Investigation about the Efficiency of the Bioaugmentation Technique when Applied to Diesel Oil Contaminated Soils

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

[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; Av. 24-A, 1515; C.P.: 199; 13506-900; Rio Claro - SP - Brasil*

ABSTRACT

This work investigated the efficiency of the bioaugmentation technique when applied to diesel oil contaminated soils collected at three service stations. Batch biodegradation experiments were carried out in Bartha biometer flasks (250 mL) used to measure the microbial CO_2 production. Biodegradation efficiency was also measured by quantifying the concentration of hydrocarbons. In addition to the biodegradation experiments, the capability of the studied cultures and the native microorganisms to biodegrade the diesel oil purchased from a local service station, was verified using a technique based on the redox indicator 2,6 - dichlorophenol indophenol (DCPIP). Results obtained with this test showed that the inocula used in the biodegradation experiments were able to degrade the diesel oil and the tests carried out with the native microorganisms indicated that these soils had a microbiota adapted to degrade the hydrocarbons. In general, no gain was obtained with the addition of microorganisms or even negative effects were observed in the biodegradation experiments.

Keywords: bioremediation, bioaugmentation, soil, diesel oil, service station

INTRODUCTION

Diesel oil leakages from the underground storage tanks, distribution facilities and various industrial operations represent an important source of soil and aquifer contamination. Diesel is a complex mixture of normal, branched and cyclic alkanes, and aromatic compounds obtained from the middle-distillate fraction during petroleum separation (Gallego et al., 2001).

Among several clean-up techniques available to remove the petroleum hydrocarbons from the soil and groundwater, bioremediation processes are gaining ground due to their simplicity, higher efficiency and cost-effectiveness when compared to other technologies (Alexander, 1999). These processes rely on the natural ability of microorganisms to carry out the mineralization of organic chemicals, leading ultimately to the formation of CO_2, H_2O and biomass.

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

*Author for correspondence: adrianomariano@yahoo.com.br

The effectiveness of bioremediation is often a function of the microbial population and how it can be enriched and maintained in an environment. According to several authors (Kataoka, 2001; Mulligan et al., 2001; Bento et al., 2003; Rahman et al., 2003), the inoculation of bacteria with hydrocarbon biodegradation capabilities may shorten the time of the treatment. However, there are studies showing that the injection of microorganisms is often an inefficient approach to enhance the treatment of soils contaminated with hydrocarbons (APS, 1989 and 1990; Alexander, 1999; Trindade et al. 2002; Pala et al., 2002). Thus, the purpose of the present study was to investigate, in laboratory scale experiments, the efficiency of the bioaugmentation technique when applied to soils contaminated with diesel oil, testing different bacterial cultures and a commercial inoculum.

MATERIALS AND METHODS

Sampling and soil characteristics

Soil samples were collected at three service stations. At the service stations ASP and SB, diesel oil spills occurred from underground storage tanks approximately ten years ago, thus samples were contaminated with diesel oil. At these places, samples were collected from the capillary fringe (depth: ASP - 1.60 m; SB - 2.60 m). At the third service station (RC), the sample was collected at 0.50 m during the replacement of underground pipes. This sample showed low level of contamination of unknown fuel, possibly due to leaks in the pipes and ground infiltrations. Until performing the biodegradation experiments, samples were stored at 5^oC. Table 1 summarizes some physicochemical characteristics of these soil samples. The total petroleum hydrocarbons concentrations (TPH) are expressed in Tables 4 to 7.

Table 1 - Soil samples characteristics

	ASP	SB	RC
pH (CaCl$_2$)	6.7	5.0	6.7
moisture content (%)	16.6	17.6	8.8
organic carbon (%)	1.39	0.99	0.29
total nitrogen (%)	0.08	0.07	0.02
available phosphorus (ppm)	6.0	3.0	2.0
C:N:P ratio	100 : 5.75 : 0.043	100 : 7.08 : 0.03	100 : 6.89 : 0.10
	(mmolc/dm^3)		
K	1.7	1.4	1.1
Ca	100	20	15
Mg	8	6	2
H+Al	11	16	10
Al	-[a]	-[a]	-[a]
CEC[b]	121.8	44.1	28.7
grain size distribution (%)			
sand	78.7	66.7	81.4
silt	16.3	18.3	7.3
clay	5.0	15.0	11.3

	micronutrients (ppm)								heavy metals (ppm)				
	S	Na	Fe	Mn	Cu	Zn	B	Co	Mo	Ba	Cd	Cr	Ni	Pb
ASP	24	24	136	9.4	1.6	1.7	0.24	-[a]	0.03	9.73	0.03	2.10	0.44	5.9
SB	14	17	150	49.1	1.1	2.2	0.21	2.29	-[a]	55.81	0.19	4.92	1.53	7.96
RC	12	13	19	3.0	0.6	7.3	0.15	0.56	-[a]	4.06	0.12	9.93	0.30	7.10

[a] not detected; [b]cation exchange capacity.

Concentrations of heavy metals were not above the most restricted levels established by Cetesb (São Paulo Environmental Agency – Brazil) and by the Dutch list (Cetesb, 2005).
The physicochemical analyses were performed by the laboratory "Instituto Campineiro de Análise de Solo e Adubo (ICASA)", according to the methodology proposed by Embrapa (1997), except the following parameters: total nitrogen (laboratory "PIRASOLO – Laboratório

Agrotécnico Piracicaba", according to Embrapa (1997)); grain size distribution (ABNT, 1984) and the moisture content (obtained by the oven drying method).

