

EX-SITU BIOREMEDIATION EFFICIENCY IN REMOVING ORGANIC AND INORGANIC COMPOUNDS FROM ARTIFICIALLY AND ANTHROPOGENIC CONTAMINATED SOILS

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Abstract: The paper presents the results of bioremediation experiment conducted in order to determine the effectiveness of this method for the decontamination of a soil historically contaminated with heavy metals and artificially controlled with two of the most aggressive group of organic pollutants: hydrocarbons from crude petroleum and polychlorinated biphenyls from transformer oil. The experiment followed how a heavy metals polluted soil respond to inoculation with heterotrophic bacteria selected for their ability to metabolize the petroleum hydrocarbons and / or polychlorinated biphenyls, the effects exerted by heavy metal pollution on soil capacity to initiate and supports its own decontamination mechanisms. To implement full-scale bioremediation, the soil from Copșa Mică, historically contaminated with heavy metals, after it has been excavated was transported and disposed on a waterproofed platform in 0.2 m thickness layers, which were controlled contaminated with crude oil and transformers oil, resulting four experimental variants to whom technological elements of the bioremediation process were applied. Monitoring soil quality parameters: pH, organic carbon content, total nitrogen, C/N ratio, mobile phosphorus and potassium contents and the soil loading degree with inorganic pollutants, heavy metals (Zn, Cu, Fe, Mn, Pb, Ni, Cr, Cu, Cd) and organic: total petroleum hydrocarbons (TPH) and polychlorinated-biphenyls (PCBs) was performed at 1 month, 3, 5 and 10 months after controlled pollution application. The data obtained have been interpreted dynamically, and comparisons were made between the four experimental variants. Biodegradation efficiency analysis of the two categories of pollutants (TPH and PCBs) applied to soil by controlled pollution, shows that ex situ bioremediation method is definitely favorable for hydrocarbons polluted soil remediation. The data obtained until 10 months from when controlled pollution of soil it was carried out, although representing a favorable method for bioremediation even in the case polychlorinated-biphenyls pollution are not yet defining and conclusive. Well known molecular recalcitrance of these compounds prevents the attack of microorganisms and their inherent toxicity affects the adaptability and multiplication of microorganisms with the ability to break-down the phenolic nuclei, and initiate biodegradation.

Keywords: *ex situ* bioremediation, soil, heavy metals, petroleum hydrocarbons, polychlorinated biphenyls

1. INTRODUCTION

Long-term impact and ecological significance of pollution on the biosphere have resulted in increased interest to assess interactions between pollutants, environment and living organisms. Soil microbial populations are under tremendous pressure due to soil contamination with a variety of toxic substances such as heavy metals and other organic

pollutants that have environmental and anthropogenic origin (McGrath et al., 1988., Chaudhary et al., 1996). It is known that heavy metals at high concentrations affects soil microbial populations and their activities directly affecting soil fertility (Smith, 1996). Concentration of a toxic metal that affects the growth and survival of different organisms varies greatly (Babich & Stotzky, 1977), on the other hand, the magnitude of microbial diversity makes it difficult to

study the entire spectrum of the population. Although often the relationship between microorganisms and soil pollution with heavy metals is complex and contradictory (Smith, 1991), however, even a short-term study, artificial contamination with metal salts to a soil with well-known physicochemical properties, evaluation of indigenous microbial populations that survived, could reveal the appearance of organisms with intrinsic ability to tolerate metal.

The wide use of polychlorinated biphenyls (PCBs), more than 1.7 million tons worldwide (Seeger et al., 2010) associated with improper storage led to their spread widely on the entire surface of the planet, thus, today, PCBs can be detected in air, water, soil, plants, animals and humans. Due to their high chemical (Singleton, 1994), and biochemical stability, and high solubility in lipids PCBs show a greater capacity to bioaccumulate in food chains, with highest concentrations accumulated in the beings from the top of the food chain (predators or humans). PCBs negative impact on the environment and health, expressed in carcinogenic diseases of the reproductive function, nervous and immune system modifications (Faroon et al., 2001), inhibition of photosynthesis and loss of biological diversity, usually are observed too late.

Bioremediation is an attractive alternative for treating soils contaminated with PCBs, but the rate of the process may be limited, among other factors, by the low presence of soil microorganisms able to degrade these compounds. Have been reported bacterial species capable of degrading PCBs, belonging to the genera *Pseudomonas*, *Burkholderia*, *Rhodococcus*, *Corneybacterium* and *Bacillus* (Seto et al., 1995; Warren et al., 2004; McLeod et al., 2006; Furukawa & Fujihara, 2008; Seeger & Pieper, 2009). Addition of exogenous microorganisms and nutrients necessary for the development and their activity can be beneficial to break down PCBs. Rate of microbial degradation of PCBs may also be limited by the hydrophobic nature of these compounds, which limits bioavailability, availability microorganisms to perform the attack (Providenti et al., 1993, Robinson & Lenn, 1994; DeVlieghe & Vestraete, 1996; Rojas-Avelizapa et al., 1999), but also their toxicity or degradation intermediates (Cámara et al., 2004; Ohtsubo et al., 2004; Agulló et al., 2007; Pieper & Seeger, 2008). Above all, shall be added strong binding tendency of PCBs from soil organic matter, which is a great limiting factor of bioremediation (Fava & Piccolo, 2002).

