”BABEŞ-BOLYAI” UNIVERSITY CLUJ-NAPOCA
FACULTY OF BIOLOGY AND GEOLOGY

INDICATOR MICROORGANISMS OF ENVIRONMENTAL POLUTION

PhD thesis summary

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CONTENT

Keywords: polluted soils, ecophysiological groups, enzymatic activities, *Azotobacter chroococcum, Pseudomonas putida*, heavy metals

I. INTRODUCTION

Soil is a dynamic system that is vital to human activities and to maintaining ecosystems. As an interface between the earth's crust, atmosphere and hydrosphere, the soil is a non-renewable resource that fulfils many vital functions: biomass production, storage, filtering and transformation of organic substances and minerals; source of biodiversity, habitats and species; physical environment for people and human activities; source of raw materials.

In the evolutionary sense, microorganisms (primarily heterotrophic microorganisms) are recycling agents responsible for maintaining the biosphere. These agents develop favorable, thermodynamic chemical reactions obtaining energy and carbon from dead biomass. As a result of microbial processes of decomposition, the essential nutrients present in the biomass of one generation of organisms are available for the next generation.

The impact of human activities on soil quality has increased over the past decades due to population growth and extensive exploitation of natural resources, including soils. The following processes may be indicated as the main sources of impact on soil quality: emissions into the atmosphere mainly from industry and traffic; agricultural techniques - especially the use of organic or mineral fertilizers and pesticides; waste deposits on soils.

The rate of production and dispersion of pollutants exceeded in the present time the natural processes of biodegradation. Developing technological remedies for environmental pollution, physical and chemical processes may be essential, but also the microbiological processes offer important perspectives.

II. SCOPE AND OBJECTIVES

The scope of this research is to argument from a scientific point of view the need to include biological parameters in studies of environmental impact assessment and in the national strategies for soil quality monitoring, practices that currently are based only on the determination of physical and chemical parameters.

Chemical tests measure the amount of pollutants but they do not reflect the environmental consequences resulting from their mobilization, accumulation along the food chain and specially their impact on key metabolic processes in the soil. Biological methods, in turn, reflect the impact on the organisms from the soil, thus showing enhancement / inhibition of the activities under stress conditions.

Given the fact that the soil is subjected to strong anthropogenic influence, it is of major importance to establish the effect of pollutants on edaphic soil communities of microorganisms.

In this context, the main objectives of the thesis were the following:

1) The study of abundance, diversity, dynamics and ecological significance of some bacterial groups (involved in the biogeochemical cycle of nitrogen, sulphur, iron and carbon) of polluted soils in Cluj county, in relation with determinant environmental factors;

2) The evaluation of enzyme activities (catalase, phosphatase, urease, actual and potential dehydrogenases) in order to determine the microbial potential of soils;

3) The determination of bacterial indicator of soil quality (BISQ) and enzymatic indicator of soil quality (EISQ), which allows comparison and ranking of the analyzed samples;

4) Analyzing existent correlations between microbiological and physical-chemical parameters, in order to assess the variation of density in bacterial groups from polluted soils and the intensity of enzymatic activities, depending on the physical and chemical factors;

5) Determining the effect that heavy metals (zinc, lead and cadmium) have on microbial populations, by the study of eco-physiologic group dynamics of bacteria and enzymatic activities in the presence of different concentrations of environmental pollutants and identification of bacterial parameters sensitive to pollution;

6) Determining the effect of heavy metals on cell viability for *Azotobacter chroococcum* and *Pseudomonas putida*, and the resistance of species to heavy metals in the culture medium (trypan blue exclusion test);

7) Determination of the effect of heavy metals on the *Pseudomonas putida* bacterium using the growth inhibition test (measuring the concentration at which cell proliferation is inhibited by 10% and 50%, respectively);

8) Identifying the most suitable biological indicators of soil pollution in order to include them in studies of impact assessment and in the monitoring strategies for contaminated soils, applicable to other ecosystems.

The thesis is structured on 11 chapters. Each chapter contains a theoretical part, a part where the working methods are described, a part where results and discussions on scientific research undertaken and associated conclusions are included.

This thesis makes a contribution of real scientific value on the effect of anthropogenic influences on soil quality, their relationship with microbial activity and enzymatic potential, but also with the physiology and viability of strains of *Azotobacter chroococcum* and *Pseudomonas putida*, all these being new in the specialized literature from the field of microbial ecology.

Microorganisms and microbial communities can be an integrated unit for measuring soil quality, an issue that cannot be obtained by physical or chemical measurements and / or analysis of large organisms. To prevent irreversible ecological consequences, bacterial parameters which proved to be sensitive to pollution with heavy metals may be included in assessment studies and monitoring strategies for contaminated soils.

III. DESCRIPTION OF SAMPLE COLLECTION POINTS

Given the main sources of pollution in Cluj county, representative soils for each type of pollution were taken for studies, in the following eight experimental variants (**Fig. 1**):

1. Polluted soil from Cluj-Napoca urban area, due to heavy traffic (Unirii Square);
2. Industrial polluted soil:
    - non-metallic materials processing industry (SC Casirom SA Turda);
    - metallurgy industry (SC Combinatul de Utilaj Greu SA Cluj);
3. polluted soil due to farming activities from bird farms in Cluj-Napoca (Popeşti) area and suid farms from Bonțida, areas vulnerable to nitrate pollution;
4. polluted soil due industrial waste disposal (hexachlorocyclohexane landfill in Turda - Turda HCH)
5. polluted soil due to household waste uncompliant landfilling (Pata Rât landfill from Cluj-Napoca);
6. unpolluted soil from Cheile Turzii natural protected area.

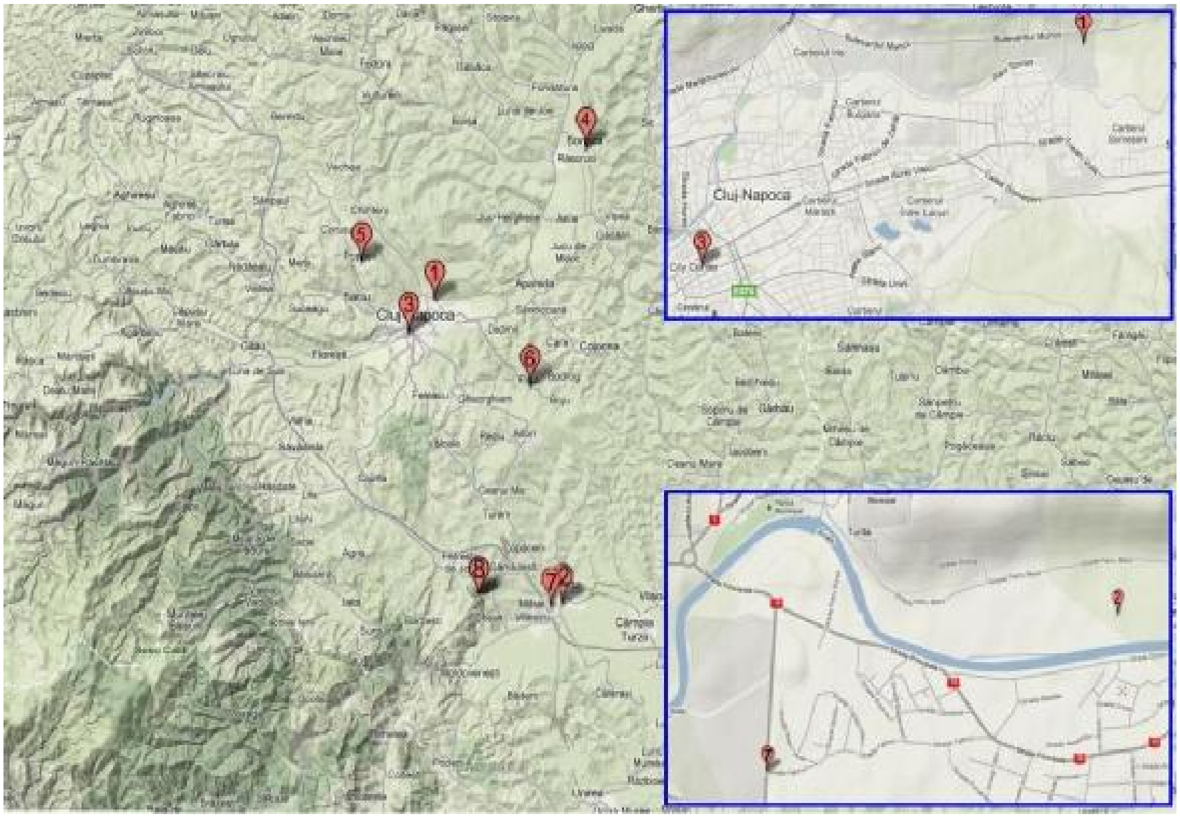

Fig. 1. Geographical placement of studied zone

IV. PHYSICAL AND CHEMICAL ANALYSIS OF SOILS

IV.1. Sample harvesting and preserving

Soil sampling for physical-chemical and biological analysis was done according to STAS 7184/1-84 methodology provided in "Soils. Sampling for soil and agrochemical studies" and were processed in accordance with ISO 10381-6:1997 standards "Soil quality. Guidelines for collecting, handling and conservation of soils for a laboratory study of aerobic microbial processes" and ISO 11464:1998 "Soil quality. Pre-treatment of samples for physical-chemical analysis”.