Determination of the total petroleum hydrocarbons (TPH) content was carried out by the "Analytical Technology" laboratory by gas chromatography with the flame ionization detector (FID) according to the US Environmental Protection Agency (USEPA) methodology SW-846/8015.

Respirometric biodegradation experiments

In order to verify the influence of the bioaugmentation technique in the biodegradation of diesel oil, batch biodegradation experiments were carried out in Bartha biometer flasks (250 mL) used to measure the microbial CO_2 production (Bartha and Pramer, 1965; Régis and Bidoia, 2005; Inazaki et al., 2005). Mineralization studies involving the measurements of total CO_2 production can provide excellent information on the biodegradability potential of hydrocarbons (Balba et al., 1998).

The experiments were divided into three parts. The first aimed to evaluate the influence of the bioaugmentation together with the biostimulation (addition of nutrients) technique, in the biodegradation efficiency of ASP and SB soils. These experimental conditions are presented in Table 2.

Soil samples were separately homogenized by a thorough mixing and divided into three aliquots, in which the amendment solution of each treatment was added. The nitrogen and phosphorus correction was performed, respectively, using $(NH_4)_2SO_4$ and KH_2PO_4 solutions to obtain a nutrient ratio (C:N:P) of 100:15:1 (Mariano et al., 2007). The bacterial consortium (*Staphylococcus hominis* and *Kocuria palustris*) was added at a concentration of 10^6 Colony Forming Units (CFU) per gram of dry soil. Considering the addition of amendments, the water content of the soil changed to 21.1% (ASP) and 20.2% (SB). At treatments 1 and 4 (controls), the water content was corrected in relation to the other treatments by adding distilled water.

In the second part of the biodegradation experiments (Table 2), a diesel oil contamination of the RC soil was simulated. Thus, diesel oil purchased from the local service station was added to the soil. Regarding the bioaugmentation with the bacterial consortium *S. hominis / K. palustris*, two conditions were simulated: a) addition of microorganisms before contamination; b) addition of microorganisms after contamination. The idea of the former strategy was to evaluate how efficient the bioaugmentation would be if it was employed as a way to "prepare" the soil to a possible future spill.

The addition of the inoculum to treatment 9 was done 71 days previously to contamination. Using the same inoculum prepared for the first biodegradation experiment, at a concentration of 10^4 CFU / gram of dry soil. Then the inoculated soil (600 g) was kept in a becker covered by a black plastic to maintain the soil in dark conditions. After this period, the diesel oil contamination was simulated by adding 6 g diesel / kg soil to treatments 8 to 12.

In treatments 10 to 12, the concentrations of inocula were 10^6, 10^9 and 10^8 CFU / g of dry soil, respectively. Considering the addition of the inoculum, the water content of the soil changed to 9.3% (treatment 9) and 28.8% (treatments 10 to 12). At treatments 7 and 8 (controls), the water content was corrected in relation to treatment 10 by adding distilled water.

The third part of the biodegradation experiments (Table 2) aimed to verify the efficiency of a commercial inoculum, a microorganism mix, called Efficient Microorganisms (EM), which was applied in the ASP soil. EM was initially diluted in water (1:10) then applied at a concentration of 0.034 mL / g of soil, which corresponded to 10^7 CFU / g of dry soil. Considering the addition of the inoculum, the water content of the soil changed to 19.0%. At treatment 13 (control), the water content was corrected in relation to treatment 14 by adding distilled water.

For all experimental parts, the biometer flasks were prepared in triplicates (3 x 50 g of soil) and incubated at 27°C in the dark for 57 (part 1), 92 (part 2) and 47 (part 3) days. 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 (1mL; 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.

Table 2 - Respirometric experiments.

treatment	soil	experimental condition
		Part 1
1	ASP	control (no treatment)
2	ASP	biostimulation (N and P addition)
3	ASP	biostimulation (N and P addition) + bioaugmentation (bacterial consortium)
4	SB	control (no treatment)
5	SB	biostimulation (N and P addition)
6	SB	biostimulation (N and P addition) + bioaugmentation (bacterial consortium)
		Part 2
7	RC	control 1 - (no contamination and no treatment)
8	RC	control 2 - (contamination with no treatment)
9	RC	bioaugmentation (*S. hominis / K. palustris*) before contamination
10	RC	bioaugmentation (*S. hominis / K. palustris*) after contamination
11	RC	bioaugmentation (*Ochrobactrum anthropi*) after contamination
12	RC	bioaugmentation (microbial consortium - R) after contamination
		Part 3
13	ASP	control (no treatment)
14	ASP	bioaugmentation (commercial inoculum)

Biodegradation efficiency calculation

The carbon dioxide analyses were used to estimate the total amount of hydrocarbons mineralized during the biodegradation experiments. Assuming that 50% of the biodegraded carbon is converted to CO_2 and the other 50% is added to the soil as humus and biomass (Cetesb, 1990), the amount of biodegraded carbon can be expressed as:

Total biodegraded carbon (µmol) = 2 x CO_2 produced (µmol)   (1)

In the case of the RC soil (treatments 7 to 12), it is necessary to subtract the CO_2 produced by the control without contamination (treatment 7):

Total biodegraded carbon (µmol) = 2 x (CO_2 produced (µmol) - CO_2 produced in the control (µmol))   (2)

The biodegradation efficiency (BE) to all experiments is:

BE% = (Total biodegraded carbon (µmol) / initial soil organic carbon content (µmol)).100   (3)

Hydrocarbons removal efficiency was also measured by gas chromatography.

