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Effects of Jet Fuel Spills on the Microbial Community of Soil†

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Hydrocarbon residues, microbial numbers, and microbial activity were measured and correlated in loam soil contaminated by jet fuel spills resulting in 50 and 135 mg of hydrocarbon g of soil^{-1}. Contaminated soil was incubated at 27°C either as well-aerated surface soil or as poorly aerated subsurface soil. In the former case, the effects of bioremediation treatment on residues, microbial numbers, and microbial activity were also assessed. Hydrocarbon residues were measured by quantitative gas chromatography. Enumerations included direct counts of metabolically active bacteria, measurement of mycelial length, plate counts of aerobic heterotrophs, and most probable numbers of hydrocarbon degraders. Activity was assessed by fluorescein diacetate (FDA) hydrolysis. Jet fuel disappeared much more rapidly from surface soil than it did from subsurface soil. In surface soil, microbial numbers and mycelial length were increased by 2 to 2.5 orders of magnitude as a result of jet fuel contamination alone and by 3 to 4 orders of magnitude as a result of the combination of jet fuel contamination and bioremediation. FDA hydrolysis was stimulated by jet fuel and bioremediation, but was inhibited by jet fuel alone. The latter was traced to an inhibition of the FDA assay by jet fuel biodegradation products. In subsurface soil, oxygen limitation strongly attenuated microbial responses to jet fuel. An increase in the most probable numbers of hydrocarbon degraders was accompanied by a decline in other aerobic heterotrophs, so that total plate counts changed little. The correlations between hydrocarbon residues, microbial numbers, and microbial activity help in elucidating microbial contributions to jet fuel elimination from soil.

In the course of a broader study on bioremediation of fuel-contaminated soils (10), it became desirable to quantify the responses of the soil microbial community to fuel spills. Changes in the microbial numbers and activity of soil in response to a pollution event that may be regarded as a selective carbon substrate addition have a certain intrinsic interest. In addition, for the reasons discussed in the preceding paper (10), poisoned soil controls underestimate the true microbial contribution to hydrocarbon disappearance. Therefore, the measurement of population dynamics and microbial activity changes also help to put into perspective the somewhat misleading information provided by poisoned soil controls. Because of the labor-intensive nature of enumerations and activity measurements, these were confined to a single fuel (jet fuel), soil type (loam), and incubation temperature (27°C). Jet fuel was applied at a low (50 mg g of soil^{-1}) and a high (135 mg g of soil^{-1}) concentration. Measurements were made primarily in surface soil, where oxygen was freely available, but for comparison, measurements were also made in the soil columns used in the previous study (10), where oxygen diffusion was probably limiting. Enumerations were performed both by direct microscopic and by cultural techniques. The fluorescein diacetate (FDA) stain used in connection with epifluorescence microscopy (6) allowed us to selectively enumerate metabolically active bacteria and mycelial fragments, since FDA itself does not fluoresce; only the metabolically liberated fluorescein fluoresces. Enumerations included plate counts for aerobic heterotrophs and most probable number (MPN) counts for hydrocarbon degraders. For activity measurements we used the quantified hydrolysis of FDA (9), the same agent that was used for determination of metabolic status in the epifluorescence microscopic counts.

MATERIALS AND METHODS

Preparation, incubation, and analysis of jet fuel-contaminated soil. The characteristics of jet fuel and loam soil (Bayway Refinery, N.J., Exxon USA) used in these studies were described in the preceding paper (10). Soil columns were prepared, contaminated, and incubated at 27°C as described in the preceding paper (10); but most of the experiments were conducted in surface soil, i.e., in an incubation system in which oxygen diffusion was not expected to limit biodegradation substantially. In the latter case, the 60-g (dry weight) soil samples were incubated in 600-ml beakers in a layer that was less than 2-cm thick. The beakers were covered with thin polyethylene film (Saran Wrap) to reduce evaporation. The covers were removed for aeration every 2 to 3 days. In every other respect, these samples were treated and incubated in the same manner as the soil column samples were. Bioremediation by liming, fertilization, and tilling was performed as described in the preceding paper (10); but for tilling of the soil in the beakers, a stainless steel wire loop was used that was an integral part of the incubation system.

For analysis of jet fuel residues, the soil of entire columns or beakers was Soxhlet extracted with CH_2Cl_2 and analyzed by quantitative gas chromatography. The details of the analytical procedures, the average recovery, and standard deviation of the analysis were described in the preceding paper (10).

