CULTURE-DEPENDENT APPROACH FOR DETERMINING MICROBIAL DIVERSITY IN SOILS FROM KCM/AGRIA REGION[1]

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Abstract

Significant shifting of the microbial communities structure was detected in three metal-polluted soils collected around the Pb-Zn smeltery KCM and the agrochemical factory AGRIA situated near the town of Plovdiv, Bulgaria. Industrial-, dumpsite- and agricultural soils were contaminated with As: 7.5-52.9 mg/kg; Hg: 0.086-0.404 mg/kg; Cd: 2.3-71.1 mg/kg; Mn: 742-1510 mg/kg; Pb: 138-2560 mg/kg; Cu: 32-268 mg/kg and Zn: 293-4490 mg/kg. Remarkable ecological disturbance was found in the agricultural soil using ecotoxicological test with type strain *Pseudomonas putida* DSM 50026 (ISO 10712). In order to assess ecologically relevant bacteria over twenty soil bacterial isolates were cultured. Six of them possess tolerance to one or more heavy metals. Four of the isolates demonstrated herbicide tolerance to 2,4-dichlorophenoxyacetic acid. Our results suggest that the microbial community responds to long-term metal- and pesticides contamination through changes in microbial community structure and selection for resistance.

Key words: polluted environments, microbial community structure, tolerant bacteria

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Introduction. Pollution of the environment with heavy metals and organic compounds took a threatening size in the last decades. The harmful effect of heavy metals like Pb, Cd, Mn, Cu, Zn is multiplied in cases when they interact with other pollutants like xenobiotics [1]. Bacteria living in high metal concentrations develop abilities to resist such stress [2–4].

Microorganisms growing in the presence of extreme metal concentrations possess specific mechanisms of resistance as exclusion of metal ions, binding of metal ions in the cell wall or intracellular sequestration in specific binding components as metallothionein. Heavy-metal ions Hg^{2+}, Cd^{2+}, Ag^+, Cu^+, Zn^{2+} and Pb^{2+} form strong toxic complexes in the cell which makes them dangerous for any physiological function [5, 6]. Different molecular and biochemical processes may cause adaptive response in microbial community exposed to heavy metals:

  (i) Induction of specific enzymes in the bacteria of microbial community resulting in an increase of degradative capacity of the total community;

 (ii) Selective growth of a specific subpopulation of a microbial community able to take up and metabolize the pollutant;

(iii) Selection of mutants which possess specific metabolic capacities.

Bacteria play a significant role in migration and detoxication of a large number of heavy metals due to bacterial ability to interact and biotransform them [7–9]. Metal-microbe interactions have an important role in several biotechnological applications including biomineralization, bioremediation, bioleaching and gained growing attention in recent years [10]. This development use indigenious isolates or microbial communities that are capable to mobilize and immobilize metal ions. The information about the diversity and activity of the indigenious microflora in metal-polluted soils is of basic importance for understanding the biogeochemical processes occurring in these environments. The problem of pollution with mixed wastes, especially environments co-contaminated with both metals and herbicides, requires isolation of pollutant-tolerant strains [11].

The aim of the present work was to derive information about the metal pollution in soils around the Pb-Zn smeltery (KCM), Plovdiv, Bulgaria and to assess ecotoxicology effect of the pollutants. In addition to isolate and characterize environmental bacteria and to test them for heavy metal and pesticide tolerance. This is the first profound investigation of the bacterial diversity in the co-contaminated area of the KCM smeltery.

Materials and methods. SITE HISTORY AND SAMPLE COLLECTION. The investigations were carried out in the region with strong industrial activity in South Bulgaria. KCM-S.A. is the biggest Pb-Zn smeltery in Bulgaria and AGRIA is a factory for producing of agrochemicals for plant protection. KCM started in 1961 and now produce 56000 t/y of zinc, 48000 t/y of lead, some precious metals and their alloys. Closely located AGRIA has output sheet containing over 40 herbicides, fungicides and insecticides. Both enterprises have been working for decades and cause co-contamination with heavy metals and pesticides of soils and waters in the area.

Out of 66 soil monitoring points three highly metal-contaminated soil samples were chosen. Soil samples were collected in March 2002 under sterile conditions, transported in ice to the laboratory and kept in refrigerator at $4\,^{\circ}\mathrm{C}$. The analyses started 24 h after collection.

Sample 1 represents soil from industrial area, sample 2 – soil from dump site and sample 3 – soil from an abandoned agricultural field.

