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Effects of oxygen, nitrogen, and temperature on gasoline biodegradation in soil *

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Abstract

Biodegradation was considered to be a feasible approach to remediate petroleum hydrocarbon-contaminated soil from a site at the University of Idaho. Before a full-scale treatment process was designed, the biodegradative capacity of the soil's indigenous microorganisms was tested. Gas chromatography was used to measure gasoline vapor components in the headspace above the contaminated soils held in closed containers. In a study of biodegradation kinetics, gasoline degradation rates under various conditions (different soil cores, temperatures, oxygen concentrations, and nutrient concentrations) were tested. It was found that gasoline hydrocarbons could be biodegraded at relatively high rates after appropriate nutrient additions. An unexpected observation was that the optimal concentration of oxygen for the gasoline-degrading microorganisms in these soils was only 10%.

Introduction

Contamination from leaks, spills, and other unplanned releases of gasoline, diesel, heating oil, and other petroleum products has become a major environmental concern. Gasoline components such as benzene, toluene, ethylbenzene, and xylene isomers (BTEX) are especially hazardous wastes, and are often found at sites on the EPA's priority list for remediation [1,2]. Leakage from underground storage tanks is a major source of gasoline contamination. It is estimated that more than 10% of the 3.5 million petroleum product storage tanks in the U.S. are leaking, and have caused at least 300,000 environmental incidents [3]. Not only does the petroleum leaked from these tanks contaminate the soil ecosystem, but it is also a potential long-term source of groundwater contamination. Both the biodegradation of petroleum in the environment [4–6] and the effect of environmental parameters on the biodegradation of oil sludge [7–10] have been well studied and reported in the microbiological literature. Many studies have shown that microbial degradation of petroleum hydrocarbons in the environment is strongly influenced by physical and chemical factors such as temperature, oxygen, nutrients, salinity, pressure, water activity, pH, and the chemical composition, physical state, and concentration of the contaminant; and by biological factors such as the composition and adaptability of the microbial population. Short-chain aliphatic hydrocarbons are generally thought to be more susceptible than other petroleum components to microbial attack [11, 12]. In experiments to evaluate and optimize the environmental parameters of 'land-farming', Dibble and Bartha [7] found that degradation was optimal at a soil water-holding capacity of 30 to 90%, pH 7.5 to 7.8, C:N ratio of 60:1; C:P ratio of 800:1, and temperature of $\geq$ 20° C. We recently initiated the cleanup of petroleum hydrocarbons that had leaked from a gasoline tank at the West Farm Operation Center, University of Idaho, Moscow, Idaho. The ability of the indigenous soil microorganisms to degrade petroleum hydrocarbons was measured as part of an initial assessment of the feasibility of bioremediation. In studies of the biodegradation of volatile organic chemicals in soil, it has been difficult to separate losses due to volatility from removal via microbial metabolism. Song and Bartha [13] studied the effects of jet fuel spills on the microbial community of soil, using soil columns with frequently opened covers and soil-containing beakers covered with thin

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polyethylene film, which were opened every 2 to 3 days. Because biodegradation and evaporation competed in removing petroleum hydrocarbons, biodegradative losses could not be differentiated from volatility losses. Other data were therefore collected, such as changes in the numbers of microorganisms and changes in microbial metabolic activities. The investigators noticed that losses through volatilization of volatile chemicals from biologically active samples could not be estimated from losses from poisoned controls, because of decreasing concentrations of components during the biodegradation process. Bioventing is a relatively new in situ soil restoration technology that removes hydrocarbons with both vacuum extraction of volatile chemicals and forced oxygenation for aerobic biodegradation. The biodegradation component of bioventing technology is not well understood, particularly in regard to laboratory investigations of hydrocarbon biodegradation in soils during vapor extraction. For some bioventing applications at petroleum-contaminated sites, data were simply recorded in the field to estimate the contribution from biodegradation. In the cleanup of petroleum-hydrocarbon-contaminated sites by bioventing, Dupont et al. [14] and Miller et al. [15] monitored the concentrations of O_2 and CO_2 in the off-gas. They assessed the hydrocarbon fraction undergoing biodegradation by measuring the consumption of O_2 or evolution of CO_2 by soil microorganisms. The volatilization of organic hydrocarbons in gasoline makes measuring their biodegradation difficult. Therefore, to assess the potential for biological treatment of petroleum contamination at the West Farm Operation Center, the rate of petroleum biodegradation by soil microorganisms in the contaminated soil was measured. The average rates of biodegradation for all petroleum hydrocarbons and some individual hydrocarbon components such as benzene, toluene, ethylbenzene, *o*-xylene, hexane, and methylethylpentane were determined. To overcome problems of measurement associated with volatility, we used gas chromatography (GC) to measure gasoline vapor in headspace above contaminated soils in closed microcosms. We also examined the nutritional and physical factors affecting the biodegradation of petroleum hydrocarbons by the soil microbial populations. Although the presence of oxygen is a main factor affecting the microbial oxidation of hydrocarbons, the effects of oxygen concentration on gasoline biodegradation have generally not been reported. We have been studying the biodegradation of hydrocarbons under various conditions (different soil cores, temperatures, oxygen concentrations, and nutrients). Direct microscopic counts and viable plate counts of microorganisms were conducted along with degradation time courses. Oxygen and carbon dioxide concentrations in the headspace, and the petroleum in the soil, were also measured. These data allowed us to reliably determine hydrocarbon biodegradation rates in a specific Idaho soil.

Materials and methods

Site description

A 500-gallon (1893 liters) underground storage tank was installed at the West Farm Operation Center, University of Idaho, in 1960 to store engine fuel for general mechanical operations. Fuel was purchased on an as-needed basis, but an inventory control record was never kept for the tank. After farm equipment was found running poorly in 1979, a check revealed that the tank had a maximum of eight inches (20 cm) of water underneath the fuel. The tank was assumed to be leaking and was excavated, cut in sections, and removed from the site in January, 1991. At the time of excavation it was found that the soil surrounding the tank had been contaminated by gasoline. The top of the storage tank was about four feet (1.2 m), and the bottom about eight feet, below the surface. Samples produced by soil drilling indicated that the gasoline and diesel fuel had leaked, diffusing into and contaminating about 800 cubic yards (610 m^3) of unsaturated soil. An asymmetrical plume of contaminated sediments occupied a volume approximately 25 feet (7.6 m) deep, 50 feet (15 m) long, and 25 feet (7.6 m) wide. The gasoline total petroleum hydrocarbon (TPH) in the soil samples ranged from 0.5 to 2900 ppm. The surface soils at the site were Palouse silt loams; some deeper samples were fine to coarse, poorly sorted granitic sands. The soils had low hydraulic conductivity. Available data also indicated that groundwater, which occurs at a depth of greater than 45 feet (15 m) had not been contaminated at this site [16].

