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**OAK RIDGE
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MARTIN MARIETTA

**Bioremediation of Petroleum-
Contaminated Soil on Kwajalein
Island: Microbiological
Characterization and
Biotreatability Studies**

H. I. Adler
R. L. Jolley
T. L. Donaldson

**MANAGED BY
MARTIN MARIETTA ENERGY SYSTEMS, INC.
FOR THE UNITED STATES
DEPARTMENT OF ENERGY**

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Chemical Technology Division

**BIOREMEDIATION OF PETROLEUM-CONTAMINATED SOIL
ON KWAJALEIN ISLAND:
MICROBIOLOGICAL CHARACTERIZATION
AND BIOTREATABILITY STUDIES**

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LIST OF ACRONYMS

API	Analytab Products, Inc.
BCYE	Buffered charcoal yeast extract
BP	Burn pit
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CFU	Colony forming units (microbial)
CWA	Clean Water Act
EMB	Eosin methylene blue
EPA	Environmental Protection Agency (U.S.)
GC	Gas chromatograph
HAZWRAP	Hazardous Waste Remedial Action Program, DOE
HC	Hydrocarbon
HPLC	High-performance liquid chromatography
IAM	Institute for Applied Microbiology (University of Tennessee)
KDB-n	Respirometry test runs (Chapter 9)
NNAE	Nonnutrient agar, with nonviable <i>E. coli</i>
ORAU	Oak Ridge Associated Universities
ORNL	Oak Ridge National Laboratory
PDB	PeeDee belemnite standard for ¹³ C
PHB	Poly hydroxy butyrate
PHC	Petroleum hydrocarbons
PHA	Poly beta-hydroxy alkanolate
PLFA	Phospholipid ester-linked fatty acid
PTYEG	Peptone, trypticase, yeast extract, and glucose with Noble Agar
PTYG	Peptone, tryptone, yeast extract, and glucose
RCRA	Resource Conservation and Recovery Act
RQ	Respiration quotient
STP	Sewage treatment plant
TCE	Trichloroethylene
TPH	Total petroleum hydrocarbons
TPnn-m	Test pit number nn, depth m inches
TSA	Trypticase soy-extract
TSCA	Toxic Substances Control Act
USAKA	United States Army Kwajalein Atoll
UT	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 Action 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.

The investigations reported in this technical memorandum were conducted to determine the biotreatability of soil samples collected on Kwajalein Island. The material included here represents the collective efforts of a large group of scientists and engineers whose names and affiliations accompany their written chapters. 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, a USAKA facility in the Republic of the Marshall Islands. The bioremediation demonstration project is being conducted by a multidisciplinary team of investigators from Oak Ridge National Laboratory (ORNL), Oak Ridge Associated Universities (ORAU), and The University of Tennessee (UT).

EXECUTIVE SUMMARY

Bioremediation technology is being evaluated for use on the Kwajalein Atoll, which is located in the Republic of the Marshall Islands. The study was undertaken by the Oak Ridge National Laboratory (ORNL) on behalf of the U.S. Army Kwajalein Atoll (USAKA). During February of 1991, a team from ORNL and The University of Tennessee (UT) visited the USAKA. In addition to making on-site observations regarding microbial abundance and distribution of petroleum contaminants, they brought back to Oak Ridge various soil and water samples for detailed analyses. This report documents the biological studies of these samples and presents observations made during the period from February to April of 1991 by investigators at ORNL, UT, and the Oak Ridge Associated Universities.

The soil and groundwater of Kwajalein Atoll contain a wide variety of aerobic and anaerobic microorganisms. Although the concentration of live organisms varies from site to site, no sterile environments were found. The number of organisms per gram of soil ranged from 10^3 to 10^8 . A few of the isolated microorganisms have the potential for being human pathogens. This is not an unusual finding during the examination of soil and water samples from many environments.

Some of the organisms present in soil samples containing petroleum contaminants have the ability to degrade certain fractions of the hydrocarbons present. The biological activity of the native populations of organisms can be stimulated by the addition of inorganic and/or organic nutrient supplements. The addition of commercially available bacterial preparations to Kwajalein soil enhances biological activity. There is some evidence that the more heavily weathered hydrocarbons (i.e., generally larger molecules with partially oxidized substituents) may be relatively difficult to degrade by biological methods.

Experiments with bioluminescent reporter strains of bacteria demonstrated that naphthalene and toluene are biologically available in some Kwajalein soil samples. The application of gene probe techniques to Kwajalein soil samples did not reveal the presence of hydrocarbon-degrading genes. However, the only probes available were derived from gram-negative organisms and may not be sufficiently homologous to the genes of the predominantly gram-positive populations present in the Kwajalein soil samples.

The assay of ester-linked phospholipid fatty acids from the Kwajalein soil samples suggests that an actinomycete population is present, along with a variety of other bacteria. No fatty acids derived from eukaryotes were observed. The fatty acid profiles suggest that most of the microorganisms were not actively dividing and may be starved for certain essential nutrients.

In summary, the biological observations made on the USAKA soil and water samples support the concept that bioremediation may be an effective way to remove at least some of the hydrocarbons contaminating Kwajalein Atoll. The technical approach should certainly include the addition of nutrients to contaminated areas. It may also be helpful to add certain microorganisms to increase the rate of hydrocarbon degradation.

1. INTRODUCTION

1.1 BACKGROUND

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 is located ~2100 nautical miles southwest of Honolulu, Hawaii, and 700 nautical miles north of the equator (Fig. 1.1). 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 mile². Kwajalein Island is ~3.5 miles long by 0.3 to 0.5 miles wide with a land surface area of ~1.2 mile². The population of the base, including army, subcontractor, and family personnel, is ~3000.

The United States and the RMI recently negotiated a Compact of Free Association allowing the U.S. 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., RCRA, CERCLA, TSCA, CWA).^{*} 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 significant 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 Action Program (HAZWRAP), that a project be initiated to evaluate the feasibility of using bioremediation for environmental restoration of contaminated sites in the atoll. If it appeared feasible, a technology demonstration would then 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 (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, ORNL/TM-11894). Siegrist and co-workers demonstrated that substantial numbers of live microorganisms are present in Kwajalein soils and water. Preliminary field experiments suggested that this indigenous population of microorganisms could degrade at least some

^{*}RCRA = Resource Conservation and Recovery Act; CERCLA = Comprehensive Environmental Response, Compensation, and Liability Act; TSCA = Toxic Substances Control Act; CWA = Clean Water Act.

ORNL DWG 92-280

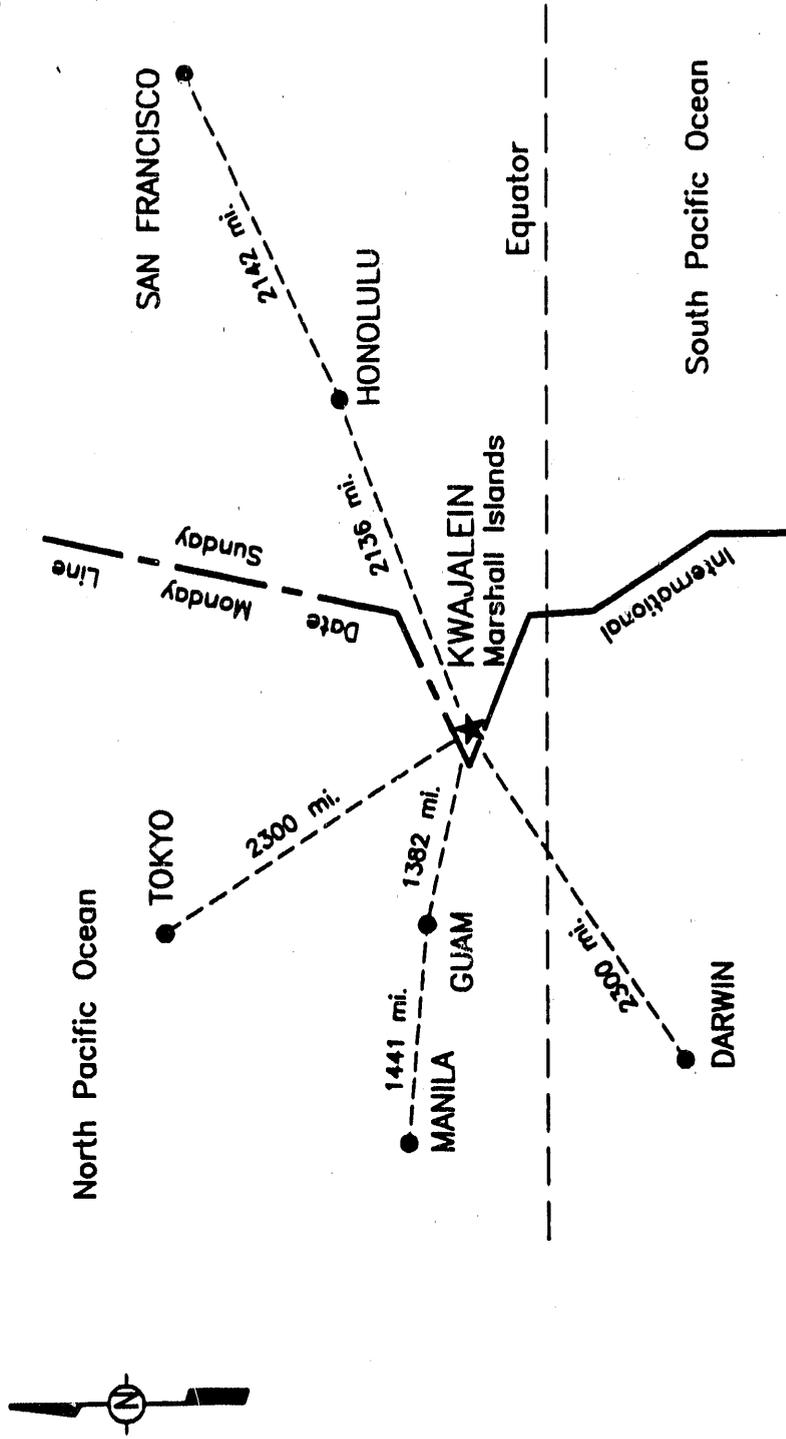


Fig 1.1. Geographical location of U.S. Army Kwajalein Atoll Base.

petroleum hydrocarbons. Several existing conditions will favor bioremediation via the growth of a large variety of microorganisms: (1) The soil is basically a coral sand with neutral to slightly alkaline pH. (2) The soil temperature does not vary appreciably and remains near 84°F throughout the year. (3) Water is readily available.

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 biological experiments that were carried out between February and April 1991, using these samples, are presented here. The overall objective was to evaluate, in a broad preliminary manner, some of the biological parameters that must be considered during the design of the potential demonstration at a site on Kwajalein. 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? (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?

1.2 ORGANIZATION OF THIS REPORT

The main body of this report consists of chapters that summarize the activities of each group participating in the collective study. The individual chapters are organized in styles commonly used for presentation in scientific journals. Table 1.1, which is provided as a general guide to the experiments, identifies the investigators responsible for the work and the locations (site and depth) from which the samples examined in this study were collected. It also indicates, where applicable, the specific compounds examined and the primary observations and recommendation made by the investigators.

Table 1.1. Guide to the experiments on Kwajalein soil and water samples

Title	Samples examined ^a	Specific compounds assayed or added	Major recommendations and observations (by researchers)
Pathogenicity Profile (Lyndall et al.)	TP01-8, -56 TP04-32, water STP ^b	•	If large amounts of aerosols are generated, respirators and goggles should be used for protection from potential pathogens.
lux Gene Bioreporters (Heitzer et al.)	TP01-8 TP04-65 (and others)	Naphthalene, toluene, and xylenes	Catabolic gene expression is possible in soils; naphthalene and toluene are biologically available.
Gene Probs-3 (Sayler et al.)	TP01-8, -33, -56 TP02, -63 TP03-12, -72 TP04-32, -65 TP05-62, -70	Naphthalene, toluene	No degradative genes were detected; degradative potential may be low. That which is present may be due to gram-positive species.
Fatty Acid Profiles (Ringelberg and White)	TP01-8, -33, -56 TP02-6, -63 TP03-12, -72 TP04-32, -65, water TP05-62, -76 STP ^b	•	Data indicate an actinomycete population. Some bacteria are in the stationary phase; some are starved bacteria; lack of eukaryotes noted.
Microbial Activity and Biomass (PHELPS et al.)	TP01-8, -33, -56, water TP02-6, -63 TP03-12, -72 TP04-10, -32, -63, water TP05-62, -70 BP10-8, -19, -31, -35, -38 BP11-10, -30, -38, -41, -52, -59 -65, -69 BP12-10, -35, -46, -51, -55 STP ^b	Benzene, toluene	Biomass is present and can be stimulated. Diesel-fuel degrading pathways are present. Hydrocarbons can be translocated by H ₂ O. Environment is probably dominated by gram-positive bacteria. Large hydrocarbons are recalcitrant. Benzene and toluene mineralization occurs but is limited.

Table 1.1 (continued)

Title	Samples examined ^a	Specific compounds assayed or added	Major recommendations and observations (by researchers)
Anaerobic Microorganisms (Adler and Suttle)	TP01-62 TP05-62 BP10-8,-35 BP11-10,-65 BP12-10,-51 STP ^b	•	Slightly fewer anaerobic than aerobic colony-forming units are present. Microscopy suggests facultative rods; no filamentous fungi are present. Free spores are observed; spore-forming rods are present in STP.
Bacterial Degradation (Strandberg, et al.)	TP01-8 TP04-65	Diesel fuel	Native bacteria from contaminated site are more capable of degradation than those from uncontaminated site. Nutrient addition greatly enhanced activity. Addition of commercially available organisms increased activity.
Respirometric Evaluation (Siegrist et al.)	TP01 TP04-composite TP04-10, -32, -65	Diesel fuel	O ₂ uptake was stimulated by fresh fuel and nutrients. Certain heavy, weathered fractions may not be readily biodegradable. Inorganic nutrients were about as effective as a mixture of inorganic and organic nutrients.

^aIn this table and in the text, TP01, TP02, etc., identify test pits on Kwajalein Island from which material was obtained. The designation BP identifies burn pits located on Roi-Namur, a small island that is part of the Kwajalein Atoll ground. The number following the dashed line (i.e., TP01-8) indicates the depth, in inches, from which the soil sample was obtained. Refer to Siegrist et al., ORNL-TM/11894, for details.

^bSTP = Sewage Treatment Plant.

^cNot applicable.

2. CONCLUSIONS AND DISCUSSION

The results reported here suggest that the soil and groundwater of Kwajalein Island contain a wide variety of aerobic and anaerobic microorganisms. Although the numbers of organisms vary from site to site and with depth at each site, no completely sterile environments were found. Among the organisms found, a few have the potential for being human pathogens, but this is not an unusual finding when examining soil samples.

The organisms present in test pits containing diesel fuel contamination do have the ability to degrade at least certain fractions of the hydrocarbons present. There is some indication that the more heavily weathered fractions may be relatively recalcitrant.

Several experiments demonstrated that existing microbial populations in some of the Kwajalein soil samples are starved for nutrients. These populations can be stimulated by the addition of nutrients. At least one experiment suggested that a further increase in biological activity can be obtained by the addition of commercially available bacterial inocula. Although this enhancement may only be short-lived, it could be repeated at intervals in order to increase the rate at which hydrocarbons are degraded. If the importation of "foreign" bacteria to Kwajalein Atoll is not possible, it may be feasible to grow large quantities of native bacteria in fermentation vessels. These mixed populations, which could be harvested, concentrated, and applied to test areas, might prove to be as effective as the commercial preparations.

Three relatively novel and sophisticated approaches for detecting and monitoring biological activity in soil samples have been evaluated. Experiments involving bacterial strains that emit light while degrading naphthalene or toluene have demonstrated that this system can operate in the environment provided by the Kwajalein soil samples. Furthermore, they established that the compounds are biologically available. The second assay involved the application of gene probe techniques to the soil samples but did not reveal the presence of hydrocarbon-degrading genes. The probes used in the experiments were derived from gram-negative organisms and may not be sufficiently homologous to the genes of the predominantly gram-positive organisms present. The third assay, which characterized the ester-linked phospholipid fatty acids present in Kwajalein soil samples, suggested that an actinomycete population was present in addition to a variety of other bacteria. No fatty acids derived from eukaryotes were observed. This assay also suggested that most of the indigenous microorganisms were not actively dividing and may have been starved for certain nutrients. The three assays have the potential, after further development, for being useful as sensitive biological monitors of the changes taking place in microbial populations during a demonstration of bioremediation.

The laboratory-based biological observations, when viewed from a general perspective, support the concept that bioremediation may be an effective approach for removing hydrocarbon contaminants from the soil of Kwajalein Atoll. 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. The moderate and relatively constant temperature (approximately 84°F) favors the growth of many organisms, and the slightly alkaline and relatively porous soil should allow the introduction of water and nutrients at optimum rates.

3. PATHOGENICITY PROFILE

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3.1 INTRODUCTION

Samples of Kwajalein soil were examined to determine the spectrum of microbial forms present. Particular attention was given to pathogenic organisms because they pose a potential health hazard.

3.2 METHODS

Soil samples were screened for coliform bacteria by using a procedure that includes inoculation of soil samples onto various media to identify the fermentative capabilities of the organisms. Organisms from the soil samples that can ferment sugars were isolated, and their purity was ensured by transfers on several different types of media. Isolated bacteria were stained and examined microscopically to determine morphological characteristics. Each isolate was tested for motility by using a wet mount or the hanging-drop technique. Following the isolation and staining procedure, biochemical tests were performed on each isolate. These tests included the determination of each organism's ability to react to oxidase, catalase, *o*-nitrophenol galactase, arginine dehydrogenase, lysine decarboxylase, ornithine decarboxylase, citrate, hydrogen sulfide, urease, tryptophane deaminase, indole formation, gelatin liquefaction, acetoin production, nitrate reduction, and growth on various carbon sources. Several carbon sources — glucose, mannitol, inositol, sorbitol rhamnose, sucrose, melibiose, amygdalin, and arabinose — were examined. This profile determined the identity of each organism in the soil sample. The procedure was also followed for potential pathogenic organisms that produced hemolysis on blood agar. Pathogenicity was then assessed, based on the organism's identity.

Soil samples were also plated on a variety of media, which included blood agar, chocolate agar, EMB media, MacConkey's media, trypticase soy extract (TSA), Sabouraud's dextrose agar, malt agar, Salmonella-Shigella agar, and a peptone-enriched trypticase-soy medium. These plates were incubated at 30°C and at 37°C, with and without CO₂ enrichment. The complex consortia that arose on these plates were isolated; and the potential pathogenicity, as well as the identity of each organism, was determined. Several of these media were also inoculated and placed in anaerobic chambers to determine the presence of pathogenic anaerobes. Identification of potential pathogenic isolates was confirmed using standard clinical microbiological techniques, as partially outlined above, and the use of API identification strips.

The presence of parasitic organisms was determined by direct microscopic examination using Lugol's iodine solution, Trichrome staining, and the formalin-ether sedimentation technique, which allows the recovery of all protozoa, eggs, and larvae present.

Assays for the presence of *Legionella* were carried out by standard procedures outlined by the Center for Disease Control (Atlanta, Georgia). This includes plating samples on buffered charcoal yeast extract (BCYE) media before and after acid treatment of various concentrations.

The presence of amoeba was determined by plating an aliquot of sample on a mineral salts medium coated with a layer of dead *E. coli*. Amoebic migrations were harvested, and the genera were determined. These samples were then transferred to ensure purity for possible pathogenicity testing; in addition, amoeba were allowed to lyse to release any bacteria residing within the amoeba for characterization and determination of their potential pathogenicity. Confirmation of potential *Naegleria* and *Acanthamoeba* isolates was determined by motility testing using distilled water, and pathogenicity was confirmed by the intranasal inoculation of mice.

Figure 3.1 depicts the methods used and indicates which Kwajalein samples were examined.