Measurement of changes in microbial numbers and activity in response to jet fuel spills. For enumeration of microorganisms in surface soil, portions of the soil were removed from the beakers at certain times during incubation. In the case of subsurface soil, one of the replicate columns was pushed out

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from its glass sleeve with a plunger. The subsurface soil sample was always taken from the bottom portion of the column and was thoroughly mixed. On a portion of the sample, the dry weight of the soil was determined by weighing out 1 g (wet weight) into an aluminum dish and drying it at 105°C for 24 h. After the soil was cooled in a desiccator, the soil was reweighed and the dry weight was calculated.

The dispersion and dilution procedure for the enumeration of soil microorganisms was as follows. A 10-g (wet weight) soil sample was placed in a sterile Mason jar containing 100 ml of sterilized 60 mM phosphate buffer (pH 7.5). This Mason jar was covered with a blending adapter and was placed in a blender (Osterizer; Oster Corp., Milwaukee, Wis.). The soil sample was homogenized for 2 min at 15,000 rpm. From this soil homogenate, serial dilutions were prepared with 60 mM phosphate buffer (pH 7.5) for direct counts, plate counts, and MPN determinations.

For direct microscopic counts (6), a 1-ml portion of diluted soil homogenate was added to 4 ml of sterilized 60 mM phosphate buffer (pH 7.5) and mixed. Twenty-five microliters of FDA (Eastman Kodak Co., Rochester, N.Y.) solution containing 2 mg of FDA ml of acetone^{-1} (final concentration, 10 μg of FDA ml^{-1}) was added and thoroughly mixed with a vortex mixer. After 3 min of staining, the soil suspension was filtered through a 25-mm-diameter, 0.2-μm-pore-size black polycarbonate filter (Nuclepore Co., Pleasanton, Calif.). The filter was placed on a glass slide and covered with nonfluorescing immersion oil (Cargille type FF) and a cover slip. The preparation that was obtained was immediately examined with an epifluorescence microscope (model BH2-RFL; Olympus Optical Co., Tokyo, Japan) with a mercury burner (HBO 100W). Optical filters for FDA were exciter IF-490 and the dichroic mirror DM500 (0-515). The supplementary exciter was EY455; a barrier filter was not used. Microorganisms were counted from at least 10 microscopic fields, which were viewed at a magnification of ×1,000, and mean numbers were calculated. The length of fungal hyphae that were relatively short and linear was measured with a calibrated ocular micrometer.

For viable counts of aerobic heterotrophs, the pour plate method was used. Portions of 1 ml of three to four serial dilutions of soil suspension were placed into sterile empty petri dishes. About 20 to 25 ml of autoclaved nutrient agar medium (Difco Laboratories, Detroit, Mich.) cooled to 45°C was poured over the inoculum. The petri dish was gently shaken to mix the inoculum with the agar medium. After solidification of the medium, the plate was incubated at 27°C for 1 week, and the numbers of colonies were counted.

A five-tube MPN technique was used to estimate the number of hydrocarbon-utilizing microorganisms. The medium was jet fuel-supplemented Bushnell-Haas broth (Difco) with resazurin (1 mg liter^{-1}). Three sets of five screw-cap tubes, each containing 5 ml of autoclaved medium (without jet fuel), were inoculated with 1, 0.1, and 0.01 ml of an appropriately diluted soil suspension. After inoculation, 50 μl of jet fuel filtered through a 0.45-μm-pore-size membrane (Flotronics, Spring House, Pa.) was added to each tube. After incubation at 27°C for 3 weeks, positive tubes were counted. The color of positive tubes ranged from pink to colorless, resulting from reduction of the resazurin by microbial oxygen consumption. The MPN values were obtained from the MPN index for five tubes (1).

Microbial activity in soil was measured by the FDA hydrolysis assay (9). FDA was dissolved in acetone (2 mg ml^{-1}) and was stored at −20°C. A soil sample (1 g [wet weight]) was placed in a 125-ml Erlenmeyer flask containing 50 ml of 60 mM phosphate buffer (pH 7.6). To this soil suspension was added 0.5 ml of FDA stock solution (final concentration, 20 μg ml^{-1}). After 1 h at 27°C on a rotary shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 200 rpm, 50 ml of acetone was added to stop further FDA hydrolysis. Soil was removed from the suspension by centrifugation for 5 min at 6,000 rpm followed by filtration through Whatman no. 3 filter paper. This produced a clear solution with a low-background absorbance. The amount of FDA hydrolyzed was measured as the A_{490} with a spectrophotometer (Spectronic 2000; Bausch & Lomb, Inc., Rochester, N.Y.). All microbial numbers and activity values were calculated per gram of dry soil.

