 for *E. coli*, 0.49 for *B. subtilis*, 0.96 for *P. fluorescens* and 4.4 for *V. fischeri*. Addition of sub-toxic concentrations of rhamnolipids (50 mg l^{-1} and EC_{20} level: 20 mg l^{-1} for *B. subtilis* and *V. fischeri,* 35 mg l^{-1} for *E. coli* and 70 mg l^{-1} for *P. fluorescens*) significantly mitigated the toxic effect of cadmium for all the used Gram-negative strains (Figure 4 A, C, D; Table 4). The most remarkable reduction of Cd toxicity was observed in case of *E. coli* followed by *V. fischeri* and *P. fluorescens* (50 mg l^{-1} of rhamnolipids reduced the toxic effect of Cd by 10, 4.8 and 2.3-fold respectively; Table 4 and Figure 4). Surprisingly, no mitigating effect of rhamnolipids on Cd toxicity was observed in case of Gram-positive bacterium *B. subtilis* (Figure 4 B), indicating that the effect of rhamnolipids may be related to the structure of the bacterial cell wall.

4.2. The effect of rhamnolipids on Cd bioavailability

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

4.3. The effect of rhamnolipids on free Cd ion concentration

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

389 measurement of low concentrations of Cd (0.001–0.03 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 Cd^{2+} 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 - 40 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 1.5mg 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 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 EC_{50}
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 EC_{50} of
460 the rhamnolipids for *P. aeruginosa* DS10-129 (EC_{50}=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 EC_{50} 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 containing1.5 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

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

Conclusion

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

Acknowledgements

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554 **References**

555 [1] Al-Tahhan R, Sandrin TR, Bodour AA, Maier RM (2000)
556 Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas*
557 *aeruginosa*: effect on cell surface properties and interaction with hydrophobic
558 substrates. Appl Environ Microbiol 66:3262-3268

559 [2] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith
560 JA, Strahl K (1987) Current protocols in molecular biology. New York: John
561 Wiely and Sons

562 [3] Benincasa M, Abalos A, Oliveira I, Manresa A (2004) Chemical
563 structure, surface properties and biological activities of the biosurfactant
564 produced by *Pseudomonas aeruginosa* LBI from soap stock. Antonie Van
565 Leeuwenhoek 1:1-8

566 [4] Bondarenko O, Rõlova T, Kahru A, Ivask A (2008) Bioavailability of
567 Cd, Zn and Hg in soil to nine recombinant luminescent metal sensor bacteria.
568 Sensors 8(11):6899-6923

569 [5] Bulich AA (1982) A practical and reliable method for monitoring the
570 toxicity of aquatic samples. Process Biochem 17:45-47

571 [6] Chandrasekaran EV, Bemiller JN (1980) Constituent analyses of
572 glycosaminoglycans. Methods in Carbohydrate Chemistry. Academic Press,
573 New York, pp 89-96

574 [7] Guo YP, Hu YY, Gu RR, Lin H (2009) Characterization and
575 micellization of rhamnolipidic fractions and crude extracts produced by
576 *Pseudomonas aeruginosa* mutant MIG-N146. J Colloid Interface Sci
577 331(2):356-363

578 [8] Haba E, Pinazo A, Jauregui O, Espuny MJ, Infante MR, Manresa A
579 (2003) Physicochemical characterization and antimicrobial properties of
580 rhamnolipids produced by *Pseudomonas aeruginosa* 47T2 NCBIM 40044.
581 Biotechnol Bioeng 81(3):316-22

582 [9] Helander IM, Mattila-Sandholm T (2000) Fluorometric assessment of
583 Gram-negative bacterial permeabilization. J Appl Microbiol 88:213-219

584 [10] Higgins DG, Sharp PM (1988) CLUSTAL: a package for performing
585 multiple sequence alignment on a microcomputer. Gene 73:237-244

