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Bioremediation of Petroleum Hydrocarbons in Soil Column Lysimeters from Kwajalein Island†

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ABSTRACT

Soil column studies were used to evaluate petroleum hydrocarbon (PHC) remediation in soils from Kwajalein Atoll. Treatments included controls, and combinations of water, air, nutrients, and bioaugmentation with indigenous microbes (W, A, N, and M, respectively). Microbial colony forming units (CFU) decreased in the control columns and in treatments without air. Treatments including W + A + N and W + A + N + M exhibited increased CFU. One third of the PHC was removed by water and another third was removed by W + A + N and W + A + N + M treatments. Bioaugmentation with indigenous PHC degraders did not enhance bioremediation. Potential for bioremediation was demonstrated by air, water, and nutrient amendments.

Index Entries: Bioremediation; petroleum hydrocarbon degradation; soil columns; lysimeter studies.

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INTRODUCTION

Considerable research has documented the effects of petroleum hydrocarbon (PH) contamination in natural environments (*1,2*) and PH degradation (*1–4*). Long-chain alkanes are more slowly degraded by microorganisms than short-chained PH (*3*) but *in situ* total petroleum hydrocarbon (TPH) degradation has been documented at rates of 1 μg to >50 mg/kg/d (*1,4*). These findings make bioremediation feasible and attractive in remote locations where other options may be problematic.

The US Army Kwajalein Atoll (USAKA), located in the Republic of the Marshall Islands, has petroleum hydrocarbon contamination resulting from years of military activities (*5*). USAKA is located in the west central Pacific Ocean, approx 2100 nautical miles southwest of Honolulu, HI, and 700 nautical miles north of the equator. Given its remoteness, the lack of sophisticated remediation technologies and the amenability of petroleum hydrocarbons to biodegradation, a project was initiated to evaluate the feasibility of using bioremediation for environmental restoration of contaminated sites in the atoll.

In 1991, a team of scientists and engineers characterized the site (*5*), and observed the presence of TPH degrading microorgamisms (*6*). On site respirometric studies indicated that fertilizer amendments increased CO_2 evolution from contaminated soils (*7*), and treatability studies indicated significant stimulation of TPH degradation (*8*). Soil column studies were performed to verify operation and performance of selected bioremediation processes at the laboratory scale before field demonstration operations were initiated. The soil column studies focused on a soil profile that included increasing water content and TPH as depth increased from 1 to 2 m. The column studies focused on the lower vadose zone, capillary fringe, and upper portion of the saturated zone where free product and TPH globules were detected. The major questions addressed by this study concerned monitoring/measurement protocols, and effects of air, water, nutrient, and bioaugmentation on the rate and extent of bioremediation of Kwajalein soils.

METHODS AND PROCEDURES

Collection of Soil Cores

Twenty-five soil columns were collected from the USAKA bioremediation demonstration area. Field screening indicated the presence of hydrocarbon contamination in highly transmissive sediments of the 1.3–2 m depth zone corresponding to the capillary fringe and upper region of the saturated zone. Crushed coral sands ranging in particle size from silts to 5 cm in diameter from the 1.0–2.0 m depths were collected in stainless steel tubes (7 cm diam × 1 m long). The columns were capped and shipped

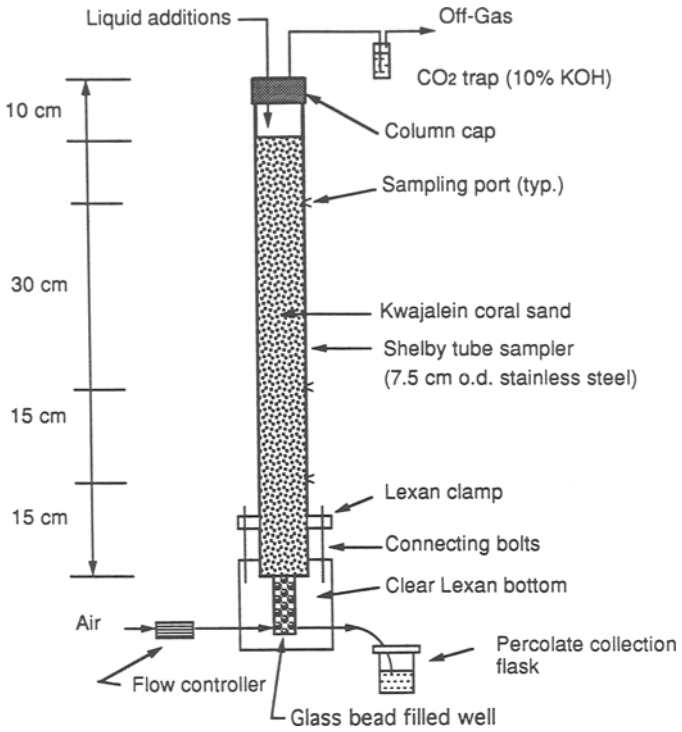

Fig. 1. Diagram of a test column.

