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Ex situ bioremediation of a soil contaminated by mazut (heavy residual fuel oil) – A field experiment

Vladimir P. Beškoski [a,*], Gordana Gojgić-Cvijović [a], Jelena Milić [a], Mila Ilić [a], Srdjan Miletić [a], Tatjana Šolević [a], Miroslav M. Vrvić [a,b]

[a] Department of Chemistry, Institute of Chemistry, Technology and Metallurgy, 11001 Belgrade, Njegoševa 12, P.O. Box 473, Serbia
[b] Faculty of Chemistry, University of Belgrade, 11158 Belgrade, Studentski trg 16, P.O. Box 51, Serbia

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ABSTRACT

Mazut (heavy residual fuel oil)-polluted soil was exposed to bioremediation in an *ex situ* field-scale (600 m^3) study. Re-inoculation was performed periodically with biomasses of microbial consortia isolated from the mazut-contaminated soil. Biostimulation was conducted by adding nutritional elements (N, P and K). The biopile (depth 0.4 m) was comprised of mechanically mixed polluted soil with softwood sawdust and crude river sand. Aeration was improved by systematic mixing. The biopile was protected from direct external influences by a polyethylene cover. Part (10 m^3) of the material prepared for bioremediation was set aside uninoculated, and maintained as an untreated control pile (CP). Biostimulation and re-inoculation with zymogenous microorganisms increased the number of hydrocarbon degraders after 50 d by more than 20 times in the treated soil. During the 5 months, the total petroleum hydrocarbon (TPH) content of the contaminated soil was reduced to 6% of the initial value, from 5.2 to 0.3 g kg^{-1} dry matter, while TPH reduced to only 90% of the initial value in the CP. After 150 d there were 96%, 97% and 83% reductions for the aliphatic, aromatic, and nitrogen–sulphur–oxygen and asphaltene fractions, respectively. The isoprenoids, pristane and phytane, were more than 55% biodegraded, which indicated that they are not suitable biomarkers for following bioremediation. According to the available data, this is the first field-scale study of the bioremediation of mazut and mazut sediment-polluted soil, and the efficiency achieved was far above that described in the literature to date for heavy fuel oil.

1. Introduction

Mazut is a low quality, heavy (chain length 12–70 C atoms) residual fuel oil (ASTM D396-09a; ISO 8217). In the United States and Western Europe mazut is blended or broken down with the end product being diesel. In Eastern Europe, however, mazut is used as a source of heating fuel. The long-term storage and use of mazut can leave hydrocarbon residues in the reservoir itself, with a high content of different mechanically-derived contaminants and water in the reservoir; this can potentially lead to dangerous pollution of the living environment (particularly soil) during cleaning, when there is a serious threat to underground water.

Among numerous technologies used for cleaning up contaminated areas, the most common is bioremediation (Forsyth et al., 1995; MacNaughton et al., 1999), using zymogenous microorganisms (Langer et al., 2004). Some defined bacterial species are able to degrade, to a limited extent, all hydrocarbons present in heavy fuel oil or oil sludge (which are complex mixtures of alkanes, aromatic hydrocarbons and NSO-asphaltene fractions) (Bossert and Bartha, 1984). A consortium of microorganisms can conduct these complex processes of degradation, while at the same time, being more resistant, on average, to changes in the ecosystem than just a single microbial species (Brenner et al., 2008).

While there is significant information in the literature about the microbiological degradation of defined individual hydrocarbons (Singh and Ward, 2004), there is significantly less data about the biodegradability of some commercial petroleum products, including mazut and heavy residual fuel oil (McMillen et al., 1995; Sugiura et al., 1997; Nocentini et al., 2000; Iturbe et al., 2004; Delille et al., 2008). Studies published to date on the bioremediation of mazut and heavy residual fuel oil contaminated soils are laboratory-based, using model systems, and have indicated the potential of bioremediation for stimulated self-cleansing (Boronin et al., 1997; Díez et al., 2005).

Environmental factors play a vital role in the bioremediation of soil contaminated with heavy oil deposits (Dibble and Bartha, 1979). The most significant physical and chemical characteristics of soil which can influence the process of bioremediation are: density and water retention capacity, pH, moisture and carbonate content, temperature, availability of oxygen and carbon-based

* Corresponding author. Tel.: +381 112637273; fax: +381 112636061.
E-mail address: vbeskoski@chem.bg.ac.rs (V.P. Beškoski).

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nutrients, nitrogen, phosphate and potassium, as well as the concentration of heavy metals (Rogers et al., 1993).

