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MICROORGANISMS -INDICATORS OF THE LEVEL OF SOIL POLLUTION WITH LEAD

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ABSTRACT

Environmental pollution with heavy metals present a real threat to wildlife because the metals cannot be naturally decomposed as is the case with organic pollutants, and as such they can survive in the environment while accumulating the heavy metals in different parts. Pollution with metals can affect different organisms in the environment, such as microorganisms, plants and animals, but the degree of toxicity depends on the species.

Microorganisms have different mechanisms of coping with a variety of toxic metals. Large number of metals is essential for growth of microorganisms, but some can be very harmful too. This is happening because heavy metals have the ability to form complexes with proteins and make them inactive, for example, inactivation on enzymes. Many heavy metals are detrimental to microorganisms even at very low concentrations. We have investigated the resistance of lead as heavy metal on microorganism populations living on soil contaminated with heavy metals. Resistance to soluble lead was investigated in two different bacterias *Pseudomonas marginalis* and *Bacillus megaterium*. The population of microorganisms showed different response to the heavy metal.

***Key words**:* microorganisms, heavy metals, lead, soil, *Pseudomonas marginalis*, *Bacillus megaterium.*

INTRODUCTION

Although some heavy metals are required as micronutrients, all heavy metals are toxic in excess. In order to avoid toxicity, metals must be eliminated quickly and efficiently from the cell. In general, there are two basic mechanisms of resistance to heavy metal ions: intracellular complexation of toxic metal ions is mainly used in eukaryotes; whereas, reduced accumulation based on active efflux of the cations is the primary mechanism developed in prokaryotes. In bacteria binding factors and enzymatic transformations (oxidation, reduction, methylation, and demethylation) play a role as defense mechanisms.

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The knowledge about heavy metal resistance mechanisms generally broadens the insights into the ability of bacteria to inhabit polluted environments, but can also be applied in biotechnology for example for bioreporter-technology or bioremediation purposes. Bacterial bioreporters, which are living microorganisms that produce a specific, quantifiable output in response to target chemicals, take advantage of heavy metal inducible transcription systems and sense bioavailable metals depending on metal flux through the cell. Thus, the bioreporter technology is closely related to the research about heavy metal resistance mechanisms and will be introduced alongside the actual resistance mechanisms.

Heavy metals

Areas surrounding historic mining and smelting operations represent some of the most highly contaminated and destructed soil habitats due to the high toxicity and widespread ecological effects associated with metal contamination. The term "heavy metal" is ambiguous and has been used inconsistently in the scientific literature for decades. The term "heavy metal" has come to be associated with a select group of metals and metalloids (i.e., lead, chromium, cadmium, copper, zinc, arsenic) that are potentially toxic to animals and the environment. Metals are naturally present in the Earth's crust, and trace levels of metals are continuously released into the environment from the geochemical weathering of rocks and minerals. However, as a result of increased mining activity and the widespread use of metal-additives in gasoline and paint, metal residues are ubiquitously distributed throughout soils and groundwater. While many metals, including iron (Fe), copper (Cu), manganese (Mn), selenium (Se), cobalt (Co), magnesium (Mg), and zinc (Zn), are known to be essential to plants and animals for normal growth and development, other metals, like lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg), have no nutritional benefit. All metals at high concentrations are toxic to living systems, and their toxicological effects on humans, wildlife, plants and aquatic species have been well characterized. In addition to the negative effects of metals on humans and wildlife, high concentrations of metals can impact soil quality and soil health, largely as a result of the negative effects of metals on soil microbes.

Environmental pollution with heavy metals present a real threat to wildlife because the metals cannot be naturally decomposed as is the case with organic pollutants, and as such they can survive in the environment while accumulating in different parts of that environment (Igwe et al., 2005; Smejkalova et al., 2003). Pollution with metals can affect different organisms in the environment, such as microorganisms, plants and animals, but the degree of toxicity depends on the species. Several trials have shown that heavy metals can cause shifts in the population of microorganisms (Barkay *et al*., 1985; Doelman *et al*., 1994; Gringell *et al*., 1976; Roane & Kellogg, 1996). Heavy metals have toxic effect on soil microorganisms (Pawlowska & Charvat, 2004) such as changes in diversity, population number and activity of soil microorganisms (Smejkalova *et al*., 2003; Gupta, 1992; Hattori, 1996; Kelly *et al*., 2003; Gasper *et al*., 2005). The toxicity of heavy metals in relation to microorganisms is perceived in the inhibition of the enzyme activity, destruction of the membrane function, as well as destruction of nucleic acids. Heavy metals can reduce metabolic activity, diversity, and number of cells. The level of enzymes in microorganisms, including bacteria, algae, and fungi serves as a constructive model for the study of the effect that heavy metals have at cell level (Avery, 2001). These microorganisms can also be used for the removal of toxic heavy metals from contaminated places because they can accumulate heavy metals and radio nucleotides from the outer environment (Ali & Wainwright1995). The resistance to heavy metals that the microorganisms have includes the acceptance of these in the form of phosphates, carbonates, and sulfides, physical exclusion on the part of electronegative components of the membrane and egzo-polymers, as well as intracellular sequestration with low molecular weight, cysteine-rich proteins.

