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Laboratory-scale bioremediation experiments on hydrocarbon-contaminated soils

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Abstract

Successful application of bioremediation technology to contaminated soil requires knowledge of the characteristics of the site and the parameters that affect the microbial biodegradation of pollutants. Here, we propose a simple protocol for biotreatability assays in two phases. In the first phase of the assays we examined the type and metabolic activity of the indigenous microorganisms at the site, and the presence of possible inhibitors. The biodegradability of contaminants in soil slurries under optimal conditions was also tested. In the second phase several parameters, such as the influence of nutrients and the addition of surfactant and specialized inocula, were evaluated in microcosms with 2.5 kg soil. The application of this protocol to two hydrocarbon-contaminated soils is described. In the first phase of the protocol, the results obtained with the first soil indicated high metabolic activity of indigenous microbial populations and a total petroleum hydrocarbon (TPH) decrease of 46%. Assays of the second soil indicated low indigenous microbial metabolic activity and limited biodegradation of TPH. In the second phase of the protocol, which lasted 360 days, assay of microcosms showed that the first soil responded to several treatments with a large decrease in TPH, while none of the treatments applied to the second soil showed a reduction in TPH. The information obtained from the results in the first phase of the protocol indicates whether a biological treatment of contaminated soil is appropriate. In the second phase of the protocol, we attempted to identify the most appropriate treatment through the evaluation of various conditions and additives.
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Keywords: Treatability assays; Bioremediation; Contaminated soil; Hydrocarbons

1. Introduction

Large quantities of organic and inorganic compounds are released into the environment every year as a result of human activities. Soil contamination is a typical side-effect of industrial activity. Among the technologies available to deal with contaminated soils, bioremediation based on the metabolic activity of microorganisms has certain advantages (Exner, 1994; Klein, 2000).

In general, transformation by microorganisms involves the complete destruction or immobilization of contaminants rather than their transfer from one environmental compartment to another, as occurs in physical–chemical treatments such as extraction or incineration, which in some instances entails the transfer of pollutants from soil to atmosphere. Also, it is cheaper than many other remediation technologies (Ulrici, 2000). Nevertheless, to assess whether a biological treatment of contaminated soil is appropriate, every location relating to its microbial populations and the biodegradability of its contaminants needs to be characterized. In addition, given the diversity of effects of additives such as fertilizers and inocula, it is necessary to evaluate the influence of various factors that may affect the bioremediation process. All this information is obtained through laboratory experiments known as biotreatability or feasibility assays (Cattaneo et al., 1997; Dibble and Bartha, 1979; Zhou and Crawford, 1995). Since such applications are often urgent, efforts should be made to simplify these assays.

We present a simple two-phase protocol for biotreatability assays. The first phase involves microbial characterization based on the enumeration of heterotrophic and hydrocarbon-degrading microorganisms and assessment of their metabolic activity. A Microtox assay as an indicator of the presence of possible inhibitors is also included, as

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is a rapid biodegradability test in slurry flasks. The results obtained in the first phase of the protocol indicate whether the soil is amenable to bioremediation technology. In the second phase, we attempt to identify the most appropriate treatment through the evaluation of various conditions and additives in microcosms of 2.5 kg soil. The application of the protocol to two types of hydrocarbon-contaminated soil is shown and discussed.

2. Materials and methods

2.1. Soil samples

Soil 1 was obtained as a composite sample from an old industrial site near Barcelona (Spain) after soil from four testing samples was mixed. Soil 1 was mainly contaminated with mineral oil. Soil 2 was collected from an abandoned site and prepared in the same way and was mainly contaminated by an unknown petroleum product. The main characteristics of both soils are shown in Table 1.

2.2. Soil analysis

Total organic carbon and nitrogen were measured by combustion, using an NA 2100 Protein Nitrogen Analyzer (Thermoquest CE Instruments, Milan, Italy). Combustion technique calculates all the carbon in a sample. In order to calculate organic carbon, carbonate was eliminated from soil samples by acidification according to the ISO 10694 method. Inorganic nutrients were determined by ion chromatography in a 1:5 (w/w) slurry with double deionized water. Nitrite, nitrate and phosphate were measured in a chromatographic system equipped with a Waters 515 pumping system, a Waters IC-PAK Anions column (Waters Corporate, Milford, USA), a UV/V Kontron model 332 detector (Kontron Instruments, Milan, Italy) and a Wescan conductivity detector (Wedan Instruments, Santa Clara, USA). Ammonium concentration was assessed by the automated phenate method (Standard methods 4500-NH_3 H, American Public Health Association, 1992) in a Technicon Autoanalyzer II (Bran and Luebbe Analyzing Technologies Inc., Elmsford, USA). The pH was measured in a 1:2.5 (w/v) soil:water slurry with a Crison micro pH 2000 meter (Crison, Barcelona, Spain). Conductivity was determined with a Crison conductimeter model 522 in a 1:10 (w/v) soil:water slurry.

