WRRC-2001-01

DECONTAMINATION OF THE FORMER GRAYLINE BASEYARD

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June 2001

Phase I Project Completion Report
for
"Decontamination of the Former Grayline Baseyard"
Project Period: 1 May 1999–30 April 2000
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ACKNOWLEDGMENTS

This research was funded by the Hawaii Department of Transportation, Airport Division and by the US Geological Survey (WRRIP grant no. 1434-HQ-96GR02666). The help of Ben Schlapak and Terry Kearny of the Airport Division is appreciated. The contents of the report reflect the view of the authors, who are responsible for the facts and the accuracy of the data presented herein. The contents do not necessarily reflect the official views or policies of the Hawaii Department of Transportation or the US Geological Survey. The paper does not constitute standard, specification, or regulation.

EXECUTIVE SUMMARY

Introduction

In situ bioremediation of petroleum hydrocarbon pollutants in aquifers is an increasingly widespread alternative, or an adjunct, to the costly pump-and-treat process to decontaminate groundwater and subsurface matrix. The former Grayline Hawaii baseyard at Honolulu International Airport, the study site, was contaminated by No. 2 diesel fuel which had been released from underground storage tanks during tank testing in 1989 and 1991. The product contaminated the groundwater and the subsurface soil in an area that is under the influence of the tide. Up to 1.4 ft of free product was found in monitoring wells at the site in 1995. In a more recent survey (1998) the depth of the free product had decreased to 0.5 cm. This small scale *in-situ* experiment was undertaken to study the effect of a bioremediation program on the reduction of the diesel fuel contaminant concentration and the concomitant changes in microbial populations, and to model the fate and transport of the product at the Grayline site.

Field Study

The experimental design consisted of three square areas (10 ft x 10 ft) located within the contaminated area identified in the 1998 survey. A monitoring well was drilled in the center of each square area on May 14, 1999. The wells in areas A and B were connected to an air compressor by hoses which delivered air to the water at a depth of 11 ft for 12 hours a day. In addition, well A received ammonium nitrate. Phosphorus was not added because it was present in excess of the microorganisms needs. Area C was untreated. Six soil cores were sampled at random throughout each square area 4 times during a one-year period. The soil cores were collected in polyacetate sleeves in two-foot increments to a depth of ca. 10 ft where coarse uncontaminated coral was encountered. The zone from 0 to 4 ft consisted of fill material which was discarded. Zone 1, from 4 to 6 ft, consisted of dry coralline sand which was uncontaminated. The tidal zone (zone 2) consisted of coralline sand and extended from 6 to 8 ft. It contained most of the contamination. The subtidal zone (zone 3) extended from ca.

8 to 10 ft and consisted of dark and densely packed sediment. Due to mechanical problems with the Geoprobe drilling equipment not all samples were obtained from that zone on the first two sampling days (Day 0, June 23, 1999 and Day 117, October 18, 1999). Subsequently a more powerful equipment, the Strataprobe, was used (Day 299, April 17, 2000 and Day 390, July 17, 2000). The concentration of total petroleum hydrocarbons in the diesel range (TPH) was determined by gas chromatography (GC) (using a flame ionization detector) after soil extraction by sonication in the presence of methylene chloride. Polycyclic aromatic hydrocarbons (PAH) were extracted from the soil with methanol and their concentration determined by immunoassay. All microbial enumerations were done under aerobic conditions. The total heterotrophic bacterial counts were determined by plating on R2A medium. The phenanthrene-degrading bacteria were counted on mineral medium with an overlay of phenanthrene in agar. The diesel and pristane degraders were enumerated by the most-probable-number technique in tissue-culture plates using 2 mL of Bushnel-Haas medium, 0.5 mL of soil suspension, and a drop of the appropriate sterile hydrocarbon. The monitoring-well water was sampled at high tide ca. once a month to monitor the levels of dissolved oxygen (D.O.), nitrate, ammonium, and free product. The levels of microbes in the monitoring-well water were determined 6 times during the whole experiment.

The GC profile of the contaminant indicated a highly weathered product devoid of *n*-alkanes. The branched alkanes pristane and phytane were the only identifiable peaks. This kind of residual product is known to contain a mixture of unresolved constituents which pose a challenge to microbial degradation. The advanced state of degradation of the product is indicative of intrinsic bioremediation which must have occurred under near anoxic conditions (the D.O. in the water of the 3 wells was ca. 2 mg/L in the absence of aeration). Aeration of areas A and B resulted in oxygen saturation level (7.4 mg/L) in the well water at the salinity of the site (0.5 %). An unexpected major recontamination of area A occurred during spring 2000 and was observed again during spring 2001. During the recontamination episode, several inches of free product (which had an identical fingerprint to that of the weathered product

present in the field) were observed in well A. This originally oily product was observed to be progressively transformed into solid strings of degraded product. Evidence that the indigenous microorganisms were capable of growing on some of the constituents of the weathered product during recontamination is provided by the observation that the counts of all types of microorganisms sharply increased in well A water at the time coinciding with the major recontamination episode.

Measurements of TPH in the soil of areas A and B indicated a high heterogeneity in the distribution of the product. Area C was found virtually free of contaminant and could not be used as no-treatment control. In the tidal zone (zone 2) of soil cores, the TPH levels in area A increased significantly with respect to the levels in soil of area B after day 0. However the levels did not exceed 1750 mg TPH/ kg of soil. The percentage of PAH in the TPH was variable in the soil samples (5 to 25%) and averaged 14.5% compared to 2% measured in fresh diesel fuel in another study. In the subtidal zone (zone 3) of areas A and B, the distribution of the product was more homogeneous and the TPH levels usually were well below 1,000 mg/kg of soil. Only two samples in area A (day 117) reached 5,000 and 8,000 mg of TPH-D/kg of soil, respectively. Thus, the hydrocarbon concentrations in soil were generally below the State action level (5,000 mg diesel/ kg of soil). In the tidal zone after day 0, the numbers of all types of microorganisms increased significantly in soil of area A as compared to the numbers in area B. These results support the notion that a recontamination occurred in the soil of area A and that soil microorganisms were capable of degrading some constituents of the contaminant. In zone 3, the numbers of all microorganisms were very low, which correspond to the generally low levels of contamination and may also reflect a technical bias since only the microorganisms able to grow aerobically were detected by our counting techniques. Eight bacteria were isolated from either well water or soil from the tidal zone and were found to degrade one or more of the following compounds: phenanthrene, pyrene, diesel fuel, hexadecane, pristane, and mineral oil. They were identified by two different techniques; the Biolog technique, which uses the patterns of growth on 95 different C sources, and the

16S rDNA sequence homology. The bacteria belonged to genera known to have members capable of hydrocarbon degradation such as *Sphingomonas, Pseudomonas, and Acinetobacter.* Collectively, these results suggest that the Grayline site harbors microorganisms that are capable of degrading not only fresh diesel contaminant but also some constituents of highly weathered diesel product. The respective effects of air and nutrients on the biodegradation of the contaminant in soil could not be fully evaluated in this study due to the recontamination of well A and the absence of contaminant in area C. No movement of nutrients from well A to wells B, C, or to a monitoring well close to the ocean was observed. These results indicate that, at the rates used, the nutrients posed no threat to the shoreline ecosystem.

Modeling

The study was also aimed at modeling the fate and transport of the product at the field site. Toward that aim, the study developed a model for hydrocarbon biodegradation in field-scale aquifers where fluctuations in environmental variables are of concern. The model is flexible in dealing with time-dependent boundary conditions, such as tides in this nearshore aquifer. Expressions for inhibition due to changes in two environmental variables, saturation and heat, are proposed. The expressions can be easily adopted to handle pH or solutes (such as salinity) or other factors that are spatially varied and can have inhibition effects. Although empirical in nature, these expressions or similar ones can be useful due to their analytical nature and ease of integration into the biodegradation model. Parameters of these equations should be estimated by fitting the expressions to available data.

The model is based on SUTRA, a popular code for modeling saturated and unsaturated flow and transport. SUTRA was modified to simulate multi-species fate and transport and was combined with a bacterial growth submodel. The hydrocarbon degradation model can simulate biotransformation reactions for any combination of electron donor and acceptor. Although simultaneous multiple degradation processes and microbial populations can be

simulated, our emphasis in the study is on the effect of heat inhibition and saturation inhibition on a single degradation process and population. The model was verified through comparison with analytical solutions for a single solute with linear sorption and with zero-order decay and first-order decay. An excellent match between numerical and analytical solutions was obtained for all cases simulated. The model was also successfully tested against laboratory data that deal with toluene and benzene transport and biodegradation in a continuous-flow, water saturated soil column. Effects of cyclic flooding and drainage of soils were also studied and the results used to explain some of the conflicting conclusions regarding efficiency of degradation. Although the degradation process is expected to be site-specific, the model demonstrated that cyclic flooding and drainage using dissolved O_2 are likely to reduce the efficiency of degradation. Constant water saturation near its optimal value is recommended for best results. However, wetting followed by periods of drying can have positive effects in cases that involve subjecting the soil to aeration as the only method for providing an O_2 supply.

Simulation results of a hypothetical field site examined the effects of cycles of soil wetting and drying associated with tides with and without saturation and heat effects. For the case of O_2-limited degradation, biomass concentration rises earlier but with lower rates and slower peaks. Inhibition causes a subdued intake of O_2. At early times, O_2 is sufficient to maintain a higher concentration and a higher rate of bacterial growth, in contrast to the case without inhibition. After reaching O_2 concentration that is large enough to sustain bacterial growth, inhibition drastically limits the rate of growth. Ultimately, and as should be expected, bacterial growth and product degradation are more efficient without inhibition. Under these conditions, growth and product degradation curves under inhibition have milder slopes and extend over longer time periods.