Although the most suitable criteria for optimizing the bioremediation process are known (control of temperature, aeration, particle size, moisture, macro and micronutrients in the mass to be composted, C/N ratio of the materials, etc. (Singh and Ward, 2004), so that the microbial activity necessary for treating this organic matter can be encouraged, very few published studies have attempted to treat mazut or heavy residual fuel oil on an industrial scale (Marín et al., 2006; Jiménez et al., 2006).

Our previous field-scale application showed that the oil pollutant mixture in the soil treated by *ex situ* bioremediation behaved in a complex way: different degradation rates and time evolutions were observed for fractions of the hydrocarbon mixture characterized by different molecular weights and structures (Jovančićević et al., 2008a,b; Beškoski et al., 2010). We also concluded that a stable microbial community had been formed after initial fluctuations and that the microorganisms which decompose hydrocarbons were the dominant microbial population at the end of the bioremediation process, with a share of more than 80% (range 10^7 colony forming units (CFU) g^{-1}) (Milic et al., 2009).

The current study was conducted in order to determine if our previous laboratory-scale and smaller field-scale study (Jovančićević et al., 2008a,b; Milic et al., 2009) (100 m^3) could be successfully up-scaled (Beškoski et al., 2010), and to determine the dynamics of field-scale *ex situ* bioremediation of soil contaminated with mazut, achieved by zymogenous inoculated microflora, including the degradation of differing hydrocarbon fractions.

We consider this to be the first field experiment designed to study the possibility of using bioremediation for treating a soil contaminated with heavy residual fuel oil such as mazut and mazut waste material. Key design considerations for bioremediation of soil contaminated with heavy hydrocarbons are intensive aeration achieved by mixing, biostimulation of zymogenous microbial consortia, re-inoculation of microorganisms that consume hydrocarbons and also having a control polluted soil for monitoring.

Indicators that are critical to the success of an *ex situ* biopile application for treatment and remediation of a heavy oil contaminated soil and that should be monitored are total petroleum hydrocarbon (TPH), moisture, pH, bulk density, water holding capacity (WHC), organic and inorganic carbon, nitrogen, available phosphorus and potassium as well as microbiological parameters such as total chemoorganoheterotrophs (TC) and hydrocarbon degraders (HD).

2. Materials and methods

2.1. Mazut and mazut sediment-polluted soil

The mazut-polluted soil (PS) was excavated contaminated soil from an energy power plant which, due to a break-down, had been polluted with mazut and sediment from a mazut reservoir for a year.

2.2. Preparation of the zymogenous consortium of microorganisms

A consortium of microorganisms was obtained from PS by enrichment in 200 mL volumes of mineral medium (10 vol.%) (Löser et al., 1998), containing mazut (2 g L^{-1}) as the only energy and carbon source in Erlenmeyer flasks (1 L).

Suspensions of the microbial consortium were used to seed four Erlenmeyer flasks (5 L), each containing 2000 mL of the medium containing 23 g of nutrient broth (Torlak, Belgrade, Serbia); 100 mL of soil extract (http://www.ccap.ac.uk/media/recipes/SE.htm); and 20 g of mazut. Commercial non-toxic and readily biodegradable surfactants, BioSolve CLEAR supplied by The Westford Chemical Corporation (Westford, MA, USA) were used as surface active agents to solubilize mazut. The original solution supplied by the manufacturer was used at a concentration of 1 mL L^{-1}. The growth conditions were as follows: temperature, 28 °C; 120 rpm; pH 7.0 (adjusted with 1 M HCl or NaOH); duration of growth, 96 h.

The microbial population from all four flasks was used to inoculate (approx 1 vol.%) a bioreactor designed by us (total volume 1000 L) with a working volume of 800 L, producing the microbial consortium. The medium used was: 12 g L^{-1} meat peptone (Torlak, Belgrade, Serbia); 0.2 g L^{-1} $(NH_4)_2HPO_4$; 25 g L^{-1} of autoclave-sterilized soil sampled from undisturbed deciduous woodland; BioSolve CLEAR original solution (1 mL L^{-1}); and 10 g L^{-1} mazut. The growth conditions were: non sterile, 25 °C, aeration and agitation 0.70 volume of air/volume of medium min^{-1}, pH 7.0 (adjusted with 10 M HCl or NaOH), duration 48 h and sunflower oil (1 mL L^{-1}) as antifoam.