Sample collection and storage

To obtain soil samples, test borings were drilled with a trailer-mounted SIMCO 2800 HS drill rig equipped with a $2^1/_4$-inch (i.d.) hollow-stem auger and a 140-lb safety hammer [16]. Soil samples from different depths, typically 5 feet (1.5 m) apart, were taken with

a 2-inch by 2-foot (o.d.) split-barrel sampler driven in advance of the auger. Four 6-foot brass liners were inserted into the sampler barrel to contain the samples. The samplers and the brass liners were cleaned and wiped with ethanol aqueous disinfectant between samplings. Samples were removed and stored in an ice cooler for transport [16] to the microbiological lab for microbiological biodegradation studies and to the University of Idaho Analytical Laboratory for contamination analyses. Microbiological samples were aseptically transferred to autoclaved Mason jars and stored at 4° C. Soil was sieved (size 10) before analysis.

Measurement of gasoline biodegradation by soil microorganisms

Five grams (wet weight) of a thoroughly homogenized soil sample were aseptically weighed and put into sterile 160-ml serum bottles. A small vial containing 10 mg of gasoline was placed into the bottle, and a Mininert standard gastight valve was immediately inserted to seal the bottle. The bottles were then incubated in the dark at varying temperatures. Enough time was allowed for the soil microorganisms to develop petroleum-hydrocarbon-degrading populations. After a 1.5-month acclimation period with periodic gasoline addition and degradation, the vial was removed, and the serum bottle with the soil sample was weighed. The bottle was flushed with a filtered (0.2-μm) air stream for about 8 min to drive part of the soil moisture (originally about 20%) and all of the gasoline residue out. After the weight of the soil sample decreased by 0.5 g, 0. 511 ml of an emulsified mixture (mixed with a vortex mixer) of 0.500 ml sterile double-distilled water (or 0.500 ml sterile nutrient solution) and 10 mg (about 0.011 ml) of fresh gasoline was added and mixed throughout the soil sample. Other concentrations were similarly prepared with different amounts of gasoline. Each bottle was immediately sealed with a Mininert valve, and the biodegradation of the gasoline hydrocarbon was monitored in the headspace of the soil sample by GC at various time points. Headspace hydrocarbons were sampled with a gas tight syringe (Pressure-Lok, Precision Sampling Corp., Baton Rouge, La.) by withdrawing 2a μl sample. Pristane was used as the internal standard for the GC measurements since its amount was relatively stable. Two weeks after biodegradation ended, petroleum residues in soil samples were extracted with methylene chloride (high purity solvent grade, Baxter Healthcare Corp., Muskegon, Mich.) in a Teflon-lined screw-cap flask at 4° C. The soil suspension was then filtered and washed with methylene chloride. The total extractant was brought to volume and the concentration of petroleum hydrocarbon was determined by GC.

Determining gasoline hydrocarbon concentration

To calculate the gasoline hydrocarbon concentration in the soil and in the vapor phase during biodegradation, a standard curve of the total peak area of gasoline hydrocarbons versus various amounts of gasoline vapor was prepared. A known amount of gasoline liquid was placed into an empty serum bottle identical to the bottles used in the soil petroleum biodegradation experiments. After 4 h the gasoline had evaporated and reached equilibrium. The total peak area of gasoline was then determined by GC. For each amount of gasoline, triplicate bottles were used for the standard curve measurements. To determine the soil adsorption isotherm of gasoline hydrocarbons, control measurements were done by GC with soil samples sterilized by autoclaving 1 h daily for three successive days. A mixture of a known amount of gasoline and sterile double-distilled water was spiked into the sterile soil, and the amount of gasoline in the soil and in the vapor phase was measured after various times by headspace GC. The amount of gasoline in the vapor phase was determined by comparing the GC peak area to the standard curve. The amount of gasoline in the soil was determined by subtracting the amount in the vapor phase from the total gasoline spiked to the soil initially. We thus obtained a control curve of the amount of gasoline in the soil versus the equilibrating amount of gasoline in the vapor phase, enabling us to determine at any time during biodegradation how much gasoline was in the soil alone and in the entire microcosm (soil and vapor) by measuring the gasoline in the vapor phase. The adsorption isotherms we obtained resembled a typical Langmuir adsorption isotherm. There were insignificant differences in the adsorption of various gasoline components that did not interfere with the utility of the method. We assumed that gasoline in the soil and in the vapor phase achieved equilibrium much more quickly than it biodegraded.

Hydrocarbon biodegradation under varying conditions

The biodegradation of petroleum hydrocarbons was measured for different samples, with varying numbers of indigenous microorganisms, from borings at four

depths at the contaminated site. The samples, designated as S1, S2, S3 and S4, came from depths of 26 ft, 5.3 ft, 5.8 ft, and 15.5 ft, respectively. For some samples, the biodegradation of petroleum hydrocarbon at different temperatures was also tested. We used temperatures of 11° C, 25° C, and 37° C, which corresponded to the approximate underground soil temperature in the Palouse region (of north Idaho/eastern Washington), room temperature, and the highest surface temperature in the summer, respectively.

Effect of oxygen concentration

To test the effect of oxygen concentration on biodegradation, mixtures of pure oxygen and pure nitrogen (this experiment was also repeated with pure oxygen and pure helium), with various oxygen concentrations (5%, 6%, 8%, 10%, 12%, 18%, 50%, and 90%) were used to flush bottles after the acclimation period. After the gasoline and double distilled water mixture was spiked to the soil, the petroleum hydrocarbon, oxygen, and carbon dioxide concentration were monitored in the headspace of the soil samples by GC.

Effect of nutrient condition

To test the effect of nutrient addition on hydrocarbon biodegradation, different amounts of ammonia vapor (from $NH_3 \cdot H_2O$) or NH_4NO_3 and K_2HPO_4 (Baker Analyzed Reagent, J.T. Baker Inc., N.J.) solutions were added to soil contaminated with 1780 ppm of petroleum hydrocarbons. For ammonia vapor, various amounts of $NH_3 \cdot H_2O$ (reagent grade, EM Science, N.J.) were placed into a small vial with final C:N ratios of 50:1, 18:1, and 1.8:1. The vial was then placed into the serum bottle before the addition of gasoline. For NH_4NO_3 and K_2HPO_4 solutions, 0.5 ml of 0.014% NH_4NO_3/0.0060% K_2HPO_4, 0.080% NH_4NO_3/0.032% K_2HPO_4, and 0.28% NH_4NO_3/0.10% K_2HPO_4, corresponding to C:N/C:P ratios of 300:1/3000:1, 50:1/560:1, and 15:1/172:1, respectively, were added directly to the soil with the gasoline. Petroleum hydrocarbon biodegradation was also measured by headspace chromatography.

Gas chromatography

Hydrocarbons were measured by using a Hewlett-Packard 5890A GC with a flame ionization detector (Hewlett-Packard Co., Palo Alto, Calif.), and a DB-1 column (50-m, 0.25-mm i.d., film thickness 0.5 μm; J & W Scientific, Folsom, Calif.). Helium was used as both carrier and make-up gas; compressed air and hydrogen supplied gas to the detector. The injector temperature of the gas chromatograph was set at 200° C and the detector temperature at 250° C. With 0.88 ml/min as the column flow rate, the initial temperature of the column remained at 40° C for 9 min, then went up to 90° C at a rate of 7° C/min. This was immediately followed by a temperature increase to 205° C at a rate of 5° C/min. The column remained at this final temperature for 9 min. A Hewlett-Packard 5890 Series II gas chromatograph with the same type of column was used to deliver samples to a particle beam vacuum desolvation interface connected to an MS quadruple detector (Hewlett-Packard 5989A MS controlled by HP 59940A MS ChemStation software, HP-UX series). The electron impact sample ionization mode was: repeller, 7 V; emission, 300 V; and electron energy, 70 eV. The source temperature was 250° C. Perfluorotributylamine was used as the calibration standard for the MS engine. The GC conditions were similar to the above measurements. Oxygen and carbon dioxide concentrations were determined with a Hewlett-Packard 5890 Series II gas chromatograph with a thermal conductivity detector (Hewlett-Packard Co., Palo Alto, Calif.), and a Haysep Q (80/100 mesh, 6 in by 1/4 in by 2 mm, Glass p/w) column (Alltech Associates, Inc., Deerfield, Ill.). The injector temperature was set at 50° C and the detector temperature at 60° C. For every measurement the column temperature was set at 40° C for 10 min. Nitrogen was used as both the carrier and reference gas. The column flow was adjusted to 30 ml/min.

