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BIOREMEDIATION OF PETROLEUM-CONTAMINATED SOIL ON KWAJALEIN ISLAND: COLUMN BIOTREATABILITY STUDIES

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and
HAZARDOUS WASTE REMEDIAL ACTIONS PROGRAM
Oak Ridge, Tennessee 37831
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ACRONYMS AND INITIALISMS

ACD	Analytical Chemistry Division
CFU	colony forming units (microbial)
GC	gas chromatograph
HAZWRAP	Hazardous Waste Remedial Actions Programs
KOH	potassium hydroxide
ORNL	Oak Ridge National Laboratory
PDB	PeeDee belemnite standard for ^{13}C
PTYEG	peptone, trypticase, yeast extract, and glucose with Noble Agar
PTYG	peptone, tryptone, yeast extract, and glucose
RMI	Republic of the Marshall Islands
TPH	total petroleum hydrocarbon
TSA	Trypticase soy-extract
USAKA	U.S. Army Kwajalein Atoll
UT	The University of Tennessee, Knoxville
YEPG	yeast extract, peptone, and glucose

PREFACE AND ACKNOWLEDGMENTS

This study was made possible by funding provided by the U.S. Army and administered by the U.S. Department of Energy Hazardous Waste Remedial Actions Program (HAZWRAP). We gratefully acknowledge the support of John J. MacNeill and Donald W. Ott, U.S. Army Kwajalein Atoll (USAKA). We also gratefully acknowledge the assistance of Richard Machanoff, Kwajalein Program Manager for HAZWRAP, and Terry Donaldson, Oak Ridge National Laboratory (ORNL), for project management. Special thanks are due Joe Walker, Angie Walker, and Doug Pickering for field efforts. Robert Mackowski, Mike Morrissey, Susan Pfiffner, and Terry Donaldson assisted in column operations. Lee Cooper, Tony Palumbo, Janet Strong-Gunderson, and Susan Pfiffner assisted in laboratory experiments. David Ringleberg and Julia Starr are thanked for hydrocarbon analyses and interpretation.

The investigations reported in this technical report were conducted to determine the biotreatability of soil samples collected on Kwajalein Island, a USAKA facility in the Republic of the Marshall Islands. The material included here represents the collective efforts of a large group of scientists and engineers whose names are emphasized in previous documents describing related studies with Kwajalein soils. The biotreatability studies are part of a field-scale project designed to demonstrate and evaluate bioremediation as an environmental restoration technique for petroleum-contaminated soil on Kwajalein Island. The bioremediation demonstration project is being conducted by a multidisciplinary team of investigators from ORNL, Oak Ridge Associated Universities, and The University of Tennessee, Knoxville.

EXECUTIVE SUMMARY

Bioremediation technologies are being evaluated for use on Kwajalein Atoll, located in the Republic of the Marshall Islands. The study was undertaken by Oak Ridge National Laboratory (ORNL) on behalf of the U.S. Army Kwajalein Atoll (USAKA). During February 1991, a team from ORNL and The University of Tennessee, Knoxville, visited USAKA. In addition to making on-site observations regarding microbial abundance and distribution of petroleum contaminants, soil samples were collected for detailed analyses. In July 1992, a second team visited the island and obtained cored sediments from the proposed field site demonstration area for detailed treatability studies. This report documents the biological studies of these soil columns.

The soil and groundwater of Kwajalein Atoll contain a wide variety of microorganisms. The number of organisms per gram of soil ranged from 10^3 to 10^7. Many of the organisms present in soil samples containing petroleum contaminants have demonstrated the ability to degrade certain fractions of the hydrocarbons. The degradative activity of the native populations of organisms was stimulated by the addition of nutrient supplements. Some evidence indicates that the more heavily weathered hydrocarbons will be relatively difficult to degrade by biological methods.

Biodegradation of diesel-fuel contaminants in Kwajalein Island soils is possible by using indigenous microbes. This suggests that bioremediation of such soils is technically feasible as an environmental restoration process on Kwajalein Island. However, the reduction of diesel-fuel contamination to residual levels below 100 mg/kg by an in situ bioremediation process may be a difficult and lengthy process because of the proportion of more slowly biodegradable, higher-molecular-weight hydrocarbons present. Nutrient loadings stimulated biodegradation rates. The greatest change from controls occurred via water flushing and mechanisms to increase water flux and leaching. Combinations with surfactants may be worthy of consideration. It would be likely that treatment of a few years with water + air + nutrients could significantly reduce the concentrations of the lighter total petroleum hydrocarbons. Results from the column studies emphasize that in situ bioremediation should include addition of nutrients and air, and enhanced water circulation should be considered. In addition, alternative types of phosphate should be considered as it was likely bound to calcium in the soil column studies.

1. INTRODUCTION

The U.S. Army Kwajalein Atoll (USAKA) Base is located in the Republic of the Marshall Islands (RMI) in the west-central Pacific Ocean. USAKA (Fig. 1) is located ~2100 nautical miles southwest of Honolulu, Hawaii, and 700 nautical miles north of the equator. The Kwajalein Atoll forms the largest enclosed lagoon in the world and consists of ~100 small islands with a total land area of 5.6 mile^2. Kwajalein Island is ~3.5 miles long by 0.3 to 0.5 miles wide with a land surface area of ~1.2 mile^2. The population of the Base, including Army and subcontractor personnel and their families, is ~3000.

The United States and RMI recently negotiated a Compact of Free Association allowing the United States exclusive use of 11 islands within the Kwajalein Atoll. The Compact stipulates that the environment of the atoll will be protected in accordance with U.S. environmental laws (e.g., Resource Conservation and Recovery Act; Comprehensive Environmental Response, Compensation, and Liability Act; Toxic Substances Control Act; and Clean Water Act). However, the mechanism for implementing and verifying compliance is not clear. For example, the U.S. Environmental Protection Agency, Region IX, has determined that it has no regulatory authority over Kwajalein Atoll.

USAKA has petroleum hydrocarbon contamination resulting from years of military activities. Given its remoteness, the lack of sophisticated remediation technologies and on-site waste disposal facilities, and the amenability of petroleum hydrocarbons to biodegradation, USAKA requested, through the Hazardous Waste Remedial Actions Program (HAZWRAP), that a project be initiated to evaluate the feasibility of using bioremediation for environmental restoration of contaminated sites within the atoll. If feasible, a technology demonstration would be initiated to document this feasibility and provide design, operation, and performance data for full-scale remediation on the atoll. In January 1991, HAZWRAP commissioned a team of scientists and engineers from Oak Ridge National Laboratory (ORNL), Oak Ridge Associated Universities (ORAU), and The University of Tennessee, Knoxville (UT) to conduct this project.

In February 1991, personnel from ORNL and UT visited the island and confirmed the existence of contaminated soil and water sites. The site characterization and on-site experiments resulting from this visit have been reported previously (R. L. Siegrist et al., *Bioremediation Demonstration: Site Characterization and On-Site Biotreatability Studies*, 1991, ORNL/TM-11894). Siegrist and coworkers demonstrated that substantial numbers of live microorganisms are present in Kwajalein soils and water and have the ability to degrade some petroleum hydrocarbons.

During this visit, samples of Kwajalein soil, groundwater, and sewage were obtained and were transported to Oak Ridge for use in experimental studies. The results of the bench-scale experiments examining treatability of soil columns are presented here. The overall goal of the soils column studies was to verify operation and performance of selected bioremediation processes at the laboratory scale before field demonstration operations. Importantly, the soils column studies focused on a soil profile with depth, which included increasing water content and total petroleum hydrocarbons (TPHs) as depth increased from 3–6 ft. 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.

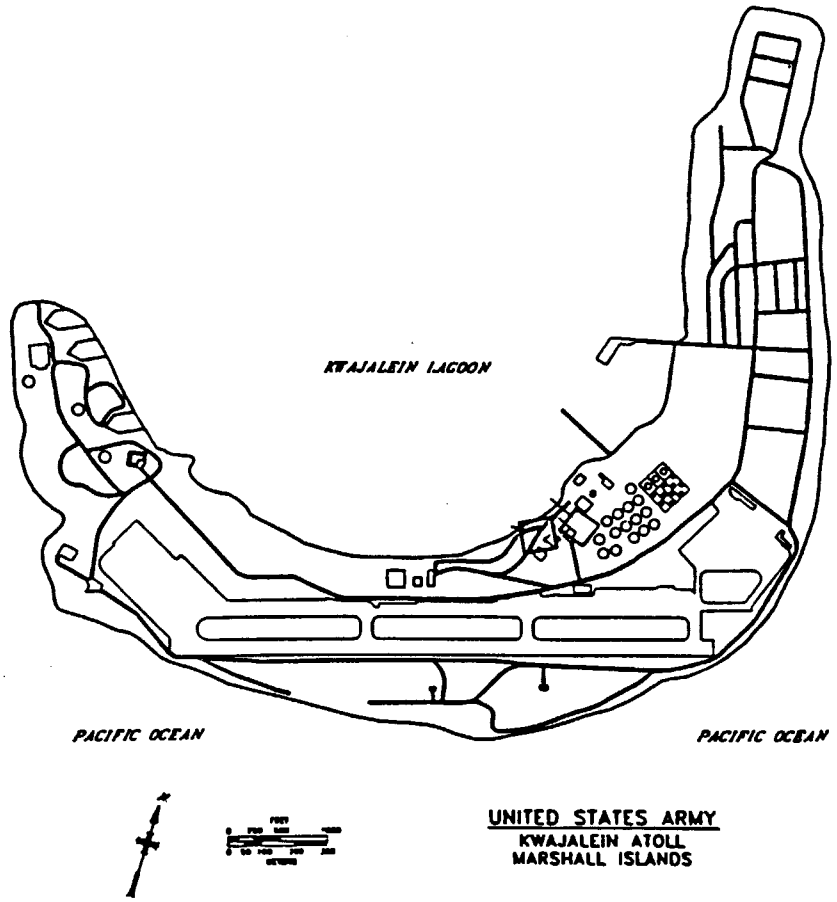

Fig. 1. Site map of the construction area on Kwajalein Island (ORNL DWG 91-12745).