2.3. Experimental biopile, design and treatment

The biopile (http://www.epa.gov/OUST/pubs/tums.htm) for bioremediation was prepared on a waterproof asphalt surface of approximately 1500 m^2 and with a 1% sloping gradient. The biopile consisted of 270 m^3 of PS mixed with 60 m^3 of softwood sawdust as the additional carbon source and bulking component and 300 m^3 of un-graded river sand added as a bulking and porosity increasing material. To ensure homogeneity, the biopile components were mixed three times with a front-end loader, and finally, raked using a tractor fitted with a harrow. The final dimensions of the biopile were 75 × 20 × 0.4 m (length × width × height), and it contained approximately 600 m^3 of material, termed the substrate for bioremediation (SB). A perimeter drain enclosed the entire treatment area and directed all leachate and runoff to a joint vessel, from where it was pumped back onto the biopile. An optimal ratio of C:N:P:K (approx 100:10:1:0.1), was achieved by spraying a solution of dissolved ammonium nitrate, diammonium phosphate and potassium chloride over the biopile using an agricultural sprayer fitted to a tractor with a trailer unit. Spraying also achieved the required moisture level in the biopile (40–60% WHC). During bioremediation, the biopile was watered, turned and mixed every 15 d to maintain the required moisture and aeration levels. The biopile was re-inoculated every 30 d during the study, with prepared microbial consortia, by spraying and mixing as described above.

After mixing, the soil was covered with plastic polyethylene foil, to prevent direct influence of weather conditions on the bioremediation material. The biopile was also amended with BioSolve CLEAR applied at a volume of 70 mL of original solution per cubic meter.

At the beginning of the study, immediately after mixing, but before adding nutrient compounds and watering, approximately 10 m^3 of the biopile mixture was set aside on the same waterproof asphalt surface, to be used as a control pile (CP). The bioremediation experiment was conducted from March to August 2009 and in this period the average temperature was 18.2 °C (range from approximately 0.3 to 35.4 °C). Chemical and microbiological indicators of the bioremediation process were followed immediately after application of the microbial biomass (time zero, sample S-0), and every 50 d during the next 150 d (designations S-50, S-100, and S-150).

2.4. Sampling, physical, chemical and microbiological analyses

Composite samples for analyses were taken at the beginning and every 50 d from the biopile and control pile by "zig-zag" sam-

pling with an Eijkelkamp auger soil sampler from 30 random places on the SB and 5 on the CP. The composite samples (approx. 20 L from the biopile, and 10 L from the control pile) were sieved (1 mm grid), collected in stopped glass jars, and stored at 4 °C. Analyses were conducted within 12–24 h after sampling (Paetz and Wilke, 2005).

Samples were analyzed for: content of clay and sand, bulk density, WHC, moisture (for original material), pH, organic and inorganic carbon, nitrogen, and available phosphorus and potassium using standard methods (Wilke, 2005; Pansu and Gautheyrou, 2006).

The minerals in the samples were detected and determined semiquantitatively by roentgen diffraction analysis powder technique. X-ray diffractometer Philips (Netherlands) type PW 1050/00. Cu $K\alpha_1$ Ni-filtered radiation. Preparations: native, with glycerol and ignited. Results interpreted using Material Phases Data System program and ASTM Joint Committed on Powder Diffraction Standards card files.

The content of TPH in the soil was extracted as per method ISO 16703 and determined gravimetrically in accordance with DIN EN 14345. GC as stated in the ISO standard, used for quality analysis and comparison of results, was conducted using an Agilent 4890D GC with FID detector: the column was HP-1MS 30 m × 0.25 mm, with a gas-hydrogen carrier, injector temperature 250 °C, initial temperature 40 °C, isothermal at 285 °C for 12 min. The results were controlled using certified European Reference Materials, and a standard mixture of *n*-alkanes, pristane and phytane by comparing retention times. Mass of samples, dilution and conditions for GC were identical for all samples.

The group composition of the organic substance of the SB from the biopile was determined by extraction and chromatographic separation on an adsorbent column, while the nitrogen–sulphur–oxygen (NSO)-asphaltene fraction was obtained by subsequent calculation (NSO-asphaltene% = 100% − (aliphatic% + aromatic%)) (Jovančićević et al., 2003). The number of microorganisms was determined by plating appropriate serial dilutions on agar plates incubated at 28 °C. The media used were: nutrient agar for TC; and a mineral base medium (Löser et al., 1998) containing 2 g of standard D_2 diesel fuel in 1 L of medium (Bossert et al., 2002) for HD. All results were calculated according to dry matter, while percentages were calculated according to mass. The results were processed by the OriginPro 8.0 program, and SPSS 11.5 software was used for statistical analysis.