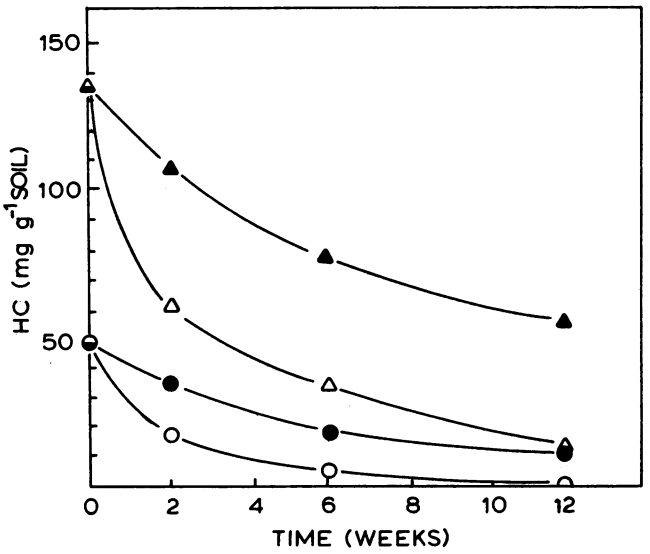

FIG. 1. Disappearance of hydrocarbon (HC) with time from surface soil and from soil columns contaminated with jet fuel. Symbols: ▲, column soil contaminated with 135 mg of jet fuel g of soil^{-1}; △, surface soil contaminated with 135 mg of jet fuel g of soil^{-1}; ●, column soil contaminated with 50 mg of jet fuel g of soil^{-1}; ○, surface soil contaminated with 50 mg of jet fuel g of soil^{-1}.

RESULTS AND DISCUSSION

Hydrocarbon degradation. Figure 1 shows a comparison of jet fuel disappearance from identically treated soil samples incubated either in beakers or in columns. As determined on poisoned controls (data not shown), the approximate two times faster disappearance rates of jet fuel from beakers as compared with that from columns could not be ascribed to evaporative losses alone but were caused, in roughly equal proportions, by higher evaporation and the faster biodegradation rates as a result of the increased oxygen availability in surface soil. To avoid oxygen limitation and to have homogeneous rather than gradient samples for our enumeration and activity measurements, we performed these primarily on surface soil incubated in beakers. However, to connect the system used in this study to the previously used soil column incubation system (10), we also made enumerations on soil from the bottom part of columns. In an entire soil column, a gradient exists between the well-aerated surface layer and the poorly aerated subsurface soil.

Direct enumerations. The responses of the soil microbial community to jet fuel differed according to oxygen availability even more strongly than expected. In uncontaminated,

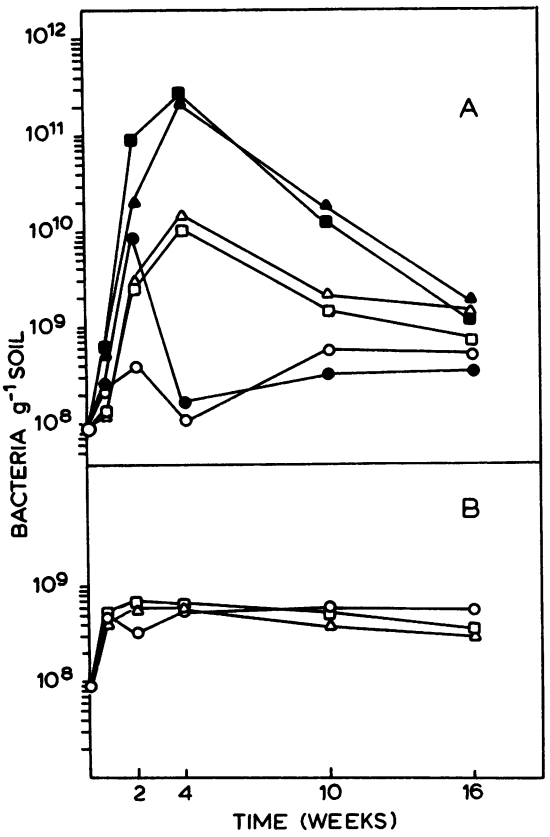

FIG. 2. Changes in the numbers of metabolically active bacteria with time as determined by FDA epifluorescence counts in surface soil (A) and in subsurface soil (B) contaminated with jet fuel. Symbols: ○, no jet fuel, no bioremediation; ●, no jet fuel, bioremediation treatment; □, 50 mg of jet fuel g of soil^{-1}; △, 135 mg of jet fuel g of soil^{-1}; ■, 50 mg of jet fuel g of soil^{-1} plus bioremediation; ▲, 135 mg of jet fuel g of soil^{-1} plus bioremediation.