2.3. Microbial populations and respirometric analysis

Heterotrophic and degrader populations were enumerated by the most-probable-number (MPN) technique (Wrenn and Venosa, 1996). The heterotrophic microbial population was enumerated on trypticase soy broth. Mineral medium with *n*-hexadecane was used as the sole source of carbon and energy for alkane degraders.

Respirometry experiments were conducted with soil, soil with nutrients nitrogen (N) and phosphorus (P) and soil with nutrients supplemented with glucose (0.2 g), an easily assimilable substrate, in order to assess the real and potential metabolic activity of indigenous microorganisms. A 20 g sample of sieved soil (< 2 mm) was placed in a plastic vial. Field holding capacity was adjusted with water to 60% in all treatments. N and P were added as NH_4Cl (150 mg) and K_2HPO_4 (20 mg). Vials containing soil were placed in closed 1-l glass jars. A glass vial containing 10 ml 0.2 N NaOH was placed in each jar to trap CO_2. The NaOH trap was periodically replaced. $BaCl_2$ (10 ml) was added to the NaOH trap and the amount of CO_2 produced by each microcosm determined by titration with 0.1 N HCl.

2.4. Toxicity assay

The Microtox™ test, which uses the luminescent bacterium *Vibrio fisheri*, was performed, using lyophilised *V. fisheri* cells which were reconstituted for testing. All assays were performed at 15°C after a 30 min contact period. Toxicity results are reported as the effective concentration promoting a 50% (EC_{50}) reduction in light emitted by the bacteria. For both soils, aqueous soil washings (lixiviate), prepared in accordance with the solid phase extraction protocol developed by Microbics Corporation (1992), and an organic extract, as detailed below, were tested.

2.5. Soil slurries

A 12.5-g sample of sieved soil (< 2 mm) was resuspended in 50 ml of deionized water in 250-ml Erlenmeyer flasks. Two flasks were not modified, two were supplemented with N and P (final concentration equivalent to a C:N:P molar ratio of 100:10:1) and a third pair of flasks were used as abiotic controls with the addition of formaldehyde (3%). Slurries were incubated at 25°C and 200 rpm, and the liquid and solid phases were extracted separately. The liquid phase was extracted three times with 20 ml dichloromethane. The solid phase was extracted as described below. Organic extracts were combined to give the extractable organic matter. Total petroleum hydrocarbon fraction (TPH) was obtained by column chromatography as detailed below and was quantified by gravimetry.

2.6. Soil microcosms

For each soil, 12 aluminium trays covered with aluminium foil were prepared as microcosms, each containing 2.5 kg sieved soil (< 6 mm). In all treatments, the water content was adjusted to 60% of the field-holding capacity. This moisture content has been used in several studies and lies within the interval recommended by Dibble and Bartha (1979). Twice a week, the microcosm content was mixed and deionized water added. Five treatments were carried out in

Table 1
Characteristics of soils

	Soil 1	Soil 2
Composition	Sand and gravel 95%, silt and clay 5%	Sand and gravel 99%, silt and clay 1%
TOC (%, w/w)	2.1	5.2
Total N (%, w/w)	0.21	< 0.01
Maximum holding capacity (%)	28.5	13.2
Conductivity (μS)	164	90
pH	7.8	8.2
Nitrate and nitrite (mg kg^{-1})	< 0.1	0.85
Ammonium (mg kg^{-1})	< 0.1	< 0.1
Phosphate (mg kg^{-1})	< 0.1	1
EOM[a] (mg kg^{-1})	23016	31700
TPH (mg kg^{-1})	20970	21840
PAH (mg kg^{-1})	Nd[b]	1987
Heterotrophs (MPN g^{-1})	3.8×10^7	2.9×10^6
Alkane degraders (MPN g^{-1})	1.8×10^6	5.6×10^5

[a]Extractable organic matter.
[b]Not detected.

duplicate trays:

(1) Basic treatment, A: aerated by mixing every week and addition of water to maintain 60% of the field holding capacity. These conditions were applied to all treatments.
(2) Nutrient treatment, N: NH_4NO_3 and K_2HPO_4 were added for the first 30 days to give a final concentration equivalent to a C:N:P molar ration of 100:10:1.
(3) Surfactant treatment, NS: nutrients and 0.85 ml Tween 80 were added.
(4) Inoculated treatment, NI: nutrients and an inoculum to reach 10^8 microorganisms g^{-1} of soil. Consortium TD specializing in gas–oil degradation (Viñas et al., 2002) was inoculated into soil 1 and consortium F1AA specializing in the degradation of degraded crude oil (Viñas et al., 2002) was inoculated into soil 2.
(5) Easily assimilable substrate, NG: nutrients and 5 g glucose were added. As control, two trays of each soil were kept without treatment throughout the experiment. At 270 days of incubation, nutrients of soil 1 were depleted. In consequence, nutrients were added to restore the molar ratio. At the same time the inoculated treatment (NI) was reinoculated.

2.7. Analyses of contaminants

At intervals between days 0 and 360, two composite samples were taken from each microcosm by sampling at various points, combining and mixing. They were sieved (< 2 mm) and dried for 16 h at room temperature. Organic matter was extracted from 10 g of soil. Before the extraction, *o*-terphenyl (0.8 mg) and d_{10}-anthracene (0.4 mg) in acetone solution (1 mg ml^{-1}) were added as surrogate internal standards. The acetone was allowed to evaporate and 10 g anhydrous Na_2SO_4 added and mixed in. This mixture underwent soxhlet extraction with dichloromethane:acetone (1:1) for 24 h. The extract was dehydrated over an Na_2SO_4 column and concentrated to 1 ml by rotary evaporation, after which 0.6 ml extract was used to obtain the aliphatic and aromatic fractions on an aluminium chromatographic column, following the EPA3611 method (US Environmental Protection Agency, 1996). Fractions were concentrated to 1 ml; and 0.6 ml of each was mixed to obtain the TPH fraction. TPH content was determined by gravimetry and by GC-FID analysis from duplicate 0.4-ml samples.

GC-FID analysis was performed in a Trace 2000 series (Thermoquest CE Instruments, Milan, Italy). Prior to analysis, α-androstane (35 μg ml^{-1}) and d_8-naphthalene (200 μg ml^{-1}) were added to each sample as internal standards. Compounds were separated on a DB-5 Column (0.025×300 cm^2 with 0.25 μm film thickness; J&W Scientific, Folsom CA, USA) with helium as a carrier gas (1 ml min^{-1}). The column temperature was held at 60°C for 5 min and increased to 320°C at a rate of 3°C min^{-1}. The final temperature was held for 13 min. The TPH content was calculated from the total peak area compared with that of an aliphatic standard (AccuStandart, New Haven, USA) curve. Light and heavy mineral oil content were calculated from areas with $< C_{28}$ and $\geqslant C_{28}$, respectively.

3. Results and discussion

3.1. Microbial populations and soil respirometry

The heterotrophic microbial population was slightly higher in soil 1 than in soil 2 (3.8×10^7 and 2.9×10^6 cells g^{-1}), but the percentage of hydrocarbon degraders (Table 1) was higher in soil 2 (19.3%) than in soil 1 (4.7%). Nevertheless, there were large differences between the soils in the metabolic activity of indigenous microorganisms, determined as production of CO_2 (Fig. 1). The evolution of cumulative CO_2 in soil 1 indicated a progressive increase

