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Biodegradation of petroleum sludge and petroleum polluted soil by a bacterial consortium: a laboratory study

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Abstract This article presents a study of the efficiency and degradation pattern of samples of petroleum sludge and polluted sandy soil from an oil refinery. A bacterial consortium, consisting of strains from the genera *Pseudomonas*, *Achromobacter*, *Bacillus* and *Micromonospora*, was isolated from a petroleum sludge sample and characterized. The addition of nitrogen and phosphorus nutrients and a chemical surfactant to both the samples and bioaugmentation to the soil sample were applied under laboratory conditions. The extent of biodegradation was monitored by the gravimetric method and analysis of the residual oil by gas chromatography. Over a 12-week experiment, the achieved degree of TPH (total petroleum hydrocarbon) degradation amounted to 82–88% in the petroleum sludge and 86–91% in the polluted soil. Gas chromatography–mass spectrometry was utilized to determine the biodegradability and degradation rates of *n*-alkanes, isoprenoids, steranes, diasteranes and terpanes. Complete degradation of the *n*-alkanes and isoprenoids fractions occurred in both the samples. In addition, the intensities of the peaks corresponding to tricyclic terpenes and homohopanes were decreased, while significant changes were also observed in the distribution of diasteranes and steranes.

Keywords Isoprenoids · Mixed culture · *n*-alkanes · Petroleum sludge · Steranes · Surfactant · Terpanes

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Introduction

Petroleum production, refining, transportation and use contribute highly to environmental pollution. The contamination of soil with petroleum hydrocarbons causes a significant decline in its quality and such soils become unusable. In order to control the environmental risks caused by petroleum products, bioremediation as an environmentally friendly technology has been established and applied, especially biostimulation and bioaugmentation of the easily to be degraded petroleum hydrocarbons (e.g., alkanes).

The primary mechanism for the elimination of hydrocarbons from contaminated sites is biodegradation by natural populations of microorganisms. To maximize the process in bioremediation technologies, two main approaches have been explored: biostimulation, in which nutrients are added to stimulate the intrinsic hydrocarbon degraders, and bioaugmentation, in which microbial strains with specific degrading abilities are added to work cooperatively with normal indigenous soil microorganisms (Alvarez and Illman 2006).

Petroleum and petroleum products are complex mixtures consisting of thousands of compounds that are usually grouped into four fractions: aliphatics, aromatics, nitrogen–oxygen–sulphur (NSO) compounds and asphaltenes. Asphaltenes are generally solvent-insoluble and resistant to biodegradation. Aliphatic hydrocarbons consist of normal alkanes (*n*-alkanes), branched alkanes (isoalkanes) and cyclic alkanes (naphthenes). Isoalkanes, naphthenes and aromatics are much less biodegradable than *n*-alkanes. According to Perry (1984), the susceptibility of hydrocarbons to microbial attack is ranked in the following order: *n*-alkanes > isoalkanes > low molecular weight aromatics > naphthenes.

A large number of microorganisms (bacteria, fungi and some algae) that are capable of using petroleum hydrocarbons as the sole source of carbon and energy have been described (Das and Chandran 2011). It is generally accepted that a single microorganism is not capable of degrading all compounds from such mixtures. Mixed cultures not only have broad substrate specificity but also degradation could be achieved in a system of cooxidation and commensalism.

Although there have been many reports on crude oil biodegradation, only a small number of the studies focused on the decomposition of heavy petroleum products or waste. Similarly, the number of strains degrading branched chain (Namio et al. 2005) and *n*-alkanes with more than 30 C atoms is limited (Wentzel et al. 2007) in comparison with the number of light *n*-alkane degraders.

In recent years, the study of the ability of defined microbial consortium/mixed cultures to degrade hydrocarbons of petroleum products or waste, such as petroleum sludge (PS) (Rahman et al. 2003; Vasudevan and Rajaram 2001), lubricating oil (Wongsa et al. 2004), turbine oil (Ito et al. 2008) mashine oil (Zvyagintseva et al. 2001) or engine base oil (Koma et al. 2003) has become of special interest.

The purpose of the present study was to isolate and characterize a mixed culture capable of degrading PS and polluted sandy soil from an oil refinery, and to optimize the degradation process by enhancement of the consortium by nutrient and surfactant addition. Furthermore, the goal was to determine the extent of degradation by comparing specific fractions of hydrocarbons in the residual oil.

Materials and methods

Substrates for biodegradation

The samples are taken from the Oil Refinery Novi Sad, Serbia. The first sample was PS from former storage fuel tank N8 and the second sample was polluted soil (S) in the vicinity of the tank N8.

Microbiological methods

Microbial enumeration

The number of microorganisms was determined by the method of a serial dilution (10^{-2}–10^{-10} CFU ml^{-1}) on agar plates. All determinations were conducted in triplicate. For bacteria nutrient agar was used, for yeasts and moulds malt agar with streptomycin (100 mg l^{-1}), and for hydrocarbon-degrading microorganisms the mineral salts medium, MM (Löser et al. 1998) with 2000 ppm diesel fuel (DM).

Isolation of microorganisms

The bacteria utilized for the preparation of consortium were isolated from sample PS. The medium consisted of mineral salt solution (NPK solution, 0.1% NH_4NO_3, 0.025% K_2HPO_4) inoculated with PS sample (20%, w/v) and incubated during 4 weeks aerobically with shaking at 200 rpm at 28°C. Every week a new portion of NPK solution was added to restore initial volume of solution and depleted nutrients.

After 4 weeks, the cultures were spread onto DM agar plates with diesel fuel as the C source. Pure cultures were obtained from selected colonies by repeated inoculation on DM medium, nutrient agar,

McConkey agar, Pseudomonas isolation agar and casein-starch agar.

Identification of strains

The identification of isolated strains was achieved by API tests (Biomeriex), fatty acid methyl ester (FAME) composition and by sequence analysis of 16S rRNA genes.

The API 50CH/E, API 20NE and Coryne kits (Biomerieux) were utilized as described in the manufacturer's instructions. Then, the data were interpreted with apiweb^{TM} software.

For FAME analysis, bacteria were cultivated on rotary shaker on tryptone soy broth (TSB). FAMEs were prepared according to the procedure described in MIDI Technical Note 101 (Haack et al. 1994) and analysed by gas chromatography/mass spectrometry (GC–MS).

Gas chromatograph/mass spectrometer system Agilent 7890A–5975C inert XL EI/CI was used. Column was HP-5 ms (30 m × 0.25 mm × 0.25 μm), gas helium and oven program: 50°C for 0 min, then 4.3–285°C for 5 min. For FAME identification, standard bacterial acid methyl esters (BAME, Supelco) and NIST5a.L database were used.

Analyses of 16S rRNA gene sequences

The genomic DNA of each bacterium was extracted as previously described (Hopwood et al. 1985). The 16S rRNA genes were amplified by PCR using 27F (5′-AGAGTTTGATCMTGGCTCAG-3′; Lane 1991), and 1492R (5′-CGGCTACCTTGTTACGACTT-3′; Liu et al. 2007) primers and amplified fragments were sequenced using Applied Biosystems 3130 genetic analyser (Foster City, USA). Taxonomic analysis was conducted by the GenBank basic local alignment search tool (BLAST) program, and 16S rRNA gene sequences of analysed strains were deposited in NCBI GenBank under accession numbers JF826526-30.