3.3 RESULTS AND DISCUSSION

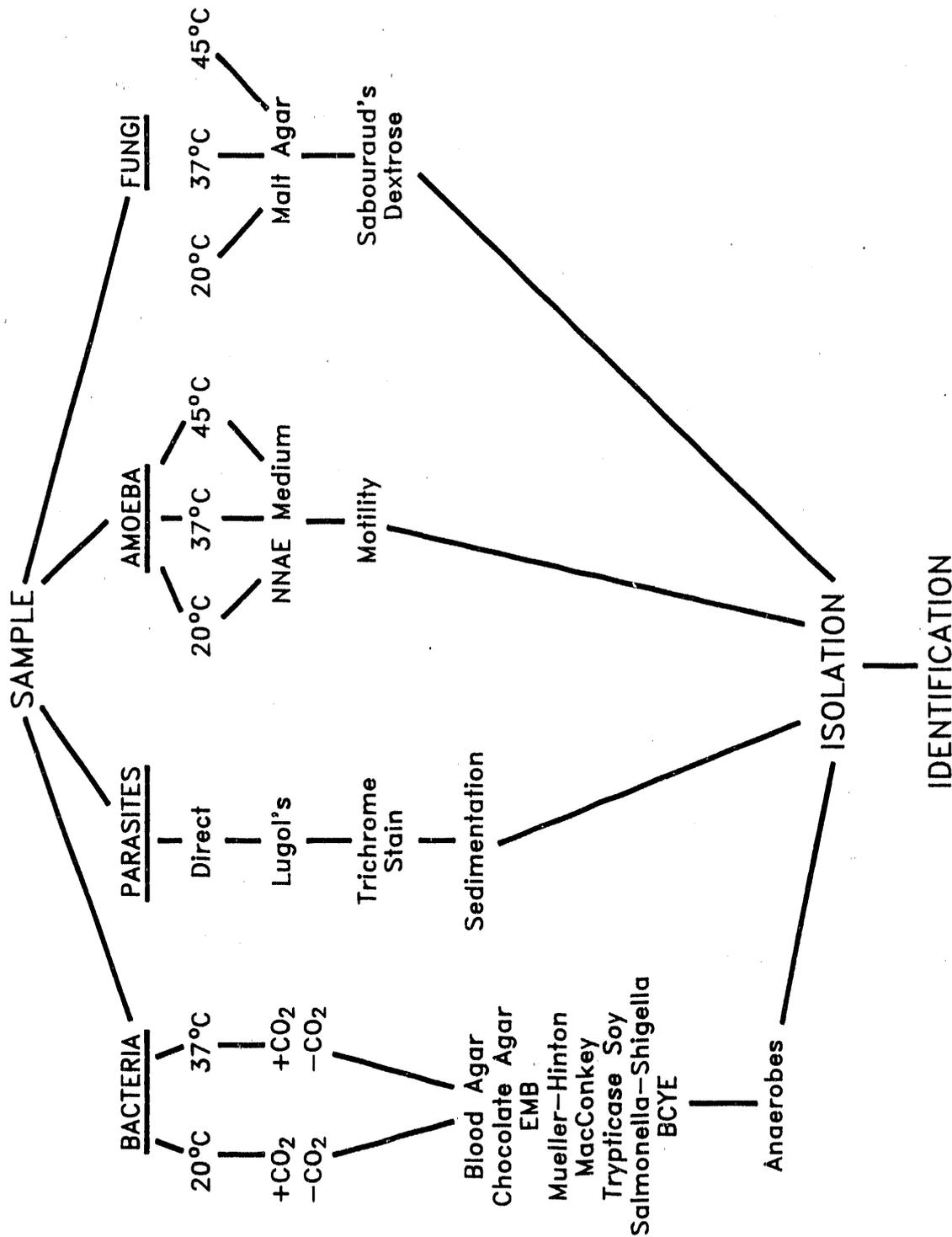
The Kwajalein microbiological profile, which is based on representative sites, yielded some very interesting organisms as well as some potential pathogens (Table 3.1). A number of hemolytic organisms, notably *Aeromonas hydrophilia*, which produced the strongest beta-hemolytic reaction yet seen in this laboratory, were found to be present. Although many of these bacteria are ubiquitous and are generally only pathogenic in immunosuppressed individuals, the potential exists for severe infections if these organisms are incorporated into wounds or damaged tissue. *Klebsiella pneumoniae* has been known to cause respiratory infections in healthy individuals. The absence of pathogenic *Naegleria* and *Legionella* reduces the health risk to workers on the island, although *Acanthamoeba* has been known to cause severe infections if incorporated into damaged eye tissue. Thermophilic fungi can always pose potential health risks and can be very difficult to treat.

The pathogenicity of these isolates has not been evaluated and may be necessary in some instances. Total bacterial counts of these organisms, especially *Aeromonas hydrophilia* and *Enterobacter cloacae*, are quite high (10^5 organisms/mL) but may be unreliable because of the length of time the samples were in transit.

Depending on the method of bioremediation chosen, certain precautions for the workers might be considered. If large amounts of aerosols are to be generated, respirators and/or eye goggles should be worn. If fertilization is chosen as the mode of action, this procedure may selectively stimulate some pathogens and enhance any potential health problems. Overall, these soils should be treated with caution.

METHODS

(STP, Water,* TP01-8, TP01-56, TP04-32)



* Drinking water supply prior to chlorination.

Fig. 3.1. Microbiological analysis of Kwajalein remediation sites.

Table 3.1. Kwajalein pathogenicity profile

Sample	Bacteria ^{ab}	Fungi	Amoeba
Water ^c	<i>Aeromonas hydrophilia</i> ^d <i>Pseudomonas alcaligenes</i> ^d <i>Pseudomonas fluorescens</i> <i>Bacillus mascerans</i> <i>Bacillus firmus</i> <i>Bacillus circulans</i> <i>Pseudomonas aeruginosa</i> ^d <i>Pseudomonas luteola</i> <i>Pseudomonas cepacia</i> <i>Pseudomonas putida</i> <i>Flavobacterium odoratum</i>		Hartmanella
TP01-8	<i>Aeromonas hydrophilia</i> ^d <i>Pseudomonas fluorescens</i> <i>Bacillus firmus</i> <i>Bacillus circulans</i> <i>Pseudomonas aeruginosa</i> ^d <i>Pseudomonas cepacia</i> <i>Pseudomonas putida</i> <i>Flavobacterium odoratum</i> <i>Bacillus stearothermophilus</i> ^d <i>Bacillus polymyxa</i> <i>Enterobacter cloacae</i> ^d <i>Pseudomonas paucimobilis</i> <i>Bacillus thuringiensis</i>		Hartmanella
TP01-56	<i>Aeromonas hydrophilia</i> ^d <i>Pseudomonas fluorescens</i> <i>Pseudomonas aeruginosa</i> ^d <i>Pseudomonas cepacia</i> <i>Pseudomonas putida</i> <i>Flavobacterium odoratum</i> <i>Bacillus stearothermophilus</i> ^d <i>Enterobacter cloacae</i> ^d <i>Pseudomonas luteola</i> <i>Klebsiella pneumoniae</i> ^d <i>Bacillus subtilis</i> ^e <i>Pseudomonas pseudoalcaligenes</i>		

Table 3.1 (continued)

Sample	Bacteria ^{a,b}	Fungi	Amoeba
TP04-32	<i>Pseudomonas luteola</i> <i>Pseudomonas aeruginosa</i> ^d <i>Pseudomonas putida</i> <i>Flavobacterium odoratum</i> <i>Pseudomonas paucimobilis</i> <i>Bacillus mascerans</i> <i>Bacillus polymyxa</i> <i>Bacillus firmus</i> <i>Bacillus circulans</i> <i>Moraxella bovis</i> ^d		<i>Hartmanella</i> <i>Acanthamoeba</i>
Sewage Treatment Plant (STP)	<i>Aeromonas hydrophilia</i> ^d <i>Pseudomonas alcaligenes</i> ^d <i>Pseudomonas aeruginosa</i> ^d <i>Pseudomonas putrefaciens</i> ^d <i>Bacillus stearothermophilus</i> ^d <i>Bacillus subtilis</i> ^e <i>Pseudomonas fluorescens</i> <i>Bacillus polymyxa</i> <i>Bacillus mascerans</i> <i>Bacillus firmus</i> <i>Bacillus circulans</i> <i>Achromobacter sp.</i> <i>Pseudomonas putida</i> <i>Flavobacterium odoratum</i> <i>Pseudomonas paucimobilis</i> <i>Enterobacter cloacae</i> ^d	<i>Mucor</i> ^f <i>Actinomycete</i> ^f	<i>Hartmanella</i> <i>Acanthamoeba</i>

^aNo *Legionella sp.* were detected in any sample; algae were present in large numbers in the water sample.

^bNo parasites were detected in any samples.

^cDrinking water supply prior to chlorination.

^dBeta hemolytic.

^eAlpha hemolytic.

^fThermophilic; potential thermophilic *Aspergillus sp.* were also present.

4. lux GENE BIOREPORTERS

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4.1 SUMMARY

Biodegradative bacterial strains harboring bioluminescent genetic constructions were used to examine the presence and bioavailability of contaminants in Kwajalein Island samples. These genetic constructions consisted of a fusion between a catabolic operon for naphthalene or toluene and the lux gene cassette. In the presence of the specific inducer contaminant, these strains emit visible light that can be accurately detected and quantified using a photomultiplier apparatus.

The results of this study demonstrate the expression of both catabolic operons and the bioavailability of inducing compounds for both operons in 2 of the 12 samples at our disposal (i.e., from the bottom of TP04 and TP05). Results from the time course of these experiments are presented in graphic form to illustrate the expression of the catabolic nah and xyl (toluene, xylene) genes.

The success of this technique encourages further experimentation to determine the optimal conditions for chemical sensing and degradative gene expression in this system and, ultimately, as an aid to *in situ* biodegradation.

4.2 INTRODUCTION

Remediation of a contaminated site is a process that can be divided into two parts: characterization of the site (e.g., description of the soil matrix, analysis of contaminants) and the bioremediation process itself. The system described below is useful for both halves of this dichotomy: it serves as a bioreporter of specific contaminant chemicals of interest, describes the bioavailability of these chemicals, and provides a useful monitor of the efficacy of bioremediation.

The system described in this report utilizes recombinant bioluminescent microorganisms as bioreporters of the contaminants naphthalene and toluene. The technology described for this system is generally applicable to many other substances such as phenol, benzene, heavy metals, etc. The efficacy of bioluminescence monitoring of naphthalene has already been established, and further reports for other contaminants are in progress.

The objectives for this study involved the examination of soil samples from Kwajalein Island through the use of these bioluminescent reporter strains. Experiments were designed to determine whether lux gene reporters are suitable for bioremediation studies with environmental samples. These experiments would report the presence and bioavailability of specific contaminants, providing very useful information that will be necessary to a large-scale bioremediation project. In addition, the versatility of the lux strains would be tested to evaluate other applications of this technology in the bioremediation process. Such tests would include optimization experiments for light generation in typical soil samples and supplementation assays to determine whether *in situ* bioremediation is a viable option.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial Strains

Three bacterial strains were used in this study. HK44 is a *Pseudomonas fluorescens* strain that contains a nah-lux fusion plasmid. It was constructed by transposon mutagenesis with TN4431; the transposon was inserted into the lower pathway of naphthalene degradation. The plasmid was moved by conjugation to a similar strain with an intact lower pathway. RB1351 contains a cloned fragment of the upper pathway, which includes the promoter. This promoter fragment is transcriptionally fused to the lux genes of *Vibrio fischeri*. This plasmid has been moved into *Pseudomonas putida* PB2440, which also contains the intact NAH7 plasmid. Strains HK44 and RB1351 produce bioluminescence in the presence of salicylate or naphthalene and degrade these compounds as well. These strains have been used to detect naphthalene in contaminated soil samples and to serve as a sensitive indicator of genetic activity directly related to biodegradation.

RB1401 contains a xyl-lux fusion plasmid; it was constructed by subcloning the upper pathway promoter from the TOL plasmid and fusing it transcriptionally to the lux genes. This plasmid was introduced into *Pseudomonas putida* mt-2, which contains the intact TOL plasmid. This strain produces bioluminescence in the presence of toluene or xylene and degrades them as well.

The structures of the relevant plasmid constructions, as well as the specific strains that harbor them, are presented in Fig. 4.1. For storage, the strains were grown overnight in rich medium and then aliquoted into sterile vials with glycerol at a 15% final concentration, frozen, and stored at -70°C.

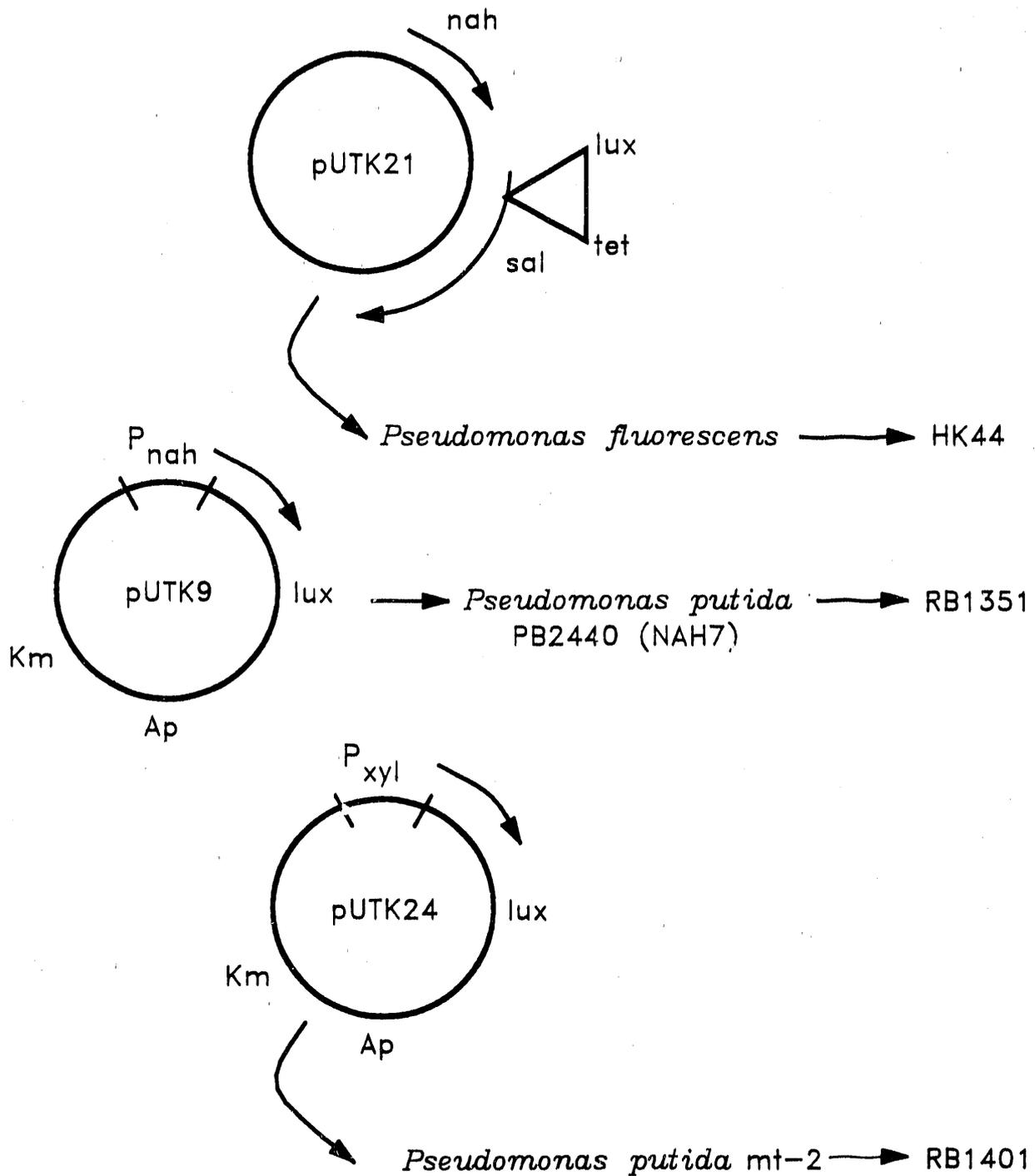


Fig. 4.1. Strain construction. HK44 was constructed by TN4431 transposon mutagenesis of the lower pathway of naphthalene degradation. RB1351 was constructed by subcloning of the NAH7 upper pathway promoter into *lux* vector plasmid pUCD615. The plasmid was then moved into *Pseudomonas putida* PB2440 (NAH7). RB1401 was constructed by subcloning the TOL upper pathway promoter into pUCD615 and subsequent introduction of the plasmid into *P. putida* mt-2 (a TOL strain).

4.3.2 Experimental Design

Formulae for media used in this study are presented in Table 4.1. The strains were prepared by thawing a frozen aliquot and allowing growth for several hours in yeast extract, peptone, and glucose medium (YEPG), followed by dilution into a minimal salts medium with glucose as the sole carbon source (1 mg/mL). This culture was grown overnight, after which the cells were diluted 1:10 into the same medium and grown for 3.5 h. Thirty-milliliter aliquots were harvested by centrifugation for 10 min at 8000 x g and then resuspended in minimal medium without glucose to starve the cells. Typically, these cultures had an optical density between 0.350 and 0.400 at 546 nm. Samples (2 g) of Kwajalein soils were weighed and distributed to sterile EPA vials with Teflon lids. The soil was mixed with 2 mL of minimal salts medium (without a carbon source) and then with 2 mL of the bacterial suspension. All vials were shaken at 27°C. Excess headspace provided sufficient oxygen. At intervals, the vials were examined for light output using an Oriel photomultiplier with a flexible liquid light pipe. Positive and negative controls were used throughout (10 mg naphthalene or 0.015 mM toluene). Duplicates of each soil sample were utilized.

4.3.3 HPLC Analysis

Samples of aqueous soil slurry supernatant were examined by HPLC analysis. They were directly injected into a Varian Vista 5500 liquid chromatograph equipped with a guard column and a 25-cm VYDAC 201 TP column. Fractions were eluted under isocratic conditions with an acetonitrile/water mixture and were analyzed using fluorescence detection (Perkin-Elmer LS-4 fluorescence spectrometer).

The data obtained from these experiments consist of *in situ* bioluminescence readings in Kwajalein soil slurries. A comparison of the light production among the various samples gives a measure for the extent of catabolic gene expression for both the *nah* and the *xyI* operons, which are essential for the biodegradation of the pollutants naphthalene, xylene, and toluene. The observed light induction correlates with the presence and bioavailability of such inducing compounds. Such data provide information on the degree of contamination of individual soil samples and the potential for genetic activation of degradative strains therein.

4.4 RESULTS

4.4.1 Optimization of the Process

In order to optimize the bioluminescence reporter system for maximum sensitivity, a series of experiments was performed to reduce background bioluminescence. The HK44 strain was selected as a model for this work. As seen in Fig. 4.2, the initial quantity of bioluminescence can be reduced by increasing the carbon substrate starvation time prior to the soil slurry assay. A low background light response is important since it reflects low catabolic enzyme levels. The initial lack of these enzymes would allow for a slow degradation of target substrate and, therefore, a longer physiological response period that could be monitored. In addition, a low light background might also reveal increases in catabolic gene expression as a result of low target substrate concentrations that would not be detected at high initial expression levels. For these reasons, a 3-h starvation period was chosen.

Table 4.1. Composition of cultivation media

Cultivation medium	Concentration (g/L)
Mineral salts medium	
MgSO ₄ · 7H ₂ O	0.1
NH ₄ NO ₃	0.2
Phosphate buffer, 0.5 M	100 (mL/L)
Trace-element solution	0.1 (mL/L)
Glucose	1
Trace-element solution	
MgO	10
CaCl ₂	2.94
FeCl ₃ · 6H ₂ O	5.4
ZnSO ₄ · 7H ₂ O	1.44
CuSO ₄	0.25
H ₃ BO ₄	0.062
Na ₂ MoO ₄ · H ₂ O	0.49
YEPG	
Yeast extract	0.2
Polypeptone	2.0
Glucose	1.0
NH ₄ NO ₃	0.2
Phosphate buffer, 0.5 M	100 (mL/L)

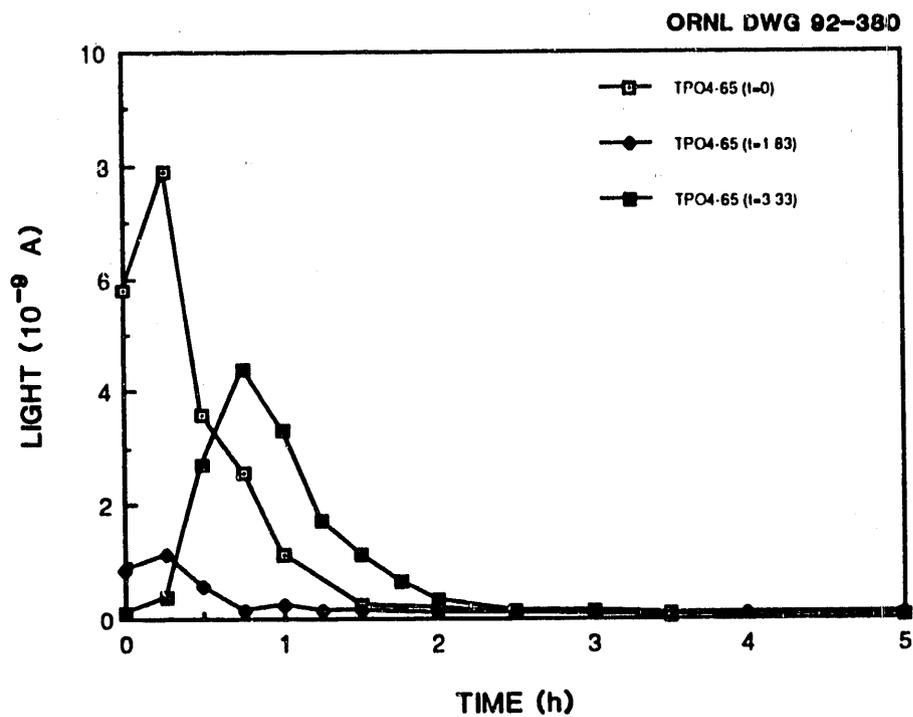


Fig. 4.2. Effect of starvation time on initial catabolic naphthalene operon expression in *Pseudomonas fluorescens* HK44 in TP04-65 soil slurry. Starvation periods prior to the soil slurry assay were 0, 1.83, and 3.33 h.