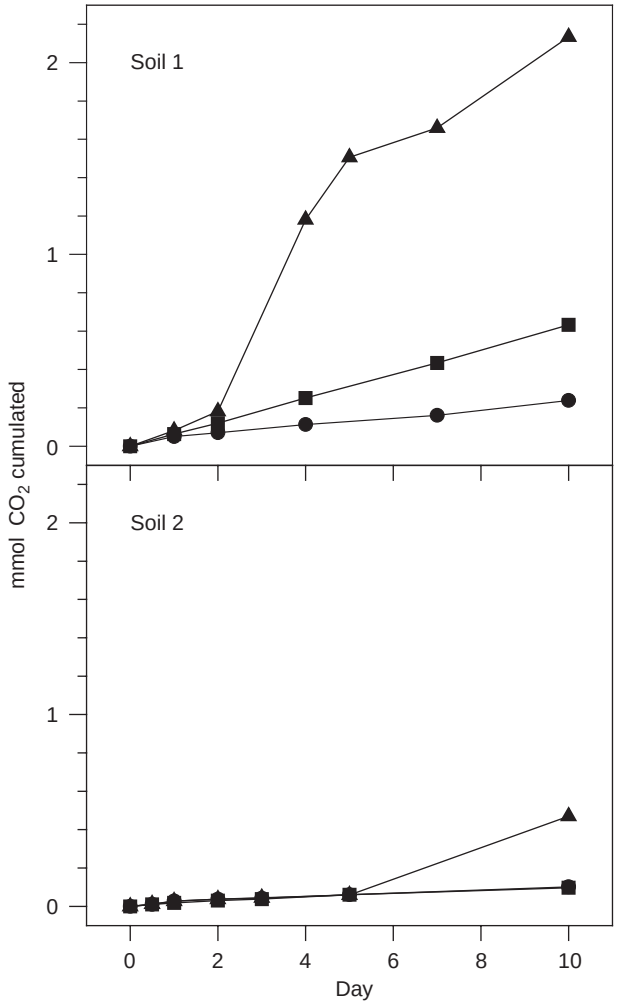

Fig. 1. Compared respirometry of soils 1 and 2. (●) soil; (■) soil and nutrients; and (▲) soil, nutrients and glucose.

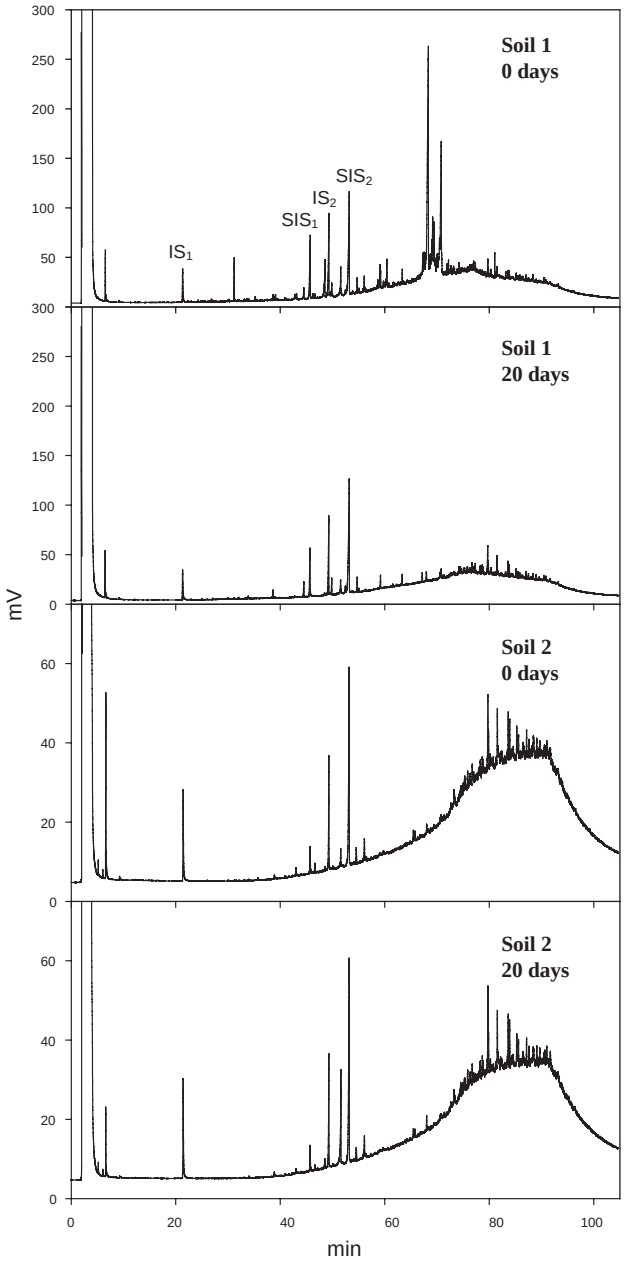

Fig. 2. Chromatograms of TPH content of soil 1 and soil 2 in slurry experiments.

in respiratory activity, especially when nutrients or glucose were added. In contrast, soil 2 showed a null response in the first 5 days, followed by a slight increase when glucose was added. This difference could be attributed to the absence of assimilable sources of carbon and energy or to a presence of toxic compounds in soil 2.

