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The role of indigenous bacterial and fungal soil populations in the biodegradation of crude oil in a desert soil

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Abstract

The biodegradation capacity of indigenous microbial populations was examined in a desert soil contaminated with crude oil. To evaluate biodegradation, soil samples supplemented with 5, 10 or 20% (w/w) of crude oil were incubated for 90 days at 30 °C. The effect of augmentation of the soil with vermiculite (50% v/v) as a bulking agent providing increased surface/volume ratio and improved soil aeration was also tested. Maximal biodegradation (91%) was obtained in soil containing the highest concentration of crude oil (20%) and supplemented with vermiculite; only 74% of the oil was degraded in samples containing the same level of crude oil but lacking vermiculite. Gas chromatograms of distilled fractions of crude oil extracted from the soil before and after incubation demonstrated that most of the light and part of the intermediate weight fractions initially present in the oil extracts could not be detected after incubation. Monitoring of microbial population densities revealed an initial decline in bacterial viable counts after exposure to oil, presumably as a result of the crude oil's toxicity. This decline was followed by a steep recovery in microbial population density, then by a moderate increase that persisted until the end of incubation. By contrast, the inhibitory effect of crude oil on the fungal population was minimal. Furthermore, the overall increased growth response of the fungal population, at all three levels of contamination, was about one order of magnitude higher than that of the bacterial population.

Introduction

Pollution of soils by crude oil poses a grave threat to the environment, as oil is toxic to terrestrial wildlife and can penetrate and contaminate groundwater aquifers (Rahman et al. 2002). Bioremediation – a treatment technology aiming at enhancing the natural biodegradation of pollutants – has long been accepted as one of the primary measures by which petroleum and other hydrocarbons may be eliminated from the environment (Atlas 1984). In recent years, there has been increasing interest in the development of economically feasible techniques for *in situ* bioremediation of oil-contaminated soils (Joergensen et al. 1995; Moeller et al. 1996). Since crude oil comprises many types of alkanes and polycyclic aromatic hydrocarbons, of which only some are soluble, a range of different oxygenases is required to degrade its diverse components. It is unlikely that a single microbial strain would feature the required array of enzymes, and in fact the efficiency of oil biodegradation exerted by indigenous microbial consortia has been found to be higher than that obtained with a single microbial isolate introduced into the soil (Rosenberg et al. 1998; Straube et al. 1999). Such autochtonous microbial consortia usually exhibit better adaptation to the physicochemical and environmental conditions specific to the different polluted habitats (Korda et al. 1997).

For these reasons, bioremediation measures usually rely on indigenous microorganisms.

A wide range of factors can affect the rate and extent of *in situ* bioremediation of oil-contaminated soil, among them environmental conditions (mainly moisture and aeration) and the availability of nutrients (nitrogen and phosphorus). Soil aeration may be problematic when crude oil contamination penetrates into deep layers where microaerophilic conditions prevail. One of the most cost-effective ways of improving soil aeration is by introducing clay minerals such as vermiculite (Rhykerd et al. 1998). Clay minerals are positively charged but can exchange cations; vermiculite, for example, can readily exchange calcium and magnesium ions. When introduced into soil, clay minerals can exchange cations with positively charged organic ions, resulting in a reduction in surface polarity and facilitating sorption of hydrophobic contaminants such as crude oil. Moreover, the introduction of clay minerals increases the soil's surface/volume ratio, thereby further promoting sorption of oil and its availability to microbial degradation (Rhykerd et al. 1999). Increased oil sorption on the minerals may reduce the concentration of the oil in the soil solution, thereby decreasing its toxicity to the microbial population (Loosdrecht et al. 1990).

In the present study we examine the biodegradation of crude oil by indigenous bacterial and fungal populations in samples of a desert soil and evaluate the kinetics of these populations during incubation of the crude oil in the soil. We also investigate the effect of vermiculite applied as a bulking agent.

Materials and methods

Soil sampling

Soil was collected from an undisturbed yard in the city of Beer Sheva, which is located in the northern part of the Negev Desert in Southern Israel. The samples were taken from the top 10 cm of the soil at five random locations at least 2 m apart. The soil was sieved through 1 mm net and mixed well in a stain mixer (Winkworth Machinery Ltd., Reading, England). The initial soil water content was determined after drying the soil at 100 °C for 24 h. Field capacity was determined by saturating the soil with water and measuring the percentage (w/w) of water remaining in the soil after free drainage ceased. In all experiments the soil water content was adjusted to 50% of field capacity.