METAL-CONTENT ANALYSIS. Metal-content analysis was performed according to Standard Methods for Examination of Soils (EC). The value of the following elements Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, Hg, In, K, Li, Mg, Mo, Na, Ni, Pb, Se, Si, Sn, Sr, Te, Ti, Tl, U, V and Zn was investigated. The analyses were carried out by HR-ICP-AES Jobin Yvon ULTIMA 2, in conformity with ISO 11885, after

dissolution of the solid phase with HNO_3 (also in exchangeable phase after extraction with $MgCl_{2)}$.

MICROBIAL COMMUNITY STRUCTURE ANALYSIS. Soil microbial counts were determined by the plate count method for viable cells. Soil suspensions were prepared by shaking 1 g soil in 9 ml sterile distilled water for 30 min. Serial dilutions from 10^{-1} to 10^{-7} were surface spread on selective agar plates. CFU were counted after 2, 5, and 7 days at 30 and $37\,^{\circ}$C. The analysis includes the following microbial groups: heterotrophic aerobes, heterotrophic anaerobes, spore-forming bacteria, denitrifying bacteria, amonifying bacteria, nitrifying bacteria, Fe(II) oxidizing bacteria, Mn(II) oxidizing bacteria, Fe(III) reducing bacteria, Mn(IV) reducing bacteria, colourless sulphur bacteria, cellulose degradating bacteria, oligocarbophiles, actinomycetes, fungi [12–18]. Additionally the presence of metal-leaching bacteria *Acidithiobacillus thiooxidans, Acidithiobacillus ferrooxidans, Acidithiobacillus denitrificans, Acidithiobacillus thioparus* was investigated. All chemicals used in the analysis were supplied by Merck and Fluka (Germany). Nutrient media were supplied by Difco (USA).

ISOLATION OF NOVEL BACTERIAL CULTURES. Bacterial cultures were selected on the basis of their morphology in nutrient agar and picked up from a single colony at the end-point serial dilution. The isolation of microorganisms was performed using methods described elsewhere [19]. Heterotrophic aerobes (named R) and spore-forming bacteria (named RG) were maintained at $4\,^{\circ}$C and stored in glycerol at $-20\,^{\circ}$C for further investigation.

ECOTOXICOLOGY TEST. The ecotoxicological test was carried out according to the international standard method EU-ISO/DIS 10712.2: *Pseudomonas putida* growth inhibition test for determining the inhibitory effect on *Pseudomonas putida* DSM 50026 (1995) [20]. 1 mL of mid-exponential phase *P. putida* was mixed with 5 mL of water extract of each soil sample and 4 mL minimal medium and further cultivated for 16 h/$22\,^{\circ}$C/180 rpm. In the control instead of contaminated water an equivalent volume of mineral medium was added. Each sample was analysed spectrophotometrically at 450 nm using Specol 11 (Carl Zeiss, Jena). Minimal medium contained for the preculture 1g $NaNO_3$, 0.24 g K_2HPO_4, 0.12 KH_2PO_4, 0.1 g yeast extract, 10 g glucose, 0.4 g $MgSO_4$, 0.0001 g Fe citrat in 1000 mL dH_2O, pH 7.0, and was autoclaved at 0.5 atm for 20 min. For the test culture yeast extract and glucose were eliminated according to ISO protocol.

TOLERANCE TO HEAVY METALS. The novel bacterial cultures were tested for metal tolerance to the most dangerous for the environment and human health metallic cations. Stock solutions of 1 mM $CuSO_4$, 1 mM $ZnSO_4$, 4 mM $Pb(NO_3)_2$, 20 mM $MnSO_4$ and 0.5 mM $CdCl_2$ were prepared in distilled water and sterilized for 20 min at 0.8 atm. Bacterial isolates were grown for 48 h at $30\,^{\circ}$C in the presence of above mentioned metals. Minimal media contained per litre 1 g $NaNO_3$, 0.24 g K_2HPO_4, 0.12 KH_2PO_4, 0.1 g yeast extract, 10g glucose, 0.4 g $MgSO_4$, 0.0001 g Fe citrat.

TOLERANCE TO 2,4-D. The isolated bacterial cultures were also tested for resistance to the chlorophenolic herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) too. 2,4-D ($C_8H_6Cl_2O_3$) is a poorly degradable and third worldwide distributed herbicide and defoliant. Bacteria were grown in mineral medium containing per litre 1 g $NaNO_3$, 0.24 g K_2HPO_4, 0.12 KH_2PO_4, 0.1 g yeast extract, 10g glucose, 0.4 g $MgSO_4$, 0.0001 g Fe citrat, with 0.1 mM and 1 mM 2,4-D added. Bacterial isolates were grown in the presence of 0.1 mM and 1 mM 2,4-D for 48 h at $30\,^{\circ}$C.