3.2. Microtox assay

The EC_{50} of the total organic extract (EOM) was 0.635 and 8.45 g for soils 1 and 2, indicating that soil 2 was less toxic. However, the EC_{50} of lixiviates was similar for both soils (21.65% and 19.98%). The results indicate that toxins were not responsible for the lower respiratory activity of soil 2.

3.3. Soil slurries

The biodegradability test also revealed a great difference between soils. TPH contents of soil slurries showed no variation when formaldehyde was added. Soil 1 showed a TPH decrease of 46% at 20 days of incubation, while soil 2 only degraded 12% of the initial TPH (Fig. 2). The chromatographic profile of the contaminants present in soil 2 corresponded to a heavy fraction of oil petroleum products, while that of soil 1 corresponded mostly to mineral oils. The high recalcitrance of contaminants of soil 2 could explain the absence of respiratory activity and the scarce biodegradation achieved in the slurry test. The results obtained in the first phase of the protocol, in which the metabolic activity and the biodegradability of contaminants were assayed,

indicated that while soil 1 was suitable for the application of a bioremediation technology, soil 2 was not. Moreover, as long as population numbers do not fall below a defined threshold level, the quantity of microorganisms does not ensure the suitability of a bioremediation process. The same applies for the percentage of degraders in the heterotrophic population. Nevertheless, the treatability assays of the second phase were conducted on both soils to confirm these predictions.

3.4. Soil microcosms

Initial TPH concentration of soil 1 was 20970 mg kg^{-1} as determined by gravimetry (the TPH content of an uncontaminated soil from the same site was 165 mg kg^{-1}) and 11975 mg kg^{-1} as shown by GC-FID using an alkane standard. Mineral oil was the main contaminant (80%), with a concentration of 9521 mg kg^{-1} determined by GC-FID using a mineral oil as standard.

The kinetics observed in the decline of TPH (GC-FID) in soil 1 was analyzed for two periods: the first 60 days and days 60–360 (Fig. 3). Basic treatment (A), without nutrients, showed a delay in the reduction of TPH concentration until the second month, which gradually decreased to 5361 mg kg^{-1} (55%) at 270 days (Table 2). However, microcosms that involved nutrient addition led to a large decrease in TPH in the first month. Since the same reduction was achieved in treatments with nutrients by the end of the first 2 months, it was established that the effect of fertilization was the abolition of the adaptation period. Although the literature contains a wide variety of responses to the addition of nutrients, our findings coincide with those described by other authors (Lewis et al., 1986; Steffensen and Alexander, 1995; Swindoll et al., 1998).

The total reduction of TPH was similar (6000 mg kg^{-1}) in treatments with no addition of nutrients during 270 days of incubation, and in those with addition of nutrients, but in this case for 60 days. The inorganic forms of NO_3^- and NH_4^+ of soil 1 were not detected, and total nitrogen was 0.21% (2100 mg kg^{-1}) of soil (Table 1). Accordingly, the microbial populations of microcosms without nutrients (A) had to use part of total nitrogen as a nitrogen source, probably through slow transformation to assimilable forms and its immediate incorporation to their biomass, and as nitrogen acquired by fixation. In fertilized microcosms, the immediate availability of assimilable forms was translated into a rapid use of hydrocarbons.

