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Evolution of humic acids during *ex situ* bioremediation on a pilot level – The added value of the microbial activity

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Abstract: Environmental pollution is a global problem, while bioremediation technology removes pollutants from the environment using microorganisms. This study was aimed at investigating how a bioremediation process affected soil humification. In soil polluted with petroleum and its derivatives that was submitted to bioremediation, besides the total petroleum hydrocarbons and the number of microorganisms, quantitative and qualitative changes of isolated humic acids were determined during the process. The bioremediation of 150 m^3 of polluted soil lasted 150 days. The level of total petroleum hydrocarbons decreased by 86.6 %, while the level of humic acids increased by 26.5 %. The elemental analysis showed the reduction of C and the H/C ratio and the increase of O and the O/C ratio of isolated humic acids during the process. The ratio of absorbencies at 465 and 665 nm also increased. Based on this and the Fourier-transform infrared spectra, it was shown that the humic acids isolated at the end of bioremediation were enriched with oxygen functional groups and aromatic structures. This study provides one of the first insights into the relationship between bioremediation and humification, as well as evidence of how hydrocarbon-degrading microorganisms have a significant influence on changes to humic acid structure during bioremediation.

Keywords: remediation; humification; total petroleum hydrocarbons.

INTRODUCTION

Petroleum and its derivatives are widely used in all domains of life. These compounds are extensively used as a energy sources for transportation, heating, and the generation of electricity.[1] Pollution of soils, sediments, and waters by petroleum products is a substantial environmental problem.[2,3] Pollution occurs

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due to accidental releases of hazardous waste during extraction, refining, transport, storage and the use of petroleum and its derivatives.[4,5] However, the persistence of these compounds in the environment and their toxic effects can cause risks for humans and other living organisms.[1,5,6] Bioremediation is a method of remediating petroleum pollution by applying selected microorganisms to polluted environmental substrates. These non-pathogenic microbes can degrade or transform toxic substances into harmless products.[1,2] Bioremediation is low-cost and environmentally friendly remediation technology. This process is in harmony with the principles of sustainable development, and it has become widely used in recent decades.[3,7]

During bioremediation of sites polluted with petroleum and its derivatives, substances similar to humic compounds are created.[8,9] Wu and colleagues[10] found similar sequential biological processes in composting. This is very important because humic substances (HSs) have a tremendously positive influence on soil quality.[9,11,12]

HSs, heterogeneous organic macromolecules, are composed of a soil fraction soluble at all pHs – the fulvic acids (FAs), plus humic acids (HAs) that are soluble in neutral to alkaline pHs, and an insoluble fraction – humin.[9,11,12] The essential components of HSs are HAs, and their study is important for understanding many processes in the environment.[13] HAs are composed of quinone, phenolic, enolic and carboxylic acid functional groups, and as a result, they have numerous uses.[11,14,15] Scientific reports about soil and sediment humification are plentiful, but there is not enough data about the humification process during bioremediation and biodegradation. Since HAs are of vital importance for soil quality, their quantity should always be determined in bioremediation processes.

The aims of this study were to: 1) investigate the relationship between bioremediation and humification processes and to determine quantitative and qualitative changes of humic acids isolated during bioremediation of soil polluted with petroleum and its derivatives and 2) confirm the hypothesis that during bioremediation of soil polluted with petroleum hydrocarbons, the evolution of humic acids will be stimulated to a higher degree than in non-polluted control soil.

EXPERIMENTAL

Experimental biopiles

The biopile for *ex situ* bioremediation consisted of 150 m^3 soil polluted with petroleum and its derivatives. The control biopile (5 m^3) consisted of non-polluted soil sampled from a location near the biopile. The geometry of the biopiles was a three-sided parallelepiped.