Characterization of crude oil

Light Arabian crude oil was provided by Eilat-Ashkelon Petroleum Pipeline Company (Ashkelon, Israel). Analysis of total petroleum hydrocarbons (TPH) was performed according to Method 1664 of the U.S. Environmental Protection Agency (http://www.epa.gov/waterscience/methods/1664f051.html). The content of heavy metals in the crude oil was tested by inductively coupled plasma emission spectroscopy (Perkin-Elmer, Wellesley, MA, USA). Both analyses were performed at the Israel Institute of Petroleum and Energy (Tel Aviv, Israel).

Soil contamination with crude oil

The soil was supplemented with crude oil at a rate of 5, 10 or 20% (w/w) and mixed well in a stain mixer (Winkworth Machinery Ltd., Reading, England). Nitrogen and phosphorus were added to the contaminated soil as KNO_3 and KH_2PO_4 at a C:N:P ratio of 100:10:1, respectively. The soil carbon content was estimated from the TPH data obtained from the soil extracts (47,900, 86,100 and 163,800 ppm for samples supplemented with 5, 10 and 20% of crude oil, respectively).

Where indicated, the contaminated soils were amended with vermiculite aggregates (50% v/v). The treated soils were placed in 1-l plastic boxes (0.5 kg per box) and incubated at 30 °C for 90 days.

In another experiment, the effect of aeration on the proliferation of microbial populations in a contaminated soil was tested. Reduced aeration was obtained by placing the contaminated soil in tubular polyethylene sleeves 5 cm in diameter. Soil was sampled either near the top of the sleeve (depth of 5 cm) or from a depth of 70 cm, where aeration level was presumably lower than near the surface. Soil humidity was maintained at 50% of field capacity throughout the incubation period.

Assessment of biodegradation of crude oil

Residual TPH values after 90 days of incubation of the crude-oil contaminated soils were determined

as indicated above. Soil samples from the same treatments were also subjected to soxhlet extraction (8 h with chloroform). One gram of contaminated soil was wrapped in Whatman (No. 4) filter paper to prevent loss of soil particles during extraction. After extraction the chloroform was evaporated and the samples were subjected to simulated distillation (Lawrence & Milind 1995) of crude oil extracts to obtain the boiling distribution of the petroleum constituents. The distilled fractions were analysed by gas chromatography using GC ASTM Method 5307D (1999) at Oil Refineries Ltd. (Haifa, Israel). The content and distribution of aromatic hydrocarbon compounds (mono-, di- and polyaromatic) were analysed by HPLC (Shimadzu Scientific Instruments, Kyoto, Japan).

Enumeration of soil bacterial and fungal populations

During the incubation period, samples were collected weekly to evaluate changes in the viable bacterial and fungal populations. Bacterial population densities were determined by plating soil dilutions, from the incubated samples, on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI, USA). Fungal population densities were determined separately on TSA amended with 200 mg/l of chloramphenicol to inhibit bacterial populations.

Results and discussion

The capacity of indigenous soil microbial populations to degrade crude oil was evaluated in a simulated contamination of a desert soil that, to the best of our knowledge, had never been in contact with petroleum. In order to evaluate the biodegradation of the crude oil and monitor its effects on the dynamics of the soil microbial populations, soil samples supplemented with 5, 10 or 20% (w/w) of crude oil were incubated for 90 days under controlled conditions. Petroleum biodegradation is known to be enhanced by high aeration level, apparently because availability of oxygen is essential for the activity of petroleum-hydrocarbon degrading enzymes (mono- or dioxygenases) (Britton 1984; Singer & Finnerty 1984). Soil aeration and consequently the biodegradation of crude oil can be improved by the addition to soil of various minerals as bulking agents (Rhykerd 1998). Early in this sudy we compared the capacity of several minerals to absorb crude oil and found that maximal oil absorption was provided by the clay mineral vermiculite (data not shown). Accordingly, we also examined the effect of amendation with vermiculite as a means of improving soil aeration and optimizing biodegradation of crude oil.