The major questions concerned subsurface hydraulics, microbiology, monitoring/measurement protocols, and the effects of air, water, nutrient, and bioaugmentation on the rate and extent of bioremediation of Kwajalein soils in vertical profiles. Some of the specific questions addressed were: (1) What range of diesel-fuel components can be degraded by the bacterial species present in the Kwajalein soil and water samples? (2) Will the rate of biodegradation be enhanced by supplying nutrients? (3) Will the rate of biodegradation be enhanced by adding commercially available microbial inocula? (4) Are hydrocarbons readily available for biodegradation? (5) Are any health risks posed by stimulating the growth of the naturally occurring microbial population? and (6) Is it possible to develop biological indicators that will allow real-time, on-site assessment of the nature and health of the microbial population during the course of the demonstration project?

2. METHODS AND PROCEDURES

2.1 COLLECTION OF SOIL CORES

Twenty-five columns of soil were collected from the USAKA bioremediation demonstration area beginning on July 13, 1992. Crushed coral sands from approximately 3- to 6-ft depths, which prior information indicated was the contaminated zone of interest, were collected in Shelby tubes (3 in. diam × 36 in. long). The sampling tubes were thin-walled, stainless steel construction with a beveled cutting edge at the boring end and holes at the top end for mounting to the coring drive-head. Cores were collected within an 8-ft × 8-ft area adjacent to the proposed field demonstration site (Fig. 2). Field measurements included temperature, pH, and field determination of approximate hydrocarbon concentrations using a Hanby test kit (Siegrist et al. 1991). Field screening indicated the presence of hydrocarbon contamination in the 4.5- to 6-ft depth zone. This depth corresponded to the capillary fringe and upper region of the saturated zone.

2.2 SOIL CORE HANDLING AND TRANSPORTATION

Field screening for TPHs was accomplished with a Hanby test kit. Field results suggest that contamination was negligible at the surface, approximately 500 mg/kg at depths of 4.5 ft, greater than 3000 mg/kg at 6-ft depths. Twenty-five columns were collected, capped with plastic end caps, taped, and stored upright. Columns were shipped in crates, which were designed to remain upright during transport. Crates and columns were express-shipped without refrigeration. Each shipping crate contained a minimum and maximum temperature recording thermometer. Air temperatures within the shipping crates remained between 18 to 37°C.

2.3 COLUMN SETUP

Upon arrival at the Center for Environmental Biotechnology, UT, on August 5, the cores were visually inspected. The 16 longest and most circular columns were selected for the treatment study. The next longest three columns were retained as control columns. Three columns were sacrificed at time = 0, and three were too damaged to use. The 16 longest columns were randomly selected for treatment regimens. Each column was cut two times with a pipe cutter. The holes at the top of the column where the drill-head attaches to the sampling barrel were cut off. In all instances, approximately 10 cm of headspace was left between the uppermost sediments and the top of the test column. In addition to cutting the top of each column, the bottom was also trimmed. Care was taken to remove as little of the sediment zone as possible yet providing a round bottom of the core liner for emplacement into the lexan bottom plate. Even in undamaged cores, it was necessary to remove at least 2 cm from the bottom of the core liner to avoid the beveled cutting edge and ensure a flat seal with the butyl rubber o-rings of the lexan bottom plate. Length of soil in the trimmed columns was 70 $\pm$ 5 cm.

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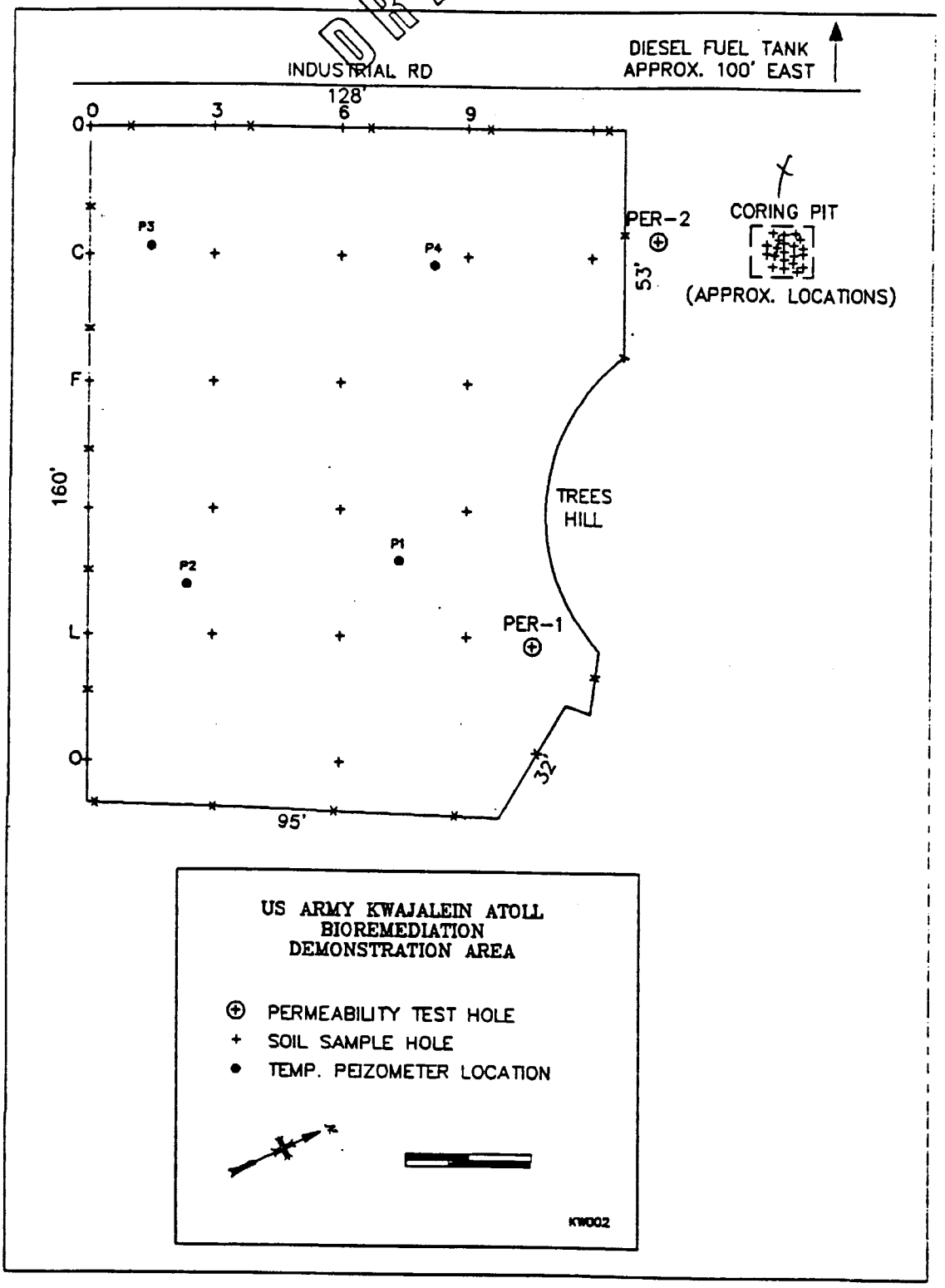

Fig. 2. Schematic of plot and sampling site.

The bottom of each column was placed into a 6 in. × 6 in. × 3 in. piece of clear lexan (Fig. 3). One side of the lexan block was polished to provide a viewing window. Each lexan block was equipped with a 3-in.-diam hole extending 1 in. 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 (Fig. 3). Beneath the sealed hole in the lexan was a 1.5-in.-diam hole that extended within 0.5 in. of the bottom of the lexan block. Within the internal diameter of the 1.5-in. hole, glass beads were packed. These glass beads held the coral sands in the core liners as well as provided mixing for entering gas phases. One side of this 1.5-in.-diam hole was polished, enabling viewing of the glass beads. Exiting the 1.5-in.-diam hole and extending out the bottom of the lexan block were two Swagelok fittings. A 1/8-in. fitting enabled gas to enter the lexan block for passage upward through the column. A second fitting was 1/4-in. Swagelok and provided a port for sampling and collecting liquid effluents exiting the column.