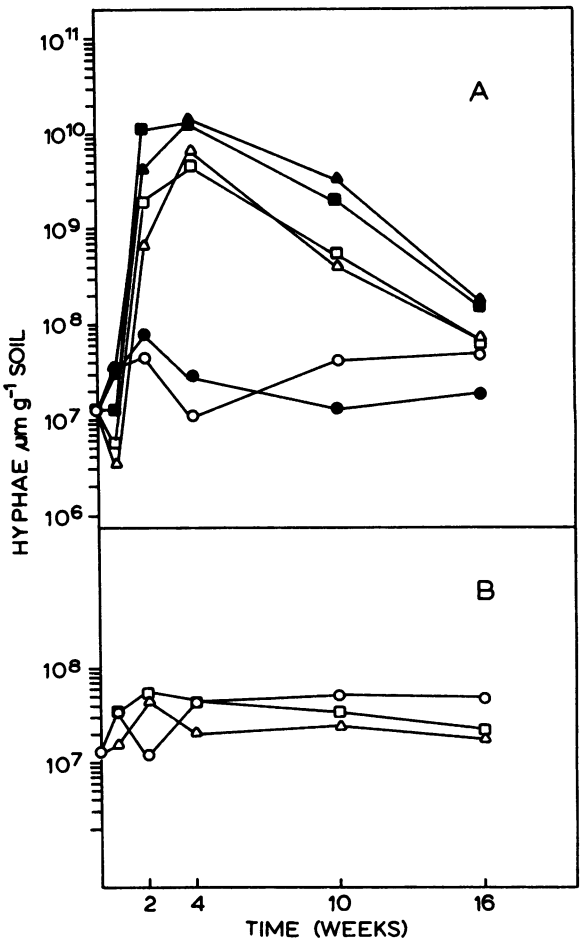

FIG. 3. Changes in the length of metabolically active fungal hyphae with time as measured by FDA epifluorescence microscopy in surface soil (A) and subsurface soil (B) contaminated with jet fuel. Symbols are as described in the legend to Fig. 2.

untreated surface soil (Fig. 2A), the numbers of bacteria determined by the FDA direct count method fluctuated between 1×10^8 and 5×10^8 g of soil^{-1} during the 16-week incubation period. Part of this fluctuation may be ascribed to the partial drying, sieving, and rewetting of the soil that increased the biodegradation of indigenous substrates in soil (5). Bioremediation treatment of uncontaminated soil caused a sharp but short-lived increase of numbers from 10^8 to 10^{10} g of soil^{-1}. By week 4, the numbers returned again to about 10^8 g of soil^{-1}. The pH adjustment, in combination with N and P fertilizer, increased the utilization of indigenous soil carbon, but the amount of this substrate was apparently quite limited. Jet fuel contamination at both levels increased bacterial numbers to 1×10^{10} g of soil^{-1}. This effect was more gradual and lasting than was stimulation by bioremediation treatment alone. Jet fuel contamination combined with bioremediation treatment increased metabolically active bacteria 2,000-fold to 2×10^{11} g of soil^{-1}. After 4 weeks, a gradual decline in numbers occurred, but after 16 weeks, the numbers were still 1 order of magnitude higher than the background. The maximum numbers (2×10^{11} g of soil^{-1}) of bacteria exceeded most reported soil counts, but numbers that were almost as high have been reported by Westlake et al. (11) in hydrocarbon-contaminated and fertilized Canadian soils, even though these soils were not cultivated.

In soil from the lower part of the columns (Fig. 2B), numbers increased both in control and in jet fuel-contaminated soils from 1×10^8 to about 5×10^8 g of soil^{-1} and were essentially level thereafter. As judged from the control numbers, this response was primarily due to soil handling rather than the jet fuel spill. Bioremediation treatment was not performed on these columns.