3. Results and discussion

Key parameters for monitoring effectiveness of bioremediation are reduction of TPH and number of HD microorganisms as crucial indicators of degradation and utilization of hydrocarbons.

3.1. Basic characteristics of the mazut-contaminated soil and the substrate for bioremediation

The basic chemical, physico-chemical and microbiological characteristics of the PS and the SB immediately after initial mixing are shown in Table 1. The concentration of heavy metals in all samples was below that of reference values (Dutch Standards, 2000), which indicates that the SB did not contain these microorganism growth and activity inhibitors (data not shown).

The proportion of HD bacteria comprised approximately 20% of the total number of bacteria in the PS, which indicates the presence of intensive biodegradative processes and the large bioremediation potential, while the SB contained nearly 6% of these bacteria; this was a consequence of dilution (Table 1). According to the concentration of clay and sand, the PS was clayified sand, and its WHC, porosity and ability to be mixed increased during formation of the SB due to the addition of sawdust and raw river sand.

In order to assess whether the analysis of composite sample was sufficiently representative and accurately represented the actual state in biopile, a key parameter for following bioremediation, TPH, was analyzed in the composite sample S-0 in five replications (five independent determinations), and in each of the 30 individual samples from which the composite sample S-0 was created. Statistical analysis of these results are shown in Table 2.

Statistical analysis showed that the analysis of composite samples was a valid technique to use in the current study, according to their coefficients of variation (Miller and Miller, 2010). Coefficients of variation, or relative standard deviation for the composite sample and for individual samples were 10% and 23%, respectively. Since the coefficient of variation indicates the homogeneity of properties of units in the set, large coefficients of variation (>30%) are often associated with increased experimental variability; otherwise the property can considered relatively homogenous. Therefore, it can be concluded that both properties were homogenous, that the biopile was well homogenized by mixing, and that analysis of a composite sample is satisfactory to monitor indicators

Table 1
Characteristics of the mazut-polluted soil and the substrate for bioremediation.

Characteristics	PS	SB
Minerals	Quartz > clay minerals (clay mica–illite, kaolinite, montmorillonite) > calcite ≈ feldspar > dolomite > chlorites	Quartz ≫ feldspar ≈ calcite ≈ clay minerals (clay mica–illite, kaolinite, montmorillonite) > chlorites > dolomite
Content of sand + clay (%)	61 + 35	77 + 17
Bulk density (kg m^{-3})	1538 ± 100[a]	1612 ± 100[a]
Moisture (%)	17.8 ± 0.3	13.9 ± 0.6
WHC (%)	18.3 ± 1.6	26.1 ± 1.4
pH	7.3–7.5	7.4–7.6
Loss on ignition (%)	8.5 ± 1.2	9.7 ± 1.1
TPH (g kg^{-1})	12.4 ± 0.5	5.2 ± 0.2
Organic carbon (%)	1.79 ± 0.06	2.44 ± 0.08
Inorganic carbon[b] (%)	1.45 ± 0.05	0.67 ± 0.06
Total nitrogen (%)	0.16 ± 0.03	0.12 ± 0.01
Available phosphorus (mg kg^{-1})	47 ± 3	25 ± 3
Available potassium (mg kg^{-1})	18 ± 3	10 ± 2
TC (CFU g^{-1})	1.2×10^6	9.7×10^5
HD (CFU g^{-1})	2.7×10^5	5.6×10^4

[a] Error of determination. In all other results: ±standard deviation for five measurements.
[b] Calculated on the basis of measured carbonates.

Table 2
Statistical analysis of single and composite samples taken at zero time.

	S-0 composite sample[a]	Single samples[b]
Parameter	TPH (g kg^{-1})	
Number of samples	1	30
Number of determinations per sample	5	1
Mean	5.18	5.21
Std. error of mean	0.23	0.22
Median	5.42	5.12
Std. deviation	0.52	1.19
Coefficient of variation (%)	10	23
Minimum	4.38	2.11
Maximum	5.61	7.54
Range	1.23	5.43

[a] Five independent determinations of one composite sample.
[b] Single determinations of each 30 individual samples.

Table 3
Changes in basic parameters during bioremediation.

