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Phosphatase activity as a parameter for assessment of the rhizodegradation potential of poplar clones: Greenhouse dose-response experiment of phytoremediation of oil contaminated soil

Andrej Pilipović[1], Saša Orlović[1], Marina Katanić[1], Jelica Simeunović[2], Saša Peković[1], Milan Matavuly[3*]

[1]*University of Novi Sad, Institute of Lowland Forestry and Environment, Antona Čehova 13, 21000 Novi Sad, Republic of Serbia*
[2]*University of Novi Sad, Faculty of Science, Department of Biology and Ecology, Trg Dositeja Obradovica 2, Novi Sad, Republic of Serbia*
[3]*Faculty of Pharmacy – European University, Novi Sad, Trg Mladenaca 5, 21000 Novi Sad, Republic of Serbia*

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Summary. During rhizo-degradation, certain microorganisms are capable of breaking down hazardous pollutants such as naphtha and its derivatives or other xenobiotics into non-toxic products. Some plant clones with improved plant-microbe interactions have been selected which should display enhanced ability to secrete exudates which stimulate microbial activity in the substrate where phyto-degradation occurs. Metabolic processes performed by microorganisms of the rhizosphere, and in particular enzymatically catalyzed bio-degradation, form the basis of bio-conversion processes for persistent and toxic pollutants into non-toxic or less toxic byproducts. Such bio-conversion is in fact the essence of both spontaneous and controlled bio-remediation processes. Thus, these processes can be monitored by measuring the activity levels of highly specific or less specific enzymes. One universal group of enzymes present in microorganisms involved in bioconversion are phosphomonoester-hydrolases: including alkaline, acid and neutral phosphatase enzymes. Phosphomonoester-hydrolase activity is a good indicator of organic load in natural freshwater, as well as during bio-remediation processes that occur in artificial and natural ecosystems. The aim of this study was to investigate the rhizo-degradation potential of different poplar (*Populus* spp.) clones in oil contaminated soil, and to measure phosphomonoester-hydrolase activity in rhizosphere microflora as an indicator of rhizo-remediation processes. The effect of crude naphtha on the number of aerobic heterotrophic, facultative oligotrophic and naphtha-oxidizing bacteria, along with the phosphomonoester-hydrolase activity present in rhizosphere microflora, was studied using a greenhouse dose-response experiment. Cuttings from three poplar clones were planted into pots with soil loaded with 6 dosage levels of oil contaminated dry soil ranging from 0-6174 mg/kg of crude naphtha. Six months after planting, both the number of bacteria and phosphatase activity in the rhizosphere zone were increased in low contaminated soil (5%), but significantly decreased with increasing of oil contamination (50%) in a dose-response manner, to a maximum 10-fold reduction in 100% naphtha contaminated soil (vs. 0% contaminated soil controls). No differences were observed between different poplar clones with respect to support of rhizosphere bacterial growth. The relationship between phosphatase activity and the abundance of different groups of bacteria suggest that this phosphatase activity is associated with a specific group of bacteria.

Keywords: indicator, oil polluted soil, phosphatase activity, poplar clones, rhizodegradation.

INTRODUCTION

Soil contaminated with oil and its derivatives represents a significant environmental problem, particularly due to the ever increasing use of fossil fuel based transportation, machinery and lubricants, as well as accidental oil spills and other environmental disasters (Petrovicy et al. 1985, 2004; Vidyikant et al. 2007). Everywhere they are processed, transported, moved, stored or used, petrochemical products contaminate the surrounding soil. In particular, the oil and natural gas industry utilize a series of complex technological processes that are potential hot-spots of environmental contamination (Rončević 2002; Yovanovicy et al. 2003, 2005,

*Corresponding author, e-mail: milan.matavulj@dbe.uns.ac.rs

2006; Popov et al. 2008; Marjanovic et al. 2009). Recent studies have demonstrated the persistence of these compounds in ecosystems, and the possibility of their migration in the soil and subsequent release into the groundwater; spreading their impact to a much greater area beyond the spill center (Petrovic et al. 2003; Petrović et al. 2001, 2004).