Contamination of soils with petroleum hydrocarbons (PH) is worldwide still one of the major environmental problems. Despite recent

technological advances, accidental spills of crude oil and its refined products occur on a frequent basis during routine operations such as extraction, transportation, storage, refining and distribution (Nikolopoulou et al., 2007). Petroleum fuel spills are classified as hazardous waste (Bossert & Bartha 1984) and are considered as the most frequent organic pollutants of terrestrial and aquatic ecosystems (Bossert et al., 1984, Margesin & Schinnur 1997; Margesin, 2000). It is estimated that 1.7-6.8 million tones of crude oil, with a best estimate of 3.2 million tones per year, are released from all sources into the environment (Saadoun & Al-Ghzawi, 2005). The petroleum can contain up to 20% benzene, toluene, ethyl benzene and xylene (BTEX), and these are on the hazardous list. The BTEX compounds, although not miscible with water, are mobile and can contaminate the groundwater, which is recognized as a serious and environmental problem (Scherr et al., 2007; Zawierucha & Malina, 2011).

Crude oil is an extremely complex and variable mixture of organic compounds in a wide range of molecular weight, from the gas methane to the high molecular weight tars and bitumen, and of molecular structure: straight and branched chains, single or condensed rings and aromatic rings. The two major groups of aromatic hydrocarbons are monocyclic, such as benzene, toluene, ethylbenzene and xylene (BTEX), and the polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, anthracene and phenanthrene.

Conventional technologies based on various physical and chemical processes have been employed for effective remediation of oil hydrocarbon contaminated soil (Khan et al., 2004; Malina & Zawierucha 2007). However, most of these techniques are very expensive, and some of them lead to the toxic by products that may cause secondary contamination of soil and water, requiring additional post-treatments, and continuous monitoring and control (Liang et al., 2009). Moreover, they do not usually result in a complete destruction of the contaminants and may negatively affect or destroy soil structure and functions, (Gouda et al., 2008).

Although, depositing of contaminated soil at landfill sites became legally permitted even stimulated in most countries, the destiny of polluted soil is still badly defined by laws in the European Economic Community. An EEC soil directive is still in discussion and its acceptance will undoubtedly open a huge market for soil bioremediation. According to Vijgen (2002) report for the Directorate General Research of the European

commission (cited by Lebeau, 2011), “only 10–25% of all excavated contaminated soils are treated. Assuming a simple equal division according to the three main techniques (thermal, physical/ chemical and biological) it would appear that only a small part of the soil (~3–8%) excavated from remediation sites has any real chance of going to biological treatment”. This same author stated that, “despite economically and ecologically positive aspects of bioremediation along with the possibility to reuse the soil after the treatment, this technology suffers from a lack of reliability”.

Biological methods, such as bioremediation is a versatile alternative to physicochemical treatments (Atlas, 1991; Bartha, 1986) and produces non-toxic end products such as CO₂, water and methane from petroleum hydrocarbons (Walter et al., 1997). This process is considered to be relatively cost-effective and environmentally friendly technology which optimizes microbial degradation activity by control of the pH, nutrient balance, aeration and substrate mixing (Desai & Banat 1997; Hosokawa et al., 2009). Bioremediation is a treatment method that exploits microorganisms and their enzymatic activities to effectively remove contaminants from contaminated sites (Scherr et al., 2007). However, the rate of hydrocarbon biodegradation in soil is affected by several physicochemical properties of the soil and contaminants, as well as biological characteristics of indigenous microorganisms. These include the number and species of microorganisms present, concentrations of hydrocarbons and environmental conditions (pH, temperature, nutrients, oxygen and moisture content) suitable for microbial degradation (Leahy & Colwell 1990; Gouda et al., 2008; Perfumo et al., 2007; Horel & Schiewer 2009).

Bioaugmentation through which could enhance the activity of microorganisms, thus increasing the biodegradation rates, is a promising and low-cost bioremediation method, in which effective bacterial isolates or microbial consortia capable of degrading petroleum hydrocarbons are introduced to the contaminated soil. This technique, are generally using multiplied indigenous microorganisms strains, inoculation of soil with exogenous or laboratory-modified bacterial cultures still generating many controversies not being acceptable by the public (Gentry et al., 2004; Fantroussi & Agathos 2005; Hosokawa et al., 2009; Zawierucha & Malina, 2011). Sometimes, the application of oil-degrading microorganisms may lead to a failure of bioaugmentation, because the survival and degrading ability of microorganisms introduced to a contaminated site are highly

dependent on environmental conditions (Vogel, 1996; Gentry et al., 2004). In many cases, potentially degrading strains isolated from one site are not necessarily applicable to the other site. Although major progress has been achieved on the lab scale, successes obtained on the field are more or less limited (Winkelmann et al., 2009). It is considered that indigenous bacteria are best adapted to the environment of the treated site (Rahman et al., 2003, Voiculescu et al., 2005; Lacatusu A.R. et al., 2009; Lacatusu R., et al., 2009). Also, the issue of valorization of the potentially of petroleum hydrocarbon degraders soil's microorganisms has insufficiently been exploited till now in Romania.

