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Bioremediation of diesel-contaminated soils: Evaluation of potential *in situ* techniques by study of bacterial degradation

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Abstract

The development of a simple laboratory methodology allows the implementation of *in situ* bioremediation of polluted soils with diesel fuel. In this investigation microbiological and chemical analyses and a suitable bioreactor design, were very useful for suggesting the best ways to improve biodegradation extents in a diesel-enriched soil. Biostimulation with inorganic nitrogen and phosphorus produced the best results in a simple bioreactor, with biodegradation extents higher than 90% after 45 days. Also, the addition of activated sludge from a domestic wastewater plant increased the degradation rate to a great extent. In both cases, microbiological studies showed the presence of *Acinetobacter* sp. degrading most of the hydrocarbons. Simultaneously, a diesel fuel release (approximately 400,000 l) was studied. Samples taken in polluted soil and water revealed that bacteria from the genus *Acinetobacter* were predominant. In plate studies, *Acinetobacter* colonies produced a whitish substance with the characteristics of a biosurfactant. Remarkably, the presence of this product was evident at the field site, both in the riverbanks and in the physical recovery plant. The study of the similarities between laboratory results and the diesel spill site strongly suggested that natural conditions at the field site allowed the implementation of *in situ* bioremediation after physical removal of LNAPL (light nonaqueous-phase liquids).

Introduction

Petroleum hydrocarbons, although not xenobiotics, are due to their large-scale use as one of the main potential sources of environmental contamination (Rosenberg & Ron 1996; Head & Swannell 1999). Diesel fuel is a complex mixture of normal, branched and cyclic alkanes, and aromatic compounds obtained from the middle-distillate, gas-oil fraction during petroleum separation (WHO 1996). Soil and groundwater are often contaminated with gasoline or diesel fuel from leaking underground storage tanks and because of accidental spills and leakage from pipelines. Due to their mobility, these compounds may cause considerable damage not only in soils but also in water intakes or groundwater reservoirs. In the last few years various new regulations have been introduced to control the environmental risks and, at the same time, biotechnological research focussed on the remediation of organic polluted land has rapidly increased. As a consequence, cost-effective bioremediation techniques have been developed during this period, especially to clean up diesel- and gas-polluted sites (King et al. 1997; Head & Swannell 1999; Atlas & Unterman 1999; Piehler et al. 1999; Hughes et al. 2000).

Bioremediation may be defined as the use of biodegradative processes (usually by means of microorganisms) to clean up soils and waters polluted by hazardous substances. *In situ* treatments, including natural attenuation – defined as *in situ* attenuation processes that reduce the hazard associated with the release of contaminant (Soo Cho et al. 1997), biostimulation and bioaugmentation, usually avoid excavation and emission control costs, and may therefore be considered

as cheap and clean technologies. In order to demonstrate the feasibility of a bioremediation technology, laboratory tests, which reproduce real environmental conditions as closely as possible, are necessary (Kerr 1994; Skladany & Baker 1994; Atlas & Bartha 1998). These experiments help to establish the usefulness of potential bioremediation treatments and complement field experiences.

The objectives of this work were to analyse the best laboratory methodology capable to stimulate, in the most simple way, the degradation of diesel fuel in a laboratory-contaminated soil. For the design of the experimental conditions authors have consulted previously reported experiences (Song et al. 1990; Walworth & Reynolds 1995; Hunt et al. 1997; Taylor & Viraraghavan 1999). The parameters to be modified and the experimental approaches to be tested were chosen focusing on a further implementation in a diesel-polluted site. Moreover, in order to find coincidences between the two experimental situations, a study of the microbial activity and populations, both in lab experiments and in a real polluted site, was carried out. The similarity of the results, specially in what refers to microbial activity and variety between the lab experiments and the diesel-polluted site supports the idea that the methods developed in the lab studies could be used in polluted sites to improve and rationalise bioremediation treatments.

Materials and methods: Laboratory experiments

Design of bioreactor

A natural sandy soil obtained near the city of Oviedo (Spain) was contaminated in the laboratory with commercial diesel (6,000 mg/kg) and then placed in a set of bioreactors (borosilicate glass recipients of 3,000 cm^3, 40 cm long $\times$ 25 cm wide $\times$ 3 cm high, each containing 2.5 kg of soil). Soils were tilled daily in order to achieve a good aeration. Bioreactors were placed in a room with natural light, with 10 °C temperature fluctuations ranging from 15–25 °C. The original soil moisture was periodically recovered with the addition of 300 ml of distilled and sterilised water after weighing the bioreactors to measure water losses. This simple way of water replacement was chosen to avoid the need for an exhaustive control of moisture and thus achieve more practical conditions.