During the 1999 NATO bombing, the oil refinery of Novi Sad suffered significant damage where, of 73,569 tons of oil and oil products, approximately 90% burned, 9.9% was spilled on the soil surface, and around 0.1% was leaked directly into the Danube river. The total surface area of gasoline contaminated soil was approximately 8,500 m^2, while 51,000 m^2 was polluted with crude naphtha, and 35,000 m^2 was contaminated with oil derivatives such as diesel and kerosene (Nježić and Ačanski 2009). Thus, oil leakage is a significant environmental problem due to the proximity of the Danube and the Renney wells, which supply the city of Novi Sad with water (Petrovic et al. 2003, Petrović et al. 2004). Because of this, soil cleanup technology is essential to prevent the further spread of pollution and penetration into groundwater and surface waterways.

The processes of soil remediation using mechanical and physical-chemical methods is very expensive, ranging between $100-$1,500 per ton of soil, depending on the purification/processing techniques utilized (Schnoor 1995).

In contrast to the above mentioned methods, an alternative may be the application of phytoremediation techniques which can cost ten-fold less. Phytoremediation is the use of plants and their associated microorganisms for environmental cleanup (Salt et al. 1995; Raskin et al. 1997). Phytoremediation technology uses the natural processes by which plants and rhizosphere microorganisms decompose and deposit organic and inorganic pollutants (Pilon-Smits 2005).

The decomposition of organic compounds via the activity of microorganisms of the plant rhizospere is called rhizodegradation. Rhizodegradation, also known as phytostimulation, is the degradation of contaminants in the rhizosphere (the area of soil surrounding plant roots) by means of microbial activity, coupled with secreted plant root exudates containing carbon and nitrogen compounds which provide the microorganisms with additional nutrients. Microorganisms such as yeast, fungi or bacteria consume contaminants as a source of energy and nutrition (Matavulj et al. 1983; Katanić et al. 2013). During this process of biodegradation, certain microorganisms are capable of breaking down hazardous pollutants, such as naphtha and its derivatives and other xenobiotics into nontoxic and harmless products. It is possible to select clones with improved plant-microbe interactions. Such plants would have enhanced ability to secrete natural substances which stimulate microbial activity (Maqbool et al. 2013; Ouvrard et al. 2014).

The term rhizosphere was originally described in leguminous plants by Hiltner 1904 and according to Curl and Truelove (1986) represents a zone of increased numbers of microorganisms and microbial activity on the root surface. The rhizosphere is a perfect example of co-operation, where a consortium of different microorganisms each contribute specific enzymatic activities required for synergistic, efficient degradation of complex organic compounds that no single microorganism is capable of degrading alone (Andretson et al. 1993; Walton et al. 1994).

Rhizosphere microorganisms exudate various enzymes which enable various processes of bio-transformation in the rhizosphere, including oxidation, reduction, hydrolysis, conjugation and others (Coats 1991; Lu et al. 2009). Plants can enhance the bio-degradation of organic pollutants in their rhizosphere, in a process called phyto-stimulation (McCutcheon and Schnoor 2003). Phyto-stimulation is useful for the biodegradation of hydrophobic organic compounds, such as polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), and other petroleum hydrocarbons, that cannot be absorbed, but can be broken down (Hutchinson et al. 2001).

The interaction of plants and microorganisms during phytostimulation is an example of mutual aid, since microorganisms create favorable conditions for the life of plants by detoxification of phytotoxic compounds, and by increasing the availability of some nutrients (Anhalt et al. 2000), while plants provide other nutrients for microorganisms through root exudates (Chappell 1997). In addition, intensive growth of plants together can increase the biodiversity of the rhizosphere and expand the range of possible biotransformations (Belden and Coats, 2004).