Specific characterization of strains

The growth of isolated strains on different hydrocarbons as the sole C source was tested on MM with 200 ppm of substrate in test tubes on rotary shakers incubated at 28°C for 15 days. The tubes (10 ml) were inoculated with 100 μl suspension (10^6 CFU ml^{-1}) of tested strain. After 15 days, growth was monitored by the increase in CFU ml^{-1} in the nutrient agar plates.

The tolerance to the presence of heavy metals was assayed by testing growth on Mueller–Hinton agar supplemented with increasing concentration of $CuSO_4{\cdot}5H_2O$, $Cd(CH_3COO)_2{\cdot}2H_2O$, $NiSO_4{\cdot}6H_2O$ and $Cr(NO_3)_3{\cdot}9H_2O$ by the method described by Nieto et al. (1989).

Biodegradation experiment

Biodegradation assays were carried out in 500 ml Erlenmeyer flasks containing 100 ml of MM and 10% (w/v) of samples PS or S. Pure cultures of isolated strains grown on TSB were centrifuged and suspended in physiological saline to 10^6 CFU ml^{-1}. 0.5 ml of each culture was employed to inoculate the flasks containing S sample in bioaugmentation treatments. BioSolve® (Westford Chemical Corporation, USA) was added as surfactant (3%, v/v). All experiments were conducted in triplicate. The control flasks were prepared with the addition of 2% $HgCl_2$. Cultures were incubated on rotary shaker at 200 rpm at 28°C.

The duration of the experiment was 3 months. Every week, sterile water was added to the flasks to restore initial volume of solution. Content of phosphate in fermentation broths was determined every week during experiment. If it was low, NPK solution was added. Specifically, NPK solution was added after 1, 2, 3, 4, 6 and 8 weeks to the PS sample, and after 1, 2, 4, and 6 weeks to the S sample. Also, surfactant was added not only in the beginning but also after 4 and 8 weeks of study.

The process was monitored by the determination of viable bacterial count, the phosphorus content in the culture broth, the pH and total petroleum hydrocarbon (TPH) content.

Chemical methods

The content of total petroleum hydrocarbons (TPH) in the samples was determined by DIN EN ISO method 14345: 2004.

The total content of carbon and nitrogen was determined with Vario EL III CHNS/O Elemental Analyzer, hydrocarbon fractions were separated by

Walker's method (Walker et al. 1975), and phosphorus and carbonate contents in samples were determined according to the recommended methods (Rump 1999). The contents of Fe, Ni and V were determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) using iCAP 6500 Duo according to EPA method 3051 (1995).

Chromatography

The GC analysis of TPH samples was performed on the instrument Agilent 4890D with FID detector. The following protocol was used: Column was HP-1MS (30 m × 0.25 mm); carrier hydrogen, injector temperature 250°C; initial temperature 40°C for 9 min; followed by 4°C min^{-1} from 40 to 65°C; 9°C min^{-1} from 65 to 286°C; and isothermal at 285°C during 12 min.

Polycyclic alkanes of sterane and triterpane types in THP samples were analysed by GC–MS. A Agilent 6890 gas chromatograph, fitted with HP-5MS column (30 m × 0.25 mm × 0.25 μm; temperature was linearly programmed from 60° to 285° at 3°C min^{-1}),-was coupled to a Agilent 5975B mass spectrometer with electron ionization. Helium was used as the carrier gas (flow rate, 1 ml min^{-1}). *n*-Alkanes and isoprenoids were identified from *m/z* 71, steranes and diasteranes from *m/z* 217 and terpanes from *m/z* 191 ion chromatograms obtained from analysis in single ion monitoring mode (SIM). The most relevant peaks have been identified based on the organic geochemical literature data (Peters et al. 2005) or on the basis of total mass spectra using data base NIST5a.L.

Mineralogical analysis

The minerals in the samples were detected and determined semiquantitatively by roentgen diffraction analysis powder technique using X-ray diffractometer Philips (The Netherlands) type PW 1050/00 (CuKα1 Ni-filtered radiation; preparations—native, with glycerol and ignited). The results were interpreted using Material Phases Data System program and ASTM Joint Committed on Powder Diffraction Standards card files.

Statistical analysis

In order to evaluate the effects of surfactant addition and bioaugmentation on the biodegradation of the samples PS and S, a two-way analysis of variance (ANOVA) with repeated measurements was performed for each sample. Two factors included: (1) the addition of surfactant for the S sample bioaugmentation or their combination, and (2) time intervals (4, 8 or 12 weeks) for both measured parameters (degree of TPH degradation and number of microorganisms). Additional tests (post hoc tests for the S sample and *t* test for the PS sample) were employed to find statistically significant differences between the particular treatments.

The analyses were performed using the software SPSS 11.5 program. The normality of distribution was checked using Kolmogorov–Smirnov test. The tests were considered statistically significant if $P < 0.05$.

Results

The substrates used in this article were selected in the preliminary experiments during the testing of the bioremediation potential of different samples with high hydrocarbon content originating from the refinery. With biostimulation and aeration, in one group of samples (PS), the degradation rate was satisfactory with a large number of microorganisms over several weeks of experiment, whereas in the second group of samples (S) the biodegradation was quickly stopped due to a drastic drop of the number of microorganisms (approximately to 10^2 CFU ml^{-1}). Since bioavailability may represent a limiting factor for biodegradation, it had to be determined whether the key cause was insufficient solubility of the hydrocarbon substrate, which could be resolved by means of adding surfactants, or a lack of microorganisms with specific degradation activities.

Chemical, microbiological and mineralogical characteristics of samples

TPH content of sample PS was 412.5 g kg^{-1} (54% saturated hydrocarbons, 27% aromatic hydrocarbons and 21% NSO and asphaltens). TPH content of sample S was 103.2 g kg^{-1} (60% saturated hydrocarbons, 20% aromatic hydrocarbons, 20% NSO and asphaltenes). Other chemical and microbiological characteristics of the samples employed in the experiments are presented in Table 1.

Table 1 Initial composition of samples used for bioremediation

Parameter (unit)	PS	S
Water content, %	4	19.3
pH (1:2.5 in H_2O)	7.9	7.7
$CaCO_3$, %	2.78	4.25
C total	36.73	8.17
N total	0.38	ND
P_2O_5, g kg^{-1}	0.049	0.009
Loss on ignition, %	53.81	16.32
Fe, g kg^{-1}	10.85	2.10
V, mg kg^{-1}	29	11
Ni, mg kg^{-1}	60	16
Bacteria, CFU g^{-1}	7.0×10^4	7.5×10^4
Hydrocarbon degraders, CFU g^{-1}	5.9×10^3	7.3×10^3
Fungi, CFU g^{-1}	3.0×10^4	6.8×10^3

Calculated on soil dry mass

ND not determined

On the basis of X-ray diffraction analysis, the following minerals were present (in the order of decreasing abundance): in PS quartz, feldspar and clay minerals (illite), calcite and dolomite, and for sample S—quartz and dolomite in equal quantities, and then as minor components—calcite, cristobalite, feldspar, reflections of zeolite, gypsum, pyrolusite and clay minerals (mica, illite, chlorite and montmorillonite).

