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RESEARCH ARTICLE

Mesocosm trials of bioremediation of contaminated soil of a petroleum refinery: comparison of natural attenuation, biostimulation and bioaugmentation

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Abstract
Purpose Contamination with petroleum hydrocarbons (PHC) is a global problem with environmental implications. Physico-chemical treatments can be used for soil cleanup, but they are expensive, and can have implications for soil structure and environment. Otherwise, biological remediation treatments are cost-effective and restore soil structure. Several remediation experiments have been carried out in the lab and in the field; however, there is the challenge to achieve as good or better results in the field as in the laboratory. In the ambit of a project aiming at investigating suitable biological remediation approaches for recovering a refinery contaminated soil, we present here results obtained in bioremediation trials. The approaches biostimulation and bioaugmentation were tested, in parallel, and compared with natural attenuation. For this purpose, mesocosm experiments were carried out inside the refinery area, which constitutes a real asset of this work.
Methods Soil contaminated with crude oil was excavated, re-contaminated with turbine oil, homogenised and used to fill several 0.5 m^3 high-density polyethylene containers. The efficiency of procedures as follows: (1) natural attenuation; (2) manual aeration; (3) biostimulation by adding (3.1) only nutrients; and (3.2) nutrients and a non-ionic surfactant; and (4) bioaugmentation in the presence of added (4.1) nutrients or (4.2) nutrients and a non-ionic surfactant were evaluated after a 9-month period of experiment. For bioaugmentation, a commercial bacterial product was used. In addition to physico-chemical characterization, initial and final soil contents in total petroleum hydrocarbons (TPH) (by Fourier transform infrared spectrophotometry) and the total number of bacteria (by total cell counts) were carried out. For TPH degradation evaluation the soil was divided in four fractions corresponding to different depths: 0–5; 5–10; 10–15; and 15–20 cm. Mean values of percentages of PHC degradation varied between 20 and 50% at surface and between 10 and 35% below 5-cm depth. Natural attenuation was as efficient as most of the tested treatments (about 30% TPH degradation) being exceeded only by bioaugmentation combined with nutrient and surfactant amendments (about 50% TPH degradation). Higher TPH degradation at surface suggests that a combination of sufficient dioxygen, propitious for aerobically degradation, with sunlight required for production of strong photochemical oxidants like ozone, contributed for enhancing degradation. Indeed, the atmosphere of the refineries is relatively rich in volatile organic compounds and nitrogen dioxide (a side-product of the combustion of residual volatile PHC released by the chimneys), which are precursors of O_3 and other photochemical oxidants produced in sunny days, which are very common in Portugal. The fact that natural attenuation was as efficient as most of the soil treatments tested was very probably a result of the presence, in the initial soil, of physiologically adapted native microorganisms, which could be efficient in degrading PHC.
Conclusions A cost-effective way to reduce half-life for the degradation of PHC of contaminated soil of the refinery

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will be a periodic revolving of the soil, like tillage, in order to expose to the oxidative atmosphere the different layers of contaminated soil. A combination of soil revolving with bioaugmentation together with nutrients and surfactant amendments may result in an additional improvement of PHC degradation rate. However, this last procedure will raise markedly the price of the remediation treatment.

Keywords Petroleum hydrocarbons · Refinery soil · Biostimulation · Bioaugmentation

1 Introduction

Soil spread contamination with petroleum hydrocarbons (PHC) is a worldwide problem, which is considered a relevant environmental concern. Among the sources of PHC, petroleum refining results in the generation of large quantities of oil sludge, consisting of hydrophobic and refractory substances.

For soil cleanup, physico-chemical treatments can be applied, but they are extremely expensive (Ouyang et al. 2005) and, in addition, they damage the structure of the soil and/or utilise organic solvents, which are harmful for the environment. In contrast, biological remediation treatments are cost-effective approaches that rehabilitate soil structure.