The metabolic processes performed by rhizophere microorganisms, such as enzymatically catalyzed biodegradation, are essential for the bioconversion of persistent and toxic pollutants into non-toxic or less toxic products: the essence of spontaneous or controlled bioremediation processes. These processes can be monitored by measuring the activity levels of highly specific or less specific enzymes. Phosphomonoester-hydrolases (including alkaline, acidic and neutral phosphatase enzymes) represent a universal group of enzymes present in rhizosphere microorganisms (Matavulj et al. 1976, 1990; Matavulj 1986, 1997; Babić et al. 2013). Experiments have shown that phosphomonoester-hydrolase enzymes are actively involved in the degradation of organic pollution in water and soil (Matavulj and Flint 1987; Song et al. 2006; Topalova et al. 2013), and are also involved in the purification of persistent, hard-to-degrade oil-based pollutants (Petrović et al. 1982, 2001; Matavulj et al. 1983; Jakšić et al. 2002). In addition, phosphomonoester-hydrolase activity has been shown to be a good indicator of organic load in natural freshwater (Matavulj et al. 1984, 1997, 2000; Matavulj 1997; Zlatković et al. 2010), as well as for bioremediation processes in artificial (Matavuly et al. 2002, 2003; Schneider and Topalova 2013) and natural ecosystems (Matavulj et al.

1989, 2007; Nemes et al. 2008; Todorova and Topalova 2013).

In light of the above, the aim of the present study was to investigate the rhizodegradation potential of different poplar (*Populus* spp.) clones in oil-contaminated soil, and to measure phosphomonoester-hydrolase activity in rhizosphere microflora as an indicator of bioremediation.

MATERIAL AND METHODS

Experiments were conducted in terrestrial cultures of different poplar clones under semi-controlled conditions. Varying amounts of oil-contaminated soil (0%, 5%, 25%, 50%, 75%, and 100% by volume) taken from the Novi Sad oil refinery was mixed with uncontaminated soil and used as a substrate for plant growth. The concentrations of pollution per treatment are shown in Table 1. Cuttings of following poplar clones: (I) *Populus nigra x maximowitzil* cl.9111/93; (II) *Populus deltoides* cl. "Bora" and (III) *Populus x euramericana* cl. "Pannonia", were planted in the above prepared soil. Plants were grown from May to October, and soil samples for microbiological investigations were collected in late September (Pilipović et al. 2005). Vessels without plants were used as a control, to determine the impact of plants on the growth of microorganisms. Microbiological studies consisted of: (I) determination of the total number of heterotrophic bacteria, facultative heterotrophic bacteria and naphtha-oxidizing aerobic heterotrophs; and (II) the phosphatase (acid, neutral and alkaline phosphomonoester-hydrolase) activity of rhizosphere microorganisms. Sampling was conducted in the rhizosphere zone in four places in each pot, and samples were used for a composite sample mixture. Samples were stored in a refrigerator at +4 °C until analysis (< 1 month).

Determination of the total number of microorganisms was performed using the standard pour-plate method (Gajin et al. 1987; Matavulj et al. 1996), by growth on solid agar nutrient medium in petri plates. Determination of the number of indicator groups of saprotrophic aerobic bacteria was conducted in four replicates on different media: (I) nutrient agar - to determine the total number of aerobic heterotrophic bacteria, (II) agar naphtha - to determine the total number of oil-oxidizing bacteria (Rodina 1972) and (III) ten-fold diluted agar nutrient medium - for determination of facultative-oligotrophic bacteria, the dominant group in habitats poor in easy-to-degrade organic substrates (Ishida et al. 1982; Gayin et al. 2003; Campbell et al. 2013). Previous studies suggest that oligotrophic bacteria are predominant in soil, even on carbon-rich sites such as root surfaces (Ohta and Hattori 1983).

Petri plates were incubated after inoculation at 22 °C for 7 days. After incubation, colonies were counted and total colony number expressed per 1 g of sample.