The residual total petroleum hydrocarbons (TPH) extracted from contaminated soil amended or non-amended with vermiculite and incubated for 90 days clearly showed that degradation of the crude oil had taken place, presumably through the action of the soil microflora (Figures 1, 3 and 4). Maximal reduction in TPH (91%) was obtained in soil supplemented with vermiculite and containing the highest concentration of crude oil (20%). This high level of degradation may apparently be attributed to the elevated soil aeration provided by the vermiculite, since in the absence of the clay mineral only 74% of the oil was biodegraded (Figure 1). This is supported by the study of Rhykerd et al. (1998), who also found that addition of vermiculite to soil improved oil biodegradation. The biodegradation rate obtained in our study is markedly higher than reported by Al-Daher et al. (1998) and Gogoi et al. (2003), who recorded petroleum hydrocarbon degradation rates of 60 and 75% over periods of 8 months and 1 year, respectively. The boiling distributions of the crude oil compounds in the soil extracts show

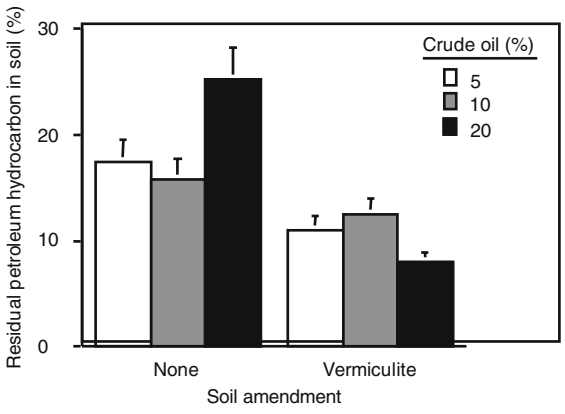

Figure 1. Effect of vermiculite amendment (50% v/v) on bioremediation of soil contaminated with 5, 10 and 20% crude oil (w/w) in terms of hydrocarbons (TPH) extracted from soil samples after 90 days of incubation at 30 °C (±SD). Initial concentrations (after 1 day) of TPH extracts from soil contaminated with 5, 10 and 20% crude oil were 47,900, 86,100 and 163,800 ppm, respectively.

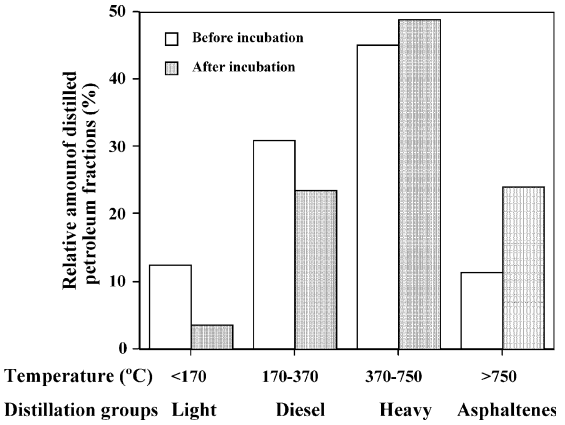

Figure 2. Boiling point distribution of distilled fractions of crude oil extracted from soil contaminated with 20% (w/w) crude oil after 1 and 90 days of incubation at 30 °C.

that the soil microflora preferentially utilized the lighter oil compounds, which are characterized by boiling temperatures lower than 170 °C. After 90 days the light oil fraction had decreased in quantity about four-fold (Figure 2). Similarly, the gas chromatogram showing the distribution of the distilled fractions of the crude oil extract clearly demonstrated that the oil composition had undergone a complete transformation after 90 days in the soil: most of the light and part of the intermediate weight fractions initially present in the oil extracts had disappeared (data not shown). This result agrees with the report by Capelli et al. (2001) of minor changes only in the level of asphaltenes. Presumably, as recently demonstrated (Pineda-Flores et al. 2004), isolation of asphaltene degrading microorganisms can be accomplished only by a specific enrichment directed towards isolation of microorganisms utilising asphaltenes as sole carbon source.