586 [11] Ivask A, Francois M, Kahru A, Dubourguier HC, Virta M, Douay F
587 (2004) Recombinant luminescent bacterial sensors for the measurement of
588 bioavailability of cadmium and lead in soils polluted by metal smelters.
589 Chemosphere 22:147-156
590 [12] Ivask A, Rõlova T, Kahru A (2009) A suite of recombinant
591 luminescent bacterial strains for the quantification of bioavailable heavy
592 metals and toxicity testing. BMC Biotech 9:41, doi: 10.1186/1472-6710-9-41
593 [13] Ivask A, Virta M, Kahru A (2002) Construction and use of specific
594 luminescent recombinant bacterial sensors for the assessment of bioavailable
595 fraction of cadmium, zinc, mercury and chromium in the soil. Soil Biol
596 Biochem 34(10):1439-1447
597 [14] Juwarkar A, Anupa N, Dubey KV, Singh SK, Sukumar D (2007)
598 Biosurfactant technology for remediation of cadmium and lead contaminated
599 soils. Chemosphere 68(10):1996-2002
600 [15] Kahru A (1993) In vitro toxicity testing using marine luminescent
601 bacteria *Photobacterium phosphoreum*: The biotox™ test. ATLA 21(2): 210-
602 215
603 [16] Kahru A, Ivask A, Kasemets K, Põllumaa L, Kurvet I, François M,
604 Dubourguier HC (2005) Biotests and biosensors in ecotoxicological risk
605 assessment of field soils polluted with zinc, lead and cadmium. Environ
606 Toxicol Chem 24(11):2973 - 2982
607 [17] Kim IS, Park JS, Kim KW (2001) Enhanced biodegradation of
608 polycyclic aromatic hydrocarbons using nonionic surfactants in soil slurry.
609 Appl Geochem 16:1419–1428
610 [18] Kimura M (1980) A simple method for estimating evolutionary rate of
611 base substitutions through comparative studies of nucleotide sequences. J Mol
612 Evol 16:111-120
613 [19] Leedjärv A, Ivask A, Virta M, Kahru A (2006) Analysis of
614 bioavailable phenols from natural samples by recombinant luminescent
615 bacterial sensors. Chemosphere 64:1910-1919
616 [20] Maslin PM, Maier RM (2000) Rhamnolipid enhanced mineralization
617 of phenanthrene in organic metal co-contaminated soils. Bioremed J 4:295-
618 308

619 [21] Matsufuji M, Nakata K, Yoshimoto A (1997) High production of
620 rhamnolipids by *Pseudomonas aeruginosa* growing on ethanol. Biotechnol
621 Lett 19:1213-1215
622 [22] Meighen EA (1991) Molecular biology of bacterial bioluminescence.
623 Microbiol Rev 55:123-142
624 [23] Mortimer M, Kasemets K, Heinlaan M, Kurvet I, Kahru A (2008) High
625 throughput kinetic *Vibrio fischeri* bioluminescence inhibition assay for study
626 of toxic effects of nanoparticles. Toxicol in Vitro 22(5):1412-1417
627 [24] Mulligan CN, Yong RN, Gibbs BF (2001) Heavy metal removal from
628 sediments by biosurfactants. J Hazard Mater 85:111-125
629 [25] Nitschke M, Costa SGVAO (2007) Biosurfactants in food industry.
630 Trends Food Sci Technol 18(5):252-259
631 [26] Ochoa-Loza FJ, Artiola JF, Maier RM (2001) Stability constants for
632 the complexation of various metals with a rhamnolipid biosurfactant. J
633 Environ Qual 30(2):479-485
634 [27] Pornsunthorntawee O, Wongpanit P, Chavadej S, Abe M, Rujiravanit
635 R (2008) Structural and physicochemical characterization of crude
636 biosurfactant produced by *Pseudomonas aeruginosa* SP4 isolated from
637 petroleum-contaminated soil. Bioresour Technol. 99**:** 1589–1595
638 [28] Rahman KSM, Rahman TJ, Lakshmanaperumalsamy P, Marchant R,
639 Banat IM (2002a) Emulsification potential of bacterial isolates with a range of
640 hydrocarbon substrates. Acta Biotechnol 23:335-345
641 [29] Rahman KSM, Rahman TJ, McClean S, Marchant R, Banat IM
642 (2002b) Rhamnolipid biosurfactants production by strains of *Pseudomonas*
643 *aeruginosa* using low cost raw materials. Biotechnol Prog 18:1277-1281
644 [30] Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-Gonzalez MI,
645 Rojas A, Teran W, Segura A (2002) Mechanisms of solvent tolerance in gram-
646 negative bacteria. Annu Rev Microbiol 56:743–768
647 [31] Rodrigues L, Banat IM, Teixeira J, Oliveira R (2006) Biosurfactants:
648 potential applications in medicine. J Antimicrob Chemother 57:609-618
649 [32] Saitou N, Nei M (1987) The neighbor-joining method: a new method
650 for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425