Phosphatase activity was determined using p-nitrophenylphosphate as a substrate (Matavulj and Flint 1987). Samples were transferred to spectrophotometric plates in three wells containing TTA buffer at pH 5, 7 or 9 respectively, and p-NPP (para-nitrophenylphosphate) was added. Spectrophotometer plates were incubated in a water bath at 30 °C for 60 minutes. After incubation, the reaction was stopped by addition of 10M NaOH and the yellow-colored p-nitrophenol was developed. Samples were read in a spectrophotometer (Thermo Multiskan EX Labsystens), and on the basis of these results phosphatase activity was calculated. Results were expressed using the phosphatase activity index (PAI) – the mean activity values of acid, neutral and alkaline phosphatase (Matavulj 1986, 1990).

Total petroleum hydrocarbon (TPH) and mineral oil content in soil samples were determined according to Škunca–Milovanović et al. (1990) using quantitative IR spectrophotometry. Soil porosity and air capacity was measured according to Bošnjak et al. (1997), while C/N ratio was determined by analysis of carbon and nitrogen using a CHN Elementar III analyzer, which works on the principle of oxidation and reduction of elements at temperatures of 900 °C and 500 °C, respectively. Calculations and statistical analysis of the data was performed using Microsoft Office Excel. The Excel Analysis tool pack was used for regression analysis.

RESULTS

In order to measure the effect of oil contaimination on rhizosphere bacterial growth, the total number of heterotrophic, facultative heterotrophic and naphtha-oxidizing aerobic heterotrophic bacteria were determined. As can be seen in Table 2, increased soil contamination by naphtha is associated with a reduction in the number of facultative oligotrophic bacteria in the rhizospheres of the tested poplar

Table 1. Concentrations of oil pollutants, total petroleum hydrocarbons (TPH), mineral oil and other characteristics of tested soils (g/kg soil).

% of contaminated soil	Moisture (%)	TPH (mg/kg)	Mineral oil (mg/kg)	C/N ratio	Porosity (%)	Air capacity (%vol)
0%	3.25	20	0	79.98	51.91	44.69
5%	0.26	578	254	72.16	46.01	30.88
25%	0.28	2812	1571	36.96	42.06	29.24
50%	0.44	6046	3769	23.69	40.63	28.32
75%	0.43	9311	5370	17.71	44.47	32.69
100%	0.42	10841	6174	14.31	46.08	36.34

Table 2. Number of bacteria and phosphomonoester-hydrolase activity in poplar rhizospheres.

% of contaminated soil added	Clone	Number of bacteria			Phosphatase activity
		Facultative oligotrophs (cfu/g)	Aerobe heterotrophs (cfu/g)	Naphtha-oxidizing (cfu/g)	PAI (μmol pNP/s/dm^3)
0%	Pannonia	1.29E+06	2.00E+06	1.21E+06	9.80
	9111/93	1.17E+06	2.75E+06	1.62E+06	6.95
	Bora	1.26E+06	2.69E+06	1.27E+06	6.16
	$\bar{x}$ clones	1.24E+06	2.48E+06	1.37E+06	7.64
	Control	1.09E+06	1.71E+06	1.06E+06	7.70
5%	Pannonia	1.95E+05	1.47E+06	1.03E+06	14.72
	9111/93	1.94E+06	2.29E+06	1.34E+06	12.55
	Bora	1.38E+06	2.67E+06	1.85E+06	6.05
	$\bar{x}$ clones	1.17E+06	2.14E+06	1.41E+06	11.11
	Control	1.42E+06	1.67E+06	1.65E+06	7.93
25%	Pannonia	1.86E+06	1.97E+06	3.95E+05	5.05
	9111/93	9.78E+05	2.29E+06	7.23E+05	3.48
	Bora	7.25E+05	2.11E+06	7.50E+05	8.26
	$\bar{x}$ clones	1.19E+06	2.12E+06	6.23E+05	5.60
	Control	1.51E+06	1.07E+06	9.43E+05	8.11
50%	Pannonia	8.55E+05	1.09E+06	8.70E+05	0.68
	9111/93	5.92E+05	6.20E+05	2.56E+05	6.10
	Bora	5.76E+05	7.22E+05	3.04E+05	5.09
	$\bar{x}$ clones	6.74E+05	8.11E+05	4.77E+05	3.96
	Control	1.85E+06	7.34E+05	2.07E+05	1.21
75%	Pannonia	4.06E+05	5.96E+05	2.01E+05	4.83
	9111/93	4.63E+05	8.12E+05	1.76E+05	2.03
	Bora	1.60E+05	6.33E+05	1.52E+05	1.11
	$\bar{x}$ clones	3.43E+05	6.80E+05	1,76E+05	2.65
	Control	1.80E+06	9.03E+05	1.76E+05	2.30
100%	Pannonia	5.32E+05	5.82E+05	2.18E+05	1.00
	9111/93	1.22E+05	6.21E+05	2.30E+05	0.17
	Bora	4.00E+05	3.97E+05	2.31E+05	0.91
	$\bar{x}$ clones	3.51E+05	5.33E+05	2.26E+05	0.69
	Control	1.23E+06	5.02E+05	7.61E+05	2.56