Although both samples used for biodegradation contain high concentrations of hydrocarbon (>10%), there are significant differences between them in the type of matrix. According to the mineralogical composition, the S sample belongs to sandy soils. The N and P content is low in both the samples, hence, the addition of N and P nutrients is a basic technique (Alvarez and Illman 2006) for stimulating growth of autochthonous microorganisms.

Microbiological analysis of the samples indicates the presence of bacteria and fungi as well as degraders of hydrocarbons in the samples, but in relatively small numbers. The cause may lie in a high concentration of contaminants, low water content in the PS sample and the matrix effect in the S sample.

Isolation and taxonomic identification of the bacterial strains

Bacterial strains used to prepare a mixed culture-consortium were isolated as pure cultures from the PS sample. An inspection of colonies grown on nutrient agar and selective media and comparison of morphological, physiological and biochemical characteristics determined the five bacterial types as dominant. Two types are Gram positive sporogenous rods, including one beta-haemolytic, Voges Proskauer negative (labelled as NS026) and the other non-haemolytic, Voges Proskauer positive (NS032). The third type represents Gram positive reddish-orange colonies with branching mycelium (NS094). The others are Gram negative rods, oxidase positive, out of which one produces a fluorescent pigment (NS009) and the other one does not (NS014).

According to physiological-biochemical properties (API tests), the strains were preliminary identified as *Bacillus cereus* 1 (NS026), *Bacillus licheniformis* (NS032), *Arthrobacter sp.* (NS094), *Pseudomonas fluorescens* (NS009) and *Achromobacter denitrificans* (NS014). The final identification was performed by sequence analysis of the 16S ribosomal RNA gene. 16S rDNA sequence similarity of the isolated strains with corresponding sequence of type strains in NCBI database was shown in Table 2. For the strains identified by API tests as *Bacilllus*, *Pseudomonas* and *Achromobacter* confirmations were obtained. Molecular characterization indicated that the strain labelled as NS0094 belongs to the genus *Micromonospora.*

The isolated bacteria were additionally characterized by the determination of the fatty acid methyl ester profiles (Table 3). The Gram negative strains show the presence of acids with cyclopropane structure and of 12-hydroxy acid in the strain *Pseudomonas*. The branched chain fatty acids dominate in the Gram positive strains.

Taxonomic position of the isolated strains is in accordance with the finding from the previously published articles (Arvanitis et al. 2008; Cameotra and Singh 2008; Verma et al. 2006; Vasudevan and Rajaram 2001), in which *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Rhodococcus*, *Arthrobacter and Corynebacterium* are the main hydrocarbon degraders isolated from the PS and waste.

The consortium members grow on the majority of the selected hydrocarbons (Table 4) as the sole source of carbon, and show tolerance to the heavy metals in the range 2.5–25 mmol l^{-1} (Table 5), which indicates a broad capacity for the degradation and ability to survive.

Table 2 Taxonomic identification of the isolated bacterial strains

Strain	Gene bank accession number	Sequence alignment		Nearest phylogenetic neighbour (Gene bank accession number)
		No. of nucleotides[a]	Identity, %[b]	
NS009	JF826528	1428	99	*P. grimontii* CFML 97-514 (NR 025102)
NS014	JF826529	1310	98	*A. spanius* LMG 5911 (NR 025686)
NS026	JF826526	1437	95	*B. cereus* JCM 2152 (AB598737)
NS032	JF826527	1402	99	*B. licheniformis* CICC 10101 (AY859477)
NS094	JF826530	1314	96	*M. aurantiaca* DSM 43813 (NR 026279)

[a] The number of 16S rDNA nucleotides used for the alignment, attached as Supplementary material

[b] The percentage identity with the 16S rDNA sequence of the nearest phylogenetic neighbour

Table 3 Cellular fatty acid composition of isolated strains, % of total detected

Fatty acid[a]	NS026	NS032	NS094	NS009	NS014
*i*12:0	ND[b]	ND	0.55	ND	ND
12:0	ND	ND	0.40	6.84	0.47
12 2OH	ND	ND	ND	6.3	ND
12 3OH	ND	ND	ND	0.98	ND
*i*13:0	9.15	ND	5.70	ND	ND
*a*13:0	ND	ND	2.15	ND	ND
13:0	0.65	ND	ND	ND	ND
*i*14:0	3.61	ND	1.82	ND	ND
14:0	3.62	0.29	2.63	0.25	2.44
*i*15:0	30.05	29.37	11.79	ND	ND
*a*15:0	9.58	33.63	4.44	ND	ND
15:0	ND	ND	0.84	ND	ND
*i*16:0	6.56	3.18	5.62	ND	ND
Σ16:1[c]	0.68	ND	1.54	11.05	16.52
16:0	26.28	9.49	28.07	46.03	42.13
*cy*17:0	ND	ND	ND	18.61	33.61
*i*17:0	8.45	15.32	8.51	ND	ND
*a*17:0	1.09	ND	6.83	ND	ND
17:0	ND	ND	1.77	ND	ND
Σ18:1[c]	ND	ND	13.14	9.37	0.97
18:0	0.27	0.58	3.97	0.57	2.89
18:0 10 methyl	ND	ND	0.24	ND	ND
*cy*19:0	ND	ND	ND	ND	0.36

[a] Fatty acids are designated in terms of the total number of carbon atoms: number of double bonds. The prefixes *a* and *i* indicate *anteiso* and *iso* branching, *cy* refers to cyclopropane fatty acids, OH indicated the presence of hydroxyl group

[b] not determined, values <0.20% are omitted

[c] Σ total sum of monounsaturated acid

Biodegradation

The course of biodegradation in the S and PS samples is presented in Figs. 1 and 2. The effect of surfactant addition on both the samples as well as bioaugmentation on the S sample was studied. The process was followed by determining the number of bacteria in the suspension (Figs. 1a, 2a) as well as through the decrease of the TPH content (Figs. 1b, 2b). The experiment was conducted in triplicate, and statistical analysis of obtained data was performed. Statistically significant differences between treatment groups over time were found for the both samples and for both measured parameters: degree of degradation and number of microorganisms. Moreover, the significant differences observed changed over the time course of the study, which was a clear indication that there were interactions between factors included in the study.

The results shown in Figs. 1a and 2a clearly indicated that biostimulation (addition of NPK) in the first week of the experiment led to an abrupt increase of the number of microorganisms in both the samples.

In the PS sample, due to its high content of organic substances (high C/N/P), the consumption of phosphates was significantly higher, and during the first month a further portion of NPK had to be added weekly, which, albeit with fluctuations, led to the growth of a stable microbial population (Fig. 1a).