In the ambit of a project aiming at investigating suitable biological remediation approaches for recovering a refinery contaminated soil, we present here results obtained in bioremediation trials. The approaches *biostimulation* (introducing suitable amounts of water, nutrients and oxygen into the soil, in order to enhance the activity of indigenous degraders) and *bioaugmentation* (introduction of PHC degrading microorganisms) were tested, in parallel, and compared with natural attenuation.

Stimulated or not, indigenous microorganisms capable of PHC biodegradation could have a crucial impact in remediation, especially if site was exposed prior to the contaminant (Bento et al. 2005; Sabaté et al. 2004). However, the composition of the hydrocarbons and the environmental conditions affect the composition and competence of the microbial population (Bento et al. 2005).

Inoculation of an enriched-specific competent strain or consortium into the soil may also improve bioremediation when competent degraders are absent or present at relatively low numbers. Bioaugmentation can use microorganisms, which are already present in the contaminated soil, but at abundances too low to effect remediation. This process requires that samples are taken from the site to be treated, and cultures are grown in the laboratory in the presence of elevated concentrations of the contaminant. Ideally, the contaminant is the sole source of energy, ensuring that only those microorganisms that consume it will grow (Devinny and Chang 2000). The attainment through local preparation of suitable and sufficiently dense cultures for bioaugmentation is expensive and difficult (Devinny and Chang 2000). Such constraints can be avoided by buying suitable commercial products. This last procedure was chosen for the present work. According to Devinny and Chang (2000), the added microorganisms may not successfully reproduce in the soil. They do not need to compete with existing soil organisms or to escape waiting predators. They need only the ability to survive for a period of time and to metabolise the contaminant and they may do so even as they are dying out. Nevertheless, bioaugmentation is still a controversial subject, remaining debatable as a scientific and as a technological endeavour (El Fantroussi and Agathos 2005). Some studies have demonstrated the utility of bioaugmentation, whereas the treatment has failed in other cases (El Fantroussi and Agathos 2005). Some additional work is needed before bioaugmentation becomes a fully reliable and predictable adjuvant to bioremediation.

Until now, major contributions of the reported experiments of soil bioremediation have been carried out in the lab (in well-controlled conditions), while the field experiments are scarce (Ouyang et al. 2005). However, the difficulty of achieving as good or better results in the field as in the laboratory is the challenge to face in bioremediation strategy (Bento et al. 2005).

This work aimed to investigate, in parallel, and down to a 20-cm soil depth, the efficiency of PHC degradation of the procedures as follows: (1) natural attenuation; (2) manual aeration; (3) biostimulation by adding (3.1) only nutrients; and (3.2) nutrients and a non-ionic surfactant; and (4) bioaugmentation in the presence of added (4.1) nutrients or (4.2) nutrients and non-ionic surfactant. The experimental conditions used, which were mesocosm experiments carried out inside the refinery area, constitute a real asset of this work.

2 Material and methods

2.1 Experimental design

2.1.1 Field study

Soil contaminated with crude oil was excavated near a tank container where an oil spill occurred. Turbine oil was added to the soil in order to increase its degree of contamination. Approximately 3 m^3 of re-contaminated soil was well-homogenised and distributed uniformly by five containers of high-density polyethylene with 1.0×1.0 m^2 of base area. Each container was divided in two equal parts, and only one part was subjected to manual aeration. The containers were differently treated/amended as described in Table 1.

Table 1 Experimental design

Container [a]	Addition of		
	Nutrients (N+P)	Non-ionic surfactant	Bioaugmentation product
NA	–	–	–
N	+	–	–
NS	+	+	–
BN	+	–	+
BNS	+	+	+

[a] *NA* natural attenuation, *N* nutrient amendment, *NS* nutrient and surfactant amendments, *BN* bioaugmentation and nutrient amendment, *BNS* bioaugmentation with nutrient and surfactant amendments

Nutrient amendment to attain a final proportion of C:N:P of about 62:5:2 was performed by periodic addition of a mixture of NH_4NO_3 and $(NH_4)_2HPO_4$ commercial grade. The nutrients were added and dissolved in water to facilitate the entrance/dissolution on N and P in the soil matrix.

