Brazilian Journal of Microbiology (2008) 39:133-142
ISSN 1517-8382

BIODEGRADABILITY OF COMMERCIAL AND WEATHERED DIESEL OILS

Adriano Pinto Mariano[1,2]*; Daniel Marcos Bonotto[1]; Dejanira de Franceschi de Angelis[2]; Maria Paula Santos Pirôllo[2]; Jonas Contiero[2]

[1]Instituto de Geociências e Ciências Exatas, Universidade Estadual Paulista, Rio Claro, SP, Brasil; [2]Departamento de Bioquímica e Microbiologia, Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, SP, Brasil

Submitted: February 15, 2007; Returned to authors for corrections: November 21, 2007; Approved: January 20, 2008.

ABSTRACT

This work aimed to evaluate the capability of different microorganisms to degrade commercial diesel oil in comparison to a weathered diesel oil collected from the groundwater at a petrol station. Two microbiological methods were used for the biodegradability assessment: the technique based on the redox indicator 2,6 - dichlorophenol indophenol (DCPIP) and soil respirometric experiments using biometer flasks. In the former we tested the bacterial cultures *Staphylococcus hominis*, *Kocuria palustris*, *Pseudomonas aeruginosa* LBI, *Ochrobactrum anthropi* and *Bacillus cereus*, a commercial inoculum, consortia obtained from soil and groundwater contaminated with hydrocarbons and a consortium from an uncontaminated area. In the respirometric experiments it was evaluated the capability of the native microorganisms present in the soil from a petrol station to biodegrade the diesel oils. The redox indicator experiments showed that only the consortia, even that from an uncontaminated area, were able to biodegrade the weathered diesel. In 48 days, the removal of the total petroleum hydrocarbons (TPH) in the respirometric experiments was approximately 2.5 times greater when the commercial diesel oil was used. This difference was caused by the consumption of labile hydrocarbons, present in greater quantities in the commercial diesel oil, as demonstrated by gas chromatographic analyses. Thus, results indicate that biodegradability studies that do not consider the weathering effect of the pollutants may over estimate biodegradation rates and when the bioaugmentation is necessary, the best strategy would be that one based on injection of consortia, because even cultures with recognised capability of biodegrading hydrocarbons may fail when applied isolated.

Key words: biodegradability, bioremediation, commercial oil diesel, weathered diesel oil.

INTRODUCTION

The composition of a product released into the environment begins to change almost immediately because of numerous biochemical and physical processes. The geochemistry related to organic compounds is described in details by Kaplan *et al.* (14). According to them, hydrocarbons released into the environment are subject to biotic and abiotic weathering reactions in the soil and groundwater media. These processes act together, with the rate of transformation being related to the chemical composition of the fuel and local environmental factors, including temperature, soil moisture and nutrient and oxygen contents. Grain size and clay-type are also important parameters for controlling weathering processes in the soil. Major abiotic reactions include hydrolysis, dehydrogenation, oxidation and polymerization (17). These reactions are often closely related to microbial (biotic) transformations in the soil profile. Biotic weathering of a hydrocarbon fuel consists of two interdependent mechanisms: microbial uptake (4) and metabolic degradation (26). These transformations are likely to occur stepwise, producing alcohols, phenols, aldehydes and carboxylic acids

*Corresponding Author. Mailing address: Instituto de Geociências e Ciências Exatas (IGCE), Departamento de Bioquímica e Microbiologia - Instituto de Biociências (IB), Universidade Estadual Paulista (UNESP). Av. 24-A, 1515 - CP 199. CEP 13506-900, Rio Claro, SP, Brasil. E-mail: adrianomariano@yahoo.com.br

in sequence. Biodegradation is the major weathering process for middle distillates, as the diesel oil (14).

Diesel oil contains 2000 to 4000 hydrocarbons, a complex mixture of normal, branched and cyclic alkanes, and aromatic compounds obtained from the middle-distillate fraction during petroleum separation (11). Some of these compounds can be used as indicators for diesel oil weathering assessment. Changes in concentration ratios of hydrocarbons as benzene, toluene, ethylbenzene and xylenes (BTEX) are due mainly to evaporation and dissolution processes. These compounds are characterized by their high vapour pressure and aqueous water solubility. Benzene and toluene preferentially dissolves in the groundwater when compared to ethylbenzene and xylenes, which have a lower solubility and are more resistant to biodegradation (14).

The poliaromatic hydrocarbons (PAH) provide another useful tool for monitoring environmental alterations. Some of the PAH compounds of diesel fuel are among the least affected by weathering. These semi-volatile compounds with a low solubility and recalcitrant characteristic may persist for a long time in the environment.

The weathering process may also be evaluated by analyzing the total petroleum hydrocarbons (TPH). In this case, chromatographic profiles of a commercial diesel, generally present a satisfactory resolution for all n-alkanes and some other isoprenoid alkanes, such as pristine (2,6,10,14-tetramethylpentadecane) and phytane (2,6,10,14-tetramethylhexadecane). Nevertheless, the major fraction of the diesel oil is not characterized because the majority of the components could not be resolved and they appear in the chromatograms as a "hump", which is called the "unresolved complex mixture (UCM)", which presumably includes branched and cyclic alkanes and polar transformation products (19, 6). The resolved hydrocarbons are called "total resolvable hydrocarbons (TRH)" and the TPH are the sum of TRH and UCM. The TRH are non-degraded hydrocarbons, and they appear as peaks in the chromatogram.

Hydrocarbon degrading microorganisms usually degrade branched alkanes and isoprenoid compounds at much slower rates than straight-chain alkanes. Therefore, the ratio of straight-chain alkanes to these highly branched biomarker compounds can reflect the extent to which microorganisms have degraded the hydrocarbons in the diesel oil (2). The hydrocarbons content in the weathered diesel is mainly characterized as isoprenoid alkanes and UCM, which are more recalcitrant than n-alkanes (11). Thus, due to the biodegradation, the UCM "hump" becomes larger and the TRH peaks decrease.