[33] Sandrin TR, Chech AM, Maier RM (2000) A rhamnolipid biosurfactant reduces cadmium toxicity during naphthalene biodegradation. Appl Environ Microbiol 66:4585-4588

[34] Shin KH, Ahn Y, Kim KW (2005) Toxic effect of biosurfactant addition on the biodegradation of phenanthrene. Environ Toxicol Chem 24(11):2768-2774

[35] Stacey SP, McLaughlin MJ, Cakmak I, Hettiarachchi GM, Scheckel KG, Karkkainen M (2008) Root uptake of lipophilic zinc-rhamnolipid complexes. J Agric Food Chem 56(6): 2112-2117

[36] Villaescusa I, Martinez M, Pilar M, Murat JC, Hostan C (1996) Fresenius J Anal Chem 354: 566–570

[37] Wang X, Gong L, Liang S, Xiurong Han X, Zhu C, Li Y (2005) Algicidal activity of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa.* Harmful Algae 4:433-443

[38] Zhang Y, Miller RM (1992) Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl Environ Microbiol 58(10):3276-3282

Legends for Figures

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

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

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

Figure 4 Inhibition of luminescence in bioluminescent bacteria after 30 minutes of exposure to Cd or Cd-rhamnolipids mixture in heavy metal MOPS (HMM) medium. $CdCl_2$ without rhamnolipids (◆), $CdCl_2$ with rhamnolipids at EC_{20} level (20 mg l^{-1} for *B. subtilis* and *V. fischeri,* 35 mg l^{-1} for *E. coli* and 70 mg l^{-1} for *P. fluorescens*) (▲) and $CdCl_2$ with 50 mg l^{-1} rhamnolipids (Δ). Dashed horizontal line indicates 50% inhibition of bioluminescence (respective EC_{50} values).

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 $CdCl_2$
720 without rhamnolipids (◆), $CdCl_2$ with 10 mg l^{-1} rhamnolipids (□), $CdCl_2$ with 20 mg l^{-1}
721 rhamnolipids (○) and $CdCl_2$ 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)
724