The model shown in Figure 1 summarizes the overall processes related to the bioremediation that conceivably took place in the bioreactors. These parameters enabled us to establish experimental models suitable for further analysis. These conditions are detailed below.

Soil and sludge analysis

Although an approximate knowledge of the presence of appropriate bacteria and soil contents in nutrients could be enough to predict the natural capabilities of any soil to biodegrade pollutants, we also assumed that other chemical and mineralogical properties (clay contents, presence of heavy metals, organic matter, and others) might play an essential role. In this investigation, some of these parameters were determined applying X-ray techniques (difractometry).

Chemical analysis of the soil used for the bioreactors was carried out in order to select the appropriate nutrients to be added. USEPA methods 3050 (hot plate, HNO_3-HCl, totally recoverable) were applied to digest the samples. Then, multi-elemental analyses of soil samples were performed by ICP-AES (Inductively Couple Plasma and Atomic Emission Spectrometry). Total S, C, H and N were quantified using a CHN-600 Leco equipment (ASTM D4239 and ASTM D3178). Periodically, pH and redox potential were also measured (method ISO 10390, "Soil quantity determination of pH") to follow the evolution of these parameters with respect to the original soil samples: pH = 8 and Eh = 100 mv. The analysis of the sewage sludge used in biostimulation experiments (C, H, N, and S) was carried out as specified above.

Experimental conditions analysed in the bioreactor

These are summarized in Table 1 and detailed below.

(a) Control experiment. This consisted of sterilised soil soaked with contaminant (commercial diesel fuel, 6,000 mg/kg as in all the experiments). The objective was the measurement of the contaminant degradation without the participation of biological processes. In addition, this sort of experiment could allow the determination of those compounds recalcitrant to degradation by comparison of results with the other experiments.

(b) Attenuation (intrinsic bioremediation). This second experiment used just natural soil soaked with contaminant. The objective of this test was the evaluation of the soil's natural ability to degrade the contaminant.

(c) Biostimulation. The aim of these experiments was the improvement of the biodegradation rate

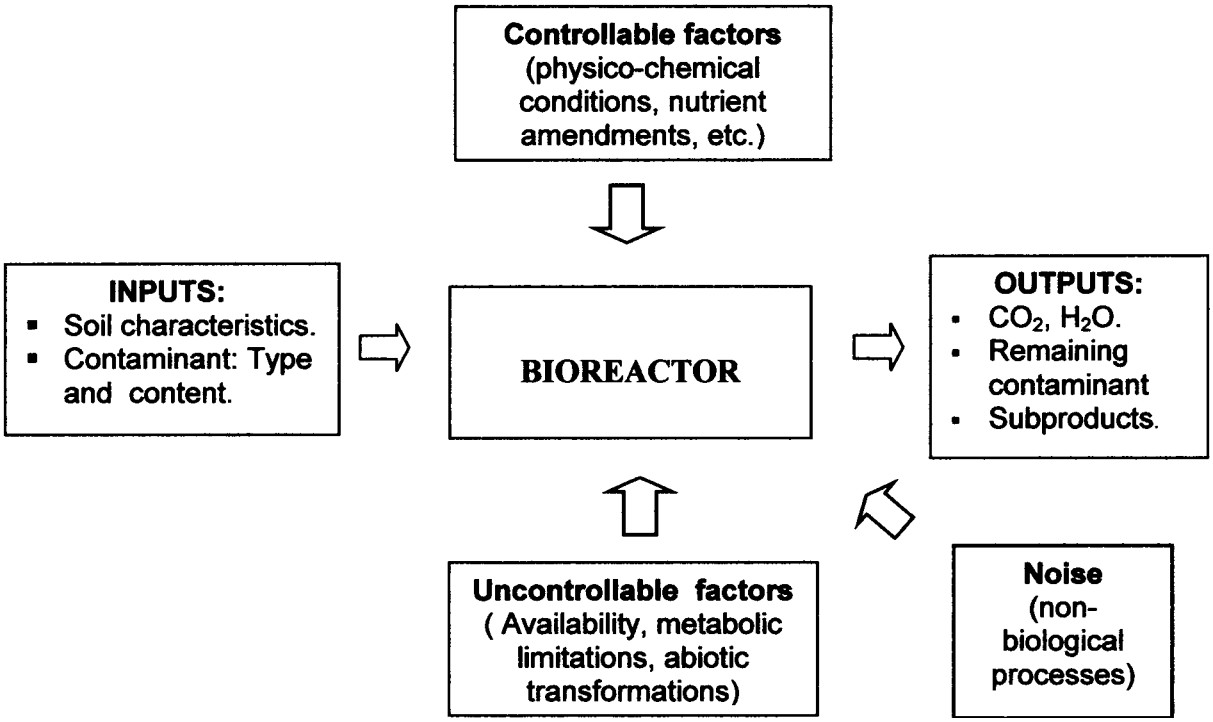

Figure 1. Bioreactor model and processes (see text).