4.4.2 Bioreporters for Naphthalene

Figure 4.3 demonstrates the clear difference in the expression of the catabolic *nah* operon between contaminated and uncontaminated soils when the HK44 strain is used. The response curve for the contaminated soil, TP04-65, shows the induction of bioluminescence followed by a gradual decrease to a background level. In contrast, the uncontaminated soil, TP01-08, demonstrates no bioluminescent response at all.

It was observed that, under these experimental conditions, a light response could also be induced in strain HK44 by substrates other than naphthalene and salicylate. Table 4.2 summarizes the results obtained when various carbon sources are used in this assay and compares these responses to the response with naphthalene. None of these other carbon sources resulted in a response comparable with the naphthalene response, even though the molar aqueous concentration of the other substrates was higher. In addition, the concentrations used for glucose, succinate, and acetate are clearly above environmentally practical levels.

Strain RB1351, which also responds to naphthalene, was used in order to obtain an independent analysis of the soils. A nonspecific bioluminescent response was also observed, but it was significantly less pronounced under these experimental conditions when compared with that for strain HK44. Figure 4.4 illustrates the positive result with the TP04-65 soils as compared with the control soil, TP01-08. Although the overall response is not as strong, it can be considered significant, whereas the slight transient increase seen for soil TP05-70 (data not shown) cannot be considered significant. This obvious difference in the response of strain RB1351 as compared with strain HK44 might be due, in part, to the fact that the RB1351 experiments were performed very late in the experimental investigation, after the soil vials had been opened many times. It is possible that substrate could have been dispersed with each opening of the vial.

In general, the results obtained by using HK44 and RB1351 are in agreement. A summary of these results is shown in Fig. 4.5, using a profile of the sample sites. Of the samples placed at our disposal, only the TP04-65 and TP05-70 demonstrated a positive response. All other samples were apparently uncontaminated with naphthalene at levels detectable by these assays, or these compounds are not bioavailable. An indication for the presence of naphthalene in soil slurry supernatants was found for the samples TP04-65 and TP05-70, while the supernatant of TP01-08 demonstrated no detectable levels of naphthalene. However, these data have to be considered as suggestive since naphthalene identity has been judged only according to retention times.

4.4.3 Bioreporter for Toluene

The RB1401 strain was utilized in an analogous experiment to determine whether toluene or xylene was present in these samples. Figure 4.6 presents data for a typical pair of samples for comparison. The data are remarkably similar to those seen with naphthalene, which confirms the contaminated nature of soils TP04-65 and TP05-70. A profile of the results seen with this strain is presented in Fig. 4.7.

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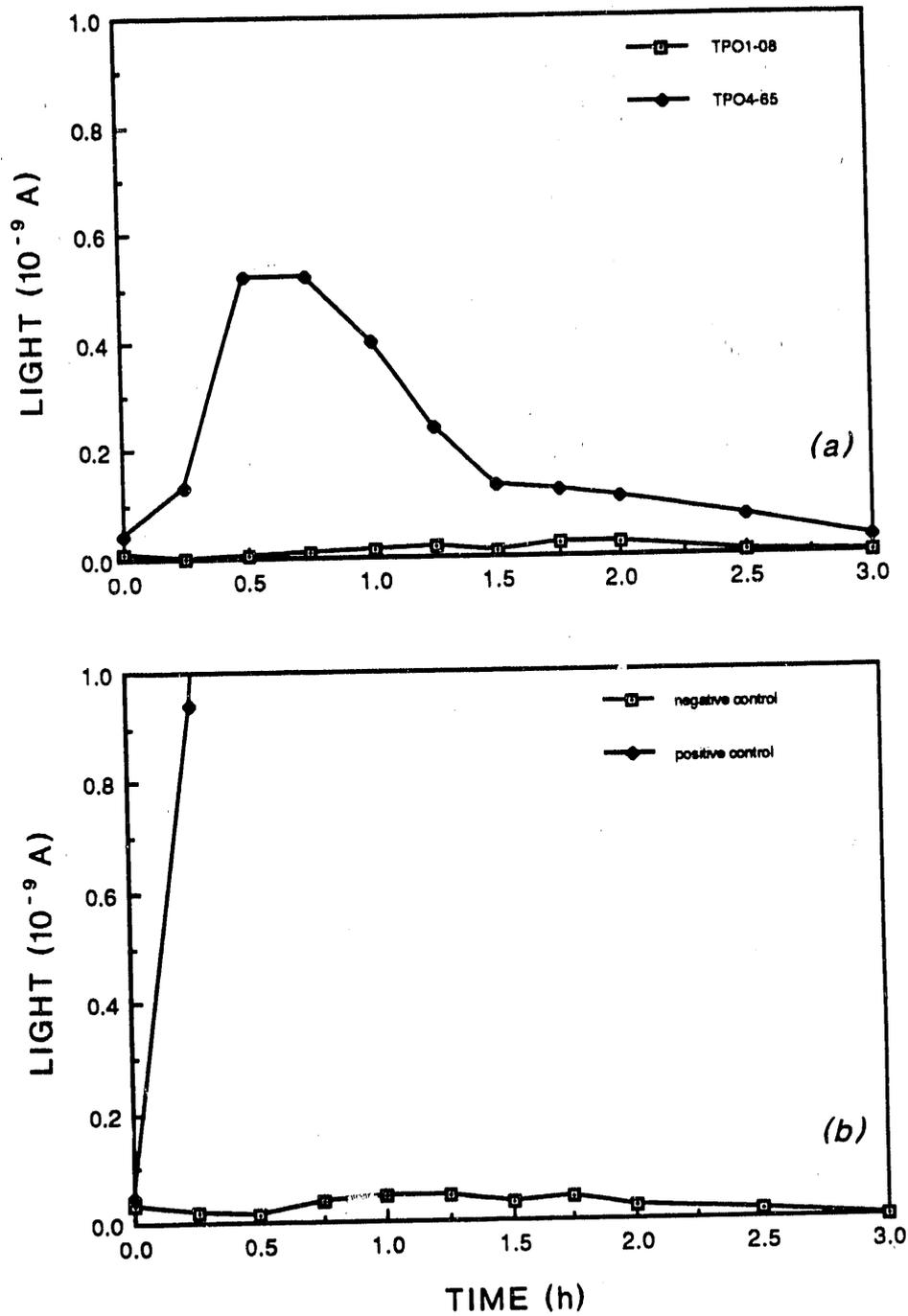


Fig. 4.3. Dynamic response of catabolic naphthalene operon expression in *Pseudomonas fluorescens* HK44. (a) Kwajalein control soil TP01-08 and contaminated soil TP04-65 amended with mineral salts medium; (b) mineral salts medium amended with naphthalene crystals (positive control) and without naphthalene (negative control).

Table 4.2. Effects of various carbon substrates on the expression of the catabolic naphthalene operon in *Pseudomonas fluorescens* HK44 and *Ps. putida* RB1351 and on the expression of the catabolic xylene operon in *Ps. putida* RB1401

Substrate	HK44 ^a	RB1351 ^a	RB1401 ^a
Glucose, 0.55 mM	++	+	+
Succinate, 0.55 mM	+	n.d. ^b	n.d.
Acetate, 0.55 mM	-	n.d.	n.d.
YEPG, diluted 20X	+	n.d.	n.d.
YEP, diluted 20X	+	n.d.	n.d.
Salicylate, 0.62 mM	+++	+++	-
Naphthalene, 0.15 mM	+++	+++	-
Toluene, 0.015 mM	-	-	+++

^a -, no expression; +, significant expression; ++, significant expression; +++, very strong expression; n.d., not determined.

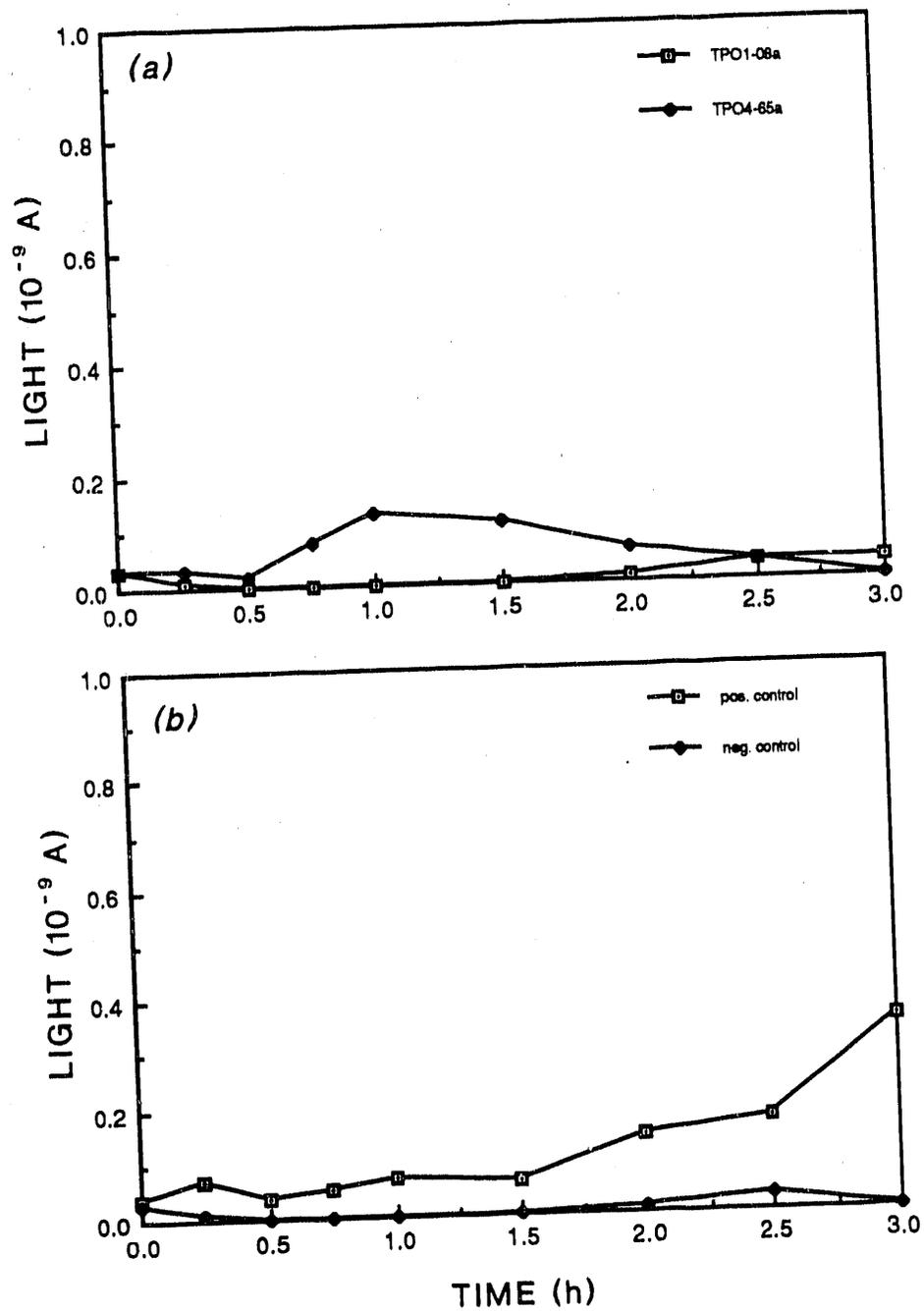


Fig. 4.4. Dynamic response of catabolic naphthalene operon expression in *Pseudomonas putida* RB1351. (a) Kwajalein control soil TPO1-08 and contaminated soil TPO4-65 amended with mineral salts medium; (b) mineral salts medium amended with naphthalene crystals (positive control) and without naphthalene (negative control).

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Site Depth	TP01	TP02	TP03	TP04	TP05
6		-			
8	-				
12			-		
32				..	
33	-				
56	-				
62					-
63		-			
65				+	
70					+
72			-		
W				-	

Fig. 4.5. Assessment of bioavailability for naphthalene operon-inducing compounds in *Pseudomonas fluorescens* HK44: cross section along the Kwajalein sample sites. Positive response (+); no response (-); no sample available (blank). All depths are in inches; W = water table.

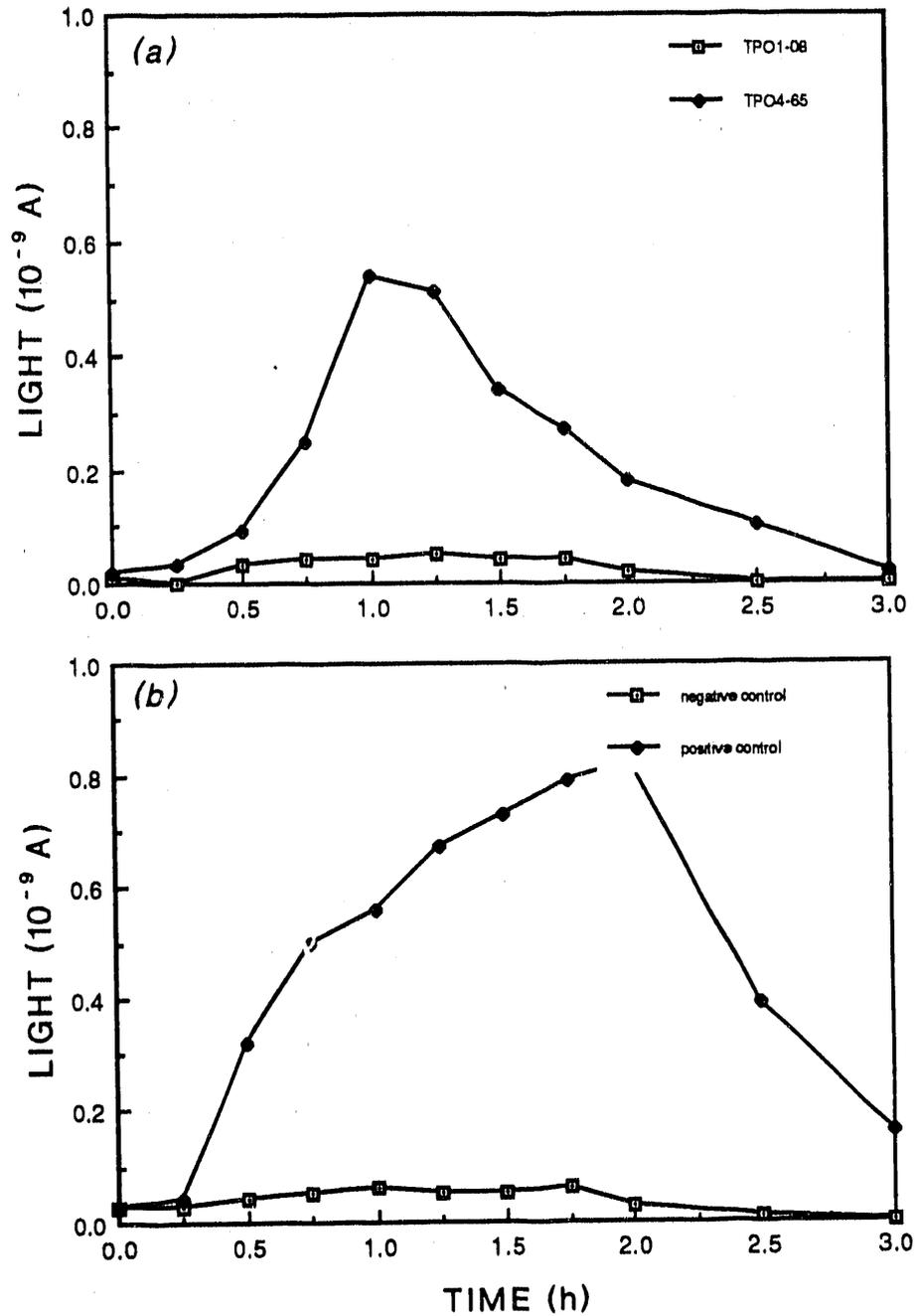


Fig. 4.6. Dynamic response of catabolic toluene operon expression in *Pseudomonas putida* RB1401. (a) Kwajalein control soil TP01-08 and contaminated soil TP04-65 amended with mineral salts medium; (b) mineral salts medium amended with toluene (positive control) and without toluene (negative control).

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Site Depth	TP01	TP02	TP03	TP04	TP05
6		-			
8	-				
12			-		
32				-	
33	-				
56	-				
62					-
63		-			
65				+	
70					+
72			-		
W				-	

Fig. 4.7. Assessment of bioavailability for toluene operon-inducing compounds in *Pseudomonas putida* RB1401: cross section along the Kwajalein sample sites. Positive response (+); no response (-); no sample available (blank). All depths in inches; W = water table.

The different responses obtained in the positive controls (compare Figs. 4.3, 4.4, and 4.6) are attributable to the crystalline structure of naphthalene. Such crystals enter solution very slowly and, thus, provide a continuous source of substrate. Since toluene is very toxic to cells, small amounts must be used (final concentration, 0.015 mM). This may result in a less intense, transient light increase.

4.5 DISCUSSION

The results presented here clearly show a distinct difference in the catabolic gene expression pattern between heavily contaminated and presumably noncontaminated soil samples. In addition, the same expression patterns were observed in independent experiments for naphthalene and toluene detection. These results indicate the presence and bioavailability of both of these inducing substrates. The finding that substrates other than naphthalene can result in gene expression under these experimental conditions, especially when strain HK44 is used, must be taken into account. However, the detection of naphthalene in soil slurry supernatants of TP04-65 and TP05-70 (but not in TP01-08) provides an indication that the response of HK44 is specific for the contaminant. In addition, a positive response due to soluble soil compounds has not been observed in uncontaminated Tennessee soils thus far. Clearly, further experiments are necessary to minimize nonspecific responses and to investigate the control of gene expression.

The response of strains RB1351 and RB1401 to glucose was less pronounced than that of strain HK44 and, consequently, seems to be less problematic. However, the response to other possible inducers would have to be investigated in further studies.

It has been suggested that bioluminescent reporter strains could be used to optimize the conditions for biodegradation, and experiments are presently under way in our laboratory to determine whether this is a practical approach. For example, the generation of light could be followed during addition of specific nutrients to the soil samples in an effort to determine the best feed stock for an *in situ* bioremediation.

In summary, the versatility, sensitivity, and cost-effectiveness of the bioluminescent reporter strains make them a potentially attractive option for determining the spread of specific contaminants and for the optimization of the bioremediation process.

4.6 CONCLUSIONS

Based on the results obtained in this study, the following conclusions can be drawn:

1. The use of bioluminescent reporter strains is an effective technique for the determination of specific catabolic gene expression in contaminated soil samples.
2. An important parameter of bioremediation, bioavailability, is directly measured with this assay. The method presented shows the bioavailability of compounds that induce specific catabolic operons.
3. The method displays a great potential for specific contaminant detection in complex environmental matrices; however, further characterization and optimization studies to address specific applications are needed.

4. Experiments in defined liquid culture systems indicate that quantitative estimates of contaminant levels can be obtained with this method.
5. Optimization experiments should continue in order to investigate other facets of these strains.
6. This method shows promise for the Kwajalein Project in two aspects: (a) it is simple, rapid, and reproducible and, therefore, very useful for on-site analyses; and (b) it provides important information that may influence decisions regarding appropriate remediation technologies.

4.7 ACKNOWLEDGMENTS

We express our thanks to Oren Webb, Department of Chemical Engineering, The University of Tennessee, for providing help with the high-performance liquid chromatography (HPLC) analysis.

5. GENE PROBES

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5.1 INTRODUCTION

These experiments were undertaken to determine whether bacteria isolated from Kwajalein soil contained DNA sequences that were identical or similar to known genes involved in the biodegradation of organic compounds. If identity or a high degree of similarity exists, it is possible to demonstrate this by hybridizing the DNA from the isolated bacteria with DNA probes prepared from well-characterized degradative genes.

5.2 METHODS

Soil samples from the Kwajalein Island were plated on agar containing peptone, tryptone, yeast extract, and glucose (PTYG). After 2 weeks, the colonies were transferred to nylon filters. Cells were lysed, and DNA was denatured with 0.5 N NaOH. DNA was extracted from 2 g of soil and fixed on nylon filters (slot blot). Three different probes were used (i.e., nah A, naphthalene dioxygenase; xyl C, benzaldehyde dehydrogenase; and tod C2C1BA, toluene dioxygenase). Naphthalene and toluene mineralization experiments were carried out with all samples.