The concentration of aromatic compounds in crude oil serves as an indicator of its toxicity. In the present study, HPLC analysis showed similar concentrations of the mono-, di- or polyaromatic compounds before and after incubation for 90 days (Table 1). This shows that the overall biodegradation levels of the various aromatic compounds were similar and that, apparently, the process of biodegradation did not increase the oil's toxicity. However, the decline in microbial population densities that was detected at the start of the incubation period could reflect the toxic effects of aromatic hydrocarbons (Figure 3). This is

Table 1. Distribution values (±SD) of aromatic hydrocarbons in crude oil extracts of soil contaminated with 20% (v/v) crude oil after incubation for 1 and 90 days at 30 °C

Incubation time (days)	Concentration of hydrocarbons (%)		
	Monoaromatic	Diaromatic	Polyaromatic
1	10.50 ± 1.26	3.31 ± 0.39	6.14 ± 0.77
90	12.25 ± 1.47	4.85 ± 0.52	7.98 ± 0.95

supported by the increase in the length of this initial decline (from 5 to 30 days) as the crude oil content of the soil increased from 5 to 20%. At all levels of oil supplementation, the period of inhibition was followed first by a phase of steep increase in population density and subsequently by a moderate increase continuing until the end of incubation (Figure 3). This recovery could have resulted from adaptation of the bacterial populations, featuring selective enrichment and genetic changes that resulted in increased proportions of hydrocarbon degrading bacteria (Lehay & Colwell 1990). The addition of vermiculite had no effect on total bacterial counts, as demonstrated by the similar bacterial population densities (upto 4×10^7 CFU/g soil) recorded in vermiculite amended and non-amended soils. Nevertheless, initiation of the recovery period – the phase characterized by a sharp increase in population densities – started earlier in the vermiculite amended soil, particularly at the high oil content (20%; Figure 3). This could be due to sorption of oil on vermiculite, resulting in reduced toxicity to the bacterial population.

By contrast, the initial suppressive effect on the fungal populations in the same oil-contaminated soil samples was minimal and lasted only 5 days (Figure 4). Furthermore, no reduction in fungal populations was observed in vermiculite amended soil, indicating that vermiculate facilitated adaptation to the hydrocarbons. The overall increase in the fungal population was higher than the corresponding increase in the bacterial populations and exceeded two orders of magnitude as compared with non-amended soil. The higher growth response of the fungal populations to the presence of oil in the soil suggests that, in this specific desert soil, fungi play a more dominant role in oil biodegradation than bacteria (Figures 3 and 4). Indeed, Palittapongarnpim et al. (1998) isolated two yeast strains from a soil contaminated with crude

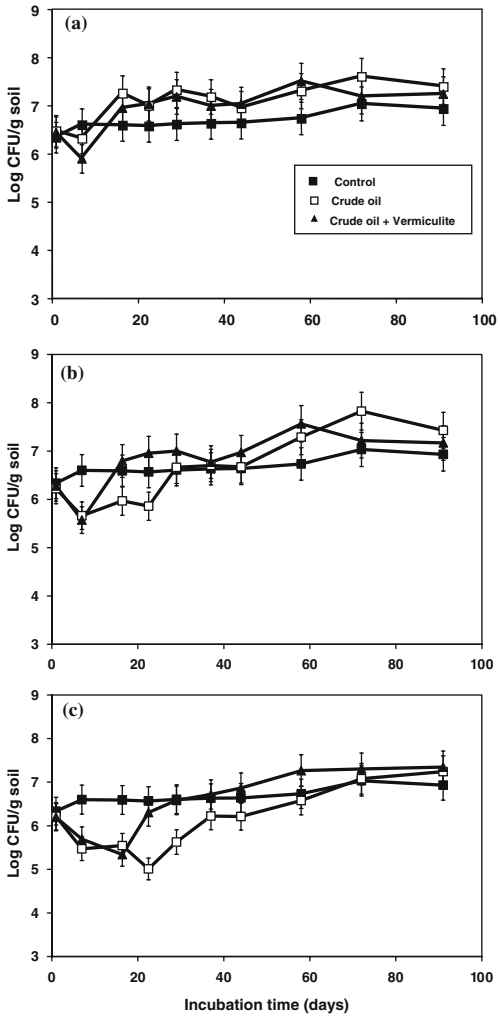

Figure 3. Effect of vermiculite amendment (50% v/v) of soil contaminated with 5% (a), 10% (b) and 20% (c) of crude oil (w/w) on changes in bacterial population densities (±SD).