The main objectives of this research has been to determine (a) the effectiveness of bioremediation method for the decontamination of a soil historically contaminated with heavy metals and artificially polluted with two of the most aggressive group of organic pollutants: hydrocarbons from crude petroleum and polychlorinated biphenyls from transformer oil and (b) how a heavy metals polluted soil respond to inoculation with heterotrophic bacteria selected for their ability to metabolize the petroleum hydrocarbons and/or polychlorinated biphenyls, the effects exerted by heavy metal pollution on soil capacity to initiate and supports its own decontamination mechanisms.

2. MATERIALS AND METHODS

In order to organize the *ex situ* bioremediation experiment, the top layer of soil (up to 20cm deep), historically polluted with heavy metals from Copșa Mică was excavated and transported on a waterproofed bioremediation platform, whose surface (2000 sqm) was divided into four equal parts, corresponding to the four experimental variants proposed (Figs 1, 2, 3). The four areas were been artificially contaminated with crude oil and transformer oil resulting in four experimental variants:

I. CRUDE OIL: historically heavy metals contaminated soil, artificially polluted with crude oil;

II. PCB: historically heavy metals contaminated soil, artificially polluted with transformer oil;

III. MIXTURE: historically heavy metals contaminated soil, artificially polluted with a mixture of crude oil and transformer oil.

IV. METALS: historically heavy metals contaminated soil, without artificial pollution with organic pollutants.

After a morphologic study of a representative soil profile, conducted at the site chosen for research

at Copșa-Mică, we proceeded to analyze the main physicochemical and microbiological characteristics of native soil as a reference base in the interpretation of any changes arising out of the application of decontamination procedures.



Figure 1. Location of historically heavy metals contaminated site from Copșa Mică, Romania



Figure 2. The excavation of the top layer of soil



Figure 3. Artificially contamination of soil with crude oil and transformer oil on waterproofed bioremediation platform

After one month from controlled pollution of soil, screening of heterotrophic microorganisms from the four experimental plots was made, and the dominant microorganisms have been isolated and identified. They have been purified by successive passages on nutrient media and tested for the ability to use hydrocarbons from crude oil or polychlorinated biphenyls from transformer oil by cultivation in mineral medium whose sole carbon source was represented by each of the two pollutant substances. After selection of bacterial strains competent in biodegradation, poly-strains inoculums' was done. This was applied in soil artificially contaminated with organic pollutants in each experimental plot.

Another technological link complementary to selected bacterial inoculation was organic fertilization, with mixture farmyard manure fermented with plant debris (corn stalks), calculated in proportion of 150 t/ha, aiming to create a nutrient medium favorable to bacteria multiplication and their activity, for both native soil inhabitants and those introduced by inoculation.

Monitoring soil quality parameters: pH, organic carbon content, total nitrogen, C/N ratio, mobile phosphorus and potassium contents and the soil loading degree with inorganic pollutants, heavy metals (Zn, Cu, Fe, Mn, Pb, Ni, Cr, Cu, Cd) and organic: total petroleum hydrocarbons (THP) and polychlorinated-biphenyls (PCBs) was performed at 1 month, 3, 5 and 10 months after controlled pollution application.

Soil samples were analyzed by ICPA methodology (Florea et al., 1987) developed to assess main physical (particle size) and chemicals soil properties: organic carbon and humus - Walkley-Black method (modified by Gogoasa), total nitrogen content, mobile phosphorus and potassium content - Egner-Riehm-Domingo method, pH (H₂O), ratio soil/water 1/2,5 – potentiometric method using glass calomel electrode. Also, microbiological analyses: quantitative determinations of heterotrophic bacteria (total bacteria number method) using traditional culturing methods and taxonomic determinations by usually identification methods, optical microscopy, determination keys and physiological tests (Sneath, 1986; Florenzano, 1983), were carried out.

Total heavy metals content (Cd, Co, Cr, Cu, Fe, Mn, Pb, Zn) was determined by atomic absorption spectrometry in air acetylene flames.

Total petroleum hydrocarbons was determined by gravimetric method (ISO 13511:2007) and polychlorinated biphenyl by gas chromatography with electron capture detection (ISO 10382:2007).

Analytical data were statistically calculated, with the grouping center parameters: arithmetic mean, geometric mean, median, module (\bar{x} , Xg, Me, Mo) - and the spreading values: minimum value, maximum value, standard deviation (x_{\min} , x_{\max} , σ) being determined.

The obtained data have been interpreted dynamically, and comparisons were made between the four experimental variants.