Over this time interval (4 weeks), the addition of surfactants had no statistically significant effect on the number of bacteria in the suspension, but in contrast, the differences in the degree of degradation

Table 4 Growth of isolated strains on selected hydrocarbon as the sole C source

	Bacillus sp. NS026	*Bacillus* sp. NS032	*Micromonospora* sp. NS094	*Pseudomonas* sp. NS009	*Achromobacter* sp. NS014
Diesel fuel	+[a]	+	+	+	+
n-Hexane	+	+	+	+	+
n-Hexadecane	+	+	+	+	+
n-Octadecane	+	+	+	+	+
Benzene	+	+	+	+	+
Toluene	+	+	–	–	–
Xylene	–	+	–	–	–
Phenantrene	–	+	+	–	–
Pyrene	+	+	+	+	+
Etylbenzene	+	+	–	–	–
Octylbenzene	+	+	+	+	+
Hexadecylbenzene	+	+	+	+	+
Sodium-benzoate	+	+	+	–	+
2-Phenylphenol	+	+	+	+	–
Dibenzothiophene	+	+	+	+	+

[a] +viable growth observed in 1×10^{-5} dilution

Table 5 Tolerance to metal ions, mmol l^{-1}

	Ni^{2+}	Cu^{2+}	Cr^{3+}	Cd^{2+}
Bacillus sp. NS026	25	2.5	25	1.25
Bacillus sp. NS032	5	2.5	2.5	1.25
Micromonospora sp. NS094	5	2.5	5	2.5
Pseudomonas sp. NS009	5	25	5	25
Achromobacter sp. NS014	12.5	12.5	2.5	12.5

Numbers in table represent maximum concentration of metal salts in Mueller–Hinton plates where visible growth is observed

of the TPH were statistically significant (Fig. 1b). Thus, in the first month, the TPH decreased from the initial 412 g kg^{-1} to 331 (19.6%), and with the addition of surfactant even down to 154 g kg^{-1} TPH (62.6%).

The effect of surfactants on the growth of the microorganisms became visible in the period between week 4 and 8 of the experiment, probably not only because the obtained biomass had a lot of available organic substance at its disposal but also because the surfactant could serve as an additional C source. Therefore, the total number of bacteria in the suspension in week 6 attained 4.6×10^9 CFU ml^{-1}. After 8 weeks, the number of microorganisms dropped, but by that time 70–80% of the hydrocarbons had already been degraded. At the 8th and 12th weeks of the study, statistically significant differences were found between mean values of number of microorganisms, but not in degradation efficiency.

Compared to the PS, a lower maximum density of the biomass ($\sim 10^6$–10^7 CFU ml^{-1}) in the S samples (Fig. 2) was attained throughout the entire study because of the lower content and availability of the organic substance. It must be emphasised that the addition of surfactants greatly increased the availability of the hydrocarbons, hence, over the course of the entire experiment, in the tests with the surfactant, the number of microorganisms was higher by almost two orders of magnitude, and consequently, the degradation rate increased as well. Therefore, in the number of microorganisms and the degradation efficiency, there were statistically significant differences between the mean values of both paired treatments (without and with surfactant, treatments 1 and 2, 3 and 4, Fig. 2) in 4th and 8th week. However, at the 12th week, statistically significant differences were found only between treatments 1 and 2 and only in the degree of degradation.

The addition of the bacterial consortium to the S sample helped to maintain the number of the viable bacterial population. After 8 weeks, similar to the PS

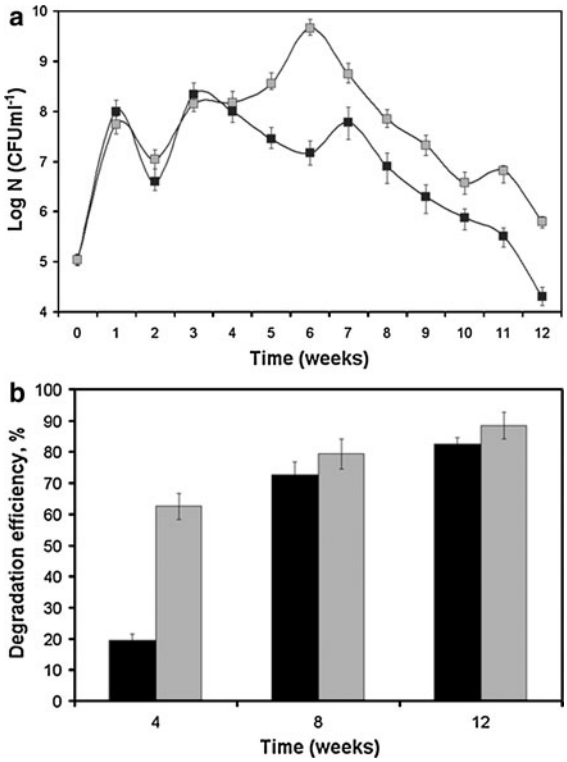

Fig. 1 Degradation of the PS sample **a** Growth of bacteria treatment: biostimulation (*filled square*), biostimulation + surfactant (*shaded square*), Values are the means from three replicates, *error bars* represent minimum and maximum values ($n = 3$) Statistically significant differences, $P < 0.05$, between treatments at the 8th and 12th week were found. **b** Reduction of TPH content treatment: biostimulation (*filled square*), biostimulation + surfactant (*shaded square*). *Bars* represent mean values ± standard deviation ($n = 3$) Statistically significant differences, $P < 0.05$, between treatments at the 4th week were found

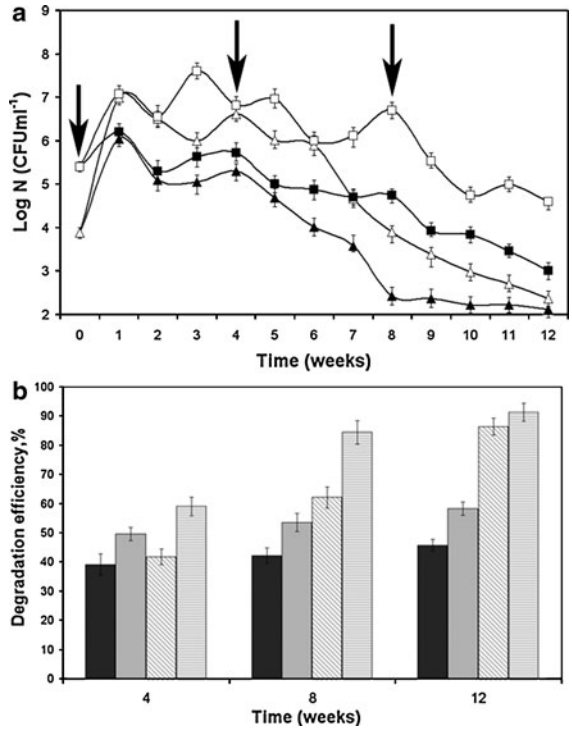

Fig. 2 Degradation of the S sample **a** Growth of bacteria treatment: 1. biostimulation (*filled triangle*), 2. biostimulation + surfactant addition (*open triangle*), 3. biostimulation + bioaugmentation (*filled square*) 4. biostimulation + bioaugmentation + surfactant (*open square*) Values are the means from three replicates, *error bars* represent minimum and maximum values ($n = 3$) The *arrows* point to the addition of the bacterial consortium. At the 4th week, statistically significant differences between treatments 1 and 3, 2 and 4 were not found ($P > 0.05$). At the 12th week, statistically significant differences between all treatments were not found ($P > 0.05$). **b** Reduction of TPH content treatments: 1. biostimulation (*filled square*), 2. biostimulation + surfactant (*shaded square*), 3. biostimulation and bioaugmentation (*cross line square*) 4. biostimulation, bioaugmentation + surfactant (*horizontal line square*) *Bars* represent mean values ± standard deviation ($n = 3$) At the 4th week, statistically significant differences between treatments 1 and 3, 2 and 3 were not found ($P > 0.05$). At the 8th week, statistically significant differences between treatments 2 and 3 were not found ($P > 0.05$). At the 12th week, statistically significant differences between treatments 3 and 4 were not found ($P > 0.05$)

sample, the number of microorganisms decreased. However, the addition of the bacterial consortium had no effect on bacterial numbers at this time, irrespective of the surfactant addition. The reduced number of microorganisms after 8 weeks in all treatments may be the result not only of decreased availability of any remaining hydrocarbons but also the accumulation of toxic metabolites.