Strategies to accelerate the biological breakdown of hydrocarbons in soil include stimulation of the indigenous microorganisms (biostimulation) by optimizing factors as nutrients, oxygenation, temperature, pH, addition of biosurfactants and through inoculation of an enriched mixed microbial consortium into the soil (bioaugmentation) (1,10).

Concerning the bioaugmentation technique, a previous evaluation of the microorganisms capability of degrading the pollutants is the first step to set up a field scale remediation project based on addition of microorganisms. However, in this phase, many studies are carried out by simulating contaminations, where, for instance, commercial fuel is added to soil or groundwater. This approach may result in wrong conclusions, because the pollutants have their characteristics altered by physical-chemical and biological mechanisms when exposed to large periods in environmental conditions, the so-called weathering effect. Thus this work aimed to evaluate the capability of different microorganisms to degrade commercial diesel oil in comparison to a weathered diesel oil collected from the groundwater at a petrol station.

MATERIAL AND METHODS

Microorganisms

Different bacteria cultures and microorganisms consortia were tested in relation to their capability of biodegrading diesel oils. The bacteria cultures of *Staphylococcus hominis* and *Kocuria palustris* were isolated from the soil of the petrol station where the weathered diesel oil was collected and identified by rDNA 16S sequence (performed by CPQBA/UNICAMP) (21). The bacterial strain *Pseudomonas aeruginosa* LBI was isolated by Benincasa *et al.* (5) from a hydrocarbon contaminated area.

The bacterial strains *Ochrobactrum anthropi* and *Bacillus cereus* were previously isolated and identified by Kataoka (15) from a landfarming at the Brazilian oil refinery Replan (Petrobras S/A). From the wastewater of the same refinery, a microbial consortium (R) was also obtained.

The commercial inoculum called Efficient Microorganisms (EM) is a microorganism mix that has demonstrated efficient performance as a biological fertilizer and as a biological amendment to wastewater pond treatments. Although EM has been applied successfully in biological wastewater treatments, it has never been tested as an agent to enhance bioremediation of hydrocarbon contaminated sites, thus originally EM has not been provided for this proposal.

From the Petrobras oil terminal (Terminal Marítimo Almirante Barroso - Tebar), located in São Sebastião (SP-Brazil), we obtained the consortium L, the activated sludge of a pilot bioreactor that has been tested to treat water of production.

Other consortia tested were ASP-S and ASP-GW, obtained respectively from the soil and the groundwater of the petrol station where the weathered diesel oil was collected; SB-S and SB-GW, respectively, from the soil and groundwater at another petrol station; RC, from the soil collected during the replacement of underground pipes of a third petrol station; U, from an uncontaminated soil collected at Unesp campus.

Inocula preparation

The inocula *S. hominis*, *K. palustris*, *O. anthropi*, *B. cereus*, *P. aeruginosa* LBI and consortium R were prepared using bacterial cells transferred from the storage culture tubes and streaked onto the surface of Petri dishes containing nutrient agar (Merck, Germany). To prepare the consortia ASP-S, SB-S, RC-S and U, 1.0 g of respective soils were added to Erlenmeyer flasks (125 mL) containing 50 mL of Bushnell-Hass (BH) medium and kept under agitation during 3 days. After this period, the medium was streaked onto the surface of Petri dishes containing nutrient agar. The ASP-GW and SB-GW were prepared by streaking 1 mL of respective groundwater onto the surface of Petri dishes containing nutrient agar.

The Petri dishes were incubated during 24 hours at 35ºC. Then cells were harvested using sterile water.

The EM and consortium L were added to the biodegradability experiments flasks, without previous preparation.

Diesel oil biodegradability experiments

The biodegradability experiments were carried out using a technique based on the redox indicator 2.6 - dichlorophenol indophenol (DCPIP) (13). The inocula *S. hominis*, *K. palustris*, *O. anthropi*, *B. cereus*, *P. aeruginosa* LBI and consortium R were added (125 µL, O.D = 0.55 at λ = 610 nm (SHIMADZU UV-1601PC)), separately, to test tubes (duplicates) that contained sterile Bushnell-Hass (BH) medium (7.5 mL) and 50 µL of diesel oil. The concentration of DCPIP was 27 mg/mL. The inoculum EM (200 µL, concentration equal to 10^9 CFU/mL), the consortium L (1.0 mL concentration equal to 10^7 CFU/mL), the inocula prepared from the native microorganisms of soils and groundwaters (1.0 mL, concentration not determined) and *P. aeruginosa* LBI again (1.0 mL, concentration not determined) were added to Erlenmeyer flasks (125 mL) (duplicates) that contained sterile BH medium (50 mL) and 1% (v/v) of diesel oil. The concentration of DCPIP was 20 mg/mL. Test tubes and Erlenmeyer flasks were kept under agitation (240 rpm) at room temperature (27±2ºC). The BH medium consists of, $g.L^{-1}$: $MgSO_4$: 0.2; $CaCl_2$: 0.02; KH_2PO_4: 1.0; K_2HPO_4: 1.0; NH_4NO_3: 1.0; $FeCl_3$: 0.05 (9).

The principle of this technique is that during the microbial oxidation of hydrocarbons, electrons are transferred to electron acceptors such as O_2, nitrates and sulphate. By incorporating an electron acceptor such as DCPIP to the culture medium, it is possible to ascertain the ability of the microorganism to utilize hydrocarbon substrate by observing the colour change of DCPIP from blue (oxidized) to colourless (reduced). This Hanson *et al.* (13) technique has been employed in several works (8,20,24).

Soil respirometric experiments

In order to compare the biodegradability of the diesel oils when released to the environment, a soil contamination was simulated by adding the diesel oils (6g / kg soil), separately, to the soil collected at the petrol station where consortium RC was obtained (RC soil).

The experiments were carried out in Bartha biometer flasks (250 mL) that were used to measure microbial CO_2 production as described by Bartha & Pramer (3). Mineralization studies involving measurements of total CO_2 production can provide excellent information on the biodegradability potential of hydrocarbons (2).

