Article:
Perelomov, L.V., Sarkar, B. orcid.org/0000-0002-4196-1225, Sizova, O.I. et al. (4 more authors) (2018) Zinc and lead detoxifying abilities of humic substances relevant to environmental bacterial species. Ecotoxicology and Environmental Safety, 151. pp. 178-183. ISSN 0147-6513

https://doi.org/10.1016/j.ecoenv.2018.01.018

Zinc and lead detoxifying abilities of humic substances relevant to environmental bacterial species

L.V. Perelomov[a*], Binoy Sarkar[b,c], O.I. Sizova[d], K.B. Chilachava[a], A.Y. Shvikin[a], I.V. Perelomova[e], Y.M. Atroshchenko[a]

[a]*Department of Chemistry, Tula State Lev Tolstoy Pedagogical University, Prospekt Lenina, 125, Tula, 300026, Russia*

[b]*Department of Animal and Plant Sciences, The University of Sheffield, Western Bank, Sheffield, S10 2 TN, UK*

[c]*Future Industries Institute, University of South Australia, Mawson Lakes, SA 5095, Australia*

[d]*Institute of Biochemistry and Physiology of Microorganisms of RAS, Prospekt Nauki, 5, Pushchino, Moscow region, 142290, Russia*

[e]*Department of Human Physiology, Tula State University, Prospekt Lenina, 92, Tula, 300026, Russia*

[*] Corresponding author:

Dr. L.V. Perelomov; Email: perelomov@rambler.ru

Abstract

The effect of humic substances (HS) and their different fractions (humic acids (HA) and hymatomelanic acids (HMA)) on the toxicity of zinc and lead to different strains of bacteria was studied. All tested bacteria demonstrated a lower resistance to zinc than lead showing minimum inhibitory concentrations of 0.1 - 0.3 mM and 0.3-0.5 mM, respectively. The highest resistance to lead was characteristic of *Pseudomonas chlororaphis* PCL1391 and *Rhodococcus* RS67, while *Pseudomonas chlororaphis* PCL1391 showed the greatest resistance to zinc. The combined fractions of HS and HA alone reduced zinc toxicity at all added concentrations of the organic substances (50 – 200 mg L^{-1}) to all microorganisms, while hymatomelanic acids reduced zinc toxicity to *Pseudomonas chlororaphis* PCL1391 at 200 mg L^{-1} organic concentration only. The HS fractions imparted similar effects on lead toxicity also. This study demonstrated that heavy metal toxicity to bacteria could be reduced through complexation with HS and their fractions. This was particularly true when the metal-organic complexes held a high stability, and low solubility and bioavailability.

Keywords: Humic acid; Hymatomelanic acid; Heavy metals; Minimum inhibitory concentration; Microbial toxicity; Metal-organic complexes

1. Introduction

The introduction of heavy metals, in various forms, in the environment can produce considerable harmful impact on microbial communities and their activities (Gadd, 2005). These elements generally exert an inhibitory action on microorganisms above specific concentrations, by blocking essential enzymes, displacing essential metal ions in biomolecule structures, and/or modifying the active conformations of biological molecules (Gadd, 2005; Giller et al., 2009,). However, at relatively low concentration, some of these elements are essential for microorganisms (e.g., Co, Cu, Zn, Ni) since they provide vital co-factors for some proteins and enzymes (Dupont et al., 2011). At polluted sites, the response of microbial communities to heavy metals depends on the concentration and bioavailability of the elements. It is dependent on the actions of complex processes which are controlled by multiple factors such as the type of an element, the properties of microbial species and the environmental conditions (Hassen et al., 1998). A wide range of soil properties, including pH, redox potential (Eh), clay, iron oxide and organic matter contents, may alter the effects of a given metal loading on the soil microorganisms (Violante et al., 2010).

Numerous studies have shown that humic substances (HS) are capable of altering both the chemical and physical speciation of trace elements and affecting their bioavailability and toxicity (Tipping, 2004; Tang et al., 2014; Zhou et al., 2005; Kostić et al., 2013; Boguta and Sokołowska, 2016). The structural complexity of HS creates opportunities for a broad range of chemical interactions with heavy metals and other pollutants. The mechanisms of these interactions include ion exchange, complexation, redox transformations, hydrophobic bonding, coagulation, peptization, etc. (Boguta and Sokołowska, 2016).

65 The high molecular weight fractions of HS may get readily adsorbed onto the plant cell
66 wall, but do not enter the cell. On the other hand, low molecular weight fractions of HS
67 were shown to reach the plasmalemma of root cells, and in parts were translocated into
68 the shoots (Perminova et al., 2006). Hence, irrespective of their molecular sizes, HS
69 hold a great potential to function as amendments for mitigating adverse impacts of
70 pollutants and as active agents in environmental remediation (Perminova and Hatfield,
71 2005).
72 Multiple interactions between HS, trace elements and living microorganisms might take
73 place in the environment: (a) binding interactions that effect on chemical speciation and
74 bioavailability of trace elements, (b) sorption interactions affecting physical speciation
75 or interphase partitioning of trace elements, (c) abiotic-biotic redox interactions that
76 impact metabolic pathways of toxicants, and (d) direct and indirect interactions with
77 various physiological functions of living microorganisms (Perminova and Hatfield,
78 2005). These interactions of HS with various microorganisms under a heterogeneous
79 contaminated environment is extremely complex, and our understanding of these
80 processes is poor. Therefore, in the present study we investigated the effect of humic
81 substances and their different fractions (humic acids and hymatomelanic acids) on the
82 toxicity of lead (Pb) and zinc (Zn) towards different strains of agriculturally and/or
83 environmentally important bacteria.
84

85 **2. Materials and methods**

86 ***2.1. Humic substances extraction***

87 Mixed sample of mesotrophic sphagnum peat (5 sampling points for each pooled
88 sample) were collected from the small sphagnum bog (0-20 cm depth) situated in Tula
89 region, Russia. Humic substances (HS) from the peats were isolated using alkaline

extraction procedure as described by Stevenson (Stevenson, 1994). For the extraction, a portion of the peat was added to a 0.5 N NaOH solution in the ratio of substrate to alkali 1:10, and the mixture was refluxed for 3 h with constant stirring, and then stored for 24 h at room temperature (25 ± 2 °C). Dark colored supernatant liquor with HS was decanted, filtered through a 0.45 μm membrane filter and dried for the preparation of HS fractions. The yield of HS in the employed procedure was 12.4%.

