ELSEVIER

International Biodeterioration & Biodegradation 44 (1999) 87–92

INTERNATIONAL BIODETERIORATION & BIODEGRADATION

www.elsevier.com/locate/ibiod

Biodegradation of crude oil in sandy sediment

Jussara P. Del'Arco, Francisca P. de França*

Departamento de Engenharia Bioquímica, Escola de Química, Centro de Tecnologia, Bloco E, Universidade Federal do Rio de Janeiro, Ilha do Fundão, 21949-900, Rio de Janeiro, Brazil

Received 25 January 1999; received in revised form 3 May 1999; accepted 10 May 1999

Abstract

The bioremediation of light Arabian oil in sandy sediment by the mixed culture designated N_D, obtained from landfarming, associated to indigenous microorganisms resulted in 42.9% reduction of the heavy fraction of oil in 28 days when a phosphate and a nitrate source were provided. Tests performed only in the presence of native flora, have achieved 11.9% removal of these compounds. These results have demonstrated the importance of utilizing cultures adapted to pollutants to enhance efficiency and productivity of the bioremediation process. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Economical and environmental considerations have forced petrochemical industries to reduce substantially the amount of hydrocarbon waste material through modifications of the operational procedures (Milne et al., 1998). Bioremediation is one of the biological treatments that has attracted special attention. It is defined as the breakdown of organic compounds by microorganisms. Mineralization is the complete conversion of organic molecules into inorganic substances. Biotransformation is the transformation of a parent compound into other metabolites, which may be more or less toxic than the parent compound. The possible benefits and advantages of bioremediation include: low cost of energy required for the biochemical transformations, possibility of treatment on site, reduction of transportation costs, permanent waste elimination reducing the long term liability risks, reduction of the contamination risks in other areas and the positive public acceptance (Prince and Sambasivam, 1993). Currently, bioremediation is approved by the US Environmental Protection Agency as a hazardous waste site cleaning-up technology. The United States Government has also developed a National Contingency Plan (NCP) to establish a set of procedures to deal with oil and hazardous materials spills or catastrophes. As a part of NCP, a list (product schedule) was issued, including different bioaugmentation and biostimulation agents (Aldrett et al., 1997). Laboratory studies have demonstrated that the majority of these products do not enhance microbial degradation of petroleum hydrocarbons. For some of the products tested, the biodegradation substantially occurred during the first 7 days. After that period, further biodegradation related to time was almost negligible due to either: (1) the recalcitrant nature of the oil in relation to the specific agent cultures; (2) to a nutrient depletion that occurred after the first week; (3) to degradation metabolite or intermediate product toxicity (Aldrett et al., 1997). In this context, selection of adapted cultures capable of degrading contaminants under biological and physicochemical conditions of the polluted site is fairly commendable.

The purpose of this work was the degradation of hydrocarbons of light Arabian oil by a mixed culture, isolated from a petroleum contaminated soil and adapted to light Arabian oil in sandy sediment supplied with inorganic nutrients.

* Corresponding author. Fax: +55-21-567-0283.
E-mail address: fpfranca@h2o.eq.ufrj.br (F.P. de França).