clones, as well as in control pots without plants (Table 2). With respect to total number of bacteria, an overall decreasing trend was observed. However, clones 9111/93 and "Bora", had an increased number of facultative oligotrophs at 5% oil contamination, and clone "Pannonia" had an increased number of facultative oligotrophs at 25% contamination. In control pots without plants, the number of oligotrophic bacteria was found to be higher than in vessels with plants only at 75% and 100% oil contamination.

Microbial phosphatase activity was also determined as a function of % oil contamination and expressed as the phosphatase activity index (PAI). As can be seen, there was an increase in microbial phosphatase activity in the rhizospheres of all of tested clones at 5% oil contamination, while reduced PAI values were observed at 25% oil contamination. Increased oil contamination in the growth substrate beyond 25% lead to a general decrease in microbial phosphatase activity in both plant containing pots and control pots without plants.

Results from regression analysis and coefficient of determination (see Fig. 1) revealed a positive correlation between the number of organotrophic bacteria and phosphatase activity index (PAI). The coefficient of determination (0.4475) and correlation coefficient (0.6689) were found to be significantly correlated between the analyzed parameters.

Determination of the total number of organotrophic (aerobic heterotrophs) bacteria (Fig. 1) also demonstrated that increased oil pollution in the growth substrate resulted in a reduction of organotrophic bacterial growth in the rhizospheres of the tested clones, as well as in control soil substrates without plants. At 75% oil contamination, the number of organotrophic bacteria in control pots without plants was higher than those planted with clones "Bora" and 9111/93, while the clone "Pannonia" supported the growth of less organotrophic bacteria at 5% oil contamination.

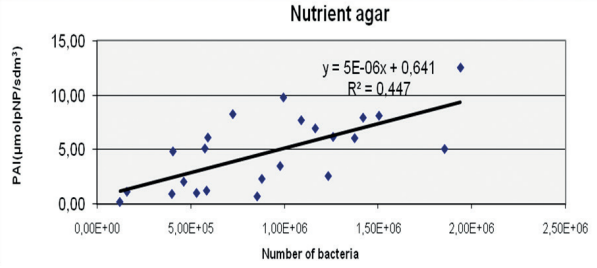

Fig. 1. Regression analysis and Coefficient of determination (R^2) between the number of aerobic organotrophic bacteria and the Phosphatase activity index (PAI).

Regression analysis and the coefficient of determination (Fig. 2) revealed a positive correlation between the number of facultative oligotrophic bacteria and phosphatase activity index. The coefficient of determination (0.408) and correlation coefficient (0.6387) suggest a significant relationship between the analyzed parameters. The total number of oil-oxidizing bacteria (Table 2) reduces with increasing soil pollution in both the rhizosphere of the studied poplar clones, as well as in control pots without plants.