Results and discussion. The experimental data shown in Table 1 demonstrate that soils in the KCM area were strongly polluted with Mn, As, Cd, Zn, Cu and Pb. The amount of the remaining elements did not significantly exceed the standards.

The highest metal concentrations were registered in agricultural soil. In this soil the content of Cd, Pb and Zn exceeded in times the maximum permissible limit (MPL). The possible reason for strong contamination of the sample is the dust emissions coming

T a b l e 1

Content of trace elements in soils from KCM-AGRIA (mg/kg)

Sample	As	Hg	Cd	Mn	Pb	Cu	Zn
Industrial soil	19.3	0.310	21.4	1340	990	268	1810
Dumpsite soil	7.5	0.086	2.3	742	138	32	293
Agricultural soil	52.9	0.404	**71.1**	1510	**2560**	256	**4490**

* Bold numerals show the values exceeding maximum permission limit

from the KCM chimney. The analysis of elements bound as exchange cations in soil phases shows that the most mobile and with potentially harmful effect element was Cd. Other elements presented in minor amounts as exchange cations were Cu, Zn and Mn. Above mentioned heavy metals considered to be of concern – As, Mn, Cd, Zn, Cu and Pb could readily include in the food chain and further damage the human health.

- heterotrophic aerobes
- heterotrophic anaerobes
- spore-forming bacteria
- denitrifying bacteria
- amonifying bacteria
- nitrifying bacteria
- Fe(II) oxydizing bacteria
- Mn(II) oxydizing bacteria
- Fe(III) reducing bacteria
- Mn(IV) reducing bacteria
- colourless sulphur bacteria
- sulphur reducing bacteria
- oligocarbophiles
- actinomycetes
- fungi
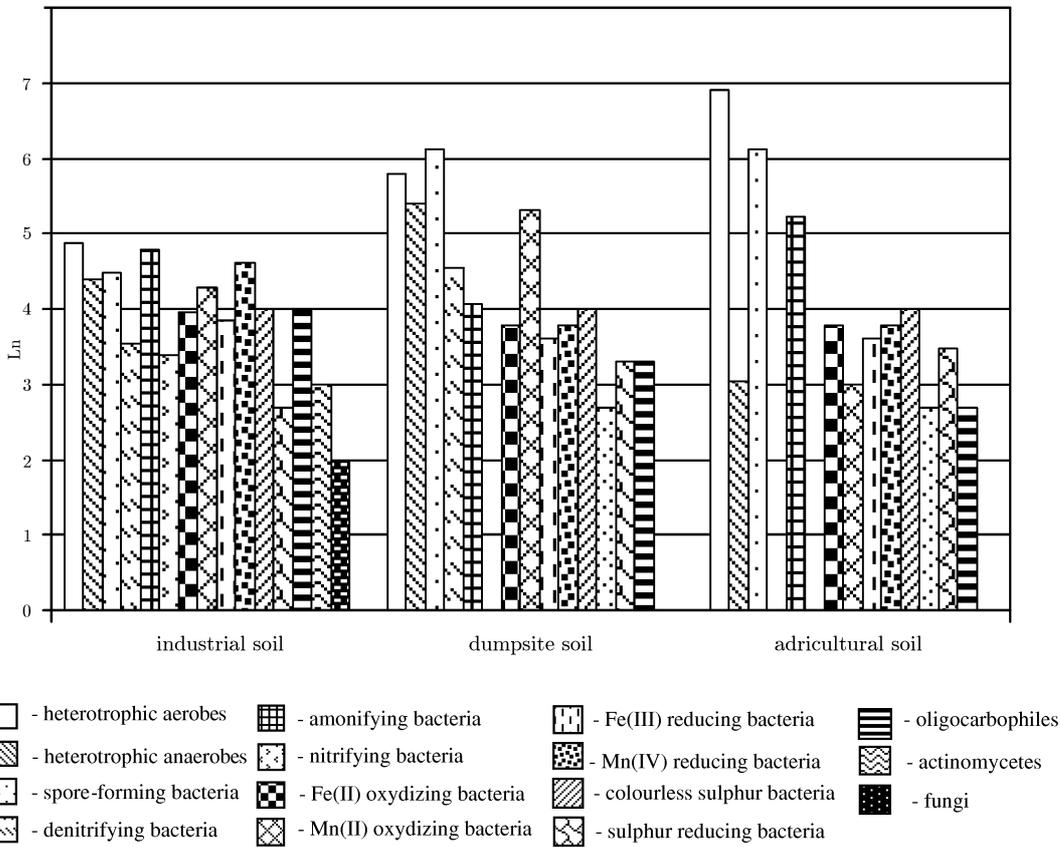
Fig. 1. Quantitative analysis of microbial communities in KCM-AGRIA soils

