SOIL QUALITY ASSESSMENT BASED ON CHEMICAL, ENZYMATIC AND BACTERIOLOGICAL ANALYSIS

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ABSTRACT
This study highlights the problem of soil pollution as the result of human activities. Soil pollutants may be either chemicals or biological in nature. Microbial enzymatic activities are often proposed as indicators of environmental stress. The soil samples were submitted by chemical, microbiological and enzymatic analyses. Chemical analyses were made for determining the heavy metals. Heavy metals from the forest soil were represented by Cu, Zn, Mn, Ni, Pb, Cd and Cr. To evaluate the concentration in heavy metals from the filtrate, we used a acetylene-nitrous oxide flame atomic absorption spectrophotometry. Potential dehydrogenase activity, the only indicator of the possible sources of pollution, excluded the presence of either chemical or biological pollution. The number of bacteria involved in the biogeochemical cycle of nitrogen in the analyzed soil indicated a high efficiency regarding the mineralization of the organic residues of plant and animal origin.

KEY WORDS: heavy metals, enzymatic activity, ecophysiological bacterial groups

INTRODUCTION
Soil pollution is the result of human activities and in order to solve this problem, prevention solutions must be taken in consideration. Soil pollutants may be chemicals (pesticides, hydrocarbons, heavy metals, heat) and of biological nature (animal and human manure, pathogens). Soil pollution with heavy metals is the consequence of the anthropogenic activities, mostly mining industry. Amongst the effects of soil pollution with heavy metals, we recall: imbalance of physical, chemical and biological processes in soil, decrease of biological activities, inhibiting nitrification processes, toxic action on plants. The toxicity and mobility of heavy metals in soil depends on many factors such as: the total concentration of metal, its chemical formula and properties, environmental factors, soil properties (pH, organic matter content, redox reactions) (Nyamangara, 1998). Heavy metals accumulate in soil in different geochemical forms. Soluble fractions are considered to be bioavailable for plants and microorganisms. Studies on heavy metals’ mobility and bioavailability were made in order to evaluate the risk of exposure of soil microorganisms (Li and Thornton, 2001; Kartal et al, 2006; Cuong and Obbard, 2006).

The soil is one of the most dynamic environments of biological interactions in nature. It also is the receiver of plenty organic and anorganic substances resulting from
human deliberate or accidental activities, such as xenobiotic treatment used in agriculture. These chemical substances might affect the growth and the dynamics of soil microorganisms. The presence in soil of different chemical substances has a negative influence on the enzymatic activities going on at this level.

Bacterial enzymatic activities in the soil provide the decomposition of organic residues of plants or animals and so they allow the biogeochemical circuit of the main chemical elements: C, N, S, P, Fe. The decomposition of organic waste is caused by intracellular and extracellular enzymatic components produced by microorganisms (bacteria, fungi) or derivatives from animal or vegetal sources (residues derived from plants, digestive tracts of small animals). The enzymatic activities are the result of microorganisms' direct action, which releases into the environment enzymes ready to desintegrate the substrate (Hicks et al, 1990). The enzymes involved in these processes might be included in the oxidases (dehydrogenase, catalase, peroxidases) or hydrolases class (invertases, proteinases, phosphatase) (Benitez et al. 2004). They can be used to measure the effect of disturbance factors in soil (Taylor et al. 2002).

Enzymatic activities are often proposed as indicators of environmental stress when pollutants are found in soil ecosystems acts (Badine et al 2001; Sannino and Gianfreda 2001). Present and potential dehydrogenase activity and the ability to reduce Fe III can be used as toxicological tests on soil specimens, knowing that the dehydrogenase activity is caused by proliferated microflora and there is a close relationship between the number of soil microorganisms, especially heterotrophic groups and dehydrogenase activity.