However, at 5%, 50% and 75% oil contamination, the total number of oil-oxidizing bacteria was greater or equal to that measured in control soil samples from pots without plants. An exception to this trend is clone 9111/93, which displayed a reduced number of oil-oxidizing bacteria with increased oil pollution, while clones "Pannonia" and "Bora" supported higher numbers of naphtha-degrading bacteria at 5% ("Bora") and 50% ("Pannonia"). In general, unplanted pots supported less growth of aerobic saprotrophic bacteria vs. pots containing plants. Thus, bacterial populations were in most cases stimulated by the rhizosphere effect, in agreement with Cartmill et al. (2013).

Regression analysis and coefficient of determination between the number of oil-oxidizing bacteria (Fig. 3) and phosphatase activity revealed a positive correlation. In addition, in this case, the coefficient of determination (0.4277) and coefficient of correlation (0.6539) suggested a significant association between the analyzed parameters.

The relationship between phosphatase activity and the abundance of different groups of bacteria suggested that this phosphatase activity was associated with a specific group of bacteria. Moreover, an increased PAI/Oil-oxidizing bacteria (PAI/Naphtha) ratio was observed with increased soil pollution, which was highly evident at 50% and 75% oil contamination. These increases were particulary striking in the rhizospheres of clones 9111/93 and "Bora" treated with 50% oil contaminated soil; and in clones "Pannonia" and 9111/93 treated with 75% oil contaminated soil (Fig. 4).

DISCUSSION

The impact on the quantitative, as well as qualitative composition of indigenous microbial communities largely

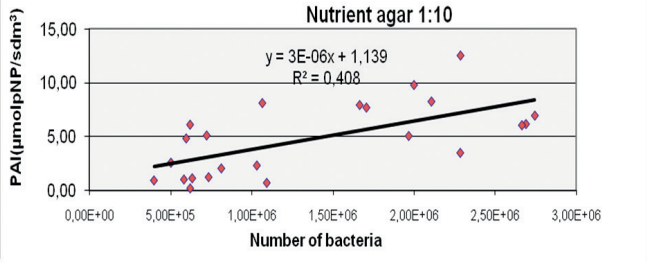

Fig. 2. Regression analysis and Coefficient of determination (R^2) between the number of facultative oligotrophic bacteria and the Phosphatase activity index (PAI).

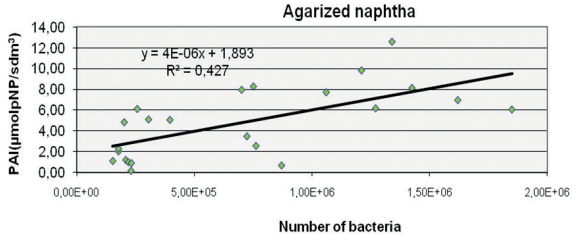

Fig. 3. Regression analysis and Coefficient of determination (R^2) between the Number of naphtha-oxidizing bacteria and the Phosphatase activity index (PAI)

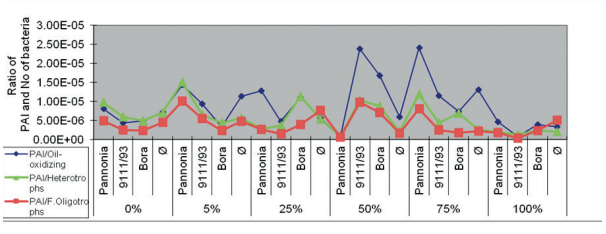

Fig. 4. The ratio of Phosphatase activity index (PAI) and number of different groups of bacteria in the rhizosphere of studied poplar clones.