For the preparation of the humic acid (HA) fraction, concentrated HCl was added to the solution of HS to adjust the pH to pH 1 following the alkaline extraction. The acid precipitated HAs were filtered through a 0.45 μm membrane filter and thoroughly washed with distilled water until a neutral pH (pH = 7) was achieved. The purification of the HA from low molecular weight impurities was performed by dialysis for 24 h in bags with a pore size of 12-14 kDa (Membrane Filtration Products Inc., Texas, USA). The hymatomelanic acid fraction of the HS was obtained by ethanol extraction. Rectified ethanol (300 mL) was added to 5 g of the previously prepared HA and boiled at 78°C under reflux condition for 4 h. The refluxing process was continued until no colored material was observed. The ethanol solution was then concentrated upon vacuum rotary evaporation to almost dryness.

2.2. IR characterization of humic substances

Infrared (IR) spectra of the extracted HA and HMA were collected on a Nikolet-380 FTIR spectrometer (Thermo Scientific, USA). Infrared spectra were obtained using the potassium bromide pellets technique, in which 2 mg of dried humic material was mixed with 200 mg of dried FTIR grade KBr. The instrument was set up with a resolution of 8 cm^{-1} and 64 scans per analysis. Scans covering the 4000-500 cm^{-1} range were recorded and averaged. The spectra were processed using the Nicolet Omnic 8 software.

115

116 ***2.3. Determination of minimum inhibitory concentrations of different HS***

117 Three non-pathogenic, easily cultivable and agriculturally and/or environmentally
118 important bacterial strains were used in this study. Two of the strains were Gram
119 negative bacteria and one strain was Gram positive bacterium. All the three strains were
120 procured from the All-Russian Collection of Microorganisms - VKM. The first bacterial
121 candidate was a Gram negative natural rhizobacterium *Pseudomonas chlororaphis*
122 PCL1391. It was isolated from roots of plants grown in unpolluted areas. This bacterial
123 strain is able to produce the antibiotic phenazine-1-carboxamide, and has active
124 colonizing ability and poses high antagonistic activity against phytopathogenic fungi, in
125 particular, *Fusarium oxysporum.* The second bacterial strain was *Pseudomonas*
126 *fluorescens* 142NF (pNF142) which is a Gram negative bacterium, isolated from oil
127 contaminated soils. It has a plasmid responsible for the degradation of naphthalene and
128 other petroleum hydrocarbon contaminants in the environment (Filonov et al., 2005).
129 The third test strain was *Rhodococsus* RS67 which is a Gram positive soil bacterium
130 able to degrade petroleum hydrocarbon contaminants. It was isolated from oil polluted
131 soils. The Gram negative *Pseudomonas fluorescens* 142NF (pNF142) and Gram
132 positive *Rhodococsus* RS67 are environmentally important for their ability to degrade
133 hydrocarbons and remediate heavy metal pollution, hence they were selected to
134 investigate in this study.

135 All the bacterial strains were initially cultivated in Lysogeny broth (LB) medium
136 (Maniatis et al., 1982) with an initial neutral pH (pH 7). LB medium contained: 10 g
137 bacto-triptone, 5 g yeast extract, and 10 g NaCl in 1 L medium. Minimum inhibitory
138 concentrations (MIC) (levels of bacterial resistance) of Zn and Pb (as their nitrate salts)
139 and MIC in the presence of HS fractions were determined in a modified mineral

140 Duxbury medium (Duxbury, 1981) by a method described previously (Podolskaya et
141 al., 2002). The original mineral Duxbury medium consists of 0.3 g KCl, 0.025 g $CaCl_2$,
142 0.2 g $MgSO_4 7H_2O$, 0.5 g $(NH_4)_2SO_4$, 1 g glucose, 1 g tryptone and 0.5 g yeast extract in
143 1 L medium. To prevent the formation of sparingly soluble $ZnSO_4$ in the culture media,
144 magnesium and ammonium sulfates were replaced by their respective chloride salts.
145 Microorganisms were first grown for 18 h in sterile LB medium until stationary phase
146 which corresponded to optical density (OD) values 0.6-0.7 and colony forming unit
147 (CFU) counts 5 x 10^{11} mL^{-1}. Then bacterial strains in LB medium (50 µL) were
148 inoculated into experimental test tubes with 10 mL of the mineral Duxbury medium
149 (OD of initial experimental medium was 0.025-0.03 and CFU counts 1-2 x 10^8 mL^{-1})
150 with corresponding additions of the trace elements (Zn and Pb) and HS. The heavy
151 metal concentrations in the experimental media ranged from 0.1 to 1.5 mM in steps of
152 0.1 mM. Test tubes without the metal addition served as the control treatments. The test
153 tubes following bacterial inoculations were incubated on a horizontal shaker with 150
154 rpm at 24°C for 24 h in the cases of *Pseudomonas chlororaphis* PCL1391 and
155 *Phodococsus* RS67, and 30 h in the case of *Pseudomonas fluorescens* 142NF (pNF142).
156 The incubation durations were decided from preliminary growth tests on the selected
157 microorganisms (data not presented). The MIC was evaluated from the growth of the
158 bacterial strains (OD of culture) in the above treatment media. All experiments were
159 performed in triplicate, and the OD values were collected on a Shimadzu
160 spectrophotometer (Japan) at a wavelength of 600 nm.
161 To study the detoxifying effect of HS, a series of solutions comprising Zn or Pb and
162 corresponding dissolved fractions of HS were prepared in deionized water and
163 simultaneously added to the Duxbury medium. Final concentrations of heavy metals in
164 the experimental test tubes were 0.1 - 1.5 mM, and the concentrations of HS were 50,

100 and 200 mg L^{-1}. After inoculation, the strains were cultured in test tubes with constant shaking as stated previously, and the growth of microorganisms was evaluated by measuring corresponding OD values as described above. A control with corresponding bacterial strains in uncontaminated HS was used as the zero point of OD determination.