The ratio of length, width and depth were 3.75:1:0.02, respectively. Both biopiles contained added sawdust and wood chips, to increase the water holding capacity and aeration, an alternative source of carbon, BioSolve®CLEAR, to increase dispersion of petroleum hydrocarbons in water, and to increase the contact surface between microorganisms and lipophilic pollutants, as well as manure from a poultry farm as a source of nitrogen and phosphorus for the biostimulation of the process. Finally, an enriched consortium of hydrocarbon-degrading

microorganisms was added every two weeks to enhance microbial activity on both biopiles. The consortium was prepared as previously described.[16] Dominant genera were *Pseudomonas*, *Nocardia* and *Rhodococcus.* This pilot bioremediation study lasted 150 days.

Sampling

In order to collect the most representative samples, biopile soil samples were taken by the zigzag sampling method[17] with an Eijkelkamp auger soil sampler. Approximately 20 soil samples were then mixed and homogenized, thereby producing one composite biopile sample. Four composite samples for analysis were collected on each sampling day, at the beginning of bioremediation (designation S0), after 60 days (S60), after 120 days (S120) and after 150 days (S150). Control samples were taken at the same time from the control biopile.

Determination of the number of microorganisms in the composite polluted biopile samples

During bioremediation of polluted soil, the number and composition of microbial consortia were monitored. The number of total chemoorganotrophs (TC), *i.e.* aerobic and facultative anaerobic and mesophilic bacteria, was determined on nutrient agar (15.0 g peptone I, 3.00 g meat extract, 5.00 g NaCl, 0.30 g K_2HPO_4, 18.0 g agar and 1 L deionized water; pH 7.30). Microorganisms that decompose hydrocarbons (HD) were determined on a mineral agar (1.00 g NH_4NO_3, 0.25 g $CaHPO_4$, 50.0 mL soil extract, 16.0 g agar and 1 L deionized water) supplemented with 2.00 g L^{-1} diesel D2 as a source of hydrocarbons.[18] Malt agar was used to determine numbers of yeasts and molds (YM, 5.00 g peptone I, 30.0 g malt extract, 15.0 g agar and 1 L deionized water; pH, 5.40). Composite biopile samples were homogenized and appropriate serial dilutions were plated on agar plates. All plates were incubated at 28 °C, for 48 h (TC and YM) and for 7 days (HD).[19,20]

The presence of genera important for the bioremediation process, *Pseudomonas*, *Nocardia* and *Rhodococcus*, were determined using the following media: *Pseudomonas* was analyzed on *Pseudomonas* isolation agar (20.0 g peptone I, 1.40 g $MgCl_2 \cdot 6H_2O$, 10.0 g K_2SO_4, 25.0 mg irgasan, 13.6 g agar, 20.0 mL glycerol and 1 L deionized water).[21] This selective medium includes irgasan, a broad-spectrum antimicrobial agent. Irgasan is not active against *Pseudomonas* spp. and it was added after sterilization. *Nocardia* and *Rhodococcus* were analyzed on M3 agar (0.47 g KH_2PO_4, 0.73 g Na_2HPO_4, 0.01 g KNO_3, 0.29 g NaCl, 0.10 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 200 μg $FeSO_4 \cdot 7H_2O$; 180 μg $ZnSO_4 \cdot 7H_2O$; 20.0 μg $MnSO_4 \cdot 4H_2O$, 0.20 g sodium propionate, 18.0 g agar and 1 L deionized water).[22] After sterilization in an autoclave, actidion (50.0 mg L^{-1}) and tiamin HCl (4.00 mg L^{-1}) were added. Actidion prevent growth of yeast and fungi. Tiamin HCl solution was sterilized through a 0.45 μm pore filter.