High metal contaminations in the studied area affected the microbial community living in the polluted area and especially acted some microbial groups. Heterotrophic bacteria which require organic supplement for growth and energy supply may contribute to metal leaching. The heterotrophic microorganisms do not have any benefit from the metal leaching. Among the bacteria members of genus *Bacillus* and genus *Pseudomonas* are the most effective in metal solubilization. The quantitative microbial analyses showed a pronounced prevalence of heterotrophic aerobes in the investigated soils: $10^4 - 10^5$ cells/g for industrial and dumpsite soils and 10^7 cells/g for agricultural soil.

Spore-forming bacteria were $10^5 - 10^6$ cells/g in all three samples (Fig. 1). The high number of the latest bacteria in all investigated soils undoubtedly determined this environment as extreme one.

Denitrifying, amonifying and nitrifying bacteria showed concentrations of $10^3 - 10^5$ cells/g but nitrifying bacteria absented in the dumpsite soil and both nitrifying- and denitrifying bacteria absented in the agricultural soil. As those bacterial groups serve as indicator of metal contamination in the environment, the results correlate with the data from ICP analysis which shows that the agricultural soil is the most polluted one.

Remarkable is the presence of Mn(II)- and Fe(II)- oxidizing bacteria presented in all samples at concentration of $10^3 - 10^5$ cells/g.

Much recent research on dissimilatory metal-reducing microorganisms has focused on their role in bioremediating of contaminated environments. Those microorganisms consume oxygen and develop anoxic conditions [8]. Fe(III) reducing microorganisms were reported to degrade organic pollutants within contaminated aquifers and can convert U(VI) to U(IV). Fe(III)- and Mn(IV) reducing bacteria lessen iron and manganese to their bivalent forms. They were presented with 10^3 cells/g in all soil samples.

Sulphate-reducing bacteria (SRB) which decrease the sulphate to hydrogen sulphide in strictly anaerobic conditions, were found between 10^2 and 10^4 cells/g. SRB enzymatically mediate the reductive precipitation of toxic metals including U(VI), Cr(VI), Tc(VI) and As(V).

Colourless sulphur bacteria, typical aerobic bacteria, showed a concentration of 10^4 cells/g soil.

Cellulose degrading bacteria and oligocarbophiles showed very poor presence of $10 - 10^3$ cells/g.

Actinomycetes were found in the industrial soil with 10^3 cells/g but they were absent in other two samples.

Fungi can remove both soluble and insoluble metal species from solution and are also able to leach metal cations from solid wastes. Many of them can produce organic acids which can also solubilize complex metal cations. Fungi were also detected only in the industrial soil and were not found in dumpsite and agricultural soil.

Actinomycetes fungi also appear as indicator for environmental pollution and their absence demonstrated a change in the microbial community structure in samples 2 and 3 and generally that the ecological balance in these soils is damaged (Fig. 1).

The most active in bioleaching bacteria, belonging to genus *Acidithoibacillus*, were subject of special investigation. These are Gram-negative, non-spore forming rods which grow under aerobic conditions. Most thiobacilli are chemolithoautotrophic species which use carbon dioxide from the atmosphere as their carbon source for synthesis of new cell material. The energy derives from the oxidation of reduced sulphur compounds, including sulphides, elemental sulphur and thiosulphate as final oxidation product – sulphate [7]. *Acidithiobacillus thiooxidans*, *Aciditthiobacillus denitrificans*, *Acidithiobacillus novelus* and *Acidithiobacillus thioparus* were not registered in any

soil sample. This fact is regular because these bacteria prefer the acidic pH while the soil samples from KCM had a neutral pH.

The biorestoration of environments polluted with mixed wastes, especially co-contaminated with metals and pesticides, requires isolation of strains that are both metal-tolerant and have a herbicide degradable capability. A total of 20 novel indigenious soil bacterial cultures were isolated. Results showed that the bacterial cultures R_4, RG_1, RG_2, R_7, RG_5, RG_6 demonstrate tolerance to one or more heavy metals. (see Table 2.)