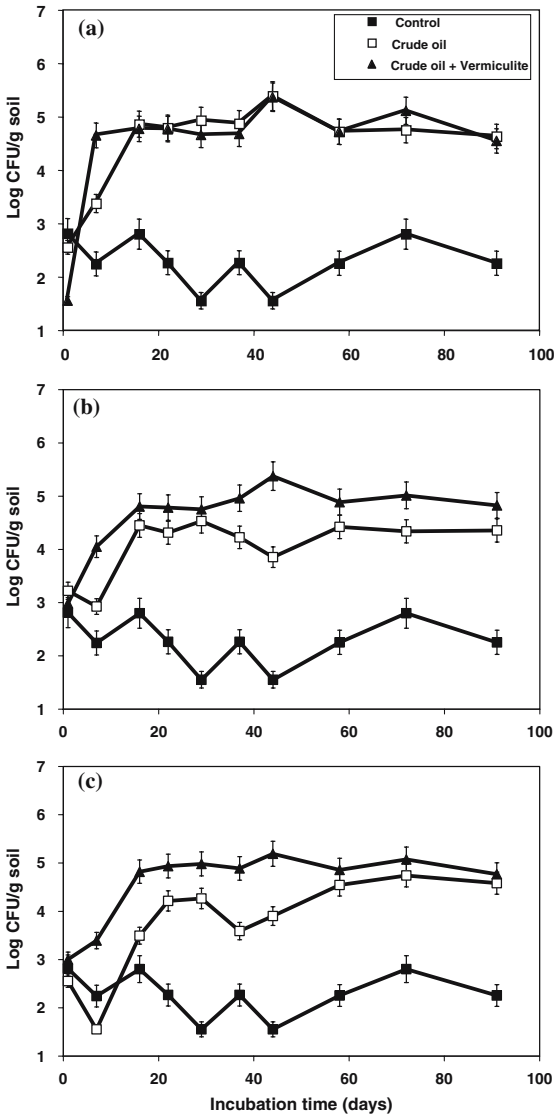

Figure 4. Effect of vermiculite amendment (50% v/v) of soil contaminated with 5% (a), 10% (b) and 20% (c) of crude oil (w/w) on changes in fungal population densities (±SD).

oil that exhibited a better oil degradation than the bacteria isolated from the same soil. One of these yeasts degraded 87% of the TPH in liquid medium. However, in other cases the rate of crude oil degradation by fungi may be much lower. Obuekwe et al. (2001) report that after 1 year of incubation of oil contaminated desert soil in Kuwait, fungi alone accounted for upto 81% degradation of crude oil; however, the fungi were less effective than the bacterial population, which degraded upto 100% of crude-oil soil contamination.

Since oil spills may percolate down to soil layers characterized by limited aeration, we also sought to compare the response of bacterial and fungal population dynamics to oil contamination at different depths. To do so, we packed contaminated soil in vertical polyethylene sleeves and examined samples from two depths, 5 and 70 cm (Figures 5 and 6). At all three contamination levels tested (5, 10 and 20% crude oil w/w), the bacterial populations proliferated throughout the incubation period (100 days), and no major differences in population density were found between the two depths (Figure 5). By contrast, the density of the fungal population in soil samples from the top of the soil column was about one order of magnitude