Determination of total petroleum hydrocarbons

The total petroleum hydrocarbons (TPH) from both the polluted and nonpolluted biopiles were extracted[23] and determined gravimetrically.[24] The values are expressed as g kg^{-1} of dry weight (g kg^{-1} d.w.). A more detailed analysis of polluted biopile samples was conducted using a gas chromatograph (Agilent 7890A) with a flame ionization detector (FID). The HP-5 column was 30.0 m long, 0.32 mm in diameter and with 0.25 μm thickness of stationary phase. The flow rate of the hydrogen carrier gas was 2.00 mL min^{-1}. The starting temperature was 40.0 °C, the injector temperature was 250 °C, and the detector temperature was 300 °C, while the temperature was ramped up at 4.00 °C min^{-1}. ChemStation, Agilent Technologies, was used to process the data. The analysis was performed in triplicate. The error in the method was about 5.70 %.

Determination of group composition of the composite polluted biopile samples

Soxhlet extraction using an Omnilab FoodALYT RS 60 was used to extract the organic compounds from the composite polluted biopile samples (acetone:*n*-hexane 1:1, 130 mL, 24 h). After extraction, the samples were evaporated to dryness and submitted to fractional separation of the organic substances based on a previously described method.[25] In short, the extracts were saponified with a solution of KOH in methanol and neutralized with 10 % hydrochloric acid. The products were dissolved in a mixture of dichloromethane and hexane and individually fractionated by column chromatography on alumina and silica gel into saturated hydrocarbons, aromatic hydrocarbons, polar fraction (alcohols and ketones) and fatty acids (the group composition). All fractions were eluted with different solvent mixtures.[25] Values are expressed as g kg^{-1} d.w. The analysis was performed in triplicate. The results were processed by Microsoft Office Excel 2007 program.

Extraction of humic acids

Air-dried composite samples (40.0 g) from both the polluted and nonpolluted biopiles were separately mixed with $NaOH/Na_4P_2O_7$ solution (200 mL).[26] The mixtures were heated (boiling water bath with shaking, 2 h) and cooled to room temperature. The supernatant was separated by centrifugation (3000 rpm, 10 min) from each mixture, and the HAs were precipitated from supernatant (with 6.00 M HCl to pH 1). After repeated centrifugation, the supernatant was discarded. The HAs were purified according to International Humic Substance Society and Jednak *et al.*[9] The analysis was performed in triplicate. The error in the method was about 4.50 %.

For quantitative and qualitative analysis of isolated humic acids from the composite polluted biopile samples, the following methods were used: elemental analysis, E_4/E_6 ratio (ratio of absorbencies at 465 and 665 nm) and Fourier-transform infrared spectroscopy (FTIR).

Elemental analysis of the isolated humic acids

Contents of C, H, N and S in dry composite polluted biopile samples (dried at 105 °C, 2 h) were determined using a Vario EL II CHNS/O elemental analyser. The oxygen percentage was obtained by subsequent calculation (O content = 100 % – (C content, % + H content, % + N content, % + S content, %).

E_4/E_6 *Ratio of the isolated humic acids*

The E_4/E_6 ratios of the isolated HAs were calculated based on the ratio of the absorbancies at 465 and 665 nm. Absorbances of solution (30.0 mg of HAs in 100 mL of 0.05 M $NaHCO_3$) were recorded using a UV–Vis spectrophotometer (Shimadzu, UV-1280).[9,27,28]

Fourier-transform infrared spectroscopy of the isolated humic acids

FTIR spectra of HAs were recorded on an FTIR spectrometer (Nicolet 6700 from Thermo Nicolet Corp., Madison, WI, USA), in the wavelength range of 4000–400 cm^{-1}. The samples were prepared before analysis (making pellets consisting of isolated HAs and KBr).

RESULTS AND DISCUSSION

Change in TPH content and number of microorganisms during bioremediation

Gravimetric analysis showed the initial level of *TPH* (0 days) was 21.6 g kg^{-1} d.w. At the end of the study (after 150 days of bioremediation), the *TPH* in the polluted biopile had been reduced by 86.6 % (concentration at the end was 2.89 g

kg^{-1} d.w.). Gas chromatograms of the samples from the polluted biopile at the start and end of bioremediation are shown in Fig. 1.