As indicated previously, for the S sample, the main goal was to study the effects of bioaugmentation alone and in combination with the added surfactant on the degree of degradation. Biostimulation and biostimulation with added surfactant (treatments 1, 2) finally achieved hydrocarbon degradation of 40–45% and 50–60%, respectively (Fig. 2b). On the other hand, bioaugmentation (repeated inoculation with consortium biomass, treatments 3 and 4) led to continual decrease in TPH values, which finally reached much lower values of 86.4–91.3% of the starting levels.

In degradation efficiency, bioaugmentation with surfactant addition (treatment 4, Fig. 2b) was found to be statistically significantly different at all times

from all other treatments except treatment 3 (bioaugmentation) at the 12th week. This confirms the advantage of adding microbial biomass with specific degradation abilities to such types of polluted soil to achieve a high degree and efficiency of degradation.

The final efficacy of THP degradation achieved in this experiment was 82.5–88.6% for the PS sample and 86.4–91.3% for the S sample, which corresponded with the data found in the literature (Ito et al. 2008; Mishra et al. 2001; Ward et al. 2003). In control flasks supplemented with $HgCl_2$, the extent of TPH degradation was approximately 6% (data not shown).

However, it should be noted that the PS and S samples used in this study contained high levels of hydrocarbons in comparison with the other published data (Ito et al. 2008; Verma et al. 2006; Wongsa et al. 2004; Zvyagintseva et al. 2001). For example, in the PS sample, the initial concentration of TPH was 4%, while in the studies described by the other authors, concentrations were usually 0.3–1%.

Although in some treatments, a high degree of degradation was observed even by the 8th week, the study lasted 12 weeks to achieve the maximum degree of degradation and to track differences between the treatments.

Biodegradation pattern

Figure 3 shows gas chromatograms of TPH extracts of the S and PS samples in the beginning and in the end of the experiment.

The results indicate that in the PS sample, *n*-alkanes in the range of C_{15}–C_{38} were initially present, and those peaks corresponding to pristane (Pr) and phytane (Phyt) were lower in intensity than the related *n*-alkanes (C_{17}, C_{18}), which is an indication of a non-biodegraded sample.

In the S sample, *n*-alkanes are degraded, and Pr and Phyt peaks are clearly differentiated, while the wide peak represents UCM (unresolved complex mixture). It is important to note that the fact that *n*-alkanes as the

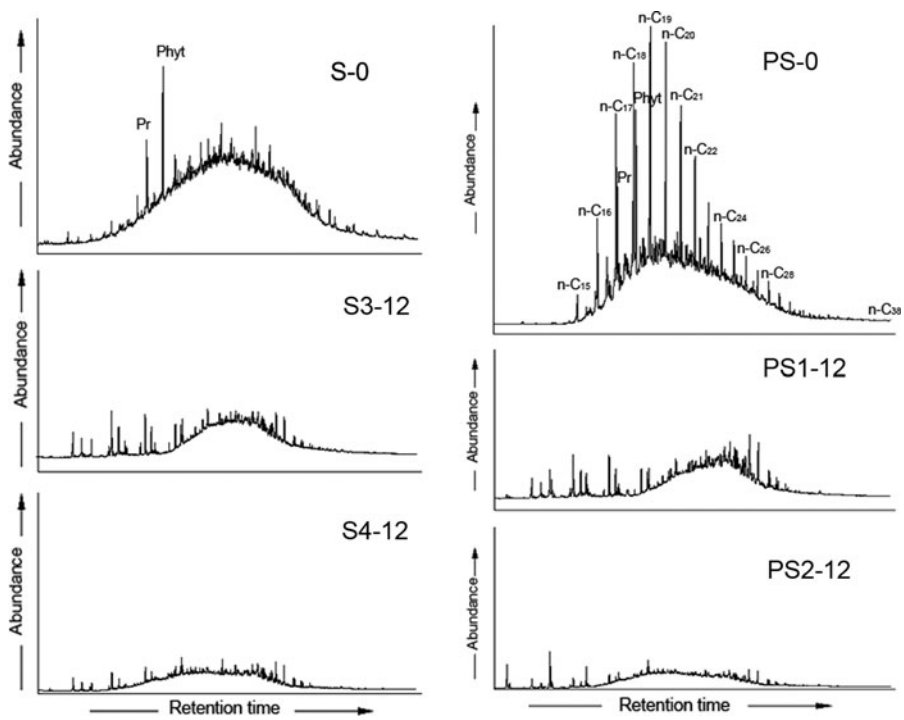

Fig. 3 Gas chromatograms of samples during experiment samples: S-0 and PS-0—samples S and PS at the beginning of experiment; PS1-12: sample PS (treatment 1: biostimulation) at the end of experiment; PS2-12: sample PS (treatment 2: biostimulation + surfactant) at the end of experiment; S3-12: sample S (treatment 3: biostimulation + bioaugmentation) at the end of experiment; S4-12: sample S (treatment 4: biostimulation + bioaugmentation + surfactant) at the end of experiment, *Pr* pristane, *Phyt* phytane

most biodegradable part of hydrocarbons were depleted in the initial sample S, together with sandy composition, contribute significantly to decreased biodegradation potential of this sample.

After 3 months, in the end of the experiment, there were no *n*-alkanes or isoprenoids in the samples.

In order to define more precisely the intensity of hydrocarbon degradation, the TPH extracts were analysed in more detail by GC–MS with respects to polycyclic alkanes of sterane and terpane types. As it has been already mentioned, these cyclic compounds are expected to be exposed to microbial degradation after *n*-alkanes and isoprenoids. Furthermore, due to the resistance to biodegradation, these compounds represent biomarkers for oil weathering and biodegradation studies (Wang and Fingas 2003).