Parameter[a]		S-0[b]	S-50	S-100	S-150
Moisture (%)		15.4 ± 0.5	13.0 ± 0.7	14.5 ± 0.2	13.4 ± 1.5
WHC (%)		26.4 ± 1.7	29.1 ± 1.8	30.2 ± 1.1	32.1 ± 1.1
pH		7.3–7.5	7.3–7.5	7.2–7.6	7.1–7.3
Loss on ignition (%)		9.9 ± 1.1	6.9 ± 1.6	6.7 ± 0.2	6.1 ± 0.7
Organic carbon (%)		2.46 ± 0.04	1.87 ± 0.08	1.19 ± 0.06	1.08 ± 0.05
Inorganic carbon (%)		0.65 ± 0.03	0.66 ± 0.04	0.60 ± 0.03	0.56 ± 0.03
Total nitrogen (%)		0.25 ± 0.03	0.23 ± 0.04	0.22 ± 0.02	0.25 ± 0.01
Available phosphorus (mg kg^{-1})		241 ± 5	239 ± 4	250 ± 3	245 ± 2
Available potassium (mg kg^{-1})		24 ± 3	25 ± 4	21 ± 2	24 ± 3
Time of bioremediation (d)		0	50	100	150
SB	TC (CFU g^{-1})	2.0×10^6	2.2×10^6	1.3×10^7	8.0×10^6
	HD (CFU g^{-1})	7.2×10^4	1.5×10^6	9.9×10^6	2.0×10^6
	HD (%)[c]	4	68	76	25
CP	TC (CFU g^{-1})	9.7×10^5	2.2×10^5	3.2×10^5	4.8×10^5
	HD (CFU g^{-1})	5.6×10^4	1.8×10^4	2.2×10^4	4.3×10^4
	HD (%)[c]	6	8	7	9

[a] Remarks are identical to those in Table 1.
[b] SB after mixing, watering, biostimulation and re-inoculation.
[c] Share of HD within the TC.

that are critical to the success of a biopile application for heavy oil contaminated soil remediation (Table 3).

3.2. Changes in basic indicators during bioremediation

The addition of ammonium nitrate, diamonium phosphate and potassium chloride achieved the required balance of C:N:P:K ≈ 102:10:1:0.1, as shown in column S-0 (Table 3). During bioremediation, the WHC increased from 26% to 32%, which was a direct result of the reduction in the concentration of hydrophobic compounds and the increased polarity of the SB. In the biopile remediation, the pH reduced somewhat due to the appearance of low molecular weight organic acids during degradation of the hydrocarbon compounds. The maximum temperature in the biopile during the bioremediation process was not greater than 55 °C, which was important for the activity of the predominating mesophiles.

The initial concentration of HD bacteria in the SB before inoculation was approximately 10^4 CFU g^{-1}. In previous studies, it was confirmed that when the population of HD bacteria is less than 10^5 CFU g^{-1} of soil-substrate, no significant degree of bioremediation will occur, and, therefore, it is necessary to increase numbers of these microorganisms (Forsyth et al., 1995).

The biopile was re-inoculated every 30 d. Changes in basic microbiological parameters during bioremediation show that the level of the active bacterial consortium, particularly active HD microorganisms, was maintained and increased. Comparing the results for the number of microorganisms in the SB and CP, it can be seen that the inoculation and re-inoculation, followed by biostimulation and aeration, affected the microbial profile variation and maintained the necessary level (number) of HD microorganisms. For example, in the SB sample S-100 the number of HD was near 10^7, in contrast to 10^4 in the CP.

After biostimulation and re-inoculation, the number of HD bacteria on 50 d was more than 20 times greater than on 0 d, and they comprised more than 68% of the TC. In the middle of bioremediation, HD bacteria increased to more than 75% of the total number of bacteria, compared to only 6% before inoculation. Before the end of bioremediation, the proportion of HD bacteria was reduced to around 25%. According to published studies, the biodiversity of contaminated soil reduces as the population of pollutant degraders increases and as a consequence of the toxic effects of the contaminants (Katsivela et al., 2005), which is confirmed by the current study. In all CP samples, a small number of HD microorganisms was detected and a small proportion, less than 10% of HD in the number of TC, which indicates a bioremediation potential which is not sufficient for self cleaning of this soil (Gojgić-Cvijović et al., 2006). Moreover, this share decreased over time. On the other hand, the technology applied to the biopile ensured that the number of HD and TC microorganisms increased in SB. This shows that re-inoculation together with aeration achieved by mixing and biostimulation will enable success of the applied bioremediation process.