The length of fungal hyphae in surface soil (Fig. 3A) closely followed the pattern described for bacteria. In uncontaminated and either untreated or bioremediation-treated soils, the initial length of the fungal hyphae was 1.3×10^7 μm and fluctuated between 1×10^7 and 8×10^7 μm g of soil^{-1}. These numbers closely agree with those reported by Pinholt et al. (8). Jet fuel contamination in untreated soil briefly decreased the length of active hyphae, but this was followed by increases of 2.5 and 3 orders of magnitude in untreated and bioremediation-treated samples, respectively. The numbers 1×10^{10} μm of hyphae g of soil^{-1}) appeared to be very high. However, Faegri et al. (3) have reported 4.12×10^9 μm of hyphae g of uncontaminated Norwegian soil^{-1}, and thus, the double value found in this study is not unreasonable. After 4 weeks, the length of active fungal hyphae gradually declined. In soil from column bottoms, the length of fungal hyphae fluctuated both in uncontaminated and in contaminated soils to between 1×10^7 and 6×10^7 μm g of soil^{-1}, with no clear trend.

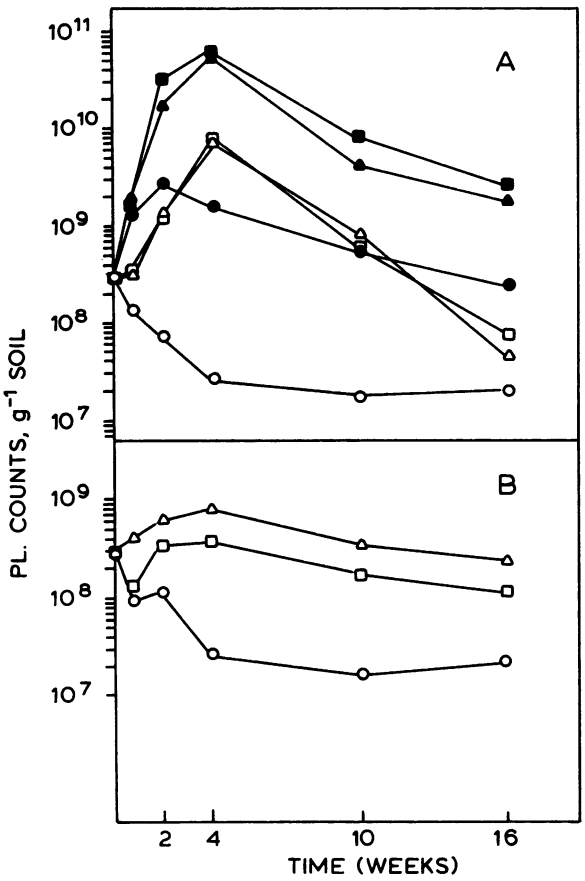

FIG. 4. Changes in the plate (PL.) counts of aerobic heterotrophs with time in surface soil (A) and subsurface soil (B) contaminated with jet fuel. Symbols are as described in the legend to Fig. 2.

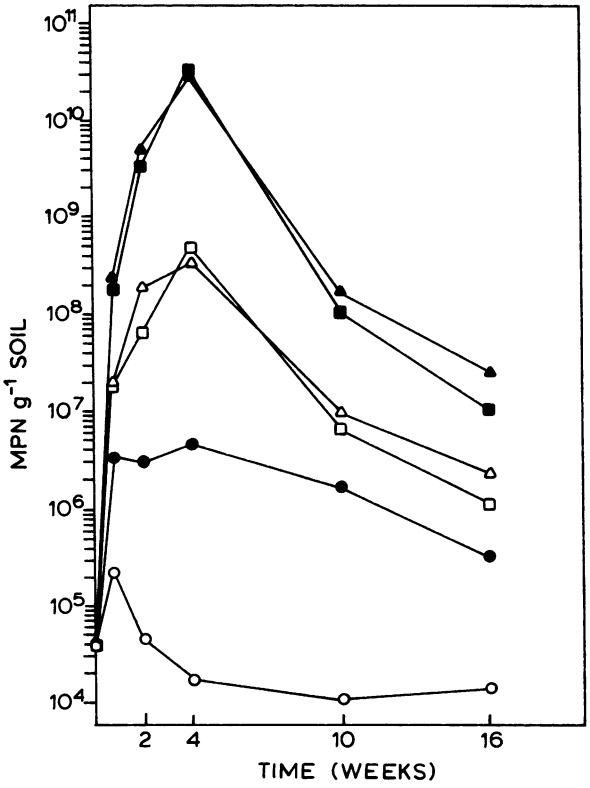

FIG. 5. Changes in the numbers of hydrocarbon degraders with time in jet fuel-contaminated surface soil as determined by MPN counts. Symbols are as described in the legend to Fig. 2.