For the physical-chemical analysis of soils, samples collected under sterile conditions from 0-20 cm depth, in the summer of 2008 were used. Individual samples were collected with a MOLE type soil sampler from three different points and mixed together to obtain a composite sample for each area analyzed. The composite samples obtained were used for all subsequent analysis. Each sample was labeled, the sampling location was specified, date and depth at which sampling was performed. Samples were kept refrigerated at a temperature of 4 ° C until processing.

IV.2. Materials and methods

The physical–chemical parameters determined were: temperature, pH, conductivity, humidity, organic composition, organochlorine pesticides concentrations by gas chromatography-mass spectrometry and heavy metals concentration (Cr, Cu, Pb, Ni, Co, Cd) by atomic absorption spectroscopy.

IV.3.Results and discussions

The pH of the analysed samples has varied between 6.66 (Unirii Square) and 7.95 (Casirom) and the conductivity between 82 μS/cm (Pata Rât) and 322 μS/cm (CUG). Humidity, likewise has varied between 7.33% (CUG) and 20.86% (Casirom). The maximal organic substance composition was recorded in Cheile Turzii sampling point (11.38%) and the minimal in Casirom area (3.51%).

All metals analysed were present in all sampling points, thus proving the existence of a natural pollution background, overlapped by the anthropogenic one. There were recorded exceedings of the maximum permissible concentration (MPC) established by Order no. 756/1997 for the approval of the Requirements for environmental pollution assessment for zinc (CUG, Pata Rât), copper (CUG, Unirii Square, HCH Turda), lead (CUG, Unirii Square, HCH Turda), nickel (CUG, Pata Rât) and cobalt (Popeşti, HCH Turda). There were no exceedances of the normal indicators of chromium and cadmium (**Fig. 2**).

The biggest exceedances of the normal values have been recorded in HCH Turda area for lead (3.2 times), cobalt (3.1 times) and copper (2.9 times) and in Pata Rât area for zinc (1.8 times) and nickel (1.2 times).

High concentrations of heavy metals (Pb, Co and Cu) in the Turda HCH landfill is the consequence of the fact that in this area were deposited waste from Turda Chemical Plant activity, whose main activity was the production of more than 18 chemicals, including: HCH (as a substitute to DDT), potassium carbonate, liquid chlorine, organic polymers, calcium chloride, calcium hypochlorite, cupric oxy-chloride and others.

High levels of heavy metals (especially Zn and Ni) in the Pata Rât municipal waste landfill is the result of storing approximately 3.5 million tonnes of household waste mixed with industrial waste without prior separation into categories of materials and without that they are treated or neutralized.

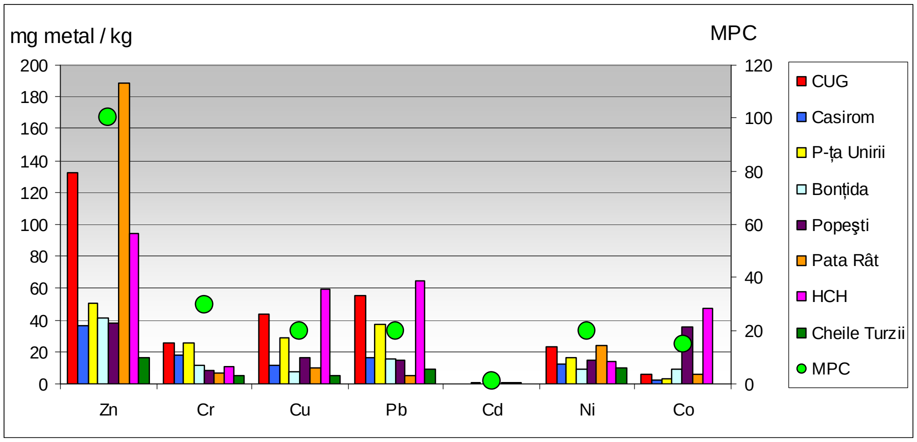

Fig. 2. Heavy metal concentrations determined in polluted soils studied in Cluj county during summer of 2008

Analysis of HCH in the industrial landfill Turda revealed a concentration of 472.9 mg / kg, or about 1.000 times greater than the threshold of intervention provided for sensitive soils by Order no. 756/1997.

High pollutant concentration in soil draws attention on the need for urgent measures for site remediation to be identified and implemented, the environment and human health hazards being real.

V. STUDY OF MICROBIAL POPULATIONS DISTRIBUTION IN POLLUTED SOILS IN CLUJ COUNTY

V.1. Materials and methods

This study performs for the first time a complex bio-monitoring of soil quality on the basis of studies of microbial population dynamics and on the abundance of seven eco-physiological groups of bacteria, which can be sensitive indicators of soil quality: aerobic heterotrophic bacteria (Atlas, 2004), ammonifying bacteria (liquid culture medium containing peptone), nitrifying bacteria (Drăgan-Bularda, 2000), denitrifying bacteria (Pochon, 1954), iron-reducing bacteria (Pârvu *et al.*, 1977) and sulphate reducing bacteria (Allen, 1957).

With the exception of heterotrophic aerobic bacteria (in which case the plate count method was used) in order to determine the most probable number of bacteria (MPN) the decimal dilutions technique was performed and the results were statistically processed using the table of Alexander (1965). To assess the general microbial potential of analyzed soils, based on the number of bacteria from different analyzed eco-physiological groups the bacterial indicator of soil quality was calculated (Muntean, 1995-1996).

The obtained data were statistically analyzed using SPSS Statistics 17 programme, by calculating the Pearson correlation coefficient at two thresholds of significance: 0.05 and 0.01.

V.2. Results and discussions

In all analyzed soils the presence of six eco-physiologic groups of bacteria was detected: aerobic heterotrophic bacteria (AHB), ammonifying bacteria (AB), ammonium oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), denitrifying bacteria (DB), iron-reducing bacteria (IRB), while sulphate reducing bacteria (SRB) were determined only in soil samples from Bonțida, Pata Rât and Cheile Turzii (**Tab. 1**).

Sulphate reducing bacteria were detected in only three sampling points of the eight points analyzed, recording the highest value in the Pata Rat deposit, due to high proportion of biodegradable waste (61%), which suffers anaerobic decomposition inside the landfill.

Tab. 1

Numerical variation limits for the ecophysiological bacterial groups in polluted soils from Cluj county during summer 2008

Bacteria	2008 (minimum - maximum)
Ammonifying bacteria (no./g dry soil)	2358 - 90520
Ammonium oxidizing bacteria (no./g dry soil)	236 - 50450
Nitrite-oxidizing bacteria (no./g dry soil)	26 – 2376
Denitrifying bacteria (no./g dry soil)	36 – 4200
Aerobic heterotrophic bacteria (CFU/g dry soil)	20000 – 9600000
Sulphate reducing bacteria (no./g dry soil)	0 – 42
Iron-reducing bacteria (no./g dry soil)	25 - 940

In order of their abundance, aerobic heterotrophic bacteria (10^4 - 10^6 CFU/g dry soil) were followed by ammonifying bacteria (10^3 - 10^4 /g dry soil), ammonium oxidizing bacteria (10^2 - 10^4 /g dry soil), denitrifying bacteria (10^1 - 10^3 /g dry soil), nitrite-oxidizing bacteria

(10^1 - 10^3 /g dry soil), iron reducing bacteria (10^1 – 10^2 /g dry soil) and sulphate reducing bacteria (0 - 10^1 / g dry soil) (**Tab. 1**).