The population of the HD bacterial consortium was stable even after 150 d following the first application of the zymogenous microbial consortium, indicating the high survivability of the introduced strains (data not shown). The reasons for that include the maintenance of suitable conditions in the biopile, including moisture and aeration levels, but above all, the fact that the microbial consortia themselves used for re-inoculation had been isolated from PS before being introduced to the bioremediation process.

3.3. Changes in TPH

The pattern of the decline in TPH content was very clear. The contamination level of TPH in S-0 was found to be 5.2 g kg^{-1} of soil. With application of bacterial consortium and nutrients, the TPH level was reduced to 2.1, 1.3 and 0.3 g kg^{-1} of soil after 50, 100, and 150 d, respectively (Fig. 1), meaning 60%, 75% and 94% of the TPH were biodegraded. The degradation was most rapid during the first 50 d and the rate declined with time. The residual concentrations of TPH after the 5-months bioremediation were less than one tenth of the initial ones.

Although the degradation was not clearly linear during the whole process, on the basis of the reducing TPH concentrations it was calculated that mazut reduction was 3.1 g kg^{-1} during the first 50 d, 0.8 g kg^{-1} during 50–100 d and 1.0 g kg^{-1} during 100–150 d.

There was a noticeable correlation between the share of HD microorganisms and decreases in TPH during the first 100 d of the study. Namely, the proportion of HD microorganisms in the number of TC in the first 50 d increased from 4% to 68% while TPH decreased to 40% of the initial value. From 50 to 100 d, the proportion of HD increased further to 76% and TPH was reduced to 25% of the initial value. In the last 50 d, though, the HD share reduced to just 25% while the TPH in the same period reduced to 6% of the initial value. Pearson correlation coefficient for these two values was 0.99641 from 0–100 d. Possible explanation for the fact

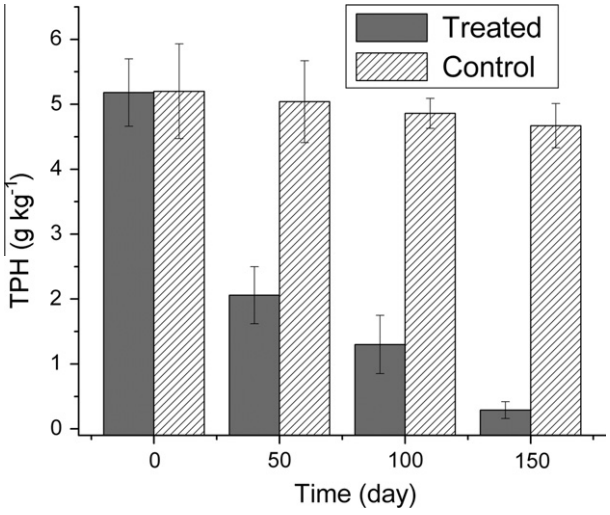

Fig. 1. Reductions in TPH concentrations during bioremediation (bar is ± standard deviation for five measurements).

that the proportion of HD microorganisms dropped from 76% of total microbes on day 100 to just 25% of the total on day 150 is increase in the TC number and also that the hydrocarbon as source of carbon for HD microorganisms were being depleted. These microbes achieved a population maximum, and then they share slowly started to decline but their number remained stable until the end of bioremediation (in the order of 10^6 CFU g^{-1}).

Slight reductions in TPH concentrations were also observed in the control pile. The initial concentration of TPH in the control pile was found to be 5.2 g kg^{-1}. The concentration decreased to 5.0, 4.9, and 4.7 g kg^{-1} during 50, 100 and 150 d, respectively. The control pile showed a clear lag period, and the maximum degradation achieved in this untreated and uninoculated control soil was 10% after 150 d. This reduction in the concentration of hydrocarbons could be ascribed to combined joint action of the indigenous microbial population together with abiotic factors (e.g. weathering). In this control pile, the total numbers of TC and HD bacteria were approximately 10^6 and 10^4 CFU g^{-1}, respectively, during this 150 d study. However, the current study did not examine whether biotic or abiotic factors were more responsible for this observed reduction in TPH concentration in the control pile. One can conclude that the zymogenous microbial population already present has the potential for mazut biodegradation, but the process is very slow.

The chromatograms (Fig. 2) gave qualitative and semiquantitative information on the changes in the composition hydrocarbons in the samples. The unresolved complex mixture of hydrocarbons (UCM) is part of the TPH (Jovančićević et al., 2008a).

As judged from GC, the abundance of *n*-C_{17} and *n*-C_{18} *n*-alkanes at time zero was somewhat smaller than the abundance of pristane (C_{19}) and phytane (C_{20}). This indicates that the hydrocarbons left in the soil were already degraded to some extent during the natural biodegradation process.