obtained by intrinsic bioremediation. Thus, natural soil was supplemented by adding ammonium nitrate (NH_4NO_3), potassium and sodium phosphates (KH_2PO_4 and Na_2HPO_4) and magnesium sulphate ($MgSO_4{\cdot}7H_2O$), as specified in Table 1. In other bioreactors, sterile domestic sewage sludge was used to fertilise soils, because of its high contents of organic matter, nitrogen and other nutrients such as S and P (Table 1). The sludge was sterilised by autoclaving to avoid the effects of its microbiological contents (see below) whereas its nutritional properties were preserved.

(d) Bioaugmentation: The same sludge – without sterilisation – used in the preceding experiment was used in this final experiment.

Chemical and microbiological analyses

Experiments were run for 45 days, and soils were sampled every fifteen days (five samples were taken simultaneously from bioreactors). Composite samples were obtained by mixing 1 cm × 1 cm × 3 cm cores extracted from different parts of the bioreactor. These soil samples were then subdivided and finally placed in sterile bottles for chemical and microbial analyses. To evaluate the presence of hydrocarbons, chromatographic techniques were used: 24 hours Soxhlet extraction with dichloromethane and ethanol (1 : 1) was performed, then the extracts were filtered and purified. Finally, the extracts were injected into a HP5890A gas chromatograph connected to a Flame Ionisation Detector (FID). To obtain the total content of every hydrocarbon corresponding with the peaks, we referred the areas to the mass of the original samples (standard normalisation method). The chromatographic analysis of the diesel fuel added to the soil is shown in Figure 2a.

Bacterial cell number was estimated by plate-count on two types of growth medium. The first was a rich complex medium designed for growing most chemoorganotroph bacteria. The composition of this medium was 3% TSB (Tryptic Soy Broth), 0.05% yeast extract, 1.5% agar. The second medium was a synthetic one in which the contaminant (commercial diesel) was the sole carbon source. The composition was 0.13% NH_4NO_3, 0.05% $MgSO_4{\cdot}7H_2O$, 0.02% $CaCl_2{\cdot}2H_2O$, 0.5% KH_2PO_4, 0.5% K_2HPO_4, 0.2% diesel. Plate-count in both cases was performed as follows: samples of 1 g of soil were added to 10 ml of a 0.1% $Na_2HPO_4{\cdot}12H_2O$ solution (Fredrickson & Balkwill 1998) and vortexed vigorously. After appropriate

Table 1. Summary of experimental conditions used in the bioreactors. The common conditions for all the soil samples were: pH = 8; Eh = 100 mV; T = 15–25 °C; 6,000 mg/kg of Diesel fuel; soil moisture (25%); aeration: regular tilling.

Experiment	Amendments
Control experiment	Heat-sterilised soil
Natural attenuation	None
Biostimulation	1. 400 ml of nutrients added to a solution containing: NH_4NO_3 (0.2%), KH_2PO_4 (0.07%), Na_2HPO_4 (0.18%) and $MgSO_4 \cdot 7H_2O$ (0.05%).
	2. 250 ml of sterilised sludge – 65% of water content – with the following composition (percentage of dry matter): 25% C, 4% H, 4% N and 0.5% S; hydrocarbon content negligible.
Bioaugmentation	250 ml of the sewage active sludge used above.

serial dilutions, 0.1 ml of the suspension were spread over the surface of duplicate agar plates with the above media and incubated at 30 °C for 72 h. The estimation of the proportion of bacteria capable of growing on diesel plates was obtained dividing the total account of colonies obtained in the two media, respectively.

Bacterial identification

The bacterial colonies obtained in the diesel plates as described above were reisolated in the plate-count medium and MacConkey agar. Those isolated were verified by direct microscopic observations with a Nikon phase-contrast microscope and by using a series of morphological-biochemical tests (von Graevenitz et al. 1995). Several of the colonies were gram-negative, pleomorphic rods or diplococci, non motile, nitrate reductase negative, catalase positive and cytochrome oxidase negative. Otherwise, as was the case of the further identified *Pseudomonas stutzeri*, were gram-negative rods, catalase positive and oxidase positive. The isolates were identified with the API 20NE strips (bioMerieux, France) with the following code number: *Acinetobacter lwoffii* (0000010), *A. haemolyticus* (0000051) and *P. stutzeri* (1044655). We also carried out an antimicrobial susceptibility test by two methods: disk method (Bauer et al. 1966) and ATB (bioMerieux, France). The isolates were sensitive to all antibiotics for gram-negative rod bacteria, with the exception of fosfomycin and cephalothin.

