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Bioremediation of soil heavily contaminated with crude oil and its products: composition of the microbial consortium

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Abstract: Bioremediation, a process that utilizes the capability of microorganism to degrade toxic waste, is emerging as a promising technology for the treatment of soil and groundwater contamination. The technology is very effective in dealing with petroleum hydrocarbon contamination. The aim of this study was to examine the composition of the microbial consortium during the *ex situ* experiment of bioremediation of soil heavily contaminated with crude oil and its products from the Oil Refinery Pančevo, Serbia. After a 5.5-month experiment with biostimulation and bioventilation, the concentration of the total petroleum hydrocarbons (TPH) had been reduced from 29.80 to 3.29 g/kg (89 %). In soil, the dominant microorganism population comprised Gram-positive bacteria from actinomycete-*Nocardia* group. The microorganisms which decompose hydrocarbons were the dominant microbial population at the end of the process, with a share of more than 80 % (range 10^7 CFU/g). On the basis of the results, it was concluded that a stable microbial community had been formed after initial fluctuations.

Keywords: bioremediation; microbial consortia; petroleum contamination.

INTRODUCTION

Bioremediation is a modern method in which the natural ability of microorganisms is employed for the reduction of the concentration and/or toxicity of various chemical substances, such as petroleum derivatives, aliphatic and aromatic hydrocarbons, industrial solvents, pesticides and metals.[1]

Some microorganisms can decompose or transform the chemical substances present in petroleum and petroleum derivatives. Hydrocarbons from crude oil represent substrates for microorganisms, hence, when an accidental oil spill occurs,

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the number of hydrocarbon degrading microorganisms in the ecosystem increases. A single microorganism can degrade only certain types of petroleum compounds, but a mixed population – microbial community enables a higher level of degradation. Moreover, some substances can be decomposed only by cometabolism. In natural conditions, the presence of microorganisms that use the products of primary degradation is of particular importance. Further decomposition of crude oil is stimulated by the removal of its degradation products.

The speed and efficiency of bioremediation of a soil contaminated with petroleum and petroleum products depends on the number of hydrocarbon-degrading microorganisms in the soil. The most important factors for population growth are temperature, oxygen, pH, content of nitrogen and phosphorus, hydrocarbon class and their effective concentration. The degree and rate of biodegradation are influenced by the type of soil in which the process occurs.[2,3]

The specific metabolic activity of microorganisms in biodegradation experiments could be monitored by molecular biological methods, fatty acid profiling and by classical microbiological methods.[4] The first two methods enable an in detail taxonomical identification and an estimation of the non-cultivatable microorganisms. Despite being time consuming, classical methods are often used not only as a community characterization method but also as a tool for the isolation of microorganisms with the desired characteristics, particularly with substrates of low degradability.

Various authors studied metabolically active microbial communities – consortia during petroleum bioremediation under laboratory condition or as field experiments.[5–8] For example, Groudeva[8] studied the composition of a metabolically active consortium during bioremediation of water and wetlands contaminated with crude oil and heavy metals.

This work represents a continuation of our research in the area of the biodegradation of hydrocarbons in water and sediments on the area of the Pančevo Oil Refinery, Serbia.[9–12] This paper examines the composition of the microbial consortium during the *ex situ* experiment of bioremediation of 150 m^3 of soil heavily contaminated with petroleum and petroleum products from the oil refinery complex. The experiment was performed from May to October 2006.

EXPERIMENTAL

In the complex of the Oil Refinery Pančevo, a 150 m^3 in volume bioremediation pile ("open bioreactor") of a watertight base of soil contaminated with petroleum and petroleum derivatives was made. The natural aeration was stimulated by a system of perforated pipes. Bioremediation was performed by adding organic amendments – poultry manure as an N and P source and sawdust to improve the soil texture. The pile was protected from direct external influences by a "green house".

Every 15 days, six composite samples were prepared by the zigzag technique and used for all determinations.

