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

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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.

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).

The high molecular weight fractions of HS may get readily adsorbed onto the plant cell wall, but do not enter the cell. On the other hand, low molecular weight fractions of HS were shown to reach the plasmalemma of root cells, and in parts were translocated into the shoots (Perminova et al., 2006). Hence, irrespective of their molecular sizes, HS hold a great potential to function as amendments for mitigating adverse impacts of pollutants and as active agents in environmental remediation (Perminova and Hatfield, 2005).

Multiple interactions between HS, trace elements and living

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microorganisms might take place in the environment: (a) binding interactions that effect on chemical speciation and bioavailability of trace elements, (b) sorption interactions affecting physical speciation or interphase partitioning of trace elements, (c) abiotic-biotic redox interactions that impact metabolic pathways of toxicants, and (d) direct and indirect interactions with various physiological functions of living microorganisms (Perminova and Hatfield, 2005). These interactions of HS with various microorganisms under a heterogeneous contaminated environment is extremely complex, and our understanding of these processes is poor. Therefore, in the present study we investigated the effect of humic substances and their different fractions (humic acids and hymatomelanic acids) on the toxicity of lead (Pb) and zinc (Zn) towards different strains of agriculturally and/or environmentally important bacteria.

2. Materials and methods

2.1. Humic substances extraction

Mixed sample of mesotrophic sphagnum peat (5 sampling points for each pooled sample) were collected from the small sphagnum bog (0–20 cm depth) situated in Tula 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.

2.3. Determination of minimum inhibitory concentrations of different HS

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

All the bacterial strains were initially cultivated in Lysogeny broth (LB) medium (Maniatis et al., 1982) with an initial neutral pH (pH 7). LB medium contained: 10 g bacto-triptone, 5 g yeast extract, and 10 g NaCl in 1 L medium. Minimum inhibitory concentrations (MIC) (levels of bacterial resistance) of Zn and Pb (as their nitrate salts) and MIC in the presence of HS fractions were determined in a modified mineral Duxbury medium (Duxbury, 1981) by a method described previously (Podolskaya et al., 2002). The original mineral Duxbury medium consists of 0.3 g KCl, 0.025 g $CaCl_2$, 0.2 g $MgSO_47H_2O$, 0.5 g $(NH_4)_2SO_4$, 1 g glucose, 1 g tryptone and 0.5 g yeast extract in 1 L medium. To prevent the formation of sparingly soluble $ZnSO_4$ in the culture media, magnesium and ammonium sulfates were replaced by their respective chloride salts.

Microorganisms were first grown for 18 h in sterile LB medium until stationary phase which corresponded to optical density (OD) values 0.6–0.7 and colony forming unit (CFU) counts 5×10^{11} mL^{-1}. Then bacterial strains in LB medium (50 μL) were inoculated into experimental test tubes with 10 mL of the mineral Duxbury medium (OD of initial experimental medium was 0.025–0.03 and CFU counts $1–2 \times 10^8$ mL^{-1}) with corresponding additions of the trace elements (Zn and Pb) and HS. The heavy metal concentrations in the experimental media ranged from 0.1 to 1.5 mM in steps of 0.1 mM. Test tubes without the metal addition served as the control treatments. The test tubes following bacterial inoculations were incubated on a horizontal shaker with 150 rpm at 24 °C for 24 h in the cases of *Pseudomonas chlororaphis* PCL1391 and *Phodococsus* RS67, and 30 h in the case of *Pseudomonas fluorescens* 142NF (pNF142). The incubation durations were decided from preliminary growth tests on the selected microorganisms (data not presented). The MIC was evaluated from the growth of the bacterial strains (OD of culture) in the above treatment media. All experiments were performed in triplicate, and the OD values were collected on a Shimadzu spectrophotometer (Japan) at a wavelength of 600 nm.

To study the detoxifying effect of HS, a series of solutions comprising Zn or Pb and corresponding dissolved fractions of HS were prepared in deionized water and simultaneously added to the Duxbury medium. Final concentrations of heavy metals in 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 Fig. 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 ν 3260 (HA) and 3240 (HMA) cm^{-1} were assigned to the N-