The top of each column was fitted with a plastic end cap equipped with two 1/4-in. Swagelok fittings (Fig. 3). One fitting was for gas exit, while the other was for liquid entry. Unused fittings were clamped closed. In addition to the end caps, holes were placed in each column 15, 30, and 60 cm from the bottom of the soil column. Holes were drilled using a nitrogen-gas-cooled drill bit attached to a drill press. Holes were 0.5 in. in diameter and sealed with 22-mm teflon-lined rubber septa. The holes provided sampling ports for obtaining subcores used for biological analyses during the course of the treatment study. The septa were held in place with 3-in. hose clamps. All columns were placed in the upright position approximately 12 in. above a laboratory bench.

Air additions were accomplished through a series of flow meters. Airflow, targeted at 10 mL/min (often 10–20 mL/min), passed through dedicated flow meters for each column. Before entering a column, the airflow was passed through a liquid and glass bead-filled sparging vial. Sparging vials were inverted 15-mL serum vials that contained 10 g of glass beads and 5 mL of water. Bubbling of gasses through the glass beads verified airflow into each designated column. Control of air addition was accomplished by a timer that actuated airflow at prescribed time intervals. The building air supply was used to charge two 15-gal pressure tanks at 60 psi. Reducing regulators and air-purifying cartridges were downstream of the pressure tanks. This system enabled air to flow to the columns for up to 16 h after any event that terminated the availability of the building air supply.

Liquid additions were accomplished by a gravity-fed, timer-controlled manifold system that was positioned 2 ft above the columns (Fig. 4). At designated time intervals, 70 mL of liquid were transferred from either the nutrient vessel or the water vessel into tygon tubing and 60-mL syringes. A delayed time release then closed the valve at the liquid reservoirs and opened the valves to the corresponding columns. Airflow was always curtailed immediately before and after the nutrient additions.

2.4 COLUMN OPERATIONS

All chemicals used were reagent grade and were obtained from Mallinckrodt (Paris, Kentucky) or Sigma Chemical Company (St. Louis, Missouri). Resi-analyzed, glass-distilled solvents were purchased from the J. T. Baker Chemical Company (Phillipsburg, New Jersey).

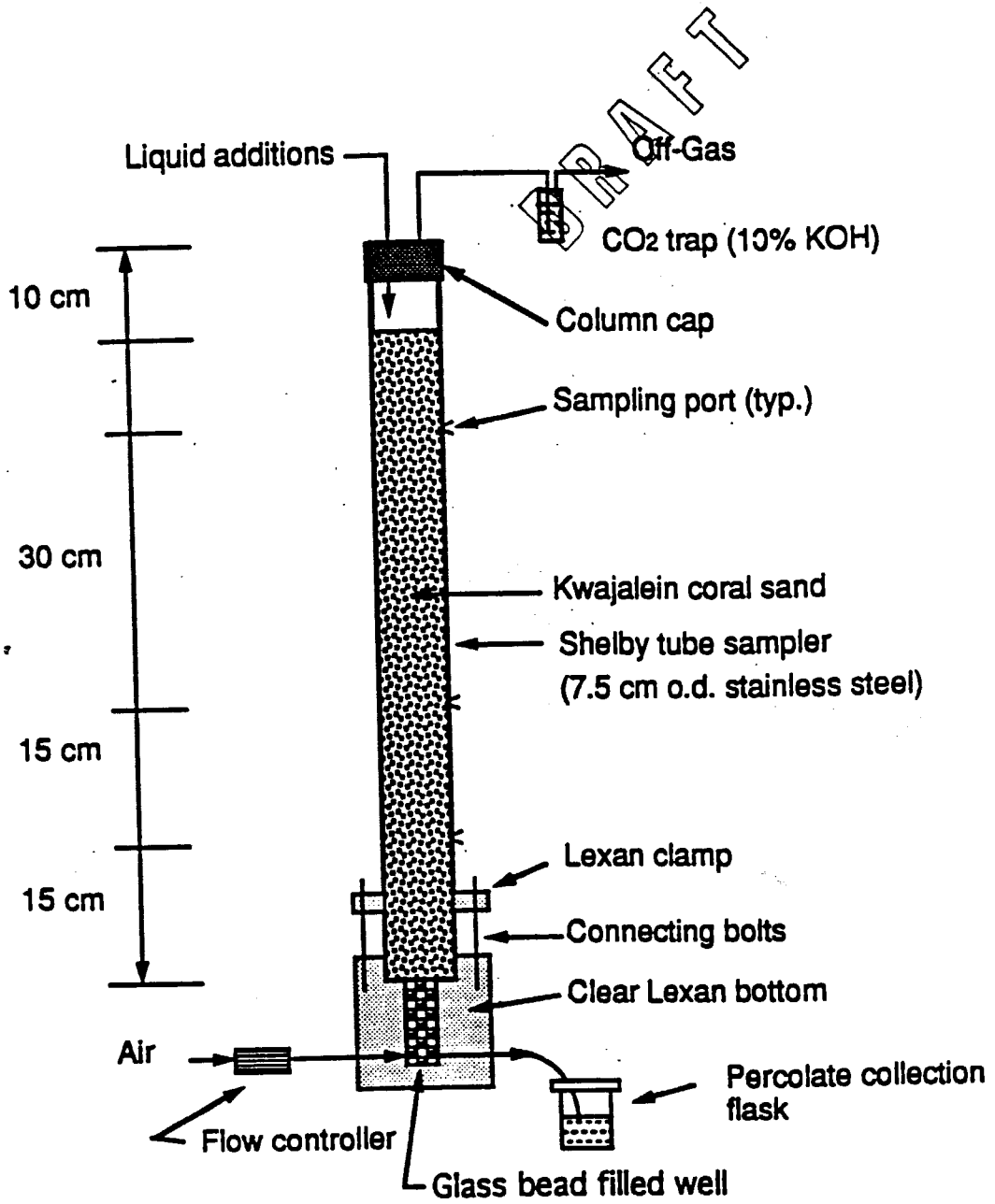

Fig. 3. Diagram of a test column.

Fig. 4. Picture of column installation.

Column studies were initiated on August 8, 1991. Conditions used for the various column treatments are shown in Table 1. Columns 1–3 received water + nutrients. Columns 4–6 received water + nutrients + air. Columns 7–9 received water + nutrients + microbial inocula, while columns 10–12 additionally received air. Columns 13–15 received water only, and columns 16–18 were controls receiving no treatment. Columns 19–21 were sacrificed at the beginning of the experiment for baseline characterization. Treatments continued from August 8, 1991, through the end of March 1992. Additions of microorganisms ended January 7, 1992.

Timed nutrient or water and air delivery were as follows: At 5:00 p.m. each day air delivery was initiated. At 8:00 a.m. air delivery ceased. At 9:00 a.m., 12:00 p.m., and 3:00 p.m., a liquid dose of 70 mL was applied to the columns for a total of 210 mL/d. At 5:00 p.m., air sparging resumed. After each power outage, the scenario was reset. Notes were kept in a log notebook, which identified power outages, plugged flows, other problems, and observations.

Nutrients were prepared in 20-L batches using reverse osmosis purified water, which was pumped into a reservoir located 2 ft above the reactor tops. Final pH of the nutrient mixture was 6.9–7.1. A commercial agricultural fertilizer was used as the base fertilizer to which supplements were added. The nutrient amendments derived from the fertilizers included three macronutrients (a mixture of organic/inorganic nitrogen plus inorganic phosphorus and potassium) and seven micronutrients (sulfur, boron, copper, iron, manganese, molybdenum, and zinc). The fertilizer solids were dissolved in deionized water to yield stock nutrient solutions. The nutrient solution included N:P:K, which were at a mass ratio of approximately 32:17:23, respectively. The objective was to apply technical-grade fertilizer in a 2.3:1.0:1.9 ratio. Unfortunately, the commercial technical-grade fertilizers did not adequately dissolve in these solutions or precipitated upon storage. Consequently, the nutrient mixture was a 1.5-mM ammonium-based fertilizer to which 0.56 mM KH2PO4 was added in addition to an extra 1.65 mM NO_3. In addition to these macronutrients, a trace mineral solution was added at 0.1 X strength normally used in microbiological media. The trace mineral solution was the same constituents as used in microbiological media. It contained μM concentrations of copper, zinc, borate, selenium, etc.

Each week microbial inocula were added to the columns that were designated to receive microbial inocula (Table 1). Commercial strains of microorganisms were not used. All microorganisms added to the columns were from enrichments established from Kwajalein contaminated sites. Enrichments were maintained on a very dilute medium, which contained mineral salts, vitamins, phosphate buffer, and 1.0 mg/L of tryptone and yeast extract. One of 11 carbon sources was added to 2–4 tubes of this basal media. Pentane, hexane, decane, or hexadecane was added at 40 μL/10 mL liquid in separate tubes. 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 month, 40 enrichments were transferred to fresh media as described below.