0964-8305/99/$ - see front matter
PII: S0964-8305(99)00063-3

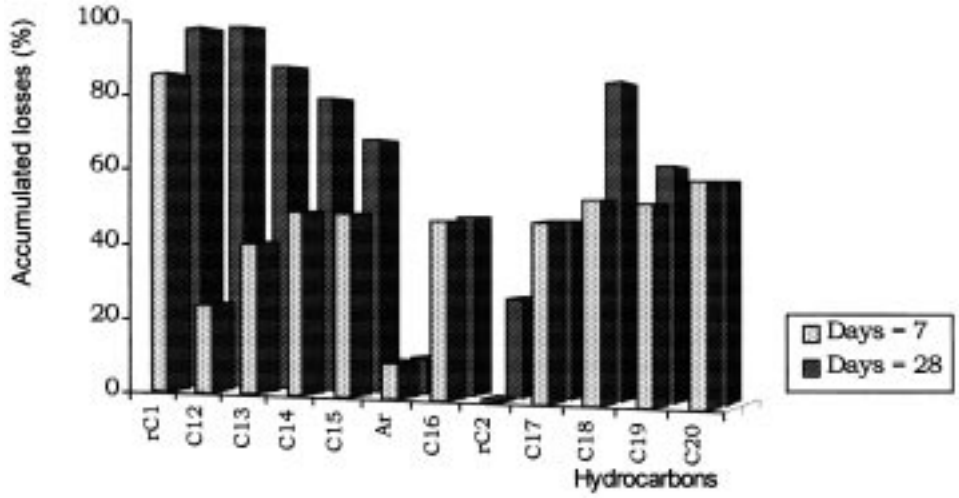

Fig. 1. Evolution of the accumulated losses in the biodegradation of the light Arabian oil by culture N_D until the 7th and 28th day of process. Where: rC1 is 2,2,4,4,6,8,8-heptamethylnonane; C_{12} is dodecane; C_{13} is tridecane; C_{14} is tetradecane; C_{15} is pentadecane; Ar is 1H-indene-2,3dihydro-1,1,3 trimethyl-3; C_{16} is hexadecane; rC2 is 2,6,10,15-tetramethylheptadecane; C_{17} is heptadecane; C_{18} is octadecane; C_{19} is nonadecane; C_{20} is eicosane.

2. Materials and methods

The light Arabian oil used in this study was provided by Petróleo Brasileiro S.A. SARA (Saturates, Aromatics, Resins and Asphaltenes) analysis indicated the following composition (%): 46.50 saturated, 32.23 aromatics and 21.27 polar compounds. Resins and asphaltenes that constitute the polar fraction are often considered to be non-biodegradable (Milne et al, 1998). In the experiments, a mixed culture designated N_D, obtained from landfarming (Refinaria Duque de Caxias, Rio de Janeiro, Brazil) was used as inoculum. That culture was selected because a preliminary investigation showed its ability to degrade hydrocarbons of light Arabian oil. The culture N_D was previously adapted to the light Arabian oil, by successive culture in 500 ml Erlenmeyer's flasks containing 100 ml of mineral medium (composition gl^{-1}: 0.5 KH_2PO_4; 1.4 Na_2HPO_4; 1.0 NH_4NO_3; 0.1 $MgSO_47H_2O$; 0.02 $CaCl_22H_2O$; 0.03 $MnSO_4H_2O$) containing 1% (v/v) of the light Arabian oil, at 150 rpm and 30°C. The experiments were performed in polyvinyl chloride (PVC) reactors with dimensions $0.45 \times 0.29 \times 0.31$ m. Each reactor received a total of 76.8 kg of beach sand in natura (Recreio of the Bandeirantes, Rio de Janeiro, Brazil). Firstly 64.0 kg of sand plus 7.8 l of mineral solution were mixed and disposed to obtain a 25 cm layer. Then, 12.8 kg of the sand was mixed with 90 g of light Arabian oil and 1.6 l of mineral medium containing the culture N_D and spread over the first layer to produce a 5 cm layer. These bioreactors were simulating the bioremediation process of an accidental oil spill (Rosenberg et al., 1992). The influence of the native microbial flora in the process was evaluated in an identical uninoculated system. Periodically, the upper contaminated sand layer, was humidified with water or mineral solution in order to replace the evaporated water and the inorganic nutrients consumed. These reactors were also tilled weekly with the purpose of stimulating biodegradation by increased availability of oxygen. The samplings were made from 5 equidistant points of the container in the upper layer. These samples were combined in a single one. The 70 g amount of the soil sample was Soxhlet-extracted with 200 ml CCl_4 for 6 h. The extract was brought to 200 ml. Analyses were performed by combined gas chromatography (Hewlett-Packard Model 5880A) and mass spectrometry (Hewlett-Packard Model 5987A) using a 30 m × 0.25 mm column HP-5 (phenyl 5% methyl silicone 95%). The heavy fraction of the extract was determined by the partition-gravimetric method (American Public Health Association, 1992). The microbial growth was determined by pour plate technique in the sandy sediment (Milne et al., 1998). Bacto-nutrient agar (Difco Laboratories, 0001) was used to quantify bacterial colony forming units (cfu) after incubation at 30°C for 48 h. Bacto-Sabouraud agar (Difco Laboratories, 0109) containing 0.04% chloramphenicol (Merck, 1023660050) was used for determination of fungi colony forming units (cfu) in the sandy sediment after incubation at 25°C for 120 h. For the determination of nitrate and phosphate, 10 g of the sample were mixed with 50 ml of acid solution (HCl 0,1N) and stirred at 150 rpm during 4 h. Ten ml of the supernat were filtered through a 0.45 micrometer membrane (Sartorius, 17598) in order to clear the sample of microorganisms. The nitrate and phosphate were quantified by spectroscopic measurements (UNICAM 5625 UV/VIS spectrometer) by the methods of cad-