3. RESULTS AND DISCUSSIONS

3.1. Native soil properties

Native soil used for the bioremediation experiment, is a Calcaric Mollic Gleyic Fluvisols (drained) belonging to the Protisols class, located to Copșa Mică, in the Târnava Mare river meadow. Soil texture is medium loamy clay with a medium carbonates content, weakly alkaline reaction (pH 8,03), medium organic carbon content in 0-20 cm (2,369%), total nitrogen ranged from lower limit of middle class (0,152%), high C/N ratio with an average of 18,3 that showing existing mineral imbalances, low average mobile phosphorus content (15,1 mg·Kg⁻¹), and high mobile potassium (289,3 mg·Kg⁻¹) (Table 1).

Table 1. Statistical parameters of the main chemical properties of the surface horizon (0-20 cm) of the soil from Copșa Mică used for experimentation

Statistical parameters	Soil properties					
	pH	C _{organic} %	Nt	C/N	P _{AL} mg·Kg ⁻¹	K _{AL} mg·Kg ⁻¹
Xmin*	7.86	1.949	0.119	15.9	7.8	162.4
Xmax	8.11	2.993	0.197	25.7	42.0	514.9
X	8.03	2.369	0.152	18.3	15.1	279.3
σ	0.06	0.266	0.019	2.3	8.5	84.5
VC (%)	0.8	11.238	12.141	12.7	56.6	30.3
Xg	8.03	2.355	0.151	18.1	13.5	268.6
Me	8.04	2.332	0.149	17.6	13.0	253.4

*Xmin – minimum value; Xmax – maximum value; X – arithmetic mean; σ - standard deviation; Xg – geometric mean; Me – median

Total petroleum hydrocarbons (TPH) determinations did not reveal the presence of this class of pollutants in the native soil, the values being under the device detection limit. Polychlorinated biphenyls (PCBs) determinations revealed the presence of only three PCBs congeners 153, 138 and 180, whose concentration, although exceeding normal values are much below the alert threshold for sensitive uses.

Heavy metal content determinations have highlighted that only three of the chemical elements analyzed presented polluting concentrations in soil, which raising environmental concerns. Thus cadmium, one of the most toxic heavy metals, with proven effects on human health and a huge bioaccumulation in the food chains capacity and biomagnification, exceeding by 12.67 times the alert threshold for sensitive uses (soil is located on arable land with agricultural destination) and 7.6 times the intervention threshold for the same uses.

Lead, another very toxic chemical, exceeding by 16.44 times the alert threshold for sensitive uses, and by 8.22 times the intervention threshold. And finally, zinc exceeds by 6.41 times the alert threshold for sensitive uses and by 3.2 times the intervention ones (Table 2).

In terms of quantitative and qualitative analysis of bacterial microflora, the data showed for the top soil layer an average value of total bacterial number (TBN) of 79.65 x 10⁶ viable cells/g dry soil, which is a medium level of colonization with heterotrophic bacteria of the soil from the contaminated site, which corresponding, in fact, to physico-chemical properties of this soil type affected by historical pollution with heavy metals and black smoke.

Table 2. The average content of heavy metals in the surface horizon (0-20 cm) of alluvial soil from Copșa Mică under artificial pollution with crude oil and transformer oil

Chemical element	Medium value (mg·Kg ⁻¹)	Exceeding times of	
		<i>the alert threshold</i>	<i>the intervention threshold</i>
	for sensitive use		
Cd	38	12,7	7,6
Cr	77	-	-
Fe	40297	-	-
Mn	848	-	-
Ni	51	-	-
Pb	822	16,4	8,2
Zn	1922	6,4	3,2

The diversity of bacterial genera and species of native soil was composed by 7 taxons: *Pseudomonas spp*, *Arthrobacter globiformis*, *Arthrobacter citreus*, *Bacillus cereus*, *Bacillus circulans*, *Mycobacterium roseum*, *Flavobacterium spp* being important to note that the *Pseudomonas*, *Arthrobacter* and *Flovobacterium* are reputed for the species with high abilities in biodegradation of petroleum hydrocarbons and *Pseudomonas* is known as the only bacterial genus capable to degrade PCBs.

3.2. Dynamics of petroleum hydrocarbons

Regarding the total petroleum hydrocarbon content, at the first measurement, obviously, the highest value was recorded in the variant controlled polluted with crude oil (Fig. 4), followed by the variant polluted with a mixture of crude oil and transformer oil. In the variant polluted only with oil transformer, the highest value of the TPH concentration was recorded, also, at one month after completion of artificial pollution.

Since the determination made at three months after pollution TPH concentration from crude oil polluted plot begins to decrease up to 38% of the value determined at 1 month, unlike the mixed polluted plot, in which the TPH concentration was still 84.14% of the previous value after 3 months. Analytical data obtained at 5 months (after inoculation) showed that the concentration of TPH from crude oil polluted plot only reached 10.66%, and that from mixed polluted lot 26.5%. To 10 months after controlled pollution, the concentration of TPH in crude oil polluted plot reached 7.66% of the initial value, while in the mixed polluted plot it was maintained at 14.79%.