Biodegradation is a natural process when the reduction of a xenobiotic substance is due to microorganisms activity. Most of these microorganisms act in the natural environment, but there are some changes that can be made in order to speed the process of degradation in a limited time (Shing, 2008). Normally, extracellular enzymes are active for a short period of time because they can be quickly denaturated, degraded or irreversibly inhibited in soil (Marx et al 2005). Nevertheless, a particular proportion of an extracellular enzyme can be stabilized by adsorbtion in humic substances, so is possible for the enzyme to persist in soil (Badine et al. 2001).

The composition of a microbial community in a given environment is strongly influenced by the environment's biogeographical and ecological properties, such as the dynamic food webs, the cycling of nutrients, and the presence of organic and inorganic matters (Becks et al, 2005; Bell et al, 2005). Although many studies on environmental microbial compositions have been conducted, most of them are focused on the microcosms found in solid substrates (Stach et al, 2003, Walker et al, 2005).

The determination of the main ecophysiological groups of bacteria involved in biogeochemical cycle of elements in nature (implicitly nitrogen which is important mostly to the land) is useful to identify structural changes in soil caused by the action of anthropogenic factors. Some groups of microorganisms are able to use different
kinds of pollutants as source of material (C, N, P) and energy required for growth and development. (Gimsing et al., 2004; Merini et al, 2007; Zabaloy et al, 2010).

Studies on soil sample allows the assessment of soil quality from chemical analysis (concentration of heavy metals), biochemical analysis (enzymatic activities) and microbiological (ecophysiological groups of bacteria involved in the biogeochemical cycle of nitrogen).

MATERIAL AND METHODS

In order to take soil samples, the vegetation was removed from a depth of approximately 5-15 cm. Afterwards, the samples were submitted to chemical, microbiological and enzymatic tests.

Heavy metals analysis. The soil samples were dried using a oven. Drying temperature was set at 105° C. Subsequently, the samples were weighed using an analytical balance. This procedure was repeated five times and only the significant value was considered. Further, the soil samples were calcinated in a burning furnace at a temperature of 550° C, for 4 hours. The resulting ash was dissolved in 20 ml HNO₃ 0.5 concentration and then filtered through a paper filter. The volume of each sample was brought to 50 mL with 30 mL of 0.5 N HNO₃ solution. The nitric acid (65%, ρ = 1.39 g/cm³), used to make digestion solution (HNO₃ 0.5 N), was provided by Sigma-Aldrich Chemie GmbH. We used flame atomic absorption spectrophotometry with an acetylene-nitrous oxide flame (Perkin-Elmer 403 AAS) in order to evaluate the concentration in Cu, Zn, Mn, Ni, Pb, Cd, and Cr existing in the filtrate. Stock solutions (1000 ± 5 mg kg⁻¹ d.w.) for each analyzed TE were purchased from May and Baker Group. They were cooked in three different concentrations in order to obtain the corresponding calibration curves. All glassware used during the process was properly treated with Pierce solution 20% (v/v), rinsed with cold tap water, treated with 20% (v/v) nitric acid and then rinsed with bidistilled water. TE analysis shows a recovery percentage between 85% and 105%. TE levels in soil were expressed as milligram per kilogram dry weight (mg kg⁻¹ d.w.).

Enzimatic activity. The permanganometric method (Dragan-Bularda, 2000) was used to determine catalase activities. The reaction mixtures was composed of 3 g soil, 2 ml H₂O₂ 3%, 10 ml phosphate buffer and was incubated at 37° C during 1 hour. Enzymatic activity was expressed in mg H₂O₂/3 g soil. Using the Casida and co. methods we were able to determine the actual and potential dehydrogenase activity. The reacting mixture consisted of 3 g soil, 0.5 ml 2,3,5 triphenyltetrazolium solution (TTC), 2 ml distilled water and 1 ml glucose solution, respectively, for potential dehydrogenase. The treated samples were incubated at a temperature of 37° C for 48 hours. Dehydrogenase activity was expressed as mg formazan/3 g soil (Casida, 1964).