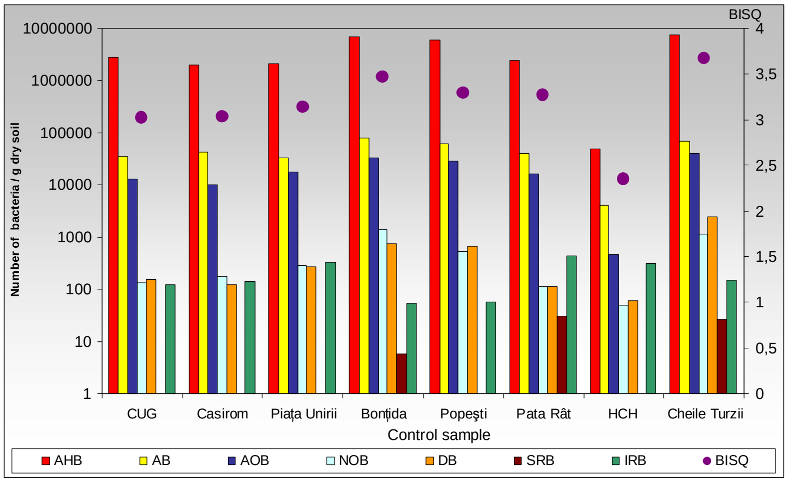

Fig. 3. Bacterial density recorded in the polluted soils studied in Cluj county during 2008

In the case of eco-physiological groups of studied bacteria there were found numerical fluctuations in function of the sampling points and seasonal fluctuations, with minimum values in winter and peaks in the warm seasons of the year (summer and sometimes autumn).

Harmful effect of emissions of pollutants due to road traffic, industrial activities or waste disposal was obvious in all studied eco-physiological groups, the values recorded in polluted areas being lower than in the control, unpolluted area (**Fig. 3**). This effect is even more meaningful pictured with the help of the bacterial indicator for soil quality (BISQ) values, calculated based on the number of bacteria belonging to all eco-physiological analyzed groups (Muntean, 1995-1996).

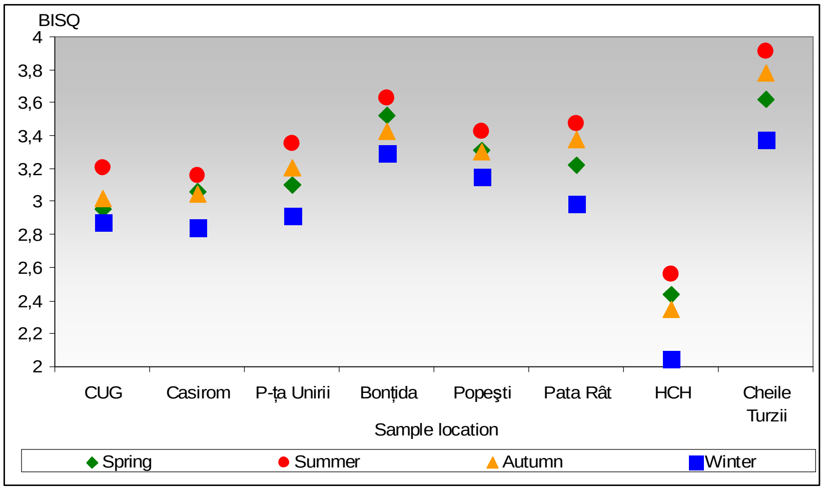

Fig. 4. Seasonal variation of BISQ in the polluted soils from Cluj county during 2008

A first finding which emerges is linked to the difference in potential recorded in the four seasons. In all sampling points, the highest values of BISQ were recorded during summer due to the presence of nutrients and high temperatures and lowest in the winter (**Fig. 4**).

Another obvious finding is the low level of potential bacterial soil in HCH Turda area compared to other sampling points. BISQ values of sampling points CUG, Casirom and Unirii Square were lower than those recorded in Pata Rat, Popesti, Bonțida and Cheile Turzii. The high levels of BISQ in the waste landfill area are probably due to the contribution of organic materials that have supported the development of microorganisms. Also, the high values of BISQ in the sampling points from Bonțida and Popesti are a consequence of the stimulating effect of manure applied to land in the area, plus loss of wastewater resulting from breeding farms, as a result of leakage of collection system purge.

Tab. 2.

Hierarchy of polluted soils studied according to the BISQ values determined in 2008

Position	**Sample location**	**BISQ**
1	Cheile Turzii	3.670
2	Bonțida	3.467
3	Popeşti	3.296
4	Pata Rât	3.266
5	Unirii Square	3.143
6	Casirom	3.028
7	CUG	3.016
8	HCH Turda	2.349

In the hierarchy of polluted soils, based on bacterial indicator values, the soil from the Cheile Turzii protected area zone was the first of the eight positions, suggesting active and balanced bacterial communities. The last position was taken by the soil located near the hexachlorocyclohexane deposit, an area partly covered with plants, because of the high concentrations of pesticides and heavy metals (**Tab. 2**).

CAP.VI. ENZIMOLOGY STUDIES ON POLLUTED SOILS FROM CLUJ COUNTY

VI.1. Materials and methods

In general, enzyme activities in soil change earlier than other parameters, so they are early indicators of soil quality changes (Dick *et al.,* 1996; Lee *et al*., 2002; Hu and Cao, 2007; Garcia-Ruiz *et al*., 2008). As a result, on the same soils that were studied from microbiological and physical-chemical point of view, enzymological investigations have been carried out.

Four enzymatic activities were quantitatively determined: the phosphatase activity (PA), catalase activity (CA), urease activity (UA) and actual (ADA) and potential dehydrogenases activities (PDA) (Drăgan-Bularda, 2000). On the basis of absolute values of each studied enzyme activity, the enzymatic indicator of soil quality was calculated (Muntean *et al*., 1996).

VI.2. Results and discussions

The studied enzymatic activities showed oscillations (**Tab.3**) in relation to season and sampling points.

Tab. 3.

Numerical variation limits of enzymatic activities determined in polluted soils from Cluj county during 2008

Soil enzymatic activity	2008 (minimum-maximum)
Actual dehydrogenase activity (mg formazan/g dry soil)	0.01 – 1.77
Potential dehydrogenase activity (mg formazan/g dry soil)	0.16 – 3.45
Phosphatase activity (mg phenol/g dry soil)	0.19 – 7.95
Catalase activity (mg H_2O_2/g dry soil)	3.31 – 37.82
Urease activity (mg NH_4^+/g dry soil)	2.0 – 28.47

The dehydrogenase activity showed values higher in control soil than in the contaminated soils, which reflects the sensitivity of this activity, regardless of source of pollution (**Fig.5**).

Phosphatase and catalase activities were more intense in the sampling points from Cheile Turzii, Bonțida, Popesti and Pata Rât, compared to areas affected by industrial pollution, traffic or industrial waste disposal, which shows the inhibitory effect of pollutants.

The first group of soils characterized by an increased enzyme activity, had a higher content of organic substances, thus ensure a better development of microorganisms.

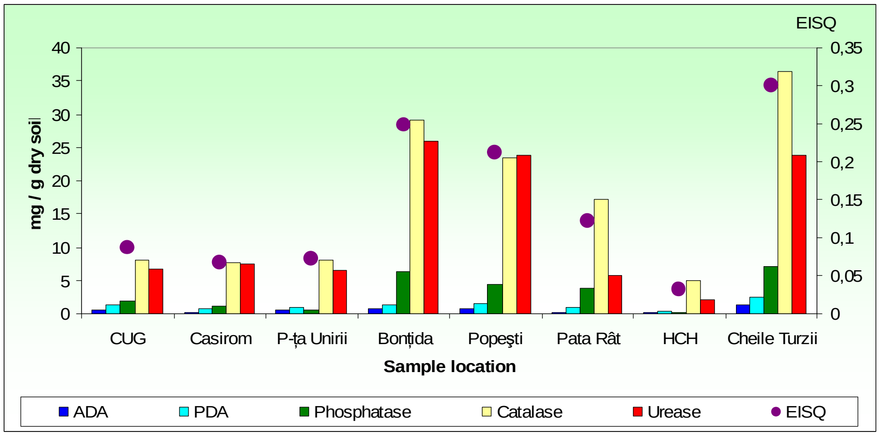

Fig. 5. Intensity of enzymatic activity**:** actual and potential dehydrogenases (mg formazan/g dry soil), phosphatase (mg phenol/g dry soil.), catalase (mg H_2O_2/g dry soil) and urease (mg $NH4^+$/g dry soil) recorded in polluted soils studied in Cluj during 2008

Urease activity was most intense in Popesti and Bonțida areas, where measured values exceeding the ones recorded in the control sample, as a result of the stimulating effect exerted by organic nitrogen compounds present in animal manure.