Diesel oil biodegradability test

Before carrying out the biodegradation experiments, the capability of the studied cultures and the native microorganisms to biodegrade diesel oil purchased from a local service station, was verified using a technique based on the redox indicator 2,6 - dichlorophenol indophenol (DCPIP) (Hanson et al., 1993).

The inocula *S. hominis*/*K. palustris*, *O. anthropi* and consortium – R (125 µL, O.D = 0.55 at µ = 610 nm (Shimadzu UV-1601PC)), were separately added to test tubes (duplicates) that contained 7.5 mL sterile Bushnell-Hass (BH) medium (Difco, 1984) and 50 µL of diesel oil. The concentration of DCPIP was 27 µg/mL. The inoculum EM (200 µL, concentration equal to 10^9 CFU/mL) and the inocula prepared from the native microorganisms of soils ASP, SB and RC (1.0 mL, concentration not determined) were added to 125 mL Erlenmeyer flasks (duplicates) that contained sterile 50 mL BH medium 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 27±2 ^{o}C.

The principle of this test 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 the hydrocarbon substrate by observing the color change of DCPIP from blue (oxidized) to colorless (reduced) (Hanson et al., 1993). This technique has been employed in several works, for instance, Roy et al. (2002), Cormack and Fraile (1997), Mariano et al. (2008a) and Pirôllo et al. (2008).

Microorganisms

The cultures of *Staphylococcus hominis* and *Kocuria palustris* were isolated from the diesel oil contaminated soil of the service station ASP and identified by rDNA 16S sequence (performed by CPQBA/UNICAMP) (Mariano et al., 2007).

In treatments 3, 6 and 9, inoculum was prepared as follow: bacteria were grown separately on liquid PCA medium (10 mL), and incubated at 35°C for 48 h. Cells were harvested by centrifugation (8,000 rpm for 10 min) and the supernatant was discharged. The resulting cell pellet was resuspended in 5.5 mL sterile saline solution (0.85%) and centrifuged as above. The cell pellets were grouped and the final cell suspension was prepared with the addition of 5.5 mL saline solution.

In treatments 10 to 12, inocula were prepared using bacterial cells transferred from storage culture tubes and streaked onto the surface of Petri dishes containing nutrient agar (Merck, Germany) with an incubation time of 24 h at 35°C. Then cells were harvested using sterile water and adjusted to an O.D. of 1.2 at 610 nm (SHIMADZU UV-1601PC). The bacterial strain *Ochrobactrum anthropi* was previously isolated and identified by Kataoka (2001) from a landfarming at the Brazilian oil refinery Replan (Petrobras S/A). From the wastewater of the same refinery, the microbial consortium – R was obtained.

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

In the biodegradability tests, the native microbial inocula were prepared by adding 1 g of respective soils to 50 mL of BH medium in Erlenmeyer flasks (250 mL) that were kept under agitation (240 rpm) at 27±2 °C for 3 days. After this period, the culture media were streaked onto the surface of Petri dishes containing nutrient agar (Merck, Germany) and incubated at 35°C for 24 h. Finally, cells were harvested using sterile water, and the concentrations of the inocula were not adjusted.

Counts of bacteria

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

RESULTS AND DISCUSSION

Diesel oil biodegradability test

The results obtained with the biodegradability test using the redox indicator DCPIP (Table 3) showed that the inocula used in the respirometric biodegradation experiments were able to degrade diesel oil. Moreover, the tests carried out with the native microorganisms indicated that these soils had a microbiota adapted to degrade hydrocarbons.

Table 3 - Diesel oil biodegradability test using DCPIP.

culture	decolourization
S. hominis	yes (after 3 days)
K. palustris	yes (after 3 days)
S. hominis + K. palustris	yes (after 3 days)
O. anthropi	yes (after 3 days)
microbial consortium-R	yes (after 4 days)
commercial inoc.-EM	yes (after 13 days)
native microorg. -ASP	yes (after 3 days)
native microorg. -SB	yes (after 6 days)
native microorg. -RC	yes (after 2 days)

Obs. During the test, no decolourization of the substrate control (without inoculum) or of the inoculum control (without diesel oil) was observed.

Respirometric biodegradation experiments
The CO_2 production in treatments 1, 2 and 3 (ASP soil) and its cumulative values are shown in Figures 1 and 2, respectively. There was no lag period in any treatment, probably due to a previous adaptation of the microorganisms (inoculum consisted of indigenous microorganisms) to the pollutants and favorable experimental conditions.

Initially, CO_2 production in treatments 2 and 3 (Fig. 1) was superior to 1. However, approximately after the 15th day, these curves showed a tendency to reach values of the control (treatment 1), presumably due to an exhaustion of the readily degradable hydrocarbons and nutrient consumption (Table 8).
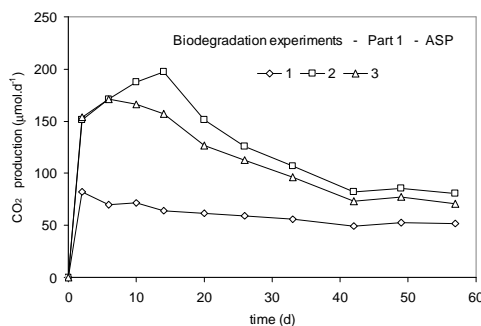
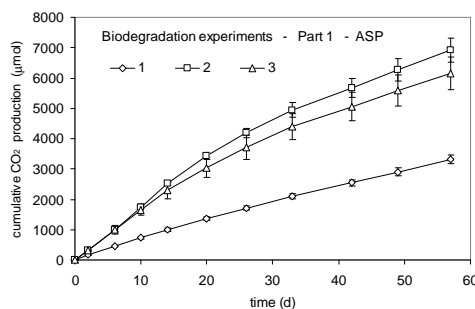**Figure 1** - CO_2 production during incubation   (ASP – part 1).