Cultural enumerations. In contaminated surface soil, the initial number (3×10^8 g of soil^{-1}) of CFU decreased to 2.2 $\times 10^7$ g of soil^{-1} during the first 4 weeks of incubation and remained level thereafter (Fig. 4A). This trend differed from that of direct enumerations. The cultural count was higher than the direct count, although the reverse is usually the rule. To resolve these apparent contradictions, it is important to remember that FDA direct counts enumerate only metabolically active microorganisms that are capable of the intake and hydrolysis of FDA and retention of the resulting fluorescein dye. Thus, endospores, fungal spores, and inactive bacterial cells or fungal hyphae would not be counted by the FDA technique; yet, at the same time, they could form colonies. In control soil, the growth response to sieving and wetting would be expected to involve primarily the autochthonous (humus-utilizing) portion of the microbial community, and these microorganisms are notoriously underestimated in counts on nutrient agar (2). At the same time, the zymogenous (opportunistic) segment of the soil microbial community was likely to decrease for the lack of readily available nutrients. Lund and Goksoyr (5) and Westlake et al. (11) have observed similar changes during the incubation of unamended soil samples and interpreted the direct counts to be more representative of the autochthonous and cultural enumeration of the zymogenous segments of the soil microbial community, respectively.

Uncontaminated but bioremediation-treated soil showed a 10-fold increase in plate counts that returned to the background level by the end of the experiment. Jet fuel contamination alone strongly increased the counts in the first 2 weeks, but by 16 weeks the numbers were lower than they were at the start of the experiment. Jet fuel in combination with bioremediation resulted in an increase in counts of more than 2 orders of magnitude that declined only gradually and that were still 1 order of magnitude higher at 16 weeks than at time zero. In subsurface soil (Fig. 4B), the plate counts of uncontaminated soil exhibited a similar trend as the corresponding curve in Fig. 4A did. However, positive responses to jet fuel were marginal; the counts of aerobic heterotrophs remained essentially level.

The maximal number of aerobic heterotrophs (6×10^{10} g of soil^{-1}) was very high when compared with most similar counts in soil, but this seems to be a characteristic response to high-energy hydrocarbon substrates. Westlake et al. (11) also reported plate counts of over 10^{10} g of soil^{-1} in hydrocarbon-contaminated and fertilized soils.

At time zero, MPN counts of hydrocarbon degraders (Fig. 5) were 4×10^4 g of soil^{-1}, and in uncontaminated surface soil, they closely followed the pattern displayed by aerobic heterotrophs. A brief increase in untreated soil at week 1 remains unexplained and may have been due to data scatter. In treated soil, an increase was expected. At 4 weeks, contamination by jet fuel increased the numbers of hydrocarbon degraders by 4 orders of magnitude. Bioremediation resulted in an additional increase of 2 orders of magnitude, to a total of about 10^6-fold. After 4 weeks, the numbers gradually declined but were still high above the background level at 16 weeks. In subsurface soils (Fig. 6), the increase of MPNs of hydrocarbon degraders was also about 4 orders of magnitude, while aerobic heterotrophs in comparable sam-

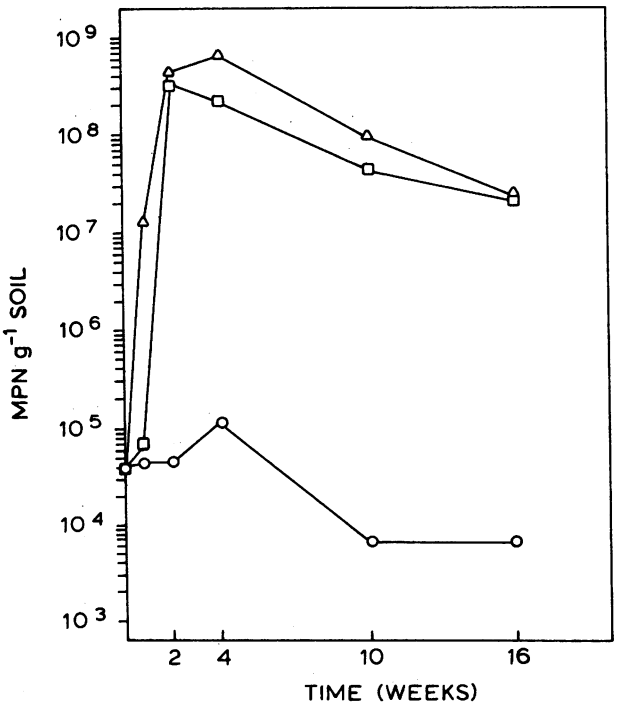

FIG. 6. Changes in the numbers of hydrocarbon degraders with time in jet fuel-contaminated subsurface soil as determined by MPN counts. Symbols are as described in the legend to Fig. 2.