For comparison of soil samples in terms of enzymology, based on measured values of dehydrogenase, phosphatase, catalase and urease activities, the enzymatic indicator of soil quality was calculated.

As the values of the enzymatic indicator of soil quality reflect, soils were characterized by lower activity in the polluted compared with unpolluted areas. The highest value of EISQ was obtained during summer, for the sampling point Cheile Turzii (0.336) and the lowest value for the HCH Turda, during winter (0.02) (**Fig. 6**).

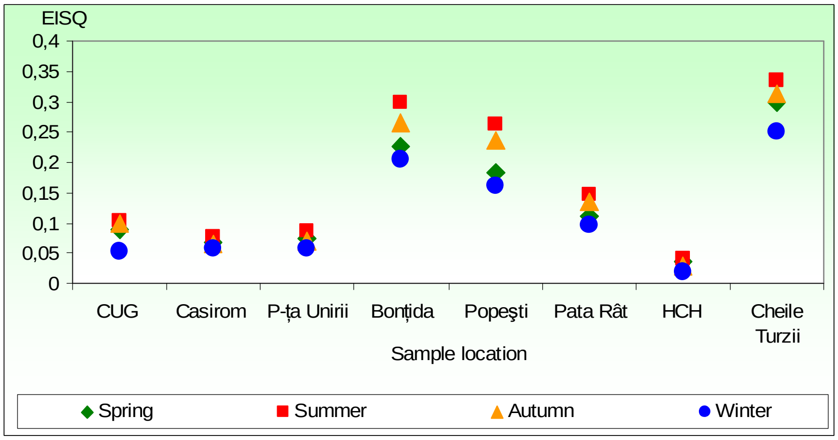

Fig. 6. Seasonal evolution of enzymatic soil potential of polluted soils in Cluj county during 2008

Comparison of the eight sampling points indicates a very low enzymatic potential in the HCH Turda area, a low one in Casirom, Unirii Square, CUG sampling points, an intermediate in Pata Rat and one higher at Popesti, Bonțida and Cheile Turzii, situation explained by the inhibitory effect of chemical pollutants (metals, pesticides) discharged into the atmosphere and stored directly on the ground.

The enzymatic indicator of soil quality (EISQ) does not reached in any one of the 8 samples analyzed, the theoretical maximum value (1), but varies between 0.032 and 0.30. After this indicator, the samples have different quality positions (**Tab. 4**).

Tab. 4.

Polluted soils hierarchy in relation to EISQ values recorded during 2008

Nr.	Sample location	EISQ
1	Cheile Turzii	0.30
2	Bonțida	0.249
3	Popești	0.211
4	Pata Rât	0.122
5	CUG	0.086
6	Unirii Square	0.072
7	Casirom	0.067
8	HCH Turda	0.032

In the HCH landfill area has been recorded the lowest enzymatic potential, but still even in this case with a seasonal variation with a winter minimum and summer maximum, which proves that the HCH effect depends on temperature.

Using statistical tests it was established that the soil is a very heterogeneous system, where the enzymatic activities interact to achieve complex biochemical transformations.

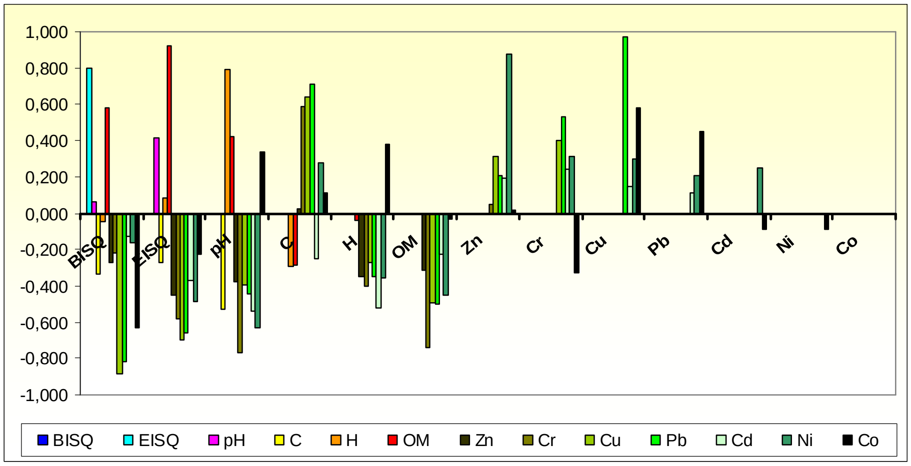

Fig. 7. Correlations between BISQ, EISQ and the physical-chemical proprieties of studied polluted soils

Between values of BISQ and of EISQ a positive correlation has been established, significant at $p < 0.05$, which reflects the parallelism between bacterial and enzymatic potential of the soils, regardless of the sampling point or the concentration of pollutants (**Fig. 7**).

The two indicators that characterize soils (BISQ and EISQ), but also the number of bacteria, respectively each enzymatic activity in part, were negatively correlated with all analyzed heavy metals (zinc, chromium, copper, lead, cadmium, nickel and cobalt), which shows strong inhibitory effect of metals on the size and activity of bacterial populations.

All enzymatic analyzed activities, the density of bacteria in each eco-physiological group, respectively BISQ and EISQ, were positively correlated with the amount of organic substances, a result that reinforces the key role played by the concentration of organic matter in edaphic ecosystems.

CAP.VII.THE IMPACT OF POLLUTION WITH ZINC, LEAD AND CADMIUM ON THE SIZE AND ACTIVITY OF MICROBIAL SOIL POPULATIONS

VII.1. Materials and methods

To assess the impact of heavy metals on microbial populations, on soil samples taken from the protected area Cheile Turzii were added salts of heavy metals (Zn, Pb and Cd), in different concentrations. The following salts of heavy metals were used in this study: $ZnSO_4 \times 7H_2O$, $(CH_3COO)_2Pb \times 3H_2O$ and $Cd(NO_3)_2 \times 4H_2O$.

Heavy metal concentrations were chosen according to their concentration in soils in Cluj County, determined in the physical-chemical analysis. Thus, a lower value than the one measured in the soil from Cheile Turzii was elected, an amount approximately equal to that measured in this soil, the maximum permitted by applicable law in force (Order no. 756/1997), an amount approximately equal to the value recorded in the soils of Cluj county and two higher values. The numbering system from I to VI for the metal concentrations tested, as detailed in **Tab. 5** is used throughout the paper to simplify the presentation of results.

In Romania, the normal concentrations limits of pollutants in soil were established by Order No. 756/1997, these being 100 mg Zn/kg, 20 mg Pb/kg and 1 mg Cd/kg.

Tab. 5

Heavy metal concentrations (Zn, Pb and Cd) used in evaluation of the impact on microbial soil population

Used concentration	**Zinc (mg/kg)**	**Lead (mg/kg)**	**Cadmium (mg/kg)**
I	10	5	0,5
II	20	10	**1**
III	**100**	**20**	5
IV	150	40	10
V	200	70	15
VI	400	140	20

The samples were kept under natural conditions for 30 days, after which was evaluated the effect of heavy metals applied on the aerobic heterotrophic bacteria, ammonifying bacteria, nitrifying bacteria, denitrifying bacteria, iron-reducing bacteria and sulphate reducing bacteria; on dehydrogenase, phosphatase, catalase and urease activities; and on soil respiration.

Overall physiological activity of microorganisms in the analyzed soil samples, expressed through soil respiration level, was determined by assessing the amount of CO_2 emitted by the total spectrum of microorganisms in the sample, using a system consisting of a room from soil respiration type SRC-1, coupled with an CO_2 analyzer tip EGM -4.

VII.2.Results and discussions

VIII.2.1. Impact of pollution with zinc, lead and cadmium on the size of microbial population from the soil

In the case of eco-physiological groups of bacteria studied, there were highlighted numerical fluctuations depending on the type and concentration of metal in which was added (**Fig. 8-10**).

The obtained results showed that the addition of pollutants in soil, different groups of eco-physiological microorganisms had different types of response. Adding Zn, Pb and Cd in low concentration had a slightly stimulatory or inhibitory effect, while application of high concentrations of heavy metals had a strong inhibitory effect. Aerobic heterotrophic bacteria were most resistant to pollution, maintaining themselves even in the presence of elevated concentrations of metals (Zn IV, Pb VI, CdVI) and nitrifying bacteria were most susceptible, followed by denitrifying bacteria and iron-reducing bacteria. Due to the sensitivity shown by nitrifying bacteria to heavy metal pollution, we recommend including this group of bacteria in assessment and monitoring of polluted soils studies.