Field site study

These took place in a rural area of Spain where a large diesel spill covered a wide strip of land including a small stream and some agricultural zones. A wide layer of free product (LNAPL) was removed by pumping the oily water and then treating it with physical methods (density separators). However, physical separators were affected by different complications due to bacterial growth (production of foams, pumps obstruction, and others) and thus the authors were required to make chemical and microbiological studies in order to evaluate the possibility of applying *in situ* bioremediation to clean up the site. To perform these analyses, water and soil samples were taken under sterile conditions from both contaminated and uncontaminated environments and from the physical separators.

The same microbiological techniques previously used in laboratory experiments were applied in this field experience. Although plate counts for total chemoorganotrophs only provide a moderate representation of *in situ* conditions (Heitzer & Sayler 1993), selective plate counts yield counts of specific catabolic phenotypes and, therefore, plate count techniques can be used for field demonstrations (Riser-Roberts 1998).

Results

Bioreactor experiments: Biodegradation extents and microbiological analysis

X-ray studies revealed that the soil used in lab experiences was mainly composed of quartz, calcite and traces of illite and kaolinite. Carbon particles and organic matter were also detected. With this soil makeup, hydrocarbons should be adsorbed in organic particles (Lyman et al. 1990; Alexander 1999). On the other hand, as shown in Table 2, the contents of elements such as arsenic, mercury and other toxic heavy metals were low and so should not affect the growth of the natural soil bacteria. Bacteria nutrient requirements include carbon, nitrogen, and phosphorus;

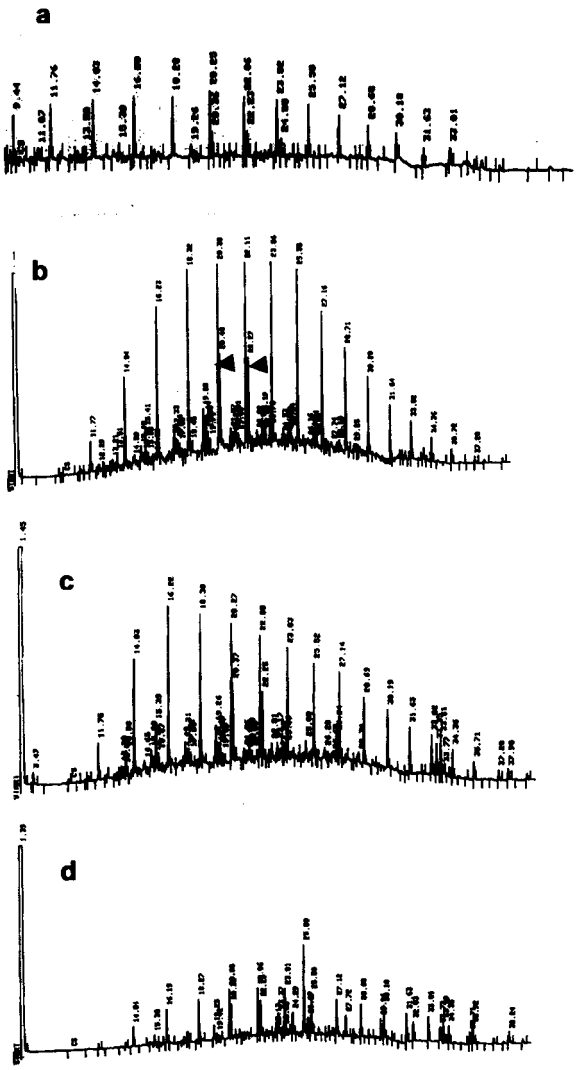

Figure 2. Chromatographic analysis of the soil samples from the sludge biostimulation experiment. (a) Diesel fuel chromatogram. Note that the scale is different because sample dilution was necessary; (b) sample taken at the beginning of the experiment; (c) sample taken after 20 days; (d) sample taken after 45 days. Arrows show the reference peaks corresponding to the hydrocarbons pristane and phytane.

in this case, carbon would be supplied by the hydrocarbons themselves. Nitrogen was present in the soil but we were unaware if its chemical forms, i.e., *N*-species in the soil, would be available to microorganisms. Added to this, the background *P*-content was negligible. As an initial conclusion, this natural soil did not provide good conditions for the attenuation of the effects of diesel pollution and some stimulation could/may be needed.