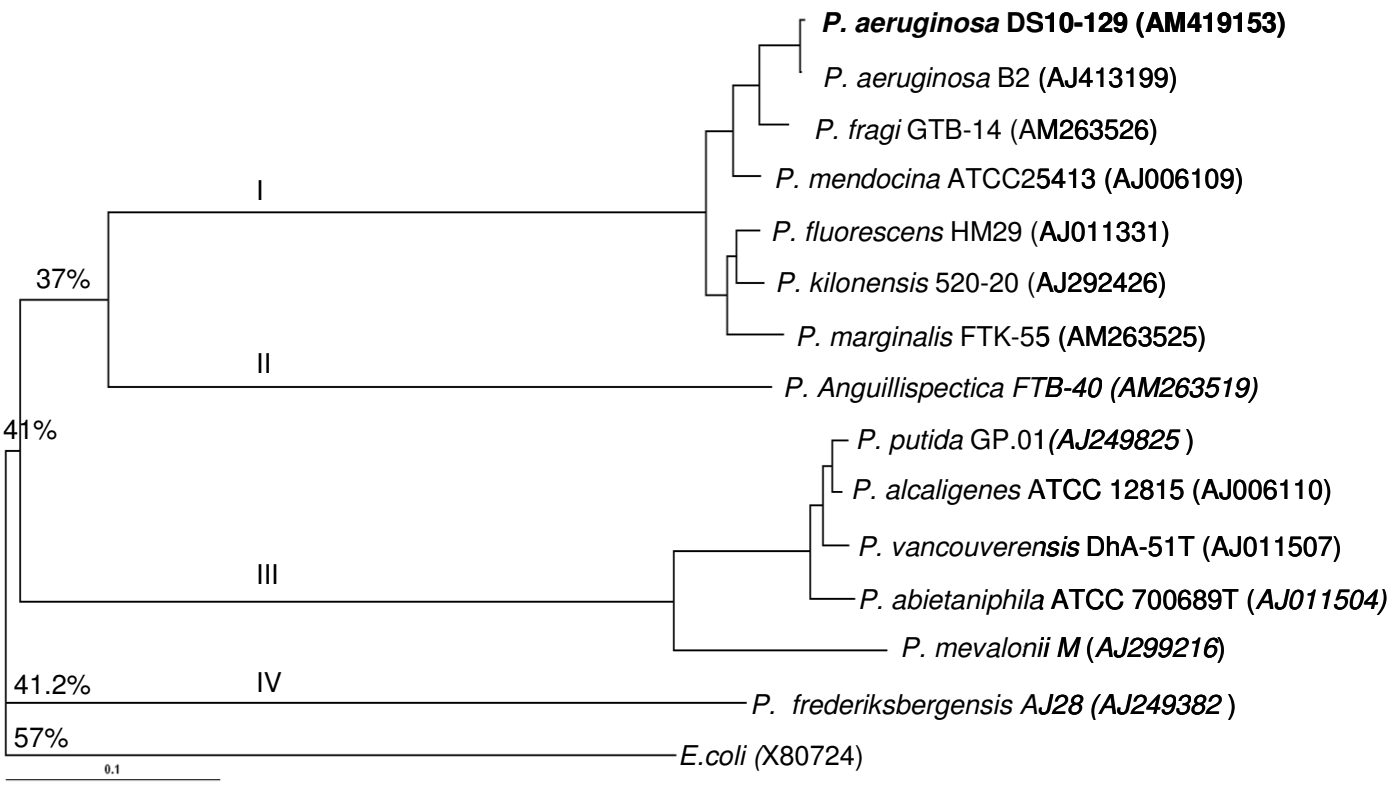
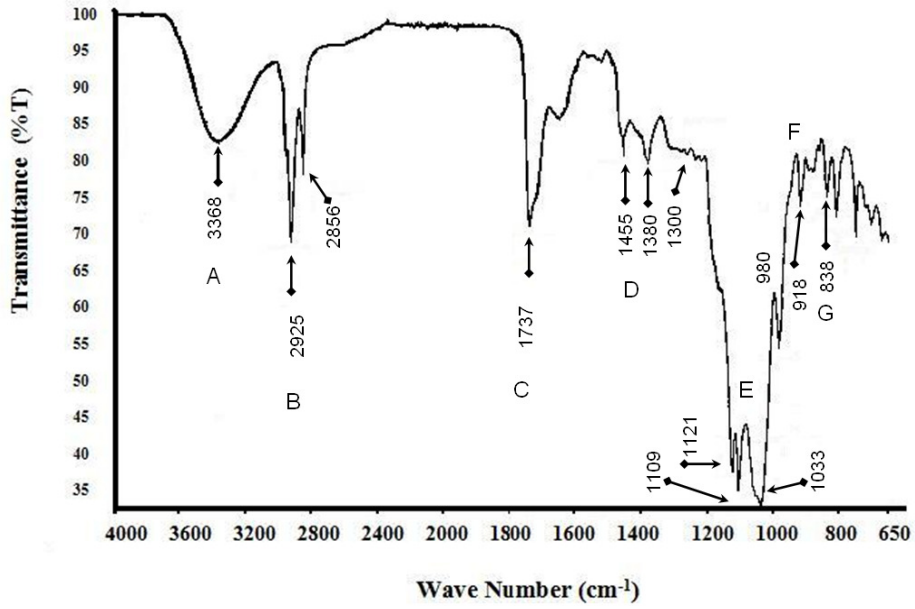

P. aeruginosa DS10-129 (AM419153)
P. aeruginosa B2 (AJ413199)
P. fragi GTB-14 (AM263526)
P. mendocina ATCC25413 (AJ006109)
P. fluorescens HM29 (AJ011331)
P. kilonensis 520-20 (AJ292426)
P. marginalis FTK-55 (AM263525)
P. Anguillispectica FTB-40 (AM263519)
P. putida GP.01(AJ249825)
P. alcaligenes ATCC 12815 (AJ006110)
P. vancouverensis DhA-51T (AJ011507)
P. abietaniphila ATCC 700689T (AJ011504)
P. mevalonii M (AJ299216)
P. frederiksbergensis AJ28 (AJ249382)
E.coli (X80724)
I
II
III
IV
37%
41%
41.2%
57%
0.1