Lead contamination in soil is a widespread problem resulting from industrial use and processing of lead ore. Bioavailable lead poses a hazard for children and can lead to mental retardation. There are limited strategies for removing lead from soil. Using bioremediation, a bacterium that could render toxic lead to non-bioavailable would provide an alternative option for detoxifying this contaminant in

the environment. The first step in devising a bioremediation strategy is to identify candidate bacterial strains capable of modifying the contaminant. The goal of this paper was to examine bacteria found in lead contaminated soil, to determine the level of lead resistance of native bacteria and to determine the mechanism of lead resistance.

Unlike other metals, lead has no biological role and is potentially toxic to microorganisms. Studies have shown high accumulation of lead in surface soil with a high rate of immobilization and accumulation as a result of the organic portion of soil, soil pH. Despite the high accumulation of lead, organic soils do not contain even 30% of the total lead of the ecosystem.The aim of this paper is different resistance to lead in microbial population that lives in soil contaminated with heavy metals. Special emphasis is shown in the resistance of two bacteria *Pseudomonas marginalis* and *Bacillus megaterium*. The influence of lead on them is investigated by monitoring the change in the number of bacterial colonies that these types form.

MATERIALS AND METHODS

Soil samples for analysis, determining the total and dissolved lead were taken along the river flow of Zletovica and Bregalnica, right tributarie of the river Bregalnica. The selection of measurement sites was made based on previously obtained results from several annual surveys made in this locality, i.e. previously made analysis of lead content in the waters of the rivers Zletovica and Bregalnica and lead content in soil. The sample for analysis was taken from the surface layers of soil. The determination of lead content was performed using a standardized method for the determination of heavy metals in soil.

DETERMINATION OF HEAVY METALS IN SOIL

Soil samples were taken from surface layers of the soil at the depth up to 5 cm. The material underwent mechanical processing to remove small roots and stones. The soil was then packed and dried in a drier for 48 hours at a temperature of 105^0C.1 gr of soil is measured on the analytical scale and is transferred into a Keldal flask for combustion. Burning is done on a sand bath with nitric, sulfuric and perchloric acid in relation 40:2:1 (ml) until ash-colored and whitish sediment is obtained. Precipitation is dissolved with hot distilled water, filtered and transferred into a 100 ml flask by adding distilled water up to the of mark.

Calculation: Values of the atomic absorber are expressed in ppm or mg/l.

In order to express the concentration in mg/100ml, the obtained concentration in mg/l should be divided with 10.

The concentration of the given element expressed in mg/gr will be calculated according to the following formula:

$$\mathrm{Pb(mg/gr)} = \frac{\text{mg Pb/100ml}}{\text{soil sample (mg)}}$$

In order to express the concentration of the given element in percentage (%), the obtained value in mg/g should be multiplied with 100:

$$\mathrm{Pb\%} = \frac{\text{mg Pb/100ml}}{\text{soil sample (mg)}} \times 100$$

METHOD FOR DETERMINING THE DISSOLVED LEAD IN SOIL

Determining the dissolved lead is done with the use of organic phosphates. Dissolved concentration of lead is detected in three organic sources in sodium pyrophosphate, phosphorus, glycerolphosphate and sodium tripolyphosphate. The dissolved lead is measured by atomic absorption.

ISOLATION OF PURE CULTURES

In nature, microorganisms exist in the form of mixed population. To study, identify and characterize a microorganism, it must be isolated in the form of a pure culture. For obtaining pure cultures from a mixed population there are two important procedures:

1. Mixed population should be diluted in a dilutient in order to separate individual organisms, so that visible colonies are formed on the agar surface after incubation, which will be isolated from colonies of other microorganisms.
2. One colony is taken from the insulation plate with inoculation loop and transferred into a new sterile medium. After incubation, all organisms in the new culture will be descendants of a single organism. In our case we used the dilution method for obtaining pure culture that is present in soil contaminated with lead.