As explained in Materials and Methods, chromatographic analyses were made to evaluate diesel degradation in the bioreactors. Moreover, we were able to determine the different degradation behaviours of diesel compounds (Figure 2). Apparently, lighter hydrocarbons were degraded at a slower rate than heavier ones after 20 days (Figure 2c). However, a more feasible explanation would be that the amount of lighter hydrocarbons initially grown as a consequence of the degradation of the heavier ones and not by slower degradation rates. On the other hand, linear alkanes were degraded first and with higher yields in all batch experiments. As a result of the sequential degradation of the constituent hydrocarbons, at the end of the experiment (45 days) all of these were degraded in order of their expected decreasing susceptibility: *n*-alkanes, isoalkanes (branched) and cycloalkanes (Figure 2d). Even biomarkers such as pristane and phytane were strongly biodegraded.

A summary of the biodegradation results is presented in Table 3. It is clear that the best scores were obtained when biostimulation experiments were carried out. In this context, the addition of inorganic nitrogen, phosphorus and magnesium, resulted in a notably higher efficiency of degradation (more than 90% of diesel degraded after 45 days) than the addition of sludge as a nutrient source (66% degraded). The microbiological analysis of the samples (Table 4) showed that the proportion of specialised microorganisms (those that grow on the diesel plates; see Methods) is higher in the soil samples supplemented with inorganic nutrients, and this corresponds with the biodegradation rates obtained in Table 3. The same correlation was obtained with the samples in which sludge biostimulation was carried out. Overall, these data pointed out that the development of bacteria capable of performing an effective biodegradation, as the direct cause of the bioremediation rates obtained. Finally, the analysis of the bacterial populations in the different soil samples showed the presence of *Acinetobacter* sp. (mainly *A. haemolyticus* and *A. baumanni*) as the dominant genus capable of growing on the diesel-synthetic medium plates.

Microbiological analysis of the diesel polluted site

Initial measurements of pH, redox and conductivity measurements of the contaminated soil showed, on average, typical values corresponding to an oil-polluted site, i.e., a reducing environment. In addition, the soil porosity was high and thus the contamination of the

Table 2. ICP-AES and Leco analysis of the natural soil elements. Values refer to percentage of dry weight.

Element	Cu	Pb	Zn	Ni	Co	Mn	As	U	Sb	V	Cr	Ba	Hg	
ppm	8	11	30	14	8	577	18	<8	<3	65	115	115	<5	
Element	Fe		Ca		Mg		Ti		Al		Na		P	K
%	1.84		2.54		0.23		<0.01		1.82		0.01		0.06	0.19
Element	C				H				N				S	
%	4.88				0.83				0.32				<0.01	

Table 3. Results of biodegradation after 45 days. Normalized data obtained from the chromatographic analysis (see Methods).

Experiment	Non-biological degradation[a] (%)	Biological degradation[b]	Global rate contaminant disappearance[c] (%)
Control	11	–	11
Natural attenuation	11	24	35
Biostimulation with nutrients	11	>80	>90
Biostimulation with sludge	11	55	66
Bioaugmentation	11	33	44

[a] Defined as the disappearance of diesel compounds in the absence of biological activity (control).
[b] Defined as the disappearance of diesel compounds due to biological activity.
[c] Defined as the total degradation.

aquifer was evident. Similar to that of the laboratory experiments described above, samples were prepared in order to evaluate the rate of specialised microorganisms. Samples were taken from natural soil and water respectively and from different points of the separation plant. The results are summarised in Table 5. As can be appreciated, the proportion of biodegradative bacteria increased notably at the end of the cleaning process.

However, in the natural polluted environment a population of more than 10^7 hydrocarbon-degrading microorganisms was found, with an average rate of specialised microorganisms of 26%. Furthermore, *in situ* determinations showed that N and P contents in soil and water were relevant and dissolved oxygen levels were not high; in addition, average temperatures throughout the year are very high (more than 20 °C). Taken together, these data indicated that there were suitable conditions for natural attenuation and/or biostimulation.

A great variety of microorganisms such as bacteria, filamentous bacteria and fungi were observed in all the samples. Finally, it was possible to identify the bacteria that were dominant on the diesel plates: *Acinetobacter lwoffii* was present in samples A, B, C and D, *A. haemolyticus* in samples A, B and C and *Pseudomonas stutzeri* in samples C and D (Table 5, Figure 3). When diesel plates with the isolated bacteria were left to stand for one week at room temperature, it was possible to observe the presence of a whitish emulsion around the colonies of *Acinetobacter lwoffii* (Figure 4), which probably corresponded to a biosurfactant. This substance clearly contributed to the formation of the foams that covered the stream and riverbanks at the field site; however, a detailed analysis of its composition was beyond the scope of this investigation.