Figure 2 - Cumulative total amounts of CO_2 produced by treatments (ASP – part 1) during incubation. Each error bar represents 1 SD of three replicate.

The production of CO_2 in the experiment with SB soil (treatments 4 to 6), represented in Figures 3 and 4, demonstrated that there was no lag phase either, regardless the non-indigenous inoculum. The addition of nutrients in SB soil had a less pronounced positive effect. It is reasonable to suppose that other aspects were limiting the biodegradation efficiency. According to Bosma et al. (1997), the rate at which microbial cells can convert chemicals during bioremediation depends on two factors: the rate of uptake and metabolism (the intrinsic activity of the cell), and the rate of transfer to the cell (mass transfer), the so-called

bioavailability. The nutritional amendment is related to the first factor, but it may have no effect when mass transfer is the limiting factor.
The decrease of the bioavailability may be a result of two factors in the case of the SB soil. As stated by Löses et al. (1999), the presence of fine particles in the soil and low levels of hydrocarbons influence negatively the bioavailability. The SB soil has a percentage of clay three times greater than ASP soil (Table 1) and a lower level of hydrocarbon concentration 7,198 and 22,607 mg.kg^{-1}, respectively.
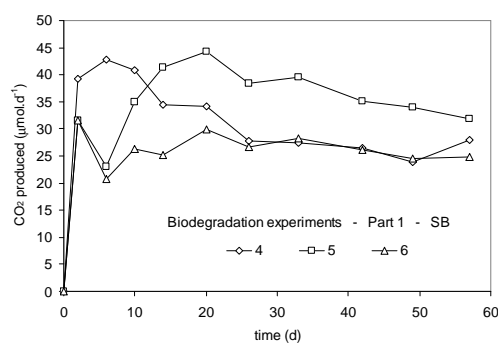
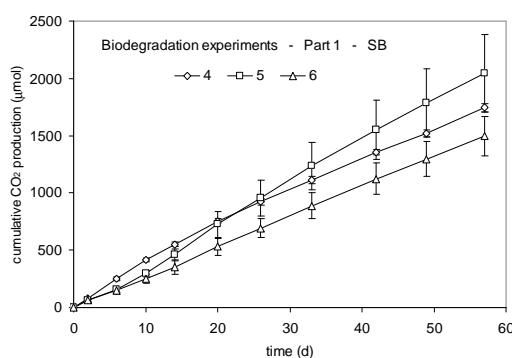**Figure 3** - CO_2 production during incubation   (SB – part 1).

Figure 4 - Cumulative total amounts of CO_2 produced by treatments (SB – part 1) during incubation. Each error bar represents 1 SD of three replicates.

Based on the CO_2 production, the biodegradation efficiency (BE) was calculated using Equation 3 (Fig. 5). This efficiency is related to the extent of mineralized hydrocarbons and excludes the portion of transformed diesel oil.

For both the soils, the addition of nutrients had a statistically significant positive effect in the biodegradation efficiency (ANOVA analysis, 95% confidence level). Biostimulation with the addition of fertilizers (N and P) has been reported as an important factor to enhance the soil bioremediation (Gallego et al., 2001; Cardona and Iturbe, 2003; Mariano et al., 2007). However, when considering the main focus of this study, i. e. the enhancement of bioremediation by inoculum injection, it had no effect in ASP soil, and in SB soil, the negative influence of the inoculum reduced the effect of the nutrient injection and this treatment obtained a biodegradation efficiency with no significant difference to the control.

In some studies, the bioaugmentation of the bacterial population already present in the soil achieved the best performance in diesel oil degradation or other hydrocarbons in soil (Kataoka, 2001; Bento et al., 2003). However, some researchers reported that inoculation had no positive or only marginal effects (Márques-Rocha et al., 2001), a behavior observed in the case of ASP soil. The result obtained with SB soil, suggests that a competition for metabolic regimes between the inoculated and the native cultures and different soil conditions (for instance, the adaptation of the inoculum to more acidic conditions of SB soil, Table 8) could have influenced negatively. This harmful effect of the bioaugmentation technique was also observed by Pala et al. (2002) and Trindade et al. (2002).
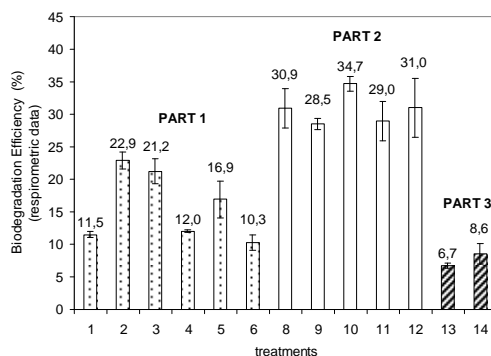
Figure 5 - Biodegradation efficiency obtained through the respirometric data. Each error bar represents 1 SD of three replicate.