Soil inoculums is taken and put in a tube with melted agar at a temperature of 50°C. After inoculation, the contents in the tube should be thoroughly mixed. Then from the content of the first test tube a sample is taken with in oculation loopwhich is inserted into tube 2 with the same characteristics. The content of the tube 1 is poured into an empty Petri dish. The procedure is repeated with the next tube. In this way, the initial number of cells is gradually diluted. In most cases to 3 dilutions are sufficient for obtaining pure cultures. Then Petri boards are left for incubation after which bacteria grown on agar can be noticed.

ISOLATION OF LEAD RESISTANT SPECIES

Bacterial species resistant to lead are identified by BIOLOG. BIOLOG system is a method for identifying bacteria that uses 96% utilization of the metabolic carbon footprint and identification of individual isolates. Growth of bacteria was observed for 24 hours over a period of 7 days. The number of lead - resistant bacteria was determined by planting crops on agar medium with the same level of lead and matching liquid culture. The isolates were examined using a medium containing: $C_6H_5O_7Na_5$ (sodium citrate), 0,5gr, $MgSO_{4X}7H_2O$, 0,1gr, $(NH_4)2SO_4$, 1,0gr, $C_6H_{12}O_6$, 1.0gr, and $Na_5O_{10}P_3$ (sodium tripolyphosphates), 0,1gr. Lead was added in the form of Pb $(NO_3)_2$ and with 5 different concentrations of 0, 0,2., 0,4., 0,6., 1,2 and 3μm.

RESULTS

In microbial analysis of the metals, total metal concentration does not always means the toxicity of the metals on microbes. That is why the interpretation of results is very difficult. In this paper, we tried to find the link of the soluble lead concentration with the observed microbes and their resistance of lead. This has shown that toxicity of available lead in conditions of increased lead concentration, will lead to a reduction in the number of colonies of microbes. Table 1 shows the difference between two bacterial isolates. Table 1 summarizes the relationship between soils and parameters of the environment and potential resistance of microbes to lead.

Table 1. Soil characterization data for *Pseudomonas marginalis* from Soil – river flow of Zletovica and *Bacillus megaterium* from Soil river Bregalnica

Soil variable	Soil - river flow of Zletovica	Soil-river Bregalnica
pH	6.2	3.8
%OC	0.76	0.96
Soil texture	sand	Loamy sand

Table 2. Total concentration heavy metals and soluble Pb (mg/kg dry weight)

	Total metal concentration mg/kg dry weight

	Soil - river flow of Zletovica	Soil-river Bregalnica
Pb	491.6	170.2
Zn	2288	733
Cu	186.7	51.1
Mn	247.9	30.9
Cd	13.8	7.4
As	4.75	1.63
Soluble Pb[a]	0.12	0.09

[a]**Atomic** absorption minimal detection limit was 0.0004 μmol g−1 Pb.

Table 3. Total cell concentration and viable cell concentration (cells g $^{-1}$ soil)

Total cell concentration (cells g $^{-1}$ soil)	
Soil - river flow of Zletovica	Soil-river Bregalnica
7.8 X10 ^{7} ± 0.9 × 10^{7}	3.8X10^{6} ±1.3X10^{6}
Viable cell concentration (cells g $^{-1}$ soil)	
Soil - river flow of Zletovica	Soil-river Bregalnica
2.6 X 10 ^{7} ± 7.9 × 10^{7}	3.2 X 10^{3} ± 2.7 X 10^{3}

Table 4. Relationship between soil parameters and microbial lead resistance

Isolate identification	PbMRLa (mM) a	Cd MRLb(mM) b	Soil pH(mM)	Soil total Pb(mM)	Soil soluble Pb
Pseudomonas marginalis	0,3	0,6	7,3	17,2	<0.0004
Bacillus megaterium	0,1	0,2	4,5	108,6	0,6

aMaximum resistance level for soluble lead in mineral salts medium, pH 6.0.
b Maximum resistance level for soluble cadmium in mineral salts medium, pH 6.0.

Discussion

In microbial analysis of the metals, total metal concentration does not always means the toxicity of the metals on microbes. That is why the interpretation of results is very difficult. In this paper we tried to find the link of the soluble lead concentration with the observed microbes and their resistance of lead. The results have shown that toxicity of available lead in conditions when the concentration of lead is increased, will lead to a reduction in the number of colonies of microbes.