Data analysis

Petroleum hydrocarbon depletion curves were analyzed with an equation similar to the Michaelis-Menten equation [17]. During biodegradation, petroleum exists in two compartments. Vapor-phase petroleum is not freely available to soil microorganisms. In soil, petroleum is assumed to be available to organisms only after emulsification and equilibration periods to dissolve and disperse the petroleum into or onto the aqueous phase. If at various initial soil petroleum concentrations S_S, the populations of the soil microorganisms and all other factors such as oxygen and nutrients are the same; and if we assume the diffusion of petroleum vapor in the soil is not limiting to petroleum biodegradation (a reasonable assumption since only a small

amount of soil was placed in the microcosm and the desorption, diffusion, and dispersion rate of gasoline vapor is very fast [18]); then the kinetics of petroleum biodegradation by soil microorganism can be written as:

$$-dS/dt = V_{max} S_S/(k_S + S_S), \qquad (1)$$

where S = S_S + S_V; S being the total petroleum in the headspace vapor and the soil. S_V and S_S represent hydrocarbon (substrate) concentration in the headspace vapor and in the soil, respectively; and k_S is a constant that represents the petroleum concentration in the soil when the rate of biodegradation is half of the maximum rate, V_{max}. V_{max} and k_S represent the microbial biodegradation rate only for petroleum in the soil, and not for the headspace. In biodegradation study the petroleum biodegradation rates were obtained from the initial slopes of the hydrocarbon degradation curves. V_{max} and k_S were obtained by best fits using a standard non-linear regression analysis computer program.

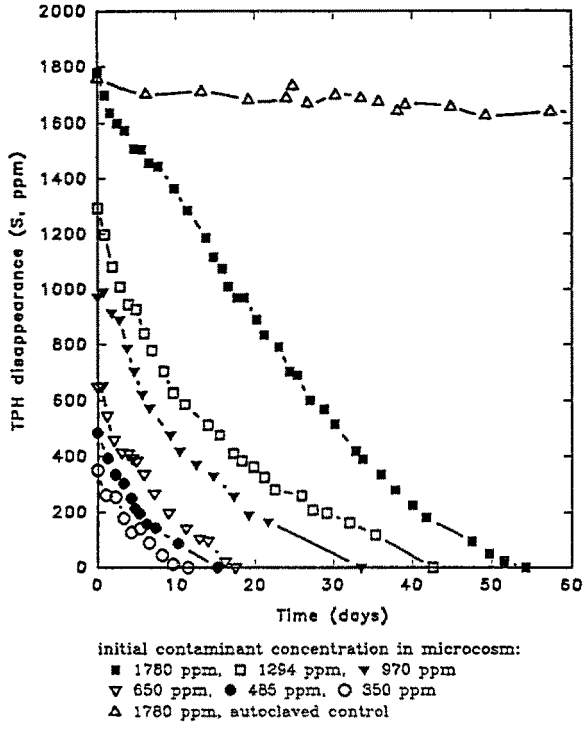

Fig. 1. Biodegradations of total petroleum hydrocarbon (S) by microorganisms in sample S3 for various initial levels of petroleum contamination, while other conditions were kept constant (25° C). Initial contaminant concentration in microcosm: ■ 1780 ppm, □ 1294 ppm, ▽ 650 ppm, • 485 ppm, and o 350 ppm. △ 1780 ppm, autoclaved control.

Results and discussion

Measurement of petroleum biodegradation in the soil

Biodegradation of petroleum hydrocarbon by adapted soil microorganisms was measured by GC of the headspace above the soil during biodegradation. The total hydrocarbon concentration in the microcosm at various times in the degradation period was then calculated. Figure 1 shows the microbial degradation of total petroleum hydrocarbon in sample S3 for various initial levels of petroleum contamination, while temperature was kept constant at 25° C. Biodegradation was measured as μg of TPH in a microcosm (in headspace and in soil) per g of soil (ppm) at different times, i.e., TPH (ppm) degraded by soil microorganisms over time. The same experiment at the highest contamination level is also shown for an autoclaved control. While the TPH in the autoclaved control was relatively unchanged during the experiment, the TPH in other microcosms gradually decreased, showing that the biodegradation of various concentrations of gasoline occurred in the contaminated soil if oxygen was available.

The biodegradation of petroleum hydrocarbons proceeded at different rates for different concentrations in the soil. Degradation rates vs. various petroleum concentrations in the soil are depicted in Fig. 2a. They were obtained from the initial slopes of the degradation curves in Fig. 1. The hyperbolic curve in the figure correlated well with the kinetics described above, confirming that equation 1 was a good model for observed petroleum degradation kinetics. The reciprocals of the TPH degradation rate and petroleum concentration in the soil, 1/V versus $1/S_S$, formed a straight line (Fig. 2b). V_{max} and k_S were obtained by best fits using non-linear regression analysis. In soil S3 the average rate of biodegradation for all gasoline hydrocarbons measured as V_{max} was about 114 μg of petroleum hydrocarbon per gram of soil per day (ppm/day), and k_S was about 120 μg of petroleum hydrocarbon per gram of soil (ppm).

The disappearance of TPH by microbial degradation was also measured at 25° C in samples S1, S2, S3, and S4 (silty clay, clay, clay loam, and clay with concretions, respectively) (Fig. 3). An initial TPH concentration of 1780 ppm was used for all these sam-