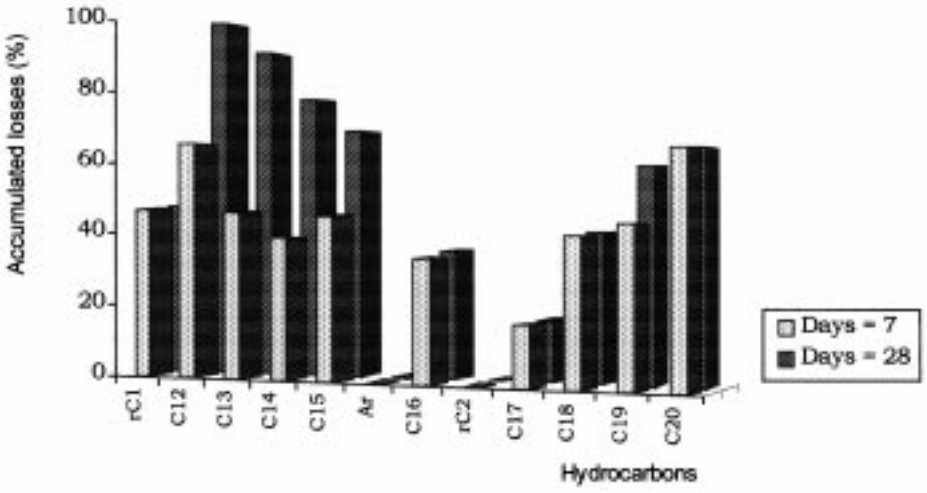

Fig. 2. Evolution of the accumulated losses in the biostimulation of the light Arabian oil until the 7th and 28th day of process in uninoculated systems. Where: rC1 is 2,2,4,4,6,8,8-heptamethylnonane; C_{12} is dodecane; C_{13} is tridecane; C_{14} is tetradecane; C_{15} is pentadecane; Ar is 1H-indene-2,3dihydro-1,1,3 trimethyl-3; C_{16} is hexadecane; rC2 is 2,6,10,15-tetramethylheptadecane; C_{17} is heptadecane; C_{18} is octadecane; C_{19} is nonadecane; C_{20} is eicosane.

mium reduction and ascorbic acid, respectively (American Public Health Association, 1992).

3. Results and discussion

The accumulated losses of the hydrocarbons within the 28 days of process reached the following levels of degradation in inoculated reactors (%): dodecane 100, tridecane 89, tetradecane 79, pentadecane 68, hexadecane 47, heptadecane 46, octadecane 82, nonadecane 60 and eicosane 56. Among the other identified hydrocarbons (2,2,4,4,6,8,8- heptamethylnonane, 2,6,10,15-tetramethylheptadecane and 1H-indene-2,3dihydro-1,1,3 trimethyl-3) 2,2,4,4,6,8,8-heptamethylnonane was the only one degraded to a level of 100% (Fig. 1). The *n*-alkanes have generally been found to be readily degraded in field and laboratory studies. Several reports indicated that biodegradation levels of these compounds increased with decreasing carbon number (de Jonge et al, 1997). Our data confirm these observation except for octadecane (Fig. 1). In general terms branched chain alkanes are degraded more slowly than their straight-chain counterparts. Commonly, the degradation of branched-chain alkanes is repressed by the presence of the *n*-alkanes. However, some recent reports (Chaîneau et al, 1995; Geerdink et al, 1996) indicate that many of these compounds are more degradable than it had previously been thought. However, it is generally true that highly branched compounds are more recalcitrant than less complex ones. Owing to steric hindrance of oxidative enzymes the β-branched and quaternary compounds are particularly recalcitrant. In the present study, the compound identified as 2,2,4,4,6,8,8-heptamethylnonane (rC1) was fully degraded within 28 days of process. Other authors have also reported the utilization of this compound by a mixed marine microbial population. The degradation was relatively rapid and the only metabolic intermediates detected were straight-chain fatty acids (Ratledge, 1994). The other branched alkane of light Arabian oil identified as 2,6,10,15-tetramethylheptadecane (rC2) had also been shown to be metabolized (Fig. 1). In this case, it has been suggested that initial metabolic attack did not only occur at the isopropyl units on the molecules but the products of initial oxidation were always terminal alcohols (Ratledge, 1994). The aromatic hydrocarbon identified as 1H-indene-2,3dihydro-1,1,3 trimethyl-3 (Ar) was the least biodegraded throughout the 28 days of process, reaching only 9.27% of removal. Some reports indicated that the high stability of these hydrocarbons is due to the high resonance of the ring (Ratledge, 1994). These results confirm that the components of petroleum showed different susceptibilities to microbial attack, corroborating results obtained by Atlas and Bartha (1973) and Leahy and Colwell (1990).