in crates which were designed to remain upright during transport. Each shipping crate contained a minimum and maximum temperature recorder which indicated that the columns were subjected to temperatures from 18 to 37°C during shipment.

Column Setup

Three columns were sacrificed at time = 0, and sixteen columns were randomly selected for treatment regimens. The bottom of each column was placed into a 15 cm × 15 cm × 7 cm lexan block (Fig. 1) equipped with a 7 cm diameter hole extending 2 cm into the block. At the bottom of the hole was a butyl rubber o-ring that sealed the lexan block against the column. The base plate was held firm against the column with interlocking lexan clamps and bolts. Beneath the sealed hole in the lexan was a 2 cm diameter hole extending within 1.0 cm of the bottom of the lexan block packed with glass beads. Exiting the bottom of the lexan block were two fittings enabling gas to enter the column and liquid effluents to exit. The top of each column contained fittings for gas exit and for liquid entry (Fig. 1). Length of soil in the columns was 70 ± 5 cm. Temperature of the columns was maintained at 23 ± 2°C, similar to the environmental conditions.

Table 1
Microbial Colony Forming Units (CFU)
30 cm from the Bottom of the Kwajalein Soil Columns

Treatment	Log CFU[a] per gram soil	
	1 Mo	7 Mo
Control	6.8 ± 0.7	5.1 ± 0.2
Water only	7.6 ± 0.2	6.7 ± 0.4
Water + nutrients	7.2 ± 0.1	6.4 ± 0.5
Water + microbes + nutrients	7.3 ± 0.2	6.6 ± 0.5
Water + air	7.3 ± 0.1	6.8 ± 0.2
Water + air + nutrients	7.5 ± 0.2	7.4 ± 0.0
Water + microbes + air + nutrients	7.8 ± 0.2	7.6 ± 0.2

[a]CFU = colony forming units per gram soils. At t = 0 the average CFU in the sacrificed column numbers was 6.75 ± 0.3. Each value represents the average of four to six soil samples obtained 30 cm from the bottom of the soil columns.

Air additions at 10 mL/min were metered into each designated column. Before entering a column the air was passed through an inverted 15 mL sparging vial containing water and glass beads. Bubbling of gases through the glass beads verified air flow into columns. A timer actuated air flow at prescribed time intervals. Liquid additions were accomplished by a gravity-fed timer-controlled manifold system positioned 0.6 m above the columns. Three times each day 70 mL of liquid was transferred from either the nutrient vessel or the water vessel into tygon tubing and into 60 mL syringes that flowed into the corresponding columns. Air flow was curtailed immediately before and after the liquid additions.

Column Operations

The column treatments are shown in Table 1. Columns 1–3 received water plus nutrients. Columns 4–6 received water, nutrients, and air. Columns 7–9 received water, nutrients, and microbial inocula, whereas columns 10–12 additionally received air. Columns 13–14 received water only, columns 15–16 received water plus air, and columns 17–18 were controls receiving no treatment. Columns 19–21 were sacrificed at the beginning of the experiment for baseline characterization.

The nutrient mixture was a nitrogen-based fertilizer containing 0.65 m*M* NH_3, plus 0.56 m*M* KH_2PO_4, and 1.65 m*M* NO_3. A trace mineral solution (*9*) was added at 0.1*X* strength normally used in microbiological media. Final pH was 6.9–7.1.

Microorganisms added to designated columns were enriched from Kwajalein soils. Enrichments were maintained on a dilute medium con-

taining mineral salts, vitamins, phosphate buffer, and 10 mg/L of tryptone and yeast extract plus one of 10 carbon sources. Pentane, hexane, decane, or hexadecane were added at 40 μL/10 mL. Benzene, toluene, ethyl benzene, and xylene were added at 2 μL/tube. Tubes receiving diesel fuel received 5, 20, or 40 μL. Naphthalene crystals were added to four test tubes to make the final concentration above solubility limits.