For each experimental condition (Table 1), the biometer flasks were prepared in triplicates (3 x 50 g of soil) and incubated at 27ºC in the dark. Produced CO_2 was trapped in a 10.0 mL solution of KOH (0.2 N), located in the side-arm of the biometer. This solution was periodically withdrawn by syringe, and the amount of carbon dioxide absorbed was then measured by titrating the residual KOH (after the addition of barium chloride solution (1 mL, 1.0 N) used to precipitate the carbonate ions) with a standard solution of HCl (0.1 N). During this procedure, the biometers were aerated during 1.5 minutes through the ascarite filters.

At the end of the experiments, replicates of each treatment were thoroughly mixed together for physicochemical and microbiological analyses. Experiment 2, carried out after experiment 1, had the purpose to confirm tendencies observed in the latter, thus only the CO_2 production was evaluated at the same conditions but in a larger period of incubation.

Enumeration of bacteria

Total heterotrophic bacteria were enumerated by using the pour plate technique on plate count agar (Acumedia, USA). Plate count of the bacterial soil population was performed as follows: samples of 1 g of soil were added to 9 mL of 0.85% sterile saline solution in essay tubes and agitated mechanically for 2 minutes. After appropriate serial dilutions, 1 mL of the suspension were spread over the surface of duplicate Petri dishes and incubated for 48 h at 35ºC. The total heterotrophic bacteria count was carried out at the beginning and at end of the first respirometric experiment.

Diesel oil composition

In order to characterize the diesel oils, superficial soil was collected in an uncontaminated area (Unesp campus) with no vegetation, and sieved (tyler 14) before being contaminated

Table 1. Soil respirometric experiments - experimental conditions.

experiment	experimental conditions	incubation time(days)
1	RC soil + commercial diesel oil RC soil + weathered diesel oil	48
2	RC soil + commercial diesel oil RC soil + weathered diesel oil	92

with commercial or weathered diesel. For the BTEX (benzene, toluene, ethylbenzene and xylenes) and PAH (poli-aromatic compounds) analyses, 8 mg diesel/kg of soil were added and for the TPH (total petroleum hydrocarbons) analysis, 3 mg/kg. The analyses were carried out by Analytical Solutions laboratory (São Paulo) according to the USEPA methods: 8021B, 8270 and 8015B, respectively.

Soil sampling and characteristics

The RC soil samples were collected at 0.50 m depth during the replacement of underground pipes at a petrol station. These samples showed low level of contamination by unknown fuel, possibly due to leaks in the pipes and ground infiltrations. Until performing the respirometric experiments, samples were stored at 5ºC. Table 2 summarizes some physicochemical characteristics of the RC soil. Values of heavy metals concentrations are not above the more restricted levels set by Cetesb (São Paulo Environmental Agency - Brazil) and by the Dutch list (7).

RESULTS AND DISCUSSION

Diesel oil characterization

The weathered diesel was collected from a thick layer above the groundwater at a petrol station where the leakage occurred approximately ten years ago. As stated by Kaplan (14), in this situation, where there is a thick free phase product, the rate of alteration is slower than for a thin layer, because these processes affect the interface fuel/water and not the body of the bulk product. Biodegradation inside the body of a free product is extremely slow, due to limitation of oxygen, water and nutrients. Thus, the fuel could remain relatively unaltered for a period of time as long as decades. The most likely alterations to occur in this situation are evaporation of the most volatile hydrocarbons and dissolution of the most soluble components. Despite these considerations, the analyses show that the diesel oil had some characteristics altered, and probably due to both biological and physical-chemical processes.

The weathered diesel has a dark green colour and a different smell from the reddish commercial diesel. As time goes by, the dyes in a released free product deteriorate, thus the colour of the fuel may change (14). Table 3 shows that the commercial diesel has a higher concentration of BTEX than the weathered diesel. The comparative chromatograms are in Fig. 1. It reflects mainly the effect of the diesel exposure to an aqueous environment, and volatilization. At the monitoring well where the oil was collected, the groundwater presented the following concentrations of BTEX: 112.1; 98.5; 115.7 and 865.8 µg/L, respectively.

The PAH concentrations are in Table 4. These recalcitrant molecules are slowly biodegraded, and as the other alterations related to the other hydrocarbons progress more rapidly, the

Table 2. RC soil characteristics.

pH ($CaCl_2$)[a]	6.7	grain size distribution (%)[a]	
moisture content (%)	8.8		
organic carbon (%)[a]	0.29	sand	81.4
total nitrogen (%)[b]	0.02	silt	7.3
available phosphorus (ppm)[a]	2.0	clay	11.3
C:N:P ratio[a,b]	100 : 6.89 : 0.10		
	(mmolc/dm^3)[a]	hydrocarbons content (mg/kg)[c]	
K	1.1	C8-C11	3400
Ca	15	C11-C14	3900
Mg	2	C14-C20	24000
H+Al	10	C20-C40	73000
Al	-[d]		
CEC[e]	28.7		

micronutrients (ppm)[a]									heavy metals (ppm)[a]				
S	Na	Fe	Mn	Cu	Zn	B	Co	Mo	Ba	Cd	Cr	Ni	Pb
12	13	19	3.0	0.6	7.3	0.15	0.56	-[d]	4.06	0.12	9.93	0.30	7.10

[a] performed by ICASA - Instituto Campineiro de Análise de Solo e Adubo;
[b] performed by PIRASOLO - Laboratório Agrotécnico Piracicaba;
[C] performed by Bioagri Ambiental (USEPA 8015);
[d] not detected;
[e]cation exchange capacity.

Table 3. BTEX concentration in diesel oils.

	commercial	weathered
	µg/kg	
Benzene	<DL	<DL
Toluene	84.90	<DL
Ethylbenzene	171.71	69.78
m,p-Xylene	565.24	112.86
o-Xylene	321.41	14.52
Total	1143.26	197.16

DL (detection limit) = 1.58 µg/kg.