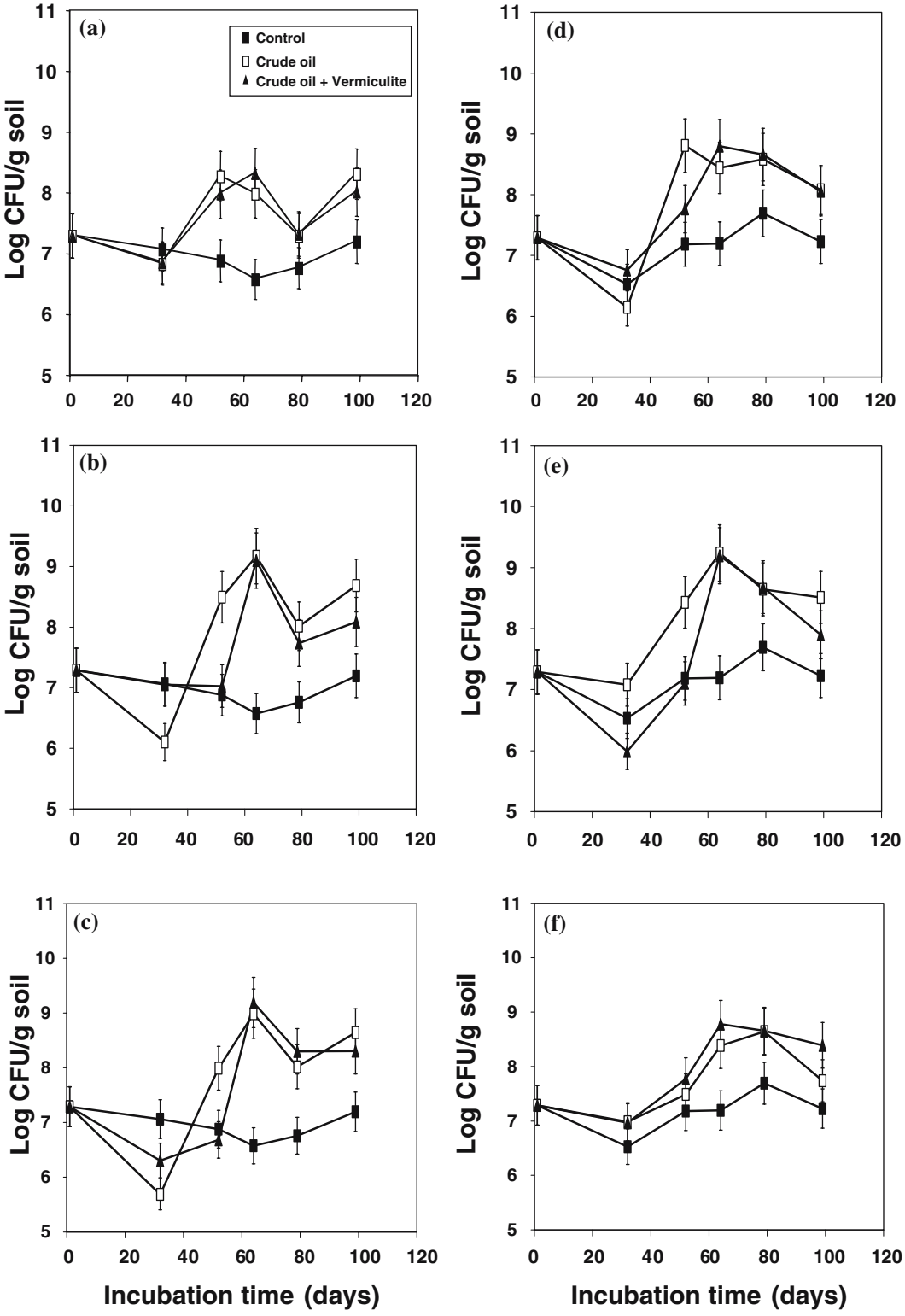

Figure 5. Effect of vermiculite amendment (50% v/v) of soil contaminated with 5% (a and d), 10% (b and e) and 20% (c and f) of crude oil (w/w) on changes in bacterial population densities at depths of 5 and 70 cm (a, b, c and d, e, f, respectively) during 100 days of incubation at 30 °C (±SD).