In summary, the study introduces a quantitative approach to assess the effects of fluctuations in water content and temperature and shows that their effects can be significant. The model can be useful in the design of *in situ* clean-up projects, either by landfarming or

under natural flow conditions. The design can include assessing amounts and frequency of solutes added and the suitability of various wetting and drying scenarios. However, it is imperative to first validate the inhibition expressions proposed in this study, in order to identify parameters of the equations based on experimental measurements.

Additional Hydrological Assessment of the Site

As mentioned above, recontamination events have prevented comprehensive assessment of biodegradation in the site. Modeling efforts are also hindered by the lack of understanding of specific causes of contamination and its extent at different times. The study also indicated that hydrocarbon concentrations in most samples did not exceed the State of Hawaii's Department of Health 5,000 mg/kg action levels for TPH-diesel in soil. This does not infer, however, that TPH-diesel concentrations in other areas of the site do not exceed the action level.

Through discussion with DOT scope of the work has been modified to include additional site investigation. The objective is to further analyze the effectiveness of *in situ* biodegradation of diesel-fuel contaminated soil. Pending further analysis, natural or enhanced biodegradation could be sufficient remediation tools for this site. The proposed additional field assessment is needed to confirm such a conclusion. The proposed phase will not include assessing bacterial activities but rather emphasize chemical and physical processes controlling the spread of diesel fuel. The work will help in evaluating the degradation process by constructing a mass budget of the product.

In this phase of the study, a maximum of 72 Strataprobe locations will be sampled. Field monitoring will be done during four quarters conducted over a one year period of performance. The sampling scheme will be similar to that used in the previous phase. It will provide a continuous core for describing the lithology and the collections of samples from the saturated and unsaturated zone. A number of micro wells (1" diameter) will be installed in some of the boreholes after finishing the Strataprobe soil sampling. Approximately 24 micro

wells will be installed during the project period. We will periodically measure water table elevation and free product thickness in the accessible wells and compare these levels with those measured in the newly installed micro-wells. We will also conduct a detailed tidal study for approximately one month in up to six wells in locations. The objective is to identify the flow patterns and aquifer properties. Specifically, we will determine the magnitude of groundwater fluctuations across the site, the hydraulic gradient, the approximate Darcy flow velocity, and the hydraulic conductivity and specific storage of the shallow unconfined aquifer. To accomplish these objectives, the tidal response at the site will be compared to the NOAA tidal data collected at Honolulu Harbor, or nearest location to the site. The data will be downloaded to a personnel computer for analysis. The tidal data will be used to evaluate the affect of the subsurface geology on groundwater movement during the rise and fall of the tides. Modeling will be used to study the response of diesel plume to tides.

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STUDY SITE

1. Site Description

This study deals with the former Gray Line Bus Depot located at 435 Kalewa Street, Honolulu, Hawai'i. A site location map is presented in Figure 1. According to a report by Rooks (1995), the site is approximately 6.2 acres in size, and is bordered to the northeast by a drainage canal and to the southeast by Keehi Lagoon. The majority of the site is paved with asphalt or concrete. A few remaining buildings are still in existence. There are four underground injection wells and four other wells within a 1-mile radius of the site. According the to the State of Hawai'i Department of Health well records, none of these wells serve as a drinking water source, and all are listed as sealed or unused. A pipeline corridor transects the site along its northeastern perimeter, which is used to deliver diesel, gasoline, jet fuel, and other petroleum products between Sand Island and the Honolulu International Airport.

2. Site History

The site housed several underground storage tanks (USTs) (Fig. 2). The Rooks report (1995) indicated the use of two 10,000 gallon diesel USTs, one 10,000 gallon gasoline UST, two 1,000 gallon motor oil USTs, and one 1,000 gallon waste oil UST. At the time of the Rooks (1995) report, four USTs were in operation. The 10,000 gallon diesel USTs were not in service, but remained in place, and the former gasoline UST was converted to a diesel UST. A diesel fuel release estimated between 200-400 gallons reportedly occurred during tank tightness testing on January 12, 1989. A second release occurred during subsequent tank testing on December 10 and 13, 1991. Fuel from both of these releases flowed primarily over the asphalt parking lot to the northern corner of the site and was cleaned up using sorbent materials. It is suspected, however, that some product may have entered the subsurface immediately adjacent to the tanks. Unitek performed an Environmental Assessment in late 1991. Five monitoring wells (installed by Engineering-Science) were inspected and

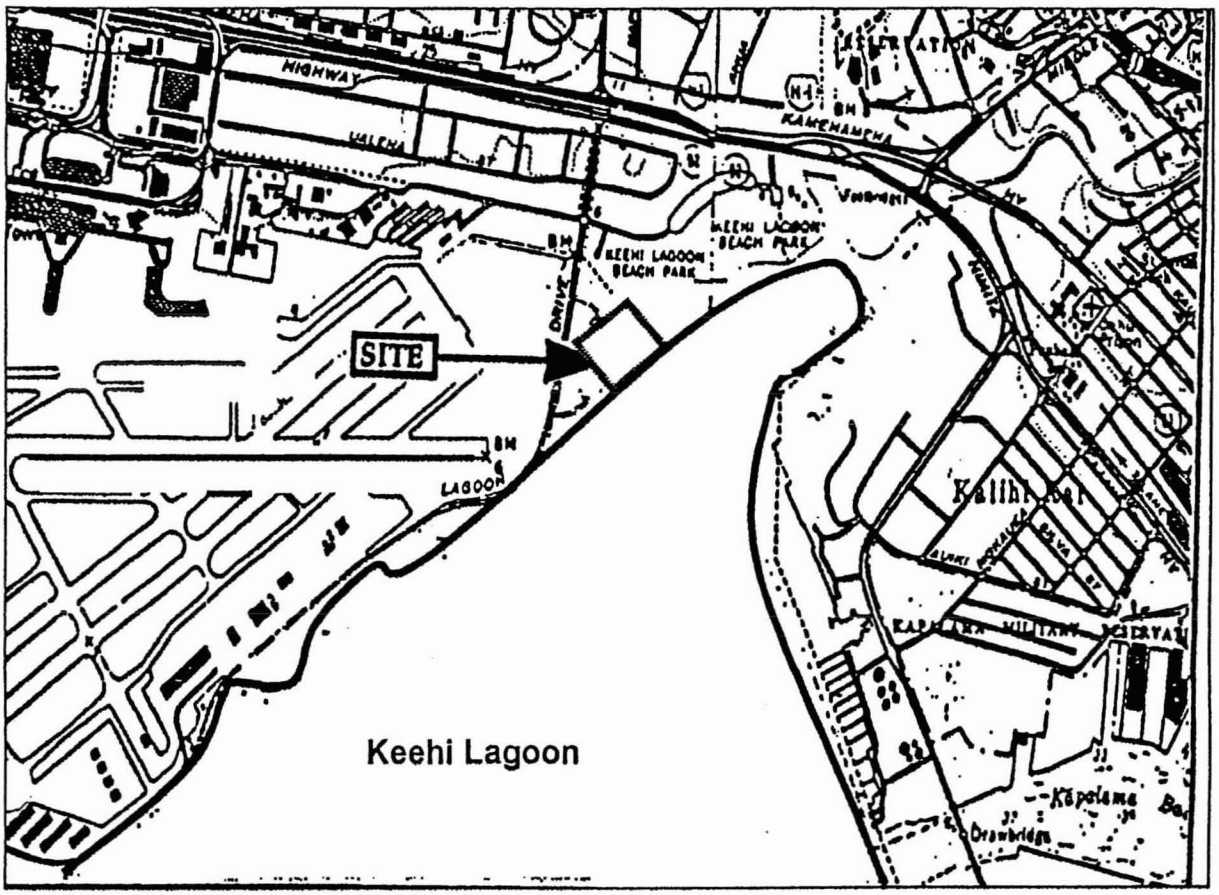


Fig.1. Location of the project site, the former Gray Line Bus Facility
Source: Rooks, 1995