Aerobic heterotrophic bacteria, ammonifying bacteria, nitrifying bacteria and denitrifying bacteria were more sensitive to Cd than for Pb, while sulphate reducing bacteria and iron-reducing bacteria showed a higher sensitivity for Pb.

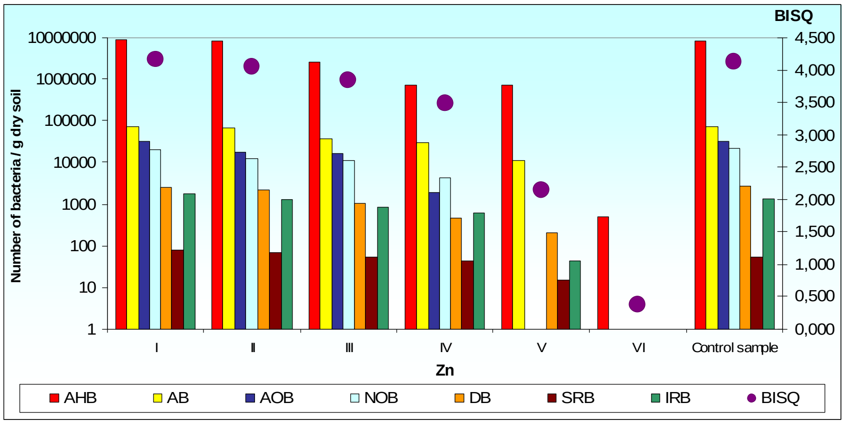

Fig. 8. Zn effect on bacteria number from samples collected from Cheile Turzii during summer of 2010
Metal concentrations: see **Tab. 5**.

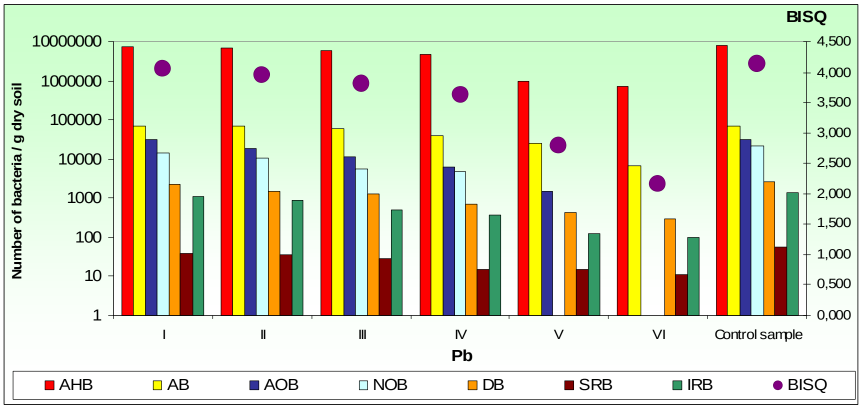

Fig. 9. Pb effect on bacteria number from samples collected from Cheile Turzii during summer of 2010
Metal concentrations: see **Tab. 5**.

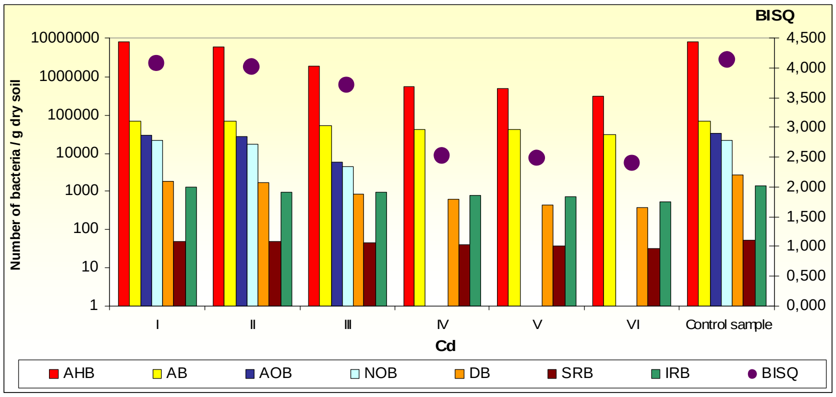

Fig. 10. Cd effect on bacteria number from samples collected from Cheile Turzii during summer of 2010
Metal concentrations: see **Tab. 5**.

The harmful effect of metals was obvious, in all eco-physiological studied groups studied values recorded in samples with high concentrations of Zn, Pb and Cd were lower than in samples with low concentrations and maximum values were recorded in the control sample, without added metals. This effect is even more meaningfully pictured by the values of the bacterial indicator of soil quality (BISQ), calculated based on the number of bacteria belonging to all analyzed eco-physiological groups (Muntean, 1995-1996) (**Fig. 11**).

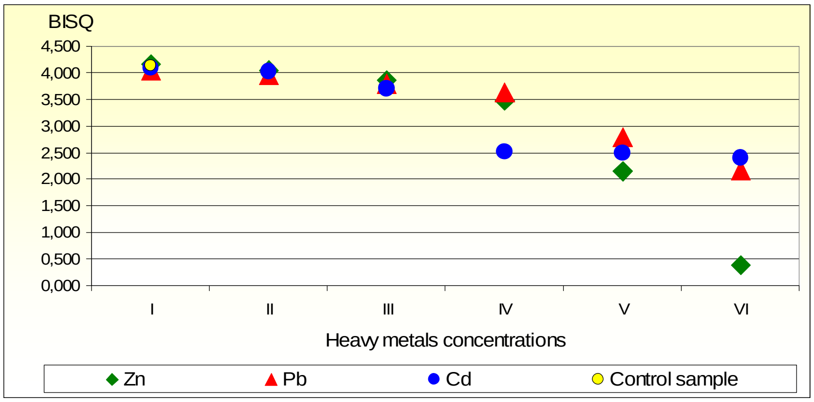

Fig. 11. BISQ evolution under the action of heavy metals (Zn, Pb, Cd)
Metal concentrations: see **Tab. 5**.

The values for BISQ are comprised between 4.127 and 0.386, with a gradual downward trend, according to the growing concentration of metal in the soil. The maximum value (4.127) was calculated for the control sample without the addition of metals and the minimum values in samples Cd VI (2.401), Pb VI (2.167) and Cd VI (0.385), respectively.

VII.2.2.The impact of pollution with zinc, lead and cadmium on enzymatic activities

All analyzed enzymatic activities were identified in all samples, with variations depending on the type of metal and its concentration.

The analyzed enzymatic activities were sensitive to pollution with Zn, Pb and Cd, aspect reflected by the analysis of EISQ evolution. However, dehydrogenase was more sensitive to pollution with heavy metals, which recommends its inclusion in soil quality assessments, along with nitrifying bacteria (**Fig. 12-14**).

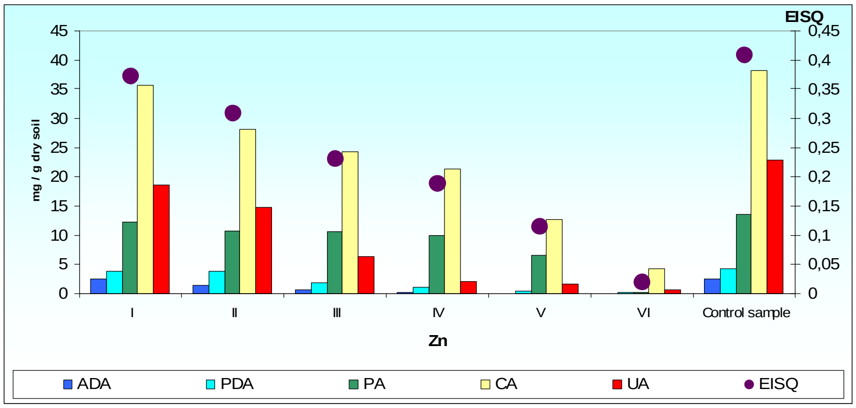

Fig. 12. Zn effect on the intensity of enzymatic activities in samples collected from Cheile Turzii during summer of 2010. Metal concentration: see **Tab. 5**.