Table 4. PAH concentration in diesel oils.

	commercial	weathered
	µg/kg	
Naphthalene	578.31	4276.21
Acenaphthylene	<DL(1)	<DL(2)
Acenaphthene	257.32	822.63
Fluorene	534.84	1221.17
Phenanthrene	257.65	2024.41
Anthracene	13.88	<DL
Fluoranthene	<DL	<DL
Pyrene	<DL	<DL
Benzo[a]anthracene	<DL	<DL
Chrysene	<DL	<DL
Benzo[b]fluoranthene	<DL	<DL
Benzo[k]fluoranthene	<DL	<DL
Benzo[a]pyrene	<DL	<DL
Indeno(1,2,3-cd)pyrene	<DL	<DL
Dibenz[a,h]anthracene	<DL	<DL
Benzo[g,h,i]perylene	<DL	<DL
Total	1641.99	8344.42

DL (detection limit) = (1) 3.16 µg/kg; (2) 31.9 µg/kg.

diesel oil become enriched with the PAH, thus the weathered diesel oil had a significant increase in the PAH concentrations. Fig. 2 shows the comparative chromatograms.

Analysing the TPH concentrations (Table 5), the weathered diesel oil presents no detectable n-alkanes and a high abundance of pristine and phytane, which is in accordance with a weathering process. Moreover, the TRH and UCM fractions are, respectively, smaller and bigger in the weathered diesel oil, which is another indicative of biodegradation. In the chromatograms (Fig. 3), it is possible to observe that the UCM "hump" becomes larger and the TRH peaks decrease in the weathered diesel oil.

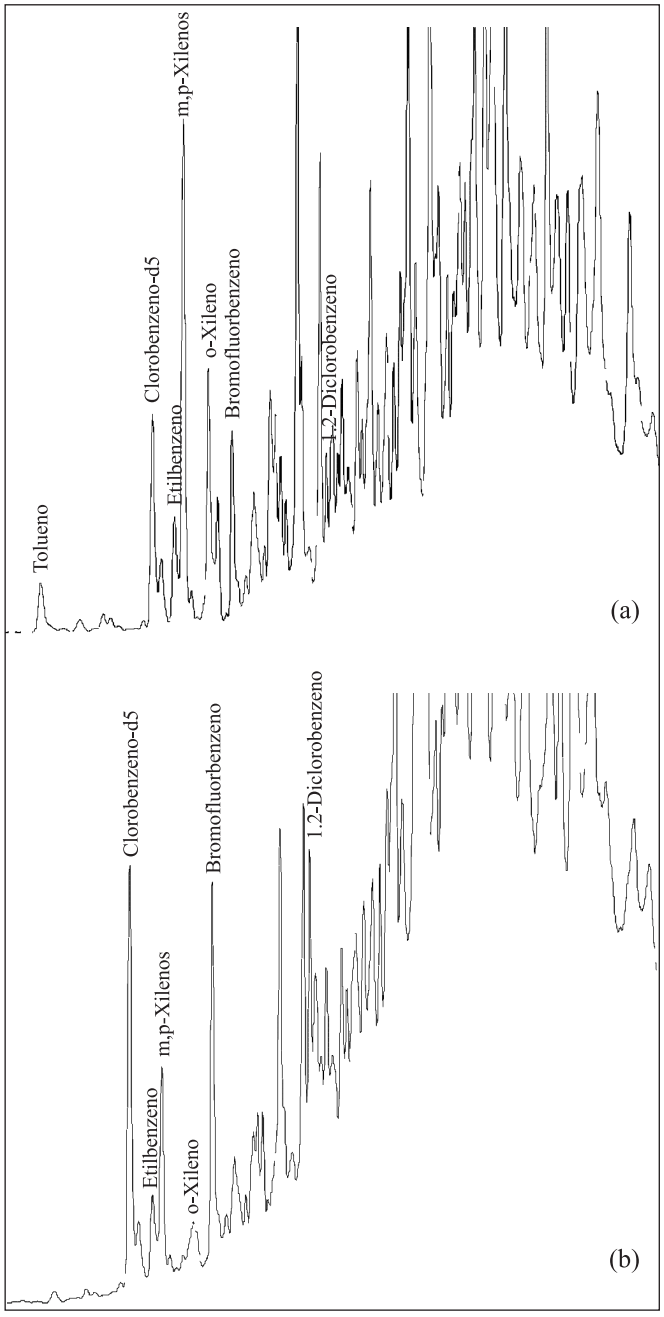

Figure 1. Chromatographic analysis (BTEX) of (a) commercial and (b) weathered diesel oils. Dilution factors: 1x.

Biodegradability experiment: redox indicator DCPIP

The results obtained with the biodegradability experiments using the redox indicator DCPIP are listed in Tables 6 and 7, related to the experiments carried out in test tubes and Erlenmeyer flasks, respectively.

Initially, as in the test tubes the inocula were added at equal concentrations, relative abilities of different cultures can be