A 0.15% (*v*/*v*) aqueous solution of the non-ionic surfactant Empilan KR6 (Huntsmann LLC) [CH_3–$(CH_2)_8$(or)$_{10}$–$(OCH_2CH_2)_6$–OH] was also used for amendment.

The commercial oil degrading bioaugmentation product MicroSolv-400 (trademark, Environmental Leverage) was used for bioaugmentation. The product was acquired as a dry powder and was hydrated with deionised water. After agitation, the mixture was allowed to sediment for 2 h and then spread on the soil. The inoculum was added so as to attain a concentration of approximately 1.6 mg kg^{-1} of soil. The product is normally used for wastewater treatment, but it can also be applied to soil remediation (information provided by the supplier). The product contains micro-organisms that use hydrocarbons as a carbon source and produce a broad spectrum of enzymes (Leverage 2007).

Manual aeration was performed with a garden hoe, down to about 10 cm of depth. The main purpose of this procedure was to increase the contact between soil, amendments and microorganisms, enhancing soil homogenisation and air circulation.

All the amendments and aeration were performed weekly.

At the beginning of the study, in July 2007, and 9 months later, composite samples from five random different locations of each container were collected. The last case sampling was performed separately at four depths (0–5, 5–10, 10–15 and 15–20 cm). The soil samples were immediately preserved in aluminium foil and frozen until analysis. Soil sampling was performed with material previously washed with detergent and deionised water.

Three or at least two replicates of each composite sample were treated, and each one was analysed in triplicate.

2.1.2 Laboratory study

With the purpose of testing specifically possible positive effects of bioaugmentation in a recently contaminated soil, a laboratory experiment was carried out. For this purpose, about 300 g of agricultural soil (grain size <2 mm) without hydrocarbon contamination was spiked with turbine oil (dissolved in acetone) and well-homogenised in order to achieve a final concentration of about 11,533 mg of contaminant per kg of soil. The soil was left in a hotte for 5 days to allow acetone evaporation (absence of perceptible odour).

Two different levels of bioaugmentation were tested, by adding two concentrations of inoculum: 1.5 and 250 mg kg^{-1} of soil. During the experiment the moisture was kept at about 15% by regular addition of suspension of microorganisms and/or deionised water, whichever is appropriate.

Bioaugmentation was performed weekly, and soil tillage was done twice a week. The experiment was carried out in duplicate, under indoor conditions with a good sunlight exposure.

Samples were collected for analysis at the end of the study. Two replicates of each sample were treated, and each one was analysed in triplicate.

2.2 Soil characterization

Soil composition, in terms of grain size, was performed by dry sieving. Nutrient and pH analyses were carried out in *"Nutrir—Serviço de análises e conselhos de adubação"* laboratory. Organic matter content was determined by loss on ignition (4 h at 500°C). Moisture content was estimated by loss of mass in an oven at 105°C, until constant weight (Jørgensen et al. 2000). The analysis of mineral oil/petroleum hydrocarbons content between C_{10} and C_{40} was performed in *"Dr. Kaiser & Dr. Woldmann GmbH"* laboratory according to DIN ISO 16703. Redox potential was determined using a Mettler Toledo pH/mV metre equipped with a Redox electrode (Inlab 501, Mettler Toledo International).