5.3 RESULTS AND DISCUSSION

None of the sites showed any reaction with the probes by colony or slot blot hybridization. Since no toluene or naphthalene degradative genes were found in the samples, it might be possible that degraders are present at very low densities and that enrichment of the soils will increase their numbers; however, direct DNA extraction and hybridization did not detect any degradative genotypes. The three DNA probes tested here were isolated from gram-negative bacteria and, on the basis of the DNA:DNA hybridization results, it seems that gram-positive bacteria populations (e.g., *Actinomyces* or *Arthrobacter*) will have a greater role in the biodegradation of toluene and naphthalene due to the absence of gram-negative degradative genes.

Percentages of mineralization ranged from 1.20 ± 0.13 to 9.0 ± 1.00 after 13 d. Table 5.1 shows that naphthalene was mineralized in sites TP01-33, TP02-63, TP03-12, and TP04-65. Toluene was only mineralized in site TP03-72. The mineralization of each compound was very low.

In conclusion, the degradative potential of the bacterial communities in Kwajalein soils appears to be very limited regarding toluene and naphthalene. Nevertheless, some mineralization was found, and it might be possible to enhance the mineralization of these compounds through the addition of different nutrients (e.g., nitrogen) since these sites are nitrogen limited. Since diesel fuel is comprised of a wide range of aromatic and aliphatic compounds, it might be possible that the majority of the microbial populations were adapted to specific components that do not include toluene and naphthalene.

Table 5.1. Naphthalene and toluene mineralization by soil samples from Kwajalein Island

Site	% of NAH and TOL mineralized ^a	Standard deviation
TP01-33	1.20 ^b	0.13
TP02-63	8.15 ^b	4.46
TP03-12	4.90 ^b	3.43
TP04-65	5.43 ^b	2.54
TP03-72	9.00 ^c	1.00

^aIncubation time, 13 d.

^b1.30 X 10⁵ dis/min of ¹⁴C-naphthalene was loaded onto 1 g of soil.

^c1.50 X 10⁵ dis/min of ¹⁴C-toluene was loaded onto 1 g of soil.

6. FATTY ACID PROFILES

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6.1 INTRODUCTION

These experiments were designed to determine the profiles of ester-linked phospholipid fatty acids present in Kwajalein soil samples. The fatty acids are derived from microbial cell membranes, and the profiles obtained provide information regarding total biomass, community structure, and nutritional conditions of the microorganisms present. This information should help in the optimization of the conditions used for the demonstration project and should also be useful in monitoring the effects of bioremediation on the microbial population changes that occur in soil.

6.2 RESULTS AND DISCUSSION

The most reliable *in situ* technology for determining the viable or potentially viable microbial biomass in soils and sediments is the determination of the phospholipid ester-linked fatty acid content (PLFA). The presence of phospholipid in viable cells is based on the demonstrated metabolic lability of phospholipids in soils and sediments. The PLFA analysis does not require quantitative recovery of cells from the soil matrix (as do many direct counting procedures) or the quantitative growth of each isolate for viable counts. Table 6.1 indicates the presence of viable bacteria in the soils (and provides estimates of their numbers, based on 10^{12} cells/g and 100 μmol PLFA/g); similar trends were shown by both PLFA and plate count analyses.

Results of PLFA analysis also provide a quantitative estimate of the microbial community structure, as different subsets of the community have different PLFA patterns. In general, the profiles showed a high degree of similarity (Fig. 6.1). The presence of 10-methyl (mid-chain) saturated PLFA (10Me-16:0,18:0) indicated that aerobic actinomycetes were present, along with gram-positive organisms, from the short, terminally branched saturated PLFA. Samples tp4-w, tp4-65, tp5-62, and stp showed high levels of anaerobic desaturase PLFA (16:1w7c, 18:1w7c) characteristic of facultative heterotrophs. High levels of these PLFA correlated with low levels of the actinomycete signatures, which indicated a low to "absent" level of oxygen in these soils. All of the samples showed a low level of polyunsaturated PLFA and relatively little 18:1w9c, a precursor in aerobic desaturation.

Changes in the ratios of specific PLFA, primarily the ratios of trans/cis and cyclopropyl/monoenoic PLFA, indicate metabolic stress. Cyclo/mono ratios increase greatly with starvation and the stationary phase of growth. This ratio was highest in samples tp1-33 (which also showed an abundance of hydrocarbons), tp4-w, and tp4-32 (Fig. 6.2). Increased trans/cis ratios indicate starvation with exposure to a more toxic environment and were seen in tp1-33, tp4-w, tp5-62, and tp5-76 (tp4-w and tp5-62 also showed the highest levels of anaerobic desaturase PLFA).

Table 6.1. Viable or potentially viable cells

Sample	Concentration (pmol/g)	Cells/g	Plate counts
tp1-08	1012.3	5.9×10^7	6.0×10^6
-33	61.7	3.6×10^6	2.0×10^4
-56	30.7	1.8×10^6	3.5×10^4
tp2-06	65.2	3.8×10^6	1.6×10^5
-63	7.8	4.6×10^5	5.0×10^3
tp3-12	29.3	1.7×10^6	1.0×10^5
-72	377.0	2.2×10^7	1.2×10^3
tp4-32	822.5	4.8×10^7	3.0×10^5
-65	5.2	3.0×10^5	2.0×10^4
-w ^a	16.7	9.8×10^5	1.0×10^4
tp5-62	40.9	2.4×10^6	5.0×10^3
-76	10.4	6.1×10^5	4.0×10^1
stp ^b	5909.0	3.4×10^8	5.0×10^6

^aSewage treatment plant.

^bWater.

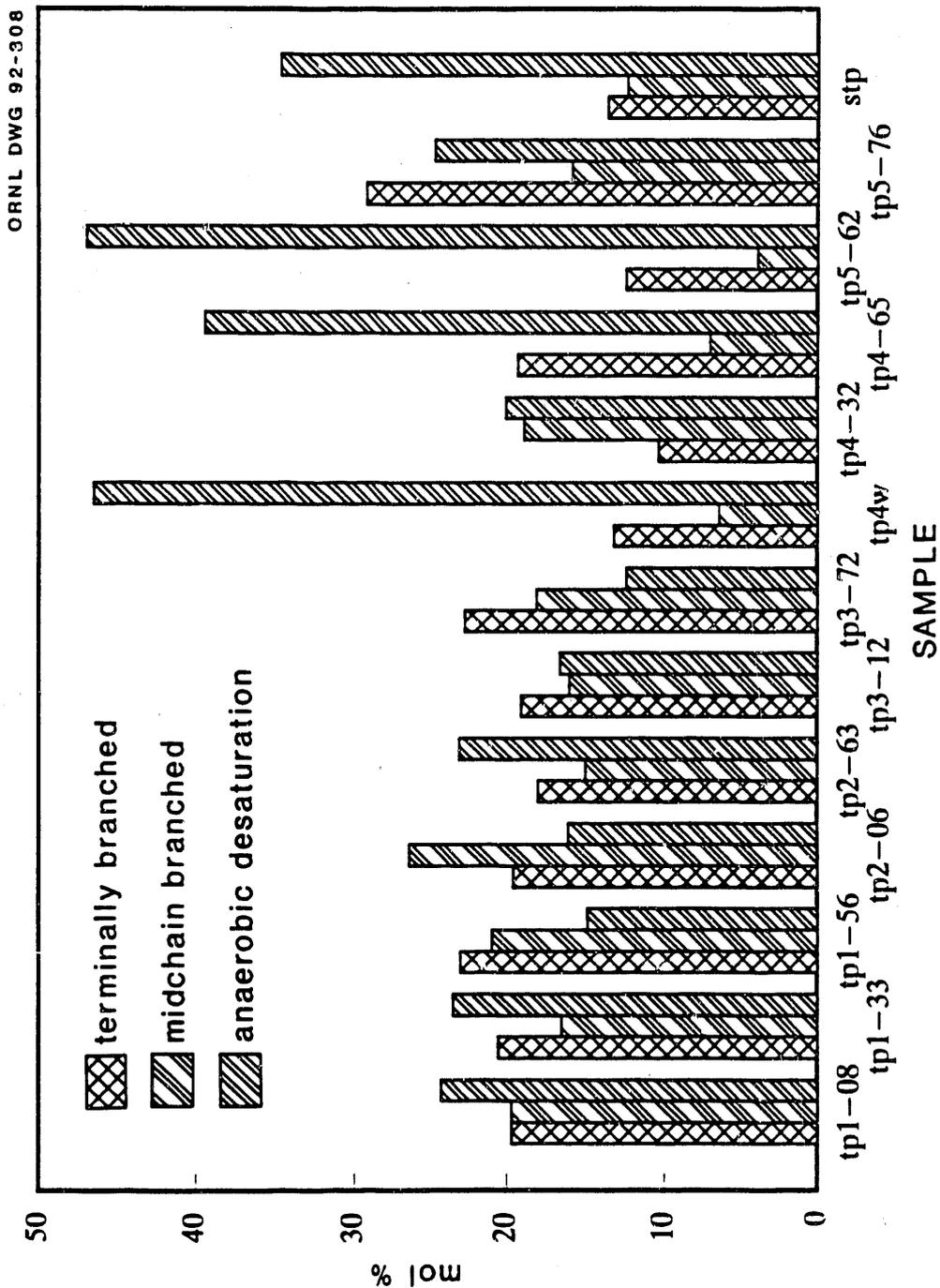


Fig. 6.1. Community structure from PLFA biosynthesis.

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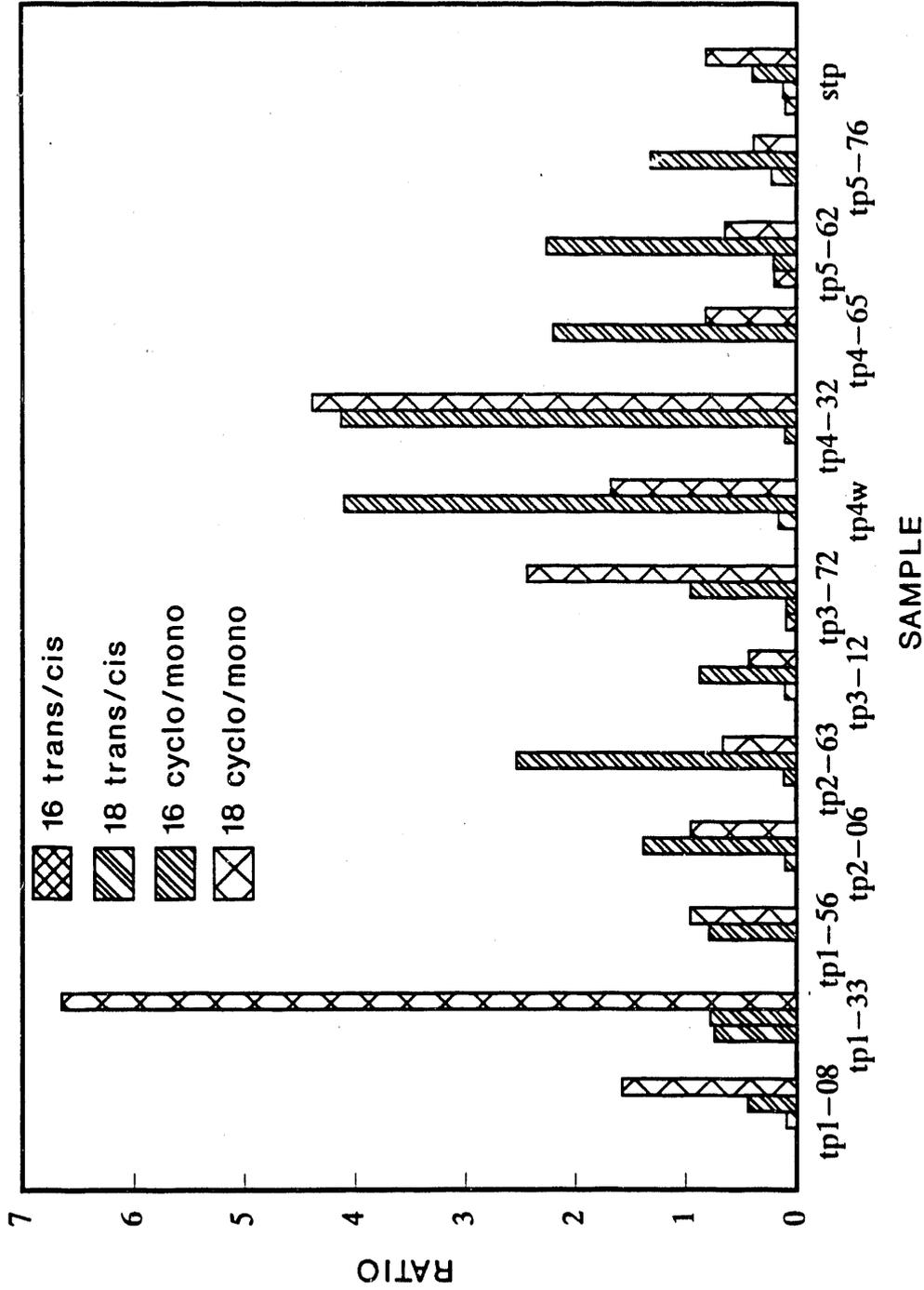


Fig. 6.2. PLFA as indicators of microbial physiology.

Formation of the endogeneous lipid storage product, poly beta-hydroxy alkanate (PHA), defines conditions for unbalanced growth. This is readily demonstrated by the ratio of PHA/PLFA, which is an important measure of the community nutritional status as high levels are correlated strongly with the ability to degrade fortuitously metabolized contaminants such as TCE. The PHA detected in these samples was the butyrate polyester (PHB). The highest levels of PHB were found in samples tp4-32 and stp (Table 6.2).

Similarities between the total community in each sample can be quantitatively related with cluster analysis in which the most closely related patterns are grouped together (Fig. 6.3). The dendrogram shows two subgroups: the anaerobic desaturase PLFA and the branched-chain PLFA. The microbial community in sample tp4-32 was significantly distinct from either of these two groups, based on the presence of two unusual PLFAs. The dendrogram shows clearly that the communities in samples tp4-w, tp4-32, tp4-62, and tp4-65 are more similar to each other than to the other samples.

Table 6.2. Production of poly beta-hydroxy alkanate

Sample	Concentration (nmol PHB/g)	PHB/PLFA ratio
tp1-08	20.36	20.11
tp3-12	5.34	182.25
-72	25.51	67.67
tp4-32	197.52	240.15
-65	4.76	915.38
-w ^a	37.60 ^b	2251.50
stp ^c	249.82	42.27

^aWater.

^bnmol PHB/mL.

^cSewage treatment plant.

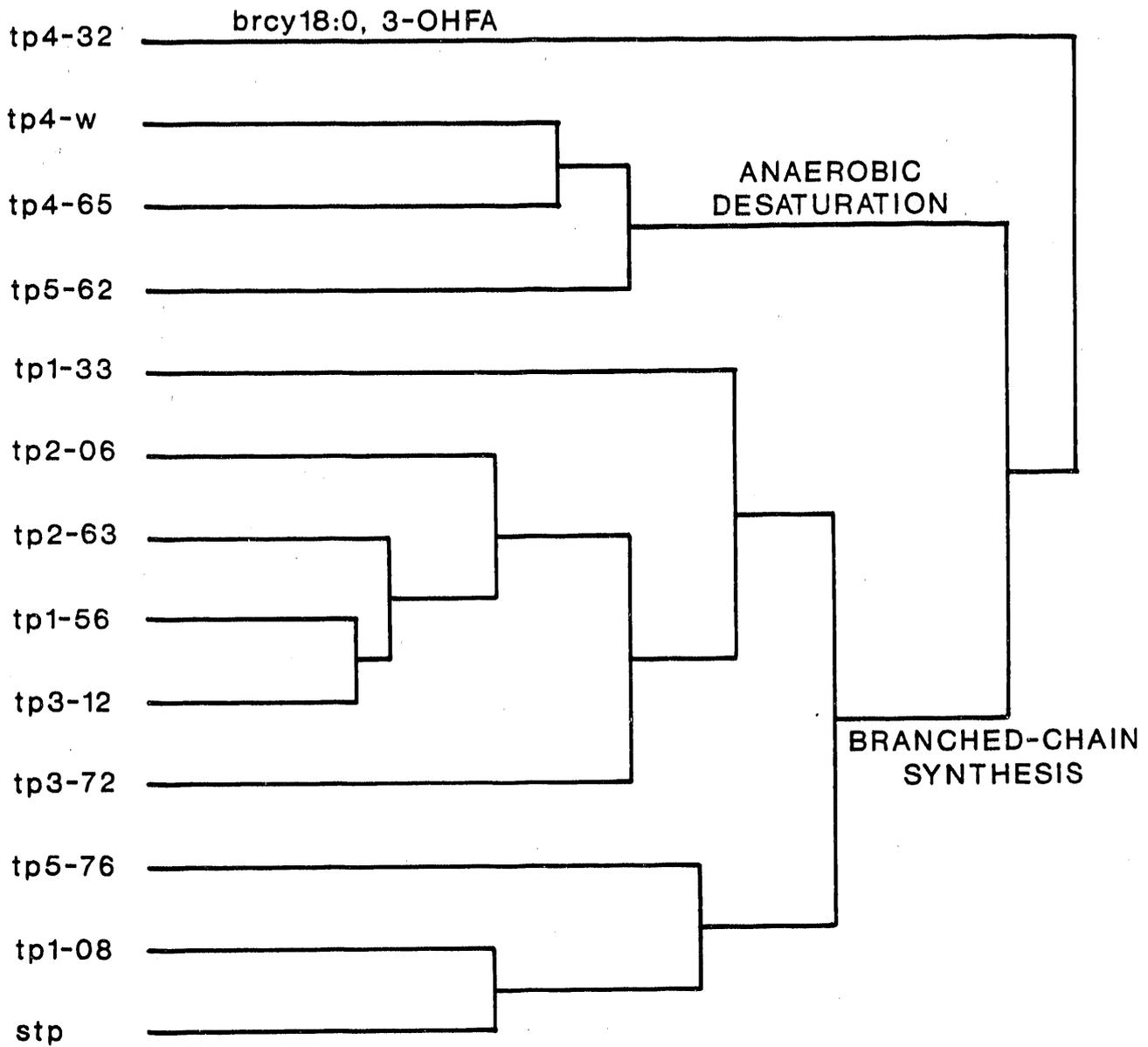


Fig. 6.3. Complete linkage: farthest neighbor cluster of sediment samples.

7. MICROBIAL ACTIVITY AND BIOMASS

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7.1 INTRODUCTION

As part of the Kwajalein characterization project, we examined microbial abundance and activity. Microbial abundance was assessed by plate-count methods and compared with on-site plate counts using both an extremely dilute and a less dilute complex medium. Activities were assessed at the general level common to microorganisms as well as at specific levels related to the degradation of hydrocarbons. The general activity of choice was [^{14}C]-acetate incorporation into microbial lipids. Nearly all life forms have the 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.

Specific activities of interest were measured by two distinct techniques: radioisotope mineralization studies and respirometry. The respirometry data will be discussed later in Chap. 10; some of the hydrocarbon analyses from those tests will be discussed here. Radioisotope mineralization of ^{14}C -toluene and ^{14}C -benzene was also analyzed in time course experiments. These results demonstrate that the biodegradation of petroleum hydrocarbons is occurring and can be augmented in the contaminated soils and sediments of Kwajalein Island.

An additional task was to investigate the bioavailability or liquid-phase availability of petroleum hydrocarbons in simple, low-technology experiments.

7.2 MATERIALS AND METHODS

7.2.1 Gases, Chemicals, and Isotopes

Nitrogen and $\text{N}_2:\text{CO}_2$ (90:10%) were greater than 99.9% pure. In the laboratory, all gases were passed through copper-filled Vycor furnaces (Sargent-Welch Scientific Company, Skokie, IL) to remove traces of oxygen. All chemicals used in the study were of reagent grade and were obtained from Mallinckrodt (Paris, KY) or Sigma Chemical Company (St. Louis, MO). Resi-analyzed glass-distilled solvents and reagents were purchased from the J. T. Baker Chemical Company (Phillipsburg, NJ).

7.2.2 Culturing

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 medium containing 1 g/L of each nutrient (Balkwill). All media contained trace minerals, including selenium and molybdate, as well as

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.

7.2.3 Radiolabeled Uptake and Transformation Experiments

[¹⁴C-1-]Acetate (56 mCi/mmol) was purchased from the New England Nuclear Corporation (Boston, MA), and [¹⁴C-U-]benzene (50.3 mCi/mmol) and [¹⁴C-U-]toluene (57.4 mCi/mmol) were purchased from Sigma Chemical. Sediment aliquots were inoculated for aerobic activity experiments. All isotope solutions (1 to 50 μCi) were frozen prior to use, thawed, and transferred with gas-tight syringes (Hamilton Company, Reno, NV). Time course experiments were performed in duplicate using sterile polypropylene centrifuge tubes for aerobic isotope incorporation experiments and anaerobic crimp-top tubes (Bellco Glass Company, Vineland, NJ) for mineralization 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 sediment, 5.0 μCi of ¹⁴C-1-acetate, and 1.0 mL 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, 2 h, 8 h, 1 d, and 3 d generally provided linear rates within two or more points. Prior to analysis, acetate incorporation experiments were thawed, and then the sediments were extracted by a modification of the single-phase chloroform-methanol method. 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).