In this research, an attempt to relate soluble lead concentrations with the observed microbial lead resistance was made. This research found that available lead, measured as total and soluble, in increased son contrition reduce the microbial growth and community numbers. Table 1 illustrates the differences between habitats for the two characterized bacterial isolates. Notable differences were seen in soil pH with Soil-river Bregalnica being both more acidic with lower manganese and zinc levels but with tree-fold elevated lead concentrations as compared to Soil - river flow of Zletovica.
Soil-river Bregalnica also had a lower culturable cell population than soil taken from river Zletovica. Direct microscopic counts were similar too. The difference between the viable counts and the direct microscopic counts was likely due to the increased metal stress imposed on Soil-river Bregalnica versus Soil - river flow of Zletovica .

Table 2 and 3 summarizes the relationship between the soil/environmental parameters and potential microbial lead resistance. For these soils, soil pH and total and soluble lead concentrations were probably the most influential factors affecting lead bioavailability and toxicity.

Two lead-resistant bacterial isolates chosen for this research were isolated from river Bregalnica and river Zletovica. Identified by BIOLOG, *Pseudomonas marginalis*could tolerate up to 2.5 mM total (0.12 mM soluble) lead in the defined minimal medium, pH 6.2 *Bacillus megaterium*, on the other hand, could tolerate 0.3 mM total (0.09 mM soluble) lead in the same minimal medium. We used a microscope to see the accumulation of lead in both microbes. The result was that *Pseudomonas marginalis* store the lead extracellular and does not accumulate the lead intracellular. *Bacillusmegaterium* accumulate the lead intracellular. In experiments to see the growth of this cells we noticed that these cells were viable in exponential phase of growth.

Atomic absorption spectroscopy in both microorganisms showed accumulation of 50% of bioavailable lead which was present in the medium. *Pseudomonas marginalis* showed extracellular mechanism of exclusion tthrough secretion of egsopolimers. The same egsopolimers were produced by *Bacillus megaterium* with the difference that *Pseudomonas marginalis* produces more egsopolimers. There is no difference in egsopolimers which were produced.

Another mechanism of resistance was noticed. Intracellular accumulation of lead in the cytoplasm of *Bacillusmegaterium*. Extracellular polymer production is not unique and is often accompanied by other metals in polluted nature. Under strong metal stress may be necessary for resistant organisms using specific targeted metal-resistant mechanism, including ATP-dependent pump discharge and intracellular sequestration, which can be more effective in detoxification, increased metal uptake across cell membranes under high concentration of available metals. Toxicity of metals to organisms is dependent on the availability of metals in general, as pH decreases and increases the solubility of metals. The toxicity of metals increases with increasing the availability of them and their mobility across cell membranes. In this paper, *Pseudomonas marginalis* has been able to reduce the toxicity of lead with general production egsopolimers. Alternatively, when is exposure to higher levels of soluble and toxic lead, *Bacillus megaterium* developed a specific mechanism of resistance to lead in the form of intracellular sequensation. As the availability of metals affect the microbial resistance, microbial detoxification / removal of metals have important implications for healing of metal-contaminated environments. The use of microbial populations specifically adapted to a specific level or range of available metals in the environment will lead to increase our capacity for purification of metal-contaminated sites.

CONCLUSION

This study suggested a possible relationship between lead resistance and environmental lead exposure. The lead-resistant *P. marginalis* used exclusion resistance and was isolated from a soil with a neutral pH and undetectable levels of soluble lead. Extracellular polymer production is not unique and is frequently encountered with other metals in polluted natural settings. *B. megaterium*, with a metal-dependent intracellular accumulation of lead, came from an acidic soil with 0.6 mM soluble lead. Under high metal stress, it may become necessary for resistant microorganisms to use specifically directed metal-resistance mechanisms, including ATP-dependent efflux pumps and intracellular sequestration, which may be more effective at detoxi-fying the increased metal penetrating cell membranes under high bioavailable metal conditions. The internal partitioning of the lead in the bacillus may have been the result of the production of a metallothionein-like protein, rendering the lead less reactive. The toxicity of a metal to microorganisms is dependent upon metal bioavailability, and, in general, as pH decreases and the solubility of the metal increases. Metal toxicity increases due to enhanced bioavailability and mobility across cell membranes.

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