2.2.1 Total petroleum hydrocarbons

For the evaluation of the efficiency of the different soil treatments and the determination of total petroleum hydro-

carbons (TPH) content an adapted procedure was used (Liste and Felgentreu 2006) and Method 8440 1996). Firstly, the soil sample was dried at room temperature, crushed and sieved (2-mm grid). Only the fraction ≤2-mm grain size was used for analysis. About 1 g of soil was mixed with 1 g of anhydrous sodium sulphate (p.a. Fluka) and with 10 mL of tetrachloroethylene (≥99%, Spectrophotometric grade, Sigma Aldrich) and an ultrasonic (Elma, Transsonic 460/H model) extraction was performed for 60 min. After settling down, the extract was decanted and mixed with 0.3 g of deactivated [for 5 h at 180°C; cooling down overnight; 2% (*w*/*w*)] silica gel (70–230 mesh, Macherey-Nagel), in order to remove interferences, like biodegradable animal greases and vegetable oils (Method 8440 1996). The mixture was well-homogenised for 10 min. Then, the liquid phase was filtered through glass wool and diluted with tetrachloroethylene, in order to obtain a concentration suitable for analysis of TPH by Fourier transform infrared spectrophotometry (Jasco FT/IR-460 Plus instrument), and the absorbance of the diluted extracts was being measured near 2,930 cm^{-1}. Soil samples collected before and after each 9-month experiment were always analysed in the same run, and the final result was expressed in percentage of the TPH that remained in the soil after the treatment: [*Final concentration (mg* kg^{-1}*)/Initial concentration (mg* kg^{-1}*)*]× *100*.

Before and after each spectrophotometric measurement, the quartz cell (Infrasil I, Starna Scientific) was washed with the solvent in an ultrasonic bath (Sonorex TK 30, Bandelin) for 3 min.

For TPH analysis, the glass material used in the sample's handling was firstly washed with Teepol and deionised water and then cleaned in a nitric acid bath for 24 h. After being washed with deionised water, vials and septa were dried and maintained in the oven at 40°C, and the remaining required material was dried at room temperature.

2.2.2 Total cell count

The enumeration of microbial cells was carried out only in soil collected at 5–10-cm depth.

Total cell count in the soil sample was performed using a modification of the 4′,6′-diamidino-2-phenylindole (DAPI) direct count method (Kepner and Pratt 1994; Porter and Feig 1980). For enumeration of microbial cells, 0.25 g of soil was added to 2.5 mL of 9 gL^{-1} NaCl solution (previously 0.2 µm-filtered) and fixed with 4% (*v*/*v*) formaldehyde (previously 0.2 µm-filtered). A 12.5% (*v*/*v*) aqueous solution of the surfactant Tween 80 was also added and, after stirring at 150 rpm for 15 min, the sample was sonicated for 20–30 s at low intensity (0.5 cycles, 20% amplitude).

In order to stain the sub-samples, an addition of DAPI was carried out followed by incubation in the dark for 12 min. Each sample was then filtered through a black Nucleopore polycarbonate filter (0.2-µm pore size, 25-mm diameter, Whatman, UK), and the still moisten filter was placed in a microscope slide and fixed with paraffin to the cover slip. The preparation was protected with aluminium foil to prevent filter dehydration and/or fluorochrome degradation. Cell counting was performed at ×1,875 on an epifluorescence microscope (Laphot, Nikon, Japan).

3 Results and discussion

The main characteristics of the initial soil are shown in Table 2. The soil was sandy, with a deficit of inorganic nutrients. The initial number of bacteria was relatively high. Values of the same order of magnitude have been used in laboratory inoculation for soil remediation tests (Sabaté et al. 2004).

The effectiveness of TPH degradation in the different experimental conditions tested (Table 1) can be seen in Fig. 1, which shows the amount of TPH remaining in the soil after the 9-month experiments.