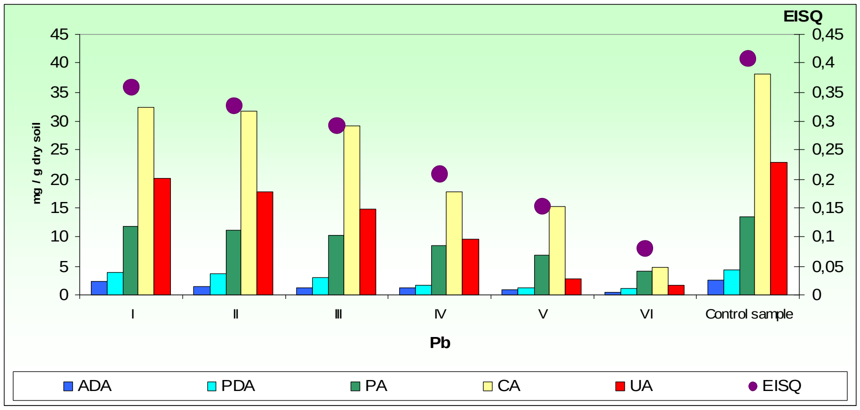

Fig. 13. Pb effect on the intensity of enzymatic activities in samples collected from Cheile Turzii during summer of 2010. Metal concentration: see **Tab. 5**.

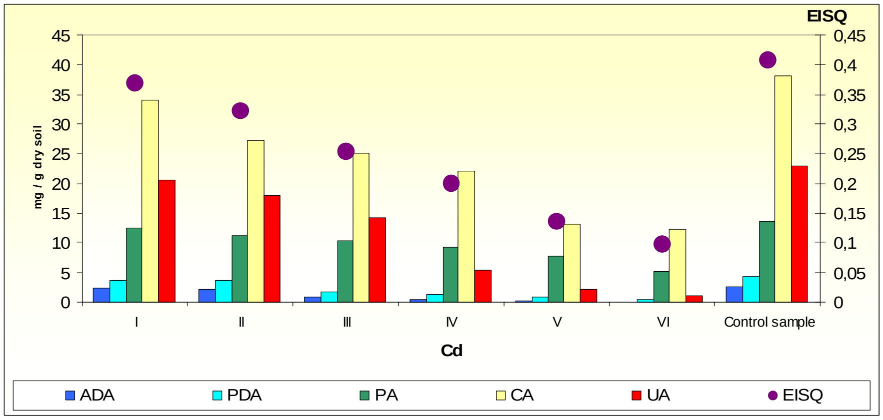

Fig. 14. Cd effect on the intensity of enzymatic activities in samples collected from Cheile Turzii during summer of 2010. Metal concentration: see **Tab. 5**.

The increased sensitivity of dehydrogenase activity to metal contamination can be explained by the fact that the dehydrogenase is active only within living cells, intact, unlike other enzymes that act outside the cell. Dehydrogenase activity was most sensitive to pollution with Cd, followed by Pb and Zn.

The phosphatase and catalase were the most tolerant, phosphatase being more sensitive than catalase in the presence of Zn and Pb and tolerant in the presence of Cd.

There was a gradual reduction of EISQ recorded, during the growing concentration of heavy metals added to the soil samples, due to the inhibitory effect of pollutants on all enzyme activities (**Fig. 15**).

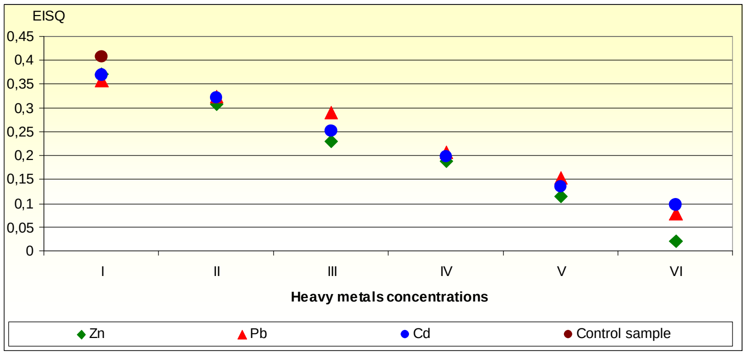

Fig. 15. EISQ evolution under the action of metals added in different concentrations. Metal concentration: see **Tab. 5**.

The enzymatic indicator of soil quality (EISQ) does not reach in any of the samples the maximum theoretic value (1), but varies between 0.407 and 0.02. The maximum value (0.407) was recorded in the control sample and the minimum in the Cd VI (0.097), Pb VI (0.079) and Zn VI (0.02) samples.

VII.2.3.The impact of pollutions with zinc, lead and cadmium on soil respirations

Soil respiration was higher in less polluted soils than in the polluted ones. However, soil respiration seems unaffected by the heavy metals present in the normal range established by Order 756/1997, the amount of CO_2 significantly decreasing only at high concentrations of heavy metals (**Fig. 16**).

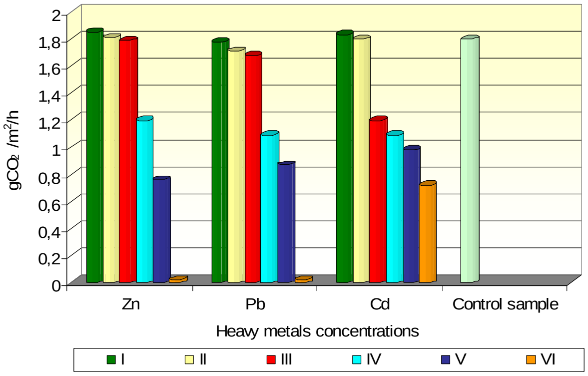

Fig. 16. The effect of different heavy metal concentrations (Zn, Pb, Cd) on soil respiration. Metal concentration: see **Tab. 5**.

Therefore, soil respiration may be a useful indicator in studies of assessment and monitoring of heavy metals impact on microorganisms, but preferably in conjunction with other indicators, because it can be stimulated in case of pollution, while other parameters are inhibited.

Statistical analysis of data showed the existence of negative correlations, statistically significant, between all eco-physiological groups of bacteria, enzymatic activities, soil respiration and concentration of heavy metals (Zn, Pb and Cd). Positive correlations, statistically significant, were detected between the size of bacterial populations and enzyme activities, respectively soil respiration.

VIII. HEAVY METALS EFFECTS ON CELL VIABILITY

VIII.1.Materials and methods

Cell viability was measured by trypan blue test, an *in vivo* method, known as dye method exclusion, which is based on the fact that the cell membrane is impermeable for some dyes, including trypan blue.

The effect of the heavy metals (Zn, Pb, Cd) on cell viability of *Azotobacter chroococcum* isolated strains from the Cheile Turzii protected area and on *Pseudomonas putida*, MIGULA strain, Berlin 33 / 2 (DSM 291) from the culture collection of Babes-Bolyai University, Cluj-Napoca, Laboratory of Microbiology.

VIII.2.Results and discussions

VIII.2.1.Establishing the effects of heavy metals on the viability of *Azotobacter chroococcum* strains isolated from Cheile Turzii soil

Analyzing the recorded data, it can be observed a maximum cell viability of 88.3% in the control sample. Cell viability had a decreasing trend in the presence of heavy metals, varying from 84.9% to 2.4% for Zn, between 78.6% and 12.9% for Pb and between 53.2% and 10.4% in samples with added Cd, which shows the inhibitory effect of three heavy metals even in low concentrations (**Fig.17**).

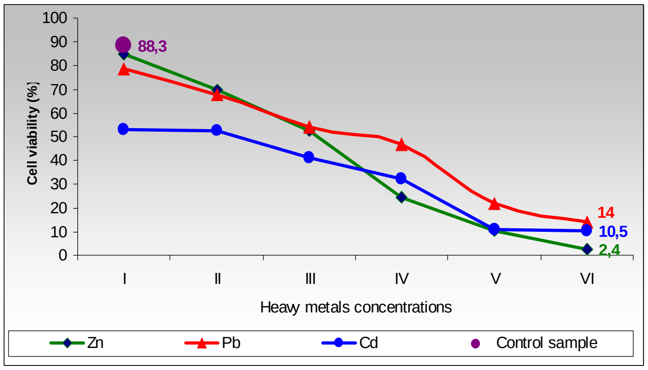

Fig. 17. The effect of different heavy metal concentrations (Zn, Pb, Cd) on cell viability of *A. chroococcum*. Metal concentration: see **Tab. 5**.