It was noticeable that a large part (around 50%) of *n*-alkanes in the size range of C_{29}–C_{35} were biodegraded during the first 50 d. This was a consequence of the preparation of the specialized mixed consortium of microorganisms able to degrade these hydrocarbons, since the only source of carbon for growth of the consortia in the bioreactor was mazut containing predominantly these hydrocarbons. *n*-Alkanes in the range of C_{14}–C_{20} were degraded completely by 100 d, followed by complete degradation of C_{20}–C_{36} by 150 d. After 100 d the GC trace also revealed a significant reduction in the UCM.

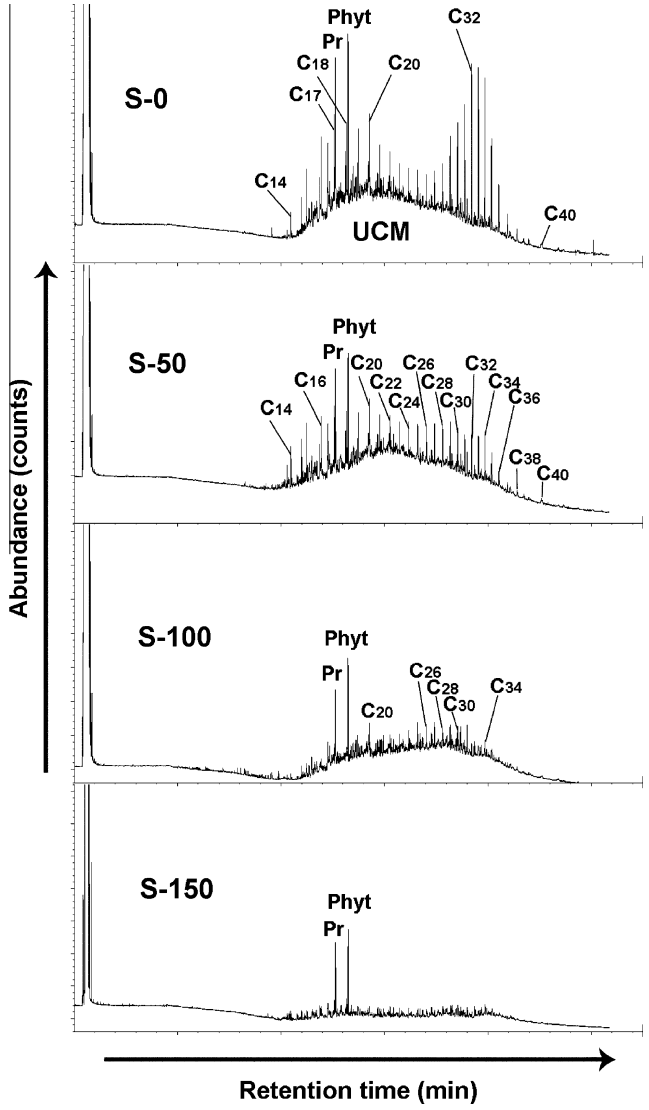

Fig. 2. Gas chromatograms of TPH during bioremediation.

GC analyses revealed that the microbial consortium biodegraded and "consumed" all components of the complex mass of hydrocarbons, although different rates of degradation were observed. Nevertheless, it ought to be mentioned that the presence of recalcitrant (Nocentini et al., 2000), non-degradable compounds was not observed, and that all constituents of the mixture were degraded to some extent, including pristane and phytane (shown in Table 4).

Bacteria degrade the *n*-alkanes faster than the isoprenoids, resulting in an increase in the ratio of pristane/*n*-C_{17} and phytane/*n*-C_{18} (Wang and Fingas, 1997). The zymogenous microbial consortia used in the current study degrade pristane and phytane,

Table 4
Indicators of bioremediation from gas chromatography.