Each week, six 1-L bottles containing 300 mL of media were used for culturing column inocula. The medium contained 300 mg/L yeast extract, 300 mg/L tryptone, plus 1000 mg/L glucose. Bottles were incubated three days at 20°C on a rotary shaker. Cells were harvested by centrifugation, washed, and resuspended in 60 mL of water. Column inocula corresponded to 120 ± 30 mg dry wt or $7 \pm 3 \times 10^{10}$ cells. At a coral density of 1.6 g/cc and average column volume of 2850 cc, the microbial inocula was $1\text{–}2 \times 10^7$ bacteria added per gram of soil in designated columns each week.

Liquid effluents were collected for one 24 h period each week. Effluents were analyzed for pH, conductivity, nutrient concentrations, and carbon dioxide. Carbon dioxide was trapped in a 10% KOH solution. The trapping solution was placed into 28 mL pressure tubes and attached to the vent line exiting each column receiving air flow. Evolved gas was bubbled through the 10 cm column of KOH before being vented to the atmosphere. Two-mL aliquots of the KOH trapping solution were placed into 28-mL pressure tubes, sealed, and acidified by injecting 1.5 mL of 30% HCL. A Shimadzu GC equipped with a thermal conductivity detector and helium carrier gas was used to quantify gaseous CO_2 (*10*).

Hydrocarbon monitoring utilized 3 g of soils, which were extracted with 2 mL of high purity iso-octane and then sonicated for 16 h in alternating 30-min cycles. Sediments were centrifuged, and the iso-octane was decanted. Another 1-mL volume of iso-octane was then added, sonicated for 4 h, centrifuged, decanted, and pooled. The iso-octane aliquots were analyzed for hydrocarbons on a Hewlett-Parkard 5890 GC at 50–250°C. C-19:0 served as the internal standard.

Total aerobic bacterial spread-plate counts were performed in duplicate with serial dilutions using a medium containing 10 mg/L each of peptone, tryptone, yeast extract, glucose, and trace minerals, plus Noble Agar (PTYEG).

RESULTS

Colony Forming Units

Total CFU found within the uppermost 10 cm of Kwajalein soils were typical of surface soils, being approx 10^7/g (data not shown). Below 10 cm, bacterial numbers decreased. The average CFU/g from the 1–2 m depth soils during the pretest sampling was log 6.75 (Table 1). After 1 mo all subcores exhibited higher CFU/g, likely reflecting stimulation of

Table 2
Carbon Dioxide Evolution from Kwajalein Soil Columns[a]

Treatment, column, wk	CO_2, μmol/d	Average CO_2 evolution rate for final 3 mo (μmol CO_2/d)
Water + air		110 ± 210
#16: wk 4	4980	
#16: wk 30	58	
Water + air + nurients		682 ± 242
#6: wk 4	4030	
#6: wk 30	1240	
Water + air + nutrients + microbes		484 ± 462
#11: wk 4	7020	
#11: wk 30	930	

[a]Carbon dioxide evolution was determined by gas chromatographic analysis of acidified KOH trapping solutions.

biological activity from sample procurement, shipping, and disturbance artifacts of column implementation (*11*). Biomass declined within the control columns through the 7-mo study. After 7 mo, the CFU/g of soil in control columns had decreased ~95%, from log 6.9 to log 5.6 (from nearly 10^7/g to 4×10^5/g). All columns not receiving air exhibited fewer CFU/g after 7 mo than the initial CFU/g of log 6.75. All treatments receiving water plus air exhibited CFU levels that were significantly greater than the initial values. These results showed the importance of air and water flow in maintaining and fostering microbial growth and survival in these petroleum-contaminated soils.

The addition of nutrients or the addition of microbes plus nutrients did not overcome the need for air and water flow in maintaining microbial density. More than 3×10^8 bacteria/g soil were added to the columns designated to receive microbial inocula over the course of 20 weekly additions. Despite the large influx of native microorganisms, the CFU/g were only slightly higher at 2.5×10^7/g after 7 mo compared to 1.6×10^7/g in treatments that received nutrients and air but no microbial inocula. These results suggested that additions of indigenously derived microorganisms did little to increase the biomass.