Each Monday, six 1-L bottles were used to prepare media for growing the enrichments used for inoculation into the designated columns. The medium contained 300 mg/L yeast extract, 300 mg/L tryptone plus 1000 mg/L glucose. The final pH was 7.0. Each bottle contained 300 mL of media. On each Tuesday, 0.2–0.3 mL of each enrichment tube was placed into one of the culture bottles used for growing the column inocula. Bottles were incubated 3 d at 20°C on a rotary shaker. On Friday mornings, the microorganisms that had grown in the bottles were

Table 1. Microbial colony forming units in Kwajalein soil columns

Treatment	Log CFU per gram soil (std. dev.)*		
	1 month	2 months	7 months
Control	6.9 (0.6)	6.2 (0.8)	5.6 (0.3)
Water only	7.6 (0.2)	7.1 (0.4)	6.3 (0.1)
Water + nutrients	7.3 (0.2)	6.9 (0.4)	6.2 (0.3)
Water + microbes + nutrients	7.2 (0.2)	7.0 (0.3)	6.2 (0.4)
Water + air	7.2 (0.2)	7.3 (0.3)	6.9 (0.1)
Water + air + nutrients	7.2 (0.5)	7.1 (0.2)	7.2 (0.2)
Water + microbes + air + nutrients	7.7 (0.3)	7.4 (0.2)	7.4 (0.2)

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 three to five soil samples per column and two or three replicate columns for each treatment. Samples were obtained from the bottom; 15, 30, and 60 cm from the bottom; and from the top of each column.

harvested by centrifugation. The concentrated cells were resuspended in reverse osmosis water and recentrifuged again. The pellet was resuspended in 60 mL of water, and optical density was determined at 660 nm on an aliquot of the final cell suspension. Each column typically received 10–12 mL of a 30-50 optical density equivalent solution as measured at 660 nm. Column inocula corresponded to 390 $\pm$ 10 OD mL unit equivalents or 120 $\pm$ 30 mg dry weight of cells. Colony forming units (CFU) of inocula averaged $7 \pm 3 \times 10^{10}$ cells added to each column each Friday morning. At a density of 1.6 and average column volume of 2850 mL, this corresponded to approximately $1\text{-}2 \times 10^{7}$ bacteria added per gram of soil in each designated column each week. The inoculation procedures continued from August 8, 1991, through January 10, 1992.

2.5 COLUMN SAMPLING

Routine samples were collected from the columns for analysis. One day each week, liquid effluents were collected and analyzed for pH, conductivity, and nutrient concentrations. One day each week, 10 mL of a 10% potassium hydroxide (KOH) trapping solution were placed into 28-mL pressure tubes, and a trap was used for CO_2 evolution. A vent line from each reactor receiving air entered a trap vessel. Stainless steel tubing (1/4 in.) connected to the column vent line and extended to the bottom of the pressure tube. Evolved gas was bubbled through the 4-in. column of KOH before being vented to the atmosphere. Contents of the KOH trap were then placed into 40-mL glass vials sealed with teflon septa for storage and analysis.

During September and October 1991, subcores were removed from the column ports and used for microbiological analyses. Three cc syringes with the tips removed served as the subcoring devices. The 3-in. hose clamps covering each of the sampling ports were removed, and 2 g of the sediments were cored. Fresh Teflon septa were placed over the holes and secured with the hose clamps.

2.6 CHEMICAL MONITORING

A rapid and simple assay for estimating TPHs was employed. Three grams aliquots of sediments 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 removed with a Pasteur pipet. Another 1-mL volume of iso-octane was then added, sonicated for 4 h, and centrifuged. The iso-octane aliquots were added and analyzed for hydrocarbons on a Hewlett-Packard 5890 or Shimadzu 9A GC at 50 to 250°C. The time sequence was 5 min at 50°C, then 5°C/min until 250°C, with a final 15 min at 250°C. Injections were in the splitless mode using a nonpolar cross-linked methyl silicone-fused silica column (50 m $\times$ 0.2 mm internal diam). Peak areas were quantified using a Nelson Analytical (Perkin-Elmer) laboratory data system operated with an internal standard program. C-19:0 served as the internal standard for all injections. The peak area of the internal standard was subtracted from all calculations.

The concentrations of nitrate, nitrite, total nitrogen, phosphorous, and phosphate in the column percolate were monitored using test kits (procured from Hach). Detection limits were <1 mg/L for all analytes. Conductivity and pH were determined electrochemically (Orion meter and electrodes for pH and YSI for conductivity determinations). Temperature was monitored daily

using thermometers, which recorded maximal and minimal temperatures that were generally between 24 to 26° C.

Carbon dioxide was measured as follows: Two-mL aliquots of the KOH trapping solution were placed into 28-mL pressure tubes, sealed with butyl rubber stoppers, and crimped with aluminum crimp seals. Contents of the tubes were acidified by injecting 1.5 mL of 30% HCL. After 30 min of equilibration, 0.4 mL of the headspace gas was removed using a gastight syringe and injected onto a gas chromatograph (GC). A Shimadzu 8A GC equipped with a thermal conductivity detector and helium carrier gas was used to quantify gaseous CO_2 from the acidified KOH. Standard curves for CO_2 concentrations were made from known standards prepared and diluted from 5, 20, and 80% CO_2-compressed gas cylinders. A second method for evaluating evolved CO_2 employed manometric techniques to quantify the CO_2 evolved after acidification of a different subsample of the KOH traps. The CO_2 was then analyzed for isotopic ratios of carbon by mass spectroscopy and reported as $d^{13}C$, the parts per thousand of ^{13}C to ^{14}C, relative to the PeeDee belemnite standard (PDB).

2.7 BIOLOGICAL MONITORING

2.7.1 Culturing of Microorganisms

Total aerobic bacterial spread-plate counts were performed in duplicate with serial dilutions using a medium containing 10 mg/L each of peptone, trypticase, yeast extract, and glucose with Noble Agar (PTYEG) and a second medium containing 1 g/L of each nutrient. All media contained trace minerals, including selenium and molybdate, as well a dilute vitamin mixture with a 10 mM bicarbonate and 2 mM phosphate buffer. Experiments were incubated at ambient temperature, which was similar to the subsurface temperature of 24 to 25° C on Kwajalein Island. Results were analyzed after 4, 14, and 30 d of incubation.

2.7.2 Radiolabeled Uptake and Transformation Experiments

[^{3}H]Acetate (3.3 mCi/mmol) was purchased from the New England Nuclear Corporation (Boston, Massachusetts). Sediment aliquots were inoculated for aerobic activity experiments. All isotope solutions (1 to 50 μCi) were frozen before use, thawed, and transferred with gastight syringes (Hamilton Company, Reno, Nevada). Time course experiments were performed in duplicate using sterile polypropylene centrifuge tubes for aerobic isotope incorporation experiments. All incubations were done at ambient temperature, which was similar to the in situ temperature of 24 to 25° C.

Acetate incorporation experiments contained 2.0 g of sediment, 5.0 μCi of [^{3}H]acetate, and 1.0 mL of sterile distilled water. At t_0 and appropriate time points, duplicate tubes were inhibited with 3.0 mL of a phosphate-buffered chloroform-methanol solution and frozen. Time points of 0, 8 h, 1 d, and 3 d generally provided linear rates. Before analysis, acetate incorporation samples were thawed, and then the sediments were extracted with chloroform-methanol. The lipid extraction was evaporated to dryness, and portions were counted by the scintillation method to determine the amount of radioactivity incorporated into microbial lipids. The earliest time points yielding measurable results were used to calculate a linear rate, which was extrapolated to disintegrations per minute per day (dpm/d).

3. RESULTS AND DISCUSSION

3.1 COLONY FORMING UNITS

Microbial abundance was assessed by plate-count methods and compared with pretest plate counts using both an extremely dilute and a less dilute complex medium. Total CFU found within the uppermost 10 in. of Kwajalein soils were typical of surface soils, being approximately 10^7/g. Below 10 in., bacterial numbers decreased. In the most contaminated zones below 3 ft, bacterial numbers were low, possibly showing effects of toxicity caused by severe hydrocarbon contamination. As shown in Table 1 and Fig. 5, the average CFU/g at the pretest sampling was log 6.75 from the 3- to 6-ft depth sediments. For these studies, cores were sampled at the bottom; 15, 30, and 60 cm from the bottom; and at the top of each sediment core. At the 1-month sampling, all subcores examined exhibited higher CFU/g. The increased biomass from the beginning of the study likely reflected stimulation of biological activity from sample procurement, shipping, and disturbance artifacts of column implementation. At the 2-month sampling date, the CFU had dropped in all treatments indicating the slow return to some new steady state. The control columns that received no air or nutrients exhibited approximately 10% of the CFU as compared to the other columns in this study. The decline in the biomass of the control columns continued through the 7-month study. After 7 months, the CFU of control columns decreased ~95%, from log 6.9 to log 5.6, or from nearly 10^7/g to 4×10^5/g. All columns not receiving air exhibited fewer CFU after 7 months than the initial CFU of log 6.75. All treatments receiving air exhibited CFU that were significantly greater than the initial values. These results suggest the importance of airflow in maintaining and fostering microbial growth and survival in these petroleum-contaminated soils.

The addition of 10^7 bacteria per cc of sediment each week did not overcome the limitation of air. Similarly, the addition of nutrients or the addition of microbes plus nutrients did not overcome the need for airflow in maintaining microbial density. The addition of nutrients did cause a significant increase in the microbial population. A further and less significant increase in CFU did occur with nutrients and the weekly addition of microbes.

More than 3×10^8 bacteria were added per gram of sediment in the columns designated to receive microbial inocula over the course of 20 weekly additions. Despite the large influx of native microorganisms, the CFU were only slightly higher at 2.5×10^7/g after 7 months compared to 1.6×10^7/g in treatments that received nutrients and air but no microbial inocula.