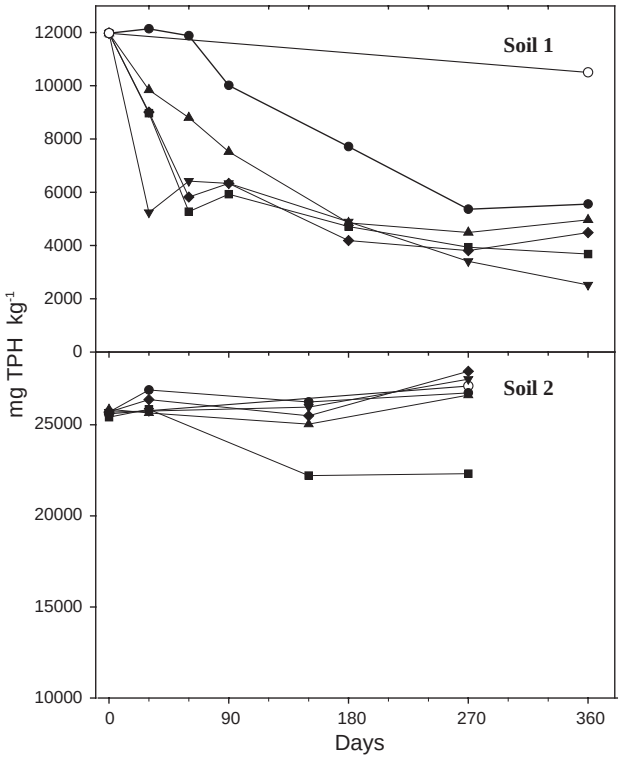

Fig. 3. Evolution of TPH concentration in microcosm experiments. (●) basic; (■) nutrients; (♦) nutrients and inoculum; (▲) nutrients and surfactant; (▼) nutrients and glucose; and (○) control.

The treatment containing nutrients and glucose (NG) was the most efficient. At 270 days, TPH content (GC-FID) was 4.887 g kg^{-1}, which represented a biodegradation of 72%. This increased to 79% at 360 days of incubation, while with the other treatments it stagnated. As described in other studies, the addition of glucose can improve the degradation of contaminants (Hendriksen et al., 1992; Hess et al., 1990). Nevertheless, a negative effect has also been described (Carmichael and Pfaender, 1997; Entry et al., 1993; Fog, 1988).

Table 2
Content of TPH in soil 1 determined by GC-FID (mg TPH kg^{-1})

Sample	Days						
	0	30	60	90	180	270	360
A	11975[a]	12140 (−1)[b]	11878 (1)	10016 (16)	7714 (36)	5361 (55)	5557 (55)
N		8971 (25)	5273 (56)	5928 (51)	4713 (61)	3936 (67)	3680 (54)
NS		9845 (18)	8802 (27)	7521 (37)	4852 (60)	4487 (63)	4962 (59)
NI		9012 (25)	5820 (51)	6333 (47)	4188 (65)	3806 (68)	4485 (63)
NG		5241 (56)	6421 (46)	6334 (47)	4887 (59)	3408 (72)	2518 (79)

[a]Mean of duplicated samples.
[b]Values in parentheses indicate percentage depletion as compared with TPH content at 0 days.

The response of TPH to treatment with surfactant Tween 80 (NS), unlike other treatments, was a gradual reduction, especially in the first 6 months. Possible competition of this product as growth substrate for microbial populations has been cited as an explanation (Liu et al., 1995; Thiem, 1994). In this case, this behavior would contradict the observed effect of glucose. Higher TPH extraction by the solvent due to presence of the surfactant could be another explanation. In relation to bioaugmentation (NI), the inoculation of consortium TD, specializing in gas oil degradation, had no significant effect, as has often been described for this class of contaminant (Venosa et al., 1996; Walter et al., 1997). The natural microbial community, especially in chronic hydrocarbon-contaminated sites such as soil 1, usually degrades oil hydrocarbons if suitable conditions are present (Alexander, 1999).

All treatments with nutrients (N, NS, NI) except the surfactant treatment (NS) had stable values between days 180 and 360. As described in other studies, the degradation pattern of organic chemicals in soil usually shows a rapid initial phase of descent followed by a period of little or no change in concentration. This kinetics is known as the "hockey stick" phenomenon (Alexander, 1999). A depletion of nutrients, a descent of microbial populations, lower bioavailability and higher recalcitrance of residual contaminants explain this kind of dynamic (Heusemann, 1997; Alexander, 1999; Manilal and Alexander, 1991). In our case, the additional supplementation of nutrients and a reinoculation of consortium TD at 270 days had no effect on this kinetics. Moreover, the level of populations at 180 days (10^7–10^8 heterotrophs g^{-1}, and 10^6–10^7 alkane degraders g^{-1} soil) was high enough to account for the absence of biodegradation.

To understand this low plateau of bioremedial activity, we examined the relative content of light and heavy mineral oil during the time course of the experiment in all treatments (Fig. 4). Light mineral oils decreased, while heavy mineral oils were significantly enriched. Therefore, the residual contaminants are more recalcitrant. Song et al. (1990) reported similar results with a residual fraction in a soil after a bioremediation experiment. These results may also account for the descent observed in microbial population at the end of the experiment, especially hydrocarbon degraders (10^4 MPN g^{-1} soil).