According to the methods presented by Drăgan-Bularda we were able to analyse the microbial iron reducing Fe³⁺ activity. This activity was expressed in mg Fe
II/3 g soil. From the interaction between/ of Fe II and α,α-dipiridil resulted a color reaction so that the solution can be photocolourmetred at 240 nm. In conformity with Dragan-Bularda’s method (2000) we determined urease activity. Reaction mixtures consisted of 3 g soil, 2 ml toluen, 5 ml phosphate buffer, 5 ml solution of urea 3%. The mixture was incubated at 37° C for 24 hrs. Activity was expressed as mg NH₄/3 g soil.

**Bacteriological analysis.** The soil samples treated with herbicides were diluted in order to allow the microbial growth. We used Key-samples of 10⁻¹ and 10⁻⁶ dilutions and stratified the studies for one gram of soil. Equal volumes of 1 ml of every soil dilution were inoculated on selective nutrient culture medium. Incubation has been made at 28°C and depending on the established ecophysiological group, the period of incubation varied from 7 to 21 days.

For the quantitative determination of nitrogen-fixing bacteria (NFB) we have used culture medium without nitrogen, i.e. Ashby culture medium, which consists in the following composition: K₂HPO₄, 0.5 g; NaCl, 0.5 g; MgSO₄, 0.2 g; K₂SO₄, 0.1 g; CaCO₃, 5 g and sucrose, 5 g. Soil samples were then incubated at 27°C during a week. On the surface of culture medium, the nitrogen fixing bacterial culture appeared as a a ring on the tubes walls, at the medium-air interface. The colour of the ring differed from fluorescent, to greenish yellow (Azotobacter vinelandii) or even brown (A. chroococum). For anaerobic fixing bacteria like Clostridium sp., in the positive tubes there could be seen gas bubbles (Zarnea, 1994).

For the cultivation of ammonifying bacteria (AMB) the following chemical composition was used: NaCl, 0.5 g; peptone, 2 g; distilled water, 1000 ml. The pH of culture environment should be 7.9. Afterwards, the samples were incubated at a temperature of 22°C for 14 days in anaerobic conditions. Using a specific color reaction (add one/two drops of Nessler reagent) we were able to highlight the ammonia which results from the activity of ammonifying bacteria. An intense yellow coloration was obtained, with or without precipitation (Cusa, 1996).

The culture environment for nitrifying bacteria (NB) has the following chemical composition: standard saline traces, 50 ml; (NH₃)SO₄, 0.5 g; CaCO₃, 1 g and 950 ml of distilled water. The samples have been incubated at 28°C for a period of 20 days. We have studied the nitrifying bacteria diphenylamine-sulfuric acid, as well. The appearance of a bluish colour showed the presence of nitrifying bacteria (Dunca et al., 2007). For denitrifying bacteria (DNB) we used a culture environment based on: standard saline solution, 50 ml; KNO₃, 20 g; glucose, 10 g; KCO₃, 5 g; oligoelements solution, 1 ml and distilled water, 1000 ml. The samples were incubated at 28°C for 7-15 days. Denitrifying bacteria were highlighted by adding diphenylamine-sulfuric acid. The tubes in which the nitrate vanished appeared colorless (Dunca et al., 2007).

**Quality indicators.** The bacterial indicator of soil quality (BISQ) has been evaluated according to the ecophysiological bacterial group. It was based on the formula proposed by Muntean (1995-1996): BISQ = 1/n x Σ log₁₀ N, where: BISQ -
bacterial indicator of soil quality, n - number of ecophysiological groups, N - number of bacteria which belongs to each ecophysiological group. The calculation of enzymatic indicator was based on the absolute values of the enzymatic activities from every sample analyzed. In order to do that we used the formula proposed by Muntean: 

$$EISQ = \frac{1}{n} \sum Vr (i) / Vmax (i)$$

where: EISQ - enzymatic indicator of the soil quality, n - number of activities, Vr (i) - real individual value, Vmax (i) - maximal theoretical individual value.