These results suggest that the considerable effort and cost of adding microorganisms did little to increase the biomass. In terms of field application, one could hypothesize that survival and impacts of exogenous microorganisms would be far greater in a laboratory experiment than in field application. Consequently, these results question the desirability of microbial supplementation but highlight needs for air and nutrient additions.

3.2 RADIOLABELED ACETATE INCORPORATION

General activities are those common to most microorganisms. The general activity chosen in this study was [^{3}H]acetate incorporation into microbial lipids. Nearly all life forms have the

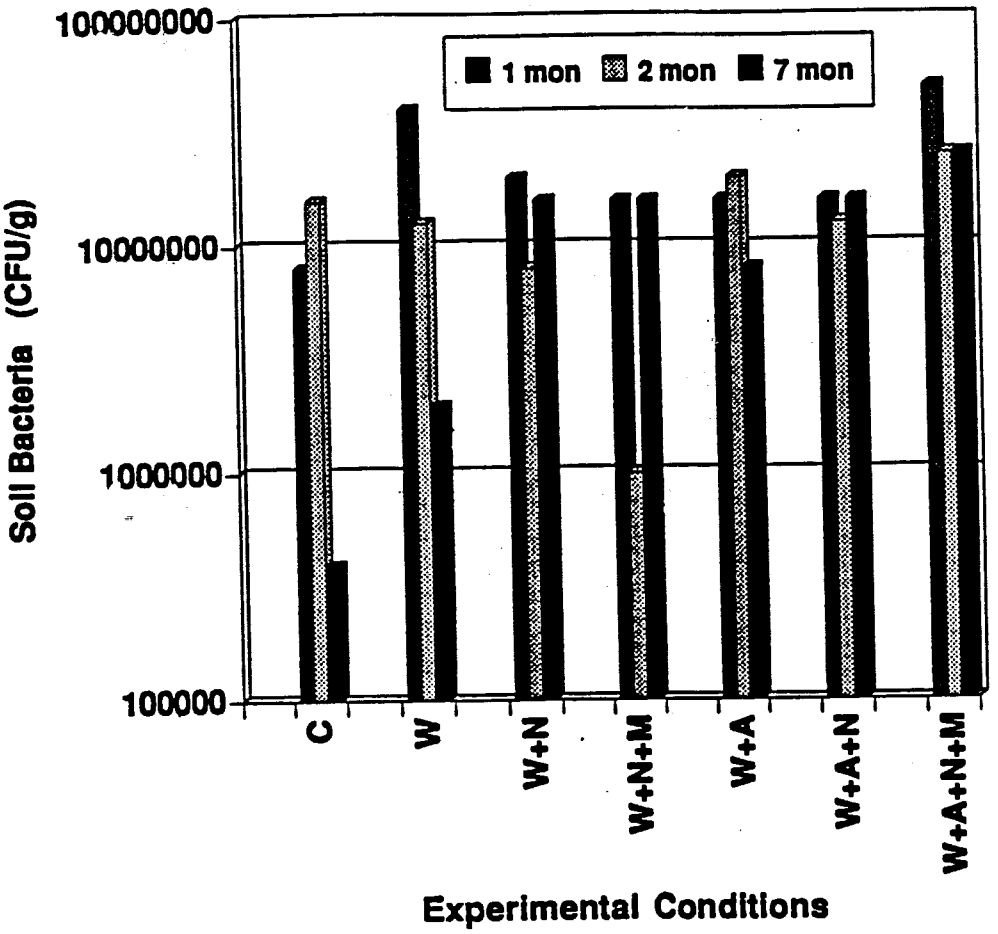

Fig. 5. Microbial colony forming units in soil columns.

ability to incorporate acetate into the lipids of cellular membranes, and the rate of incorporation is indicative of metabolism within that environment. The assay is extremely sensitive, yet can be used to evaluate activities spanning six or more orders of magnitude. Results from these experiments are used to categorize the general state of microbial activity in the environment and compare the effects of bioremediation on general bacterial metabolism.

Surface soils typically exhibit activities $>10^6$ dpm/d. Shallow sediments at the site reflect typical surface values (Table 2). For an unknown reason, the control values were extremely high in this study. Such anomalous values could be associated with stimulation of microbial activities during the dismantling of columns or during storage of samples for a couple of hours before initiating experiments. Because the control samples were the first to be dismantled, they may have been stimulated during 3–4 h of storage before initiation of the incorporation experiments. Control column 19 appeared anomalously high, while test column 5 appeared anomalously low in activity. In general, treatments that did not receive air exhibited lower activities than those receiving air. The greatest activity was observed in the water + air + nutrients + microbes treatment. Water + air was the second best treatment again, highlighting the importance of air for stimulating microbial activity and survival.

3.3 CARBON DIOXIDE EVOLUTION

Results of analyses indicated that CO_2 evolution was rapid during the first month and decreased dramatically in the final 4 months of the column studies (Table 3). During the first four weekly sampling periods (each sampling period was 24 h long), nearly all columns exhibited >1000 umol CO_2/d. In contrast, during months 5–7 less than 15% of the analyses revealed >1000 umol/d. The initial high CO_2 degassing rate may have been related to displacement of bicarbonates associated with the sediments or liberation of trapped CO_2. During months 2 and 3, a new state was approached that was likely reflective of in situ biological processes. During months 5, 6, and 7, columns receiving water and air exhibited very little CO_2 evolution despite their viable biomass (Table 1). Columns that received water + air + nutrients, with or without added microorganisms, exhibited considerable CO_2 evolution.

Table 4 and Fig. 6 show the isotopic ratios for d^{13} C-CO_2 evolved and the average CO_2 evolution rate for the final 4 months of column operations during which a new steady state was approached. Two different methods were used to quantify CO_2 evolution. Routine analyses were accomplished by GC using a helium carrier gas and thermal conductivity detector. Alternatively, CO_2 was quantified manometrically. The CO_2 was then analyzed for isotopic ratios of carbon (reported as $d^{13}C$ relative to the PDB) by mass spectrometry. As shown in Table 4 and Fig. 5, there was generally good agreement between the techniques. The average similarity was 96 $\pm$ 29%, and >66% of the analyses differed <20% between the two methods.

The CO_2 evolved at the beginning of column lysimeter operations was similar for the different treatments; the quantity ranged from 5890 to 6670 umols/d (manometric measurements) and the $d^{13}C$ varied from -13.94 to -19.59. The CO_2 evolved was probably from a variety of sources including atmospheric CO_2 in the injected air ($d^{13}C$ = -7.9), and gas dissolved from the dissolution of carbonates ($d^{13}C$ = 0) and the degradation of TPH ($d^{13}C$ = -27.8). After 5–7 months of operation, the CO_2 evolved varied in content and character as a function of operating conditions. For example, the columns receiving only air and water yielded very little

Table 2. Radiolabeled acetate incorporation into cellular lipids as indicator of microbial activities in samples from Kwajalein soil column studies

Treatment	[^{3}H]Acetate incorporation into microbial lipids thousands of dpm/day (std. dev.)*
Control	720 (80)
Water only	515 (177)
Water + nutrients	417 (36)
Water + nutrients + microbes	600 (85)
Water + air	708 (180)
Water + air + nutrients	509 (122)
Water + air + nutrients + microbes	759 (177)

*Each value represents the mean of five subsamples per column and two or three replicate columns per treatment. These results are posttreatment analyses of subsamples from the bottom; 15, 30, and 60 cm from the bottom; and from the top of each column.

Table 3. Carbon dioxide evolution from Kwajalein soil columns

Treatment (column number)		CO_2 evolved per day at designated sampling dates (total umol per day)				
		1 month	2 months	3 months	5 months	7 months
Water + air	(15)	3700	25	260	25	15
	(16)	4980	690	110	25	26
Water + air + nutrients	(4)	6150	2030	1560	56	35
	(5)	4980	78	321	260	30
	(6)	4030	2400	1980	240	1240
Water + air + nutrients + microbes	(10)	1190	53	75	10	710
	(11)	7020	2750	1660	1950	925
	(12)	1300	1860	420	40	26

Table 4. Comparison of $d^{13}C$ and evolution rates of CO_2*

Treatment (column, week)	Results from individual days: CO_2 by TCD	CO_2 by manometry	$d^{13}C$ (o/oo)	Average CO_2 evolution rate for final months (umol CO_2/L/day)
Water + air				
15:30	15	165	-12.12	49 (95)
16:4	4980	6190	-17.99	
16:30	26	22	-16.78	
Water + air + nutrients				
5:27	320	210	-24.24	310 (110)
6:4	4030	6670	-19.59	
6:30	1240	1210	-23.39	
Water + air + nutrients + microbes				
11:4	7020	5890	-13.94	220 (210)
11:27	1110	880	-24.24	
11:30	930	800	-20.09	
12:27	560	410	-24.46	

*The CO_2 evolution rate is per liter of soil column volume.