The autochthonous microorganisms also metabolized the identified hydrocarbons in the light Arabian oil, except 1H-indene-2,3dihydro-1,1,3trimethyl-3 and 2,6,10,15-tetramethylheptadecane (Fig. 2). Partial degradation of these compounds and degradation enhancement of 2,2,4,4,6,8,8-heptamethylnonane, hexadecane, heptadecane and octadecane was observed when mixed culture was introduced in the system compared to the results obtained in the system containing autochthonous microorganisms only.

Despite the technical possibility of periodic addition of inoculum, we have chosen only one inoculation at the beginning of the experiment. This alternative

Table 1
Degradation of hydrocarbons for bioaugmentation of the mixed culture N_D in sandy sediment with 0.7% of light Arabian oil

	Microorganisms (cfu/g sandy sediment)		Physical-chemical parameters				
			Heavy fraction of the oil		Moistur (%)	Addition (ml)	
Days	Bacteria	Fungi	Residual content (% w/w)	Accumulated losses (%)		H_2O	M_1[a]
0	$4.8^{b} \pm 0.5^{c} \times 10^4$	$1.6 \pm 0.4 \times 10^5$	0.42 ± 0.01	–	20.3 ± 0.2		1000
7	$4.8 \pm 0.6 \times 10^6$	$9.9 \pm 0.9 \times 10^4$	0.34 ± 0.03	19.0	21.0 ± 0.3		1000
14	$3.0 \pm 0.4 \times 10^7$	$4.2 \pm 0.5 \times 10^4$	0.29 ± 0.02	30.9	20.7 ± 0.3	700	
21	$5.1 \pm 0.6 \times 10^8$	$4.4 \pm 0.6 \times 10^4$	0.25 ± 0.02	40.5	20.9 ± 0.2		1000
28	$5.8 \pm 0.7 \times 10^7$	$6.1 \pm 0.7 \times 10^3$	0.24 ± 0.02	42.9	12.2 ± 0.2		

[a] M_1 = mineral medium (gl^{-1}): 0.5 KH_2PO_4; 1.4 Na_2HPO_4; 1.0 NH_4NO_3; 0.1 $MgSO_47H_2O$; 0.02 $CaCl_22H_2O$; 0.03 $MnSO_4H_2O$; cfu = colony forming units.
[b] Mean value of four experiments.
[c] Standard deviation of mean.

allowed the bacterial proliferation associated with the hydrocarbons consumption. In practice a simple inoculation reduces extra expenses for inoculum preparation (Table 1). Minimizing the expenses makes the process more economically attractive for field scale application.