Discussion

Petroleum-contaminated soils and aquifers represent more than half the sites subjected to bioremediation (Rosenberg & Ron 1996; Atlas & Unterman 1999). Although most of the constituents of spilled oil are degradable (reviewed in Head & Swannell 1999), the overall rates of hydrocarbon degradation, as with those of xenobiotic compounds in general, are limited by temperature, water content, oxygen, pH (hydrocarbons are mineralised most rapidly at 6.5–8.0) and inorganic nutrients, because petroleum contaminants are deficient in nitrogen and phosphorus (Providenti et al. 1993; Baker 1994). Other variables that influence the rate of degradation are cometabolic require-

Table 4. Plate-count results of the diesel-degrading bacteria in the different soil samples.

Sample	Days elapsed after experiment initiation	Total cell number per gr. (TSB medium)	Cell number per gr. (synthetic medium)	Rate of specialised microorganisms (%)
Natural soil	0	9.10×10^6	9.80×10^5	10.7
Natural attenuation	45	1.15×10^6	1.50×10^5	13.1
Biostimulation with nutrients	45	1.51×10^7	1.03×10^7	68.4
Biostimulation with sludge	45	1.22×10^6	4.30×10^5	35.0
Bioaugmentation	20	9.21×10^7	4.91×10^6	5.4
	45	1.33×10^6	3.99×10^5	30.0

Table 5. Microbiological analysis of the contaminated field samples and separators (see text).

Group of Samples	Comments	Total cell number per ml (TSB medium)	Cell number per ml (synthetic medium	Average rate of specialised microorganisms[a] (%)
A	Soil and water samples taken from the natural polluted environment	3.45×10^7	9.00×10^6	26
B	Polluted water samples after pumping (dilution effect)	1.21×10^4	2.43×10^3	2
C	Oily water samples after decantation of pumped water.	1.10×10^6	0.79×10^6	72
D	Oily water samples with foams collected from the physical separator	3.50×10^7	3.45×10^7	>95

[a]See Materials and Methods.

ments, contaminant availability or abiotic transformations (Figure 1). Laboratory studies are easier and less costly to perform than field studies and with adequate interpretation still provide valuable information for field assessments and applications (Bossert & Kosson 1997). In our work, an unsophisticated soil experimental bioreactor was designed to simulate conditions in all the batch experiments modelling a hypothetical situation in the field. With this purpose, measures were limited to control pH, redox potential, temperature and moisture. To evaluate non-biological degradation and other uncontrollable factors adequate control experiments were introduced. Non-biological processes could cause an apparent disappearance of the pollutant, thus mimicking the biodegradative activity (Shannon & Unterman 1993). Some of these processes are related to the soil characteristics mentioned above, as for example, the catalytic participation of inorganic and organic colloids in the alteration of the organic contaminant (Morra 1996). In the context of this work, non-biological processes related to volatilisation and photo-oxidation are also important (Swannell et al. 1996; Atlas & Bartha 1998) especially in what refers to the field site because of high temperatures.

Given the low supply of available carbon normally present in soil, nitrogen and phosphorus are not usually limiting in soil (Alexander 1999). However, the situation changes in the presence of an organic pollutant, as for example, at the interface between the potentially degradable compound and the surrounding en-

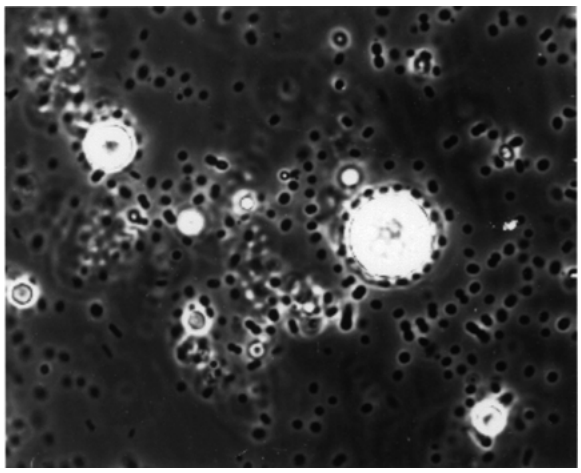

Figure 3. Acinetobacter sp. Surrounding diesel droplets (phase-contrast observation, 1000×).