Mineralization experiments contained 2.0 g sediment, 1.0 mL sterile water, and carrier-free isotope in 25-mL crimp-top tubes. Benzene was added at 1.1 μCi/tube, while toluene was added at 0.66 μCi/tube. Time course experiments were performed in duplicate with multiple time points ranging from t_0 , 1 h, and up to 1 month, with a minimum of four time points per isotope examined. All tubes utilized Teflon septa and were incubated in the dark at ambient temperature. At selected time points ($t = 0, 3 \text{ d}, 10 \text{ d}, \text{ and } 30 \text{ d}$), duplicate tubes were inhibited with 0.5 mL of 2.0 M sodium hydroxide. Each tube was acidified with 0.5 mL of 6 M HCl at 1 h prior to analysis.

Radioactive ¹⁴C-carbon dioxide and ¹⁴C-methane from mineralization and time course experiments were examined by gas chromatography—gas proportional counting. A Packard 417 gas chromatograph (GC) equipped with a thermal conductivity detector was connected to a Packard 894 gas proportional counter. The GC, which was operated at 85°C and a helium carrier gas flow of 45 mL/min, had a 0.32 cm x 1.83 m stainless steel column packed with Carbosieve (80-100 mesh). Radioactive aromatics were measured by hooking the gas proportional counter to the gas chromatograph containing the photoionization detector and switching the carrier gas from nitrogen to helium. Results were calculated as dpm/d, based on the initial slope of product evolution.

7.2.4 Analysis of Petroleum Hydrocarbons

A rapid and simple assay for estimating total petroleum hydrocarbons was explored and is being developed. Aliquots of sediment were extracted with isooctane and then sonicated for 16 h in alternating 30-min cycles. Sediments were centrifuged, and the isooctane was removed with a Pasteur pipet. Another volume of isooctane was then added, sonicated for 4 h, and centrifuged. The isooctane aliquots were added and analyzed for hydrocarbons on a Hewlett-Packard 5890 or Shimadzu 9A GC at 50°C in the splitless mode using a nonpolar cross-linked methyl silicone fused silica column (50 m x 0.2 mm internal diameter). Peak areas were quantified using a Nelson Analytical (Perkin-Elmer) laboratory data system operated with an internal standard program.

7.2.5 Liquid-Phase Availability and Bioavailability Experiments

To examine liquid-phase availability and bioavailability of petroleum hydrocarbons, we took 20-g sediment aliquots and performed experiments in triplicate using 50-mL Teflon-lined screw-capped tubes. The experiments included sediment and water in a stationary mode, sediment and water in a rocking platform, sediment and water in a sonicator, and then sediment and isooctane in each of the conditions. After 1.5 h, 5 h, 1 d, 2 d, 4 d, and 6 d, the sediment was pelleted by centrifugation and the liquid phase was removed by Pasteur pipetting. The liquid phase was replaced, and the experimental treatment continued. The liquid phase that was removed from the test bottles was then sonicated-extracted as described above with 2 mL isooctane and analyzed for hydrocarbons by GC.

7.3 RESULTS AND DISCUSSION

7.3.1 Colony Forming Units

Total colony forming units (CFU) found within the uppermost 10 in. of Kwajalein soils are typical of surface soils. Below 10 in., their numbers decrease (Table 7.1). In the most contaminated zones below 60 in. in test pits 4 and 5, the numbers are <100,000/g, possibly showing effects of toxicity caused by severe hydrocarbon contamination. Counts made in the laboratory for many samples likely reflect growth after sample collection. Lipid and probing data also suggest a gram-positive environment. Future efforts should ascertain if gram-positive organisms predominate.

7.3.2 Radiolabeled Acetate Incorporation

Surface soils typically exhibit activities $>10^6$ dpm/d. Shallow sediments at the site reflect typical surface values (Table 7.2). Activities for deeper sediments from pits 4 and 5 likely reflect the toxicity of the environment and are in the range of 10^5 dpm/d. Values of 100,000 dpm/d would be typical of subsurface aquifers >100 ft deep; these values are atypically low for pristine environments near the surface. In many aquifers, stimulation is observed over longer incubation times. The lack of stimulation in these samples suggests that either they had already been stimulated to the maximum amount during storage or that stimulation would require additional nutrients. Respirometry results showed that stimulation of >10 fold could be achieved with nutrient additions.

Table 7.1. Microbial CFU observed in Kwajalein soils and sediments

Sample number	Depth (in.)	Field analyses (log CFU)	Laboratory analyses	
			IAM agar (log CFU)	Balkwill agar (log CFU)
Test pit 01	08	6.78	5.98	5.80
	33	4.30	4.90	4.85
	56	5.54	5.73	5.70
	Water		5.86	5.83
Test pit 01	06	5.20	4.52	4.59
	63	3.69	5.08	5.20
Test pit 03	12	5.00	7.46	7.43
	72	3.08	5.08	5.36
Test pit 04	10	6.85	ND ^a	ND ^a
	32	5.48	5.43	5.45
	65	4.30	7.47	7.47
	Water	4.00	4.96	4.98
Test pit 05	62	3.70	4.95	4.94
	70	1.60	3.15	3.11
Burn pit 10	08		6.93	6.93
	19		7.15	7.23
	29	5.30	6.66	7.15
	31		6.88	6.91
	35		7.66	7.69
	38	>6	7.18	7.40
Burn pit 11	10		6.97	7.18
	30		6.76	7.15
	38	5.48	6.46	6.62
	41		7.23	7.18
	52	>4.48	7.72	7.89
	59		7.53	7.56
	65		6.70	7.00
	69		7.11	7.00
Burn pit 12	10		6.93	6.00
	35		5.48	5.27
	56		6.30	6.30
	51	>6	6.96	7.00
	55	>5	3.76	3.78
Sewage drying bed			6.40	6.20

^aND = not determined.

**Table 7.2. Radiolabeled acetate incorporation into cellular lipids
as indicator of microbial activities**

Sample pit	Depth (in.)	[¹⁴ -C-]Acetate incorporation into microbial lipids (10 ³ dpm/d) ^a	Stimulation after longer incubation times ^b
1	8	1540	+
	33	132	
	56	520	
	56 water	456	
2	6	624	
	63	186	
3	12	316	
	72	171	
4	10	1020	
	32	390	
	65	700	
	65 water	120	
5	62	260	
	70	324	
STP ^c		>2500	

^aAverage $t = 0$ was 0.4.

^b3-5 fold stimulation = +; 5-10 fold stimulation = ++; and >10 fold increase in the rate of acetate incorporation = +++.

^cSewage treatment plant.

7.3.3 Benzene and Toluene Mineralization

Mineralization of benzene and toluene was ubiquitous (Table 7.3), but rates were much less than desired. Turnover times for the isotopes have not been calculated but would be estimated to be 1 week in the most rapid instances; 3 to 6 months would be estimated for many samples. Benzene mineralization was generally faster than toluene mineralization in all samples except those from test pits 4 and 5, possibly suggesting selection in those habitats. Surprisingly, near-surface samples exhibited faster rates than did deeper sediments, possibly due to smaller pool sizes and, in part, likely due to toxicity effects of the severe contamination in deeper sediments. The conclusion is that mechanisms for mineralization are in place, and results from respirometry experiments suggest that the rates can be stimulated.

7.3.4 Liquid-Phase Availability of Petroleum Hydrocarbons

Tests showed the effect of sonication and standing (set) water on the removal of petroleum from the solid phase to the liquid phase. All samples containing water resulted in chromatograms that were four times as concentrated as those with isooctane as a result of the extraction process. Isooctane, coupled with sonication, extracted the sediments within 5 h, in contrast to sonicated water treatments that were still extracting hydrocarbons after 4 d. Interestingly, water appeared to contain above-saturation levels of petroleum hydrocarbons.

Results suggested that water could translocate hydrocarbons by two mechanisms: (1) dissolution from sediment into the aqueous phase; and (2) diffusive scouring of hydrocarbons from the solid matrix and through the aqueous phase, followed by formation of a thin hydrocarbon film on top of the water. Fuel added to water contained far more hydrocarbons than could be soluble, but they remained in the liquid phase of the experimental tube and, consequently, were carried into the extracting solvent. Stationary water could translocate hydrocarbons to the liquid phase, although it took longer than when sonicated or if isooctane were used as the stationary liquid. These results show that water can be used as an agent for making the hydrocarbons available to the biomass.

7.4 SUMMARY

Based on the results obtained in this study, the following conclusions can be drawn:

1. Biomass is present, is active, and can be stimulated.
2. Biological mechanisms are present to degrade diesel fuel.
3. Water can translocate hydrocarbons from the solid matrix into the liquid phase.
4. The Kwajalein Island environment is likely dominated by gram-positive organisms.
5. Many heavy hydrocarbons are recalcitrant, particularly those left attached to vadose sediments.
6. Benzene and toluene mineralization occurs but could be showing effects of toxicity and should be capable of being stimulated.

Table 7.3. Mineralization of radiolabeled benzene and toluene by microorganisms residing within the construction area sediments of Kwajalein Island

Sample pit	Depth (in.)	$^{14}\text{CO}_2$ from [$^{14}\text{C-U-}$]benzene (10^3 dpm/d)	$^{14}\text{CO}_2$ from [$^{14}\text{C-U-}$]toluene (10^3 dpm/d)
1	8	108	12.5
	33	7.5	3.3
	56	16	3.8
	56 water	53	2.5
2	6	9.7	10.6
	63	23.6	22.6
3	12	152	12.5
	72	15	
4	10	36	162
	32	43	30
	65	21	88
	65 water	17	54
5	62	3.7	3.8
	70	3.5	8.8

8. ANAEROBIC MICROORGANISMS

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8.1 INTRODUCTION

Although primary emphasis is being placed on the aerobic population of microorganisms in Kwajalein soil and water, it seemed reasonable to make some evaluation of the anaerobic population as well. One attempt to isolate members of the anaerobic genus, *Clostridium*, has been reported in Chap. 3. No organisms were detected in that test. This chapter describes some examinations of Kwajalein soil samples by anaerobic techniques based on the use of "Oxyrase." Oxyrase is an enzyme preparation that reduces dissolved oxygen to water and has proved to be very effective in promoting the growth of anaerobes, particularly those that are sensitive to other reducing agents such as cysteine and thioglycollate.

8.2 METHODS

Samples were obtained from the same dilution tubes containing Kwajalein soil that were used in the microbial activity and biomass study, reported in Chap. 7. These samples were plated using a pour plate technique. The agar medium consisted of yeast extract, 10 g; Oxyrase, 20 mL; Difco Agar, 15 g; 1 M sodium lactate, 10 mL; demineralized water, 970 mL. Measured aliquots from the dilution tubes (usually 0.1 mL) were placed in 609-mm-diam petri dishes. Oxyrase-containing agar (10 mL) at 45°C was added, and the contents were mixed by gently swirling the dishes. After the agar had hardened, a 5-mL overlay of agar, containing no Oxyrase, was introduced to each dish. The dishes were incubated in Torbal jars containing 95% N₂ and 5% CO₂ at 30°C for 2 weeks, and colony counts were then taken.

Direct phase microscope observations were made of selected soil samples by thoroughly mixing ~0.2-g samples with 5 mL of dilution buffer, allowing the large particles to settle, and transferring a drop of the supernatant to a microscope slide. The slide was then examined using the 100X oil immersion phase contrast objective on a Zeiss photomicroscope.

8.3 RESULTS AND DISCUSSION

The plate counts obtained (see Table 8.1) were slightly lower than, but similar to, those obtained from the same samples as reported in Chap. 7. It would appear that many of the organisms present in Kwajalein soils are capable of growth under both aerobic and anaerobic conditions.

The direct microscopic examinations of the samples yielded the results presented in Table 8.2. These microscopic observations indicate that a wide variety of anaerobic microorganisms were present in the soil samples. In some cases, larger populations were found at greater depths. Although some indirect approaches, such as fatty acid analysis, indicate that an actinomycete population may have been present, this was not confirmed by direct observation. The presence of free spores in some samples suggests that members of the genus *Clostridium* might have been present; in fact, one colony obtained from the anaerobic platings did consist of spore-containing rods having the club shape typical of these organisms.

Table 8.1. Summary of plate counts

Sample source	Anaerobic plate count
BP10 - 38	$1.56 \times 10^6/\text{mL}$
BP11 - 60	$1.4 \times 10^6/\text{mL}$
BP12 - 55	$5 \times 10^2/\text{mL}$
STP	$2.8 \times 10^4/\text{mL}$
TP05 - 70	$2.9 \times 10^3/\text{mL}$

Table 8.2. Results obtained from direct microscopy

Sample source	Morphology
BP10 - 8	Rods; several sizes and slight variation in shapes
BP10 - 38	Rods; several sizes; some motile and some free spores
BP11 - 10	Very few cells; those present very small; may be rods or cocci
BP11 - 65	Large numbers of rods; varied morphology; some extremely thin rods, some motile rods
BP12 - 10	Variety of small rods and cocci; some larger rods
BP12 - 51	Great variety of sizes and morphological types; some very thin rods; several motile types
TP01 - 62	Small motile rods, not much variety
TP05 - 62	Several types of nonmotile rods; free spores; possibly some cocci, some rods with rounded ends

9. BACTERIAL DEGRADATION

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9.1 INTRODUCTION

This part of the study was undertaken with the following objectives: (1) to determine whether Kwajalein soils contaminated by diesel fuel contain native organisms that have the ability to degrade diesel at accelerated rates, particularly when compared with organisms from noncontaminated Kwajalein soils; (2) to determine whether the biodegradation of diesel fuel in Kwajalein soils could be enhanced by addition of a standard inorganic nutrient mixture; and (3) to evaluate the utility of two commercial bacterial cultures in degrading diesel fuel in these soils.

9.2 APPROACH

Both contaminated (sample TP04-65) and noncontaminated (sample TP01-08) Kwajalein soils, as well as a sample of diesel fuel representative of that used on the island, were examined. Commercial diesel-degrading bacterial cultures were obtained from the Sybron Corporation and from the Osprey Corporation. Eight different experimental conditions were set up in duplicate in 500-mL glass bottles. These eight conditions represented different combinations of nutrients, bacteria, and diesel content (Table 9.1). Each sample bottle contained 25 g of contaminated or noncontaminated soils and 250 mL of sterile, distilled water. To each sample was added 1000 ppm (mg/kg Kwajalein soil) of a commercial inorganic nutrient mixture (Restore®, kindly provided by IT Corporation, Knoxville, Tennessee). One of the experimental setups contained Sybron bacteria, and another contained the Osprey culture, in doses approximately equal to those recommended by the manufacturers for field use. These bacterial inocula were added to soils already containing viable native bacteria. In addition to the eight experimental setups described above, two samples were set up (in duplicate) containing water, nutrients, and either diesel-contaminated Kwajalein soil or clean soil spiked with Kwajalein diesel; these samples were then kept frozen until analyzed and were used to determine baseline levels of diesel hydrocarbons in the samples before incubation. The clean soil samples were spiked with diesel fuel at a level of 400 mg/kg soil, which was the original estimate for the contaminated soil. After the experiment had been initiated, the actual level in the contaminated soil was determined to be ~8000 mg/kg.

The samples were incubated in a Comput-Ox respirometer for 286 h (nearly 12 d); this instrument, which was made available for our use by IT Corporation, monitors the oxygen utilization rate and total oxygen consumption by bacteria resulting from aerobic degradation of hydrocarbons. The data are collected by a computer and stored on a floppy disk. Data points were collected at 2-h intervals and were subsequently incorporated into Lotus 1-2-3 for further analysis and plotting.

Table 9.1. Experimental design

Experiment number	Contaminated soil	Clean soil	No nutrient	IT nutrient (1000 ppm)	250 mL H ₂ O	Osprey bacteria	No additional bacteria	Sybron bacteria	Diesel added	No diesel added
KDB 1	X			X	X		X			X
KDB 2	X			X	X		X			X
KDB 3	X			X	X	X				X
KDB 4	X			X	X	X				X
KDB 5	X			X	X			X		X
KDB 6	X			X	X			X		X
KDB 7		X		X	X		X			X
KDB 8		X		X	X		X			X
KDB 9	X		X		X		X			X
KDB 10	X		X		X		X			X
KDB 11		X		X	X		X		X	
KDB 12		X		X	X		X		X	
KDB 13		X		X	X	X			X	
KDB 14		X		X	X	X			X	
KDB 15		X		X	X			X	X	
KDB 16		X		X	X			X	X	
KDB 17		X		X	X		X		X	
KDB 18		X		X	X		X		X	
KDB 19	X			X	X		X			X
KDB 20	X			X	X		X			X

After the respirometry experiments were completed, the contaminated samples were analyzed by the ORNL Analytical Chemistry Division for both the solid- and aqueous-phase diesel hydrocarbons. These data reveal the original level of contamination as well as the extent of degradation after treatment.

9.3 RESULTS AND DISCUSSION

The data summarizing the results of the respirometry experiments are contained in Figs. 9.1 and 9.2. These data points represent the averages of the duplicate samples; oxygen uptake generally differed between duplicates by <10%. Data from two sets of samples were discarded because of leaks. The samples from which the data were discarded were (1) KDB-4, containing contaminated soil, nutrients, and Osprey bacteria; and (2) KDB-9, containing contaminated soil, no nutrients, and native bacteria only.

Several conclusions can be drawn from these data. First, it is apparent that native bacteria that had been exposed to diesel spills for some period of time were much more competent at degrading diesel fuel than bacteria from uncontaminated environments (Fig. 9.1 vs Fig. 9.2). Additionally, it is apparent that the added commercial cultures were capable of degrading the diesel fuel, as was expected. The Sybron culture was more active in every instance than any other culture examined. The rate of degradation by native bacteria was not as rapid as in the commercial cultures; this slower rate may be due to differences in the numbers of bacteria present in the different experiments.

The addition of inorganic nutrients seems to be essential to achieving good rates of degradation of diesel fuel in these samples; in the absence of nutrients, virtually no oxygen consumption was observed.

In general, the analytical data from experiments with contaminated soil samples agree with the respirometry data (Table 9.2). Lower hydrocarbon concentrations after 12 d correspond to greater oxygen consumption values. A relative value for degradation efficiency was calculated by dividing the value for total petroleum hydrocarbons in the samples after 12 d by the total volume of O₂ consumed during respirometry. The lowest values represent the best biodegradation performance. Based on these data, the most complete biodegradation was performed by Sybron bacteria. Excellent results were also obtained in samples where only native bacteria were present.

Data from duplicate contaminated samples frozen at time zero reveal contamination levels of 5100 and 10,900 µg/g soil. These data differ by approximately a factor of 2, indicating that the contamination was not uniformly dispersed in the samples obtained from Kwajalein Island.

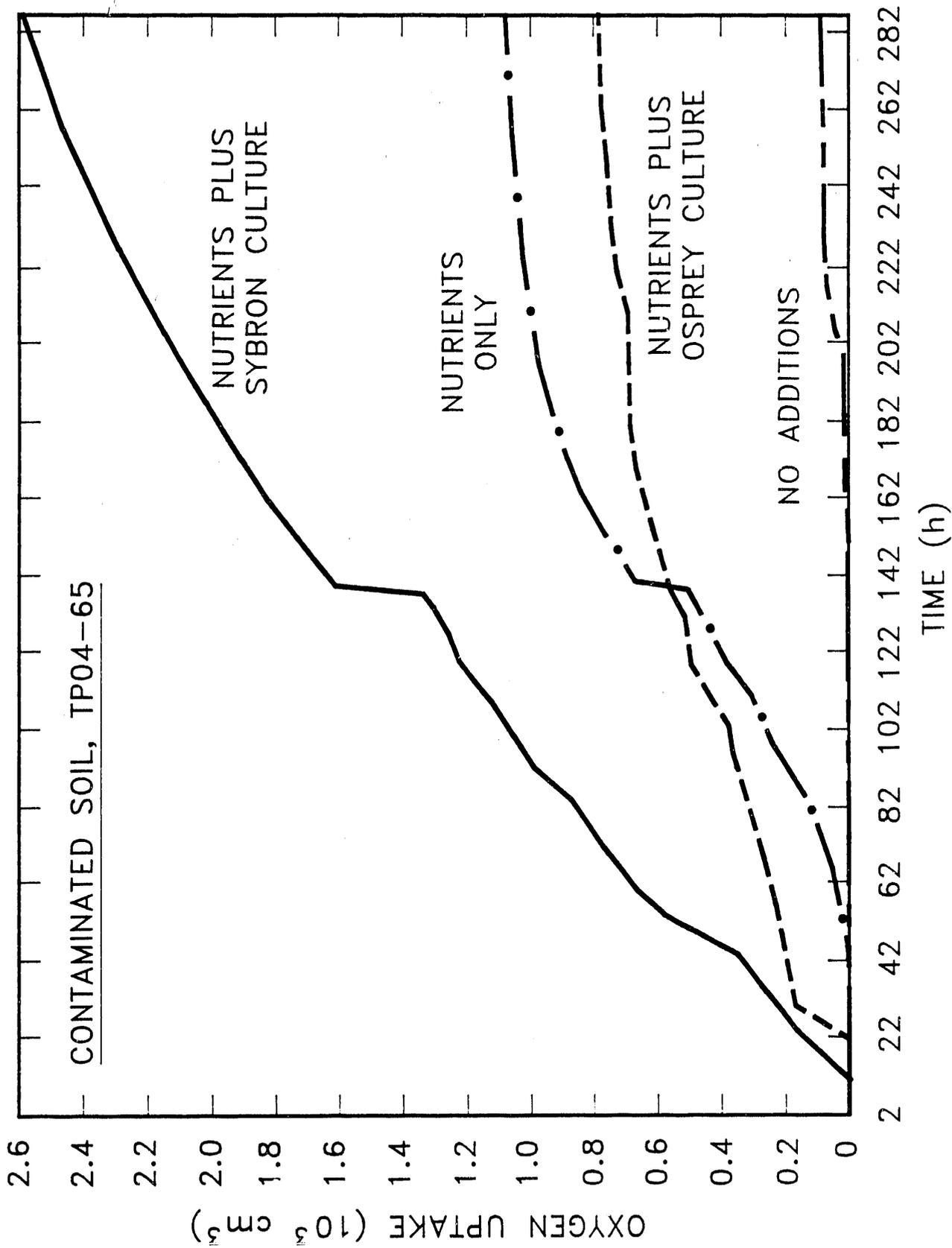


Fig. 9.1. Contaminated-soil sample TP04-65.