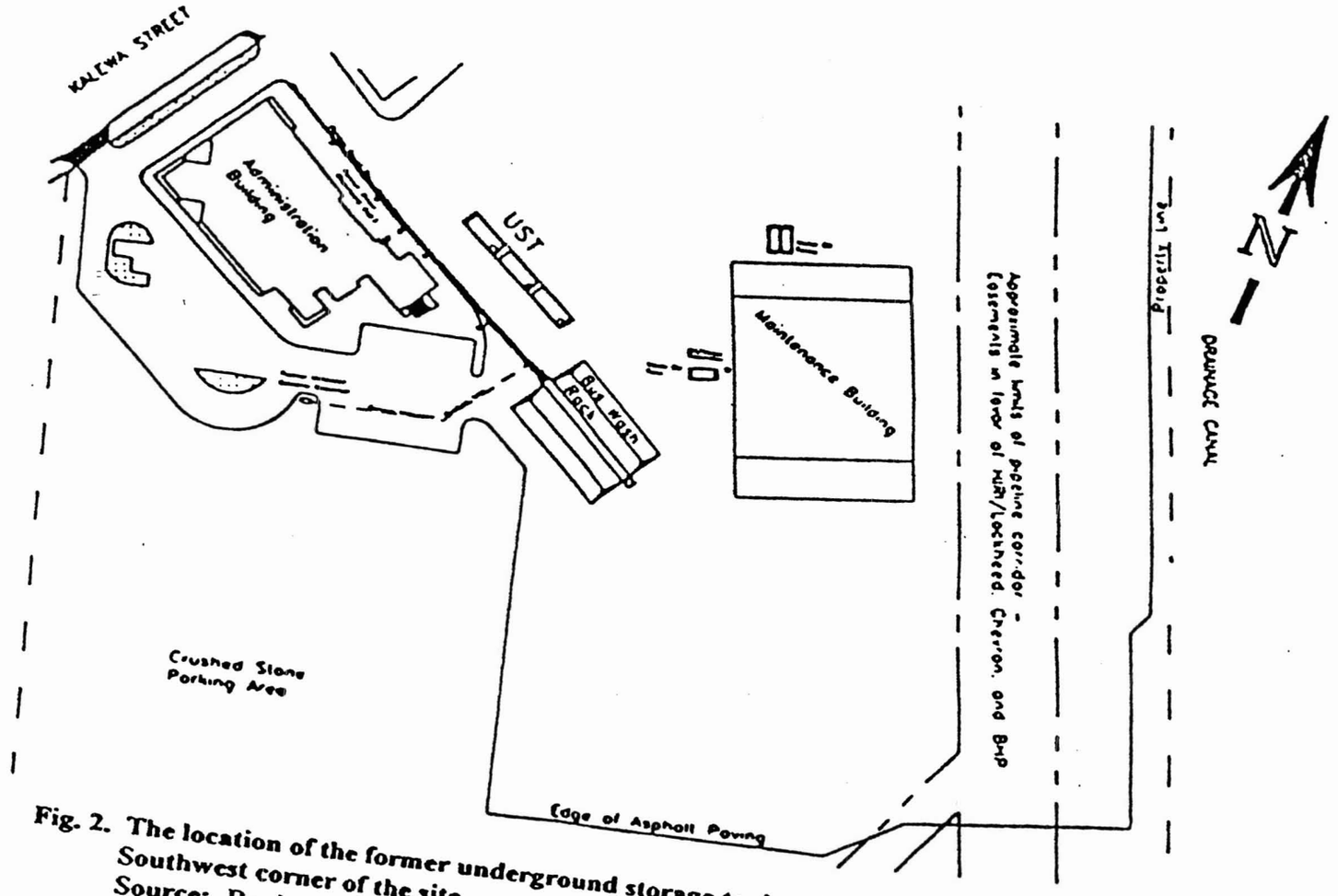


Fig. 2. The location of the former underground storage tanks of diesel fuel near the Southwest corner of the site
Source: Rooks, 1995

approximately seventeen inches of floating product in the well immediately west of the maintenance building was discovered. Approximately 0.25 inches of product was measured in the well to the southwest of the diesel/gasoline tanks. No free product was detected in the remaining wells.

In 1995, Rooks from Cotton and Frazier Consultants, Inc. assessed the lateral and vertical extent of the petroleum hydrocarbon contamination in the subsurface. Twenty-six borings, spread over the site (Fig. 3), as well as ground water from additional locations, were sampled as part of that assessment. Results indicated that there was a free-phase diesel product plume on the site with a maximum thickness of approximately 14 inches (well W2). A contour map showing measured free product thickness and the estimated extent of the free product contamination is shown in Fig. 4. A tidal assessment was also included, and indicated that the rise and fall of the water table was tidally influenced. The tidal fluctuations showed two daily tidal cycles. Rooks expected that the measured thickness of the free product would vary over the course of the tidal cycle. The tidal fluctuation study suggested that the prevailing average hydraulic gradient was very shallow and sloped downward toward the east. These findings were supported by the observed migration of the free product.

3. Scope of the Project

This research project addresses the decontamination of the former Gray Line Bus Depot, a near-shore site, on O'ahu, Hawaii. An *in situ* bioremediation experiment will be implemented at the site in an area where the diesel fuel contaminant reached the water table, about six feet below the ground surface at high tide. The project evaluates the effect of bioremediation on the biodegradation of the diesel fuel contaminant in this tidally influenced aquifer.

4. Objectives of the Project

The overall goal of this project was to study the fate of the diesel contaminant in this tidally-influenced aquifer under bioremediation treatment conditions. Using the results of this

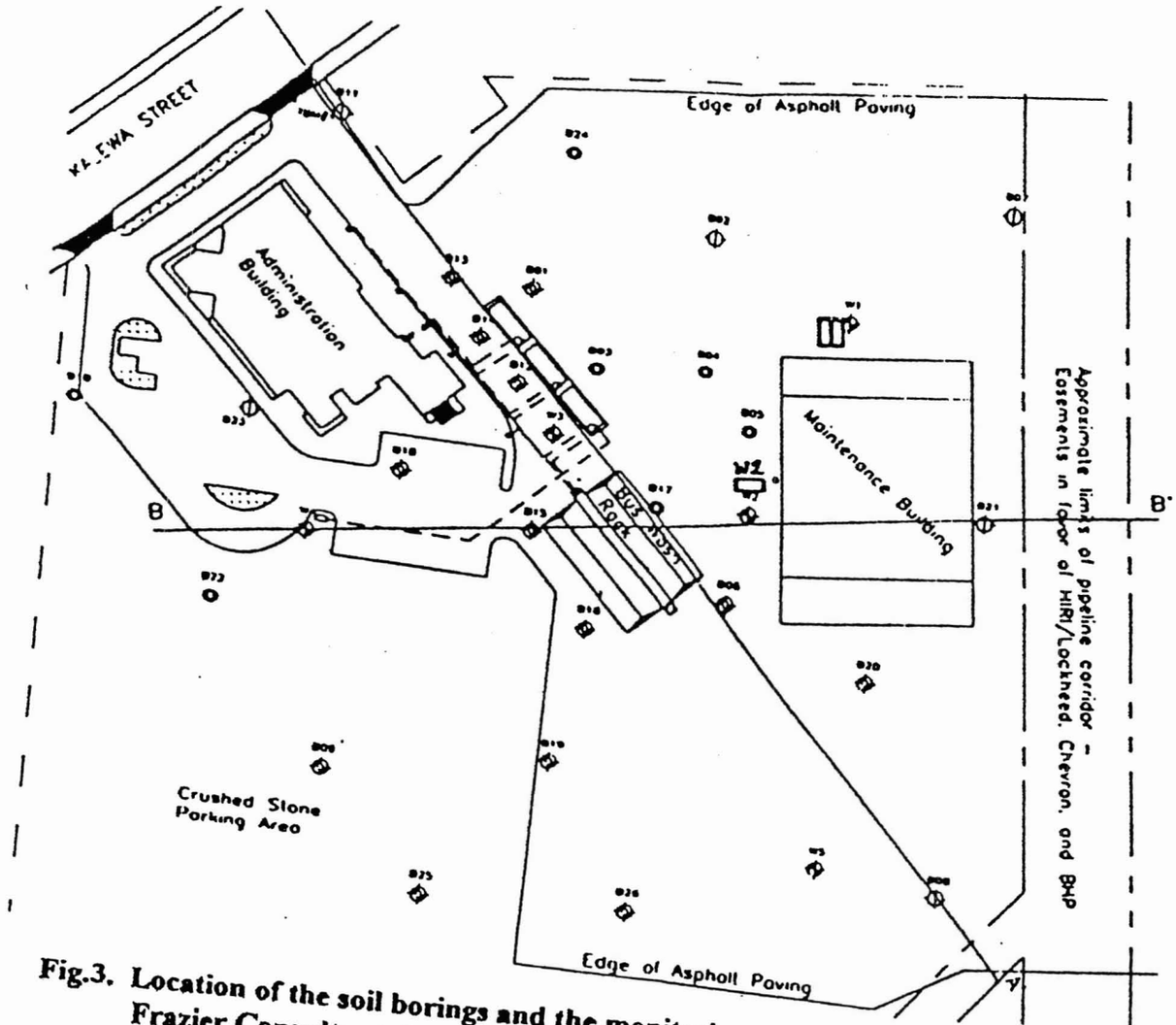


Fig.3. Location of the soil borings and the monitoring wells used by Cotton and Frazier Consultant, Inc., for their site characterization study. Rooks, 1995