depends on the particular plants in the vicinity: where different species of plants can have different effects on the local microbial community (Kirk et al. 2005). However, results of microbiological studies do not provide a complete picture of the impact of plants on microbial growth, or the degradation of oil pollution. In part this is due to the inherent complexity of the process, since interactions within the rhizosphere may greatly hamper research, understanding and subsequent conclusions (Kirk et al. 2002; Lin et al. 2002, Nie et al. 2011). Qianxin et al. (2002) found that increased oil content in soil had a greater impact on the development of *Vibrio fisheri* bacteria in the rhizospheres of plants than on the production of aboveground *Spartinia alterniflora* biomass, which can lead to underestimation of the impact of oil pollution in the soil. Wyszkowska and Wyszkowski (2008, 2010) reported that the quantitative composition and phosphatase activity of soil microorganisms depends on the dose of petroleum-derived substances. Although high doses of petroleum-derived substances cause a significant increase in the number

of microorganisms in soil, the highest dose tested (10 g per kg of soil) appears to cause a decrease in the number of oligotrophic sporulating bacteria in the examined soil.

In the present study, the total number of microorganisms and their associated phosphatase activity decreased upon increasing oil pollution more than 5%, regardless of the presence or absence of plants. These results are consistent with Chekol et al. (2004), who noted a negative correlation between the number of bacteria and levels of organic contamination with Aroclor 1248 (a PCB). Similarly, Kirk et al. (2005) reported the toxic effects of diesel on the growth of mycorrhizal fungus. Also, according to Lipińska et al. (2013), urease activity was strongly inhibited by the presence of polycyclic aromatic hydrocarbons in contaminated soil; while Ohiri et al. (2013) reported strong inhibition of acid and alkaline phosphatase activity in soil contaminated with bonny light crude oil. Pérez-Leblic et al. (2012) also found that the highest values of microbial enzyme activities correspond to landfill soil areas with the lowest concentration of hydrocarbon pollutants.

In some cases the results of these investigations suggest only a weak effect due to the presence of plants on the total number of microorganisms. For example Ionescu et al. (2009) showed that the rhizospheres of tobacco, black nightshade, horseradish and willow (*Salix caprea*) had no significant impact on the total number of microorganisms present in PCB contaminated soil vs. contaminated soil without plants. In fact, there was no apparent correlation between the total number of heterotrophs and the number of microorganisms that degrade PCBs, which was explained by variations in exudates, soil moisture and the presence of other microorganisms related to plant species (e.g. mycorrhizal fungi). In the present study, greater or equal numbers of microorganisms were found in unplanted pots compared with pots in which poplar clones were grown. This can possibly be explained by the toxic influences of very high concentrations of pollutants on the vitality of the plants, which could result in decreased development of bacteria in the rhizosphere.

The total number of microorganisms, determined via the number of viable colonies on inoculated agar media, may not be the most reliable indicator of biodegradation potential. According to Margesin et al. (2000a), only a small fraction of the total bacteria can be isolated and grown on nutrient media. Therefore, in order to monitor the intensity of microbial metabolism it is necessary to quantify biological activity directly in the soil through the determination of soil (microbial) enzymatic activity and/or soil respiration. Determination of soil enzyme activity provides information on the metabolic activity of microorganisms present, and indicates the impact of stress on a microbial population (Matavulj, 1986, Matavulj et al. 1984; Margesin et al. 2000b). These facts could explain our observed increase in the rate of PAI and the number of oil-oxidizing bacteria. Small differences in terms of the number of bacteria and enzymatic activity between vessels with and without plants can be explained by the fact that samples were taken at the very end of the experiment, which according to Lee et al. (2008), is a period when accessible, easy-to-degrade hydrocarbons are already consumed through microbial metabolism, resulting in a reduction in their numbers: the same investigators showed a reduction in enzymatic (dehydrogenase) activity due to the increased presence of PAHs in soil.