The number of microorganisms was determined by the method of serial dilution on agar plates incubated on 26 °C. For the total count of bacteria, Nutrient agar was used; for fungi, Malt agar with streptomycin; for Gram-negative bacteria, McConkey agar[13] and for microorganisms which decompose hydrocarbons, mineral base medium[14] with different carbon sources: 2000 ppm diesel fuel, 375 ppm toluene or 200 ppm phenanthrene.[15] Actinomycetes, *Nocardia* and *Pseudomonas* as well as cellulose decomposing bacteria and fungi were determined using selective media.[16,17]

The content of total petroleum hydrocarbons (TPH) in the soil was determined according to DIN EN 14345:2004. Gas chromatography was performed using an Agilent 4890D instrument with an FID detector (column: HP-1MS 30 m×0.25 mm; carrier gas: hydrogen; injector temperature: 250 °C; initial temperature 40 °C, isothermal at 285 °C for 12 min).[18]

RESULTS AND DISCUSSION

The examined soil contaminated with petroleum and petroleum derivatives originated from the Oil Refinery Pančevo, where pollution is high and chronic. The soil initially contained 29.99±1.88 g TPH/kg. Since preliminary experiments (the results are not given) revealed the presence of hydrocarbon degraders in the soil, the growth of microorganisms was stimulated by the addition of N and P sources, aeration and mixing. In this way by application of bioremediation techniques, the concentration of total petroleum hydrocarbons (TPH) was reduced to 3.29 g/kg (89 %) during the five-and-half month experiment. The changes of the TPH as well as the GC chromatogram of the initial and the last sample are shown in Fig. 1.

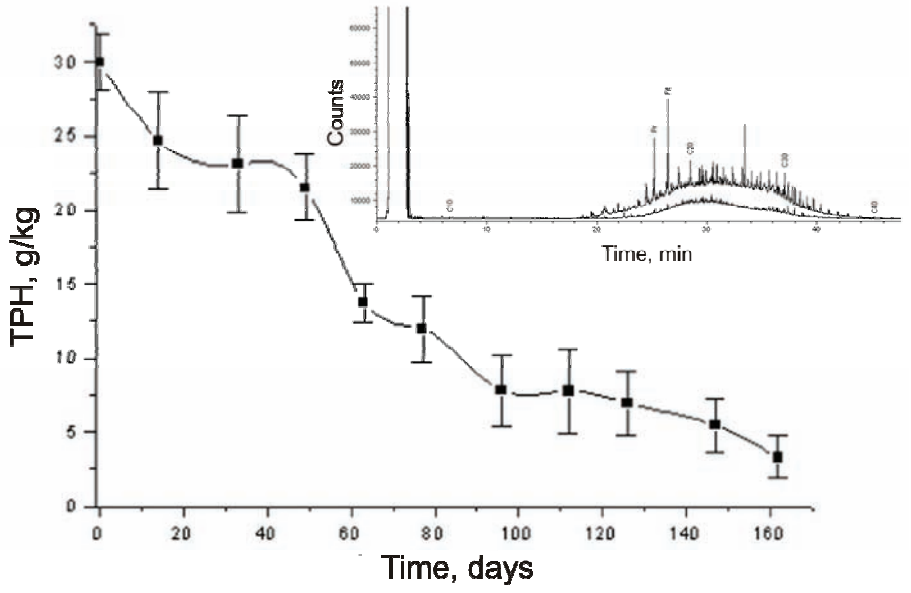

Fig. 1. Time course of the hydrocarbon concentration during the biodegradation process (all results are expressed on the basis of dry weight; mean±*SD*, *n* = 6).

It is noticeable that the highest reduction of TPH occurred in the period from the 49th to the 96th day. Apart from that, it should be pointed out that until the 63rd day, practically 50 % of the hydrocarbons had been decomposed. In initial sample, the hydrocarbons were partially biodegraded so that the part of easily degradable alkane fraction of crude oil was removed, which generally reduces the rate of further degradation.[3,6,12]

As mentioned in the introduction, studies of microbial consortia are of great importance for the best understanding of the biodegradation process. In this study, specific microorganisms groups were chosen on the basis of the general role of microorganisms in the cycling of carbon, nitrogen and phosphorus in soil. In addition, it is well known that the best hydrocarbon degraders are bacteria from the genera *Nocardia*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Arthrobacter*, *Corynebacterium*, *Achromobacter*, *Rhodococcus*, *Alcaligenes*, *Mycobacterium* and *Bacillus* and the fungi *Rhodotorulla*, *Fusarium*, *Aspergillus*, *Mucor*, *Penicillium*, *Candida* and *Sporobolomyces*.[19]