Figure 12 represents the plate count of the total soil bacterial population performed before and after the experiments. In ASP soil, the treatments with addition of nutrients (2 and 3) had a final bacterial population greater than control and the inoculum showed to be inefficient in treatment 3, whose final value was equal to treatment 2, regardless of the higher initial population. In SB soil, the final value of treatment 5 was higher than treatment 4, confirming the positive effect of the nutrients that provided a significant growth in relation to the initial time. This behavior was not observed in treatment 6 possibly due to a competition caused by the exogenous microorganisms.

Chromatographic analyses of ASP and SB samples (Table 4) revealed that at these sites, the diesel oil was already weathered, i. e. the fuel had their characteristics altered by physical-chemical and biological mechanisms due to the large period of exposure of these compounds to the environmental conditions (Mariano et al., 2008b).

Table 4 - Concentration of hydrocarbons at ASP and SB soils

soil	total n-alkanes	pristine	phytane	TRH	UCM	TPH
			(mg.kg^{-1})			
ASP	<DL	266	162	2844	19763	22607
SB	81.8	65.0	43.8	941	6256	7198

DL (detection limit) = 0.4 mg.kg^{-1}; TRH - Total Resolvable Hydrocarbons; UCM - Unresolved Complex Mixture; TPH - Total Petroleum Hydrocarbons.

Diesel oil contains 2000 to 4000 hydrocarbons, a complex mixture of normal, branched and cyclic alkanes, and aromatic compounds (Gallego et al., 2001), which cannot be totally separated by gas chromatography. In fact, only n-alkanes and a few branched hydrocarbons can be identified as separated compounds. Chromatographic profiles of commercial diesel, as used in treatments 8 to 12 (Fig. 9), 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", called as the "unresolved complex mixture (UCM)", which presumably included the branched and cyclic alkanes and polar transformation products (Marchal et al., 2003; Bregnard et al., 1996). The resolved hydrocarbons are called "total resolvable hydrocarbons (TRH)" and the "total petroleum hydrocarbons (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 the

microorganisms have degraded the hydrocarbons in the diesel oil (Balba et al., 1998). In the chromatograms, the UCM "hump" becomes larger and the TRH peaks decrease, due to the biodegradation.

The hydrocarbons content in ASP and SB soils has been mainly characterized as isoprenoid alkanes and UCM, which are more recalcitrant than n-alkanes (Gallego et al., 2001), characteristics of a weathered diesel oil.

The concentration of hydrocarbons determined at initial and final times to treatments 1 to 6 are shown in Table 5. Only the TRH fraction was determined at the beginning and the concentrations of pristine and phytane were not determined for these treatments. The results are in agreement to respirometric data, i. e. greater efficiency removal of TRH and smaller final concentrations of the other fractions in the treatments with better biodegradation efficiencies. These results also indicated that the autochthonous microorganisms were adapted to the weathered diesel oil present in the ASP and SB soils and a misbalanced content of nutrients was limiting their action. Chromatograms of the final analyses can be seen in Figure 6.

Table 5 - Concentration of hydrocarbons at the beginning (1) and end (2) of the treatments 1 to 6. Values between parentheses indicate the removal efficiency (%)

treatments	TRH		total n-alkanes	UCM	TPH
	1	**2**	**2**	**2**	**2**
			$mg.kg^{-1}$		
1	4084	3620 (11)	<DL[1]	20112	23732
2	4084	2650 (35)	<DL[1]	15970	18620
3	4084	2988 (27)	<DL[1]	17157	20145
4	877	565 (36)	<DL[2]	3287	3851
5	877	383 (56)	<DL[2]	2185	2568
6	877	752 (14)	<DL[2]	4423	5175

DL (detection limit) = (1) 21 $mg.kg^{-1}$; (2) 8 $mg.kg^{-1}$. TRH - Total Resolvable Hydrocarbons; UCM - Unresolved Complex Mixture; TPH - Total Petroleum Hydrocarbons.
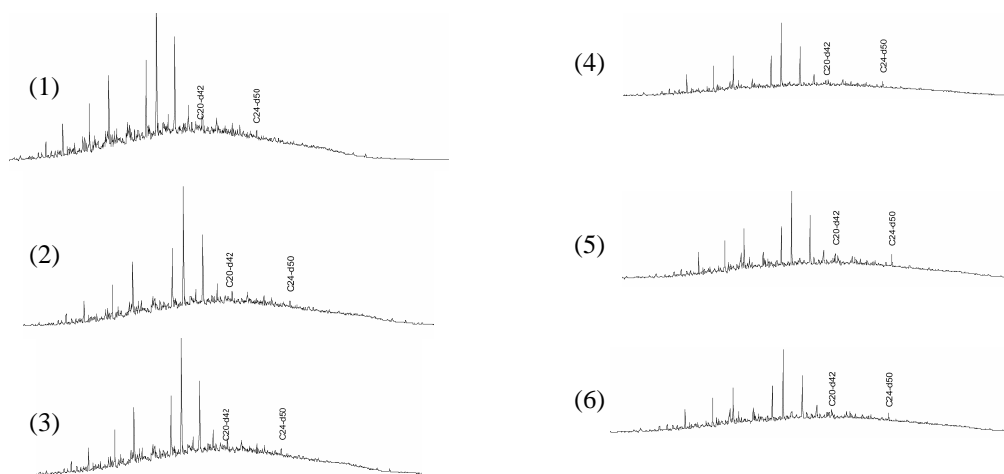

Figure 6 - Chromatographic analysis (TPH) at final time in treatments with ASP (1 to 3) and SB (4 to 5) soils. Dilution factors: 50x (1; 2 and 3); 20x (4 and 6); 10x (5). C20-d42 and C24-d50: recovery-species.