3. Results and discussion

3.1. IR characterization of humic substances

The IR spectra of the humic acid (HA) and hymatomelanic acid (HMA) fractions of the humic substances are shown in Figure 1. The FTIR spectra of the isolated HA exhibited similar absorption bands as reported elsewhere (Rodrigues et al., 2009; Kar et al., 2011). The signals centered at *v* 3260 (HA) and 3240 (HMA) cm^{-1} were assigned to the N-H/O-H stretching vibrations, confirming the presence of free and intermolecular bonded alcohols/phenols, amines/amides and possible carboxylic acids (Rodrigues et al., 2009). Bands at 2920 and 2860 cm^{-1} were attributed to aliphatic asymmetric and symmetric C-H stretching, respectively (Rodrigues et al., 2009). A weak signal near 2620 cm^{-1} was attributed to thiol groups. A peak at 1710 cm^{-1} in HMA spectra was due to the C=O stretching of ketonic and carboxylic groups. This peak was diffused in the HA spectra. Peaks at *v* 1640, 1605 and 1505 cm^{-1} could be assigned to aromatic C=C stretching. A couple of peaks at 1450 cm^{-1} and 1370 cm^{-1} were due to C-H stretching; they were more expressed for HMA than HA. Spectral bands at 1220 and 1025 cm^{-1} were attributed to the stretching vibration of the C-O bond in ethers (Rodrigues et al., 2009; Kar et al., 2011). The presence of different functional groups gives the HA and HMA the ability to form complexes with cations. Many acids have two or more of these

groups arranged so as to enable the formation of chelate complexes that are important aspect of the biological role of soil organic matter (Kar et al., 2011).

[Figure 1]

3.2. Minimum inhibitory concentration (MIC) determination

The average of three replicates MICs (levels of bacterial resistance) of Zn and Pb (as their nitrate salts) in the Duxbury medium for the selected bacterial strains are shown in Table 1. Results showed that all the three strains had a low resistance to Zn (0.1 - 0.3 mM) and a slightly higher resistance to Pb (0.3 - 0.5 mM) (Table 1). The heavy metal Zn might appear toxic in liquid media sometimes at very low doses except for some bacterial strains that were found to be relatively Zn-tolerant (e.g., *Acinetobacter calcoaceticus*, *Citrobacter freundii* and *Pseudomonas aeruginosa*) (Hassen et al., 1998). On the other hand, (Kungolos et al. 2006) showed that the toxicity of Zn was lower than Pb to the photobacterium *Vibrio fischeri* in the case of free ion species. In the current study, the highest resistance to Pb was the characteristic of the strains *Pseudomonas chlororaphis* PCL1391 and *Rhodococcus* RS67 (MIC = 0.5 mM). The strain *Pseudomonas chlororaphis* PCL1391 showed the greatest resistance to Zn also (MIC = 0.3 mM). So, in our study Zn was more toxic element because MIC for Zn was lower than that of Pb for all strains. Obviously, toxicity of the elements depended on the type of studied strains. The toxicity pattern for Zn was in the order: *Pseudomonas fluorescens* > *Rhodococcus* > *Pseudomonas chlororaphis;* while for Pb the pattern was: *Pseudomonas fluorescens* > *Pseudomonas chlororaphis* > *Rhodococcus*.

[Table 1]

The determination of MICs using the traditional approach (in growth media) cannot be related directly to actual metal concentrations in the habitat from which these bacteria

were isolated. In spite of this limitation, this technique of MIC measurement remains a valid approach to evaluate the microbial toxicity of heavy metals in polluted habitats such as agricultural soils, sludge-amended soils, marine sediments and municipal refuse (Hassen et al., 1998).

Mechanisms of bacterial tolerance to heavy metals could vary and might include: binding of the metal by proteins, extracellular polymers or to the cell wall, compartmentation inside cells, formation of insoluble metal sulphides, decreased uptake, enhanced export from cells and volatilization (Giller et al., 2009). Kosinkiewicz (1977) found that some *Pseudomonas* species could produce dark brown pigments which are humic-like polymers. The formation of humic-like substances would start in the bacterial cells and was accompanied by the presence of phenyloxidase enzymes in the bacterial cultures (Kosinkiewicz, 1977). However, often, these mechanisms begin to work only after a long-term presence of the microorganisms in the polluted environment. Campbell et al. (1995) found a higher level of metal tolerance in *Pseudomonas* isolated from soil around industrial sites compared with isolates taken from uncontaminated agricultural soils. In our work we used MIC determination as a baseline approach to assess the bacterial resistance to heavy metals in the presence of HS.

3.3. Zn detoxifying ability of HS

The HS and their fractions reduced Zn and Pb toxicity and increased bacterial resistance to these toxicants in different degrees. The combined fractions of HS (humic acid plus hymatomelanic acid) reduced the Zn toxicity at all studied concentrations of the organic substances in case of all the microbial strains (Figure 2a). The MIC at the highest organic matter (HS) concentration (200 mg L^{-1}) was increased by 5 times for

239 *Pseudomonas fluorescens* strain, by > 3 times for *Pseudomonas chlororaphis* strain and
240 by 4 times for the *Rhodococcus* strain.
241 [Figure 2]
242 The HS are known to form stable complexes with trace elements, mediate redox
243 reactions of transition metals and influence the interphase distribution of the
244 contaminants (Perminova et al., 2006). The HS could have an impact on heavy metal
245 toxicity to microorganisms in the soil solution, converting them into less-toxic
246 complexed forms. According to Tonelly and Ciavatta (1997) and Benedetti et al. (1996),
247 about 90% Cu and 70% Cd was decontaminated in the presence of natural HS.
248 Similarly, Perdue (1984) studied the interactions between HS of terrigenous origin with
249 a high content of aromatic structures and trace elements, and found that the carboxyl
250 groups of HS played a decisive role in making up the two main types of binding sites:
251 salicylate and phthalate. In addition, Ephraim (1991) also pointed out the significant
252 contribution of catechol-type functional groups of HS in binding heavy metals. It was
253 reported that HS from natural waters were prevalent in their carbon skeleton aliphatic
254 fragments and the interaction with heavy metals was mainly determined by carboxylate
255 ions, ester groups, and various combinations of functional groups (Piotrowicz et al.,
256 1984). The functional groups containing heterocyclic amine or nitrogen could also
257 participate in the metal binding process (Orlov, 1990). Moreover, HS could strengthen
258 the resistance of living microorganisms against non-specific stress factors as analogues
259 of biologically active substances (Perminova and Hatfield, 2005).
260 The chemical properties of HS are diverse and determined by their fractions with
261 different compositions, molecular weights and chemical structures (e.g., humic, fulvic
262 and hymatomelanic acids). Fulvic acid (FA) has a lower molecular weight, a higher
263 functional group density and higher acidity than HA. The molecular weights for FA are