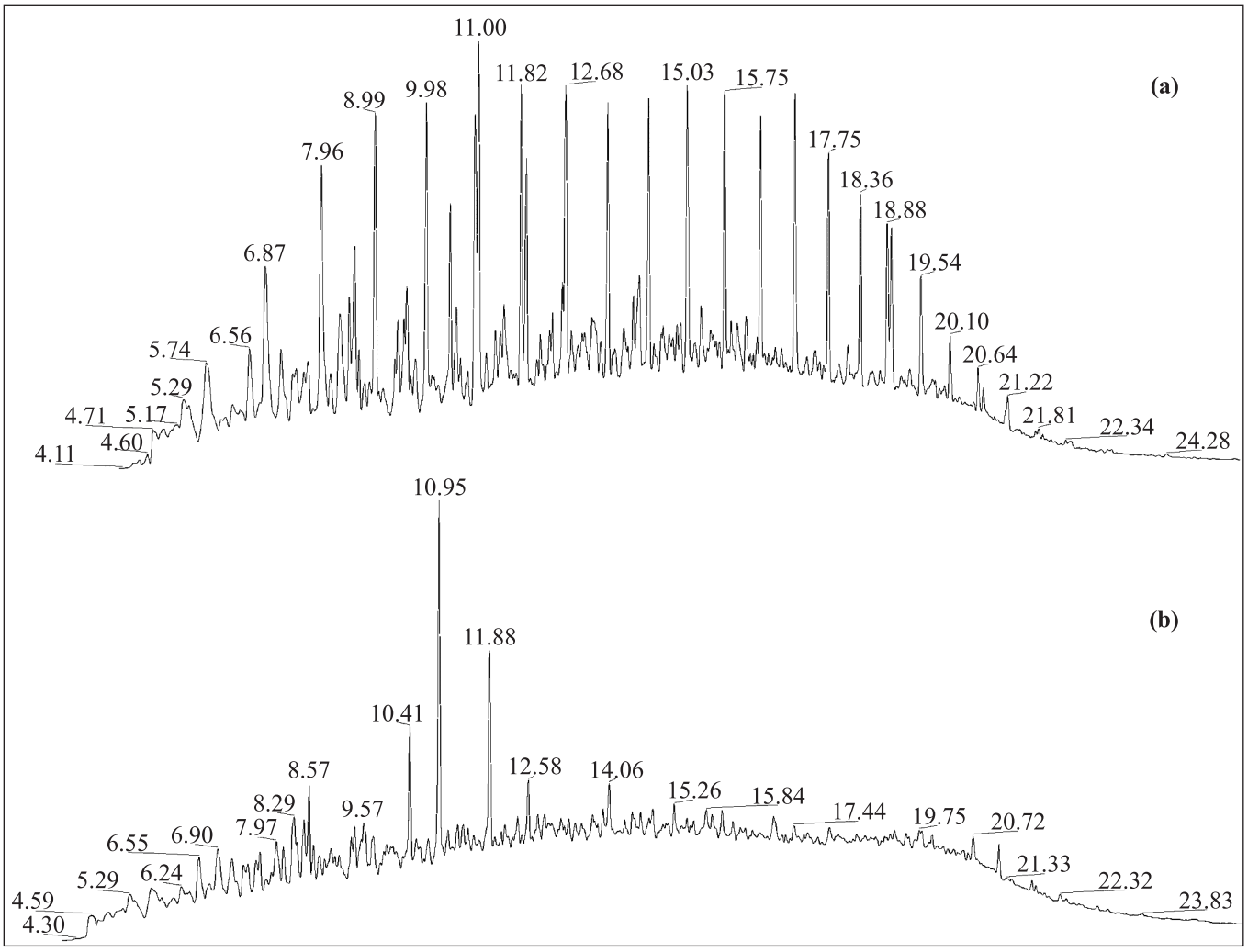

Figure 2. Chromatographic analysis (PAH) of (a) commercial and (b) weathered diesel oils. Dilution factors, respectively: 1x and 10x.

ascertained depending upon the time taken for the change in colour (13). Thus, all cultures had similar capability of biodegrading the commercial diesel oil, but only the consortium R, from the refinery wastewater, degraded the weathered diesel oil (Table 6). Even native cultures (*S. hominis* and *K. palustris*) and the genera *Bacillus* and *Pseudomonas*, known to be responsible for oil degradation (2), were not able to biodegrade the weathered fuel. No study relating the cultures *S. hominis* and *K. palustris* to hydrocarbon biodegradation was found. Nevertheless, Gomes *et al.* (12) describe the biosurfactant production by a strain of *S. aureus* utilising hydrocarbons as carbon source. The other cultures have demonstrated to be able to degrade hydrocarbons. *P. aeruginosa* is a well know producer of the rhamnolipid biosurfactant (18). The strain *P. aeruginosa* LBI was capable of producing biosurfactant using soapstock (22), mannitol and glycerol (25) and kerosene, diesel oil, crude oil and oily sludge (23). The same culture *O. anthropi*, in Kataoka (15), enhanced the biodegradation of oily sludge and was capable of biodegrading hexane, heptane, hexadecane and commercial diesel oil.

In the experiments carried out in Erlenmeyer flasks (Table 7), again *P. aeruginosa* LBI demonstrated no ability to degrade the weathered diesel oil. All consortia were able to degrade both diesel oils, except the commercial inoculum EM. As mentioned previously, EM has never been tested in hydrocarbons before. Here, it was only able to biodegrade the commercial fuel, as the other microorganisms with recognised biodegradation ability.

Experiments carried out with the native microorganisms (ASP-S, SB-S, RC-S, ASP-GW and SB-GW) indicate that the contaminated soils or groundwaters already had a microbiot adapted to degrade recalcitrant hydrocarbons. Moreover, the result obtained with the consortium U shows, as in other works

Table 5. TPH concentration in diesel oils.

	commercial	weathered
	µg/kg	
C10	<DL	<DL
C11	<DL	<DL
C12	<DL	<DL
C13	21703.5	<DL
C14	21654.5	<DL
C15	29535.3	<DL
C16	25213.5	<DL
C17	20737.7	<DL
pristane	26837.2	37916.4
C18	<DL	<DL
phytane	<DL	18833.5
C19	<DL	<DL
C20	<DL	<DL
C21	<DL	<DL
C22	<DL	<DL
C23	<DL	<DL
C24	<DL	<DL
C25	<DL	<DL
C26	<DL	<DL
C27	<DL	<DL
C28	<DL	<DL
C29	<DL	<DL
C30	<DL	<DL
C31	<DL	<DL
C32	<DL	<DL
C33	<DL	<DL
C34	<DL	<DL
C35	<DL	<DL
C36	<DL	<DL
TRH	592987.1	424207.6
UCM	2601550.3	2900513.6
Total (TPH)	3194537.4	3324721.2

DL (detection limit) = 17712.8 µg/kg;
TRH - Total Resolvable Hydrocarbons;
UCM - Unresolved Complex Mixture;
TPH - Total Petroleum Hydrocarbons.