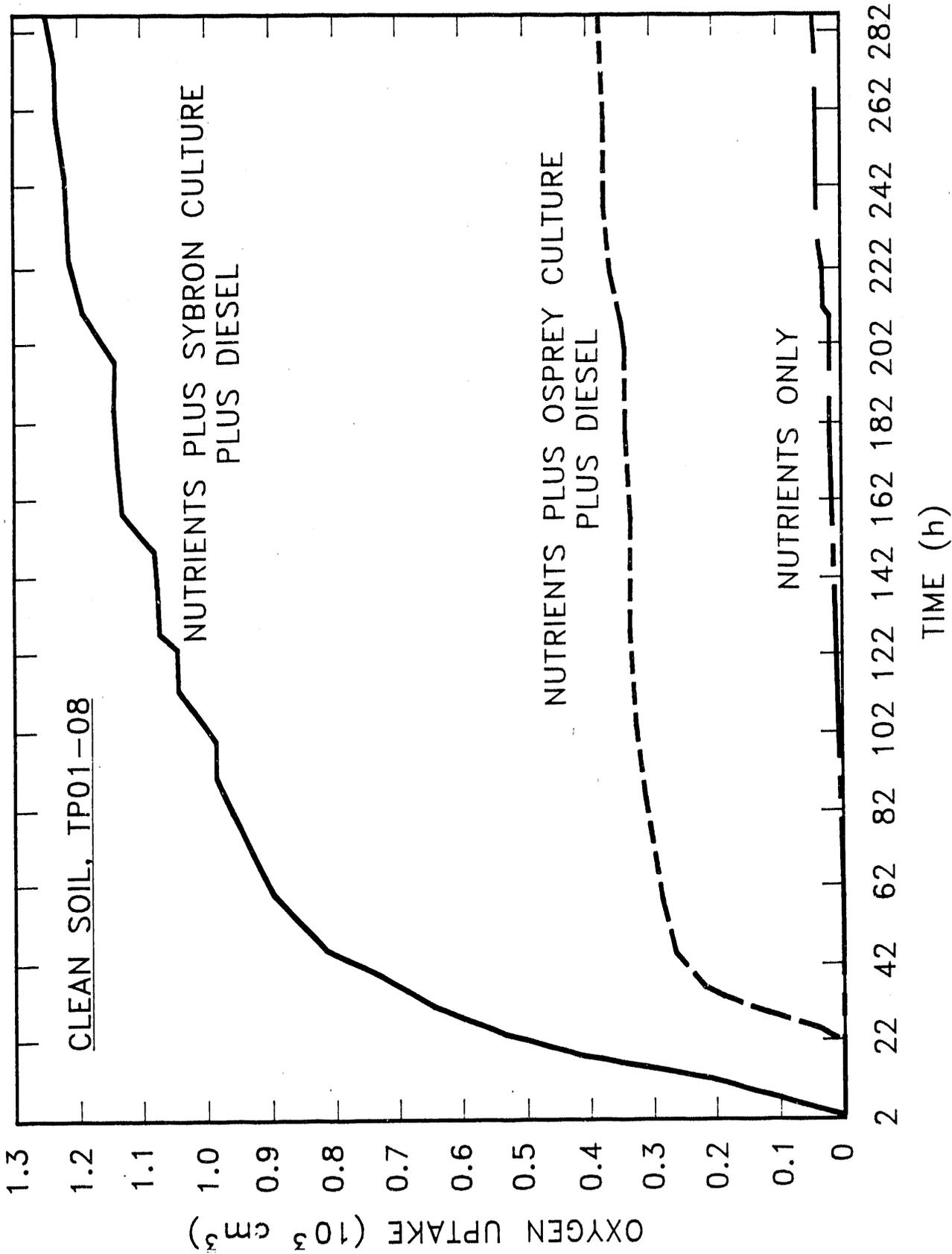


Fig. 9.2. Clean-soil sample TP01-08.

Table 9.2. Conditions for respirometry experiments after 12 d of incubation

Sample number	Treatment/ bacteria	Petroleum hydrocarbon content ($\mu\text{g/g}$ soil)	Total O ₂ consumed (mL) (respirometry)	PHC/ total O ₂ ^a
KDB-1	Native	210	1307	0.16
KDB-2	Native	310	841	0.37
KDB-3	Osprey	880 ^b	761	1.16
KDB-4	Osprey	210 ^b	NRD ^c	NRD ^c
KDB-5	Sybron	180	2641	0.068
KDB-6	Sybron	230	2544	0.090
KDB-9	Native; no added nutrients	2,000	NRD ^c	NRD ^c
KDB-10	Native; no added nutrients	2,200	91.4	24.1
KDB-19	Frozen at time zero	5,100	---	---
KDB-20	Frozen at time zero	10,900	---	---

^aPHC = petroleum hydrocarbons. This quotient gives a numerical value representing the effectiveness of the particular treatment used. The lower the value, the better the treatment.

^bProblems were experienced with both Osprey setups. The analytical data are different enough to suggest that KDB-3 was oxygen-deprived, while vessel KDB-4 leaked, giving excessively high O₂ usage readings.

^cNRD = No respirometry data. These two vessels leaked during respirometry, giving artificially high O₂ usage values.

10. RESPIROMETRIC EVALUATIONS

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10.1 INTRODUCTION

Knowledge regarding appropriate environmental conditions that stimulate the biodegradation of organic contaminants by indigenous microbes is required to design an *in situ* bioremediation process. This section presents a synopsis of the methods and results of respirometric studies to assess the potential for *in situ* bioremediation of Kwajalein Island soils that are contaminated with diesel fuel. The primary objectives of this experiment were (1) to determine if weathered existing diesel fuel contamination, as well as fresh fuel, would be biodegraded in natural, unamended Kwajalein soils; and (2) to determine the relative effects of different nutrient amendments on the rate and short-term extent of biodegradation. The experimental work was conducted between March 28 and April 19, 1991, in the laboratories of the Environmental Sciences Division at ORNL, while the hydrocarbon analyses were performed by staff members of the Institute for Applied Microbiology at the University of Tennessee.

10.2 MATERIALS AND METHODS

All of the soil sample materials used in these experiments were collected on Kwajalein Island during February 1991. After collection, all materials were stored at $\sim 4^{\circ}\text{C}$ until preparation for the respirometer experiments.

This treatability experiment was conducted using a constant-pressure respirometer. Small samples of field moist soil (5 g) from Kwajalein Island were studied both with and without a variety of amendments as outlined in Tables 10.1 and 10.2. Volume changes required to maintain constant pressure were equated with oxygen consumption and interpreted as a measure of fuel biodegradation. Six experimental runs were made, each of which involved four to six treatment conditions and measurements of oxygen uptake over a period of 3 to 10 d. A selected number of samples were also analyzed for carbon dioxide (CO_2) evolution, including total CO_2 and isotopic ratios of carbon. Hydrocarbon measurements of one or more replicates from each treatment were made at the beginning and end of each experimental run. Further details regarding the materials and methods are provided in the following paragraphs.

10.2.1. Soil Materials and Ambient Contamination

Several different soil materials from Kwajalein Island were used in these experiments. A sample of relatively clean soil was obtained from test pit 1 (TP01), which is located within the proposed desalination plant construction site. Field observations suggested that this test pit had no hydrocarbon contamination; in addition, laboratory analyses did not detect any total petroleum hydrocarbons (TPH). Therefore, the sample from this pit was considered "clean."

Table 10.1. Conditions evaluated during this experiment^a

Parameter	Range of conditions studied
Soil material and ambient fuel contamination level	TP04-32 TPH = 216 mg/kg
	TP04-composite TPH = 2500 mg/kg
	TP04-65 TPH = 8920 mg/kg
	TP01 TPH = 0 mg/kg
Diesel fuel amendments	None
	Fresh fuel spikes of 500 and 5000 mg/kg
Nutrient amendments	None
	A mixture of organic/inorganic N (28% NO ₃ , 20% NH ₄ , 52% urea) and inorganic P, K, S, B, Cu, Fe, Mn, Mo, and Zn
	A few treatments included only inorganic N, P, K, S, B, Cu, Fe, Mn, Mo, and Zn
	Mass loading rates of 0.1×, 1×, 10×, 30×, and 100× were used, where 1× loading = 6.0 mg N/kg, 2.6 mg P/kg, 5.0 mg K/kg, and 15.8 µg S/kg, 1.5 µg B/kg, 3.4 µg Cu/kg, 7.9 µg Fe/kg, 8.6 µg Mn/kg, 0.05 µg Mo/kg, and 4.7 µg Zn/kg
Water	35 to 60 wt %

^aThirty-one replicated treatments were studied, as outlined in Table 10.2.

Table 10.2. Features of individual experimental runs and replicated treatments

Treatment identification	Run ^a	Flask	Soil	Diesel fuel		Rate	Nutrients added		Water	
				Ambient (mg/kg)	Added (mg/kg)		Mass (mg N/kg)	Conc. (mg N/L)	Ambient (mL)	Added (mL)
TP04C-0x	1	1-3	TP04C	2550	0	0x	0	0	0.7	0
TP04C-01x	1	4-6	TP04C	2550	0	1x	6	31.6	0.7	1.0
TP04C-01x-5000	1	7-9	TP04C	2550	5000	1x	6	31.6	0.7	1.0
TP04C-0.1x-5000	1	10-12	TP04C	2550	5000	0.1x	0.6	3.1	0.7	1.0
Blank	1	13-14	-	-	-	-	-	-	-	-
TP04C-0x	2 ^a	1-2	TP04C	2550	0	0x	0	0	0.7	0
TP04C-01x-5000	2 ^a	3-4	TP04C	2550	5000	1x	6	31.6	0.7	1.0
TP04C-10x-5000	2 ^a	5-7	TP04C	2550	5000	10x	60	31.6	0.7	1.0
TP04C-01x-5000	2	8-10	TP04C	2550	5000	1x	6	9.4	0.7	3.2
TP04C-10x-5000	2	11-13	TP04C	2550	5000	10x	60	9.4	0.7	3.2
Blank	2	14	-	-	-	-	-	-	-	-
TP04C-10x-500	3 ^a	1-2	TP04C	2550	500	10x	60	31.6	0.7	1.0
TP04C-100x-5000	3	3-4	TP04C	2550	5000	100x	600	3160	0.7	1.0
TP04C-30x-5000	3	5-6	TP04C	2550	5000	30x	180	950	0.7	1.0
TP04C-100x-5000	3	7-8	TP04C	2550	5000	100x	600	760	0.7	3.95
TP04C-30x	3 ^a	9-10	TP04C	2550	0	30x	180	950	0.7	1.0
TP04C-0x	3	11-12	TP04C	2550	0	0	0	0	0.7	0.0
TP01	3 ^a	13	TP01	0	5000	10x	60	316	0.7	1.0
Blank	3	14	-	-	-	-	-	-	-	-
32-01x	4	1-3	TP04-32	216	0	1x	6	31.5	0.7	1.0
32-10x	4 ^a	4-6	TP04-32	216	0	10x	60	15.4	0.7	2.0
32-1x-500	4	7-9	TP04-32	216	500	1x	6	15.4	0.7	2.0
32-10x-500	4 ^a	10-12	TP04-32	216	500	10x	60	31.5	0.7	1.0
32-0x	4 ^a	13-14	TP04-32	216	0	0	0	0	0.7	0.0
32-30x	5	1-3	TP04-32	216	0	30x	180	950	0.7	1.0
32-100x	5 ^a	4-6	TP04-32	216	0	100x	600	1540	0.7	2.0
32-30x-500	5 ^a	7-9	TP04-32	216	500	30x	180	460	0.7	2.0
32-100x-500	5 ^a	10-12	TP04-32	216	500	100x	600	3160	0.7	1.0
32-0x	5	13-14	TP04-32	216	0	0	0	0	0.7	0.0
32-10x ^b	6	1-3	TP04-32	216	0	10x ^b	60	156	0.7	2.0
65-10x	6 ^a	4-6	TP04-65	8920	0	10x	60	156	0.7	2.0
65-10x ^b	6	7-9	TP04-65	8920	0	10x ^b	60	156	0.7	2.0
65-10x-500	6 ^a	10-12	TP04-65	8920	500	10x	60	156	0.7	2.0
65-0x	6 ^a	13-14	TP04-65	8920	500	0x	0	0	0.7	0.0

^aResults graphically depicted in Figs. 10.1, 10.2, and 10.6.

^bNitrogen in the amendment was comprised of inorganic nitrogen only.

Samples of diesel fuel-contaminated soil were obtained from test pit 4 (TP04), which is located adjacent to a diesel fuel tank. The analytical results indicated that petroleum hydrocarbons were present in this pit at increasing concentrations with depth (i.e., TPH = 1 mg/kg at 10 in., 216 mg/kg at 32 in., 8920 mg/kg at 65 in.). The overall texture was sand with some fine content. Roots were evident in the upper 12 in. of the soil profile. Discrete samples from depths of 32 in. (TP04-32) and 65 in. (TP04-65) were studied, as well as a composite sample from depths of 10, 32, and 65 in. (TP04 composite, or TP04-C). This latter sample was the same as that used in the respirometer experiments on Kwajalein Island and was prepared by mixing the following materials at the indicated weight percentages: 21.9% soil from a 10-in. depth, 50.7% soil from a 32-in. depth, and 27.4% soil from a 65-in. depth. The composite had a calculated TPH of 2550 mg/kg, exuded a strong petroleum odor, and had an oily "feel" when handled.

10.2.2 Soil Amendments

The amendments used for this experiment consisted of nutrients, water, and fresh diesel fuel. The nutrient amendments (both macronutrients and micronutrients) used for the first five of the six experimental runs were derived from a commercial agricultural fertilizer (Peters General Purpose Water Soluble Fertilizer, W. R. Grace & Co., Allentown, PA). A commercial agricultural fertilizer was used since it represented a likely source of nutrients for bioremediation purposes on Kwajalein Island. The nutrient amendments derived from the fertilizers included three macronutrients [a mixture of organic/inorganic nitrogen (N) plus inorganic phosphorus (P) and potassium (K)] and seven micronutrients [sulfur (S), boron (B), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), and zinc (Z)]. The fertilizer solids were dissolved in tapwater to yield stock nutrient solutions. Small aliquots of a desired nutrient solution were then added to the soil samples to yield a range of mass loadings from 1× to 100×. The 1× loading rates were designed to provide a minimal level of nutrients (Table 10.1). A nutrient amendment yielding the same mass loading rate but containing only inorganic nitrogen salts was prepared for the last run. This amendment was tested to confirm that nitrogen source had no marked impact on oxygen uptake and apparent biodegradation.

The addition of the nutrient amendments was made in 1 mL of water per 5 g of soil, which resulted in near saturation of the soil matrix (water content = ~35 to 40 wt %). Additional water (distilled, deionized) was added to a select number of soil samples to further increase the soil water content (water content = ~60 wt %). This was done to determine whether the soil water content had a marked effect on oxygen uptake and apparent biodegradation.

The nutrient amendment concentrations were constant between treatments at the same mass loading rate conditions. That is, 1× nutrients were equivalent to ~6 mg N per kg of soil; the 10× equivalent to 60 mg/kg; etc. The ratio of individual nutrient constituents was also constant (e.g., N:P:K = 2.3:1.0:1.9), with the micronutrients at a fraction of these (Table 10.1). While the nutrient mass loadings increased from 1× to 10× to 30× to 100×, the concentrations varied between some treatments at the same mass loading rate due to the water added to increase the soil saturation level. Thus, treatments at the same nutrient mass loading but different water additions could have different nutrient concentrations. For example, the approximate concentration ranges for nitrogen associated with the different mass loadings were: 0.1× nutrients = 3.1 mg N/L; 1× nutrients = 9.4 to 31.5 mg N/L; 10×

nutrients = 94 to 316 mg N/L; 30× nutrients = 462 to 947 mg N/L; and 100× nutrients = 948 to 3158 mg N/L. The concentrations of the other nutrients would be a constant ratio of these. For these relatively short-term respiration experiments, the nutrient mass loading rate was assumed to be most important in controlling the rate and extent of oxygen uptake and biodegradation.

Fresh diesel fuel (500 and 5000 mg of diesel fuel per kg of soil) was added to some treatments in order to determine the relative difference in oxygen uptake and biodegradation in Kwajalein soils previously contaminated with diesel fuel when impacted by fresh diesel fuel. It was anticipated that fresh diesel fuel would stimulate oxygen uptake and biodegradation since such fuel would be more biodegradable than the existing fuel. (The existing fuel was presumed to be older and weathered.) The fresh fuel was collected on Kwajalein Island in February 1991 and brought back to ORNL for laboratory studies.

The pH levels of the various amendment solutions prior to addition to the soil varied from ~5.1 to 6.5. To determine the change in soil pH as a result of the nutrient amendments, 1:1 (volume:weight) soil slurries were prepared, representing the range of conditions within this experiment. After 30-min equilibrations, the pH varied from 6.4 to 8.0, versus 8.5 for soil (TP04 composite) with only distilled water added. Thus, the nutrient solutions added to Kwajalein soil appeared to decrease the pH of the soil solution somewhat.

10.2.3 Respirometer Operation and Monitoring

A differential respirometer equipped with a shaker and a temperature-controlled water bath was used for these experiments (Gilson Differential Respirometer, Middleton, WI). Prior to the first experimental run, the water bath was filled with distilled water and the temperature control was adjusted to 24°C, approximately the temperature of the soils on Kwajalein Atoll. The temperature was monitored to verify that it remained constant within $\pm 1^\circ\text{C}$. The respirometer was equipped with 14 reaction flask positions. Each reaction flask had a volume of ~16 mL. Prior to each run, each respirometer flask was cleaned with detergent and rinsed successively with distilled water, methanol, and distilled water; it was then baked at 100°C for at least 30 min. Each of the reference flasks on the respirometer was filled with 7 mL of distilled water to equalize the wetted air volume in the reference flask with that in the reaction flask. A few drops of hydrochloric acid (10%) were also added to inhibit biological activity.

Immediately before each of the six experimental runs, Kwajalein soil material (either TP01, TP04-32, TP04-65, or TP04-C) was amended with nutrients, water, and/or fuel to yield a treatment condition (Table 10.2). The stepwise procedure was as follows: A 20-g sample of field moist soil was removed from storage at 4°C and placed in a 50-mL glass beaker. This weight of soil was designed to yield triplicate 5-g subsamples for the respirometer as well as a single 5-g sample for base-line hydrocarbon analyses. The desired amount of given nutrient stock solution was then added to the 50-mL beaker. This was typically 0.6 mL of macronutrient solution and 0.1 mL of micronutrient solution, plus 0.3 mL of distilled water, for a total addition of 1 mL of liquid. If a larger volume of water was required for a given treatment, it was added directly to the reaction flask. Fresh diesel fuel was added as required by dripping fuel over the surface of the soil within the beaker. After all amendments had been added, the soil was thoroughly mixed with a precleaned stainless steel spatula. The

center well of each respirometer reaction flask was temporarily plugged with tissue, and 5-g increments of a given soil and amendments were added. Then the center well plug was removed, and ~0.5 mL of 10% KOH solution was added to the well via a syringe for absorption of CO₂.

The prepared reaction flasks were subsequently attached to the respirometer using a water-soluble grease sealant. The flasks were partially immersed in the water bath, and the shaker was started. The reaction flasks were allowed to equilibrate with the reference flasks for 30 to 60 min; then the manometer valves were closed, and readings were initiated. Over the next 3 to 10 d, oxygen uptake over time was measured manometrically with a manually operated digital micrometer attached to each reaction flask-reference flask unit. The micrometer readings, given digitally in microliters, were converted to standard conditions based on the barometric pressure and temperature at the start of the experiment. This conversion yields the microliters of gas exchanged at standard conditions.