Carbon Dioxide Evolution

Carbon dioxide evolution was rapid during the first month and decreased dramatically in all columns during the final four months (Table 2). During the first month nearly all columns evolved >1000 μmol CO_2/d, likely as a result of degassing. In contrast, during months 5–7 less than 15% of the analyses revealed >1000 μmol CO_2/d. During mo 5–7 the columns

receiving water and air exhibited very little CO_2 evolution (Table 2) despite their viable biomass (Table 1). After 5–7 mo of operation, the CO_2 evolved varied as a function of operating conditions. For example, the columns receiving only air and water yielded less CO_2 (<200 μmol/d). Columns that received water + air + nutrients, with or without added microorganisms exhibited >400 μmol CO_2 evolution/d (Table 2). Columns that received bioaugmentation exhibited similar CO_2 evolution as those that did not receive additional microorganisms. This finding suggested that nutrient additions were required to sustain microbial populations and TPH biodegradation. Bioaugmentation did not appear to be required and did not increase the rate of CO_2 evolution.

It was suspected that the CO_2 traps were not 100% efficient at collecting CO_2. Evidence of this could be garnered from the CO_2 collection rate of the water + air treatment. If air flow had been 10 mL/min or 10,000 mL/16 h of air flow/d at 0.031% atmospheric CO_2, then 130 μmol of atmospheric CO_2 should have been collected each day. Extrapolation of results from the final four months of the water + air treatment suggested that the trapping efficiency was <70%. A lower trapping efficiency was substantiated by the $d^{13}C$ average of −14.45 (0/00) vs −7.9 for atmospheric air (data not shown), which suggested that a fraction of the 110 μmol of CO_2 from the air + water treatment during the final month of treatment (Table 2) was TPH derived (d = −27.8) while >66% may have been atmospheric derived CO_2 (d = −7.9) (data not shown). Trapping efficiencies were probably <70%. Accordingly, the CO_2 evolved from the water + air + nutrient treatments may have approached 1000 μmol/d.

One could estimate a hydrocarbon degradation rate based on knowledge of air flow, biodegradation stoichiometry, and carbon dioxide evolution. Air flow through the columns was targeted at approximately 10 mL/min. Assuming the TPH represented straight chained saturated aliphatics one could examine $C_{15}H_{32}$ biodegradation as a model compound. If a C_{15} aliphatic were oxidized to CO_2 a reaction could be: $23O_2 + C_{15}H_{32} \rightarrow 15CO_2 + 16\ H_2O$. In such a reaction 3 g of CO_2 would be evolved per gram of TPH degraded. In the case of the water + nutrient + air treatment an average of 660 μmol CO_2 was trapped each day, of which >530 μmol (660–130 μmol CO_2 from air) (25 mg CO_2) was derived from TPH. This would represent 75 mg TPH degraded each day for a daily degradation rate >25 mg/kg. If the CO_2 trapping efficiency was considered, the biodegraded TPH could approach 50 mg/kg each day.

Hydrocarbon Analyses

TPH concentrations in columns sacrificed at the initiation of the column studies were similar to the amounts seen in post-test characterizations (data not shown). Table 3 shows results of the TPH screening procedure as applied to subcores 30 cm from the bottoms of columns. TPH analyses

Table 3
Posttreatment Petroleum Hydrocarbon Concentrations of Subsamples Obtained 30 cm from the Bottom of Soil Columns[a]

Treatment	Petroleum hydrocarbons (PH) from screening procedure (mg/kg soil)	
	Total PH	Short-chained PH
Control	36,000 ± 44,000	6700 ± 2200
Water only	24,000	1300
Water + nutrients	11,000 ± 6000	2800
Water + microbes + nutrients	33,000 ± 3000	2600 ± 1400
Water + air	33,000	5100
Water + air + nutrients	30,000 ± 15,000[b]	2000 ± 700
Water + microbes + air + nutrients	22,000 ± 26,000[b]	1700 ± 1300

[a]Peaks eluting from 7–39 min were used for the total PH calculations, whereas peaks eluting from 7–20 min (approx C_6–C_{14}) were used for the short-chained PH calculations.
[b]If one outlying value was eliminated from each set both treatments would average 27,000 mg/kg.