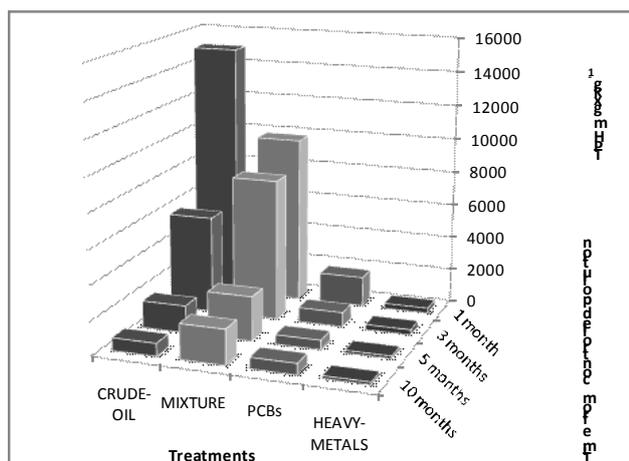


Figure 4. Dynamics of *ex-situ* bioremediation in removing petroleum hydrocarbons (TPH %) from artificially and anthropogenic contaminated soils

Following the same reasoning, in the exclusively transformer oil (PCB) contaminated plot, the percentage of TPH recovered in the soil at successive determinations have been: 1 month = 100%; 3 month = 48.57%; 5 months = 31.42%; 10 months = 28.57%.

Obviously, one can notice that, in a mixture, the rate of petroleum hydrocarbons disappearance from soil is lower than in the variant where the soil is polluted with crude oil exclusively.

TPH determinations performed in the plot historically polluted with heavy metals have shown a

slight soil contamination with petroleum hydrocarbons generated by application of artificially pollution in neighboring lots.

3.3 Dynamics of polychlorinated biphenyls

Determinations of PCBs content shows as it was expected the highest initial concentration in the transformer oil contaminated plot, followed by concentration determined in mixed polluted plot (Fig. 5).

Analyses performed at three months after pollution shows that the PCBs concentration in transformer oil contaminated plot begins to decrease, reaching up to 14.97% of the value determined at one month, unlike the mixed polluted variant, where the PCBs concentration after 3 months was still 42.93% of the previous value.

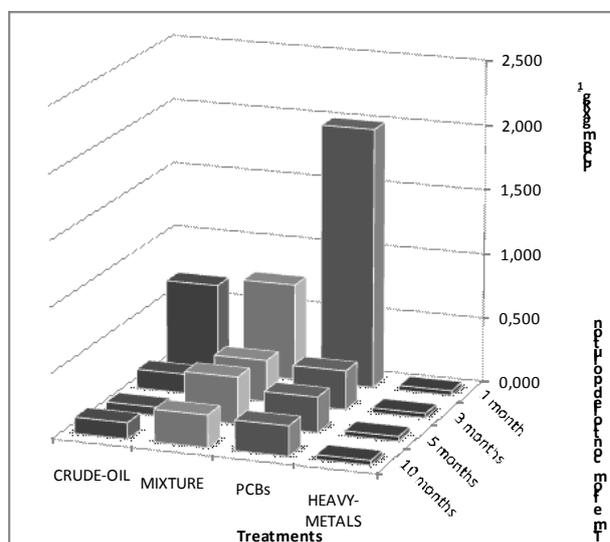


Figure 5. Dynamics of *ex-situ* bioremediation in removing polychlorinated biphenyls (PCB $\text{mg}\cdot\text{kg}^{-1}$) from artificially and anthropogenic contaminated soils

Analyses performed at 5 months (after inoculation) showed that the PCBs concentration in transformer oil contaminated plot reached 13.57%, and that from mixture polluted plot is maintained relatively high 48.72%. Here, can be observed a slight increase in the PCBs content due to non-uniformity of the soil material, practically, it is about a strong slowdown in pollutant degradation process. To 10 months from artificially pollution, the concentration of PCBs in transformer oil contaminated plot reaches only 11.58% of the initial value, while in mixture polluted plot, still, being 33.24%.

It can be stated that after a rapid disappearance of the pollutant in the first three months from the controlled pollution of the soil, the PCBs degradation is becoming very slow, remaining so until the last measurement performed at 10 months.

3.4. Heterotrophic bacteria communities

Monitoring of the soil heterotrophic bacteria communities in the four experimental variants have been showed significant variations of the total bacterial number (TBN) values, by 2 orders of magnitude (Fig. 6).

Since the first determination, there was a significant increase in the TBN values in variants controlled polluted with organic pollutants compared with those historically polluted only with heavy metals. The strongest multiplication of heterotrophic bacteria was recorded in the crude oil polluted soil.

Analytical data obtained by the determination carried out at 5 months from controlled pollution reflects inoculums application by a significant multiplication of bacteria, by 2 orders of magnitude difference, compared with the variant polluted with heavy metals, which do not received inoculation.

Heterotrophic bacteria proliferation quantified by TBN value shows, first of all, the survival rate of inoculated bacteria, which, in this case being very good, represents a favorable premise for the success of the bioremediation process application for soil decontamination.