T a b l e 2

Metal resistance of novel bacterial isolates

Environmental bacteria	Soil sample	Metal tolerance
R_4	1	1 mM $CuSO_4$
		1 mM $ZnSO_4$
		20 mM $MnSO_4$
RG_1	1	20 mM $MnSO_4$
RG_2	1	0.5 mM $CdCl_2$
R_7	3	1 mM $CuSO_4$
RG_5	3	1mM $CuSO_4$
		4 mM $Pb(NO_3)_2$
		20 mM $MnSO_4$
RG_6	3	1 mM $CuSO_4$

All novel bacteria were also screened for herbicide tolerance to 2,4-D. Results shown in Table 4 demonstrated that the bacterial isolates R_1, R_3, RG_3 and RG_5 possess tolerance to 0.1 mM 2,4- D.

Ecotoxicological test showed that all three soil samples inhibited to a different extent the growth of type strain *Pseudomonas putida* DSM 50026. Soil from agricultural soil (sample 3) had the greatest inhibiting effect on the growth of *Pseudomonas putida* – 82%, followed by dumpsite soil (sample 2) and industrial soil (sample 1) with inhibition of 70% and 54%, respectively (see Fig. 2)

Conclusions. The study is providing information about the extent of pollution in soils near the biggest Bulgarian Pb-Zn smeltery KCM. The change in the microbial communities structure in the studied soil samples correlates with the ICP data of metal content. The results demonstrated that the structure of microbial communities was affected in all investigated soils. Six of the novel bacterial isolates: R_4, RG_1, RG_2, R_7, RG_5, RG_6 showed tolerance to one or more heavy metals. Four of the newly discovered bacteria R_1, R_3, RG_3, RG_5 demonstrated slight herbicide tolerance. Our results suggest that the microbial community responds to long-term metal- and pesticides contamination through changes in microbial community structure and selection for tolerance. Further experiments of microbial degradation of organic pollutants together with metal cations are nessessary to clarify the influence of heavy metals on the biorestoration potential.
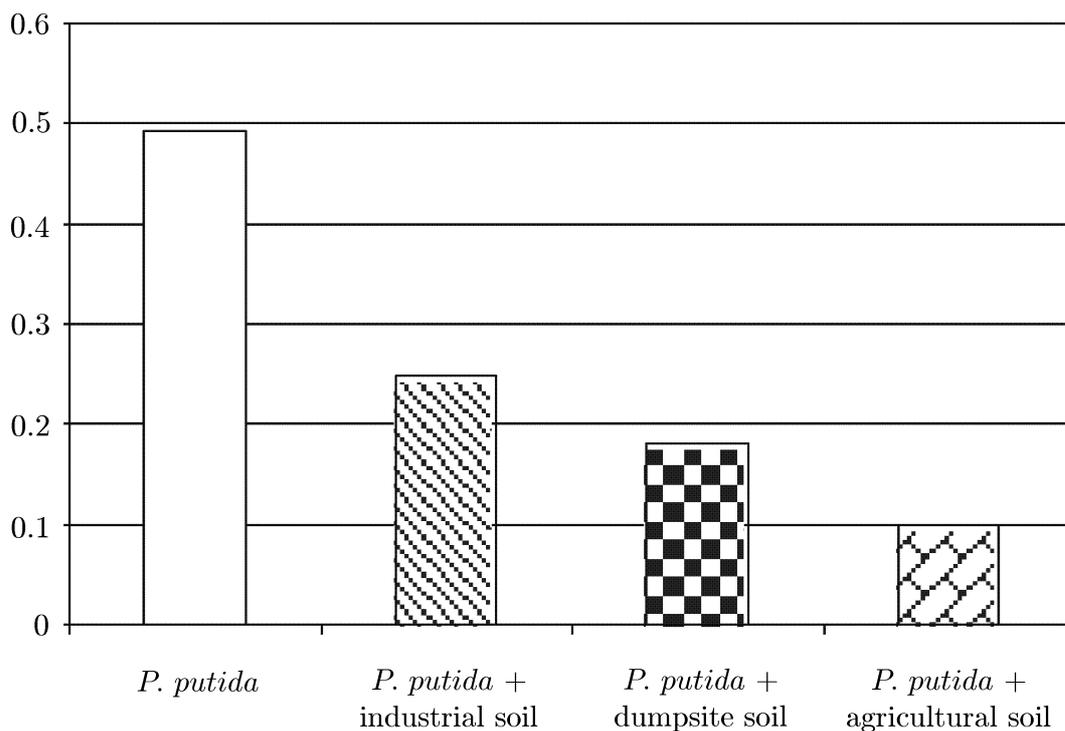
Fig. 2. Ecotoxicological test – ISO/DIS 10712.2

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