264 in the range of 0.5-2 kDa, while they extend from 2 to 1300 kDa for HA. The oxygen
265 content is reported as 32.8-38.3% for HA, and 39.7-49.8% for FA (Steelink, 1985).
266 Heavy metals complexed by FA presumably are more available to plant roots and soil
267 biota than those complexed by HA which can form both water-soluble and water
268 insoluble complexes with metal ions (Kabata-Pendias, 2010). Thus, our experiments
269 indicated that the HS extracted from peat formed stable complexes with Zn that were
270 then inaccessible to the microorganisms. This was the reason for the significant shift of
271 MIC and increased resistance of microorganisms to Zn in the mineral medium. A
272 contribution of FA in the formation of complexes was apparently insignificant.
273 The HA fraction reduced Zn toxicity maximally to the bacteria in the growth medium
274 for all the tested strains (Figure 2b). The Zn MIC for *Pseudomonas fluorescens* at the
275 highest organic matter (HA) concentration (200 mg L^{-1}) was increased by 8 times as
276 compared to no HA treatment, while the same for *Pseudomonas chlororaphis* and
277 *Rhodococcus* R67 increased by 4 and 5 times, respectively. Such toxicity reduction by
278 HA was higher than the combined HS. Thus, at increasing concentrations of HA and HS
279 in the growth medium, there was reduction of Zn toxicity to microorganisms, which was
280 also demonstrated by their increasing MICs. The reported molecular mass of HA
281 generally vary between 2 – 1300 kDa. The interaction of HA with Cu^{2+}, Fe^{2+}, Co^{2+},
282 Zn^{2+}, Pb^{2+}, and Ni^{2+} was reported extensively, and is based on the formation of metal-
283 humate compounds through both covalent bonds and electrostatic interactions (Senesi
284 and Loffredo, 2005). All these mechanisms explain the greatest resistance of the tested
285 microorganisms to Zn in the presence of HA in our experiments.
286 The hymatomelanic acid fraction of HS had the least effect on Zn toxicity in this study
287 (Figure 2c). At all the added concentrations of hymatomelanic acids, no effect on the Zn
288 toxicity to *Pseudomonas fluorescens* 142NF (pNF142) and *Rhodococcus* R67 was

289 observed. Similarly, no effect was also observed in the case of *Pseudomonas*
290 *chlororaphis* PCL1391 when hymatomelanic acid was added to the media at a
291 concentration of 50 and 100 mg L^{-1}. However, the addition of hymatomelanic acid at
292 the concentration of 200 mg L^{-1} showed a significant increase in the microbial
293 resistance to Zn.
294 Hymatomelanic acids hold an average molecular mass between 5 and 10 kDa
295 (Ziechmann, 1993). They contain methoxyl, carboxyl and hydroxyl functional groups,
296 and have characteristically high carbon content (more than 60%) (Kononova, 1966).
297 Pyrolysis-gas chromatography-mass spectrometry studies revealed that contributions
298 from fatty acids and other aliphatic materials were important and predominant. Grimalt
299 and Saiz-Jimenez (1989) showed that fatty acids constituted the predominant
300 components of all hymatomelanic acids encompassing distributions in the C_{12}-C_{34} range
301 where microbial and higher plant contributions could be recognized. Authors found that
302 despite a wide diversity of soil samples was analyzed, no major qualitative differences
303 were found in hymatomelanic acid extracts sampled (Grimalt and Saiz-Jimenez, 1989).
304 Clearly, at low concentrations of hymatomelanic acid, the processes of the formation of
305 unstable or low-molecular Zn complexes that are able to penetrate through the cell
306 membrane, were possibly dominated. The formation of complexes that are inaccessible
307 to microorganisms apparently took place at the highest concentration of hymatomelanic
308 acids only. Overall, the strain *Pseudomonas chlororaphis* PCL1391 was the most
309 responsive to HS additions in the media contained Zn. The resistance of all the strains
310 increased with the introduction of combined HS, or HA and hymatomelanic acid alone.
311
312 ***3.3. Pb detoxifying ability of HS***

313 The combined HS and the HA fractions caused an increase in Pb MICs (Figure 3a,
314 Figure 3b). Christl (2000) reported significant differences in Pb^{2+} binding behavior of
315 HA and FA at pH 4 only, but not at pH 6 and 8 (i.e., conditions of the growth medium).
316 This suggested that the Pb binding to HS was almost unaffected by the difference in the
317 chemical composition of HS.
318 [Figure 3]
319 The HA fraction decreased the toxicity of the heavy metals (both Pb and Zn) at all
320 concentrations of HA and for all the bacterial strains. However, the shift (multiply) in
321 MIC was lower for Pb than Zn (Figure 2b, Figure 3b). The stability constant of Pb-
322 humate complexes is reported to be greater than that of Zn–humate complexes (Kostić
323 et al., 2013). Thus, Pb might form more stable complexes with organic components than
324 Zn in the growth media.
325 The hymatomelanic acid fraction showed a Pb detoxifying effect on all the studied
326 bacterial strains, but only at the maximum concentration of the organic substances (200
327 mg L^{-1}) (Figure 3c). So, their effect on the binding of Pb was significantly lower than
328 the HA fraction, and was shown only at the highest concentration. There are many
329 contradictory data on the elemental composition and chemical structure of
330 hymatomelanic acid. However, there is no doubt about their differences in the
331 molecular weights as compared to HA and FA. Hymatomelanic acids are regarded as
332 the intermediates between HA and FA with molecular weights in the order of 5-10 kDa.
333 Zdanova (2011) reported separate fractions of HS dialyzed through a biological
334 membrane where the molecular masses of the fractions increased in the order of humic
335 acids > humus acids-hymatomelanic acids > fulvic acids. The bioavailability of HS
336 could increase in the presence of metal ions and with increasing pH of the system. So,

337 the metal-hymatomelanic acid complexes might partly penetrate to the cytoplasm of
338 microorganisms and cause a toxic effect (Zdanova, 2011).
339 All the strains of microorganisms increased the resistance to Pb with application of HS
340 in this study. The increase of resistance was different depending on the strain of
341 microorganisms and organic substances used. The mechanisms of increasing resistance
342 of microorganisms to heavy metals possibly involved the formation of stable complexes
343 as well as biological availability of these complexes.
344

345 **4. Conclusions**

346 The strains of microorganisms used in this work (*Pseudomonas chlororaphis* PCL1391,
347 *Pseudomonas fluorescens* 142NF (pNF142) and *Rhodococsus* RS67) demonstrated a
348 lower resistance to Zn than Pb showing minimum inhibitory concentrations (MIC) of
349 0.1 - 0.3 mM and 0.3-0.5 mM, respectively. The humic substances and humic acids
350 reduced the Zn and Pb toxicity at all the added organic matter concentrations
351 irrespective of all the microbial strains. On the other hand, the addition of
352 hymatomelanic acid only at the maximum concentration (200 mg L^{-1}) showed a
353 significant increase in the resistance of *Pseudomonas chlororaphis* PCL1391 to Zn and
354 all the three studied microorganisms to Pb. Thus, under certain conditions, metal ion
355 toxicity might be reduced through complexation with humic substances and their
356 fractions. This is particularly true when the metal-organic complexes hold high stability
357 and low solubility and bioavailability.
358