Data in Tables 1 and 2 show that the incorporation of mixed culture N_D promoted the increase of microbial density in the system. In the system containing the mixed culture N_D an increase of approximately 10^4 cells/g occurred, whereas an increase of only 10^2 cells/g was observed in the system which contained autochthonous microorganisms. The introduction of mixed culture N_D in the sandy sediment increased by 31% the bioremediation efficiency of the heavy fraction of light Arabian oil compared to the efficiency reached in uninoculated reactors (Table 2). Thus, only 11.9% of the heavy fraction of light Arabian oil was degraded within 28 days of process in the uninoculated reactors as compared to 42.9% in the inoculated ones. Parallel measurements of hydrocarbon residues and of microbial activity indicated that the biodegradation was the dominant component of the remediation process (Wang and Bartha, 1990). However, in this study, we used chemical reagents to supply the mineral required to support growth of microorganisms involved in the process. Our purpose was to reduce possible interferences caused by potential contaminants of lower grade commercial products such as chemical fertilizers used in agriculture. As shown in Fig. 3, consumption of nitrate and phosphate was high. This observation suggests that replenishment of these nutrients during the process is a condition to achieve a high level of biodegradation. Therefore other sources of mineral nutrient will be needed if this process is applicable in the field to reduce its costs.

Periodic tilling of the upper layer of the reactors, might not have been sufficient to replenish the oxygen consumed in the process. The water required to maintain a high moisture content in the sandy sediment can fill empty spaces of the dry soil decreasing the oxygen transfer in the system. To complement the lack of oxy-

Table 2
Degradation of hydrocarbons for biostimulation of the autochthonous microorganisms in sandy sediment with 0.7% of light Arabian oil

	Microorganisms (cfu/g sandy sediment)		Physical-chemical parameters				
			Heavy fraction of the oil		Moisture (%)	Addition (ml)	
Days	Bacteria	Fungi	Residual content (% w/w)	Accumulated losses (%)		H_2O	M_1[a]
0	$3.5^{b} \pm 0.6^{c} \times 10^4$	$5.2 \pm 0.6 \times 10^2$	0.42 ± 0.01	–	20.2 ± 0.2		1000
7	$3.2 \pm 0.7 \times 10^5$	$9.1 \pm 0.9 \times 10^2$	0.39 ± 0.02	7.1	20.9 ± 0.2		1000
14	$3.7 \pm 0.5 \times 10^6$	$8.9 \pm 0.9 \times 10^3$	0.38 ± 0.03	9.5	20.5 ± 0.2	700	
21	$4.1 \pm 0.8 \times 10^6$	$5.4 \pm 0.7 \times 10^3$	0.37 ± 0.02	11.9	20.4 ± 0.3		1000
28	$3.5 \pm 0.5 \times 10^5$	$4.8 \pm 0.6 \times 10^3$	0.37 ± 0.03	11.9	13.0 ± 0.2		

[a] M_1 = mineral medium (gl^{-1}): 0.5 KH_2PO_4; 1.4 Na_2HPO_4; 1.0 NH_4NO_3; 0.1 $MgSO_47H_2O$; 0.02 $CaCl_22H_2O$; 0.03 $MnSO_4H_2O$; cfu = colony forming units.
[b] Mean value of four experiments.
[c] Standard deviation of mean.

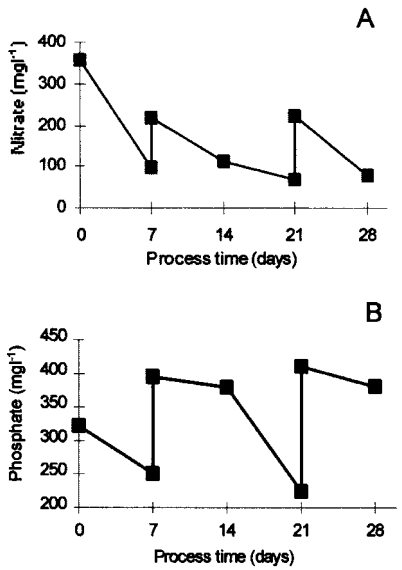

Fig. 3. Evolution of nitrate (A) and phosphate (B) during the biodegradation of light Arabian oil by mixed culture N_D associated with indigenous microorganisms present in sandy sediment.

gen, electron acceptors can be provided by oxidized inorganic compounds such as nitrate (Hutchins et al, 1998). We controlled and monitored the phosphate and nitrate added to the system in order to prevent the metabolic inhibition caused by very high concentrations of these inorganic nutrients which was observed by other authors (Morgan and Watkinson, 1989). Thirteen different products were evaluated for their effectiveness to stimulate biodegradation of petroleum hydrocarbons. Three of the 13 products tested enhanced microbial degradation of the petroleum. For some of the products evaluated, microbial growth was associated to hydrocarbon degradation for the first 7 days only. After that period, the level of biodegradation did not change significantly (Aldrett et al., 1997). However, in the present study nitrogen and phosphorus monitoring showed that the nutrients were active up to 28 d, allowing an increase of the level of hydrocarbon biodegradation. Inorganic nutrients addition were made when the amount of nitrate was lower than 100 mgl^{-1} in the system, in order to prevent nitrogen depletion (Fig. 3).