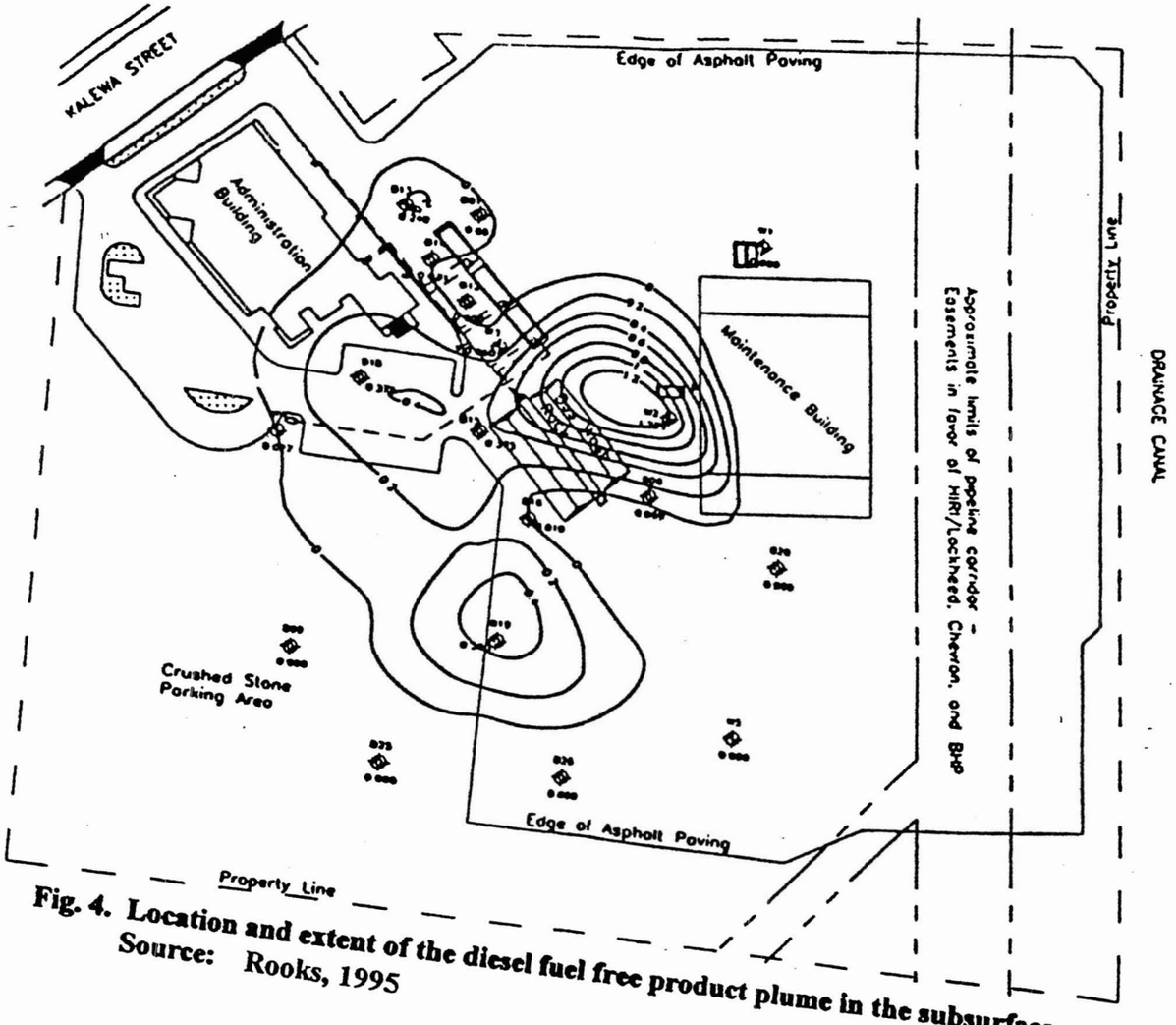


Fig. 4. Location and extent of the diesel fuel free product plume in the subsurface
Source: Rooks, 1995

study, there is the potential to develop and apply a remediation strategy for *in situ* bioremediation of petroleum hydrocarbons in Hawaiian aquifers, as well as for other coastal regions under similar conditions. The specific objectives of the project are:

1. To set up an *in situ* bioremediation experiment,
2. To evaluate the effects of the bioremediation treatment on the diesel fuel contaminant, by monitoring decreases in hydrocarbon concentrations and increases in microbial numbers throughout the experiment,
3. To monitor well-water chemistry in relation to microbial numbers and activity, and potential movement of the contaminant and nutrients toward the ocean,
4. To enumerate hydrocarbon-degrading microorganisms in the contaminated soil and well water,
5. To isolate, characterize, and identify selected hydrocarbon-degrading bacteria from the site, and
6. To model the fate and transport of the product at the field site.

SECTION II

Chapter 1
INTRODUCTION

1.1. Overview

Remediation of environmental contaminants has recently become an important concern. Petroleum is one of many contaminants that can have adverse, long-term effects on an ecosystem (Overton et al., 1994). Oil and fuel contaminants generally enter the environment through accidental spills, leakage from storage sites and pipes, or deliberate dumping (Riser-Roberts, 1992). It is estimated that out of approximately 750,000 existing underground storage tanks (UST) facilities in the United States, about 350,000 have leaked or are currently leaking, and approximately 30,000 new releases occur each year (Eweis et al., 1998). After release from an underground storage site, the liquid-phase hydrocarbons primarily move vertically under gravitational influence and by capillary forces. The liquid hydrocarbons interact with soil particles, and are retained in pore spaces or adsorbed onto the solid soil matrix. Some will also volatilize and create a vapor phase. If the released liquid volume is large, or the release is deep within the subsurface, the hydrocarbons may reach the water table and accumulate on the surface of the denser water. The soluble portion of the leaked hydrocarbons will dissolve in the ground water and migrate within it (Reisinger et al., 1994). Toxicological effects from petroleum occur through ingestion or epidermal contact, mainly with the aromatic hydrocarbon forms. These forms are comprised of one-to five-ring structures in crude oils, and two or more rings may fuse together to form polycyclic aromatic compounds (PACs). These compounds are more persistent in the environment and the larger ones are potential human carcinogens (Overton et al., 1994).

Contaminants in the environment can be transformed or removed through physical processes such as volatilization, solubilization, or dispersion. These physical processes alone, can remediate contaminated sites, but can be enhanced by biological processes. Bioremediation has been defined as a managed or spontaneous process in which biological,

especially microbial, catalysts breakdown on pollutant compounds, thereby reducing or eliminating environmental contamination. These are normal processes in the environment, and under certain conditions, petroleum hydrocarbons can be bioremediated rapidly and at a low cost. Bacteria capable of degrading petroleum hydrocarbons may commonly be found in subsurface soils, but natural breakdown of the compounds usually occurs too slowly to prevent accumulation of the pollutants to unacceptable levels. Bioreclamation or bioremediation refers to the enhancement of this native capability of the microorganisms. Indigenous microbes can be stimulated, or specially developed microbes can be added to the site to degrade, transform, or attenuate organic and organometallic compounds to low levels and nontoxic products. Oxygen and nutrients are sometimes added to the system to support biological growth and improve the degradation (Riser-Roberts, 1998).

In situ bioremediation of petroleum contaminated sites has recently become widely accepted. It is now actively supported by the U.S. EPA as an effective method of remediating contaminated soils and aquifers (Norris and Falotico, 1994). *In situ* treatment occurs on site and does not require removal of the contaminated materials off of a site. It offers an alternative to the traditional approach of site remediation involving excavation and redisposal, or on-site isolation or treatment. This process is based upon the technologies developed and used in conventional water and wastewater treatment, in mining, oil and gas, and chemical process industries. These methods use biological, chemical, physical, or thermal methods to degrade, detoxify, extract or immobilize contaminants, and they include biodegradation, air/stream stripping, neutralization, solidification/stabilization, and oxidation (Riser-Roberts, 1998).

1.2. Bioremediation Principles

Bioremediation involves the addition of nutrients and oxygen to stimulate the growth of indigenous hydrocarbon utilizing microorganisms. Diesel fuel and other petroleum by products are essentially composed of carbon and hydrogen and lack other nutrients required

for microbial growth. The ultimate goal of bioremediation is to convert these organic wastes into biomass and harmless by-products of metabolism such as carbon dioxide, water and inorganic salts. The use of microbial processes to transform or eliminate chemicals is neither a novel idea, nor a new technology. These processes have been used for decades for the elimination of chemicals from many waste-treatment systems. More recently a greater variety of chemicals have been eliminated from contaminated environments because of condition optimizations for the biodegradative microflora (Alexander, 1999). By increasing the supply of limiting nutrients, such as nitrogen and phosphorus, treatment strategies can be formulated by specific empirical developments that ensure optimal nutrient amendments for a given value of hydrocarbon contamination (Head and Swannell, 1999). Various authors provide ratios for these nutrients, and although there is some disagreement on the exact ratios they are relatively similar. Three of the major nutrients, nitrogen, phosphorus, and potassium, can be supplied in common inorganic fertilizers (Riser-Roberts, 1998). The amount of nitrogen and phosphorus to be added can be calculated from the amount of carbon in the material to be degraded. It is assumed that a certain percentage of carbon, for example 30%, is assimilated into the biomass of cells carrying out bioremediation and that the resulting biomass has a specific C:N:P ratio (e.g., 100:10:1) (Alexander, 1999; Norris and Falotico, 1994). Hydrocarbons are generally assumed to contain 85% of carbon (Shen and Bartha, 1994).

Measurements of soil organic carbon, nitrogen, and phosphorus allow the determination of the background C:N:P ratio, and the nutrient availability. This evaluation will determine the amount of supplemental nitrogen and/or phosphorus, which should be added in a readily available form for microbial uptake. To maintain optimal growth conditions for the degradative microorganisms there should be periodic monitoring of the nutrient levels, so that overloading does not cause toxicity, or leaching problems. Also, excessive precipitation of phosphorus compounds at the injection point may occur and cause plugging (Riser-Roberts, 1998). To avoid such problems, alternative forms of nutrients may be used. For, instance, studies have shown that the use of inorganic phosphorus sources can cause precipitation of

calcium and iron phosphates, making the phosphorus unavailable. Organic phosphorus forms have been found to be much more mobile in soil, and to not cause precipitation with alkaline earth cations (Mills and Frankenberger, 1994). Another limiting factor in the application of specific ratios of nitrogen and phosphorus is that it may impose conditions that select for certain microorganisms. These microorganisms may not be those that are best suited to remove the contaminants. Different organisms have different requirements for nitrogen and phosphorus, and studies demonstrate that there are different optima for biodegradation of specific compounds. For example, a recent study shows that there also appears to be two distinct optima for phenanthrene biodegradation corresponding to different supply ratios of N:P (Head and Swannell, 1999). This implies that either two different phenanthrene-degrading populations with different nutrient requirements were selected, or that different catabolic pathways with different kinetic properties were induced in the same population. Thus, a different nutrient supply may be required to achieve optimum degradation of particular components of petroleum hydrocarbon mixtures (Head and Swannell, 1999).