359 **Acknowledgments**

360 The study was supported by Russian Foundation for Basic Research.
361

362 **References**

363 1. Benedetti, M.F., Riemsdijk, W.H., Koopal, L.K., 1996. Metal ion binding by
364 natural organic matter: From the model to the field. Geochim. Cosmochim. Acta. 60,
365 2503-2513.
366 2. Boguta, P., Sokołowska, Z., 2016. Interactions of Zn(II) ions with humic acids
367 isolated from various type of soils. Effect of pH, Zn concentrations and humic acids
368 chemical properties. PLoS ONE. 11(4), 1-20
369 3. Campbell, J.I.A., Jacobsen, C.S., Sùrensen, J., 1995. Species variation and
370 plasmid incidence among fluorescent *Pseudomonas* strains isolated from agricultural
371 and industrial soils. FEMS Microbiol. Ecol. 18, 51-62.
372 4.
373 5. Christl, D.I., 2000. Influence of humic substances on the sorption of heavy metal
374 cations on mineral surfaces. Dissertation. Swiss Federal Institute of Technology, Zürich.
375 6. Dupont, C.L, Grass, G., Rensing, C., 2011. Copper toxicity and the origin of
376 bacterial resistance-new insights and applications. Metallomics. 3, 1109–1118.
377 7. Duxbury, T., 1981. Toxicity of heavy metals to soil bacteria. FEMS Microbiol.
378 Let. 11(2-3), 217–220.
379 8. Ephraim, J., 1991. Europium binding by an aquatic fulvic acid. Interaction
380 functions for the "average" sites in the fulvic acid molecule. Sci. Total. Environ. 108,
381 261-273.
382 9. Filonov, A.E., Akhmetov, L.I., Puntus, I.F., Eiskova, T.Z., Gafarov, A.B.,
383 Izmalkova, T.Y., 2005. The construction and monitoring of genetically tagged, plasmid-
384 containing, naphthalene degrading strains in soil. Microbiology. 74, 453-458.

385 10. Gadd, G.M., 2005. Microorganisms in toxic metal-polluted soils, in: Buscot, F.,
386 Varma A. (Eds), Microorganisms in soils: roles in genesis and functions. Springer,
387 Berlin; New York, pp. 325-356.
388 11. Giller, K.E., Witter, E., McGrath, S.P., 2009. Heavy metals and soil microbes.
389 Soil. Biol. Biochem. 41, 2031-2037.
390 12. Grimalt, J.O., Saiz-Jimenez, C., 1989. Lipids of soil humic acids. I. The
391 hymatomelanic fraction. Sci. Total. Environ. 81-82, 409-420.
392 13. Hassen, A., Saidi, N., Cherif, M., Boudabous, A., 1998. Resistance of
393 environmental bacteria to heavy metals. Bioresource Technology. 64, 7-15.
394 14. Kabata-Pendias, A., 2010. Trace elements in soils and plants. 4th edition. CRC
395 Press/Taylor & Francis Group, Boca Raton.
396 15. Kar, S, Maity, J.P., Jean J-S., Liu, C-C., Nath, B., Lee, Y-C., Bundschuh, J.,
397 Chen, C-Y., Li, Z., 2011. Role of organic matter and humic substances in the binding
398 and mobility of arsenic in a Gangetic aquifer. J. Environ. Sci. Heal. A. 46, 1231-1238.
399 16. Kononova, M.M., 1966. Soil organic matter; its nature, its role in soil formation
400 and in soil fertility. Oxford Pergamon Press, New York.
401 17. Kosinkiewicz, B., 1977. Humic-like substances of bacterial origin. I. Some
402 aspects of the formation and nature of humic-like substances produced by
403 *Pseudomonas*. Acta Microbiol. Pol. 26, 377-386.
404 18. Kostić, I.S., Anđelković, T.D., Nikolić, R.S., Cvetković, T.P., Pavlović, D.D.,
405 Bojić, Alj., 2013. Comparative study of binding strengths of heavy metals with humic
406 acid. Hemijska industrija. 67, 773-779.
407 19. Kungolos, A., Samaras, P., Tsiridis, V., Petala, M., Sakellaropoulos, G., 2006.
408 Bioavailability and toxicity of heavy metals in the presence of natural organic matter. J.
409 Environ. Sci. Heal. A. 41, 1509–1517.

20. Maniatis, T., Fritsch, E.F., Sambrook, J., 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, New York.

21. Orlov, D.S., 1990. Humus acids of soils and the general theory of humification. MSU Press, Moscow. (In Russian).

22. Perdue, E.M., 1984. Analytical constraints on the structural features of humic substances. Geochim. Cosmochim. Acta. 48, 1435-1442.

23. Perminova, I.V., Hatfield, K., 2005. Remediation chemistry of humic substances: theory and implications for technology, in*:* Perminova, I.V., Hatfield, K., Hertcorn, N. (Eds.), Use of humic substances to remediate polluted environments: from theory to practice. Springer, Dordrecht, Netherlands, pp 3-36.

24. Perminova, I.V., Kulikova, N.A., Zhilin, D., Grechischeva, M., Kovalevskii, D.V., Lebedeva, G.F., Matorin, D.N., Venediktov, P.S., Konstantinov, A.I., Kholodov, V.A., Petrosyan, V.S., 2006. Mediating effects of humic substances in the contaminated environments. concepts, results, and prospects, in: Twardowska, I., Allen, H.E., Haggblom, M.H., Stefaniak, S. (Eds)., Viable methods of soils and water pollution monitoring, protection and remediation. Krakow, Poland, pp. 249–273.

25. Piotrowicz, S.R., Harvey, G.R., Boran, D.A et al., 1984. Cadmium, copper, and zinc interactions with marine humus as a function of ligand structure. Marine Chem. 14, 333-346.

26. Podolskaya, V.I., Gruzina, T.G., Ulberg, Z.R., Sokolovskaya, A.S., Grishchenko, N.I., 2002. Effect of arsenic on bacterial growth and plasma-membrane ATPase activity. Appl. Biochem. Microbiol. 38, 48–52.

27. Rodrigues, A., Brito, A., Janknecht, P., Proenca, M.F., Nogueira R., 2009. Quantification of humic acids in surface water: effects of divalent cations, pH, and filtration. J. Environ. Monit. 11, 377–382.