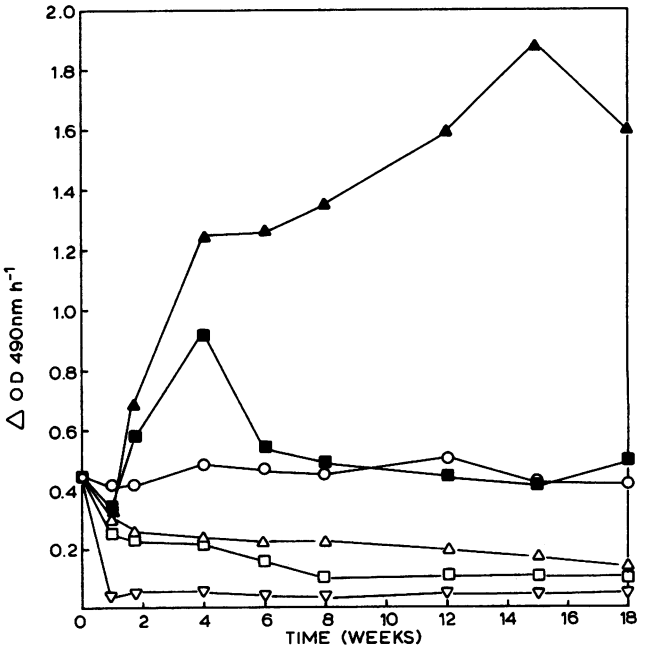

FIG. 7. Changes in FDA hydrolysis activity with time in surface soil contaminated by jet fuel. Symbols: ○, no jet fuel, no bioremediation; □, 50 mg of jet fuel g of soil^{-1}; △, 135 mg of jet fuel g of soil^{-1}; ■, 50 mg of jet fuel g of soil^{-1} plus bioremediation; ▲, 135 mg of jet fuel g of soil^{-1} plus bioremediation; ▽, poisoned control (1% $HgCl_2$).

ples (Fig. 4B) remained essentially unchanged. The comparison indicated that the unchanged plate counts concealed a large shift in community composition. As shown in Table 1, up to 90% of the aerobic heterotrophs were hydrocarbon degraders 2 weeks after jet fuel contamination, although the initial percentage of hydrocarbon degraders was only 0.01. Although this shift appears to be rather extreme, Mulkins-Phillips and Stewart (7) have suggested that such ratios can reach 100%. As calculated from the data in Fig. 5, during the first 2 weeks, the net average doubling rates by hydrocarbon degraders in contaminated and bioremediation-treated soils were 20 h. This compared with about 30 h in contaminated but untreated soil. The latter figure corresponds reasonably well to the 1.8- to 2-day net doubling times reported for degraders in an oil-contaminated but unfertilized silt loam (4). These values also clearly demonstrate the benefit of the bioremediation treatment.

FDA hydrolysis activity. As evident from Fig. 7, in uncontaminated surface soil, FDA hydrolysis activity remained essentially unchanged during 18 weeks of incubation. In bioremediation-treated soils, jet fuel contamination first depressed FDA hydrolysis activity, but this brief depression was followed by a strong increase. The increase was of brief (3-week) duration at a contamination level of 50 mg g of soil^{-1}, but it kept increasing for 14 weeks in the case of a contamination level of 135 mg g of soil^{-1}. With no bioremediation, jet fuel contamination resulted in a lasting depression of FDA hydrolysis activity at both contamination levels. In a poisoned control (2% $HgCl_2$), a 90% inhibition of FDA hydrolysis activity was evident. While the benefit of bioremediation in terms of FDA hydrolysis activity was obvious from the results of this experiment, the depression of activity in contaminated but not bioremediation-treated samples was unexpected and required some explanation.