RESULTS AND DISCUSSIONS
The soil samples collected from the forest were brought to the laboratory and subjected to chemical, enzymatic and microbiological analysis. Chemical analyses were made for determining the heavy metals. Heavy metals from the forest soil were represented by Cu, Zn, Mn, Ni, Pb, Cd and Cr. For the analysis and comparison we use the maximum values allowed in the Romanian legislation regarding the content of heavy metals coming from cultivated soils. From the comparison of results we considered those values of soil sample analyzed for all 7 items, which did not exceed the maximum allowed by the legislation in force (ppm mineral/1 g sol) (Fig. 1.).

The tested enzymatic activities (catalase, current and potential dehydrogenase, urease and the reduction capacity of Iron III) were detected in all soil samples which have been analyzed. Potential dehydrogenase activity is best represented, being the only one which gives us indication regarding the possible sources of pollution. The current and potential dehydrogenase activities are the ones which reflect the numerical density of the microorganisms existing in soil. The more these types of activities recorded higher values the higher the number of microorganisms is in soil, so pollution is reduced (Fig. 2.). The analyzed forest soil shows a urease activity well represented which means a great content of organic residues of plant or animal containing nitrogen compounds. The enzymatic activities which have been tested decreased as follows: the potential dehydrogenase activity, the urease activity, the current dehydrogenase activity, the catalase activity and the capacity of reduction the Fe III (Fig. 5.).

Ecophysiological groups of bacteria that have been analyzed were those involved in the biogeochemical cycle of nitrogen and were represented by: aerobic nitrogen fixing bacteria - *Azothobacter vinellandi* and *Azothobacter chroococcum*, anaerobic nitrogen fixing bacteria- *Clostridium sp*, ammonifying bacteria, nitrifying bacteria, denitrifying bacteria.
Ammonifying bacteria were best represented numerically in the analyzed soil sample, their number being of 38,000 /1 g sol. Ammonifying numerical abundance of bacteria related to the large amount of urease enzyme activity indicates and confirms the existence, in the analyzed soil sample, of a great amount of decaying organic material of natural and animal origin. An ammonification process entails intense stimulation of nitrification, as confirmed by nitrifying bacterial count of 4,500 bacteria/1 g soil. The anaerobic nitrogen fixing bacteria of the genus *Clostridium* are also well represented in the analyzed soil sample, their number being of 4,000 bacteria/1 g soil.

Note the absence of denitrifying bacteria in the analyzed soil sample which indicates a positive effect regarding the efficiency of the biogeochemical flow of nitrogen in enriching the soil with nitrogen. The ecophysiological groups which improve the nitrogen content accessible to plant mineral nutrition are well represented, but the denitrifying bacteria which ensure loss of nitrogen in the soil for the purposes of making them inaccessible compounds for plant mineral nutrition and for other groups of microorganisms in the soil are missing.
The ecophysiological groups of bacteria register in forest soil sample decreased as follows: ammonifying bacteria, nitrifying bacteria, anaerobic nitrogen fixing bacteria (Clostridium sp.) > Azothobacter vinellandi > Azothobacter chroococcum (Fig. 3.).

Based on the absolute values of the 5 enzymes activities the enzymatic indicator of soil quality was determinated, its value being of 0.211. On the basis of the number of bacteria values for each eco-physiological group we determined the bacterial indicator of soil quality (IBCS), its value being of 0.781 (Fig. 4.).

**CONCLUSION**

The analyzed soil samples do not presents chemical pollution from the perspective of the heavy metals content. The dehydrogenase activity value (ecotoxicological test) does not indicate the presence of a chemical or biological pollution. The number of bacteria involved in the biogeochemical cycle of nitrogen in the analyzed soil indicates a high efficiency regarding the mineralization of the organic residues of plant and animal origin.
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