At the end of a given experimental run, the reaction flasks were removed from the respirometer and the KOH was extracted from the center well and either neutralized for disposal or containerized to permit analyses of carbon dioxide evolution. On a selected number of samples, CO₂ evolution measurements were made to enable computation of a respiration quotient (R.Q. = mol CO₂ per mol O₂) and to help confirm that the evolved CO₂ was generated by the degradation of diesel fuel. This was accomplished by reducing the pH of the KOH from a given flask and measuring the CO₂ evolved by manometric techniques. The CO₂ was then analyzed for isotopic ratios of carbon [reported as d¹³C relative to the PeeDee belemnite standard (PDB)] by mass spectrometry.

The soil within the reaction flask was transferred to a 40-mL VOA vial with a Teflon-sealed cap. The soil subsamples (5 g) collected at the beginning of each experimental run and the contents of each reaction flask collected at the end were stored at 4°C for subsequent hydrocarbon analyses. These analyses were made by solvent extraction (isooctane) followed by gas chromatography with a packed column. Details of the analytical procedures may be found in Chap. 7.

Finally, the reaction flasks were cleaned (according to the above described procedure) in preparation for the next experimental run.

10.3 RESULTS AND DISCUSSION

10.3.1 Soil Respiration

In summary, six experimental runs were made during this study (Table 10.2). In each run, four to six different treatments (i.e., nutrients, water, fresh fuel amendments, etc.) were studied in duplicate or triplicate. Unamended soils and empty reaction flasks were run as controls.

Oxygen Uptake

Variations in oxygen uptake within treatment replicates were very low. Coefficients of variation (CV) between replicates within a given treatment were typically <0.20 and seldom >0.30 . The average oxygen uptake vs time profiles for a selected number of treatments are shown in Figs. 10.1 and 10.2. These and the other oxygen uptake profiles were evaluated for rate and extent of oxygen consumption vs treatment conditions (i.e., soil material, nutrients, water, fresh fuel). A discussion of the key results and observations of the individual experimental runs is given first, followed by a series of summary statements based on consideration of all runs collectively.

The objective of the first run was to compare the effects of minimal nutrient addition ($0\times$, $0.1\times$, and $1\times$) to the composite sample (TP04-C) of contaminated soil from test pit 4, both with and without the addition of 5000 mg/kg of fresh fuel. During 66 h of respiration, the total oxygen uptake ranged from 300 μL for TP04-C alone to 600 μL for TP04-C with $1\times$ nutrients and 5000 mg/kg fresh fuel. The limited stimulation associated with fresh fuel and $1\times$ nutrients suggested that nutrients at the $1\times$ loading rate were insufficient to achieve marked stimulation of biodegradation in these samples.

The second run was made with the same soil material (i.e., TP04-C) to compare the effects of higher nutrient addition and water content on fresh fuel-amended soil (e.g., Fig. 10.1: TP04C- $0\times$, TP04C- $0.1\times$ -5000, TP04C- $10\times$ -5000). During 86 h of incubation, the total uptake ranged from 600 μL for the unamended soil to 1900 μL for the soil amended with $10\times$ nutrients and 5000 mg/kg fuel. Thus, higher nutrient loadings did stimulate oxygen uptake. Water content did not exert any measurable effect on oxygen uptake.

The third run was made with TP04-C to compare even higher nutrient loadings than used in run 2. Nutrient loadings of $30\times$ and $100\times$ were used with fresh fuel amendments of 5000 mg/kg. Due to rapid oxygen uptake during the initial 24 h of the run, a respirometer malfunction occurred and these treatments had to be aborted. Apparently, the fresh fuel and higher nutrient loadings stimulated oxygen uptake beyond that observed in run 2. An additional treatment in this run included TP04-C with $30\times$ nutrient addition. This level of nutrients appeared to significantly stimulate the oxygen uptake since it reached 1500 μL after only 63 h (Fig. 10.1: TP04C- $30\times$). A final treatment of this run also included samples of clean soil from TP01 that had been amended with $10\times$ nutrients and 5000 mg/kg fresh fuel. After 63 h of incubation, the oxygen uptake in these samples was only 90 μL (Fig. 10.1: TP01- $10\times$ -5000), which suggested that the indigenous microbes in clean soil were not adapted and, thus, not readily able to biodegrade diesel fuel.

The fourth run was made using soil material from test pit 4 — not the composite, simply the material from the 32-in. depth. In TP04-32, the ambient TPH contamination was 216 mg/kg. Despite this, little oxygen uptake occurred during 209 h ($<200 \mu\text{L}$), even after adding $10\times$ nutrients and water. The addition of 500 mg/kg of fresh fuel stimulated uptake in all cases, with greater stimulation occurring at $10\times$ nutrients vs $1\times$ (1800 vs 1000 μL) (Fig. 10.2: 32- $0\times$, 32- $10\times$, 32- $10\times$ -500). After respiration had leveled off at 120 h, nutrients were added again at 168 h and oxygen uptake was resumed. This suggests that intermittent nutrient addition is required to maintain high oxygen uptake rates (and presumably high biodegradation rates).

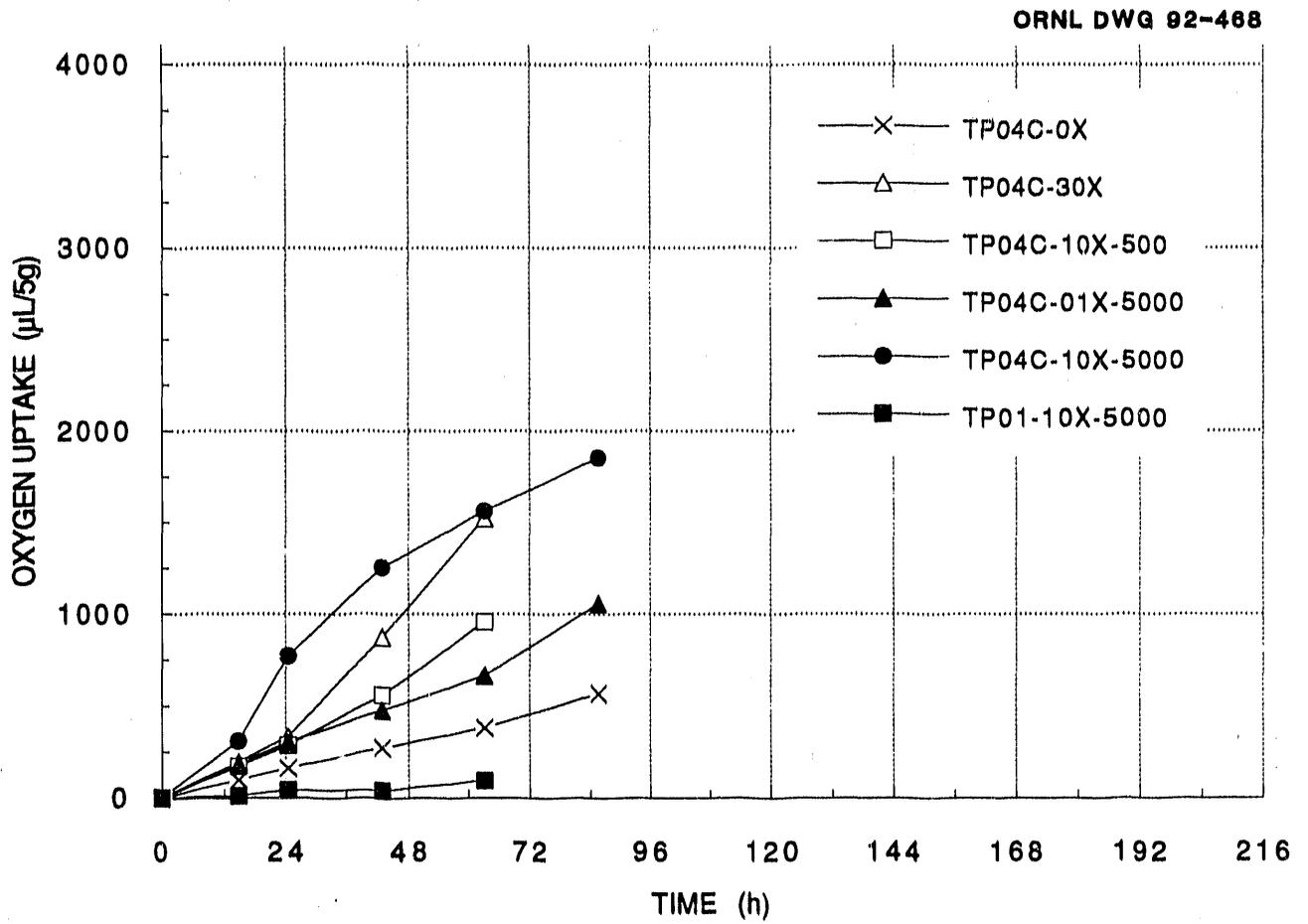


Fig. 10.1. Oxygen uptake profiles for selected treatment conditions used in experimental runs 1 through 3.

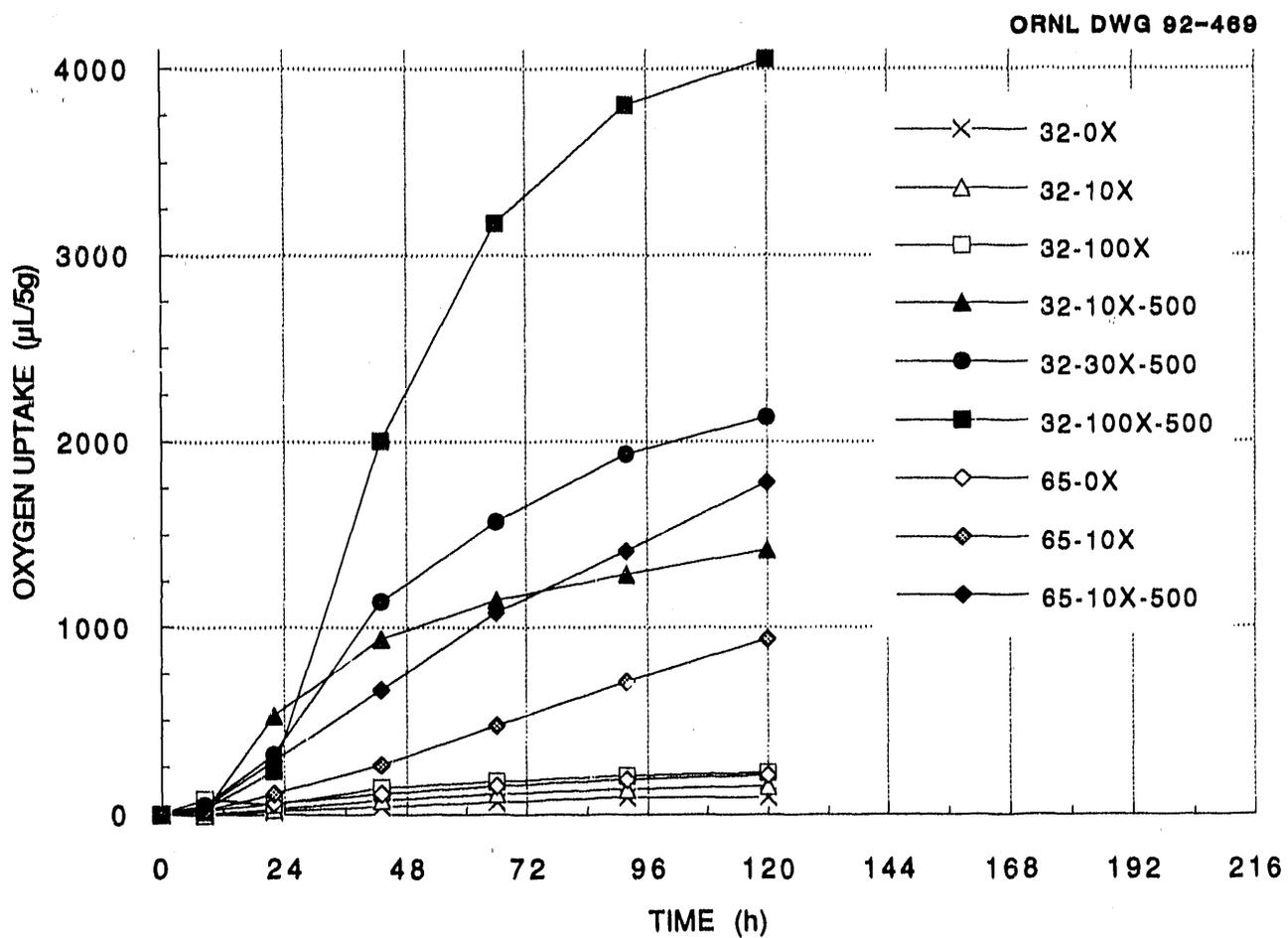


Fig. 10.2. Oxygen uptake profiles for selected treatment conditions used in experimental runs 4 through 6.

The fifth run was made using TP04-32 again, but with higher nutrient loadings and lower fresh fuel loadings (500 mg/kg rather than 5000 mg/kg). During nearly 115 h of incubation, dramatic differences were observed between treatments. In the samples without fresh fuel, there was little oxygen uptake and no substantial effect due to nutrient addition at 30× or 100× (e.g., 220 μL at 100×, 150 μL at 0×; Fig. 10.2: 32-100×). The addition of fresh fuel stimulated uptake dramatically, the magnitude of which was related to nutrient amendment (2100 μL at 30×, 4000 μL at 100×; Fig. 10.2: 32-10×-500). These data suggest that higher nutrient loadings can provide a pool of nutrients that maintain higher stimulated activity.

The final run was made using one treatment with TP04-32; all remaining runs were made with TP04-65. The soil from the TP04-65 sample was most contaminated (TPH = 8920 mg/kg). This run was made to evaluate oxygen uptake in more contaminated soil and to confirm that fertilizer type had no effect (inorganic fertilizers vs the mixed inorganic/organic used up to this point). The oxygen uptake profiles for TP04-65 were somewhat different than those of the earlier runs made with less contaminated soil (e.g., TP04-32 with TPH = 216 mg/kg). The uptake profiles with the more-contaminated TP04-65 were more linear and did not reach a plateau after ~120 h. The oxygen uptake for TP04-65 alone is similar to that for the other unamended soils (e.g., 210 μL at 119 h; Fig. 10.2: 65-0×), but the degree of stimulation by 10× nutrient addition was greater in the more highly contaminated TP04-65 as compared with TP04-32 (630 μL vs 190 μL). This suggested that greater amounts of biodegradable organics may be present in TP04-65 and higher nutrient additions may be required to enhance biodegradation in TP04-65 as compared with TP04-32. Treatments to compare the effects of nutrient types (i.e., mixed inorganic/organic vs inorganic nitrogen) revealed no measurable effect in samples of TP04-32 and TP04-65 at the 10× nutrient loading rate.

When considered collectively, the results of all six experimental runs reveal trends associated with ambient contamination level, nutrient loading, and fresh fuel addition. The oxygen uptake in soil samples from the diesel fuel-contaminated depths of TP04 were relatively low. For example, for the moderately contaminated soil, TP04-32 (TPH = 216 mg/kg), the average uptake was 90 μL after 120 h, while for the more-contaminated soil, TP04-65 (TPH = 8920 mg/kg), the average uptake was only moderately higher at 210 μL after 120 h (Fig. 10.1). In contrast, for the composited soil sample, TP04-C (TPH = 2550 mg/kg), the uptake was significantly higher at 570 μL after only 86 h (Fig. 10.2). The higher rate associated with the composite sample might be attributed to the introduction of adapted microbes from the shallow soil zone (i.e., 10-in. depth).

The addition of nutrients stimulated oxygen uptake in all of the contaminated soil samples from TP04 (i.e., TP04-32, TP04-65, TP04-C) (Figs. 10.1 and 10.2). The degree of stimulation increased with the degree of ambient contamination. For example, in the case of TP04-32, nutrient additions >1× (i.e., 6 mg N/kg) had no further effect on oxygen uptake; however, for TP04-65, which had 40 times more TPH contamination, nutrient addition at 10× (i.e., 60 mg N/kg) had a marked effect as compared with that at 6 mg N/kg (Fig. 10.2).

The addition of fresh fuel (500 and 5000 mg of diesel fuel per kg of soil) stimulated oxygen uptake by an order of magnitude or more in all contaminated soil materials (i.e., TP04-32, TP04-65, and TP04-C) (Figs. 10.1 and 10.2). The effect of fresh fuel on uptake rate was greater than that of ambient contamination level or nutrient addition alone (Fig. 10.3); however, in the fuel-spiked samples, higher nutrient loadings were required to sustain higher oxygen uptake rates. For example, for TP04-32 with fresh fuel added at 500 mg/kg, the oxygen uptake rates increased with increasing nutrient loadings. During 120 h of incubation, oxygen uptake values were 1400, 2100, and 4000 μL for the TP04-32 samples with 500 mg/kg fresh fuel and nutrient loadings of 10 \times , 30 \times , and 100 \times (600 mg N/kg), respectively.

In the clean-soil sample, TP01, the oxygen uptake was limited (90 μL after 63 h), even though the soil was spiked with fresh diesel fuel (5000 mg/kg) and nutrients (60 mg N/kg) (Fig. 10.1).

While the extent of uptake varied widely among treatments, the maximum uptake rate was typically similar and occurred during the early hours of incubation. There was normally a lag period during the first 12 h, followed by maximum uptake during the subsequent 24 h (Figs. 10.1 and 10.2).

Carbon Dioxide Evolution

Results of analyses indicated that CO_2 evolution increased with increasing nutrient concentration, but the effects were most pronounced in those samples spiked with fresh diesel fuel (Fig. 10.3). In the contaminated soil without fresh fuel, the calculated respiratory quotients (R.Q. typically >2.0) and the isotopic ratios ($d^{13}\text{C}_{\text{PDB}}$ typically >-21) indicate that the source of the carbon dioxide was probably not from degradation of the ambient diesel fuel contamination (TPH = 216 mg/kg). If the carbon were derived from the biodegradation of fresh diesel fuel, the R.Q. should have been closer to 0.65 (theoretical value for diesel fuel organics) and the $d^{13}\text{C}_{\text{PDB}}$ <-28 (measured value for Kwajalein diesel fuel). In contrast, with the addition of 500 mg/kg of fresh diesel fuel to TP04-32, the CO_2 evolution increased, the respiratory quotient declined toward 0.65, and the $d^{13}\text{C}_{\text{PDB}}$ values were reduced toward that of the diesel fuel (-28).

The CO_2 evolution data are consistent with the results of the oxygen uptake measurements described earlier. Relatively low (e.g., TPH = 216 mg/kg), but appreciable, concentrations of older diesel fuel components in Kwajalein soils are slowly biodegradable, and the addition of nutrients has a limited effect on enhancing the apparent biodegradation. In contrast, when fresh diesel fuel is added to previously contaminated soil, biodegradation is stimulated and fuel components are rapidly oxidized.