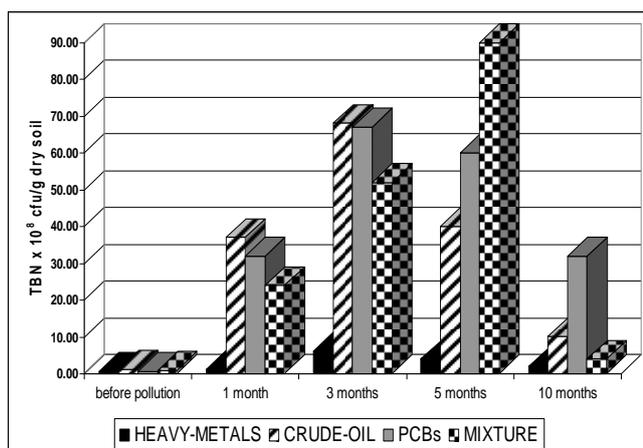


Figure 6. Dynamics of heterotrophic bacteria from artificially and anthropogenic contaminated soils

In November, at 5 months from soil controlled pollution, bacteria multiplication model already revealed from previous determinations has been reversed, in the sense that there was an explosive proliferation of bacteria in the variant contaminated with mixture of the two types of organic pollutants, petroleum hydrocarbons and polychlorinated biphenyls. Probable that in a mixture the two categories of pollutants: petroleum hydrocarbons and polychlorinated biphenyls are more aggressive against microorganisms, which thus require a period of time (lag) to adapt to substrate longer than the

necessary time of adaptation to pollution conditions generated by each pollutant separately.

At last measurement, performed in early spring, when the soil temperature was still well below the minimum necessary to 15°C, in all variants artificially polluted with organic pollutants bacterial communities appeared to be better represented from quantitative point of view, compared to variant only polluted with heavy metals.

Quantitative analysis of heterotrophic bacteria clearly shows the process of bioremediation of soil artificially contaminated with organic pollutants in experimental conditions.

3.5. Bioremediation process efficiency

3.5.1. Ex-situ bioremediation efficiency in removing petroleum hydrocarbons

Intermediate yields were calculated in terms of petroleum hydrocarbons (THP%) disappearance from soil from one determination stage to another, in order to be highlighted decontamination speeds in the each of the three experimental plots controlled polluted with organic pollutants respectively crude oil, transformer oil and mixture of the two complex chemical substances.

From Figure 7 it can be seen that in the plot exclusively polluted with crude oil, petroleum hydrocarbons degradation begins in the initial period from July to September (which is the interval between one month and three months after controlled pollution) with an efficiency of 62%, which reported to the TPH initial concentration of 15 000 mg·kg⁻¹ means over 9000 mg·kg⁻¹. Efficiency related to this period does not represent exclusively the result of bioremediation, but rather, that of volatile light crude oil component volatilization, combined with chemical and biological degradation of easily degradable fraction, represented by alkanes with low C atoms and unbranched chains or less branched.

In the time interval between September to November (from 3 to 5 months from controlled pollution) decontamination efficiency increases to 71.9%. This value is in real the biodegradation efficiency because corresponding to consecutive period of poly-strains bacterial inoculums application. This time includes both the period necessary for adaptation of inoculated microorganisms as well as the maximum proliferation and biodegradation of hydrocarbons from crude oil. In addition, the September-November period benefited from a warm weather

which has ensured an optimum soil temperature for microorganisms involved in biodegradation.

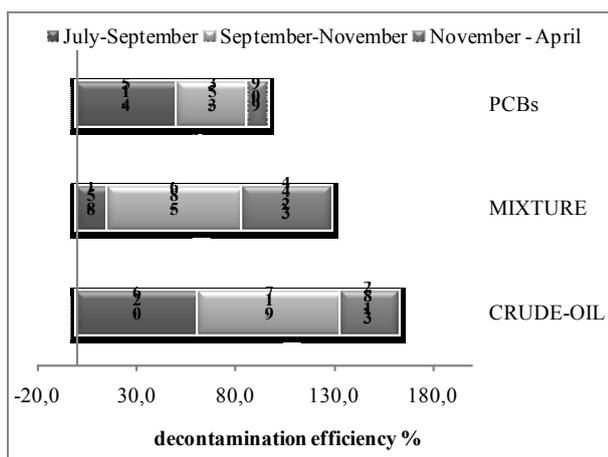


Figure 7. *Ex-situ* bioremediation efficiency in removing petroleum hydrocarbons (TPH %) from artificially and anthropogenic contaminated soils

The third period, between November and April next year (5 - 10 months from the application of controlled pollution) includes winter, when soil temperatures reached values improper for multiplication and activity of heterotrophic bacteria, which thus pass into the inactive resistance forms. However, biodegradation of crude oil has continued until the coming of winter with a rather high efficiency, 28.13% respectively.