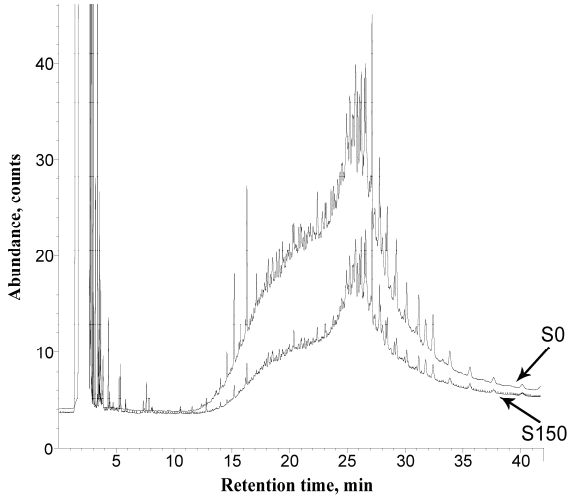

Fig. 1. Gas chromatograms of samples from the polluted biopile, S0 – at the start and S150 – at the end of bioremediation.

In the control biopile, there was a decrease of *TPH* from 0.36 to 0.34 g kg^{-1} d.w. (the *TPH* were reduced by 5.55 %). The content of *TPH* and the numbers of hydrocarbon-degrading microorganisms during bioremediation are shown in Fig. 2.

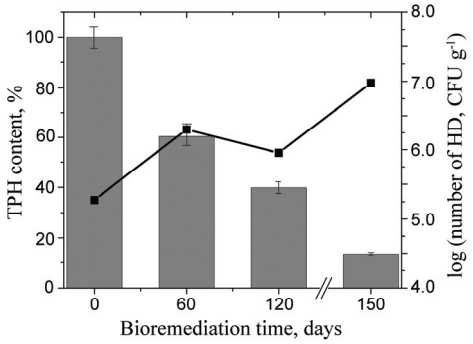

Fig. 2. The histogram shows the content of *TPH*; the line shows the increase in the number of hydrocarbon-degrading microorganisms during bioremediation of the polluted biopile (*CFU* – colony-forming units).

After the initial biopile inoculation, a stable microbial community was formed (*TC* 1.05×10^6, *HD* 1.88×10^5, *YM* 2.00×10^3 CFU g^{-1}). At the end of the bioremediation of polluted soil, 79.2 % of the microorganisms were hydrocarbon degraders, so these formed the dominant microbial population (Table S-I of the Supplementary material to this paper). In the microbial consortium, the presence of the genera *Nocardia*, *Pseudomonas* and *Rhodococcus* was confirmed. The lite-

rature shows these genera degrade petroleum and its derivatives with high efficiency.[1–3,5]

Determination of group composition of the composite polluted biopile samples

Fractional separation of organic matter extracted from composite polluted biopile samples resulted in three classes of compounds (Fig. 3): saturated hydrocarbons, aromatic hydrocarbons, and the alcohols and ketones. A fatty acid fraction was not obtained.

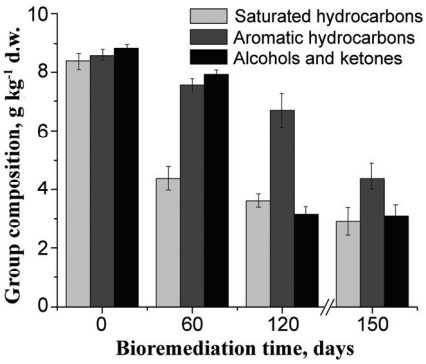

Fig. 3. Aliphatic, aromatic, and polar (alcohols and ketones) fractions of the composite polluted biopile samples during bioremediation.