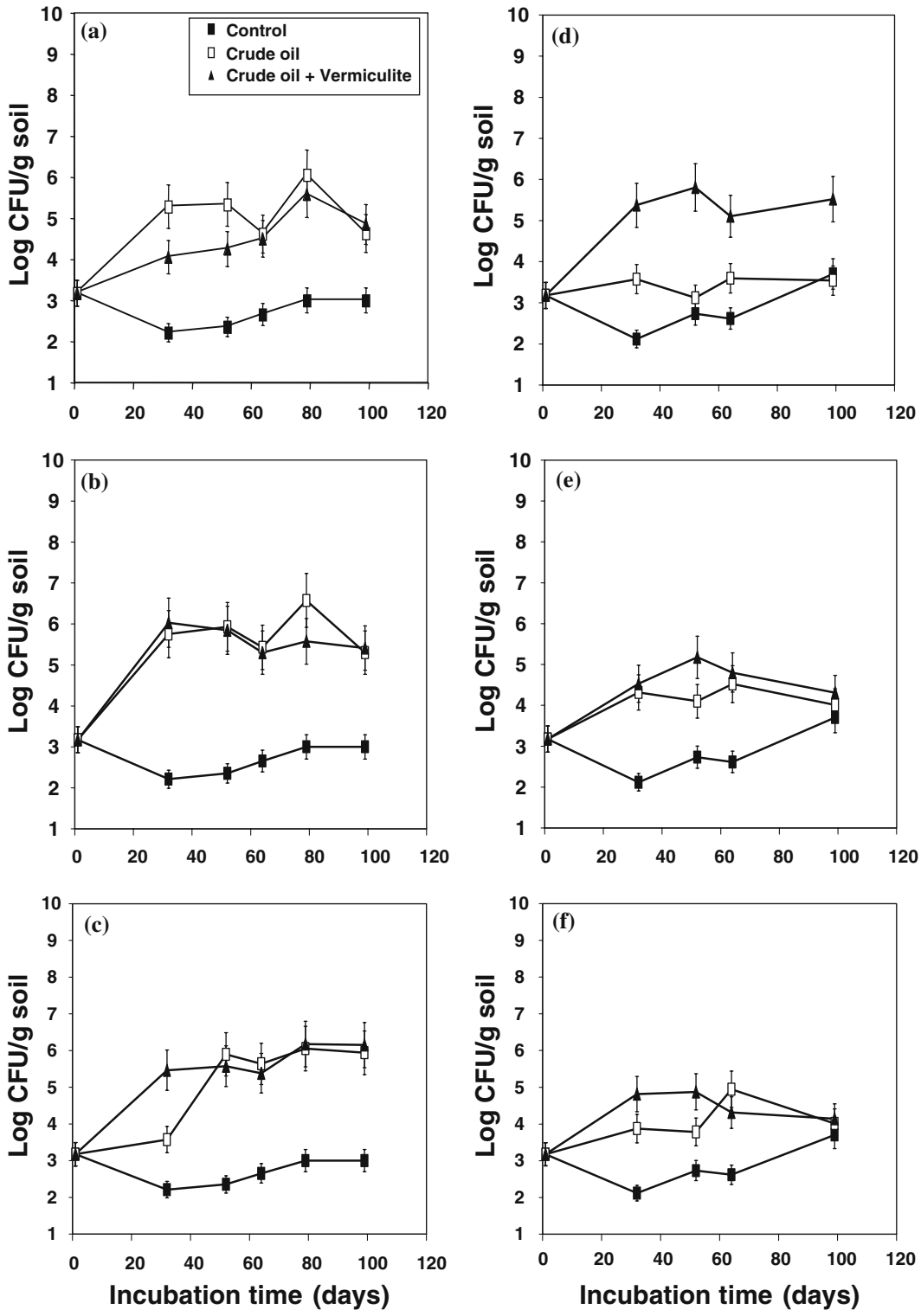

Figure 6. Effect of vermiculite amendment (50% v/v) of soil contaminated with 5% (a and d), 10% (b and e) and 20% (c and f) of crude oil (w/w) on changes in fungal population densities at depths of 5 and 70 cm (a, b, c and d, e, f, respectively) during 100 days of incubation at 30 °C (±SD).

greater than that from the 70 cm depth; the same pattern was observed for all three levels of soil contamination (Figure 6). It is therefore seems that, unlike the bacterial population, the ability of the soil's fungal population to utilize and degrade crude oil is affected by soil aeration. Addition of vermiculite resulted in an increase in fungal soil populations, mainly in the samples from the deep layer (70 cm); however, this effect was observed only in soil amended with the lowest amount (5%) of oil.

During incubation of oil contaminated soils, a gradual shift in the composition of fungal communities was observed. The most prevailing genera obtained in the course of the first 3 weeks were *Penicillium* and *Aspergillus*. However, starting from the fifth week and throughout the experiment the most dominant isolate was identified as *Memnoniella* sp. When this strain was cultured in a liquid medium containing crude oil as the sole carbon source, 61% of the oil was degraded after 24 days of incubation (data not shown). Ecological and physiological characterization of this strain, in order to optimize its degradation activity will be the subject of a future research.

Conclusions

In this study we demonstrate the capacity of indigenous desert-soil microbial populations to degrade crude oil in a relatively short time. Biodegradation took place despite the fact that, to the best of our knowledge, the soil we examined had never been in contact with petroleum. This finding is supported by a report by Delille et al. (2002) of a similar phenomenon in an Antarctic soil. The increased pace of microbial population growth on exposure to the crude oil was probably due utilization of the oil as a source of carbon and energy. The greater growth response of the fungal population as compared with the bacterial population suggests that the former plays a major role in the biodegradation of petroleum in this specific desert soil. At the same time, the increase in fungal proliferation observed in the lower section of the soil column containing 5% crude oil when amended with vermiculite apparently reflects a demand for aeration that is provided by this bulking agent.

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