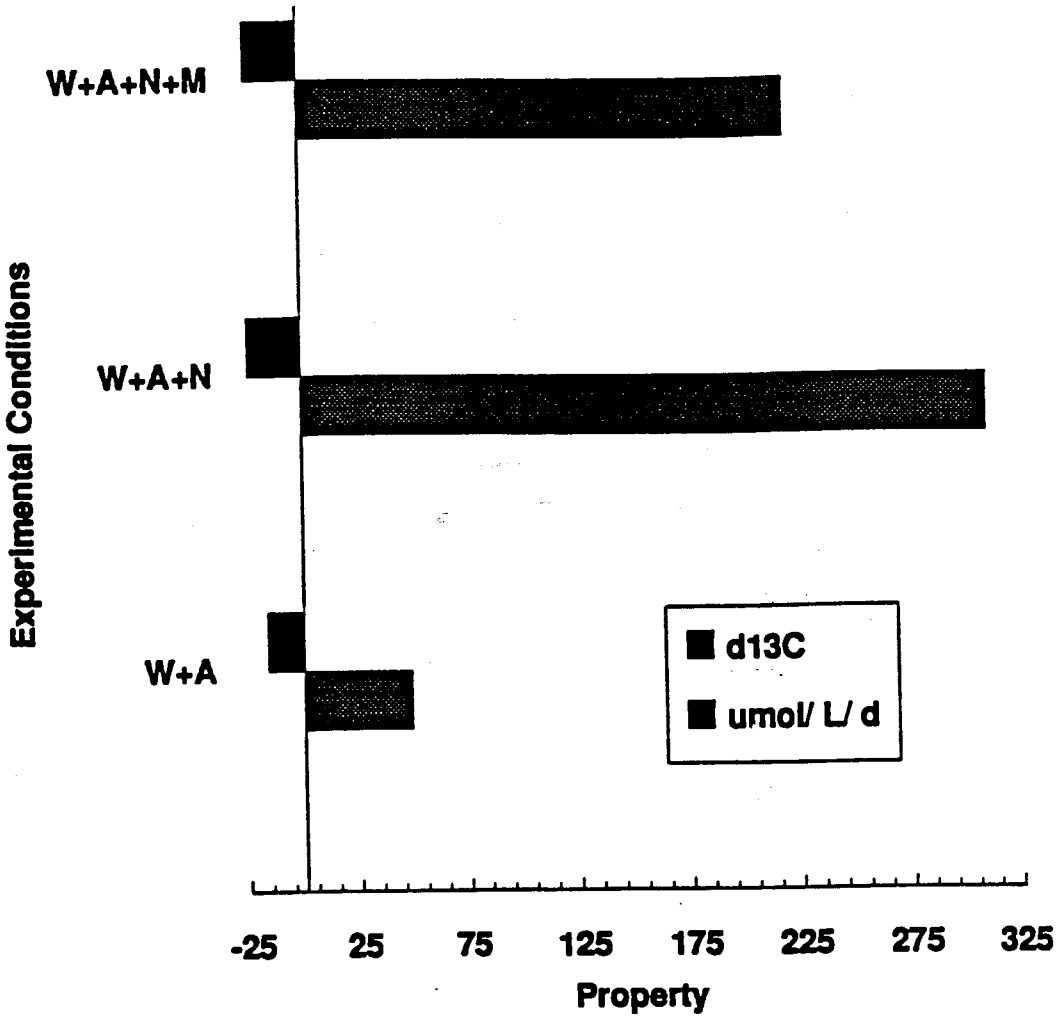

Fig. 6. Comparison of d^{13} and CO_2 evolution.
(Values to the left represent d ^{13}C and values
to the right represent CO_2 evolution in umol/L/day.)

CO_2 (<30 umol/d), and their $d^{13}C$ ratio (-12.2 to -16.78) suggested that they had a significant atmospheric component. In contrast, those columns receiving nutrients in addition to water exhibited higher CO_2 evolution (210 to 1210 umol/d) and the CO_2 resembled the $d^{13}C$ of petroleum hydrocarbons (-24.46 to - 24.44). Columns that received bioaugmentation exhibited similar CO_2 evolution data as those that did not receive additional microorganisms. This finding suggested that nutrient limitation developed after time and 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.

One could estimate a hydrocarbon degradation rate based on knowledge of airflow, biodegradation stoichiometry, the isotopic ratios, and CO_2 evolution. Airflow through the columns was targeted at approximately 10 mL/min. Because of difficulties in regulating the flow of air accurately through the 3-L columns at low pressures, the columns typically experienced airflow of 10 to 20 mL/min. It was suspected that the 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 airflow had been 10 mL/min or 10,000 mL per 16 h of airflow per day at 0.031% atmospheric CO_2, then 130 umol of atmospheric CO_2 should have been collected each day. Extrapolation of results could suggest that 17 umol CO_2/L was TPH-derived (d = -27.8), while 32 umol/L may have been atmospheric-derived CO_2 (d = -7.9). Accordingly, at 10 mL/min of airflow through the length of the 3-L column, the trap accounted for 90 umol, suggesting that the maximum trapping efficiency may have been 70%. Had airflow been 20 mL/min the trapping efficiency would have been 35%. Trapping efficiencies were probably between 35 to 70%.

Assuming the TPH represents straight-chained saturated aliphatics, one could examine C15H32 biodegradation as a model compound. If a C15 aliphatic were oxidized to CO_2, a reaction could be: $2302 + C15H32 \rightarrow 15CO_2 + 16\ H_2O$. In such a reaction, 3.5 g of CO_2 would be required per gram of TPH degraded, and 3.1 g of CO_2 would be evolved per gram of TPH degraded. In the case of the water + nutrient + air treatment, an average of 310 umol CO_2 was evolved per liter of column volume each day with an average isotopic ratio of -23.9. This isotopic ratio could be achieved by 250 umol of CO_2 derived from TPH (d = -27.8) and 60 umol from atmospheric CO_2 (d = -7.9). The estimated 60 umol from atmospheric-derived CO_2 compares well with the values obtained from the water + air treatments. Accordingly, the 250 umol CO_2 derived from TPH would represent 11 mg/L of CO_2/d or 3.6 mg TPH degraded/L each day (11/3.1). Correcting for the 1.6 density mass of Kwajalein sediments yields a daily degradation of 6 mg/kg. If the CO_2 trapping efficiency was considered, the TPH biodegraded per day could range from 6 to 20 mg/kg each day.

3.4 HYDROCARBON ANALYSES

GC analyses of the soil samples for each of the treatment conditions, before and after incubation, were completed to enable qualitative evaluation of changes in hydrocarbon contamination as a function of initial contamination level and treatment conditions. Each of the GC chromatograms was reviewed to determine the nature and extent of any loss of hydrocarbons as a result of the treatment. Standard curves of Kwajalein diesel fuel and commercially available diesel fuel procured in Knoxville, Tennessee, were compared. Figures 7a and 7b show standard curves. Figure 7a is a standard curve of TPH as integrated from 7–39 min from the GC analyses.

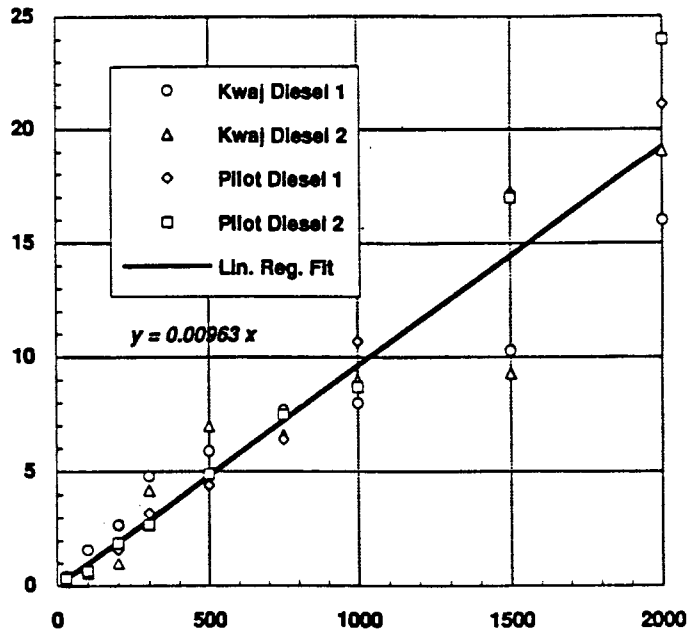

Fig. 7a. Standard curves for diesel fuel at 7–39 min.
(The y-axis is thousands of peak area units from the gas chromatograph analyses, while the x-axis depicts diesel-fuel concentrations in mg/L.)