Indicator	S-0	S-50	S-100	S-150
Dominant *n*-alkane	C_{32}	C_{20}	C_{20}	–
Pristane/*n*-C_{17}	1.47	1.42	2.16	3.8
Phytane/*n*-C_{18}	1.75	1.70	2.9	4.17
Biodegradation of TPH (%)	0	60.2	74.9	94.4
Biodegradation of pristane (%)	0	31.8	50.9	56.8
Biodegradation of phytane (%)	0	32.0	42.0	55.8

hydrocarbons which are termed recalcitrant since they are resistant to biodegradation, and which are commonly used as chemical markers to measure the degree of biodegradation of other hydrocarbon compounds and the maturation of oil (Wang et al., 1998; Olivera et al., 2000). After 150 d, bioremediation degraded 57% and 56% of pristane and phytane, respectively. The ratio pristane/n-C_{17} and phytane/n-C_{18} can be used to differentiate physical weathering and biodegradation (Wang et al., 1998). As the volatility of n-C_{17} and pristane and n-C_{18} and phytane are similar, weathering should be attributed to a reduced concentration of these substances over time, if their ratio remains constant. An increase in the pristane/n-C_{17} and phytane/n-C_{18} ratio over time is likely to be a consequence of bioremediation, since n-C_{17} and n-C_{18} degrades more rapidly than pristane and phytane, respectively. Biodegradation of pristane and phytane, as found in the current study, mean that these compounds are not suitable as markers for following a bioremediation process. In fact, comparison of the content of other hydrocarbons in relation to pristane and phytane could underestimate the degree of their biodegradation.

GC of TPH revealed that TPH concentrations in samples of control pile did not change significantly during the 150 d (data not shown).

3.4. Changes in particular hydrocarbon fractions

Biodegradation of some hydrocarbon fractions are shown in Fig. 3. The aliphatic fraction present in the highest quantity was noticeably degraded the most. In the S-0 sample, the proportion of aliphatic, aromatic and NSO-asphaltene fractions were 72%, 17% and 11%, respectively, and after 50 d, the ratio was 68%, 13% and 19%, respectively. As the n-alkane fraction is dominant and most susceptible to bioremediation, the process continued, so that the ratio of the fractions was 62%, 13% and 25% after 100 d, and 55%, 10% and 35% respectively after 150 d. The increased proportion of the NSO-asphaltene fraction was accompanied by a reduction in the absolute levels of all fractions, as illustrated in Fig. 3. Based on the analysis, the group composition of the CP soil samples at the start and after 150 d did not show significant change within the individual fractions. As the proportion of aliphatic, aromatic and NSO-asphaltene fractions after 150 d were 69%, 18% and 13%, it can be concluded that reduction in TPH in the CP samples was a consequence of the reduction in the aliphatic fraction.

On the basis of the reduced concentrations of some fractions over the course of the current study, after 150 d there were 96%, 97% and 83% reductions of the aliphatic, aromatic, and NSO-asphaltene fractions, respectively. The NSO-asphaltene fraction is the most recalcitrant and the most persistent in the living environment, as it has the slowest degradation rate (Nocentini et al., 2000).

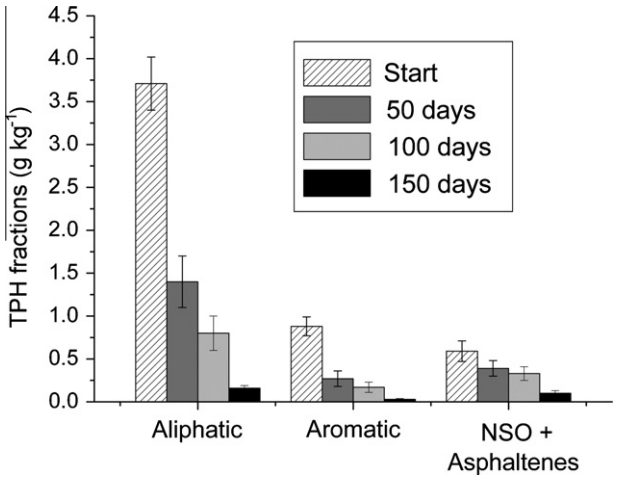

Fig. 3. Reductions in the concentration of specific TPH fractions during bioremediation (bar is ±standard deviation for five measurements).

Our previous investigation concluded that the biodegradation of petroleum type pollutants by surface water microorganisms, under natural conditions, will be restrained to the n-alkanes and isoprenoids (Antic et al., 2006). The current study has shown that the biodegradation of mazut, containing recalcitrant fractions like the aromatic and NSO-asphaltene fractions, will occur during bioremediation if stimulated with zymogenous microorganisms.

Compared with other reported soil studies, the degree of hydrocarbon reduction in this PS was relatively high. According to one study, between 10% and 30% of the initial soil pollution remains in the soil after bioremediation techniques have been applied (Margesin and Schinner, 1998). The high degree of biodegradation of TPH (more than 90%), observed in the current study, was primarily a consequence of the activity of the zymogenous microbial consortia and the dominant proportion of the aliphatic fraction (more than 70%).

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