TABLE 1. Hydrocarbon utilizers as a percentage of aerobic heterotrophs in jet fuel-contaminated subsurface soil[a]

Time (wk)	% Hydrocarbon utilizers with the following levels of jet fuel (mg g of soil^{-1}):		
	None	50	135
0	0.01	0.01	0.01
0.7	0.05	0.02	3.10
2	0.04	90.80	70.60
4	0.46	58.30	81.90
10	0.04	25.30	26.90
16	0.03	18.80	9.40

[a] Calculated from the data in Fig. 4B and 6. Bioremediation was not applied to any of these soil samples.

Correlations between hydrocarbon residue levels, enumerations, and microbial activity. Correlations between various enumerations were discussed above, but it was also of importance to correlate enumerations and activity measurements with each other and with the levels of hydrocarbon residues prevailing at the time these measurements were taken.

In surface soil, more than half of the added jet fuel disappeared within the first 2 weeks of incubation. This period coincided with the fastest increase in microbial numbers and hyphal length, regardless of the enumeration techniques used. Microbial numbers and hyphal length peaked at about 4 weeks after the spill and gradually declined thereafter. Interestingly, the contamination level (50 or 135 mg of jet fuel g of soil^{-1}) made little difference in these two samples. Figure 1 shows jet fuel residues of 7.5 and 33.7 mg g of surface soil^{-1}, respectively, at week 4, when microbial numbers started to decline. The almost fivefold concentra-

TABLE 2. FDA hydrolysis activity of individual bacteria in jet fuel-contaminated soil

Jet fuel (mg g of soil^{-1})	Bioremediation	FDA hydrolysis activity at the following times (wk)[a]:					
		0	0.7	2	4	10	16[b]
None	No	4,000	2,000	1,000	4,000	750	810
135	Yes	4,000	600	36	6	73	1,000
50	Yes	4,000	510	8	4	33	310
135	No	4,000	2,000	77	16	79	100
50	No	4,000	1,000	98	19	77	130

[a] Units of FDA hydrolysis activity are 10^{-12} optical density units bacterium^{-1} h^{-1}. Calculated from the data in Fig. 2A and 7.
[b] Bacterial numbers were extrapolated from the data in Fig. 2A.

tion difference makes it unlikely that the exhaustion of hydrocarbon substrate brought about the decline in numbers. However, one may calculate that at the time of the downturn, about 80% of the originally applied jet fuel disappeared from both samples. We theorize that the remaining 20% of the jet fuel consists of hydrocarbons that are structurally less available for biodegradation, causing a downward trend in microbial abundance well in advance of the exhaustion of all hydrocarbons.

FDA hydrolysis activity, considered to be a broad and nonspecific microbial activity indicator (9), at first assessment, correlated poorly with enumerations. Correlation seemed to be restricted to the fact that bioremediation treatment of jet fuel-contaminated soils increased both numbers and activity. However, jet fuel alone increased numbers but apparently inhibited activity. We began to suspect that products of hydrocarbon biodegradation interfered with the FDA assay. Considering that FDA is cleaved by esterases and other hydrolytic enzymes (9), competition of hydrocarbon biodegradation products with FDA for the same enzymes is not an unreasonable assumption. When we calculated the amount of FDA activity per bacterium from the data in Fig. 2A and 7 (Table 2), this inhibition appeared to be severe. Nevertheless, the tremendous increase of microbial numbers in the bioremediation-treated samples overcame this inhibition and resulted in a net increase of FDA activity. This was not the case in samples without bioremediation treatment. The inhibition of the FDA assay by hydrocarbon biodegradation products caused the highest net FDA activities to be measured when most of the jet fuel biodegradation products were already mineralized (Fig. 7). At a contamination level of 50 mg g of soil^{-1}, this occurred at about 4 weeks; at a contamination level of 135 mg g of soil^{-1}, this occurred at about 15 weeks. The depletion curves of Fig. 1 support this deduction.

Our studies revealed a strong positive response by the microbial community of surface soil to jet fuel contamination up to the highest amount (13.5% [wt/wt]) that the moist soil was able to retain by capillary forces. Bioremediation treatment of the contaminated soil further increased bacterial numbers and hyphal length to the highest numbers ever reported in soil. These increases in numbers, in conjunction with activity measurements, point to the importance of biodegradative removal of jet fuel from soil, a process that tends to be underestimated in poisoned controls (10). The strong attenuation of the microbial responses in poorly aerated subsurface soil stresses the importance of ensuring an adequate oxygen supply during bioremediation of hydrocarbon-contaminated soils.

ACKNOWLEDGMENT

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