included peaks integrated over a 7–39 min retention time while shorter-chain PH representing carbon fractions of approx C_6–C_{14} were integrated from 7 to 20 min retention times. Both TPH and short-chained fractions were highest in the control samples. The greatest reduction in TPH occurred when water was flushed through the columns. TPH contamination decreased approx 40%, presumably because of excessive water flushing. Other differences were minor when compared to water flushing. The addition of air and/or nutrients had an effect in some treatments, but the addition of microbes did not appear to facilitate TPH biodegradation. Control samples also exhibited the greatest contamination by short-chained PH. The water plus air treatment likely represented a bioventing scenario plus an estimate for evaporative losses or oxidative degradation of TPH. Short-chained PH accounted for half of the TPH in fresh fuels, but they represented <20% of the TPH in these weathered contaminated sediments. As with TPH, flushing with water resulted in the greatest impact. Additions of water + air + nutrients likely increased biodegradation of the lighter TPH. The addition of microbes had little effect on TPH biodegradation.

As shown in Table 4, most treatments did not significantly reduce the total number of PH peaks. TPH analyses of fresh diesel fuel and control columns revealed >330 separate peaks, 140 of which were in the short-chained PH grouping. The only treatment which reduced the TPH peaks to <300 was the water + air + nutrient treatment (data not shown). Flushing with water resulted in fewer short-chained PH peaks observed and in a greater percentage of the peak area fraction of the short-chained

Table 4
Number of Hydrocarbon Peaks Integrated from Subcores Analyses[a]

Treatment	Number of peaks integrated from subsamples 30 cm from bottom: Petroleum hydrocarbons, 7–39 min	Short-chained hydrocarbons, 7–20 min	Short-chained fraction, percent of peak area
Fresh diesel fuel	332	140	>33
Control	341	140	11
Water only	315	111	14
Water + nutrients	335	150	15
Water + microbes + nutrients	312	107	12
Water + air	313	101	24
Water + air + nutrients	314	73	6
Water + microbes + air + nutrients	320	115	15

[a]Peaks eluting from 7–39 min were used for petroleum hydrocarbon calculations, whereas peaks eluting from 7–20 min (approx C_6–~C_{14}) were used for the short-chained hydrocarbon calculations.

components. Increased peak area percent of the short-chained fraction was likely caused by the flushing of nonaqueous phase TPH flushed from the soils rather than solubilization of TPH into effluents. The only treatment that reduced the number of short-chained peaks was water + air + nutrients which resulted in <100 short-chained PH peaks. Addition of microorganisms did not result in fewer peaks integrated or in decreased short-chained PH fraction of the soil contents.

Differences between phosphorus additions, phosphorus removal, and the phosphorus content of biomass suggested that >99.9% of the phosphorus was unavailable to the biomass and never exited the column. All phosphorus analyses indicated effluent concentrations <2.5 mg/L; >90% were <0.5 mg/L (data not shown). Phosphorous was likely bound as calcium phosphate. Nitrate concentrations in the effluent ranged from 0 to 15 mg/L, but the average was less than 2.5 mg/L. Nitrite ranged from 0 to 6 mg/L, but >90% of the analyses were below detectable limits, and the average was <0.05 mg/L.

DISCUSSION

The results reported here suggest that the soil and groundwater of Kwajelein Island contain a wide variety of microorganisms which possess the ability to degrade fractions of the hydrocarbons. Soil column experiments demonstrated that existing microbial populations in Kwajalein

soils can be stimulated by the addition of nutrients and can degrade many of the hydrocarbons present. The nutrient stimulation and the low concentration of nutrients exiting columns indicate that the bacteria are likely starved for available nutrients. A further increase in biodegradative activity was not obtained by bioaugmentation.

Carbon dioxide evolution, changes in carbon isotope ratios of evolved carbon dioxide, decreases in TPH and short-chained PH in post-test analyses, and loss of chromatographic peaks in post-test PH analyses all indicated TPH biodegradation. These results agree well with literature that short-chained PH are more readily degraded and degradation rates of 10–50 mg/kg/day fit well within observed rates (*1–4*).

The laboratory-based biological observations, when viewed from a general perspective, support the concept of bioremediation for removing several hydrocarbon contaminants from the soil of Kwajalein Atoll. The climate and soil conditions of the atoll also favor a bioremediation approach. Based on these and previous findings, a technology demonstration has been initiated to test the feasibility of TPH bioremediation with a field-scale demonstration. Importantly, through this research, procedures for hydrocarbon analyses and QA/QC were developed and technologies transferred to the field, enabling on-site analyses of the bioremediation demonstration despite the remote geographic location.

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