Figures 4 and 5 present SIM-chromatograms of steranes (*m/z* 217) and terpanes (*m/z* 191). The obtained data show that in the initial samples the distribution (presence and abundance) of sterane and terpane components was very similar, which clearly indicates the same origin. Moreover, the biodegradation pattern was very similar in both the investigated soils. The following order of steranes degradation was observed: C27 > C28 > C29. Diasteranes were seriously depleted. Terpanes were the most abundant compounds in both the residual oils at the end of the indicated period. Significant biodegradation was

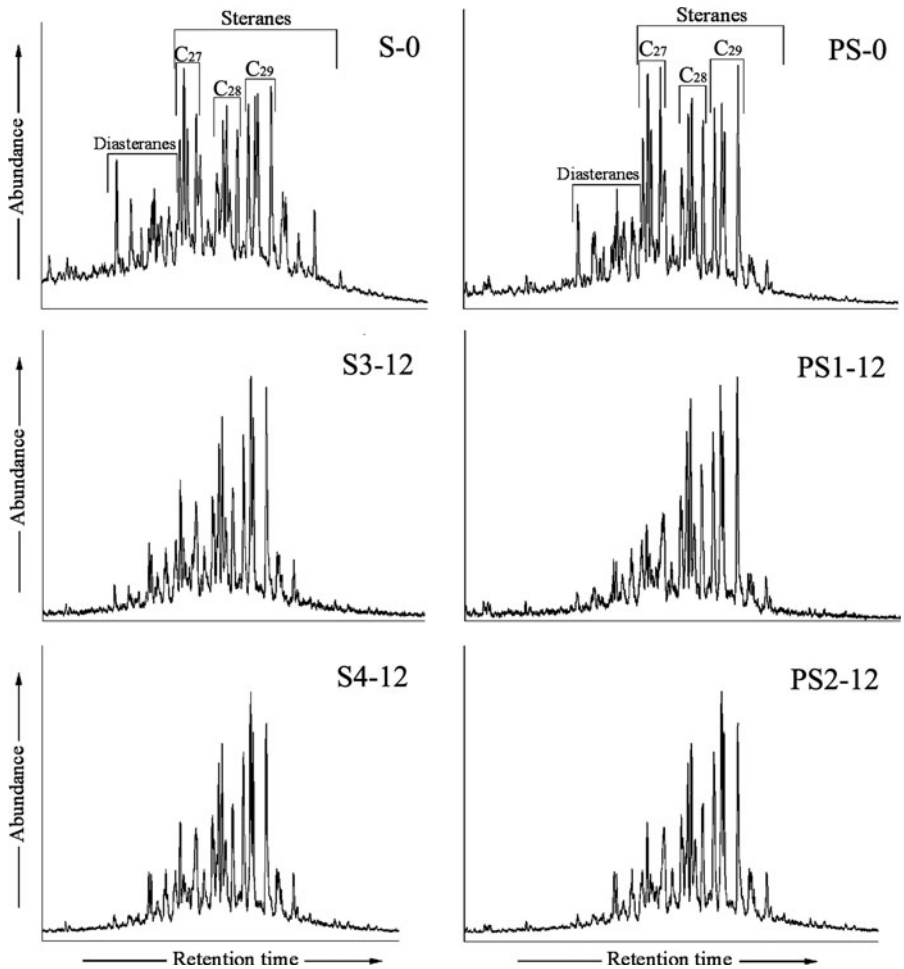

Fig. 4 GC–MS ion fragmentograms of steranes (*m/z* 217) from samples: S-0 and PS-0—samples S and PS at the beginning of experiment; PS1-12: sample PS (treatment 1: biostimulation) at the end of experiment; PS2-12: sample PS (treatment 2: biostimulation + surfactant) at the end of experiment; S3-12: sample S (treatment 3: biostimulation + bioaugmentation) at the end of experiment; S4-12: sample S (treatment 4: biostimulation + bioaugmentation + surfactant) at the end of experiment

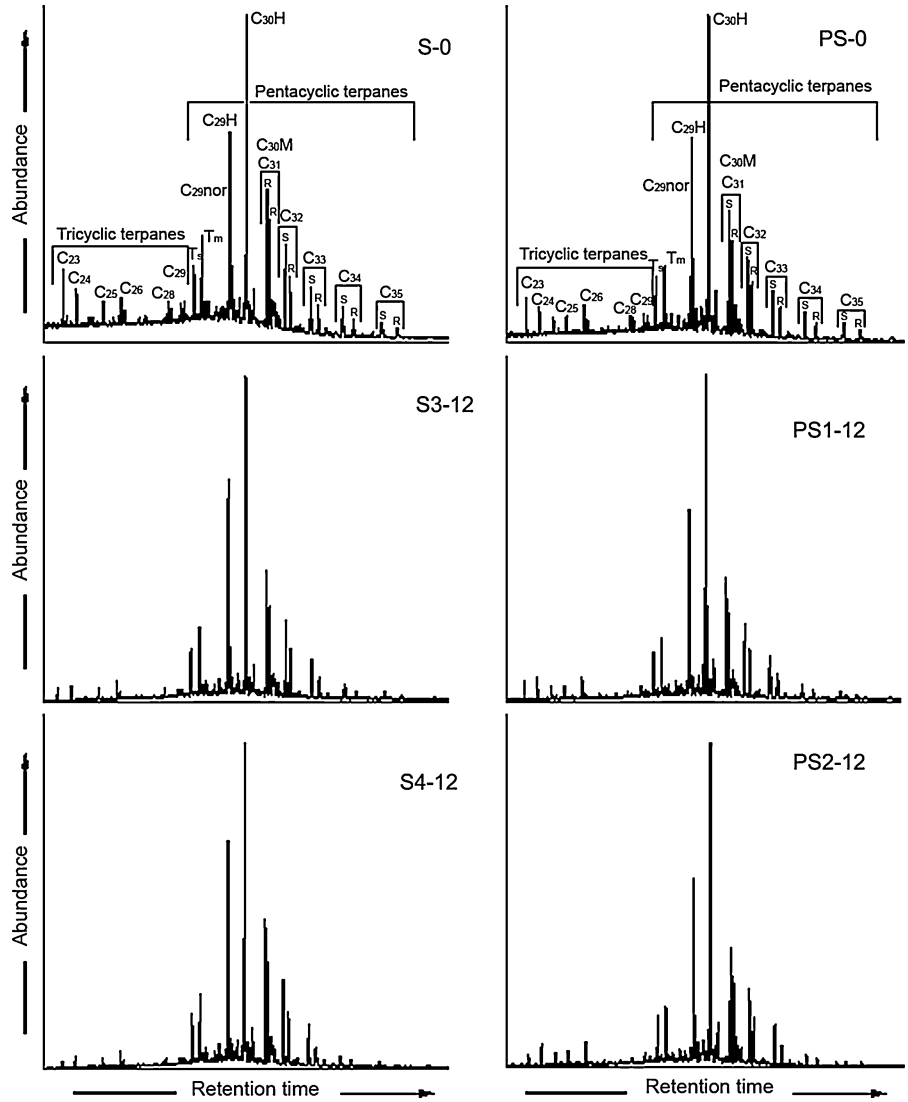

Fig. 5 GC–MS ion fragmentograms of terpanes (*m/z* 191) from samples: S-0 and PS-0—samples S and PS at the beginning of experiment; PS1-12: sample PS (treatment 1: biostimulation) at the end of experiment; PS2-12: sample PS (treatment 2: biostimulation + surfactant) at the end of experiment; S3-12: sample S (treatment 3: biostimulation + bioaugmentation) at the end of experiment; S4-12: sample S (treatment 4: biostimulation + bioaugmentation + surfactant) at the end of experiment identification of peaks: Ts—C_{27} 18 α(H)-22, 29, 30-trisnorneohopane; Tm—C_{27} 17 α(H)-22,29,30-trisnorneohopane; C_{29}H—C_{29}17α(H)21 β(H)-hopane; C_{29}nor—C_{29}18α(H)-30-norneohopane; C_{30}H—C_{30}17α(H)21β(H) hopane C_{30}M—C_{30}17β(H)21α(H)-moretane

observed with tricyclic terpanes and homohopanes in the C35 > C34 > C33 sequence, but all the other terpanes under these conditions remained intact.