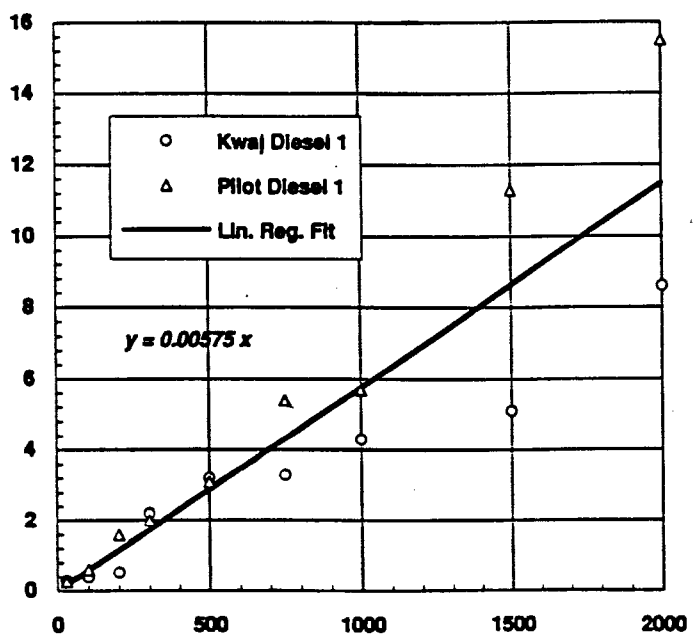

Fig. 7b. Standard curves for diesel fuel at 7–27 min.
(The y-axis is thousands of peak area units from the gas chromatograph analyses, while the x-axis depicts diesel-fuel concentrations in mg/L.)

Figure 7b is a standard curve more typical of lighter components of diesel fuel based on integration of peaks eluting from 7–27 min. An internal standard on all injections was C19, which eluted at 27.4 min. Consequently, Fig. 7b corresponds to those compounds eluting before C19. Significant differences existed between Kwajalein and the commercial diesel fuels. Diesel from Knoxville exhibited 29 $\pm$ 7% of the peak area units after C19. In contrast, 52 $\pm$ 5% of the Kwajalein diesel peak area eluted after C19. This corroborates the propensity for larger recalcitrant TPH in Kwajalein than expected from diesel fuel. The Kwajalein fuel also was darker and appeared more viscous than the locally procured materials.

Table 5 shows results from the on-site characterization and the laboratory characterization of TPH in control columns. Figure 8 shows a graphic representation of TPH analyses of control columns. The Hanby field tests (Table 5) underestimated the TPH contamination but did serve as a good indicator of the presence of TPH. Pretest characterization was also performed in laboratories of the Analytical Chemistry Division (ACD) at ORNL. Posttest screening was performed by the abbreviated screening procedure developed by UT personnel. The values from 7–39 min represent the entire TPH fraction eluting from the GC analyses, while the 7–27 min values represent TPH that eluted before the C19 internal standard. As noted from the results, the postscreening analyses of UT gave higher values than the ORNL ACD laboratory results. A number of factors may have contributed to the higher values. First, >340 peaks were integrated into the UT analyses. Second, a low-peak-reject threshold was used. Third, samples were compared to the uncontaminated surface soils, and any peak that eluted from the contaminated sites, which was not present in the uncontaminated sites, was assumed to be TPH. Fourth, samples were not serially diluted. Had the samples been serially diluted and high thresholds remained in the peak reject category of the integration system, lower values would have been recorded.

The following tables compare results from the 15- and 30-cm locations within the soil columns. These locations were chosen because they were similarly contaminated in all columns. Subcores located 60 cm from the bottom were heavily contaminated in some cores and only slightly contaminated in others.

Table 6 and Fig. 9 show results of the TPH screening procedure as applied to subcores 15 and 30 cm from the bottom of columns and peaks integrated over the entire 7–39 min. In both the 15- and 30-cm subcores, the control samples exhibited the most contamination. The greatest difference occurred when water was flushed through the columns. TPH contamination decreased approximately 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.

Table 7 shows the results of the TPH screening procedure for the same subcores, but results were integrated from 7–20 min corresponding to TPH likely less than C14. Again, the controls exhibited considerable contamination. If biodegradation was occurring, one could expect the light TPHs to be biodegraded most significantly. Table 7 suggests that additions of water + air + nutrients did result in the most biodegradation of light TPHs. The addition of microbes did not have any stimulatory effect on TPH biodegradation.

Table 5. Characterization of hydrocarbon contamination

	Total petroleum hydrocarbons (TPHs) (mg/kg)			
		Pretest TPH	Posttest TPH	
			Noncertified screening analysis	
Sample number (posttest)	Pretest field test kit	ACD (ORNL) laboratory	7–39 min	7–27 min
19(17)-10	>16000	17000	41000	30000
19(17)-20	1000	15000	75000	54000
19(17)-30	3000	13000	55000	39000
19(17)-40	6000	28000	nd	nd
19(17)-60	1	160	<50	<50
20(18)-0	>1600	7700	nd	nd
20(18)-10	2500	24000	49000	37000
20(18)-20	>1600	22000	38000	26000
20(18)-30	1500	12000	20000	12000
20(18)-40	2000	11000	nd	nd
20(18)-60	1	4	490	280
21(19)-10	4000	37000	37000	32000
21(19)-30	12000	24000	33000	23000
21(19)-40	>1600	7900	nd	nd
21(19)-60	1	1	<20	<20

nd = not determined.

Field tests used Hanby test kits. TPH screening analysis performed as described in Sect. 2.6. Integration range of 7–27 min stopped at approximately elution time for C19 hydrocarbons. The 7–39 min included larger and heavier recalcitrant fractions common to Kwajalein diesel but atypical for commercial product.

Sample designation: Columns 19, 20 and 21 were sacrificed at time = 0 and used for pretest analyses. Columns 17, 18, and 19 were analyzed posttest. Subsamples were obtained 10, 20, 30, 40, and 60 cm from the bottom of each column.

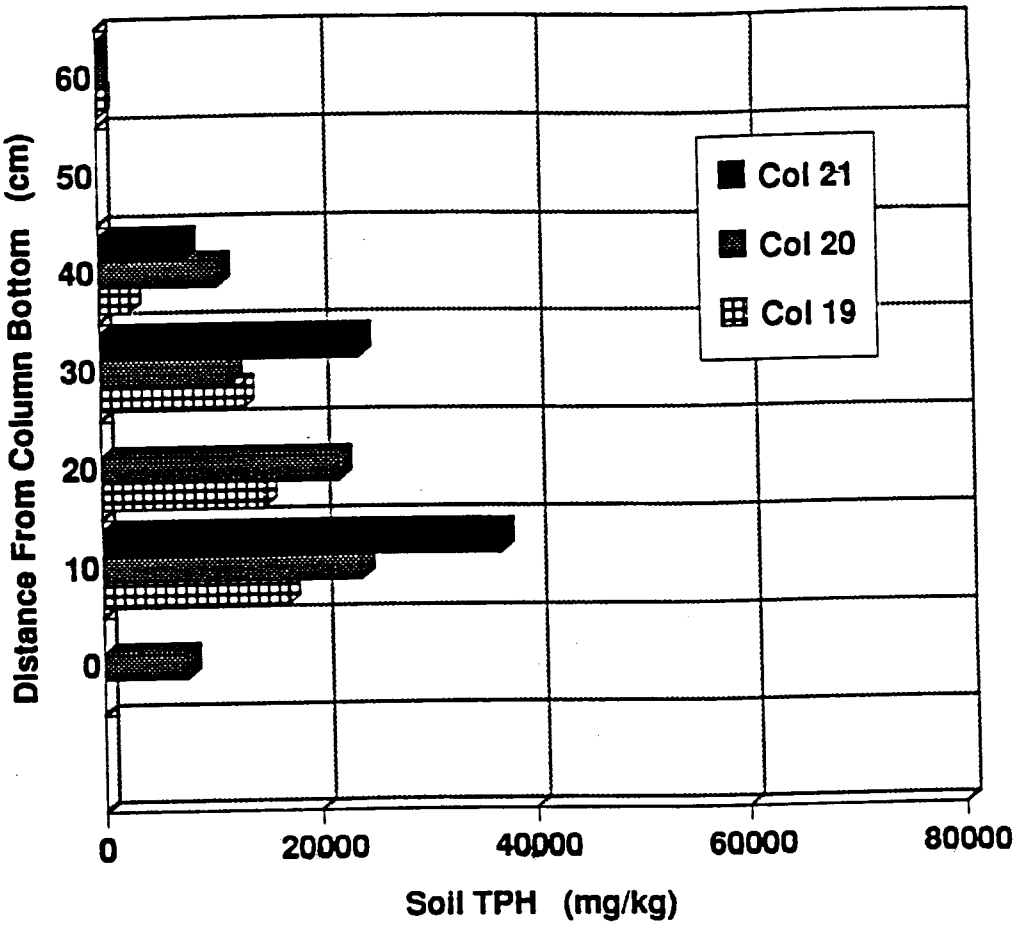

Fig. 8. Comparison of pretest and posttest total petroleum hydrocarbons of control columns.

Table 6. Posttreatment total petroleum hydrocarbon concentrations of column samples

	Petroleum hydrocarbons from screen procedure mg/kg (std. dev.)	
Treatment	15 cm from bottom	30 cm from bottom
Control	48000 (24000)	36000 (44000)
Water only	2600	24000
Water + nutrients	16000 (7000)	11000 (6000)
Water + microbes + nutrients	42000 (19000)	33000 (3000)
Water + air	33000	33000
Water + air + nutrients	23000 (6000)	3000 (15000)*
Water + microbes + air + nutrients	27000 (18000)	22000 (26000)*

Peaks eluting from 7–39 min were used for these calculations.

*If one outlying value was eliminated from each set both treatments would average 27000 mg/kg.