Oxygen has traditionally been the limiting factor in achieving maximum growth, but now many methods exist to effectively deliver adequate amounts of oxygen (Brown and Norris, 1984).

1.3. Oxygen Sources

1.3.1. Air or Oxygen Gases

Many methods of oxygen supply exist for *in-situ* bioremediation. The choice of the best method depends on the specific site. Each site has varying physicochemical, hydrogeological, and microbiological factors that control biodegradation and transport of nutrients and oxygen (Brown and Norris, 1984).

Traditionally, soil and ground water contamination were treated by excavation of the contaminated soils and/or by pumping and treating the contaminated ground water. Soil excavation, however, often is not practical or cost-effective, and follow-up ground water

treatment is usually still required. In the late 70's and early 80's, *in-situ* breakdown of dissolved contaminants in the saturated zone and some adsorbed-phase contamination used the pump-and-treat process to remove the dissolved and free-phase contamination in ground water. This approach provided long-term protection and reduced the potential for the contamination to leach back into the ground water. Closure of the site was routinely attained within three to five years with costs between $1-2 million (Brown and Jasiulewicz, 1992).

The second generation remedial technology used vapor extraction, or "soil venting" to treat contaminants adsorbed to the soil in the vadose zone. This process draws a vacuum through the soil to vaporize and extract volatile adsorbed contaminants such as gasoline, diesel fuel, and many chlorinated solvents. With this soil vapor extraction addition the length of treatment was reduced to two to three years with costs decreasing to $700,000 to $800,000 (Brown and Jasiulewicz, 1992).

Treatment of soil contamination below the water table with soil vapor extraction is essentially unfeasible because it requires extensive dewatering of the contaminated area to create unsaturated conditions. A third technology allows volatilization in the saturated zones by sparging air under pressure through soils below the water table. Sparging combined with soil vapor extraction is emerging as the third generation remedial technology. The addition of air sparging to venting can result in tremendous improvements in mass removal rates, with site closure times in a year or less and further reduced total costs between $400,000-$500,000 (Brown and Jasiulewicz 1992).

Air sparging is the introduction of air beneath the water table to enhance bioremediation (Hinchee, 1994). With sparging, air bubbles travel horizontally and vertically through the soil column, creating a transient air-filled porosity in the saturated zone. It also creates turbulence and increased mixing in the saturated zones, which increases the contact between ground water and soil. This will allow higher concentrations of the volatile organic carbons (VOC's) to dissolve in the ground water. In addition, the sparged air maintains a high dissolved oxygen content, which enhances natural biodegradation (Brown and Jasiulewicz, 1992).

Sparging relies on two mechanisms of contaminant breakdown, biodegradation and volatilization. Air sparging is divided into two techniques, in-well aeration and air injection (Hinchee, 1994). Both are used to treat saturated soils (Brown and Crosbie, 1994). In-well aeration injects air into the bottom of a well, with the air traveling upwards and removing volatile contaminants, while also aerating the water. The upward movement of water creates an in-well airlift pump effect, causing ground water to flow into the well at the bottom and flow out in the upper regions. A circulation pattern is created that treats and aerates the water as it passes through the well. The air injection technique injects air, under a pressure greater than that of the water depth, into the ground water-saturated soil. The high pressure forces air through the contaminated material and provides oxygen for bioremediation and volatilization of contaminants (Hinchee, 1994). For both techniques the amount of oxygen able to be introduced is limited to the solubility of air at the temperature of the ground water. Typically air solubility is 40-50 mg/L, which corresponds to 8-10 mg/L of oxygen (Brown and Norris, 1984).

1.3.2. Oxygen-Releasing Compounds

Hydrogen peroxide can be used as an alternative source of oxygen to enhance microbial activity during bioremediation of soil and ground water. The hydrogen peroxide is able to overcome the limitation of oxygen solubility encountered with air sparging. It is an oxygen concentrate (Lu, 1994), viewed as a chemical oxygen source miscible with water, which decomposes to generate oxygen. One part of hydrogen peroxide generates one-half part of oxygen, so in theory it can provide greater available oxygen levels. Two limiting aspects of hydrogen peroxide reactivity are decomposition and biological effects. Rapid decomposition of 100 mg/L will exceed the solubility of oxygen and result in gas formation. Using levels of hydrogen peroxide over 100 mg/L could lead to gas blockage and loss of permeability. Hydrogen peroxide is also well known for its bactericidal effects, concentrations as low as 200 mg/L have been reported to be toxic to microorganisms (Brown and Norris, 1984; Lee and Raymond, 1991).

Another alternative oxygen source is oxygen-releasing chemicals (ORC). These act as a diffusion source of oxygen since they release oxygen at a controlled rate. ORC's include calcium peroxide, urea peroxide, and magnesium peroxide. Magnesium peroxide is more popularly used because it is stable and maintains a uniform oxygen-releasing rate (Kao and Borden, 1994). ORC is a formulation of magnesium peroxide (MgO_2), the active agent, and magnesium oxide. The chemical is supplied in a powder form that, upon contact with water, releases oxygen. The magnesium peroxide and magnesium oxide are both converted to magnesium hydroxide [$Mg(OH)_2$]. The magnesium hydroxide endpoint is relatively safe, as a suspension of magnesium hydroxide in water is the commonly used Milk of Magnesia (ORC Technical Bulletin, Regenesis Bioremediation Products, CA.).

1.4. Bioremediation Systems and Processes

A method used for ground water remediation is known as pump and treat, where the ground water is pumped to the surface, treated, and either used directly or returned to the aquifer. Surface treatment usually involves aeration, the addition of nutrients, and occasionally seeding with microbial cultures capable of degrading contaminants in the ground water. In some cases the surface treatment is physical as it may involve adsorption onto activated carbon or air stripping. Generally, pump and treat systems have not been successful in remediating ground water back to drinking water standards (Eweis et al., 1998).

Ground water can also be remediated *in situ*, by stimulating microbial populations in the aquifer by introducing oxygen and nutrients. There are many methods of oxygenating the subsurface, such as air sparging or the use of hydrogen peroxide. Nutrients are usually introduced in an aqueous solution through infiltration galleries or injection wells. Subsurface conditions are not easily controlled, and problems associated with *in situ* treatment, such as isolation of the contaminants, and delivery of the oxygen or nutrients to the microbes, commonly occur (Eweis et al., 1998).

Soil remediation can also be carried out *ex situ*. *Ex situ* methods involve removal of the contaminated soil and include land treatment (landfarming), composting, and treatment of a slurry-phase in bioreactors. Landfarming involves aeration and mixing of contaminated soil by tilling, addition of nutrients (and sometimes microorganisms), and control of moisture by additions of water. The degradation processes in landfarming are principally biological, but photochemical oxidation may be significant in some cases. With composting, the contaminated material is mixed with organic bulking agents, such as manure, and formed into piles or windrows. The bulking agents increase porosity so airflow is increased. Bioreactors are slurry-phase operations in which the contaminated soil is placed in a containment vessel and enough water is added to allow continuous mixing. Oxygen can be added as required and off-gas controls are often used to prevent loss of volatile organic compounds through stripping (Eweis et al., 1998).

In situ methods include soil vapor extraction (SVE) and bioventing. SVE is a process in which volatile organic carbons (VOCs) are removed from the vadose zone by installing wells and applying negative pressure. Bioventing is used for treatment of less volatile biodegradable contaminants in the vadose zone and is an alternative to excavation and *ex situ* soil treatment. Transformation and degradation of the contaminants in the vadose zone are carried out at the point of contamination. One objective of bioventing is to minimize contaminant migration (Eweis et al., 1998).

1.5. Difficulties With Aquifer Remediation

Most of the early bioremediation research consisted of laboratory studies simulating microbial activity in shallow aquifer systems. These studies were informative and successful, because they were carried out under controlled conditions. However, the difficulties of attempting *in situ* remediation of ground water systems were not anticipated (Chapelle, 1999). Field studies demonstrate that site heterogeneities in the subsurface can lead to difficulties in nutrient and oxygen delivery (Bouwer and Zehnder, 1993). Hydrologic

constraints were found to be limiting factors, for instance, amended recharge water was not delivered efficiently to less permeable sediments. Hydrogeologic factors, such as seasonal fluctuations in ground water levels and changes in ground water flow directions, affected the efficiency of nitrate delivery to zones of contamination. In addition, variations in hydraulic conductivity could lead to "stratigraphic trapping" of fuel in the subsurface. Such trapping made it more difficult to introduce amended water to the most contaminated sediments. Incomplete mixing of injected treatment water with contaminated water was also often found to be a problem (Chapelle, 1999).

Early studies also showed that monitoring the effectiveness of nitrate-based bioremediation was extremely difficult. In particular, it was difficult to separate the effects of hydrodynamic processes (such as dilution) from biological degradation processes on concentrations of contaminants and nitrate (Chapelle, 1999). Conventional approaches for proving *in situ* biodegradation of organic pollutants in aquifers have severe limitations because accessibility and variability precludes implementation of replicated, statistically valid experimental designs. The majority of attempts to document *in situ* biodegradation in aquifers have used indirect observations (Madsen et al., 1991).