Discussion

In this article the difference in the biodegradability of the two samples was observed. The PS and S samples differ not only in the content and type of petroleum hydrocarbons but also in the mineral composition.

Optimization of biodegradation process was carried out in several phases, the first of which was biostimulation, following the addition of surfactant, and finally, if necessary, bioaugmentation was applied.

The experiment was designed to determine the need for increasing the bioavailability of hydrocarbon

substrate by using surfactant or for the introduction of microorganisms with specific degradation abilities.

Application of biostimulation to the PS sample resulted in more than 80% of hydrocarbons being degraded. Surfactant increased the number of microorganisms and accelerated the process but did not affect the final outcome. On the other hand, bioaugmentation had to be applied to the S sample, because with the addition of nutrients and surfactant only, the degree of degradation remained at the 45–60% level. Similar to the PS sample, the surfactant did not affect the final outcome but only accelerated the process of degradation.

The PS sample was chosen as a source of bacteria used for bioaugmentation for the S sample. Indigenous microorganisms present in this sample can efficiently degrade hydrocarbons even without surfactant, and so, a high level of degradation can be obtained even in 4 weeks. Biodegradation efficiency of the isolated strains has proven not only by the decrease in total hydrocarbon content of the samples but also by biomarker analysis.

The presence and distribution of biomarkers are used in organic geochemistry for determination of the biological origin, the degree of similarity or the identification of pollutant source. In biodegradation studies, the biomarker analysis shows which compounds are degraded, to what extent and in what order. Furthermore, the changes in biomarker peak intensities and their relationships depends on the biodegradation ability of microorganisms to which pollutants are exposed (Peters et al. 2005; Wang and Fingas 2003).

In the present study, based on the gas chromatography results, it can be concluded that the initial samples differ in the degree of degradation, because the S sample did not contain the most easily degraded fraction—the *n*-alkanes. However, GC–MS analysis shows that the patterns of the other hydrocarbon fractions, steranes and terpanes are identical, which indicates their similar origins.

At the end of the experiment, not only total gas chromatograms but also sterane and terpane profiles were identical regardless of the sample type and the addition of surfactant. Since sterane and terpane decomposition starts after *n*-alkane and isoprenoids depletion, the decrease of the intensity of these biomarker peaks in the final fragmentograms confirm the excellent biodegradation abilities of the bacteria used for bioaugmentation. Moreover, identical sterane and terpane profiles at the end of the study provide a unique fingerprint of biodegradation capacity of used strains.

On the basis of results published in the literature, the application of bioaugmentation has no positive effects in some cases (Gentry et al. 2004), although the ability to degrade pollutants has been proven in the applied microbial cultures (either by means of techniques of molecular biology or through growth on a specific substrate). The unpredictability is, thus, often highlighted as one of the weakness of this technique. Despite the complexity of the biological systems, it is considered that the problem largely results from the initial selection of strains (Thompson et al. 2005), and that besides the degradation ability, important factors in the selection of strains are the ability to survive in an unfavourable medium, relative abundance on the site, as well as tolerance to co-contaminants.

In this study, degradation capacity of the isolated bacterial strains is proven by means of growth on individual hydrocarbon substrates as well as through the achieved level of TPH degradation and certain fractions of hydrocarbon. The sample (PS) from which the strains were isolated represents an extremely unfavourable environment because of the lack of humidity, high concentration of hydrophobic substrate and presence of heavy metals.

An advantage to the use of mixed cultures is a broader degradation capacity, synergic effect and co-metabolism (Rahman et al. 2003; Mishra et al. 2001). Also, the consortium members should preferably belong to different taxonomic groups as they have, thus, developed different adaptation and survival mechanisms.

Besides the genera of bacteria from which the strains in this study were isolated, other authors mention the consortium members, *Acinetobacter* and *Rhodococcus* strains (Arvanitis et al. 2008; Ward et al. 2003), as efficient in the degradation of petroleum hydrocarbons. An efficient combination is the one of fast-growing strains (*Pseudomonas*) with the slow-growing strains (*Rhodococcus*). The consortium members should have different physiological response to hydrocarbons; thus, *Pseudomonas* grows quickly in the water phase, while *Rhodoccocus* grows more slowly and in association with oil (Ward et al. 2003), and therefore, given the role in the soil, at least one of the cultures should be *Actinobacteria*.

Micromonospora species are widely distributed in a variety of habitats: in soils, sediments and aquatic environments. They have incredible metabolic versatility, and their spores are highly resistant to desiccation stress. Although *Micromonospora* is not among the genera that are usually mentioned among the microorganisms that break down hydrocarbons, in previous studies, they were well documented as efficient lignocellulose (Kausar et al. 2011), pesticide (Fuentes et al. 2010) and hydrocarbon (Arafa 2003) degraders.

The presence of *Bacillus* is frequent in samples with high concentration of hydrocarbons (30–40%), and their survival is presumed to be due to resistant endospores (Ijah and Antai 2003). Nonetheless, the capacities that have been proven include growth on individual aliphatic and aromatic hydrocarbons (Verma et al. 2006; Ghazali et al. 2004), degradation of crude oil (Ijah and Antai 2003) as well as oily sludge, where during degradation certain *Bacillus* strains exceed the capacities of strains *Pseudomonas* and *Acinetobacter* (Verma et al. 2006).

Microorganisms in mixed culture may have a different relationship to hydrocarbon substrates such as (a) direct interaction with soluble hydrocarbons, (b) assimilation of dispersed (emulsified) hydrocarbons and (c) attachment to the hydrocarbon drop by hydrophobic cell surface. The surfactants not only increase the bioavailability of hydrocarbons by emulsification but also by altering the cell surface, and thus, increasing the cell affinity for hydrocarbons (Verma et al. 2006).

Chemical surfactants have different impacts on the growth of mixed cultures and TPH degradation depending on the species, electric charge, HLB (hydrophilic–lipophilic balance), CMC (critical micellar concentration) value, where this impact may be simulative, without effect, or inhibitory. Based on the data provided in the manufacturer's instructions and publications (Mulligan et al. 2001), BioSolve both stimulates the growth and increases the degradation of hydrocarbons, which has been confirmed in this article. A similar increase of the bacterial growth and degree of hydrocarbon degradation has been confirmed in the studies with Igepal CO-630 (Ward et al. 2003) and Tween 80 (Bautista et al. 2009).

The present article has established that the chemical surfactant in PS and S samples has an effect on the degradation rate, while in the S sample it also affects availability given the mineralogical composition.

It should be noted that due to biodegradability, the biosurfactants are the best solution for the environment. However, the question is whether the cultures in the consortium may produce a sufficient quantity of surfactants in situ. A microorganism produces a surfactant only in the surroundings of its cell, and hyperproduction would take special conditions. Thus, in the rhamnolipids, for instance, the best laboratory yields are achieved on a medium without phosphates. One of the solutions may be the addition of raw biosurfactants produced in a separate procedure (Cameotra and Singh 2008).