435 28. Senesi, N., Loffredo, E., 2005. Metal ion complexation by soil humic
436 substances, in: Chemical processes in soil. Soil Science Society of America, Madison,
437 WI, pp 563–618.
438 29. Steelink, C., 1985. Elemental characteristics of humic substances, in: Aiken,
439 G.R., McKnight, D.M., Wershaw, R.L., MacCarthy, P. (Eds.), Humic substances in soil,
440 sediment, and water. Geochemistry, Isolation and Characterization. John Wiley, New
441 York, pp. 457-476.
442 30. Stevenson, F.J., 1994. Humus Chemistry: Genesis, Composition, Reactions.
443 John Wiley, New York.
444 31. Tang, W.W., Zeng, G.M., Gong ,J.L., Liang, J., Xu, P., Zhang, C, Huang, B.B.,
445 2014. Impact of humic/fulvic acid on the removal of heavy metals from aqueous
446 solutions using nanomaterials: a review. Sci. Total Environ. 468-469, 1014-27.
447 32. Tipping, E., 2004. Cation binding by humic substances. Cambridge University
448 Press, New York.
449 33. Tonelli, D., Ciavatta, C., 1997. Extraction of humic acids from a natural matrix
450 by alkaline pyrophosphate. Evaluation of the molecular weight of fractions obtained by
451 ultrafiltration. Fresenius J. Anal. Chem. 359, 555-561.
452 34. Violane, A., Cozzolino, V., Perelomov, L., Caporale, A.G., M. Pigna, M., 2010.
453 Mobility and bioavailability of heavy metals and metalloids in soil environments. J.
454 Soil. Sci. Plant Nutr. 10 (3), 268 – 292.
455 35. Zhdanova, A.V., 2011. Study of structural components and physico-chemical
456 properties of humic substances of low-mineralized silt sulfide mud as a source of
457 antioxidant drug. Dissertation. Samara State Medical University, Samara. (In Russian).
458 36. Zhou, P., Yan, H., Gu, B., 2005. Competitive complexation of metal ions with
459 humic substances. Chemosphere. 58, 1327–1337.

460 37. Ziechmann, W., 1993. Humic Substances. BI Wissenschaftsverlag, Mannheim.

461

462 **Table caption**

463 Table 1. Minimum inhibitory concentrations of zinc and lead for the tested bacterial
464 strains grown in Duxbury medium

465

466 **Figure captions**

467 Figure 1. Infrared spectra of humic acids (a) and hymatomelanic acids (b) extracted
468 from sphagnum peat

469

470 Figure 2. Minimum inhibitory concentrations of zinc obtained for 3 strains of bacteria in
471 the absence or presence of 50, 100 or 200 mg L^{-1} of (a) humic substances, (b) humic
472 acids, and (c) hymatomelanic acids.

473

474 Figure 3. Minimum inhibitory concentrations of lead obtained for 3 strains of bacteria in
475 the absence or presence of 50, 100 or 200 mg L^{-1} of (a) humic substances, (b) humic
476 acids, and (c) hymatomelanic acids.

477

478 Table 1.

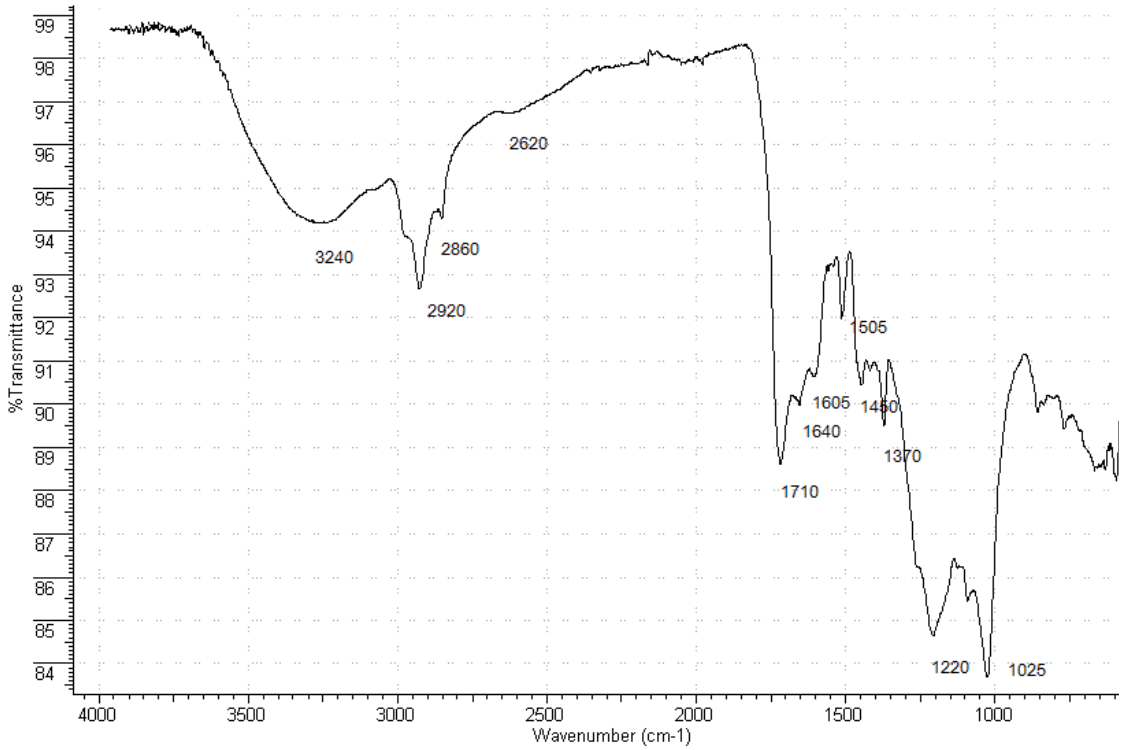
Bacterial strain	Zn (mM)	Pb (mM)
Pseudomonas chlororaphis PCL1391	0.3	0.5
Pseudomonas fluorescens 142NF (pNF142)	0.1	0.3
Rhodococcus RS67	0.2	0.5