1.6. Factors Affecting Bioremediation in Soil-Water Systems

1.6.1. Chemical and Physical Factors

Organic compounds differ widely in their solubility, and many synthetic chemicals have low water solubilities. The availability of a compound to an organism will dictate its biodegradability. For example, degradation of PAHs is generally limited because of their low water solubility. In fact, the order of degradation of PAHs is related to their water solubility. Mass transfer from the solid phase to the liquid phase is rate limiting for growth. Microorganisms use various mechanisms to metabolize organic substrates present at concentrations that exceed their water solubility. Emulsification can be employed to provide greater surface area of the substrate, and some organisms produce their own emulsifiers. Some

microorganisms may also modify their cell surface to increase its affinity for hydrophobic substrates (Riser-Roberts, 1998).

Solutes can be retained by the surfaces of solid material, such as soil, either by adsorption or absorption. This is one of the most important factors affecting the behavior of organic chemicals in the soil environment. Sorption will affect the rate of volatilization, diffusion, leaching, and the availability of chemicals to microbial degradation. For instance, soil organic matter will slow biodegradation of bound PAHs that would otherwise be readily metabolized. Phenanthrene is less degradable when sorbed to organic soil than when in liquid culture, and some humic acids can decrease mineralization of pyrene (Riser-Roberts, 1998).

The concentration of hydrocarbons can affect their biodegradability. High concentrations of hydrocarbons can be inhibitory to microorganisms, through oxygen or nutrient limitations, or through toxic effects from volatile hydrocarbons (Leahy and Colwell, 1990). There also appears to be a minimum concentration at which an organic contaminant can be degraded under steady-state conditions. Subthreshold concentrations may be characteristic of some pollutants that are found entirely in the aqueous phase of some environments. Microorganisms may not be able to assimilate carbon from chemicals present in these low amounts. Energy derived from low concentrations of the substrate may be inadequate for maintenance of the bacterial cell, or if higher concentrations are required to activate the transport and metabolic systems of the cell. Hence, the bacteria may not grow or produce the large, acclimated populations needed for enhanced biodegradation. The threshold can be lowered if the microbes have certain alternative carbon sources available. Sometimes microbes can mineralize contaminants below threshold by utilizing other compounds for growth (Riser-Roberts, 1998).

1.6.2. Other Environmental Factors

Biodegradation of contaminants in soil requires water for microbial growth and for diffusion of nutrients and by-products during the breakdown process. Aerobic hydrocarbon degradation is diminished under saturated soil moisture conditions because of low oxygen

supply, while under very dry conditions, microbial activity is hindered due to insufficient moisture levels necessary for microbial metabolism (Riser-Roberts, 1998). Optimal biodegradation is reported at a soil water-holding capacity of 50% to 70% (Atlas and Bartha, 1993).

Temperature is another important factor controlling microbial activity. Temperature affects the physical nature and composition of petroleum contaminants, the rate of microbial hydrocarbon metabolism, and the composition of the microbial communities (Leahy and Colwell, 1990). Generally, raising the temperature increases the rate of degradation of organic compounds in soil. Microbial growth usually doubles for every 10°C increase. There is also a decrease in adsorption with rising temperatures, which makes more organics available for the microorganisms to degrade. Temperatures that are too high, however, will cause rapid decreases in growth and metabolism. Increasing temperatures may also become lethal to the microbes, as enzymes begin to break down. Conversely, a lowering of the temperatures is associated with a slowing down of microbial growth rate. It can also lengthen the acclimation period and delay the onset of biodegradation. The majority of soil hydrocarbon utilizers are most active in the mesophilic range of 20-37°C, so optimum temperatures for biodegradation are within this range for most contaminated sites. Although the optimum is within the mesophilic range, utilization of hydrocarbons by microbes can occur at temperatures ranging from -2 to 70°C (Riser-Roberts, 1998). Local environmental conditions may select for a population with a lower or higher optimum temperature (Margesin and Schinner, 1997).

Biological activity in the soil is also greatly affected by the pH, through the availability of nutrients and the tolerance of organisms to pH variations. The optimum pH for rapid decomposition of wastes and residues is usually between 6.5 and 8.5. Hydrocarbon contaminated soil could also contain a number of heavy metals that are potentially toxic to the environment. Higher pH levels foster the formation of insoluble precipitates, which result in the immobilization of certain heavy metals. Also, the solubility of nutrients (i.e.,

phosphorus) is maximized at pH 6.5, and microorganisms commonly grow well in the range of 6.5 to 7.5 (Riser-Roberts, 1998; Joshi and Lee, 1996).

1.7. Chemistry of Diesel Fuel

Diesel fuels are classified as middle distillates and are denser than gasoline. They consist of hydrocarbons with carbon numbers ranging from C_9 to C_{20}, and boiling points in the range of 163° to 357°C. Diesel fuel No. 2 is essentially equivalent to fuel oil No. 2. It contains normal and branched chain alkanes (paraffins), cycloalkanes (naphthenes), and aromatic cycloalkanes, with a carbon number range of C_{11} to C_{20}. Some polycyclic aromatic hydrocarbons present in diesel fuel No. 2 are phenanthrene, methylphenanthrene, and pyrene. Generally, the total saturated hydrocarbon fraction comprises 75% of the diesel by volume, and the total aromatic hydrocarbon fraction comprises 25% by volume (Millner et al., 1994). Diesel fuel quality is based on specifications determined by analyses of physical properties that differentiate petroleum products. One test that is unique to diesel fuels is the cetane (C_{16}) number determination. The cetane number is similar to the octane number for gasoline, both being measures of the fuels ignition characteristics in an internal combustion engine. The higher the cetane number, the better the ignition characteristics. The chemical constituents of gasoline that give it a high octane number (such as aromatics) tend to give diesel a low cetane number. Gasoline and low quality diesel fuels (low cetane number with higher contents of heavy and mid-range aromatics) have a greater potential for toxicological impacts than do high cetane diesels (Block et al., 1991).

Diesel fuel can change chemically in the environment. This process is termed "weathering", and over time the diesel fuel will no longer contain many of the chemicals present in the parent mixture. Weathering is a complex process involving several environmental processes such as volatilization, biodegradation, biotransformation, and dissolution. These processes cause the removal of the more mobile toxic chemicals originally present (*e.g.*, chemicals which are volatile, have increased water solubility, low molecular

weights and low soil binding affinities), while leaving behind higher molecular weight chemicals which are resistant to biodegradation and volatilization, and bind strongly to soil. Through weathering, the composition of diesel is so altered that the mixture no longer has the same chemical or toxicological characteristics as fresh diesel fuel (Millner et al., 1994).

1.8. Specific Components of Diesel Fuels

1.8.1. Alkanes

Normal, branched, and cyclic alkanes (paraffins) are the most abundant components of diesel fuel (Block et al., 1991). Alkanes are saturated hydrocarbons, containing only carbon-hydrogen and carbon-carbon single bonds. In a study of the fate of petroleum hydrocarbons in soils, it was found that the *n*-alkanes, or straight-chain alkanes were lost by abiotic and biotic processes. The *n*-alkanes are the most widely and readily utilized hydrocarbons, with those between C_{10} to C_{25} the most susceptible components in oil to microbial attack (Riser-Roberts, 1998). The branched alkanes, also known as isoalkanes, are predominantly monomethyl, dimethyl, and trimethyl substituted paraffins (Block et al., 1991). Isoalkanes are degradable by many microorganisms, although they are less susceptible than the *n*-alkanes as growth substrates. Extensive branching, as well as positioning of the side chain, affects degradability (Riser-Roberts, 1998). In this category, pristane (2,6,10,14-tetramethylpentadecane) and phytane (2,6,10,14-tetramethylhexadecane) are of particular interest. Both are often present in significant concentrations and are easily identified by gas chromatography. The ratio of pristane to heptadecane and of phytane to octadecane for a given source of diesel fuel is frequently distinctive enough to identify the source of a fuel spill if weathering is not significant. The ratios increase during biological degradation and are useful in evaluating the age of environmental contamination (Block et al., 1991). Cycloalkanes are more resistant to microbial attack than the *n*-alkanes, and are highly toxic. Complex alicyclic compounds, i.e., hopanes, are among the most persistent compounds in petroleum spills. A

mixture of co-oxidation and commensalism by mixed microbial communities appear to be important in the degradation of cycloalkanes (Riser-Roberts, 1998).

1.8.2. Alkenes

Alkenes are unsaturated, meaning that in addition to the carbon-hydrogen bonds, one or more carbon-carbon double bonds are also present. Unsaturated 1-alkenes are generally oxidized at the saturated end of the molecule by the same mechanism used for alkanes. The presence of a terminal methyl group at each end of the molecule appears to make the molecule more degradable, since 2-alkenes are more readily attacked than 1-alkenes (Riser-Roberts, 1998). Although degradable, alkenes are not common in crude oil and are rarely identified in crude distillate products. Only when yield-enhancing steps are incorporated in the production of diesel fuel are these components found, and are predominantly branched and cyclic structures (Block et al., 1991).