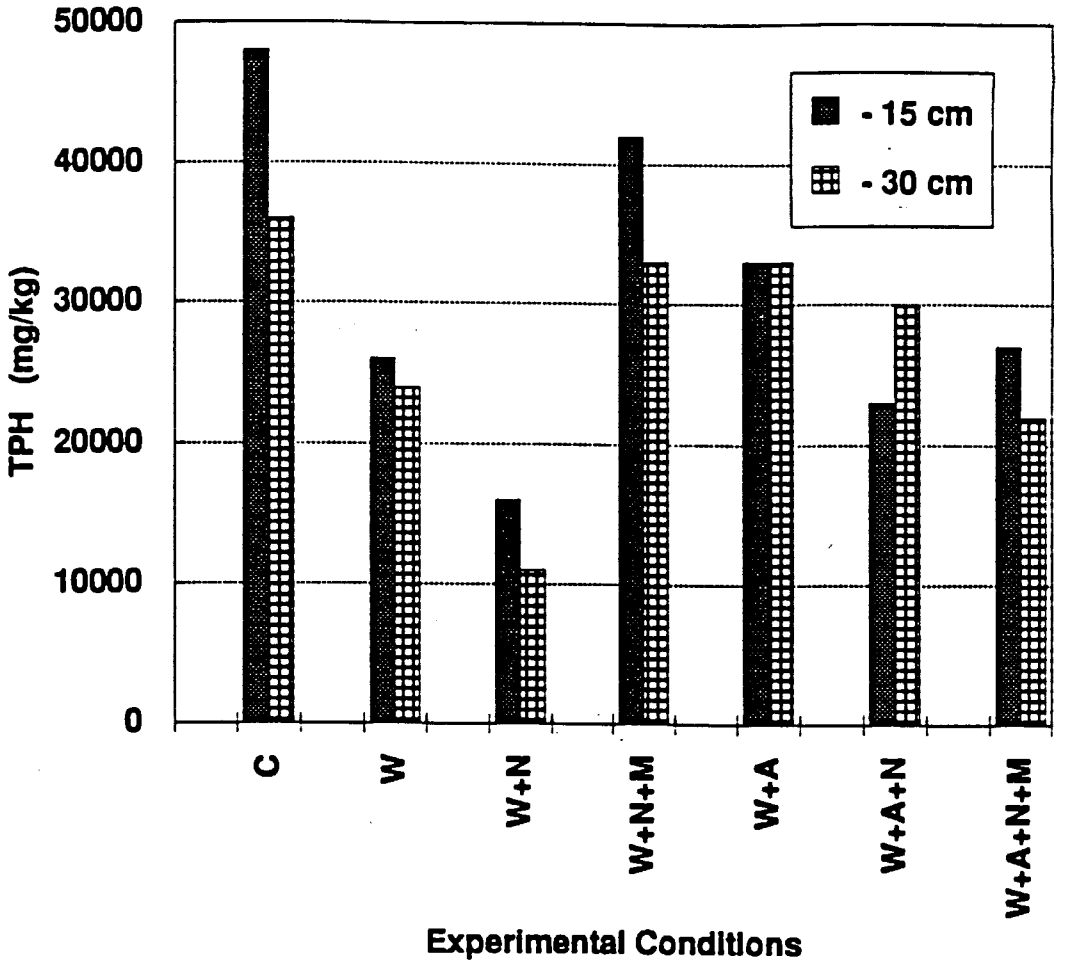

Fig. 9. Comparison of posttest total petroleum hydrocarbons.

Table 7. Short-chained hydrocarbons remaining in column samples

	Petroleum hydrocarbons from screening procedure mg/kg (std. dev.)	
Treatment	15 cm from bottom	30 cm
Control	8800 (5500)	6700 (2200)
Water only	6600	1300
Water + nutrients	4000 (1500	2800
Water + microbes + nutrients	13000 (5500)	2600 (1400)
Water + air	5100	5100
Water + air + nutrients	2200 (2800)	2000 (700)
Water + microbes + air + nutrients	3500 (1400)	1700 (1300)

Peaks eluting from 7–20 min (approximately C6– ~ C14) were used for these calculations.

Table 8 shows the number of peaks integrated by the GC integration system from analyses of the subcores located 15 cm from the bottom of the columns. The lowest number of peaks was integrated from the water + air + nutrient treatment for both the 7- to 39-min and 7- to 20-min integration schemes. Also, the smallest percentage or fraction of short-chained-to-total TPHs occurred in the water + air + nutrient treatment. Again, the addition of microbes did not appear to enhance biodegradation. Table 9 shows corroborating results from samples obtained 30 cm from the bottom of the columns. Again, the addition of microorganisms did not enhance biodegradation.

In general, the results of the hydrocarbon analyses were consistent with those of the soil respiration measurements reported previously (Siegrist et al. 1991) and CFU and CO_2 evolution that found that addition of water and nutrients resulted in enhanced biodegradation. Results of the analysis of the GC chromatograms are available upon request. Routine procedures for hydrocarbon analyses were developed, and procedures are being implemented in the field-scale demonstration.

Tables showing nitrogen and phosphorous results were not included because little was observed in the effluents. Ninety percent of all phosphate and total phosphorus samples analyzed from effluent fluids were negative. All phosphorus analyses were <2.5 mg/L. Differences between phosphorus additions, phosphorus removal, and the phosphorus content of biomass enabled one to estimate that >99.9% of the phosphorus was unavailable to the biomass and never exited the column. It likely was bound as calcium phosphate. Nitrate concentrations in the effluent ranged from 0–15 mg/L, but the average was <2.5 mg/L. Nitrite ranged from 0–6 mg/L, but >90% of the analyses were negative, and the average was <0.05 mg/L. It is likely that many of the positive results may have been false positives caused by TPHs in the effluents. It was believed that few nutrients eluted from the columns and routine monitoring would provide effective controls to ensure that effluents were not exceeding regulated limits for N or P. The pH of the eluted waters remained between 7–8.4 regardless of treatment.

Table 8. Number of peaks integrated in the hydrocarbon analyses of subcores located 15 cm from the bottom of columns

Treatment	Number of peaks integrated (std. dev.)		
	Petroleum hydrocarbons 7–39 min	Short-chained hydrocarbons 7–20 min	Short-chained fraction % of peak area
Fresh diesel fuel	332	140	see text
Control	339 (2)	140	11
Water only	332	128	17
Water + nutrients	313 (30)	103	21
Water + microbes + nutrients	341 (6)	132	20
Water + air	313	101	17
Water + air + nutrients	291 (45)	81	7
Water + microbes + air + nutrients	306 (31)	105	11

Table 9. Number of peaks integrated in the hydrocarbon analyses of subcores located 30 cm from the bottom of columns

Treatment	Number of peaks integrated (std. dev.)		
	Petroleum hydrocarbons 7–39 min	Short-chained hydrocarbons 7–20 min	Short-chained fraction % of peak area
Fresh diesel fuel	332	140	see text
Control	341 (7)	140	8
Water only	315	111	14
Water + nutrients	335	150	15
Water + microbes + nutrients	312 (33)	107	12
Water + air	314	101	24
Water + air + nutrients	314 (33)	73	6
Water + microbes + air + nutrients	320 (17)	115	15

4. SUMMARY AND CONCLUSIONS

The results reported here suggest that the soil and groundwater of Kwajalein Island contain a wide variety of microorganisms. The organisms present at sites containing diesel-fuel contamination do have the ability to degrade fractions of the hydrocarbons. Soil column experiments demonstrated that existing microbial populations in Kwajalein soils are starved for nutrients, can be stimulated by the addition of nutrients, and can degrade many of the hydrocarbons present. A further increase in biodegradative activity was not obtained by bioaugmentation. In addition, these studies developed, implemented, and transferred procedures to the field demonstration project for the routine monitoring of hydrocarbons, microbial biomass, CO_2 evolution, and the monitoring of coliforms as an indicator of potential health implications during the field-scale tests.

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. This report supports and expands on results reported earlier by Adler et al., *Bioremediation of Petroleum-Contaminated Soil on Kwajalein Island: Microbiological Characterization and Biotreatability Studies*, May 1992, ORNL/TM-11925, and Siegrist et al. (1991). In addition to the results of the laboratory investigations reported here, the climate and soil conditions that exist on Kwajalein Atoll favor a bioremediation approach.

Biodegradation of diesel-fuel contaminants in Kwajalein Island soils is possible by using indigenous microbes. This suggests that bioremediation of such soils is technically feasible as an environmental restoration process on Kwajalein Island. However, the reduction of diesel-fuel contamination to residual levels below 100 mg/kg by an in situ bioremediation process may be a difficult and lengthy process because of the proportion of more slowly biodegradable, higher-molecular-weight hydrocarbons present, particularly in subsurface zones contaminated by older, weathered fuel. While higher nutrient loadings can stimulate higher biodegradation rates, high nutrient application must be considered in light of potential groundwater quality impacts associated with potential nutrient leaching. The greatest change from controls occurred via water flushing. Mechanisms to increase water flux or leaching or combinations with surfactants may be worthy of consideration. It is likely that treatment of a few years with water + air + nutrients could significantly reduce the concentrations of the lighter TPHs.

Attempts at bioremediation on Kwajalein should include addition of nutrients and air, and enhanced water circulation should be considered. In addition, alternative types of phosphate may be worthy of consideration as well as a role for water flushing, possibly with surfactants.

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