In this study, statistically significant influence of *tillage* was not observed. Tillage is recommended both in agriculture and remediation processes (Gogoi et al. 2003) to provide oxygen, to reduce soil heterogeneity and increase contact with small clumps. However, in this case the potential redox, measured at the end of the experiments, revealed values closer or higher than 400 mV in all the treatment units at different depths, which are characteristic of aerobic conditions. Probably the periodic watering to maintain moisture integrity and the supplying of additives helped to physically open channels and, at the same time, released enough dioxygen molecules from those dissolved in water to soil environment. Indeed, oxygen content depends on several factors, like water supply (which has dissolved oxygen), soil pores and microorganisms (Vasudevan and Rajaram 2001). Specifically for PHC remediation, benefits of

Table 2 Initial soil characteristics

Parameter	Values
Fraction silt and clay	<9%
pH	6.4
Total cell counts (g^{-1} soil)	3.1×10^{8}
Phosphorus (mg kg^{-1})	11.8
Mineral N (mg kg^{-1})	7.7
Total N (mg kg^{-1})	1,000
Organic matter (%)	7.5
Moisture content (%)	12
Mineral oil (C_{10} to C_{40}) (mg kg^{-1})	11,000

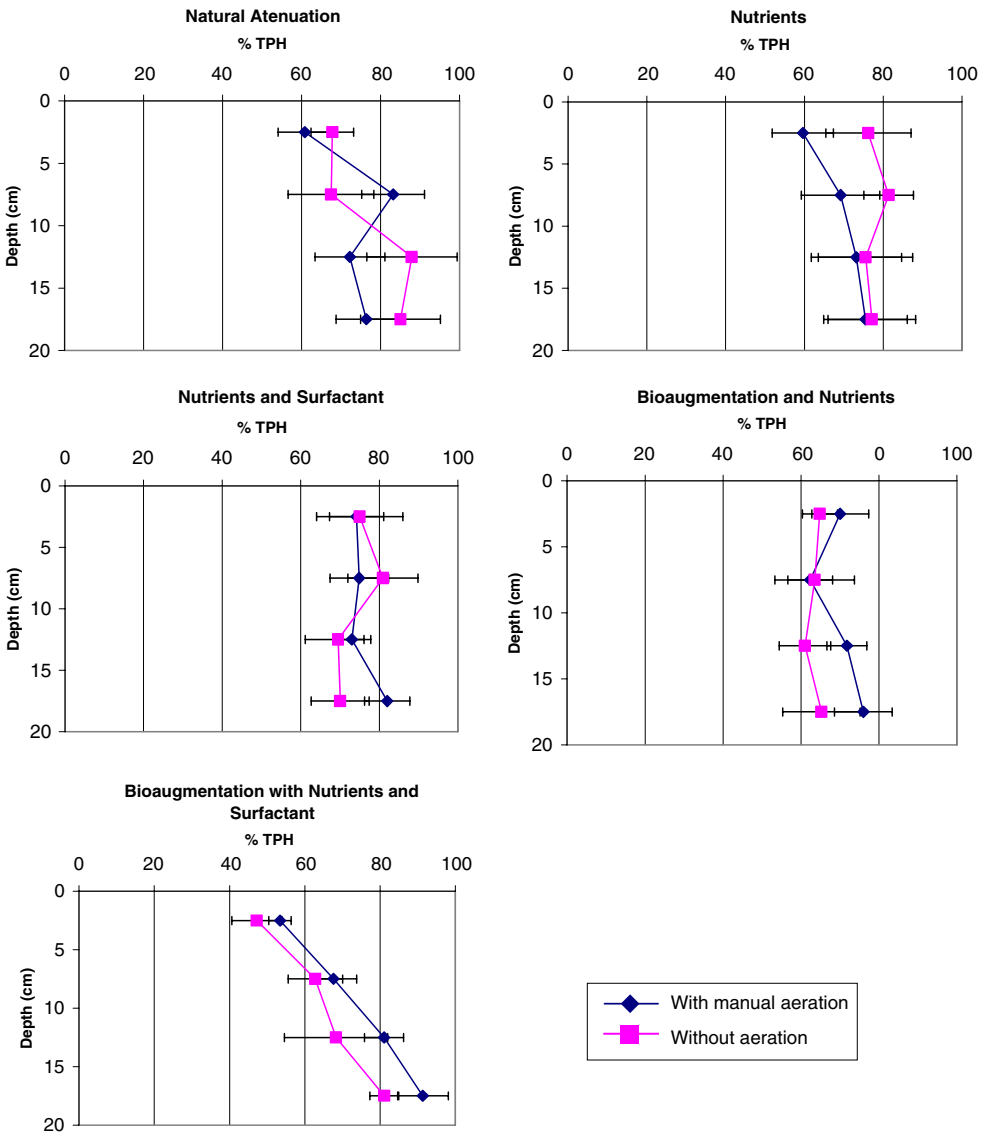

Fig. 1 Percentage of TPH [mean value and confidence limits (P=0.05)] that remained in the soil after a 9-month period of experiment in different conditions

tillage for oil redistribution, increasing its bioavailability, has been mentioned (Vasudevan and Rajaram 2001), but did not show to cause significant benefit in the present case.