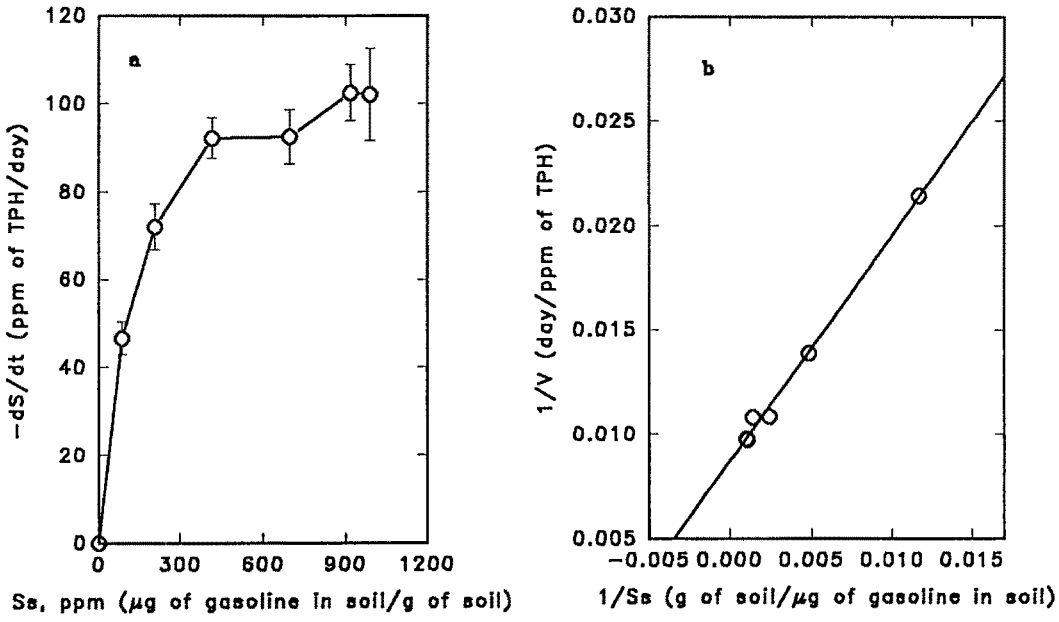

Fig. 2. Kinetics of petroleum biodegradation in soil. a. Biodegradation rates in the soil for various initial petroleum concentrations. b. Relationship of reciprocals of the TPH degradation rate and petroleum concentration in the soil: 1/V versus $1/S_S$.

Table 1. Microbial degradation rates of petroleum hydrocarbon in different soils (25° C).

Soil	Depth (ft)	No. of gasoline degraders[1] (CFU/g of dry soil)	V_{max} (ppm/day)	k_S (ppm)
S3	5.8	2.5 (± 0.2) E6	114 (± 6)	120 (± 2)
S2	5.3	2.9 (± 0.2) E5	33 (± 2)	196 (± 9)
S1	26	5.0 (± 0.5) E4	24 (± 2)	140 (± 5)
S4	15.5	1.2 (± 0.1) E6	55 (± 2)	141 (± 3)

[1] Colonies growing on mineral salts medium (solidified by purified agar) under an atmosphere containing gasoline vapor were counted by standard plate-count procedures.

ples, and other conditions were kept constant. The calculated V_{max} and k_S values and the numbers of gasoline-degrading microorganisms in the soil samples show that, under constant experimental conditions, petroleum degradation rates were directly related to the indigenous microbial population numbers [Zhou & Crawford, unpublished data] (Table 1. Numbers in parentheses are standard deviations). Other factors such as soil depth and soil particle sizes did not obviously influence biodegradation rate. For example, the deeper soil S4 had a higher biodegradation rate than the shallower S2 soil, even though nutrient supplies usually tend to be more limited the deeper the origin of the soil [19]. Further, even though the particle size is smaller for clay than for silt, the clay soil S4 had a higher biodegradation rate than silty clay soil S1. However, in Table 1 the cell-density-specific maximum rates (V_{max}/number of gasoline degraders) are not constant. One reason could be that numbers of gasoline degraders here were determined by viable cell counts, which are not as accurate as direct counts. Degradation rates and cell counts were, however, directly related. These data might also suggest that soil factors had certain effects on hydrocarbon biodegradation. They might have a cumulative effect on petroleum biodegradation rates via their effects on microbial metabolism. Various soil properties, e.g., specific surface area, pH, cation exchange capacity, clay mineral content, mois-

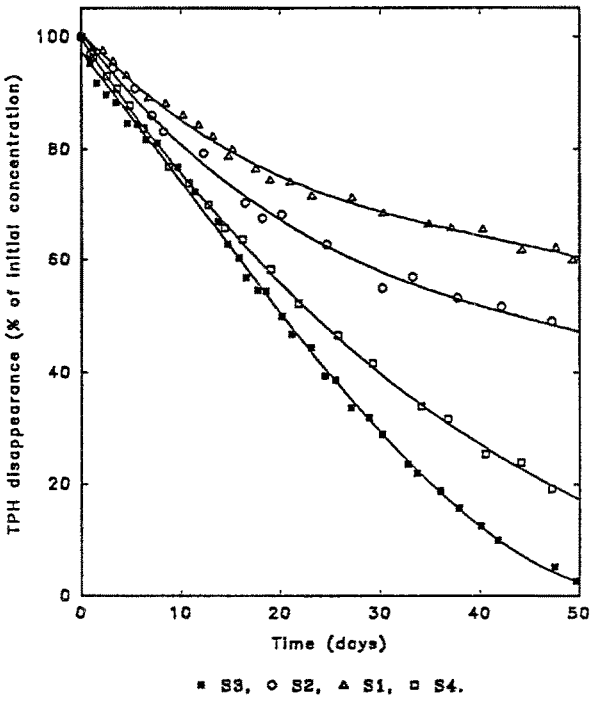

Fig. 3. TPH disappearance (S) by microbial degradation in soil samples S3, S2, S1, and S4, which represented clay loam soil, clay soil, silty clay soil, and clay soil with concretions, respectively. ■S3, ○ S2, △ S1, and □ S4.

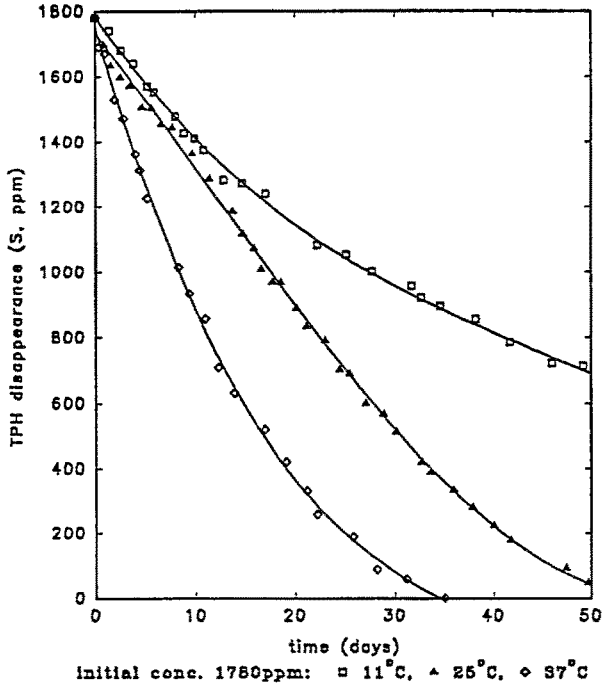

Fig. 4. Soil TPH biodegradation rates at 25° C, 11° C, and 37° C. Initial concentration 1780 ppm: □ 11° C, ▲ 25° C, and ◇ 37° C.

ture content, and nutrient absorbed on the soil particles, could affect the soil chemical biodegradation rate indirectly by affecting microbial population and activities in the soil [20].

Effect of temperature

Since soil temperature fluctuates widely during the course of bioremediation, we studied its effect on biodegradation of petroleum hydrocarbons in soil at temperatures corresponding to the approximate underground soil temperature in the Palouse (11° C), room temperature (25° C), and the highest surface temperature in the summer (37° C) (Fig. 4). Initial TPH concentration was 1780 ppm and other factors were kept constant. V_{max} and k_S values show that degradation rates increased with the temperature (Table 2).