Figure 1


Transmittance (%T)
100
95
90
85
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75
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3368
A
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B
2856
1737
C
1455
1380
D
1300
980
E
1121
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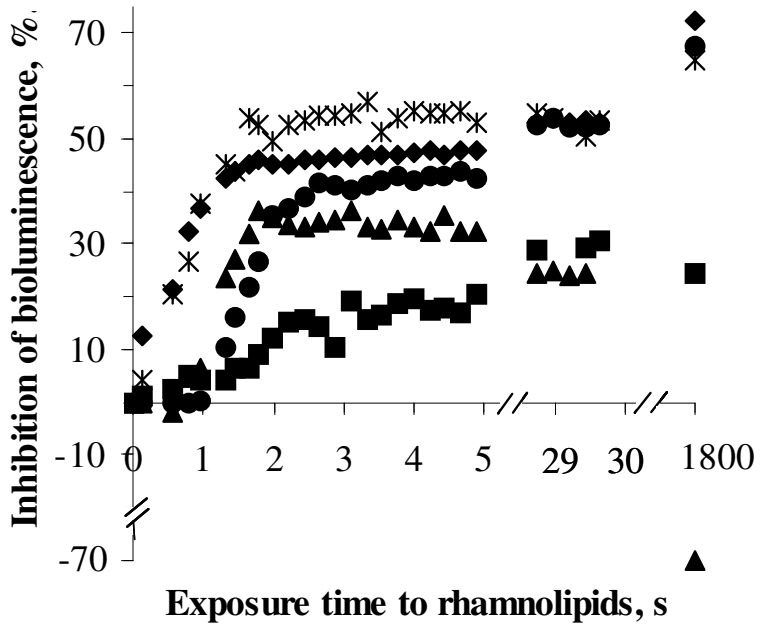
1033
F
918
838
G
4000 3600 3200 2800 2400 2000 1800 1600 1400 1200 1000 800 650
Wave Number (cm-1)

Figure 2


Inhibition of bioluminescence, %
70
50
30
10
-10
-70
0
1
2
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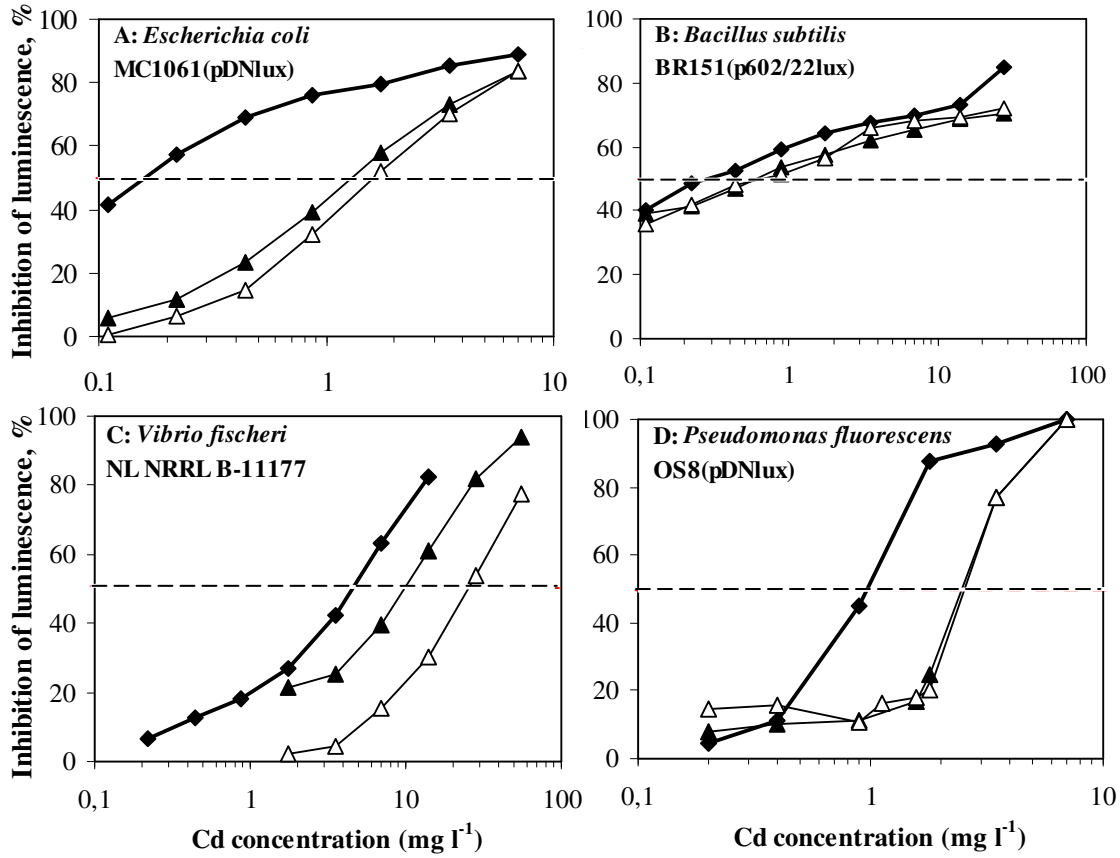
29
30
1800
Exposure time to rhamnolipids, s