In soil 2, the homogenized sample used to fill the microcosms was initially contaminated with 25.7 g TPH kg^{-1} as determined by gravimetry. Except for the basic treatment (A), which decreased TPH concentration slightly, none of the treatments showed a reduction in this parameter throughout the 270 days of incubation (Fig. 3). It should be emphasized that, in this case also, the inoculation of consortium F1AA, specializing in the biodegradation of heavy fractions of petroleum products, had no effect. Microbial consortium F1AA was obtained from enrichment cultures established in mineral medium containing 0.5% (v/v) of the saturated fraction of Cantel Aptiano-Albiano oil, inoculated with a sandy soil contaminated with crude oil. Cantel Aptiano-Albiano is a Cuban crude oil from the Varadero field. A general characteristic of the crude oils from Varadero is that they are highly degraded, with no *n*-alkanes and acyclic isoprenoids in their saturated fraction (Viñas et al., 2002). The absence of response to any of the treatments assayed was consistent with those obtained when evaluating the metabolic activity of indigenous microorganisms and the biodegradability of contaminants in soil 2.

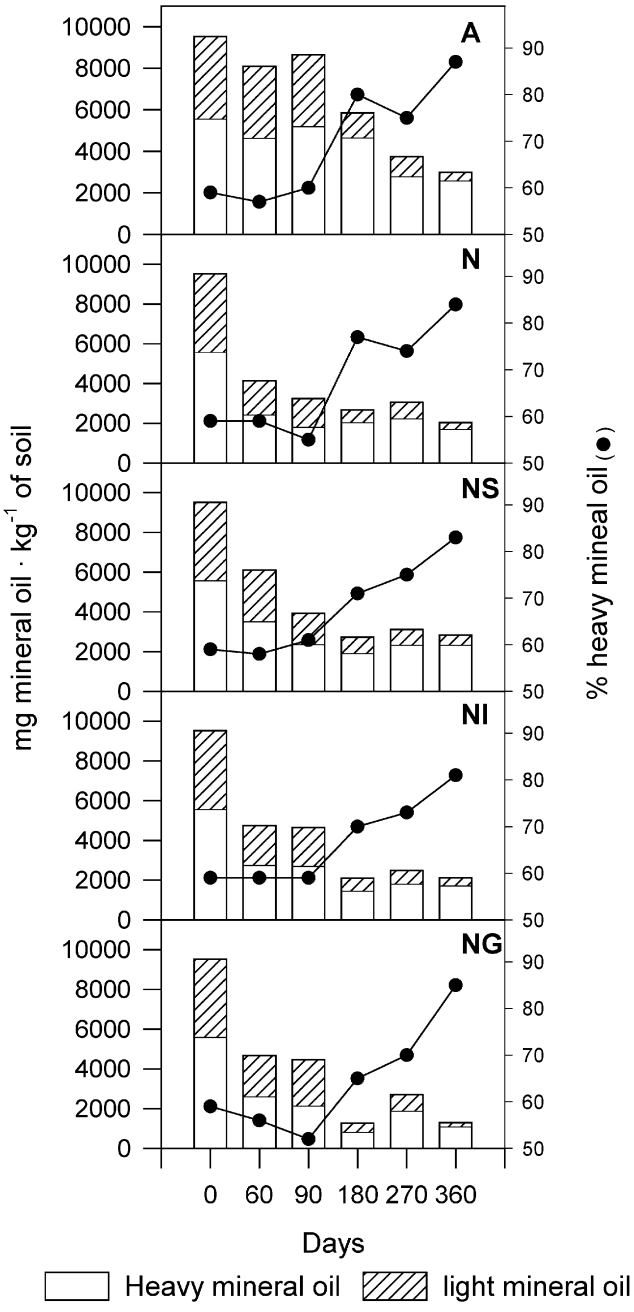

Fig. 4. Concentration of total, heavy mineral oil (white bars) and light mineral oil (grated bars) concentration in soil 1 during incubation in microcosm experiments determined by GC-FID.

Thus, the information obtained in the first phase of treatability assays helps decide whether the application of bioremediation technology to a contaminated soil is appropriate. This phase consists of simple and rapid assays that take, at most, 1 month. If the results recommend the application of bioremediation technology, environmental factors that might restrict the microbial biodegradation of pollutants must be evaluated in the second phase with microcosms. Since the effects of different parameters described in the literature vary, this second phase of assays should be performed for each site. According to the results obtained with microcosms, and given the urgency of decontamination and cost evaluation of each treatment, the type and conditions of bioremediation technology to apply must be decided in each case.

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