These data primarily reflects the efficiency of poly-strain bacterial inoculums applied in order to accelerate the petroleum hydrocarbons biodegradation processes in the contaminated soil, and secondly that the inoculated microorganisms have been judiciously selected, showing a rapid acclimatization, abundant multiplication and action for target substrate attacking, hydrocarbons from crude oil respectively.

In the experimental lot polluted by mixture of the two types of pollutants, crude oil and transformer oil, petroleum hydrocarbons degradation dynamics differs from that described above in soil polluted with crude oil. In the first period, between the first and the third month after controlled pollution, disappearance efficiency of petroleum hydrocarbons in soil is much lower, of almost 4 times compared to variant polluted with crude oil only, being only 15, 8%. This shows that the transformer oil caused, somehow, the initial adsorption of the light fraction of crude oil on soil organic matter. The next time interval after soil inoculation with selected bacteria, a noticeably acceleration of the petroleum hydrocarbons degradation was occurred, with an efficiency of 68.5%, almost comparable with that from variant polluted with crude oil exclusively. In the interval

from November to April, the degradation process showed 44.23% efficiency, achieved through significant biodegradation activity carried out by inoculated microorganisms until winter.

3.5.2. *Ex-situ* bioremediation efficiency in removing polychlorinated biphenyls

In the first stage, ranging from one month at three months after the controlled pollution, in the variant exclusively contaminated with transformer oil was recorded the highest rate of PCBs disappearance from soil, with an efficiency of 85.02% (Fig. 8).

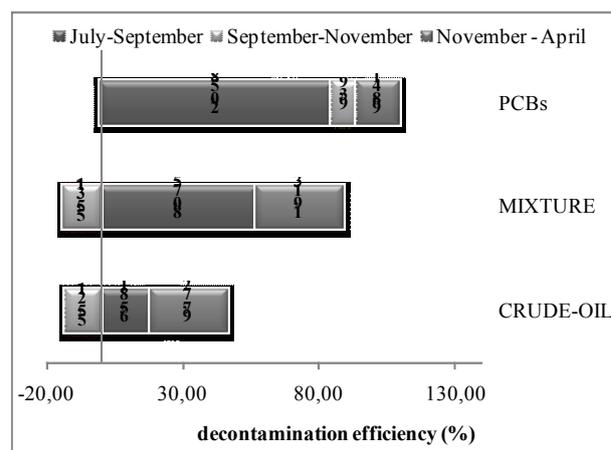


Figure 8. *Ex-situ* bioremediation efficiency in removing polychlorinated biphenyls (PCBs mg·kg⁻¹) from artificially and anthropogenic contaminated soils

Thus, content values determined at 3 months after controlled pollution have been about 7 times lower than the initial concentration. This fact, can not be explained by the degradation of chemical compounds, PCBs being some of the most recalcitrant and persistent pollutants. Perhaps, the transformer oil applied has been fast and very strong adsorbed by soil organic matter their extraction with organic solvents used in the chromatographic method determination becoming very difficult.

Between September and November, after inoculation with selected bacterial strains (inoculums included two *Pseudomonas* strains that were been developed on mineral medium with transformer oil as the sole carbon source) bioremediation efficiency was 9, 39%. Further, until installing the adverse conditions associated with winter, biodegradation efficiency was 15.07%.

In the experimental plot polluted with a mixture of crude oil and transformer oil, the behavior of PCBs in soil presented the same pattern in the first stage, disappearing from the soil with a rate of 57.08%. It is obviously about a strong polychlorinated biphenyls adsorption in soil organic-

mineral matrix, phenomenon that has resulted in analytical determination of low concentrations of PCBs. In stage from September to November, after the application of bioremediation measures, a phenomenon of polychlorinated biphenyls desorption from soil matrix occurred, which has generated an increase in the contents of PCBs compared to the previous stage and which has resulted in a negative bioremediation efficiency related to this intermediate stages.

Analysis of final efficiencies data, calculated by reporting the final concentrations to the initial concentrations of the two categories of organic pollutants utilized for controlled pollution of soil demonstrates that each of the two categories of pollutants, applied at higher concentrations, inhibits the biodegradation of the other category (Fig. 9).

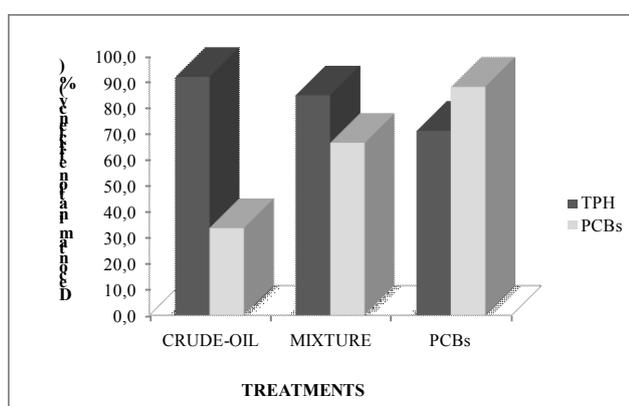


Figure 9. *Ex-situ* bioremediation efficiency in removing organic compounds (petroleum hydrocarbons and polychlorinated biphenyls) from artificially and anthropogenic contaminated soils

Thus, in the crude oil polluted variant, the final biodegradation efficiency of PCBs which appeared by dispersion in this lot is very low, of only 33.81% compared with the final PCBs biodegradation efficiency in the transformer oil contaminated plot, by 88.48%. Similarly, in the oil transformer contaminated plot the petroleum hydrocarbons biodegradation final efficiency was 71.4% compared with 92.3% reported in the crude oil only polluted plot.