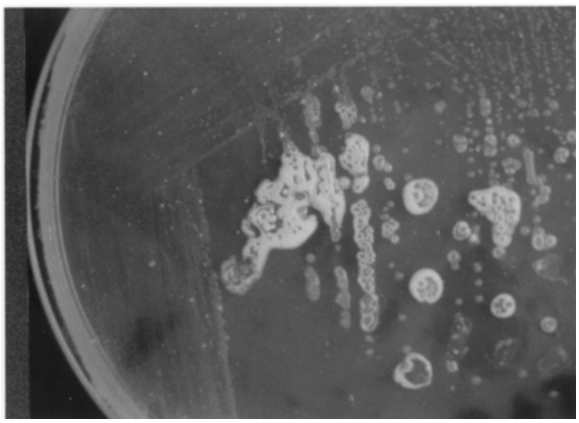

Figure 4. Agar-plates with *Acinetobacter lwoffi* growing in diesel-synthetic medium. The biosurfactant appears as a white emulsion surrounding the colonies.

vironment (Alexander 1999). This is clearly shown in our biostimulation experiments. Chromatographic determinations of the hydrocarbon components showed the highest level of diesel degradation when the soil was supplied with inorganic nitrogen and phosphorus (Table 3) and to a lesser degree when domestic sewage sludge was added. As shown in our bioreactor experiments and in batch cultures (Geerdink et al. 1996), diesel can be completely mineralized in different experimental conditions, mainly concerning the system utilized (bioreactor or batch), temperature, humidity and the origin of the microbial. From a chemical point of view, the results shown in Figure 2 suggest that linear alkanes are degraded first, but without significant differences between lighter and heavier ones. Linear alkanes produce a higher yield of biomass than branched ones and thus our results also coincide with the biodegradation rates of diesel components reported in batch cultures (Geerdink et al. 1996).

Attenuation of natural organic compounds such as those present in hydrocarbon fuels is predictable (Table 3), because the responsible microorganisms are ubiquitous in soil (Rosenberg & Ron 1996; Head & Swannell 1999). Therefore the process could be self-sustaining and limited only by the absence of electron acceptors or inorganic nutrients (Spain 1997, Atlas & Bartha 1998; reviews in Head & Swannell 1999 and in Atlas & Unterman 1999). The beneficial effect of fertilisation with these compounds has been conclusively demonstrated in marine and terrestrial oil spills, including gasoline-contaminated ground water and oil-polluted refinery sites (Baker 1994; Walworth & Reynolds 1995; Rosenberg & Ron 1996; Swannell et al. 1996; Alexander 1999). Thus, oleophilic nitrogen and phosphorus fertilisers have been developed in the form of microemulsions and applied to contaminated land and water (Rosenberg & Ron, 1996; Swannell et al. 1996; Atlas & Unterman 1999). Application of domestic sewage sludge as a potential source of nutrients is an interesting possibility, as this material is readily available from activated sludge domestic wastewater plants. In the present work, the results of biostimulation using sludge were not as good as those obtained with inorganic nutrients (Table 3). This could be explained by the significant amount of organic compounds present in the sludge (Table 1), which constitute a second, alternative choice of carbon source for microorganisms. Another possible explanation is the presence of less readily metabolizable nitrogen sources. Although the amount of total N in the sludge is high (4% in dry matter, Table 1), this is likely present mainly in organic form, because most of the soluble inorganic compounds would have been already used by the sludge microorganisms. Also, some kind of interference could appear related to hydrocarbon contents of sludge but further analysis showed that the amount of hydrocarbons in the sludge used in lab experiments was negligible. In any case, the addition of the sludge still doubled the biodegradation rate obtained in natural attenuation experiments.

Bioaugmentation (i.e., seeding with pollutant-degrading bacteria usually obtained after enrichment culture) can be used to increase the biodegradative capabilities of the indigenous microbial populations (Walter 1997; Atlas & Unterman 1999). However, the success of this approach in soils is scarce/little, as in these habitats the microorganisms must, in order

to be effective in contaminant biodegradation, move or be transported to the contaminated zone, adhere and survive against predators in an alien matrix, compete for limiting nutrients and grow and maintain their degradative capabilities (Alexander 1999). Thus, the survival of introduced bacteria is low and in general, the cost/effectiveness relation of this approach (when commercial products have to be used) would condition its utilisation. This is exemplified by the results obtained in our work when "live" sludge was added to the soil. This produced an increase over the attenuation experiment of about 10% of the rate of contaminant disappearance. However, the values were 20% lower than those obtained when the sterile sludge was added in the biostimulation experiments. This could be explained by a competition phenomenon between the indigenous microbial population in soil and the population held in the sludge, which depleted the samples from available nutrients and also delayed the development and selection of the adapted populations of degradative microorganisms (Table 4). An alternative approach would be the previous isolation of adapted microorganisms, from soil samples taken in older polluted sites. Bioaugmentation would be useful mainly against highly recalcitrant chemicals or when intrinsic bioremediation or biostimulation does not work because of insufficient or inadequate bacterial populations (Atlas & Unterman, 1999). It would also be useful when the concentration of the contaminant, or the environment is toxic for the indigenous microorganisms (Walter 1997).