Temperature generally affects the physical nature and composition of the petroleum, the rate of microbial hydrocarbon metabolism, and composition of the microbial communities [4]. Higher temperature increases the evaporation of short-chain alkanes and other low-molecular-weight hydrocarbons, which usually cause solvent-type membrane toxicity to microorganisms, and decreases the viscosity of the petroleum hydrocarbons and their solubility in the soil aqueous phase [4]. Low temperature sometimes lengthens the acclimation period and delays the onset of biodegradation. It also generally decreases microbial enzymatic activity, the so-called 'Q_{10}' effect.

Table 2. V_{max}, k_S, and Q_{10} values at various temperatures.

Temperature	V_{max} (ppm/day)	k_S (ppm)
11° C	62 (± 2)	281 (± 3)
25° C	114 (± 6)	120 (± 2)
37° C	135 (± 2)	117 (± 2)

Effect of oxygen concentration

Most biodegradation of petroleum hydrocarbons is aerobic since hydrocarbon oxidation processes generally require oxygenases [12]. Although it does occur, anaerobic biodegradation of petroleum hydrocarbons is usually negligible [4]. Generally, the greater the oxygen

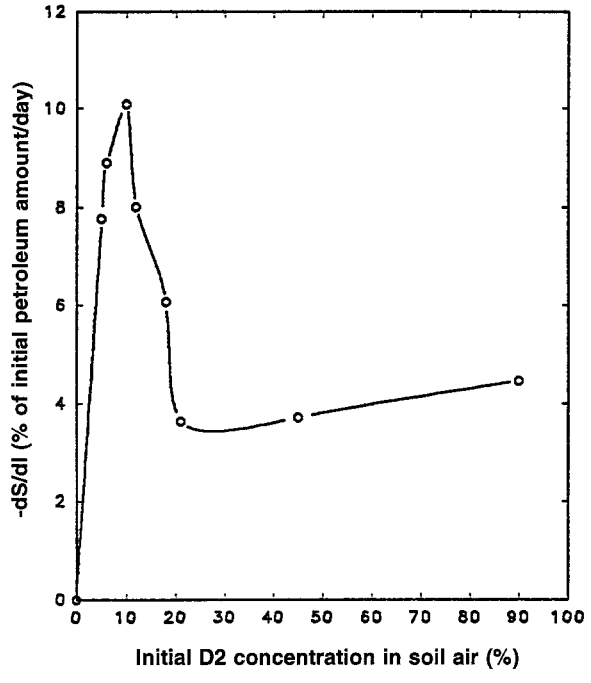

Fig. 5. Relationship between TPH soil microbial degradation rate (% of initial concentration per day) and the oxygen concentration in the supplied air (%).

supply, the faster biodegradation takes place, but our experiments with the relationship between soil microbial degradation rate (% of initial TPH concentration per day) and oxygen concentration in the supplied air (%) showed that, for gasoline, higher oxygen concentrations did not necessarily result in higher rates of aerobic biodegradation by soil microorganisms (Fig. 5).

Instead, there was an optimal oxygen concentration of about 10% oxygen in the air, which is about half of the atmospheric oxygen concentration. It is known that nitrogenase activity is higher, and that many aerobic nitrogen-fixing bacteria have higher nitrogen fixation rates when oxygen concentration is below the normal atmospheric level [21]. To determine whether a synergic effect between nitrogen and oxygen was affecting the rate of aerobic hydrocarbon biodegradation, and whether nitrogen played a role in providing nutrients throughout nitrogen fixation, helium instead of nitrogen was mixed with oxygen in the experiments. However, similar results were observed. A possible explanation is the adaptation of microorganisms to a decreased (less than the 21% found in air) oxygen soil environment through selective enrichment and genetic changes. The optimal oxygen concentration could also be genetically regulated, since certain gene networks, such as the *oxyR* and *arc* gene system, are known to regulate the effect of oxygen as an electron acceptor [22]. In practical terms, when bioventing is applied to a contaminated site, the venting rate might have to be controlled to increase the biodegradation of petroleum hydrocarbons, as well as to reduce the amount of gasoline released to the atmosphere.

Effect of nutrient concentrations

After heavy contamination by petroleum hydrocarbons, soil with low inorganic nutrients will have excessively high C:N and C:P ratios. Nutrients such as N and P then become the limiting factors for microbial degradation. Since N could be added to the soil air via vapor-phase ammonia, experiments were done to determine the effects of nutrients on petroleum degradation and the optimal concentrations of added N (Fig. 6). Biodegradation of 1780 ppm of TPH in soil S3 was studied with and without the addition of ammonia vapor (25° C) (Fig. 6a). Various amounts of ammonia vapor were added, corresponding to C:N (hydrocarbon:ammonia) ratios of 50:1, 18:1, and 1.8:1. In similar experiments with soil S2, the additions of ammonia vapor corresponded to C:N ratios of 50:1 and 18:1 (Fig. 6b). The appropriate amount of added nutrient clearly stimulated petroleum biodegradation in the soil. When C:N ratios were brought to 50:1 or 18:1, degradation normally taking 70 days when oxygen was available was completed in less than 20 days (Fig. 6a). Similar results were observed with added NH_4NO_3 and K_2HPO_4 solutions (Fig. 6c). These amendments brought the C:N/C:P ratios to 300:1/3000:1, 50:1/560:1, and 15:1/172:1, respectively. V_{max} and k_S values observed under different nutrient loads (Table 3) show that nutrients supplied either in vapor or solution enhanced the biodegradation of petroleum hydrocarbon in the soil. Ammonia vapor probably became available to soil microorganisms by diffusing to the soil and dissolving into the soil solution.

The optimal C:N ratio for S3 was around 50:1 (Fig. 6). However it might not be universal, since generally these ratios were different for different soils. Although petroleum hydrocarbons could be used as the carbon source for microbial growth, other nutrients such as nitrogen and phosphorus are also needed to biosynthesize primary metabolites, proteins, and other building blocks for cell biomass and microbial growth.