The decrease in microbial enzymatic activity at elevated oil pollution levels observed in this experiment can also be explained by a lack of nitrogen and phosphorus, which was confirmed earlier by Margesin et al. (Margesin et al. 2000a, Margesin 2007), who reported an increase in lipase and catalase activity after addition of different fertilizers to soil contaminated with naphtha. With respect to the utility of stimulus measures for enhanced degradation of petroleum hydrocarbons, similar results were obtained by Hawrot and Nowak (2006), who determined that additional soil fertilization coupled with periodic agitation (for aeration) had a significant impact on the degradation of pollutants in contaminated soils inoculated with microorganisms.

Soil enzyme activity can be used as an indirect indicator of the impact of additional measures on contaminant degradation. For example, Wyszkowski et al. (2006) found that increased fertilization or addition of organic matter (in the form of pine sawdust) resulted in increased microbial enzymatic activity in oil polluted soil: specifically increased dehydrogenase, urease and alkaline phosphatase activity. Thavasi et al. (2011) discovered that the addition of fertilizers and surfactants results in increased degradation of crude oil by cultured marine strains of three different types of bacteria. Onneby et al. (2006) showed that the rhizosphere of basketwork willow (*Salix viminalis*) grown in soil contaminated with creosote had a greater abundance of bacteria and a higher degradation of some PAHs compared with pots without plants. This phenomenon was explained by the increased presence of surface-active substances of biological origin.

Other factors also impact microbial populations and associated enzymatic/degradation activities, including watering regimens, treatment duration and the addition of stimulatory supplements. In a study conducted from 2003 to 2005, Truu et al. (2009) showed that watering willows using secondary treated wastewater increased the number of microorganisms, soil enzyme activity and nitrogen mineralization in the willow rhizosphere compared to the rhizospheres of untreated control plants. Also, an increase in the tested parameters correlated with the duration of treatment.

A possible cause of the small number of microorganisms and low degradation activity in contaminated soils could be the bioavailability of petroleum hydrocarbons in soil, which decreases with the age of the substrate. Thus, older contaminated soils are less toxic to plants and microorganisms since contaminants are more or less bonded to soil particles, making them less accessible for degradation (Tesar

et al. 2002). In the present study, the contaminated soil used was the product of many years of pollution from a Novi Sad oil refinery. Therefore, a smaller number of microorganisms and reduced enzymatic activity is inevitable in treatments containing a higher proportion of polluted soil.

Taking into account results observed in the present study, in addition to plants whose exudates stimulate the growth of microorganisms, inoculation of a consortium of microorganisms capable of more rapid degradation of petroleum hydrocarbons is necessary to improve petroleum hydrocarbon degradation in contaminated soil. In addition to the degradation of contaminants, such inoculated microorganisms also improve conditions for plant development.

Interactions in the rhizosphere can be divided into two groups (Barea et al. 2005): (I) those based on dead plant material (decomposing interactions), which affect the circulation of energy and nutrients in the soil; and (II) those based on the living plant root. Both types are of great importance in agriculture and ecology. For example, Gunderson et al. (2007) inoculated the rhizosphere of a hybrid poplar with the fungus *Pisolithus tinctorus*, resulting in increased degradation rates and increased root biomass productivity in treated plants, as well as increased nitrogen and phosphorus absorption in plant leaves. However, inoculated microbial communities are often overgrown with autochthonous microflora already adapted to conditions in the rhizosphere. Therefore, as the best solution, isolation and laboratory amplification of indigenous microflora is imposed. Subsequent inoculation of contaminated soil and the plant rhizosphere with extra stimulation by the addition of supplements would provide an initial source of carbon for survival in the initial stages of rhizodegradation.

In general, in the present study differences in support of the growth of rhizosphere bacteria between poplar clones was not observed. These results are consistent with the findings of Jordahl et al. (1997), who reported that microorganisms in the rhizosphere of poplars were not exposed to selection pressure in terms of the proportions of specific groups of microorganisms in thte consortium. According to Tesar et al. (2002), a greater impact is due to the presence of diesel, rather than clonal specificity, while Banks et al. (2003) came to a similar conclusion using different genotypes of *Sorghum bicolor* in experiments investigating phytoremediation of soil contaminated with crude oil.

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