As concerns the TPH v*ariation with depth*, the mean values of TPH degradation were, in general, between 20 and 50% at surface and between about 10 and 35% below 5-cm depth. A tendency towards higher degradation at surface (lower final TPH levels) was observed in the treatment units as follows: natural attenuation (NA); biostimulation with nutrients (N) and tillage; and, particularly, bioaugmentation combined with biostimulation with nutrients and surfactant addition (BNS). In the last case, the TPH levels were significantly lower in the surface. Higher TPH degradation at surface suggests that a combination of sufficient dioxygen, propitious for aerobic degradation, with sunlight required for production of strong photochemical oxidants like ozone, contributed to enhance degradation. Indeed, the atmosphere of a refinery is relatively rich in volatile organic compounds and nitrogen dioxide (a side-product of the combustion of residual volatile PHC released by the chimneys) which are precursors of O_3 and other photochemical oxidants produced in sunny days.

This study also showed that *natural attenuation* (NA) was as efficient as several soil treatments tested. This fact was probably a result of the presence in the soil, which contained old PHC contamination, of physiologically adapted native microorganisms (Bento et al. 2005; Sabaté et al. 2004), which could be efficient in degrading the present contamination.

It was also observed that amendment with *inorganic nutrients* did not cause, by itself, a marked enhancing of PHC remediation through stimulation of indigenous microorganism activity, despite the fact that successful cases have been reported in literature (Gogoi et al. 2003). Nevertheless, laboratorial studies have led to the conclusion that addition of nutrients can enhance microorganism activity

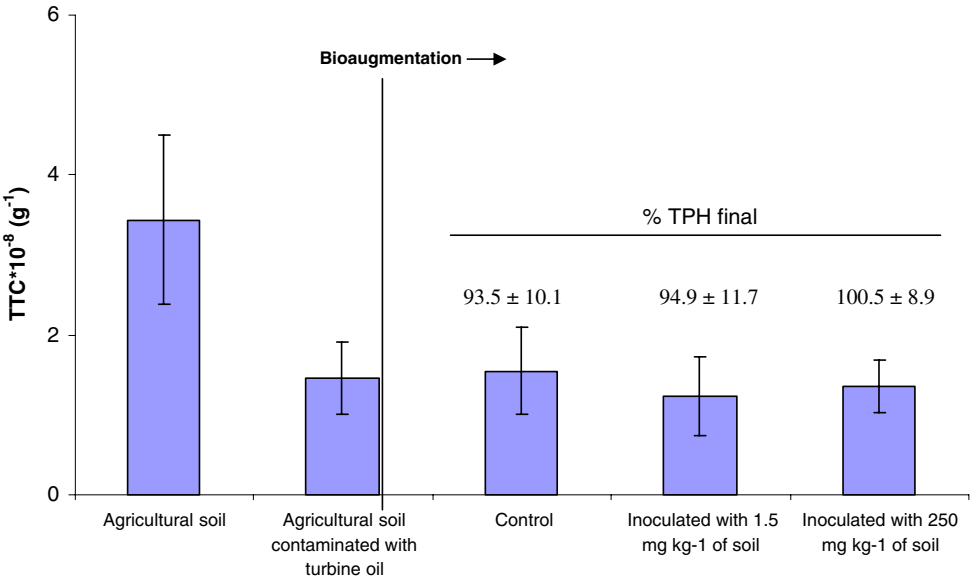

Fig. 2 Mean values and confidence limits (P=0.05) of the total cell counts in an agricultural soil: without contamination, 5 days after contamination with about 11,533 mg of turbine oil per kg of soil and 44 days after bioaugmentation