The concentration of the aliphatic fraction gradually decreased during the bioremediation process and at the end of the study was significantly lower than at the beginning (decreased from 8.28 to 2.80 g kg^{-1} d.w.). This fraction degraded the most, *i.e.*, by 66.2 %, which is in accordance with the literature.[16] The concentration of the aromatic fraction was also reduced by the end of the bioremediation process, down from 7.68 to 4.50 g kg^{-1} d.w. This 41.4 % reduction in the aromatic fraction could have been caused by microorganisms that used hydrocarbons as a source of carbon and energy or caused by microorganisms that used the aromatic fraction for the synthesis of polymeric, humic-like substances.[8,9] The alcohols and keto fraction decreased by 64.4% (from 8.84 to 3.15 g kg^{-1} d.w.). Microorganisms with different rates of catabolism under varying conditions degrade various fraction of hydrocarbons.[29,30] The rates of decrease (mg kg^{-1} per day) measured from the start to the end of the process in the current study were 36.5 for the aliphatic, 21.2 for the aromatic, and 37.9 mg kg^{-1} per day for the alcoholic and keto fraction. These results show that microorganisms consumed all the components of the hydrocarbon mixture. A significant reduction in the concentration of organic pollutants in the soil indicates successful bioremediation.

Quantitative and qualitative analysis of humic acids

Elemental analysis and E_4/E_6 *ratio of isolated HAs*. At the beginning of bioremediation, the polluted biopile contained 2.16 % HAs, while at the end of bioremediation, the biopile contained 2.94 % HAs. The increase in the level of HAs

during the bioremediation process was 26.5 %. This indicates the processes of petroleum hydrocarbon biodegradation and humification occurred in parallel. The rates of humification were: 0–60 days, 38.3; 60–120 days, 55.0, 120–150 days, 73.3 mg kg^{-1} per day. In the control, non-polluted biopile, the HAs increased by 2.80 % during the study.

TABLE I. Content of humic acids in samples of the composite polluted biopile; result of elementary analysis and H/C, O/C, E_4/E_6 ratios contained in the isolated humic acids

Bioremediation day	Amount of isolated HAs, %	Elemental composition of the isolated HAs, %					Atomic ratio		E_4/E_6 ratio	Duration of treatment
		C	H	N	S	O	H/C ratio	O/C ratio		
0	2.16	63.11	8.76	3.40	1.50	23.23	1.67	0.28	2.25	–
60	2.39	61.85	8.48	3.56	1.60	24.51	1.65	0.30	2.35	–
120	2.72	60.74	8.19	3.23	1.36	26.48	1.62	0.33	2.48	–
150	2.94	59.80	7.54	3.33	1.36	27.97	1.51	0.35	3.91	–
Literature values										
Jednak *et al.*[9]						26.25–28.93	1.56–1.69	0.32–0.37	1.97–2.30	90 days of bioremediation
Amir *et al.*[8]		47.50–48.90	6.55–6.90	6.80–7.30	0.70–1.20	36.20–37.60	1.66–1.73			135 days of composting
Yang *et al.*[31]		44.66–58.46	5.31–8.01	4.26–6.66	1.07–1.22	26.33–38.29	1.36–1.77	0.34–0.64		Thermal sludge treatment, 30 min

For extracted HAs at the start of the process, elementary analysis showed the content of carbon and the calculated H/C ratio were higher than for HAs extracted at the end of the process (Table I). This could be attributed to an increase of aromatic structures during bioremediation.[8,9,31] During bioremediation, the oxygen content increased from 23.23 to 27.97 %, the calculated O/C ratio increased from 0.28 to 0.35 and the E_4/E_6 ratio increased from 2.25 to 3.91, which indicates enrichment of HAs with oxygen functional groups.[9,15,31,32] Table I also shows some comparative values extracted from published literature. They show significant variations in values, most likely due to the diversity in the structure of humic acids.