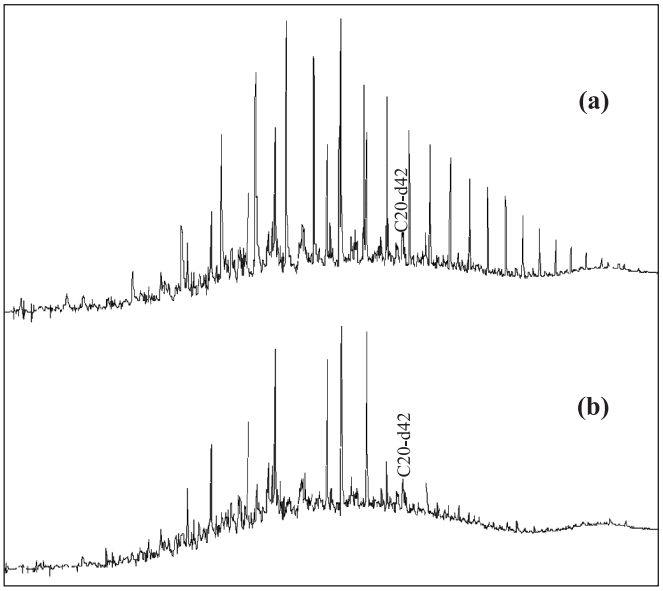

Figure 3. Chromatographic analysis (TPH) of (a) commercial and (b) weathered diesel oils. Dilution factors: 50x.

Table 6. Biodegradability experiment (DCPIP) in test tubes.

culture	decolourization	
	commercial	weathered
(1) *O. anthropi*	yes (after 3 days)	no
(2) *B. cereus*	yes (after 3 days)	no
(3) *S. hominis*	yes (after 3 days)	no
(4) *K. palustris*	yes (after 3 days)	no
(5) consortium R	yes (after 4 days)	yes (after 15 days)
(6) *P. aeruginosa* LBI	yes (after 3 days)	no
(1) + (2)	yes (after 3 days)	no
(3) + (4)	yes (after 3 days)	no
(1) + (2) + (3) + (4)	yes (after 3 days)	no

Obs: during the 18 days of experiment, no decolourization of the substrate control (without inoculum) or of the inoculum control (without diesel oil) was observed.

(27,16), that the presence of hydrocarbonoclastic microorganisms in soils is ubiquitous, even in not polluted soils.

The better performance of the consortia demonstrate the importance of considering the role of the commensalism when treating more recalcitrant pollutants as a weathered diesel oil, where each species has a specific function in the enzymatic reaction sequences responsible for the breakdown of complex hydrocarbons chains.

Biodegradability experiment: respirometric

The CO_2 production of experiment 1 and its cumulative values are represented, respectively, in Figs. 4 and 5. These results show that until the 10th day approximately, the biodegradation rates were very similar, but after this period, the rate of consumption of the commercial oil increases until the 27th day (maximum value), while the rate in relation to the weathered diesel keeps almost constant. Certainly, the labile hydrocarbons, in greater quantity in the commercial oil, were responsible for this difference.

Table 7. Biodegradability experiment (DCPIP) in Erlenmeyer flasks.

culture	decolourization	
	commercial	weathered
P. aeruginosa LBI	yes (after 3 days)	no
EM	yes (after 13 days)	no
L	yes (after 5 days)	yes (after 5 days)
ASP-S	yes (after 3 days)	yes (after 9 days)
SB-S	yes (after 6 days)	yes (after 6 days)
RC-S	yes (after 2 days)	yes (after 2 days)
U	yes (after 2 days)	yes (after 4 days)
ASP-GW	yes (after 1 day)	yes (after < 1 day)
SB-GW	yes (after 3 days)	yes (after 3 days)

Obs: during the experiments, no decolourization of the substrate control (without inoculum) or of the inoculum control (without diesel oil) was observed.

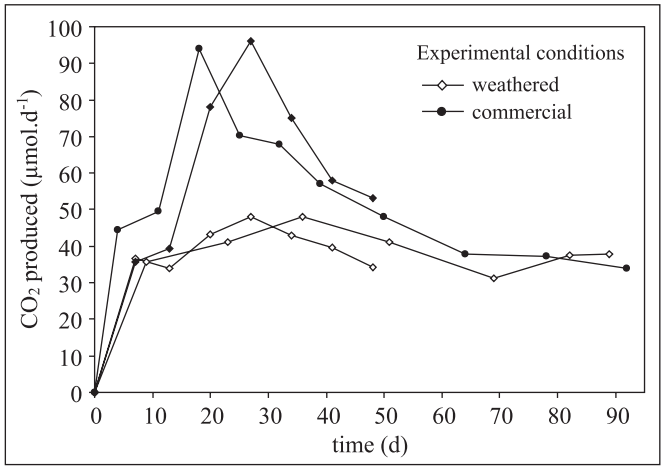

Figure 4. CO_2 production during incubation: 48 days (experiment 1) and 92 days (experiment 2).

Results of experiment 2, which was carried out at the same conditions but in a larger period of time, are plotted together with experiment 1 (Figs. 4 and 5). This comparison confirms that after 70 days the rates become equal, indicating the consumption of the labile hydrocarbons during this period in the commercial diesel oil. Moreover, the similarity between the curves shows that the respirometric technique has a good reproducibility.

The concentrations of hydrocarbons at the beginning and at the end of respirometric experiment 1 are listed in Table 8. These values are in agreement with the respirometric results. The biodegradation efficiencies of the labile hydrocarbons (total n-alkanes and the TRH fraction) are clearly higher in the commercial diesel oil, which explains the increase of the CO_2 production rate, and the concentrations of the main hydrocarbons fractions (TRH and UCM) are similar at the end of the experiment, confirming the tendency of equalization of the CO_2 production rates. Figure 6 shows that the population of the total heterotrophic

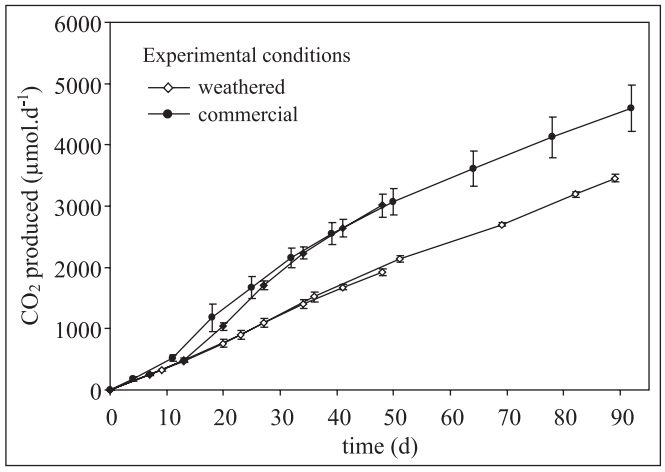

Figure 5. Cumulative total amounts of CO_2 produced by experiment 1 (48 days) and 2 (92 days) during incubation. Each error bar represents 1 SD of three replicate experiments.