In variant with mixed pollution, final efficiency of crude oil degradation is 85.2% and those of the polychlorinated biphenyls 66.81%.

From all the above, especially from biodegradation efficiency analysis of the two categories of pollutants, applied in soil by controlled pollution, results that the *ex situ* bioremediation method is clearly favorable for the decontamination of soils polluted with petroleum hydrocarbons.

The data obtained until this level of experimentation, respectively, up to 10 months after

the controlled pollution of soil, representing a favorable premise for applying the bioremediation method, even in the case of polychlorinated biphenyls polluted soils. Because of high molecular recalcitrance of these chemical compounds, enzymatic attack of microorganisms is very difficult, and their intrinsic toxicity, affects the adaptation and multiplication ability of microorganisms, which are able to breaking down phenolic nuclei, and to initiate the biodegradation processes.

4. CONCLUSIONS

Native soil used for the bioremediation experiment, a historically polluted with heavy metals, is a Calcaric Mollic Gleyic Luvisols (drained) belonging to the Protisols class, located to Copșa Mică, in the Târnava Mare river meadow.

Three heavy metals presented polluting concentrations in soil, which raising environmental concerns: Cd, one of the most toxic heavy metals, exceeded by 12.67 times the alert threshold for sensitive uses (soil is located on arable land) and 7.6 times the intervention threshold for the same uses; Pb, another very toxic chemical, exceeded by 16.44 times the alert threshold, and by 8.22 times the intervention threshold; Zn exceeded by 6.41 times the alert threshold and by 3.2 times the intervention threshold for sensitive uses.

The diversity of bacterial genera and species of native soil was relatively high, composed by 7 taxons: *Pseudomonas spp*, *Arthrobacter globiformis*, *Arthrobacter citreus*, *Bacillus cereus*, *Bacillus circulans*, *Mycobacterium roseum*, *Flavobacterium spp*, between that *Pseudomonas*, *Arthrobacter* and *Flovobacterium* are reputed for the species with high abilities in biodegradation of petroleum hydrocarbons, *Pseudomonas* being known as the only bacterial genus capable to degrade PCBs.

Since the first determination, there was a significant increase in the total bacterial number values in variants controlled polluted with organic pollutants compared with those historically polluted only with heavy metals.

The strongest multiplication of heterotrophic bacteria was recorded in the crude oil polluted soil.

Analytical data obtained at 5 months from controlled pollution reflects inoculums application by a significant multiplication of bacteria, by 2 orders of magnitude difference, compared with the variant which do not received inoculation.

As mixture, petroleum hydrocarbons and polychlorinated biphenyls are more aggressive against microorganisms, which required a lag period to adapt to substrate longer than the necessary time

of adaptation to pollution conditions generated by each pollutant separately.

Analytic data primarily reflects the efficiency of poly-strain bacterial inoculums applied in order to accelerate the petroleum hydrocarbons biodegradation processes in the contaminated soil, and secondly that the inoculated microorganisms have been judiciously selected, showing a rapid acclimatization, abundant multiplication and action for target substrate attacking.

In the experimental plot polluted by mixture of crude oil and transformer oil, disappearance efficiency of petroleum hydrocarbons in soil is much lower, of almost 4 times compared to variant polluted with crude oil only, showing that the transformer oil stimulate the initial adsorption of the light fraction of crude oil on soil organic matter.

In the first analytic stage, from one to three months after the controlled pollution, the highest rate of PCBs disappearance from soil, with an efficiency of 85.02% was recorded in the variant exclusively contaminated with transformer oil.

This fact, can not be explained by the degradation of chemical compounds, PCBs being some of the most recalcitrant and persistent pollutants, but, the transformer oil applied has been fast and very strong adsorbed by soil organic matter.

The bioremediation measures application, induced polychlorinated biphenyls desorption from soil matrix, which has generated an increase in the contents of PCBs compared to the previous stage, resulted in negative bioremediation efficiency.

Analysis of final efficiencies data, calculated by reporting the final to the initial concentrations of the two categories of organic pollutants utilized for controlled pollution of soil demonstrates that each of the two categories of pollutants, applied at higher concentrations, inhibits the biodegradation of the other category.

Biodegradation efficiency analysis of the two categories of pollutants, applied in soil by controlled pollution, revealed that the *ex situ* bioremediation method is clearly favorable for the decontamination of soils polluted with petroleum hydrocarbons.

The data obtained until this level of experimentation, respectively, up to 10 months after the controlled pollution of soil, representing a favorable premise for applying the bioremediation method, even in the case of polychlorinated biphenyls polluted soils.

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