(Bento et al. 2005) and degradation rate (Sabaté et al. 2004) in the earlier part of the experiments, due to readily available nutrients, but later on, at the end of the experiments, similar degradation yields in soil amended or not amended with nutrients have been observed. In other cases, metabolic inhibition of microorganism activity caused by high-nutrient concentrations in general has been also reported (Del'Arco and de França 1999).

The treatment with both *nutrients and surfactant* (NS) also did not enhance remediation relative to natural attenuation. Contradictory effects of surfactant addition have been reported in the literature, since increase of contaminant bioavailability and remediation (Haigh 1996) to degradation inhibition due to, for instance, the use of the surfactant by bacteria as carbon source instead of PHC (Haigh 1996).

Addition of the exogenous microorganisms with supply of nutrients (BN) did not enhance significantly TPH degradation as well. However, the simultaneous addition of exogenous microorganisms, nutrients and surfactant (BNS) significantly improved the remediation efficiency at the surface layer of soil, which was the maximum attained in this study (mean value about 50%). To note that when commercial preparations are used, it is not clear that the chosen microbial species are appropriated for the local environment (e.g. soil pH, temperature, salinity, etc.) (Devinny and Chang 2000). To have any effect at the remediation site, the added microorganisms should be able to, at least, survive for a period of time and to metabolise PHC. In the present case, it seems probable that a combination of aerobic/strong oxidative conditions with abundance of inorganic nutrient and in the presence of a surfactant constituted favourable conditions to reduce the half-life for degradation of the PHC. The possible role of the non-ionic surfactant is unknown, but, either an improvement of PHC bioavailability or co-metabolism or both are reasonable hypotheses.

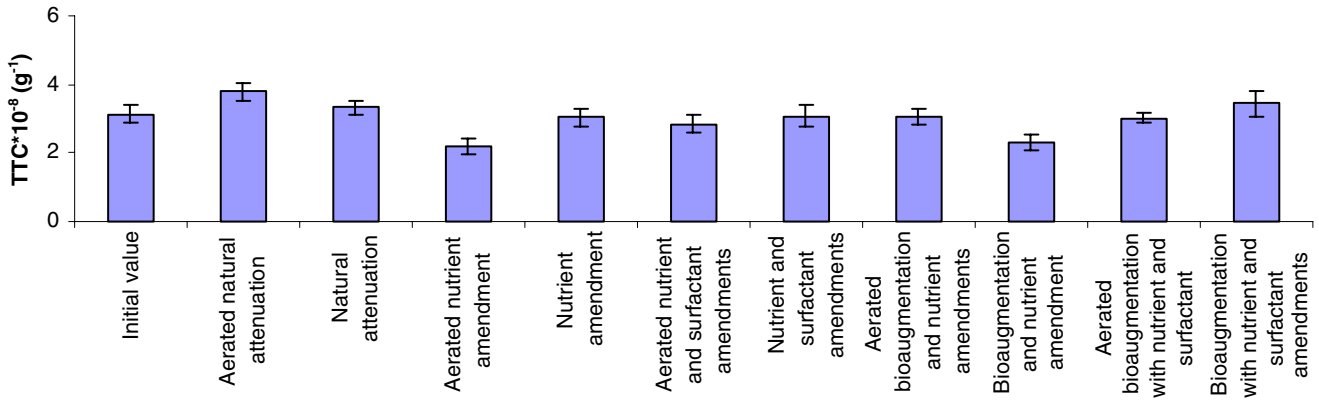

Fig. 3 Mean values and confidence limits (P=0.05) of the total cell counts in the initial soil and after 9 months of experiment in different conditions