1.8.3. Aromatics

Aromatics represent an estimated 10-30% of No. 2 diesel fuel. Flash point specifications of No. 2 diesel fuel are high enough that only trace quantities of BTEX compounds are present. Alkyl benzenes, particularly C_3 and C_4 alkyl benzenes, are common light end components of diesel fuel (Block et al., 1991). Polynuclear aromatic hydrocarbons (PAHs), containing two or more fused benzene rings, are on the U.S. EPA priority pollutant list, since some are known carcinogens and mutagens (Riser-Roberts, 1998). PAHs found in diesel No. 2 include: phenanthrene, fluoranthene, pyrene, and benz[a]anthracene (Millner et al., 1994). They are hydrophobic and most are practically insoluble in water, factors that contribute to their persistence in the environment. Microorganisms are found, however, that can degrade or partially transform PAHs, either individually or through interdependent communities (Riser-Roberts, 1998).

1.9. Methods for Measurements of Diesel Fuel Contamination

In most environmental assessments, the diversity and complexity of diesel fuel components require analytical techniques that measure the mixture as a whole. The general name for such an approach is Total Petroleum Hydrocarbon (TPH) analysis (Block et al., 1991). Petroleum hydrocarbons detected within specified ranges, as determined by the number of carbons present, are labeled as "TPH-gasoline" or "TPH-diesel" and are often described by a single numerical standard. Petroleum mixtures may undergo various physical, chemical, and biological processes that alter their composition, mobility, and toxicity. As a result, the actual weathered TPH concentration may differ from the reported TPH concentration because the measured concentration is based on non-weathered standards (Michelsen and Boyce, 1993).

1.9.1. Gravimetric Technique

The gravimetric technique is the simplest and least expensive method. It involves extracting the hydrocarbons with a solvent and determining the weight of the residue remaining after solvent evaporation. The disadvantage of this method is that many diesel components are co-removed with the solvent, which may result in low recoveries. The method is useful, however, for determining gross contamination (Block et al., 1991).

1.9.2. Infrared Technique

With the infrared technique the petroleum hydrocarbons are extracted and measured in a solvent. The sample extract infrared absorbance is determined at the maximum absorbance. This absorbance is a carbon-hydrogen bond stretching absorbance and is related to hydrocarbon concentration in the extract. Concentration is determined by direct comparison with a calibration curve prepared using a hydrocarbon standard. The method is sensitive but subject to interference from non-TPH contaminants in the sample. The carbon-hydrogen bond measured by this method occurs not only in petroleum hydrocarbons, but also in organic acids, esters, and alcohols. During bioremediation of contaminated soils, increasing

TPH concentrations measured by this method have been attributed to the generation of organic acid intermediates as the petroleum compounds degrade (Block et al., 1991).

1.9.3. Gas Chromatography (GC)

Capillary gas chromatography is an alternative to the gravimetric and infrared techniques and is well established in the petroleum industry and environmental community. The method involves separation and detection of various components of the petroleum hydrocarbon contaminant in a sample. The method of detection can utilize a simple conventional detector such as a flame ionization detector (FID), or when substantial qualitative information is necessary, it can be coupled with a mass spectrometer (Block et al, 1991).

Chromatography is an analytical technique that can be used to identify components of petroleum products. A GC trace can thus be used as a "fingerprint" analysis to determine the type of product present, the degree of weathering that has taken place, and in some cases, the specific origin of the hydrocarbons (Michelsen and Boyce, 1993). Unfortunately, chromatography does not resolve (and thus identify) a substantial proportion of some hydrocarbons in petroleum. The unresolved components are referred to as the unresolved complex mixture (UCM), or "hump", which is especially pronounced for biodegraded petroleum and certain refined fractions such as lubricating oils. Previously, the UCM was assumed to contain highly branched and/or cyclic hydrocarbons, which were considered to be responsible for its resistance to biodegradation. Studies that fractionated the UCM of several lubricating oils indicated that the UCM consists mainly of simple compounds such as normal and branched monocarboxylic acids, and minor quantities of cyclic hydrocarbon products (Gough and Rowland, 1990).

1.9.4. Immunoassay

There are current methods of screening soils to determine TPH and PAHs by immunoassay (EPA Methods 4030 and 4035 respectively). These methods can determine if these compounds are present in soil, and can provide estimated concentrations of TPH and

PAHs by comparison with standards. The sensitivity of the tests is influenced by the binding of the target analyte to the antibodies used in the kit. The TPH test is most sensitive to small aromatic compounds such as ethylbenzene, xylenes, and napthalene, which are found in fuels. The PAH test is most sensitive to the three-ringed compounds phenanthrene, anthracene, and fluorene, and the four-ringed compounds benzo(a)anthracene, chrysene, fluoranthene, and pyrene. Both methods are influenced by the nature of the hydrocarbon contamination and any degradation processes occurring at the site, and compounds chemically similar to petroleum hydrocarbons and PAHs may cause false positives. Information regarding the lower limits of detection for these compounds and cross reactivity with other compounds is provided by the manufacturer of the kits.

1.10. Degradation of Oil By Microorganisms

Microorganisms capable of utilizing hydrocarbons are widespread in nature, and are not confined to hydrocarbon containing soils. Organisms that do not normally utilize hydrocarbons may do so after a period of adaption. Many microorganisms will use a variety of hydrocarbons, just as some hydrocarbons can be used by many species. It is generally accepted that the normal alkanes are most readily degraded, and in this group, activity is highest for the alkanes from nonane to hexadecane (C_9-C_{16}). Alkanes of lower molecular weight may be toxic to microorganisms, probably because of their lipid-dissolving action. Isoalkanes are degraded less readily than the corresponding *n*-alkanes, the rate of degradation depending on the structure of the particular isomer. Cycloalkanes are degraded even less readily, although rapid growth on cyclic alkanes has been reported. Studies on individual hydrocarbons are not always a good indication of the microbial response to a mixture since there are several reports of co-oxidation.

Normal alkanes may be oxidized completely to carbon dioxide and water, but aldehydes, primary and secondary alcohols, mono- and di-carboxcylic acids, diols and methyl ketones may be formed. The attack on *n*-alkanes usually starts at a terminal methyl group and

proceeds along the chain. There is similar initial attack on isoalkanes, but oxidation frequently stops at a branching point, leading to incomplete oxidation. Cyclic hydrocarbons can give rise to a variety of compounds including ethyl cyclohexanol, catechols, cresols, and salicylic, toluic, benzoic, and phenylacetic acids (Blakebrough, 1977).

1.10.1. Uptake of Hydrocarbons

Before biodegradation can occur, the relatively low water-soluble hydrocarbons must enter the cytoplasm of microorganisms. There are two main theories on how this proceeds. The first involves uptake of hydrocarbon that has become solubilized in the aqueous phase. The second involves physical adhesion between the microorganism and oil droplets with the hydrocarbon being taken up directly without solubilization. The second theory is favored for liquids, however there is uncertainty about the size of the droplets involved. It appears that micro-micelles may play an important role. In some cases, the hydrocarbons may dissolve directly in the lipophilic regions of the membrane (Higgins and Gilbert, 1977).

1.10.2. Aerobic and Anaerobic Degradation of Hydrocarbons

Oxidation of contaminants can occur aerobically or anaerobically, and the fate of aliphatic and aromatic compounds depends primarily on the presence or absence of oxygen. Oxygen serves two distinct functions. It can act as the terminal acceptor of electrons that are released during the oxidation of electron donors, or it can react directly with the organic molecule. As an electron acceptor, oxygen can be replaced by other oxidized inorganic compounds, although the energy gains are smaller. Other compounds that can serve as alternative electron acceptors are nitrate, metal ions, sulfate, or carbon dioxide. As a direct reactant, no other compound can substitute for this function (Bouwer and Zehnder, 1993).

Anaerobic microbial transformations of organic compounds are known to occur in anoxic environments, but less cell growth results under anaerobic conditions. Through the inefficiency of anaerobiosis, the consumption rate of the compound can be many times higher than it is under aerobic conditions. Thus, the substrate may disappear faster (Riser-Roberts,

1998), however, most studies report anaerobic hydrocarbon degradation to be generally slower than under aerobic conditions (Head and Swannell, 1999).

1.10.3. Alkanes

For alkanes, the general strategy is to convert the alkane chain into a fatty acid. In the presence of oxygen, the alkane chain is attacked by a monooxygenase, which introduces one atom of oxygen from molecular oxygen into the molecule, forming a fatty alcohol (Bouwer and Zehnder, 1993). Of the complex monooxygenase enzyme systems, two enzyme systems have been described. The first involves cytochrome-P_{450}, a multicomponent enzyme extensively studied in *Corynebacterium*, *Acinetobacter*, and several yeasts. The second enzyme is a different, broad substrate specificity alkane monooxygenase, of the type that has been well studied in *Pseudomonas*. This monooxygenase is described as a three-component system involving the non-haem iron protein, rubredoxin, and the genetic information for biosynthesis encoded in a transmissible plasmid (Higgins and Gilbert, 1977).

The primary alcohol is then further oxidized via the aldehyde to the corresponding carboxylic acid by alcohol and aldehyde dehydrogenases which are mostly NAD-linked. The most common fate for the resulting fatty acid is thought to be activation to the fatty acyl-CoA followed by β-oxidation to acetyl-CoA. There is also some evidence that α-oxidation occurs during the degradation of *n*-alkanes by some microorganisms. This involves the formation of α-hydroxy fatty acids and their subsequent decarboxylation (Higgins and Gilbert, 1977).