Table 8. Concentration of hydrocarbons at the beginning (1) and at the end (2) of respirometric experiment 1. Values between parentheses indicate the biodegradation efficiency (%).

diesel oil	Total n-alkanes		pristine		phytane		TRH		UCM		TPH	
	1	2	1	2	1	2	1	2	1	2	1	2
	(mg.kg^{-1})											
commercial	499	< DL[1] (>96.3)	43.5	38.3 (12.0)	23.8	22.0 (7.6)	1231	519 (57.8)	4199	4131 (1.6)	5431	4650 (14.4)
weathered	< DL[2]	< DL[1]	58.9	67.0 (-13.8)	35.7	38.0 (-6.4)	711	550 (22.6)	4444	4313 (2.9)	5155	4864 (5.6)

DL (detection limit) = (1) 18.5 mg/kg; (2) 3.6 mg/kg;
TRH - Total Resolvable Hydrocarbons; UCM - Unresolved Complex Mixture; TPH - Total Petroleum Hydrocarbons.

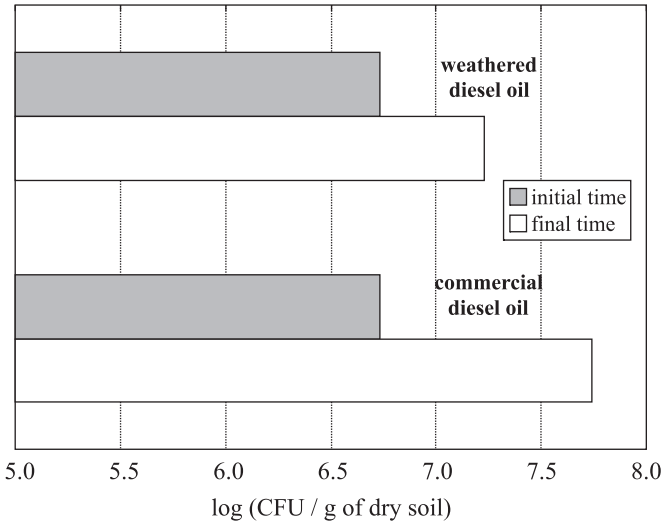

Figure 6. Total heterotrophic bacteria count at initial and final time of the respirometric experiment 1.

bacteria was favoured by the relative higher abundance of the labile hydrocarbons in the commercial diesel oil.

Finally, although the native microorganisms in the RC soil (consortium RC-S) were able to biodegrade both diesel oils, as demonstrated by the redox indicator experiments, in a condition of simulated contamination (respirometric experiments), the biodegradation efficiency of TPH was approximately 2.5 times greater when the commercial diesel oil was used, a difference that can be significant when estimating the time to clean up an old polluted site.

In conclusion, biodegradability studies that do not consider the weathering effect of the pollutants may over estimate biodegradation rates and when the bioaugmentation is necessary, the best strategy would be that one based on injection of consortia, because even cultures with recognised capability of biodegrading hydrocarbons may fail when applied isolated.

ACKOWLEDGMENTS

The authors acknowledge the Agência Nacional do Petróleo, Gás Natural e Biocombustíveis (ANP) (PRH-05); the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP); the petrol stations owners and Petrobras S/A.

RESUMO

Biodegradabilidade de óleos diesel comercial e intemperizado

Este trabalho objetivou avaliar a capacidade de diferentes microrganismos em degradar óleo diesel comercial em comparação com um óleo diesel intemperizado coletado da água subterrânea em um posto de combustíveis. Dois métodos microbiológicos foram usados para a avaliação da biodegradabilidade: a técnica baseada no indicador redox 2,6-diclorofenol indofenol (DCPIP) e os experimentos respirométricos usando os respirômetros de Bartha. No primeiro, testamos as culturas bacterianas *Staphylococcus hominis*, *Kocuria palustris*, *Pseudomonas aeruginosa* LBI, *Ochrobactrum anthropi* e *Bacillus cereus*, um inóculo comercial, consórcios obtidos do solo e da água subterrânea contaminados com hidrocarbonetos e um consórcio de uma área não contaminada. Nos experimentos respirométricos, foi avaliada a capacidade dos microrganismos nativos do solo de um posto de combustíveis em biodegradar os óleos diesel. Os experimentos com o indicador redox mostraram que apenas os consórcios, mesmo aquele de uma área não contaminada, foram capazes de biodegradar o diesel intemperizado. Em 48 dias, a remoção de hidrocarbonetos totais de petróleo (HTP) nos experimentos respirométricos foi aproximadamente 2,5 vezes maior quando o óleo diesel comercial foi usado. Esta diferença foi causada pelo consumo de hidrocarbonetos facilmente biodegradáveis, presentes em maior quantidade no óleo diesel comercial, como demonstrado pelas análises cromatográficas. Assim, resultados indicam que estudos de biodegradabilidade que não consideram o efeito de intemperização dos poluentes pode sobre estimar as taxas de biodegradação e quando o bioaumento é necessário, a melhor estratégia seria aquela baseada na injeção de consórcios, pois mesmo culturas com reconhecida capacidade de biodegradar hidrocarbonetos podem falhar quando aplicadas isoladamente.

Palavras-chave: biodegradabilidade, biorremediação, óleo diesel comercial, óleo diesel intemperizado.