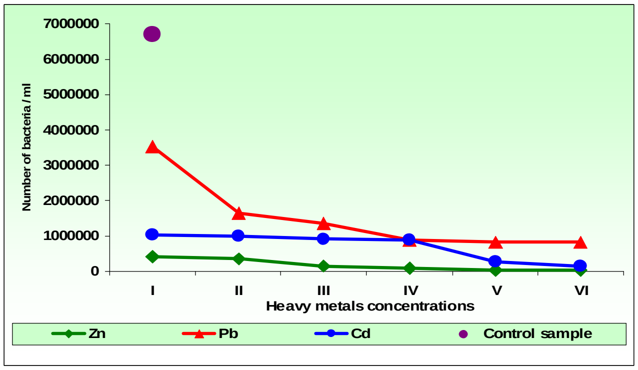

Fig. 18. The effect of different heavy metal concentrations (Zn, Pb, Cd) on cell viability density of *A. chroococcum*. Metal concentration: see **Tab. 5**.

The highest density of cells was recorded in the samples with Pb I (3.52 x 10^6/ml), Pb II (1.65 x 10^6/ml), Pb III (1.35 x 10^6/ml) and Cd I (1.03 x 10^6/ml), the order of magnitude of the number of viable cells gradually reducing to 10^5 in samples Zn I-III, Pb IV-VI and Cd II-VI and respectively to 10^4 in samples with Zn IV-VI. Maintenance of viable cells at relatively high level (10^{6}-10^4) in all samples proves the development of resistance mechanisms to metals (**Fig. 18**).

VIII.2.2. Establishing the effect of heavy metals on cell viability of *Pseudomonas putida*

Cell viability showed varying percentages depending on the type of metal and metal concentration applied. As with *A. chroococcum* cells, cell viability of *P. putida* decreased with the increase in concentration of heavy metals (Zn, Pb, Cd), from 90.5% to 25.5% in the presence of Zn, from 85.8% to 32.4% for Pb and respectively 87% from 43.4% in samples with added Cd. As expected, the maximum cell viability was recorded in the control sample, with no added heavy metals (96.7%) (**Fig. 19**).

Although heavy metals had an inhibitory effect, it is found that the percentage of cell viability was maintained at a relatively high level (25.5% - 43.4%), even in the highest concentrations of metals (Zn VI, Pb VI, Cd VI).

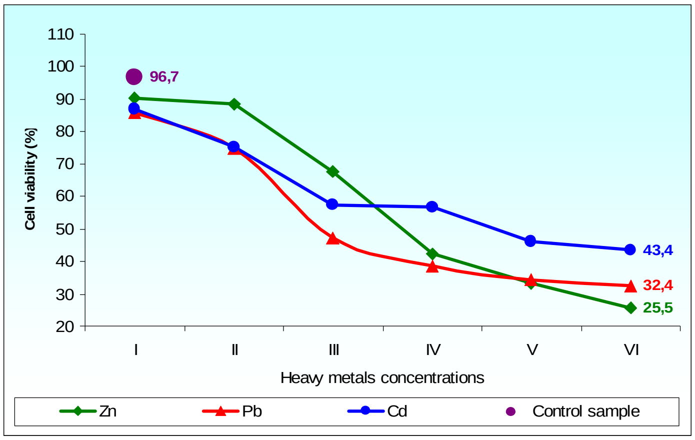

Fig. 19. The effect of different heavy metal concentrations (Zn, Pb, Cd) on cell viability of *Pseudomonas putida*. Metal concentration: see **Tab. 5**.

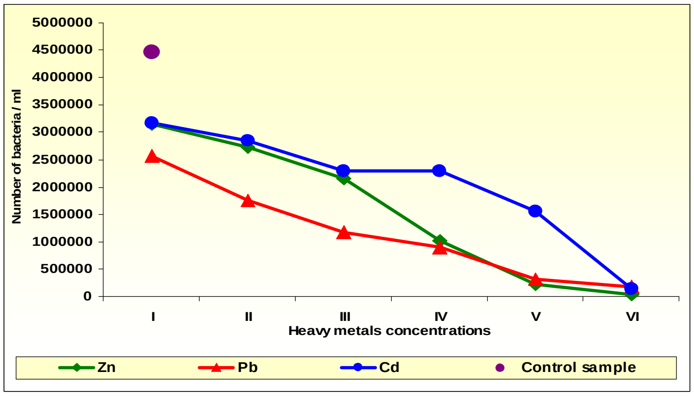

Fig. 20. The effect of different heavy metal concentrations (Zn, Pb, Cd) on cell viability density of *P. putida*. Metal concentration: see **Tab. 5**.

Assessment of cell viability by trypan blue has revealed the presence of a maximum number of viable cells in the control sample (4.47 x10^{6}/ml), this number being reduced with increasing concentrations of heavy metals, up to 1.7 x 10^{5}/ml (Pb VI), 1.42 x 10^{5}/ml (Cd VI) and 3.22 x 10^{4}/ml (Zn VI). From the analysis of these results it can be noticed that the number of bacteria remained at a level of 10^{6} / ml in samples I-IV Zn, Pb I-III and Cd I I-III-V, gradually decreasing to 10^{5} in samples Zn V, Pb IV-VI and Cd VI, and respectively at 10^{4} in the samples with Zn VI. Maintenance of viable cells at relatively high level (10^{6}-10^{4}) in all samples shows the development of mechanisms of resistance to all three metals analyzed, allowing survival under stressful conditions (**Fig. 20**).

VII.2.2.Comparison of the effect produced by heavy metals (Zn, Pb, Cd) on *Azotobacter chroococcum* and *Pseudomonas putida* cells

Cell viability showed varying percentages depending on the microorganism, the type of metal and metal concentration. Cell viability had a downward trend in both microorganisms as a result of the inhibitor effect of heavy metals present in the culture medium. Cell viability was higher in *Pseudomonas putida* than in *Azotobacter chroococcum*, which demonstrates a greater resistance of *Pseudomonas putida* species to heavy metal pollution (**Fig. 21**).

Comparing the effect of the three metals at equal concentrations it is showed that the most toxic was cadmium, followed by lead and zinc, both for *A. chroococcum* and *P. putida*.

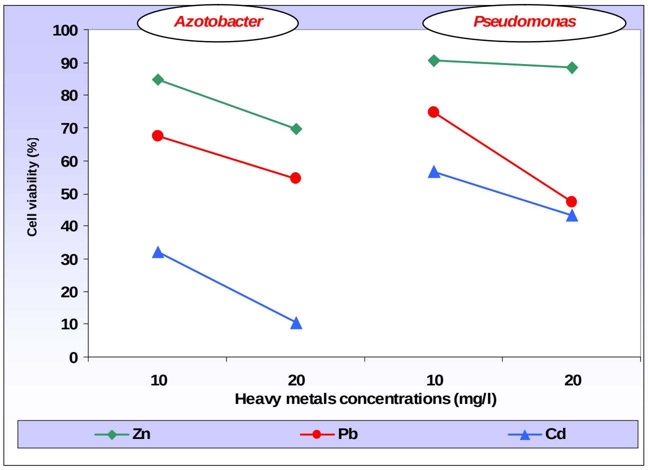

Fig. 21. Heavy metal effect (Zn, Pb, Cd) in even concentrations (10 mg/kg, 20 mg/kg) on cell viability of *A. chroococcum* and *P. Putida.* Metal concentration: see **Tab. 5**.

Reported to the control sample, there was a decrease in the number of viable cells and an increase in the number of non-viable cells, as the concentration of heavy metals in the culture medium was increased, due to exerted toxicity of pollutants on microorganisms.

The average diameter of *A. chroococcum* cells ranged from 1.5 to 2.2 µm and the *P. putida* cells between 0.5 - 1 µm. In response to heavy metal toxicity, increased heavy metal concentration in the culture medium was accompanied by a reduction in the diameter of viable cells, more evident in the strains of *A. chroococcum*, due to its ability to form cysts in unfavorable environmental conditions.

Comparative dynamics show that both species of microorganisms have retained viability in the presence of all metals at all concentrations without falling below the level of 10^4 cells/ ml (**Fig. 18, 20**). This demonstrates that bacteria have developed resistance mechanisms to all three heavy metals. Strains of *Azotobacter sp*. have the ability to form cysts, which make it possible to survive in adverse conditions (Malcolm, 1994) and *Pseudomonas sp*. has a versatile metabolism that can cope with fluctuations in environmental conditions (Berlanga *et al*., 2006).

IX. ASSESMENT OF HEAVY METAL EFFECTS ON THE GROWTH OF *PSEUDOMONAS PUTIDA*

IX.1.Materials and methods

The growth inhibition test of *Pseudomonas putida* was done in accordance with SR EN ISO 10712/2001. Water quality. Growth inhibition test for *Pseudomonas putida*. The method is based on the determination of concentration of samples in which cell proliferation is inhibited by 10% and 50% in 16 ± 1 h.