Figure 3



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825 Figure 4

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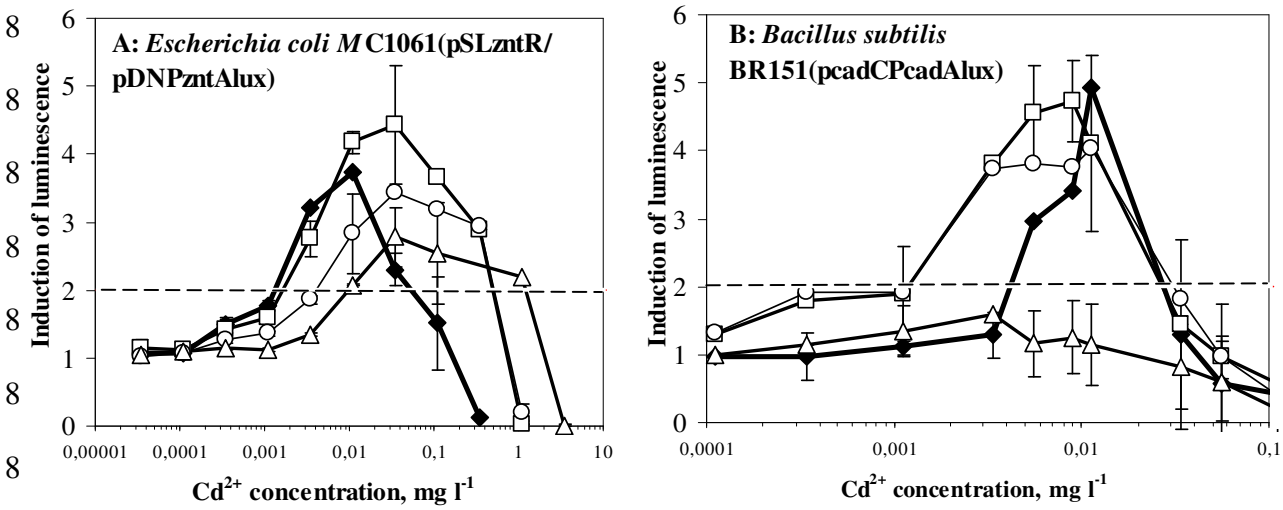

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849 Figure 5

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856 **Table 1** Luminescent bacterial strains used in this study

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

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

858 [b] (*araD*139 Δ(*ara, leu*)7697 Δ*lac*X74 *galU galK hsdR*2 *strA mcrA mcrB*1) ~~[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] (*trpC*2 *lys*-3 *metB*10) ~~[52]~~

864

865 **Table 2** Toxicity (EC_{50}) of rhamnolipids to bioluminescent bacteria as calculated by
866 inhibition of bacterial luminescence

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

867 [a] Tested at 20°C in heavy metal MOPS medium (HMM) supplemented with 2% NaCl
868 [b] Tested 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 Cd^{2+} ions in aqueous environment

Toxicity: bioluminescence inhibition test[a]; 30-min EC_{50}, mg Cd l^{-1} ± SD

	E. coli	*B. subtilis*	*V. fischeri*	*P. fluorescens*
	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

	E. coli	*B. subtilis*
	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 Cd^{2+} 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). EC_{50} 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 Cd^{2+} concentration in soil

Bioavailability: induction of Cd-induced bacteria[a], minimal inducing concentration, mg Cd l^{-1} of soil-water suspension ± SD

	E. coli	*B. subtilis*
	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

Concentration of free Cd^{2+} ions: Cd selective electrode[b], mg Cd^{2+} l^{-1} of soil-water extract ± 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

Desorption of Cd from soil: AAS[c], mg Cd l^{-1} of soil-water extract

	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%

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