Remarkably, members of the genus *Acinetobacter* were the bacteria predominant both in lab experiments after biostimulation and in samples taken at the polluted site. This suggests that the ability of this genus to degrade hydrocarbons is very important. In fact, it has been reported that they are ubiquitous in nature and are able to use a wide variety of carbon sources, including crude oil and fuels, aromatic hydrocarbons and dioxin-related compounds, (Towner et al. 1991; Cerniglia 1992; Amund 1996; Lal & Khanna 1996; Halden & Dwyer 1997; Alexander 1999; Yuste et al. 2000). Additional laboratory experiments (J.L. Gallego and J. Sánchez unpublished data) carried out in our lab have confirmed that *Acinetobacter* sp. quickly degrades crude oil and diesel, and compete very efficiently with other known hydrocarbon-degrading genus such as *Pseudomonas* sp.

Limited availability of the contaminant is a major factor that affects biodegradation (Providenti et al. 1993; Baker 1994; Head 1998). In particular, the low aqueous solubility of hydrocarbons in water makes it essential for bacteria to be adhered to the hydrocarbon/water interface via hydrophobic interactions, as the hydrocarbon oxygenases involved in the first step of hydrocarbon degradation, are always membrane-bound enzymes (Rosenberg & Ron 1996). Once the bacteria are adhered to the surface of the hydrocarbon they begin to multiply (Figure 3) and thus the growth can become limited by the surface available. Consequently, hydrocarbon-degrading microorganisms produce a variety of surface-active agents, including the *Acinetobacter* strains isolated from the polluted field (Figure 4). These compounds reduce surface and interface tensions in both aqueous solutions and hydrocarbon mixtures (Desay & Banat 1997). If the bacteria can split the oil droplets (emulsification), new surfaces become available for growth. Another important role of emulsifiers is to desorb from the oil droplets once these become depleted from the particular compounds used by the bacterium – as occurs with the crude-oil *n*-alkanes used by emulsifier-producer *Acinetobacter calcoaceticus* (Rosenberg & Ron 1996). At a chemical level, biosurfactants produced by *Acinetobacter* sp. and *Pseudomonas* sp. are polymeric molecules and rhamnolipids, respectively (reviewed in Desai & Banat 1997; Vardar-Sukan & Kosaric 2000). In the polluted site studied, biosurfactants contributed to the formation of foams that covered the stream and riverbanks and made the recovery of the spilled diesel using physical methods very difficult but, at the same time, this situation revealed that a natural attenuation process was working properly.

The results of diesel biodegradation in the soil bioreactors showed that it is possible to obtain conclusive results in a bioremediation project in about seven or eight weeks. Thus, it would be advisable to develop this approach as a previous step to the implementation of other standard and more expensive treatments (bioventing, sparging or similar) in a polluted site. Nitrogen and phosphorus biostimulation of naturally selected hydrocarbon-degrading bacteria (such as *Acinetobacter* and *Pseudomonas*, isolated in our work) seems the best choice considering cost and performance. On the other hand, the biosurfactants produced by the isolated soil bacteria could be potentially useful for bioremediation applications (although no general conclusion has been obtained from the literature; see Volkering et al. 1998). However, in the context of the physical treatment used to clean the contaminated soils, these substances very often cause several problems related to pump obstruction

and foams. Concretely, in our field site study, an integrated management was proposed: firstly, the physical removal of as much diesel fuel as possible; and then, a tilling and fertilising biostimulation treatment aimed at enforcing natural attenuation in soils. N and P should be carefully monitored to avoid very high contents and, probably, just tillage would have been enough to obtain some good results. In this context, lower costs would be achieved with the application of cheaper disposable fertiliser, i.e. domestic sewage sludge. Moreover, since the spill was recent, the soil contamination was limited to the top two meters (water table was shallow) and therefore, it was decided that all soils should be treated *in situ*. Finally, in order to remove biologically originated foams and aggregates from separation equipment, some chemical or even microbiological options could be considered.

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