The CO_2 production of treatments 7 to 12 (second part of the biodegradation experiments – RC soil) and their cumulative values are shown, respectively, in Figures 7 and 8.

There was no lag period in the experiment, probably due to a previous adaptation of the microorganisms to hydrocarbons, since the RC soil already showed a low level of contamination

before the contamination with diesel oil (Table 6). After approximately the 20th day, the CO_2 production, in all the treatments where diesel oil was added, began to decrease, which could represent a shortage of labile carbon sources as n-alkanes, presumably initially consumed. In the treatments with inoculum addition, the biodegradation efficiency (Fig. 5) was not affected (ANOVA analysis, 95% confidence level) in comparison to the control (treatment 8).
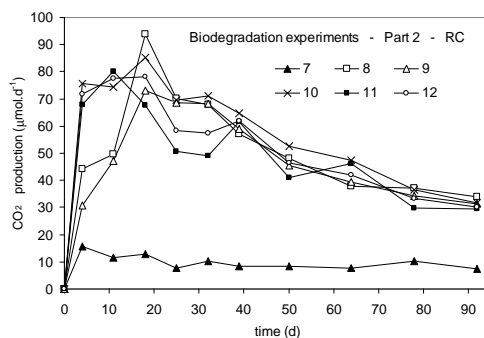

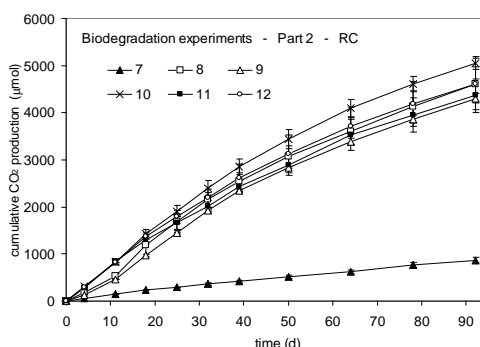**Figure 7** - CO_2 production during incubation (RC – part 2).

Figure 8 - Cumulative total amounts of CO_2 produced by treatments (RC – part 2) during incubation. Each error bar represents 1 SD of three replicate.

Table 6 - Concentration of hydrocarbons at the beginning (1) and the end (2) of the treatments with RC soil. Values between parentheses indicate the removal efficiency (%).

treatment	total n-alkanes		pristine		phytane		TRH		UCM		TPH	
	1	2	1	2	1	2	1	2	1	2	1	2
						(mg.kg^{-1})						
7	-^{a}	-	-	-	-	-	104	-	-	-	-	-
8	499	38 (92.0)	44	25 (43.2)	24	15 (39.1)	1232	341	4199	2717 (35.3)	5431	3058 (43.7)
9	499	90 (81.9)	44	22 (50.1)	24	13 (45.8)	1232	339	4199	2564 (38.9)	5431	2903 (46.5)
10	499	86 (82.7)	44	15 (66.4)	24	9 (64.3)	1232	262	4199	1984 (52.8)	5431	2246 (58.6)
11	499	186 (62.8)	44	21 (52.9)	24	11 (52.5)	1232	463	4199	2292 (45.4)	5431	2755 (49.3)
12	499	194 (61.1)	44	21 (50.8)	24	12 (45.4)	1232	503	4199	2449 (41.7)	5431	2952 (45.6)

[a]not analyzed. TRH - Total Resolvable Hydrocarbons; UCM - Unresolved Complex Mixture; TPH - Total Petroleum Hydrocarbons.

The biodegradation efficiency in terms of TPH removal (Table 6) showed similar behavior to respirometric data. The efficiencies in the latter were approximately 30% for all the treatments (8 to 12). Following this tendency of no difference between the treatments, the TPH removal was about 50% for all the treatments, although in treatment 10, the removal was slightly higher (58.6%).

Comparing the chromatograms of the initial time to those of the final time (Fig. 9), the decrease of the TRH peaks was very clear. Table 6 shows that the diesel oil compounds were degraded following the order of their expected decreasing susceptibility (Gallego et al., 2001): n-alkanes, isoprenoid alkanes (pristine and phytane) and UCM. In treatments 9 to 12 (especially in treatment 10), where inocula were added, the removal of the most recalcitrant compounds was higher compared to treatment 8, while the removal of the n-alkanes had an inverse behavior. This information obtained from the chromatographic analyses showed that, regardless of the treatments similar respirometric values, the injection of microorganisms from polluted environments increased the biodegradation of recalcitrant compounds. Similar results were obtained by Marchal et al. (2003).
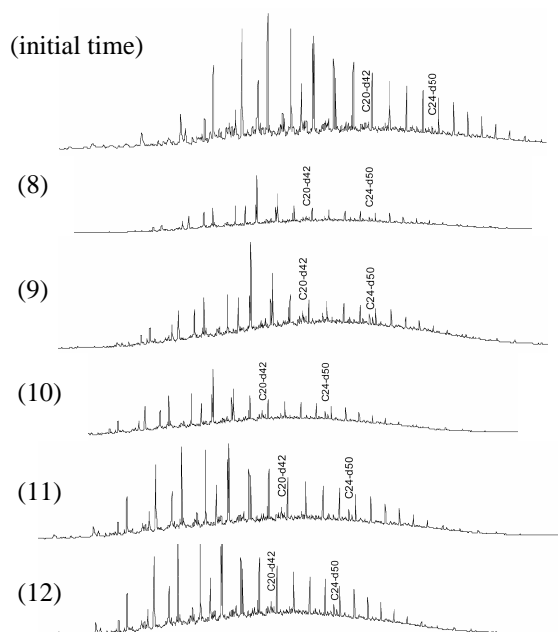

Figure 9 - Chromatographic analysis (TPH) at initial and final time in treatments with RC soil (8 to 11). Dilution factors: 20x (initial and 8); 10x (9 to 12). C20-d42 and C24-d50: recovery-species.