IX.2.Results and discussions

For all metals analyzed, a gradual increase in the percentage of inhibition growth of *P. putida* was recorded, along with the increase in metal concentrations (**Fig. 22-24**). Effective concentrations that caused growth inhibition of *P. putida* bacteria in percentage of 10% (EC10), after 16 hours of incubation, were 18.663 mg Zn / l; 8.842 mg Pb / l and 0.417 mg Cd / l. The growth inhibition of *P. putida* strain with 50% (EC50) was recorded at concentrations of 129.344 mg Zn / l; 31.914 mg Pb / l and 2.513 mg respectively Cd / l (**Tab. 6**).

In order of toxicity, cadmium was the most toxic metal for *P. putida*, followed by lead and zinc. Zinc was the least toxic, this element being involved in a wide variety of cellular processes.

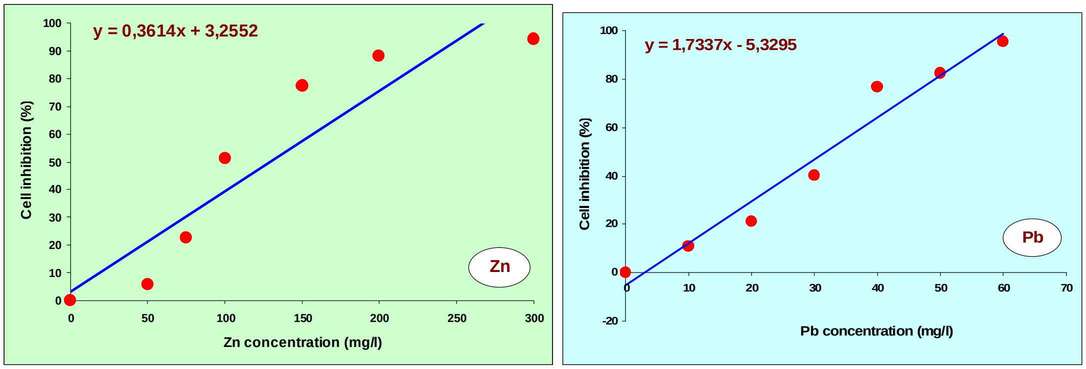

Fig. 22, 23. The effect of Zn and Pb on growth of *P. putida*

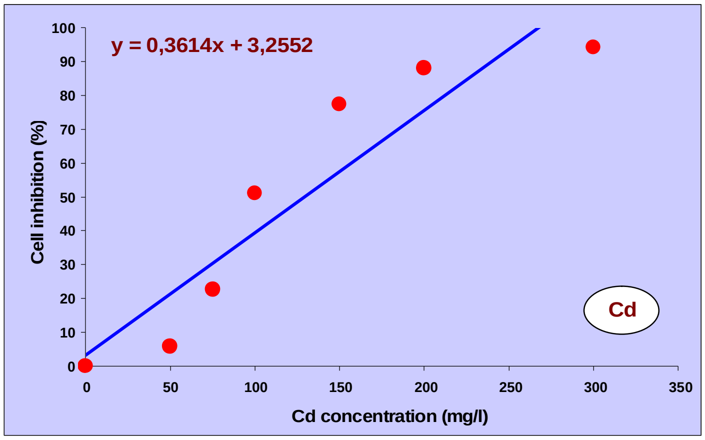

Fig. 24. The effect of Cd on growth of *P. putida*

Tab.6.

Effective concentration values that determine growth inhibition of *P. putida* with 10% and 50%

Effective concentrations	Zn concentration (mg/l)	Pb concentration (mg/l)	Cd concentration (mg/l)
EC 10	18.663	8.842	0.417
EC 50	129.344	31.914	2.513
Maximum allowed value (Order 756/1997)	**100**	**20**	**1**

For all three metals analyzed the effective concentrations that caused growth inhibition of *P. putida* with 50% (EC50) exceeded the normal values provided by Order 756/1997 to approve the Guidelines on environmental pollution assessment. EC50 was 1.3 times higher than the normal value limit for zinc (100 mg / l), 1.6 times higher than the normal limit for lead (20 mg / l) and 2.5 times higher than normal limit for cadmium (1 mg / l).

Statistical analysis of data revealed a significant positive correlation at $p < 0.01$ between the percentage of growth inhibition of *P. putida* strain and zinc concentration (r = 0.927), lead (r = 0.982) and cadmium respectively (r = + 0.985) in the culture medium, this result confirming the negative effect of metals on the multiplication of bacteria.

X. GENERAL CONCLUSIONS

A. Microbiological assessment of polluted soils in Cluj county

- The number of bacteria and the intensity of enzyme activities showed oscillations depending on season and sampling point of sample.
- In order of abundance, heterotrophic aerobic bacteria were followed by ammonifying bacteria, ammonium oxidizing bacteria, denitrifying bacteria, nitrite-oxidizing bacteria, iron-reducing bacteria and sulphate reducing bacteria.
- The number of bacteria and the intensity of enzymatic activities in polluted areas are significantly lower than in the control area. However, the presence of bacteria in polluted soils show natural development of tolerance in the presence of metals.
- Negative correlations were established, statistically significant between metal concentration, on the one hand, and the number of bacteria, respective the intensity of enzymatic activities on the other hand
- In the hierarchy of polluted soils, based on bacterial indicators values, the soil from the Cheile Turzii protected area was the first of the eight positions, suggesting balanced and active bacterial communities while in the last position was the soil from the hexachlorocyclohexane landfill area because high concentrations of pesticides and heavy metals, an area partly covered with vegetation.

B. Assessment of impact of pollution with Zn, Pb and Cd on soil microbial populations

- Bacterial density and intensity of enzymatic activities showed quantitative variations depending on the type and concentration of metal.
- The most sensitive to pollution were found to be nitrifying bacteria and dehydrogenase activities.
- The presence of elevated concentrations of metals had a strong inhibitory effect on resistant species and caused the death of those susceptible to pollution. These toxic effects show that resistance mechanisms do not provide protection from high levels of metals.

C. Effects of heavy metals on cell viability of *A. chroococcum* and *P. putida*

- Cell viability, quantified by trypan blue test, varied depending on the microorganism, the type of metal and metal concentration applied.
- Cell viability has not reached 100% in any of the samples, evolution being downward, while growing concentration of metals, this being the result of the effect of heavy metals present in the culture medium. Cell viability was higher for *P. putida* than for *A.*

chroococcum; Azotobacter can thus be considered an indicator of pollution levels more sensitive, inclusive to low concentrations of metals.

- Comparative dynamics showed that both species of microorganisms have retained viability in the presence of all metals, at all concentrations, without descending below 10^4 cells/ ml. This is a consequence of the development of mechanisms of resistance to all metals analyzed, allowing the survival of microorganisms in polluted environments.

D. Heavy metal effects on *P. putida* growth

- The percentage of growth inhibition had an upward trend, with the increase in metal concentrations.
- In order of toxicity, depending on the concentrations of metals which determined *P. putida* growth inhibition in proportion of 10% (EC10) and respectively 50% (EC50), after 16 hours of incubation, cadmium was the most toxic metal followed, by lead and zinc
- For all three metals analyzed EC50 was recorded at concentrations higher than the normal values provided by the Order 756/1997, which indicates that *P. putida* responded to excess of Zn^{2+} or presence of Cd^{2+} and Pb^{2+} through resistance mechanisms.
- In conclusion, because of their many capacities, the use of microorganisms in the assessment and monitoring programs are necessary, changes in the micro-flora of a specific site indicating changes in environmental quality.
- To capture as closely as possible changes caused by human impact several indicators must be used (measurements of microbial biomass, respiration, key microorganisms, enzyme activities, etc.).
- Enzyme activities in the soil change earlier than other parameters. Therefore, the determination of enzyme activities are more appropriate, thus providing suggestive data in a much shorter time than microbiological analysis on biodegradable processes in soil
- Having regard the crucial role of soil, which is actually an essential active mediator of performing processes that is at the basis of life on earth, is necessary and extremely important to biomonitoring in a complex way soil quality in order to identify and remove the sources of pollution and thus maintain a maximum ecological potential. A precise bio-monitoring is essential in anticipating risks for the environment and human health.
- However, deep future studies are needed for understanding of the genetic diversity of microbial populations sensitive and tolerant to metal and metal-microorganism interactions in soil in natural conditions.

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