It should be added that the experiments with bacteria, members of the consortium, did not show a significant production of biosurfactants in a mineral medium supplemented with 2% diesel oil (measured through the change of the surface tension with a stalagmometer), which does not exclude the emulsification ability or any other effects that may be present, although they were certainly not present to a sufficient extent.

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References

Alvarez PJJ, Illman WA (2006) Bioremediation and natural attenuation: process fundamentals and mathematical models. Wiley, Hoboken

Arafa MA (2003) Biodegradation of some aromatic hydrocarbons (BTEXs) by a bacterial consortium isolated from polluted site in Saudi Arabia. Pak J Biol Sci 6:1482–1486

Arvanitis N, Katsifas E, Chalkou KI, Meintanis C, Karagouni AD (2008) A refinery sludge deposition site: presence of nahH and alkJ genes and crude oil biodegradation ability of bacterial isolates. Biotechnol Lett 30:2105–2110

Bautista LF, Sanz R, Molina MC, Gonzales N, Sanchez D (2009) Effect of different non-ionic surfactants on the biodegradation of PAHs by diverse aerobic bacteria. Int Biodeterior Biodegrad 63:913–922

Cameotra SS, Singh P (2008) Bioremediation of oil sludge using crude biosurfactants. Int Biodeterior Biodegrad 62:274–280

Das N, Chandran P (2011) Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol Res Int. doi:10.4061/2011/941810

DIN-EN 14345 (2004) Characterization of waste—determination of hydrocarbon content by gravimetry

EPA SW-846 method 3051 (1995) Microwave assisted acid digestion of sediments, sludges, soil and oils. In: Test methods for evaluating solid waste, 3rd edn, 3rd Update, US Environmental Protection Agency, Washington

Fuentes MS, Benimeli CS, Cuozzo SA, Amoroso MJ (2010) Isolation of pesticide-degrading actinomycetes from a contaminated site: bacterial growth, removal and dechlorination of organochlorine pesticides. Int Biodeterior Biodegrad 64:434–441

Gentry JT, Rensing C, Pepper IL (2004) New approaches for bioaugmentation as a remediation technology. Crit Rev Environ Sci Technol 34:447–494

Ghazali FM, Rahman RNZA, Salleh AB, Basri M (2004) Biodegradation of hydrocarbons in soil by microbial consortium. Int Biodeterior Biodegrad 54:61–67

Haack SK, Garchow H, Odelson DA, Forney LJ J, Klug MJ (1994) Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. Appl Environ Microbiol 60:2483–2493

Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Smith CP, Wards JM, Shrempf H (1985) Genetic manipulation of Streptomyces: a laboratory manual, 1st edn. John Innes Foundation, Norwich

Ijah UJJ, Antai SP (2003) Removal of Nigerian light crude oil in soil over a 12-month period. Int Biodeterior Biodegrad 51:93–99

Ito H, Hosokawa R, Morikawa M, Okuyama H (2008) A turbine oil-degrading bacterial consortium from soils of oil fields and its characteristics. Int Biodeterior Biodegrad 61:223–232

Kausar H, Sariah M, Mohd Saud H, Zahangir Alam M, Razi Ismail M (2011) Isolation and screening of potential actinobacteria for rapid composting of rice straw. Biodegradation 22:367–375

Koma D, Sakashita Y, Kubota K, Fujii Y, Hasumi F, Chung SY, Kubo M (2003) Degradation of car engine oil by *Rhodococcus* sp. NDKK48 and *Gordonia* sp. NDKY76A. Biosci Biotechnol Biochem 67:1590–1593

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow MM (eds) Nucleic acid techniques in bacterial systematic. Wiley, Chichester, pp 115–175

Liu JS, Xie XH, Xiao SM, Wang XM, Zhao WJ, Tian ZL (2007) Isolation of *Leptospirillum ferriphilum* by single-layered solid medium. J Cent South Univ Technol 4:467–473

Löser C, Seidel H, Zehnsdorf A, Stottmeister U (1998) Microbial degradation of hydrocarbons in soil during aerobic/anaerobic changes and under purely aerobic conditions. Appl Microbiol Biotechnol 49:631–636

Mishra S, Jyot J, Kuhad RC, Lal B (2001) Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. Appl Environ Microbiol 67:1675–1681

Mulligan CN, Yong RN, Gibbs BF (2001) Surfactant-enhanced remediation of contaminated soil: a review. Eng Geol 60:371–380

Namio K, Haruki M, Takano S, Morikawa M, Kanaya S (2005) Isolation and characterization of *Rhodococcus* sp. strains TMP2 and T12 that degrade 2,6,10,14-tetramethylpentane (pristane) at moderately low temperatures. J Biotechnol 115:129–136

Nieto JJ, Fernandez-Castillo R, Marquez MC, Ventosa A, Quesada E, Ruiz-Berraquero F (1989) Survey of metal tolerance in moderately halophilic eubacteria. Appl Environ Microbiol 55:2385–2390

Perry JJ (1984) Microbial metabolism of cyclic alkanes. In: Atlas RM (ed) Petroleum microbiology. MacMillan Publishing Company, New York, pp 61–98

Peters KE, Walters CC, Moldovan JM (2005) The biomarker guide. Cambridge University Press, Cambridge

Rahman KSM, Rahman TJ, Kourkoutas Y, Petsas I, Marchant R, Banat IM (2003) Enhanced bioremediation of *n*-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Bioresour Technol 90:159–168

Rump HH (1999) Laboratory manual for the examination of water, waste water and soil. 3rd edn, Wiley-VCH Verlag GmbH, Weinheim, pp 144–190

Thompson IP, van der Gast CJ, Ciric L, Singer AC (2005) Bioaugmentation for bioremediation: the challenge of strain selection. Environ Microbiol 7:909–915

Vasudevan N, Rajaram P (2001) Bioremediation of oil sludge-contaminated soil. Environ Int 26:409–411

Verma S, Bhargava R, Pruthi V (2006) Oily sludge degradation by bacteria from Ankleshwar, India. Int Biodeterior Biodegrad 57:207–213

Walker JD, Colwell RR, Petrakis L (1975) Microbial petroleum degradation: application of computerized mass spectrometry. Can J Microbiol 21:1760–1767

Wang Z, Fingas MF (2003) Development of oil hydrocarbon fingerprinting and identification techniques. Mar Pollut Bull 47:423–452

Ward O, Singh A, Van Hamme J (2003) Accelerated biodegradation of petroleum hydrocarbon waste. J Ind Microbiol Biotechnol 30:260–270

Wentzel A, Ellingsen TE, Kotlar HK, Zotchev SB, Throne-Holst M (2007) Bacterial metabolism of long chain *n*-alkanes. Appl Microbiol Biotechnol 76:1209–1221

Wongsa P, Tanaka M, Ueno A, Hasanuzzaman M, Yumoto I, Okuyama H (2004) Isolation and characterization of novel strains of *Pseudomonas aeruginosa* and *Serratia marcescens* possessing high efficiency to degrade gasoline, kerosene, diesel oil, and lubricating oil. Curr Microbiol 49:415–422

Zvyagintseva IS, Suroviseva EG, Polglazova MN, Ivoilov VS, Belyaev SS (2001) Degradation of machine oil by nocardioform bacteria. Microbiology 70:270–276