Similar values of initial bacterial populations in treatments 7 and 9 (Fig. 12) indicated that the microorganisms added previously to the contamination (treatment 9) did not increase the microbial population probably due to a shortage of additional carbon source. This observation and the fact that there was no increase in the biodegradation efficiency, suggest that this strategy has even better chances to fail in real situations, where during a possible long period between the injection of the microorganisms and a spill, microorganisms would have to survive against the predators, compete for limiting nutrients and maintain their degradative capabilities. Moreover, as demonstrated in the previous item, the presence of hydrocarbonoclastic

microorganisms in soils is ubiquitous, even in not polluted soils (Venkateswaran and Harayama, 1995; Ron and Rosenberg, 2002 *apud* Lee et al., 2006).

In treatments 11 to 12, where the inocula were more concentrated, the soil population initially increased to values ranging from 10^8 to 10^9 CFU / g of dry soil. However, as the other treatments, including treatment 7 (control, without addition of diesel oil), the microbial populations were about 10^7 at final time. This similarity showed (as also observed by Hickman and Novak, 1989) that generally total microbial biomass can be a poor indicator for determining the biodegradation potential, mainly because the active biomass can differ in the species composition and in metabolic regimes.

Table 8 shows the values of pH and nitrogen and phosphorus consumption at the end of the experiments. The values of pH were stable at neutral values in all the treatments (7 to 12), which was a favorable condition for biodegradation. In relation to the nutrients, there was no consumption of N or P, which suggested that the majority of the chemical forms of these compounds were not available to the microorganisms. Probably,

balanced addition of easy assimilated forms, as those employed in the first biodegradation experiment ((NH_4)$_2SO_4$ and KH_2PO_4), would increase the biodegradation efficiency.

The CO_2 production in treatments 13 and 14 (third part of the biodegradation experiments - ASP soil) and their cumulative values are shown in Figures 10 and 11, respectively. There was no lag period in the experiment.

After approximately the 10th day, the CO_2 production began to decrease in both the treatments, which may represent a shortage of more labile (n-alkanes) or available carbon sources.

Statistical analyses (ANOVA analysis, 95% confidence level) of the biodegradation efficiencies (Figure 5) showed that the addition of the commercial inoculum EM had no influence in comparison to the control (treatment 13). This behavior was also observed in the concentration of hydrocarbons (Table 7), whose chromatograms are presented in Figure 13. The inefficiency of inoculum EM could be related to its incapacity to survive in the ASP soil as indicated by the lower final value of the total soil bacterial population in relation to the control (treatment 13) (Figure 12).
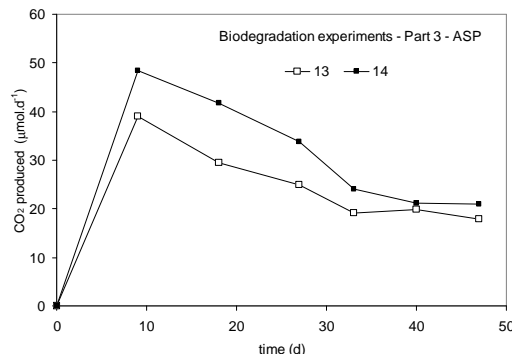

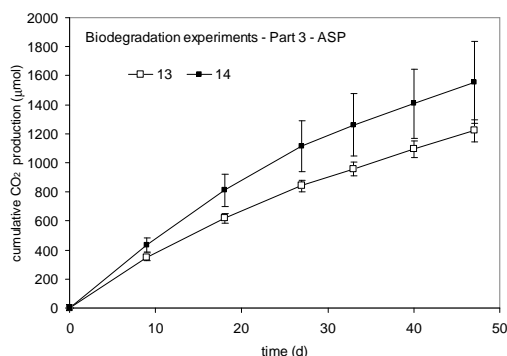**Figure 10** - CO_2 production during incubation (ASP – part 3).

Figure 11 - Total cumulative amounts of CO_2 produced by the experiment (ASP – part 3) during incubation. Each error bar represents 1 SD of three replicate.
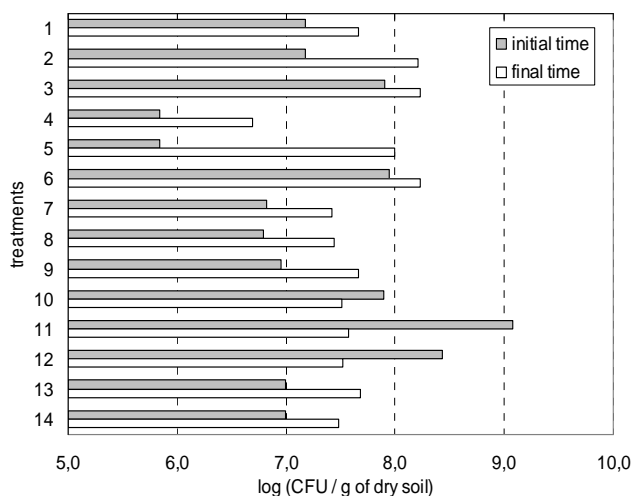
Figure 12 - Total heterotrophic bacteria counts at initial and final times of the respirometric experiments.