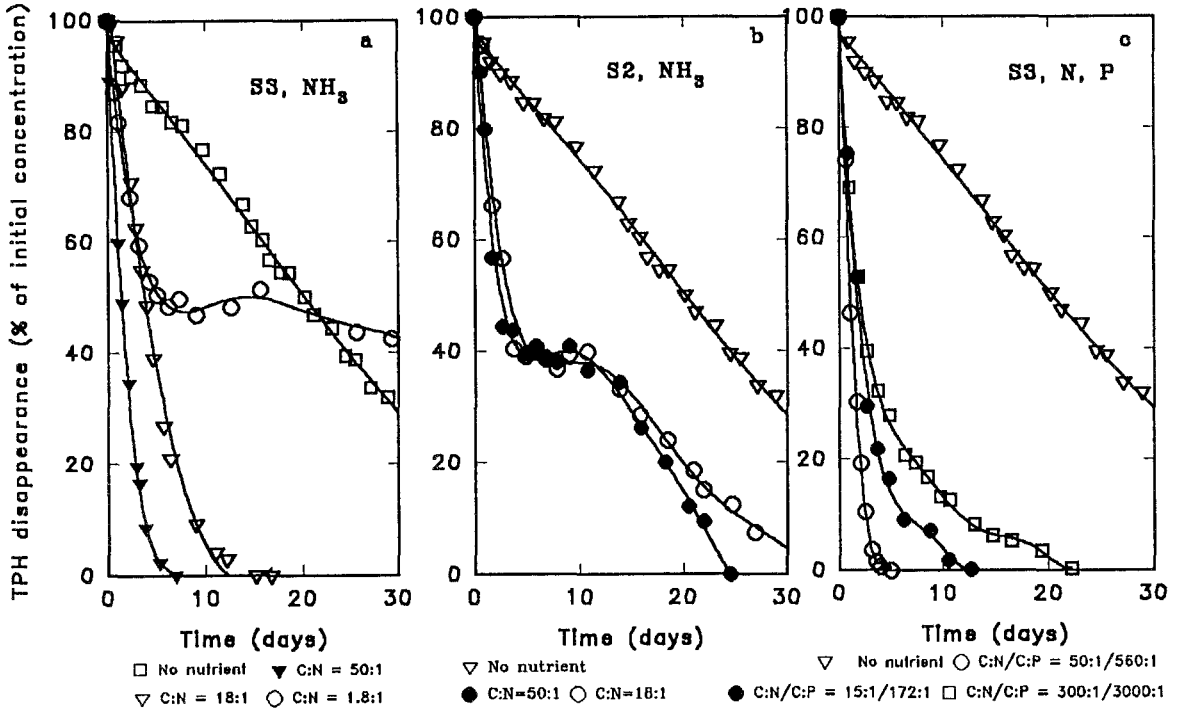

Fig. 6. Results showing the nutrient effects on petroleum hydrocarbon biodegradation. a. □ no nutrient, ▼ C:N = 50:1, ▽ C:N = 18.1, and ○ C:N = 1.8:1. b. ▽ no nutrient, • C:N = 50:1, and ○ C:N = 18.1. c. ▽ no nutrient, ○ C:N/C:P = 50:1/560:1, • C:N/C:P = 15:1/172:1, and □ C:N/C:P = 300:1/3000:1.

Table 3. V_{max} and k_S values observed under different nutrient loads.

Soil S3	V_{max} (ppm/day)	k_S (ppm)
No nutrient	114 (± 6)	120 (± 2)
C:N = 50:1	784 (± 3)	196 (± 3)
C:N = 18:1	243 (± 3)	145 (± 4)
C:N/C:P = 50:1/560:1	1046 (± 9)	137 (± 3)

Under low nutrient conditions, microorganisms did not have enough nutrient for optimal growth (C:N = 300:1); under higher nutrient conditions, the low C:N ratio probably favored microbial growth (C:N = 50:1, 18:1, or 15:1). Excessive addition of nitrogen (C:N = 1.8:1) almost stopped biodegradation, possibly due to ammonia toxicity that inhibited soil microbial growth, although this was not checked. However, a greater amount of fertilizer in itself has been shown not to inhibit biodegradation [7]. In that case, after large amounts of mineral nutrients were added, the addition of more hydrocarbons to adjust the C:N ratios to their optimum prevented any observable loss of soil microbial activity. Thus, C:N ratio might be the primary factor in determining the nutrient effect. These experiments indicated that the addition of a controlled amount of ammonia vapor to enhance the biodegradation fraction would be beneficial to bioventing.

Microbial degradation of priority pollutants

Experiments were done on the biodegradation of individual components of petroleum hydrocarbons that are U.S. EPA priority pollutants, including benzene, toluene, ethylbenzene, xylene (BTEX), and others. These components were identified by gas chromatography coupled with mass spectroscopy. Since the concentrations of different components of petroleum vary, for purposes of comparison the removal rate of each compound was measured as the percent of its initial concentration within the petroleum biodegraded in the soil (Fig. 7, Table 4).

Because rates were not V_{max}, only the rank order but not the specific values for degradation rate were considered. If the compounds are arranged accord-

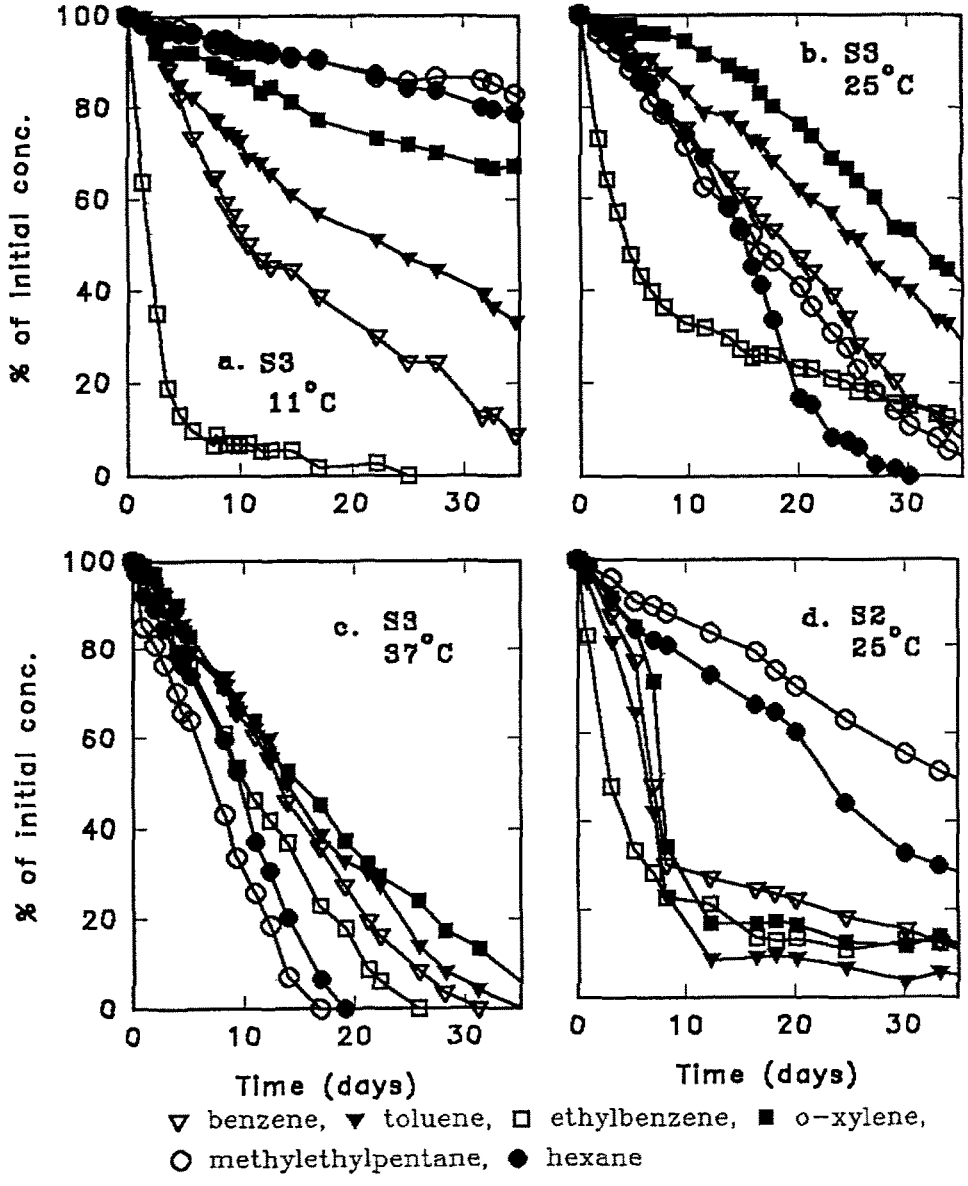

Fig. 7. Biodegradation of benzene, toluene, ethylbenzene, *o*-xylene, methylethylpentane, and hexane in two soils. ▽ benzene, ▼ toluene, □ ethylbenzene, ■ *o*-xylene, ○ methylethylpentane, and ● hexane.