479

480 Figure 1.

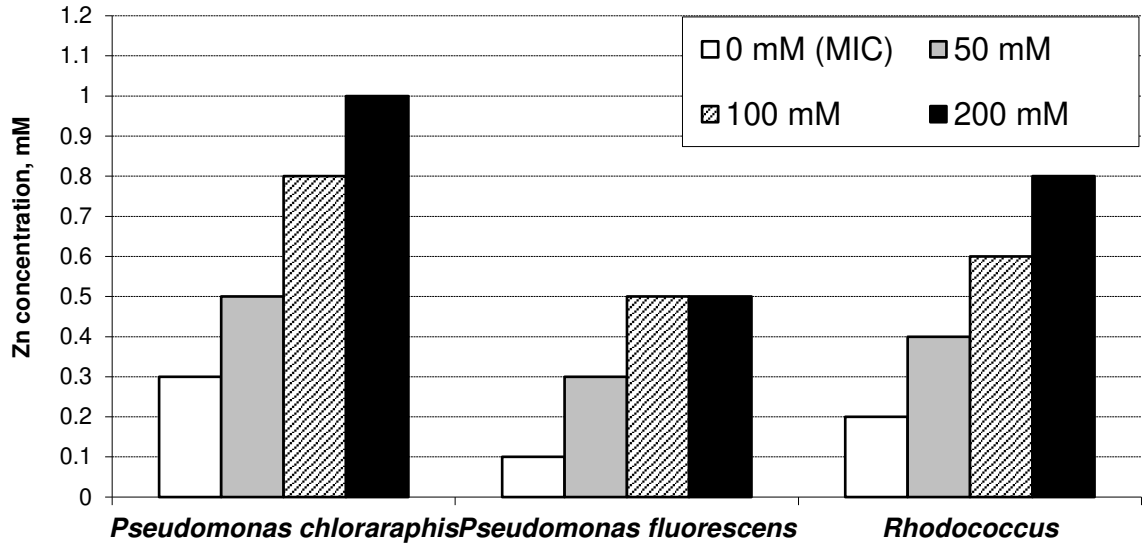
a

b



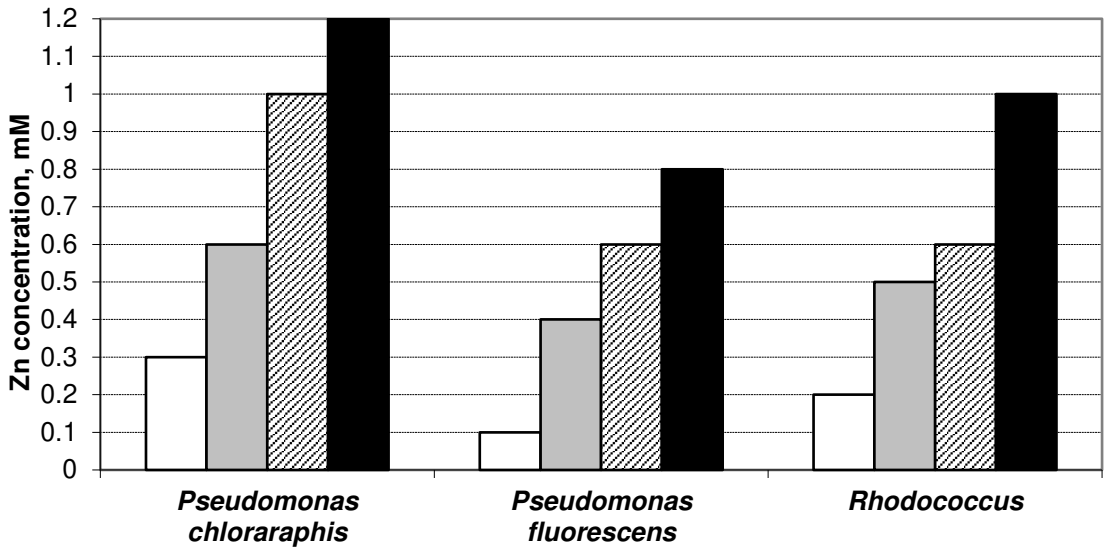
481

a

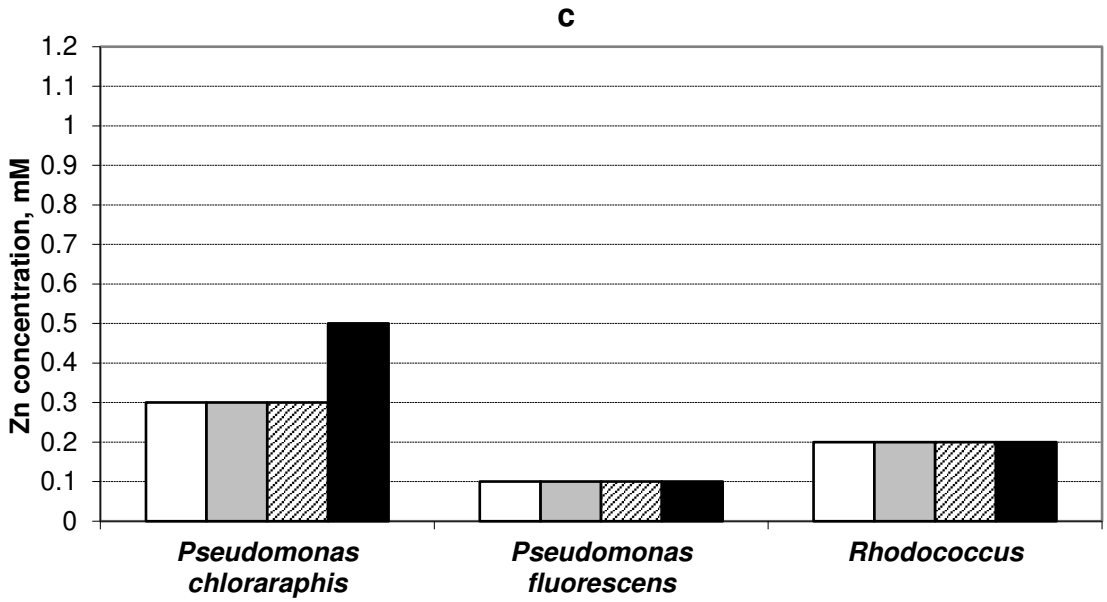

1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Zn concentration, mM
0 mM (MIC)
50 mM
100 mM
200 mM
Pseudomonas chloraraphis
Pseudomonas fluorescens
Rhodococcus