REFERENCES

1. Alexander, M. (1994). *Biodegradation and Bioremediation*. Academic Press, San Diego.
2. Balba, M.T.; Al-Awadhi, N.; Al-Daher, R. (1998). Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation. *J. Microbiol. Methods*, 32, 155-164.
3. Bartha, R.; Pramer, D. (1965). Features of a flask and method for measuring the persistence and biological effects of pesticides in soil. *Soil Sci*., 100, 68-70.
4. Baughman, G.L.; Paris, D.F. (1981). Microbial bioconcentration of organic pollutants from aquatic systems – a critical review. *Crit. Rev. Microbiol.*, 8(3), 205-228.
5. Benincasa, M.; Contiero, J.; Marnresa, M.A.; Moraes, I.O. (2002). Rhamnolipid production by *Pseudomonas aeruginosa* LBI growing on soapstock as the carbon source. *J. Food Eng.*, 54, 283-288.
6. Bregnard, T.P.A.; Höhener, P.; Häner, A.; Zeyer, J. (1996). Degradation of weathered diesel fuel by microorganisms from a contaminated aquifer in aerobic and anaerobic microcosms. *Env. Tox. Chem*., 15, 299-307.
7. Cetesb – Companhia de Tecnologia de Saneamento Ambiental. (2005). Relatório de estabelecimento de Valores Orientadores para Solos e

Águas Subterrâneas no Estado de São Paulo. [Company of Environmental Sanitation Technology]. Technical report.
8. Cormack, W.P.M.; Fraile, E.R. (1997). Characterization of a hydrocarbon degrading psychrotrophic Antarctic bacterium. *Antarctic Sci*., 9, 150-155.
9. Difco Manual. (1984). Detroit: Difco Laboratories.
10. Duarte da Cunha, C.; Leite, S.G.F. (2000). Gasoline biodegradation in different soil microcosms. *Braz. J. Microb*., 31, 45-49.
11. Gallego, J.L.R.; Loredo, J.; Llamas, J.F.; Vázquez, F.; Sánchez, J. (2001). Bioremediation of diesel-contaminated soils: Evaluation of potential in situ techniques by study of bacterial degradation. *Biodegradation*, 12, 325-335.
12. Gomes, R.V.; Martins, S.C.S.; Melo, V.M.M. (2004). Produção de biossurfactante por *Staphylococcus aureus* isolado de uma amostra de petróleo pesado. IX Enama – Encontro Nacional de Microbiologia Ambiental, Curitiba, PR.
13. Hanson, K.G.; Desai, J.D.; Desai, A.J. (1993). A rapid and simple screening technique for potential crude oil degrading microorganisms. *Biotechnol. Tech.*, 7, 745-748.
14. Kaplan, I.R.; Galperin, Y.; Lu, S.; Lee, R. (1997). Forensic environmental geochemistry: differentiation of fuel-types, their sources and release time. *Org. Geochem*., 27, 289-317.
15. Kataoka, A.P.A.G. (2001). *Biodegradação de resíduo oleoso de refinaria de petróleo por microrganismos isolados de "landfarming"*. Rio Claro, Brasil (D.Sc. Thesis. Instituto de Biociências. Unesp).
16. Lee, M.; Kim, M.K.; Singleton, I.; Goodfellow, M.; Lee, S.T. (2006). Enhanced biodegradation of diesel oil by a newly identified *Rhodococcus baikonurensis* EN3 in the presence of mycolic acid. *J. Appl. Microbiol.*, 100, 325-333.
17. Lyman, W.J.; Reidy, P.J.; Levy, B. (1992). *Mobility and degradation of organic contaminants in subsurface environments*. C. K. Smoley, Inc., Chelsea, MI.
18. Maier, R.M.; Soberon-Chavez, G. (2000). *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. *Appl. Microbiol. Biotechnol.*, 54, 625-633.
19. Marchal, R.; Penet, S.; Solano-Serena, F.; Vandecasteele, J.P. (2003). Gasoline and diesel oil biodegradation. *Oil & Gas Science Technology*, 58, 441-448.
20. Mariano, A.P.; Bonotto, D.M.; Angelis, D.F. (2007). Monitoramento de indicadores geoquímicos e avaliação de biodegradação em área contaminada com óleo diesel. *Engenharia Sanitária e Ambiental*, 12(3), 296-304.
21. Mariano, A.P.; Kataoka, A.P.A.G.; Bonotto, D.M.; Angelis, D.F. (2007). Laboratory study on the bioremediation of diesel oil contaminated soil from a petrol station. *Braz. J. Microb*., 38, 346-353.
22. Moraes, I.O.; Benincasa, M.; Monte Alegre, R. (2002). Production and characterization of rhamnolipids produced by a newly isolated strain of *Pseudomonas aeruginosa*. *Braz. J. Food Technol*., 5, 145-149.
23. Pirôllo, M.P.S. (2006). *Estudo da produção de biossurfactantes utilizando hidrocarbonetos*. Rio Claro, Brasil (M.Sc. Dissertation. Instituto de Biociências. Unesp).
24. Roy, S.; Hens, D.; Biswas, D.; Kumar, R. (2002). Survey of petroleum-degrading bacteria in coastal waters of Sunderban Biosphere Reserve. *World J. Microbiol. Biotechnol.*, 18, 575-581.
25. Silva, B.H. (2005). *Influência das fontes: carbono e nitrogêneo da produção de biossurfactantes por Pseudomonas aeruginosa ATCC 31479 e Pseudomonas aeruginosa* LBI. Rio Claro, Brasil (Final Paper Term. Instituto de Biociências. Unesp).
26. Singer, M.E.; Finnerty, W.R. (1984). *Microbial metabolism of straight-chain and branched alkanes*. In: R. Atlas (ed) Petroleum Microbiology McMillan Publishing Co., NY.
27. Venkateswaran, K.; Harayama, S. (1995). Sequential enrichment of microbial populations exhibiting enhanced biodegradation of crude oil. *Can. J. Microbiol.*, 41, 767-775.