ing to their average biodegradation rate, for the same soil the order of removal rates of different compounds at various temperatures was not the same. For soil S3 at 11° C, the degradation rates of different compounds ranged from high to low in the following order: ethylbenzene > benzene > toluene > *o*-xylene > hexane > methylethylpentane. At 25° C, the order was ethylbenzene > hexane > methylethylpentane > benzene > toluene > *o*-xylene, and at 37° C, it was methylethylpentane > hexane > ethylbenzene > benzene > toluene > *o*-xylene. Although the ranges of standard error overlapped, the biodegradation order of the compounds shown above remained true for each replicate experiment. This indicated that light aliphatic hydrocarbons such as hexane and methylethylpentane disappeared more slowly than aromatic hydrocarbons at low temperatures, but faster at higher temperatures. This might imply that as the temperature decreased, both the solubility of the short-chain aliphatic hydrocarbons in aqueous soil solutions and their toxicity to soil microorganisms increased, and their volatilization was reduced, so the microbial

Table 4. Effect of temperature on soil biodegradation rate.

Compound	Biodegradation rate (% of initial conc. in THP/day)			
	Soil S3			Soil S2
	11° C	25° C	37° C	25° C
Benzene	5.6 (± 0.5)	1.6 (± 0.5)	4.0 (± 0.6)	4.2 (± 0.6)
Toluene	2.5 (± 0.6)	1.5 (± 0.6)	1.9 (± 0.9)	5.6 (± 0.8)
Ethylbenzene	26 (± 1.8)	17 (± 1.4)	6.3 (± 0.5)	20 (± 1.9)
o-xylene	1.4 (± 0.5)	1.2 (± 0.5)	2 (± 1.0)	3.3 (± 0.5)
Methylethylpentane	0.8 (± 0.5)	2.5 (± 0.4)	23 (± 2.7)	1.8 (± 0.3)
Hexane	1.2 (± 0.5)	3.2 (± 0.4)	11 (± 4.8)	2.7 (± 0.5)

degradation rate decreased [4]. It might also imply that the activation energy for the rate-limiting reactions of aliphatic metabolism was higher than that of aromatic metabolism. In addition, it was found that the order of biodegradation rates was relatively similar for aliphatic and aromatic hydrocarbons. Ethylbenzene generally had the fastest, and *o*-xylene the slowest, biodegradation rate. It has generally been accepted that at normal temperatures short-chain aliphatic hydrocarbons are more susceptible to microbial attack and have faster biodegradation rates [11], as seen in S3 at 25° C, but some samples gave the opposite result. The removal rates of the above compounds in soil S2 at 25° C decreased in the order of ethylbenzene > toluene > benzene > *o*-xylene > hexane > methylethylpentane (Table 4). The aliphatic hydrocarbons were degraded more slowly than the aromatic hydrocarbons, which might come from the fact, as shown in other work [Zhou & Crawford, unpublished data], that S2 had a larger population of aromatic degraders. Probably microbial and soil factors as well as the properties of the specific chemicals determined their degradation rates. Short-chain aliphatic hydrocarbons might be toxic to soil microorganisms [12, 23], but with more microorganisms adapted to degrade them, as in soil S3, their degradation rate would be stimulated, since they were structurally simpler. However, if the soil microorganisms were not adapted to the aliphatic hydrocarbon contamination, their rate of biodegradation might be slow, which may be the case in soil S2.

In experiments with the effect of nutrient on soil microbial degradation of petroleum components at two temperatures, 11° C (Fig. 8a) and 25° C (Fig. 8b, 8c), the C:N ratio was 50:1 and other conditions were the same as previously. The sequence of removal rates (Table 5) for petroleum components with added ammonia vapor was ethylbenzene > benzene > toluene > *o*-xylene > hexane > methylethylpentane for S3 at 11° C; ethylbenzene > toluene > benzene > hexane > *o*-xylene > methylethylpentane for S3 at 25° C; and ethylbenzene > toluene > benzene > *o*-xylene > hexane > methylethylpentane for S2 at 25° C. In all experiments, the biodegradation rates of all compounds were faster than in experiments without added nutrient, and the biodegradation rates of most aromatic hydrocarbons had much higher increases than for aliphatic compounds. Therefore, for soil S3, even at a relatively higher temperature (25° C), degradation rates of the aromatic hydrocarbons (except *o*-xylene) were higher than those of the aliphatic hydrocarbons. The petroleum degradation curve (Fig. 6c) has two segments with different degradation rates, apparently reflecting the fact that the degradation of lightweight aromatic hydrocarbons was very fast after nutrient enhancement. The onset of aliphatic and other hydrocarbon biodegradation by a smaller aliphatic-degrading microbial population was delayed for some time after aromatic hydrocarbon degradation. The data in Table 5 imply that the addition of ammonia favored the biodegradation of aromatic hydrocarbons. Increased biodegradation could be caused by better availability of aromatic compounds, since the solubilities of the small aromatic hydrocarbons are higher than *n*-alkanes. When the population of petroleum-degrading microorganisms was stimulated by nutrient addition, the effect of the differing susceptibilities of aliphatics and aromatics was less obvious, due to the increased biomass available for processing petroleum compounds.