10.3.2 Hydrocarbon Analyses

Gas chromatography (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 (i.e., nutrient loadings and fresh fuel addition). Each of the GC traces was reviewed to determine the nature and extent of any loss of hydrocarbons as a result of the treatment. In those treatments that exhibited stimulated soil respiration as described

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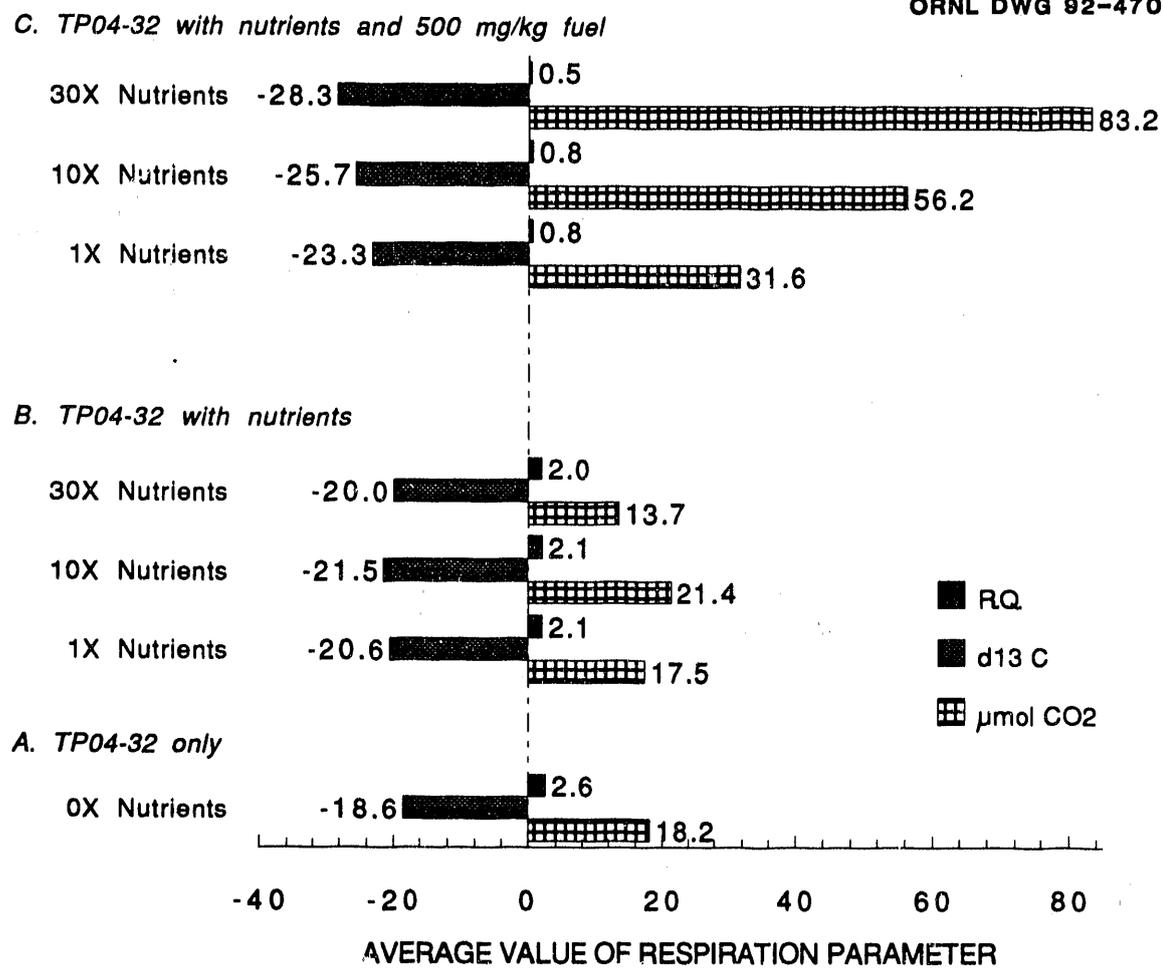


Fig. 10.3. Carbon dioxide evolution characteristics for contaminated soil from TP04-32.

above, a reduction in hydrocarbons would confirm that biodegradation had taken place. In general, the results of the hydrocarbon analyses were consistent with those of the soil respiration measurements. Results of the analysis of the GC traces are highlighted below, while a selected set of GC traces may be found in Figs. 10.4 and 10.5.

There was no evidence of hydrocarbon (HC) degradation when $10\times$ nutrients and 5000 mg/kg fresh fuel were added to clean soil (i.e., TP01). This finding is consistent with the soil respiration measurements made at ORNL, as well as those observed on Kwajalein Island during the respirometry experiment there. In the case of the contaminated soil from TP04-C without nutrient amendments, there was some loss of HC during the 63- and 86-h incubation periods. The HC loss at $1\times$ nutrients was not measurably more than that without nutrient amendments; however, the loss at $30\times$ nutrient loading was markedly higher than that at the $1\times$ loading. The HC contamination for the more lightly contaminated TP04-32 was due to more higher-molecular-weight HCs as compared with TP04-C. Incubation of the soil alone from TP04-32 revealed little, if any, HC loss. Results for TP04-32, which had added nutrients, showed limited degradation of the heavier HCs over time (115- to 209-h incubation).

Marked HC loss during incubation was observed for contaminated soil amended with nutrients and spiked with fresh fuel. The loss of HC in TP04-C with a 5000-mg/kg diesel fuel spike occurred even at $0.1\times$ nutrient loading. In the less-contaminated shallow soil, TP04-32, HC loss occurred at increasing nutrient loadings.

10.3.3 Hydrocarbon Degradation

Hydrocarbon degradation rates were calculated by assuming that the diesel fuel could be represented by a straight-chain, saturated aliphatic compound ($C_{15}H_{32}$) and that it was biodegraded to CO_2 and H_2O . The theoretical oxygen demand for this bioreaction was calculated to be 3.5 g of O_2 per g of HC. At this rate, each microliter of O_2 uptake was equivalent to the degradation of $0.08\ \mu\text{g}$ of HC. The oxygen uptake rate at 24 h of incubation (average rate between ~ 19 and 29 h) was then used to calculate theoretical hydrocarbon degradation rates (Fig. 10.6).

The calculated HC degradation rates for unamended contaminated soil from TP04 were ~ 0.3 , 2.0, and $2.7\ \mu\text{g}$ of HC per g of soil per day ($\mu\text{g HC g}^{-1} \text{d}^{-1}$), for TP04-32, TP04-65, and TP04-C, respectively. These rates, which are very low, help to explain why diesel fuel contamination can persist for extended periods on Kwajalein Island. Nutrient amendments can increase the degradation rate by a factor of 2 at the $10\times$ nutrient loading rate and a factor of 3 at the $30\times$ rate.

The HC degradation rates for contaminated soil amended with fresh fuel were substantially higher than those for the same soil with no fresh fuel added. For example, the calculated HC degradation rate for TP04-32 with $10\times$ nutrients plus 500 mg/kg fresh diesel fuel was $12.2\ \mu\text{g HC g}^{-1} \text{d}^{-1}$ as compared with $0.3\ \mu\text{g HC g}^{-1} \text{d}^{-1}$ for TP04-32 with no fresh fuel added. Higher nutrient loadings to TP04-32 containing 500 mg/kg fresh fuel stimulated even higher degradation rates: 13.8 and $23.4\ \mu\text{g HC g}^{-1} \text{d}^{-1}$ for the $30\times$ and $100\times$ loadings, respectively.

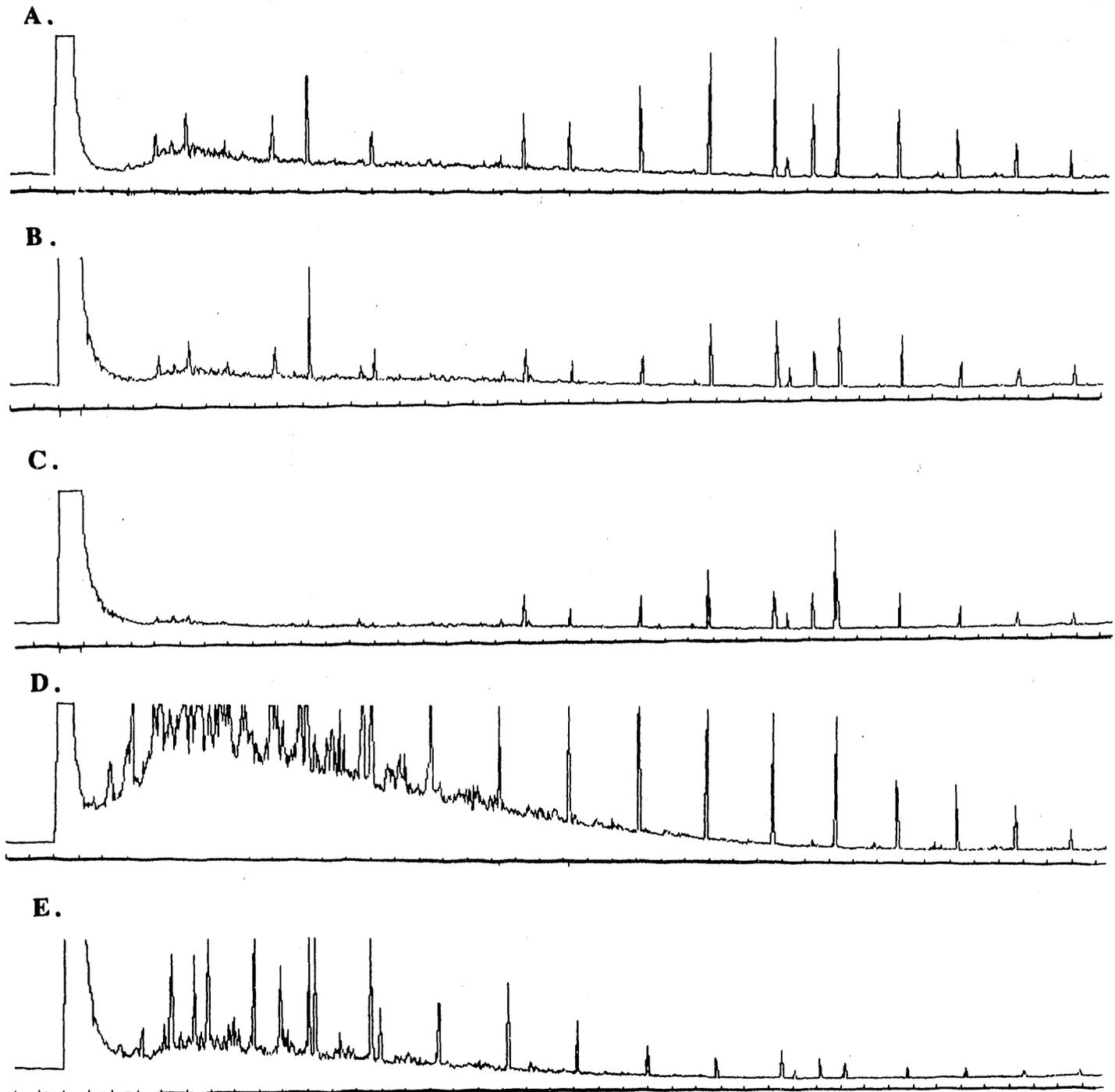


Fig. 10.4. Gas chromatography traces for soil samples from TP04-C before and after incubation: A = unamended soil before incubation; B = same soil as A after 86-h incubation; C = soil amended with 30 \times nutrients after 63-h incubation; D = soil amended with 10 \times nutrients and 5000 mg/kg fresh fuel prior to incubation; E = same soil as D after 85 h of incubation.

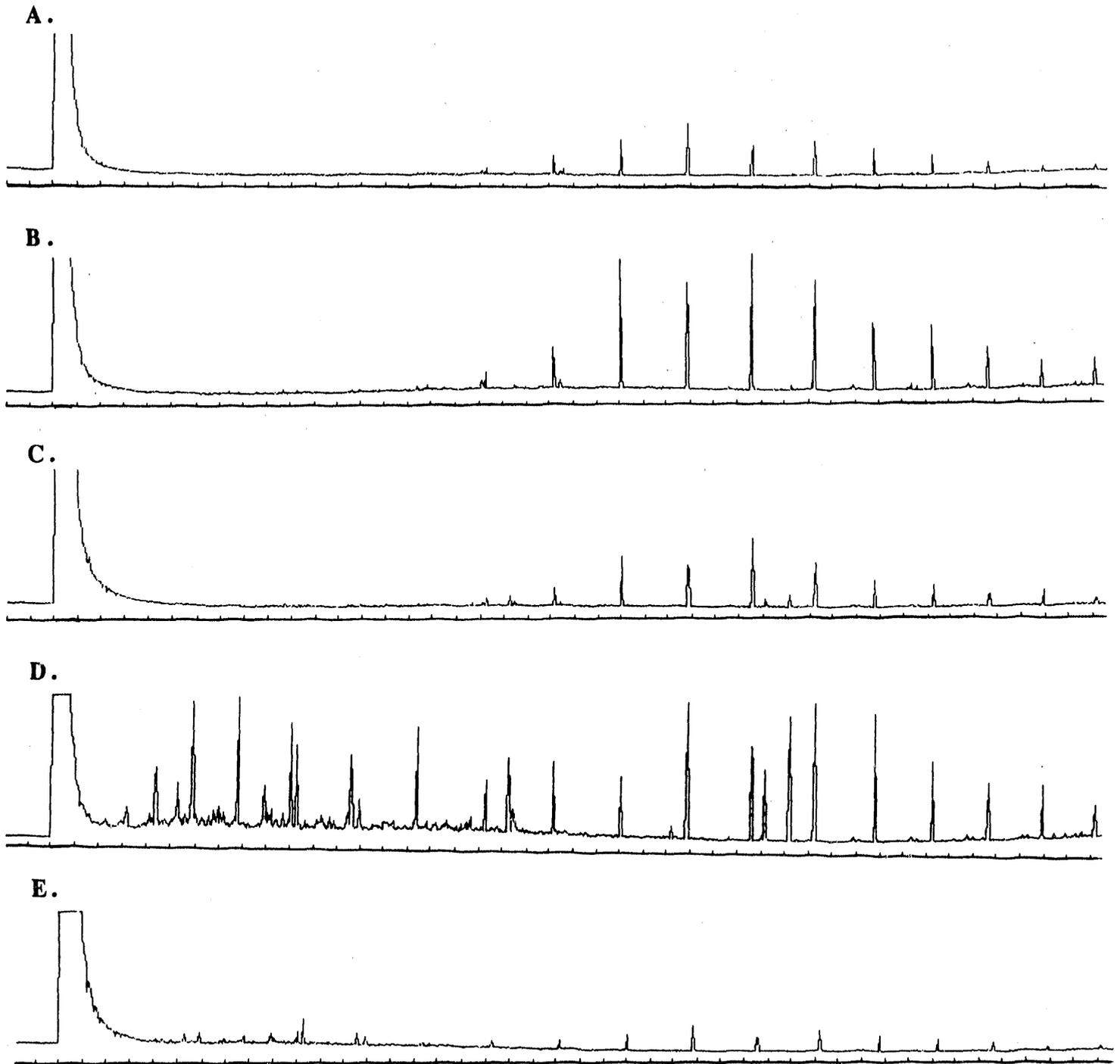


Fig. 10.5. Gas chromatography traces for soil samples from TP04-32 before and after incubation: A = unamended soil before incubation; B = unamended soil after 209 h of incubation; C = soil amended with 100× nutrients after 115 h of incubation; D = soil amended with 100× nutrients and 500 mg/kg fresh fuel prior to incubation; E = same soil as D after 115 h of incubation.

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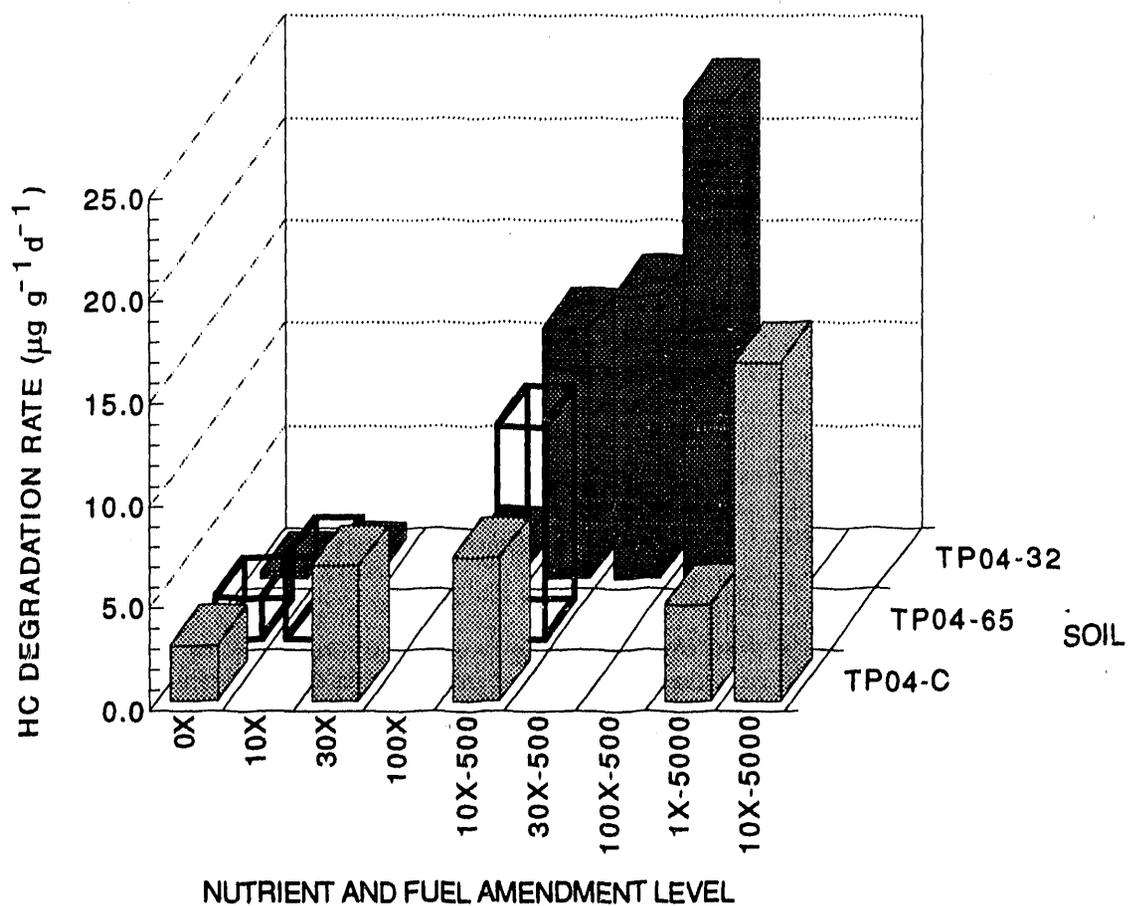


Fig. 10.6. Calculated hydrocarbon degradation rates versus nutrient amendments and fresh diesel fuel addition. [Note: hydrocarbon degradation rates were calculated based on theoretical oxygen demand for biodegradation of $C_{15}H_{32}$ and average oxygen uptake rates after 24 h of incubation (average uptake rate between ~19 and 29 h of incubation).]

The addition of fresh fuel appeared to yield higher degradation rates than those associated with the ambient contamination alone. For example, at the $10\times$ nutrient loading rate and with 500 mg/kg of fresh fuel, the HC degradation rates for TP04-32 (ambient TPH = 216 mg/kg; $12.2 \mu\text{g HC g}^{-1} \text{d}^{-1}$) and TP04-65 (ambient TPH = 8920 mg/kg; $10.4 \mu\text{g HC g}^{-1} \text{d}^{-1}$) were similar. At the $10\times$ nutrient loading in TP04-C (ambient TPH = 2550 mg/kg), the HC degradation rate was $7.0 \mu\text{g HC g}^{-1} \text{d}^{-1}$ when 500 mg/kg fresh fuel was added as compared with $16.5 \mu\text{g HC g}^{-1} \text{d}^{-1}$ when 5000 mg/kg was added.

10.4 SUMMARY AND CONCLUSIONS

Respirometric evaluations of soil samples obtained on Kwajalein Island in February 1991 were conducted at ORNL with and without varying concentrations of different amendments, including both macronutrients and micronutrients, water, and fresh diesel fuel. Six experimental runs were made, each involving four to six treatment conditions, in which the oxygen uptake was measured during incubation at 24°C for periods of 3 to 10 d. For a selected number of the samples, analyses were made of the CO_2 evolved during the respiration period, including total CO_2 and isotopic ratios of carbon, to verify that the evolved carbon was produced by the degradation of diesel fuel. Hydrocarbon measurements were made on one or more replicates from each treatment condition at the beginning and the end of each run, again to confirm that diesel fuel was being degraded in those treatments exhibiting high oxygen uptake rates. The oxygen uptake rate at 24 h of incubation (average rate between ~ 19 and 29 h) was used to calculate theoretical hydrocarbon degradation rates. Based on the results of this experiment, the following observations have been made and conclusions drawn.

Significant differences were observed in the oxygen uptake profiles obtained for the different soil materials and amendment conditions examined in this experiment. Many of the differences are believed to be significant since the variation within replicates of a given treatment was low; the coefficient of variation was typically <0.2 . Oxygen uptake rates were correlated with the nature and magnitude of diesel fuel contamination, fresh fuel addition, and nutrient loading; interactions were observed among these variables. In general, higher uptake rates were observed with higher nutrient loading rates; this effect was even more pronounced when fresh diesel fuel was added. Analyses of the CO_2 evolved during soil respiration were used to calculate respiratory quotients and to quantify carbon isotopes. These data confirmed the oxygen uptake measurements described above. Analyses of hydrocarbon contents before and after treatment and incubation revealed losses of hydrocarbons consistent with those suggested by the respiration measurements.

Biodegradation of fresh diesel fuel added to previously uncontaminated soil from Kwajalein Island was very slow ($<0.5 \mu\text{g HC g}^{-1} \text{d}^{-1}$). The indigenous microbes in this soil may be incapable of degrading diesel fuel components when initially exposed to them; however, continued exposure and acclimation would probably overcome this initial inability.

Biodegradation of higher concentrations of relatively fresh diesel fuel (e.g., 500 to 5000 mg/kg) added to previously contaminated soil can occur at substantial rates (e.g., 23 $\mu\text{g HC per g of soil per day}$), provided nutrients are added at rates of 60 to 180 mg N/d or more. In contrast, the biodegradation of relatively old, weathered diesel fuels (TP04-32 with TPH = 216 mg/kg; TP04-C with TPH = 2550 mg/kg; TP04-65 with TPH = 8920 mg/kg) is

significantly slower (e.g., 0.5 to 2.7 $\mu\text{g HC g}^{-1} \text{d}^{-1}$). The addition of nutrients can enhance the biodegradation rates significantly (e.g., a factor of 2 increase with 60 mg N/kg and a factor of 3 increase with 180 mg N/kg).

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 due to 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.

10.5 ACKNOWLEDGMENTS

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