FTIR spectra of isolated HAs. In the FTIR spectrum (Fig. S-1A–D of the Supplementary material), the following signals were present: aromatic C=C, C=O in carboxyl; ketone, quinone groups and amide I (the signal 1600–1660 cm^{-1}); aromatic C=C and amide II (the signal about 1508 cm^{-1}); OH of phenols, COO^- and amide II (signals between 1460 and 1370 cm^{-1}); aromatic ethers C–O–C and amide III (signals between 1260 and 1200 cm^{-1}).[8,31]

Based on the intensity of the peaks in the FTIR spectra, transformation of HAs was observed during bioremediation. Changes were monitored by com-

paring the peak intensity and calculating the ratio of peaks 1654.1/2920.5 (aromatic C/aliphatic C) and 1654.1/2850.8 (aromatic C/aliphatic C, Table II. The ratio of aromatic carbon to aliphatic carbon clearly increased during bioremediation. This indicates that HAs contained more aromatic structures at the end of bioremediation, which was also observed in the elementary analysis (Table I). This slight increase in the aromaticity of HAs could be caused by microorganisms that can transform aliphatics to aromatics and can form polymer humic compounds.[8,9,31]

TABLE II. The ratio of the intensity of the FTIR analysis peaks during bioremediation of the polluted biopile

Ratio between the intensities of peaks at 2920.5, 2850.8 and 1654.1 cm^{-1}	Bioremediation day			
	0	60	120	150
1654.1/2920.5	1.10	1.10	1.17	1.30
1654.1/2850.8	1.31	1.36	1.56	1.70

CONCLUSION

This study highlights the correlation between the bioremediation of soil polluted with petroleum and its derivatives and the process of humification. With increasing duration of bioremediation, the level of TPH decreases. It should be emphasized that sawdust and wood chips, together with an important part of poultry manure (straw) are lignocellulosic wastes, the biodegradation of which produces humic substances. Based on our hypothesis, it is expected that the polluted soil after treatment will be cleaned from hydrocarbons while simultaneously, the content of humic substances will be increased. It is known that white and gray rot mushrooms degrade lignin and co-metabolic polycyclic aromatic hydrocarbons. However, it has here been confirmed that microorganisms that are hydrocarbon degraders also play a role in co-metabolic degradation of lingocellulosic material and increased humification of the soil. Based on the obtained results, it could be concluded that not only did the amount of HAs increase, but their structures underwent profound changes. This was demonstrated by structural analyses, elemental analysis and FTIR spectroscopy. These results show that at the end of the bioremediation process, both the aromaticity and level of oxygen groups in the HAs isolated increased. In further work, the emphasis will be on detecting enzymes responsible for the degradation of aromatic compounds and co-metabolic production of humic acids.

SUPPLEMENTARY MATERIAL

Additional data are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

НАСТАНАК ХУМИНСКИХ КИСЕЛИНА ТОКОМ *ex situ* БИОРЕМЕДИЈАЦИЈЕ НА ПИЛОТ НИВОУ – ДОДАТА ВРЕДНОСТ МИКРОБНЕ АКТИВНОСТИ

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Загађење животне средине представља светски проблем, а биоремедијација је технологија којом се контаминанти уклањају из животне средине употребом микроорганизама. Циљ овог рада био је да се испита како процес биоремедијације утиче на хумификацију. У земљишту загађеном нафтом и њеним дериватима које је подвргнуто процесу биоремедијације, осим укупних нафтних угљоводоника и броја микроорганизама, одређене су квантитативне и квалитативне промене изолованих хуминских киселина. Биоремедијација 150 m^3 загађеног земљишта је трајала 150 дана. Количина укупних нафтних угљоводоника током биоремедијације смањена је за 86,6 %, док се ниво хуминских киселина повећао за 26,5 %. Елементарна анализа је показала смањење количине угљеника и H/C односа, али и повећање кисеоника и O/C односа у изолованим хуминским киселинама током процеса. Такође је повећан однос апсорбанци на 465 и 665 nm. На основу ових резултата и инфрацрвених спектара показано је да су хуминске киселине са краја биоремедијације обогаћене функционалним групама кисеоника и ароматичним структурама. Студија показује један од првих увида у однос између биоремедијације и хумификације, као и доказ да микроорганизми који разграђују угљоводонике имају значајан утицај на структуру хуминских киселина.

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