Table 7 - Concentration of hydrocarbons at the beginning (1) and the end (2) of the experiment with ASP soil (Part 3). Values between parentheses indicate the removal efficiency (%)

treatment	total n-alkanes		pristine		phytane		TRH		UCM		TPH	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
							$(mg.kg^{-1})$					
13	<DL	4.1	<DL	2.4	<DL	1.3	62	35 (43.5)	722	228 (68.4)	784	263 (66.4)
14	<DL	3.5	<DL	1.9	<DL	1.1	62	38 (38.7)	722	260 (64.0)	784	297 (62.1)

DL (detection limit) = 4.0 $mg.kg^{-1}$. TRH - Total Resolvable Hydrocarbons; UCM - Unresolved Complex Mixture; TPH - Total Petroleum Hydrocarbons.
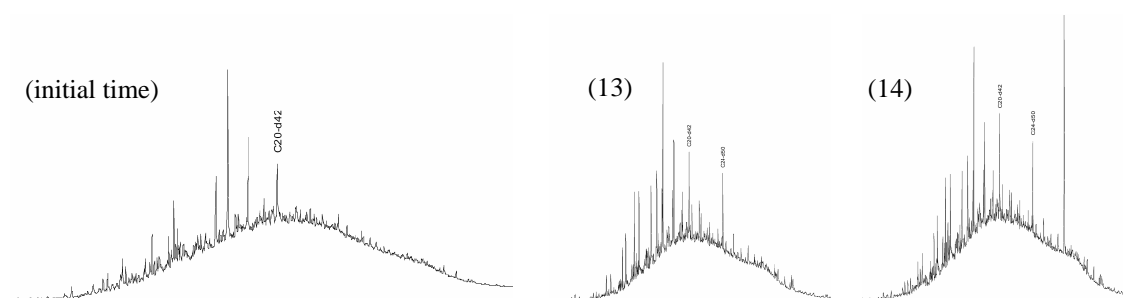

Figure 13 - Chromatographic analysis (TPH) at initial and final times in treatments with ASP soil (13 and 14). Dilution factors: 10x (initial); 1x (13 and 14). C20-d42 and C24-d50: recovery-species.

Table 8 - Values of pH, nitrogen and phosphorus at the experiments

treatment	pH[1]		N		P	
	initial	final	initial	final	initial	final
					(mg.kg^{-1})	
1	6.7	7.5	800	210	6	6
2	6.7	6.5	2090	1050	139	66
3	6.7	7.1	2090	1050	139	56
4	5.0	4.9	700	350	3	2
5	5.0	5.0	1480	1260	99	55
6	5.0	5.0	1480	1260	99	48
7	7.4	7.1	210	210	2	2
8	7.1	7.3	210	210	2	2
9	7.2	7.2	210	210	2	2
10	7.3	7.3	210	210	2	2
11	7.1	7.3	210	210	2	2
12	7.1	7.4	210	210	2	2
13	4.6	4.5	350	350	-[2]	-
14	4.6	4.5	350	350	-	-

[1] CaCl$_2$; [2] not analyzed.

CONCLUSIONS

Although the bioaugmentation was carried out with microorganisms able to degrade diesel oil, in general, no gain was obtained with this bioremediation technique when treating the contaminated soils with diesel oil. A reasonable explanation for this inefficiency could be that, when added into soils, microorganisms were subjected to ecological principles of competition and predation, which would decrease significantly their chances of survival in the natural environments. Moreover, the soils collected from the three service stations presented a native microbiota already adapted to degrade the hydrocarbons, which suggested that the use of the bioaugmentation, in order to accelerate the bioremediation process, would achieve better results in cases of more recalcitrant chemicals or when the local microbial population was insufficient or inadequate. In any case, the development of techniques to assure the survival of the microorganisms in the natural environment would be essential.

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RESUMO

Este trabalho investigou a eficiência da técnica do bioaumento quando aplicada a solos contaminados com óleo diesel coletados em três postos de combustíveis. Experimentos de biodegradação foram realizados em frascos de Bartha (250 mL), usados para medir a produção microbiana de CO$_2$. A eficiência de biodegradação também foi quantificada pela concentração de hidrocarbonetos. Conjuntamente aos experimentos de biodegradação, a capacidade das culturas estudadas e dos microrganismos nativos em biodegradar óleo diesel comprado de um posto de combustíveis local, foi verificada utilizando-se a técnica baseada no indicador redox 2,6 – diclorofenol indofenol (DCPIP). Resultados obtidos com esse teste mostraram que os inóculos empregados nos experimentos de biodegradação foram capazes de biodegradar óleo diesel e os testes com os microrganismos nativos indicaram que estes solos previamente apresentavam uma microbiota adaptada para degradar hidrocarbonetos. Em suma, nenhum ganho foi obtido com a adição dos microrganismos ou mesmo efeitos negativos foram observados nos experimentos de biodegradação.

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