4. Conclusions

The autochthonous microorganisms from sandy sediment were able to degrade the petroleum hydrocarbons identified in this study, with the exception of 1H-indene 2,3 dihydro-1,1,3trimethyl-3 and 2,6,10,15-tetramethylheptadecane. The degradation of these compounds and the increase in the degradation of 2,2,4,4,6,8,8-heptamethylnonane, hexadecane, heptadecane and octadecane were achieved when mixed culture N_D obtained from landfarming, was incorporated in the system.

The increase observed in the number of viable cells in the inoculated system in relation to the uninoculated system suggests the successful implantation of the mixed culture N_D into the system.

A single inoculation of mixed culture N_D in the sandy sediment, at the beginning of the experiment, resulted in an increase of 31% of efficiency of bioremediation of the heavy fraction of light Arabian oil compared to one obtained in uninoculated soil, showing that the introduction of the exogenous culture stimulates the biodegradation process.

Acknowledgements

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico and Centro de Pesquisas e Desenvolvimento Leopoldo A. Miguez de Mello/PETROBRAS. The authors wish to thank Dr Francisco Radler de Aquino Neto and Alberto dos Santos Pereira for their assistance in the chromatographic analyses of the work.

References

Aldrett, S., Bonner, J.S., Mills, M.A., Autenrieth, R.L., Stephens, F.L., 1997. Microbial degradation of crude oil in marine environments tested in a flask experiment. Water Research 31, 2840–2848.

American Public Health Association, 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington, DC.

Atlas, R.M., Bartha, R., 1973. Fate and effects of polluting petroleum in the marine environment. Residue Review 49, 49–83.

Chaîneau, C.H., Morel, J.L., Oudot, J., 1995. Microbial degradation in soil microcosms of fuel oil hydrocarbons from drilling cuttings. Environmental Science and Technology 29, 1615–1621.

de Jonge, H., Freijer, J.I., Verstraten, J.M., Westerveld, J., Van der Wielen, F.W.N., 1997. Relation between bioavailability and fuel oil hydrocarbon composition in contaminated soils. Environmental Science and Technology 31, 771–775.

Geerdink, M.J., van Loosdrecht, M.C.M., Luyben, K. Ch. A.M., 1996. Biodegradability of diesel oil. Biodegradation 7, 73–81.

Hutchins, S.R., Bantle, J.A., Schrock, E.J., 1998. Effect of nitrate-based bioremediation on contaminant distribution and sediment toxicity-column study. Environmental Toxicology and Chemistry 17, 349–361.

Leahy, J.G., Colwell, R.R., 1990. Microbial degradation of hydrocarbons in the environment. Microbial Reviews 9, 305–315.

Milne, B.J., Baheri, H.R., Hill, G.A., 1998. Composting of a heavy oil refinery sludge. Environmental Progress 1, 24–27.

Morgan, P., Watkinson, R.J., 1989. Hydrocarbon degradation in soils and method for soil biotreatment. CRC Critical Reviews in Biotechnology 8, 305–333.

Prince, M., Sambasivam, Y., 1993. Bioremediation of petroleum wastes from refining of lubrificant oils. Environmental Progress 12, 5–11.

Ratledge, C., 1994. Bichemistry of microbial degradation. Kluwer Academic Publishers, Netherlands.

Rosenberg, E., Legmann, R., Kushmaro, A., Taube, R., Adler, E., Ron, E.Z., 1992. Petroleum bioremediation — a multiphase problem. Biodegradation 3, 337–350.

Wang, X., Bartha, R., 1990. Effects of bioremediation on residues, activity and toxicity in soil contaminated by fuel spills. Soil Biology and Biochememistry 22, 501–505.