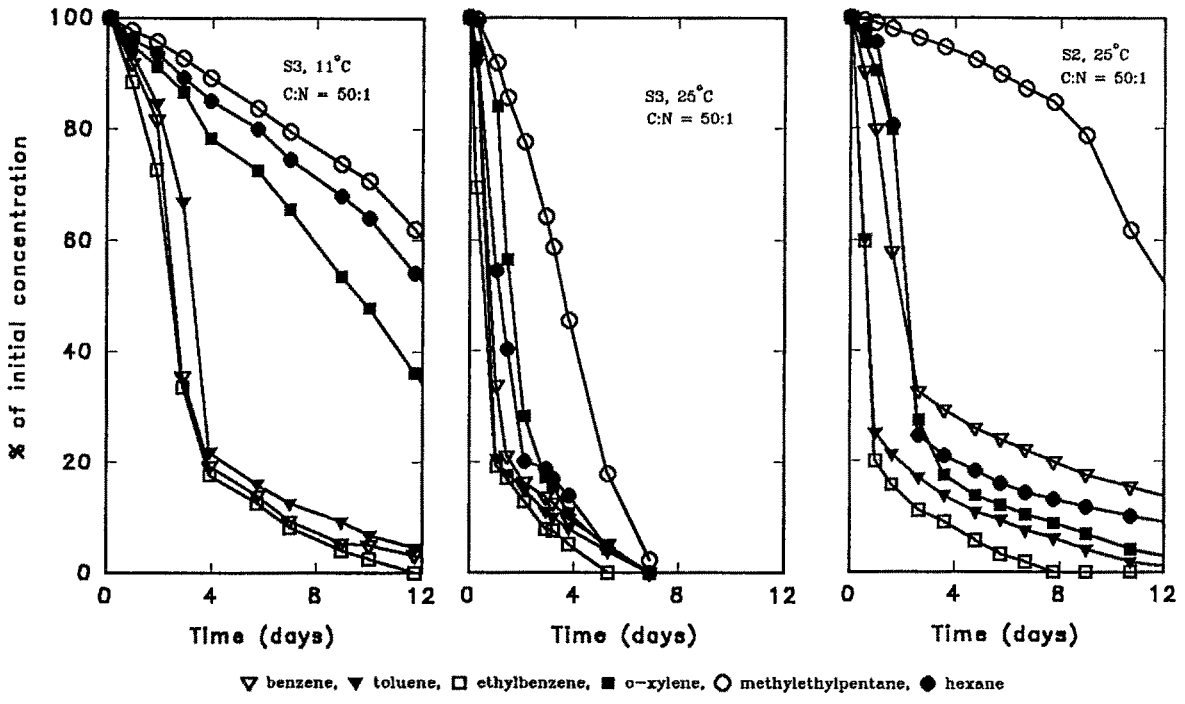

Fig. 8. Soil microbial degradation of benzene, toluene, ethylbenzene, *o*-xylene, methylethylpentane, and hexane when ammonia vapor was added; ▽ benzene, ▼ toluene, □ ethylbenzene, ■ *o*-xylene, ○ methylethylpentane, and • hexane.

Table 5. Soil biodegradation rate of petroleum components with added ammonia vapor (C:N = 50:1).

Compound	Biodegradation rate (% of initial conc. in TPH/day)		
	Soil S3		Soil S2
	11° C	25° C	25° C
Benzene	10 (± 1.8)	31 (± 4.8)	18 (± 3.2)
Toluene	8 (± 1.8)	35.9 (± 0.3)	75 (± 2.6)
Ethylbenzene	14 (± 1.9)	131 (± 1.9)	76 (± 2.2)
o-xylene	6 (± 1.0)	4 (± 1.1)	9 (± 1.0)
Methylethylpentane	2.9 (± 0.3)	1.3 (± 0.9)	0.9 (± 0.4)
Hexane	4.6 (± 0.7)	24 (± 6.0)	4.3 (± 0.8)

Measurement of oxygen

Metabolism of petroleum hydrocarbons generally requires oxygen as the electron acceptor. Petroleum-hydrocarbon-degrading microorganisms can use the hydrocarbons for biosynthesis and energy production, oxidizing them to carbon dioxide with the introduction of oxygen-utilizing oxygenase enzymes. In experiments on oxygen utilization during biodegradation with (Fig. 9a) and without (Fig. 9b) nutrient addition, about 7% of oxygen was left in the vapor in the microcosms (originally about 21%) after 1780 ppm of TPH were biodegraded in the soil. This was additional evidence that biodegradation did occur and that oxygen was required, which in turn implied that after the initial oxygen was consumed (< 5%, Fig. 5), it became a limiting factor, and the biodegradation of petroleum slowed, since actually in subsurface soil, oxygen amount in

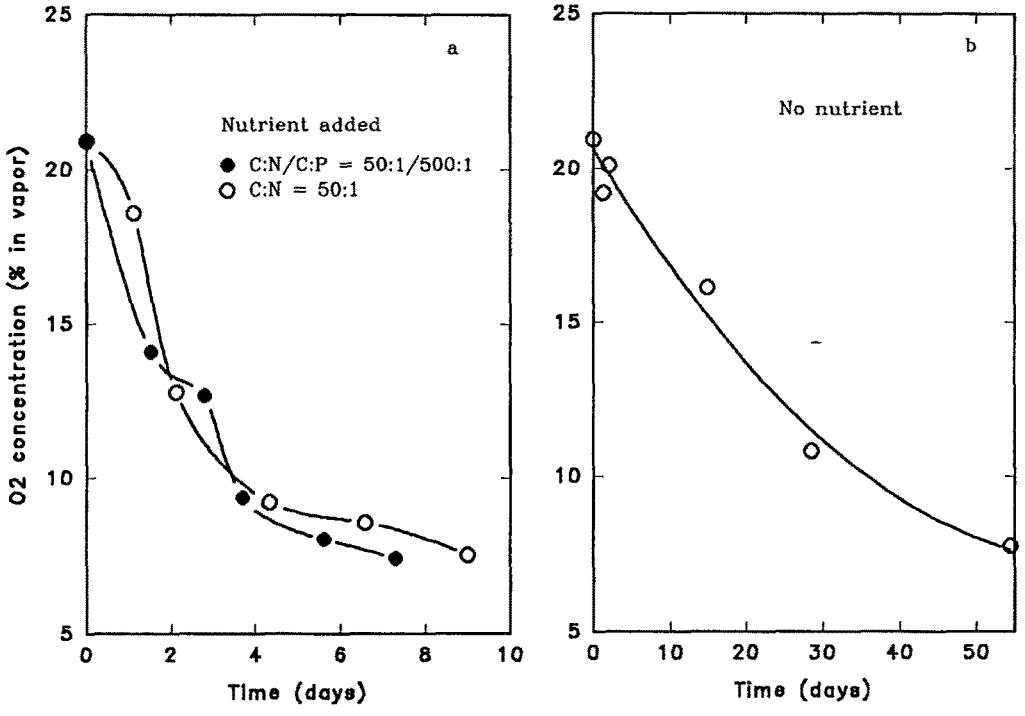

Fig. 9. Utilization of oxygen for the biodegradation of petroleum hydrocarbon in soil (a) with nutrient addition; (b) without nutrient addition.

soil vapor is very limited. As a final assessment of the extent of biodegradation of petroleum hydrocarbon in the soil, methylene chloride was used to extract the hydrocarbon residues from the soil two weeks after biodegradation appeared to be completed. Less than 10 ppm of TPH residue could be found in the soil, along with very small amounts of some nonvolatile polyaromatic hydrocarbon residues that were recalcitrant to biodegradation. However, since these concentrations were very low, nearly complete biodegradation of petroleum hydrocarbons in the soil was indicated.

Conclusions

Bioremediation should be an effective, ecologically acceptable, and economically feasible technique to remediate the petroleum-hydrocarbon-contaminated site at the West Farm Operation Center of the University of Idaho. The presence of petroleum-degrading microorganisms and the level of their metabolic activity were the main determinants of the feasibility of biotreatment. Studies were performed to determine and optimize the factors affecting metabolic activities of soil microorganisms. Kinetic studies showed that if oxygen was supplied to the soil microorganisms, the microbial community could adapt to the contaminated environment through selective enrichment and degrade the petroleum hydrocarbon at relatively fast rates. Studies of biodegradation under various conditions (different soil cores, temperatures, oxygen concentrations, and nutrients) showed that the contaminated soil at the West Farm Operation Center is nutrient limited for the biodegradation of petroleum hydrocarbon, and indicated optimal oxygen and nutrient concentrations for microbial growth and degradation of the contaminant. Some of these conditions were unpredicted (e.g., 10% oxygen) from previously published data.

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