482

b

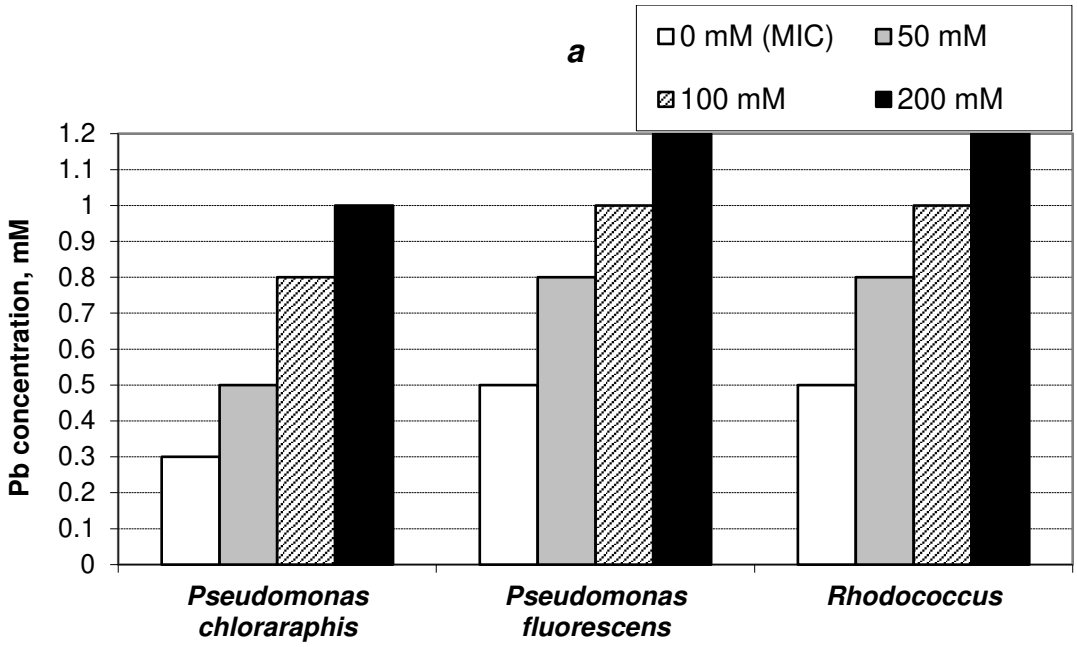

1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Zn concentration, mM
Pseudomonas chloraraphis
Pseudomonas fluorescens
Rhodococcus

483

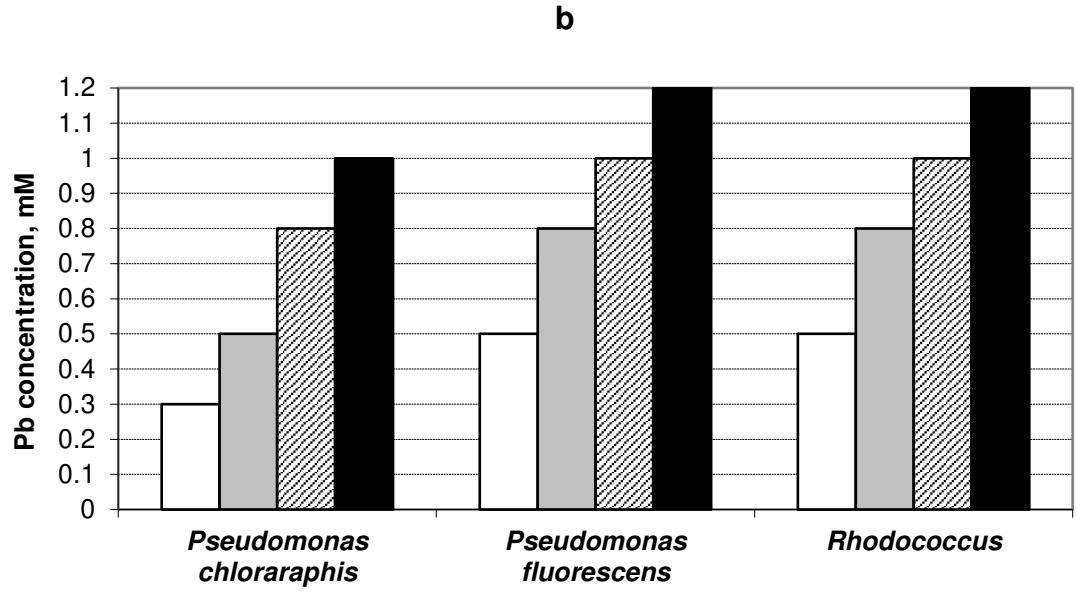


484

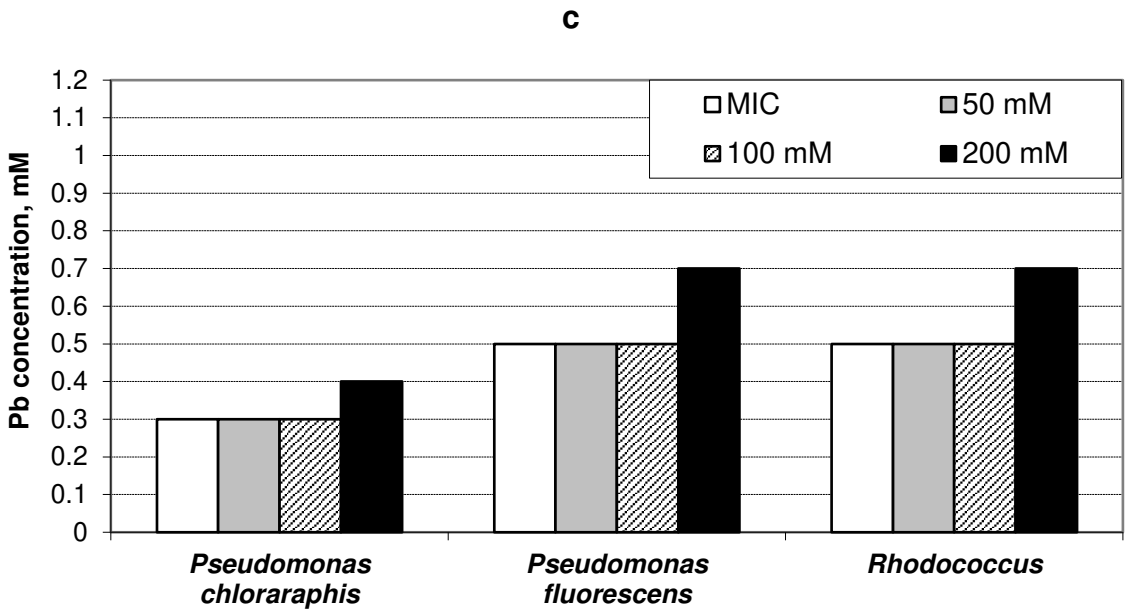
485 Figure 2.


a
0 mM (MIC)
50 mM
100 mM
200 mM
Pb concentration, mM
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Pseudomonas chloraraphis
Pseudomonas fluorescens
Rhodococcus

486


b
Pb concentration, mM
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Pseudomonas chloraraphis
Pseudomonas fluorescens
Rhodococcus

487



488

489 Figure 3.

490