It is worthy to mention that a laboratory study of bioaugmentation was carried out by us, in parallel with the field study. In the laboratorial study an agricultural clean soil was contaminated with turbine oil (see Section 2.1). The same commercial bioaugmentation product, which was used in the field experiments, was periodically added to the soil at two different concentrations, without addition of either nutrients or surfactant. Figure 2 shows that the absence of any PHC remediation was observed after a period of 44 days of experiment. The number of microorganisms present in the soil, which was subjected to treatment, was not significantly different from that of the control (Fig. 2). However, an increase of the final number of microorganisms is not a necessary condition for a successful bioaugmentation, as discussed above.

Therefore, a favourable conjugation of biotic and abiotic factors conducted to enhance PHC remediation in the described bioaugmentation experiment was carried out in the field. The same was not verified in the laboratory study, despite the fact that only recent contamination was present in the soil in this case.

It deserves also to be reported that the addition of PHC to clean agricultural soil in this laboratory study caused a reduction of about 50% of the indigenous microorganisms of the soil. The observed toxic effects could be caused by the added PHC and/or by the acetone used in the preparation of a suitable PHC solution to contaminate the soil. Despite being a very common practise (Hamdi et al. 2007; Lee et al. 2008), the use of acetone, in order to spike the soil and ensure hydrophobic organic contaminants dissolution, has also showed negative effects of acetone on the activity of soil microorganisms (Klimkowicz-Pawlas and Maliszewska-Kordybach 2008).

Concerning the number of bacteria present in the treatment units of the field study, the initial and final total cell counts are presented in Fig. 3.

This figure shows that the initial number of bacteria was already relatively high and similar to those present in the soil at the end of the experiments. Maxima positive and negative variations were, in general, of 30% or lower. These results corroborate the already published conclusions (Bento et al. 2005) that in previous contaminated soils the number of heterotrophic population, diesel degrading microorganisms and the ratio between diesel oil degrading microorganisms and heterotrophic microorganisms are not influenced by bioremediation treatments. In case of bioaugmentation, a rapid decline in population size of active exogenous inoculated microbial cells in the soil has been observed before (Van Veen et al. 1997) and attributed to both biotic and abiotic factors (El Fantroussi and Agathos 2005). Soil is a very complex and dynamic biotope and, among others, predation by protists and competition with autochthonous microorganisms for nutrients or electron acceptors are biotic elements that adversely impact bioaugmentation, acting as a buffer against incoming microorganisms. Abiotic factors, like temperature, pH, moisture, organic matter, clay contents, etc., can also condition the viability of the exogenous inoculated microbial cells (Devinny and Chang 2000).

4 Conclusions

In the studied sandy soil of the refinery, higher percentages of PHC degradation were observed at the surface soil layer (0–5-cm depth), despite aerobic conditions (redox potential close or higher of 400 mV) observed down to 20-cm depth (the maximum depth attained in this study). It can be admitted that, probably, the photochemical oxidants, present in sunny days (which are very common in Portugal) in refinery atmosphere (these areas use to be rich in volatile organic compounds and nitrogen dioxide), favoured PHC degradation.

Among the different biostimulation and bioaugmentation treatments tested, only a combination of bioaugmentation with inorganic nutrients and surfactant amendments could enhance PHC degradation relatively to natural attenuation (about 50 vs 35% after a 9-month period of experiment).

Therefore, a cost-effective way to reduce half-life for degradation of PHC of refinery's contaminated soil can be a periodic revolving of the soil, like tillage, in order to expose the different layers of contaminated soil to the atmosphere. A combination of soil revolving with bioaugmentation, together with nutrients and surfactant amendments may result in an additional improvement of PHC degradation rate. However, this last procedure will raise markedly the price of the remediation treatment.

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