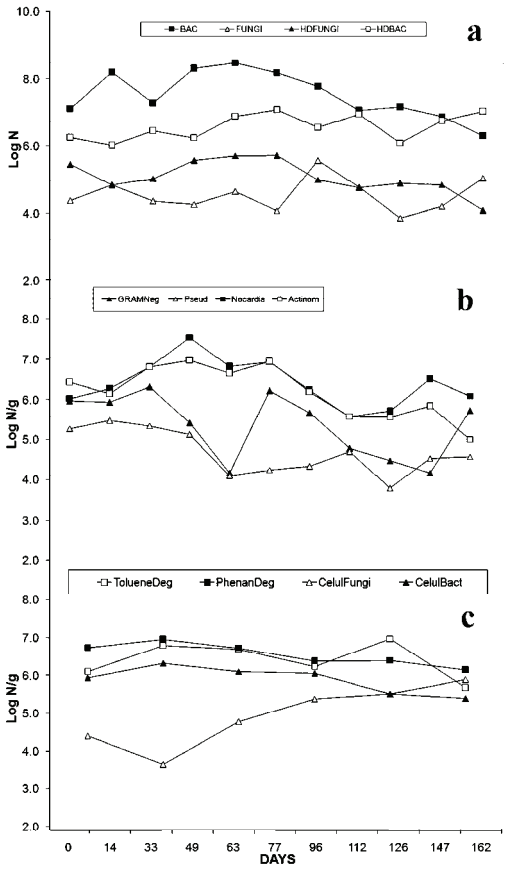

Fig. 2. Changes in the number of microorganisms during the biodegradation process (BAC-total, count of bacteria; FUNGI-total, count of fungi; HDBAC, hydrocarbon degrading bacteria; HDFUNGI, hydrocarbon degrading fungi; GRAM-Neg, Gram-negative bacteria; Pseud, *Pseudomonas*; Nocardia, *Nocardia*; Actinom, *Actinomycete*; ToluneneDeg, toluene degraders; PhenanDeg, phenanthrene degraders; CeluFungi cellulolytic fungi; CeluBac, cellulolytic bacteria).

The changes in the number of microorganisms in specific physiological-biochemical groups are given in Fig. 2. Most of these microorganisms were members of the indigenous microflora and some were introduced into the system with the organic amendments added to the soil in order to stimulate biodegradation.

The highest number of microorganisms was attained between 7–14 weeks of the process. The total number of bacteria in the soil during bioremediation was in range 10^7–10^8 CFU/g dry soil, and yeast and molds 10^4–10^5 CFU/g. The number of hydrocarbon-degrading bacteria was 10^6–10^7 CFU/g, and hydroarbon-degrading fungi 10^5–10^6 CFU/g (Fig. 2a). The number of toluene and pheanthrene degraders was 10^5–10^6 CFU/g (Fig. 2c). The dominant population in the soil comprised Gram-positive bacteria from the actinomycete-*Nocardia* group (10^6–10^7 CFU/g), while the total number of Gram-negative bacteria (10^5–10^6 CFU/g) and members of genus *Pseudomonas* (10^4–10^5 CFU/g) was slightly smaller (Fig. 2b). The number of cellulolytic bacteria was in the range 10^5–10^6 CFU/g, and that of cellulolytic fungi was 10^4–10^5 CFU/g (Fig. 2c).

On the basis of the results presented in Fig. 2, it is apparent that a stable microbial community was formed during the bioremediation experiment.

CONCLUSIONS

The metabolically active microbial community played the key role in the hydrocarbon biodegradation. The maximum number of microorganisms in the consortium was achieved from the 49th to the 96th day of the experiment and simultaneously, the highest rate of hydrocarbon degradation occurred. During the bioremediation experiment, there were no significant changes in the relationships between particular microbial populations, probably because the soil used in the experiment had a high hydrocarbon content and the contamination was aged and chronic.

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И З В О Д

БИОРЕМЕДИЈАЦИЈА ЗЕМЉИШТА ТЕШКО КОНТАМИНИРАНОГ НАФТОМ И НАФТНИМ ДЕРИВАТИМА: САСТАВ КОНЗОРЦИЈУМА МИКРООРГАНИЗАМА

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Биоремедијација, процес који користи способност микроорганизама да разлажу токсични отпад, представља савремени тренд у пречишћавању загађеног земљишта и подземних вода. Ова технологија је веома ефикасна у уклањању контаминација нафтног загађивача. Циљ овог рада је био карактеризација конзорцијума микроорганизама при *ex citu* биоремедијацији земљишта тешко контаминираног нафтом и њеним дериватима из Рафинерије нафте,

Панчево. После 5,5 месеци експеримента уз биостимулацију и биовентилацију концентрација укупних угљоводоника нафте је смањена са 29,80 на 3,29 g/kg (89 %). Доминантна популација у земљишту укључује Грам-позитивне бактерије из групе актиномицета-*Nocardia*. Микроорганизми који разграђују угљоводонике на крају процеса су били доминантна микробна популација са уделом преко 80 % (ред величине 10^7 CFU/g). На основу ових резултата се може закључити да је, након почетних промена, дошло до формирања стабилне микробне заједнице.

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