It is obvious that the initial oxidation step with molecular oxygen is not possible under anoxic conditions, so it has been suggested that alkanes are anaerobically recalcitrant (Bouwer and Zehnder, 1993). More recent evidence, however, shows that alkanes can be degraded under anaerobic conditions, specifically under nitrate, or sulfate reducing conditions, but the pathways involved have yet to be elucidated (Head and Swannell, 1999; Coates et al., 1997; Bregnard et al., 1997).

Branched chain alkanes were thought to be resistant to microbial degradation, because the β-oxidation enzymes were unable to degrade the branched chain substrates (Higgins and Gilbert, 1977). More recent evidence shows that mixed as well as pure microbial cultures can utilize branched compounds, such as pristane, as the sole carbon source and energy source. Numerous metabolites of pristane have been identified, and were found to be further biodegraded through β- or ω-oxidation. Other branched isoprenoid alkanes, such as phytane, norpristane, and farnesane, can also be degraded aerobically (Bregnard et al, 1997). In addition to aerobic degradation, it has now been reported that pristane can be degraded under nitrate, and sulfate-reducing conditions, although again, degradation pathways remain unknown (Bregnard et al., 1997; Head and Swannell, 1999).

1.10.4. Alkenes

Unsaturated aliphatic compounds (alkenes and alkynes) are degraded aerobically by mechanisms similar to those by which alkanes are degraded. The double and triple bonds make these compounds more reactive and can undergo additional reactions, such as epoxidation and hydration. There is some evidence that the double bond of the alkenes can be hydrated anaerobically to form an alcohol, which is then further converted as in the aerobic pathway (Bouwer and Zehnder, 1993).

1.10.5. Aromatic Compounds

Aerobic biodegradation of many classes of aromatic compounds is common and proceeds through the key intermediate, a catechol. Prokaryotes introduce an oxygen molecule via the dioxygenase enzyme, which forms first a *cis*-diol. Then through non-enzymatic reactions, the catechol intermediate forms. The aromatic ring of the catechol is opened by another dioxygenase reaction by either an *ortho-* or *meta-* fission (Bouwer and Zehnder, 1993).

Anaerobically, the aromatic ring is not oxidized but reduced. The key intermediate in this pathway is a cyclohexanone, and ring opening is achieved through hydration of the intermediate (Bouwer and Zehnder, 1993). Numerous studies demonstrate anaerobic

degradation of monoaromatic hydrocarbons (i.e. benzene and toluene), and now recently, a number of PAHs, such as napthalene, phenanthrene, methylnaphthalene, fluorene, and fluoranthene, have been found to be degraded anaerobically in marine sediments under sulfate-reducing conditions (Coates et al., 1997; Head and Swannell, 1999). Under anoxic conditions, substituted aromatic compounds appear to be more easily degraded than nonsubstituted ones. Ease of degradation is suggested to depend on the functional group and the terminal electron acceptor present (Bouwer and Zehnder, 1993).

Chapter 2
MATERIALS AND METHODS

2.1. Design of the Field Experiment

The site design consisted of three square areas (10 ft. x 10 ft.) with a monitoring well centered in each area (Fig. 5a). The three experimental areas were located within the contaminated area identified by the latest survey of the site in 1998 (Fig. 5b). The wells and square areas were designated A, B, and C, with C being the most easterly and closest to the shoreline. Different treatments were assigned to the wells. Well A received air and nutrients, well B received only air and well C was designated as the no-treatment control. The monitoring wells were installed on May 14, 1999 by Valley Drilling Co., Honolulu, HI. The installation required a large drill rig which bore down to 13 feet. The wells were lined with screened poly-vinyl chloride (PVC), 13 feet in length and two 2 inches in diameter (wells B and C), or 4 inches in diameter (well A). Once the wells were installed they were capped with PVC covers 10 cm below the surface. Heavy iron covers were installed above the PVC well covers at the surface. The implementation of the air and nutrient treatments started in July 1999, after the construction of a shed to protect the air compressor (Craftsman, 5.5HP, 30 gallons) and the connection of the air compressor to the air line (400 ft. in length). Air was supplied to the wells through hoses attached to the air line fitted with diffusers eleven feet below the surface. Figure 5a shows the orientation of the wells in respect to the air line. The air was injected daily at low pressure (lowest setting) in wells A and B for 12 hours. Both wells A and B were fitted with pressure gauges, which measured 5 psi and 3 psi respectively throughout the experiment. The air compressor tank was drained of water condensate monthly.

Calculations of the amount of fertilizer to add to well A were based on soil analyses (Appendix 2A) performed by the University of Hawaii Agricultural Diagnostic Service Center. Soil collected from the Day 0 (June 23, '99) sampling was used to determine the amount of nitrogen and phosphorus that should be added to attain a ratio of C:N:P of

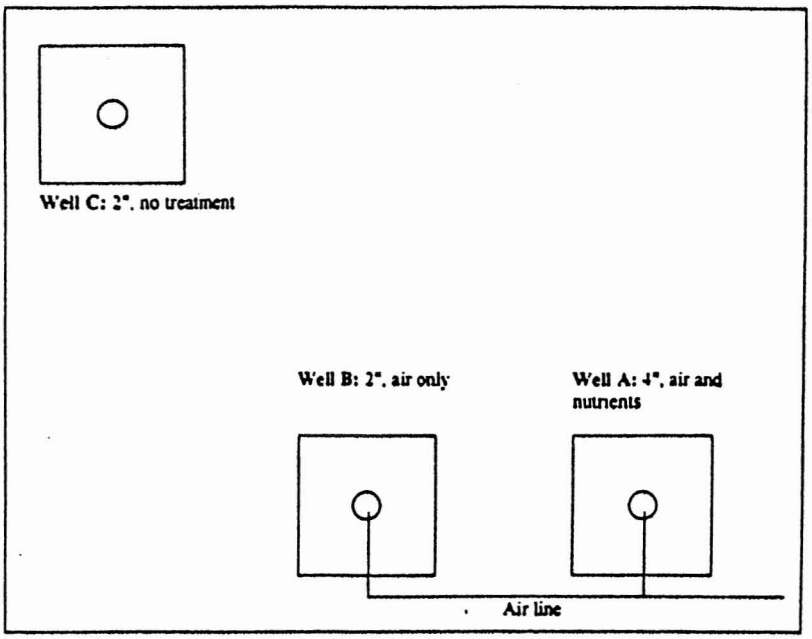


Fig. 5a. Relative position and diameter of each monitoring well. Treatments for each well are indicated. Each square size is 10 ft. x 10 ft.

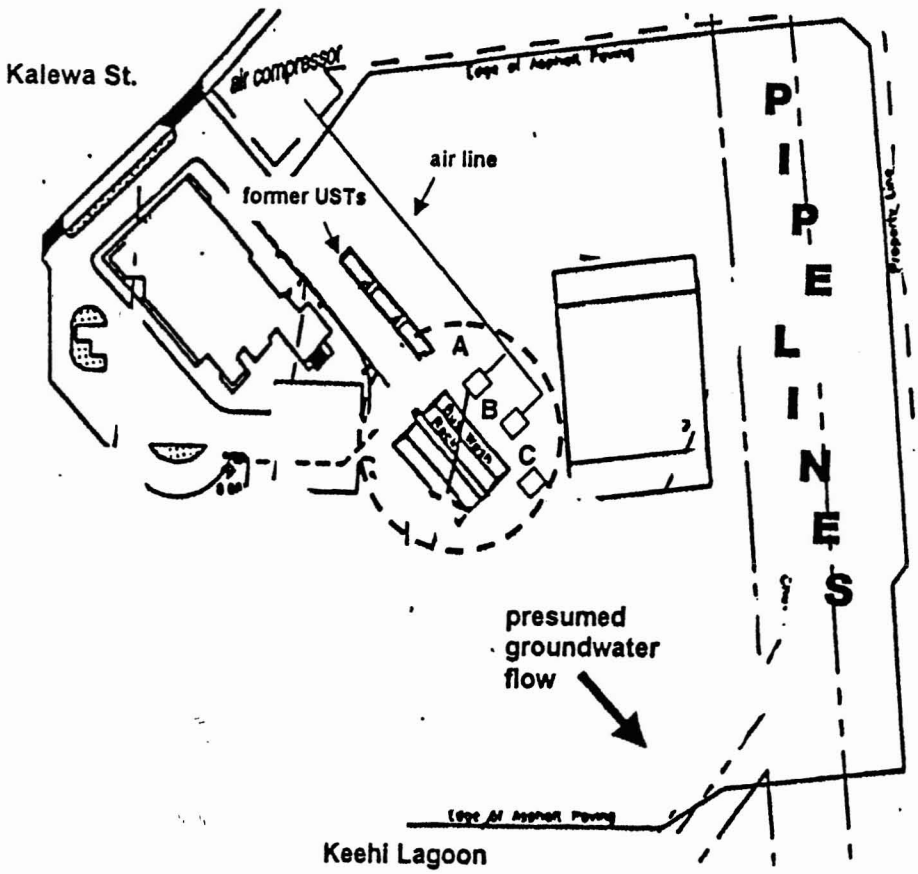


Fig. 5b. Location of the 3 experimental squares A, B, and C, on the latest map (1998) of the extent of the contaminated area (dotted-line circle) at the former Gray Line site.

100:4:1. This ratio (modified from Douglas et al., 1996) had been used in a preliminary lab experiment using sand from the site (Robert et al, 1998). The amount of total nitrogen available in the soil was based on the ammonium-N concentration in the analyses, since the nitrate-N measurements were zero. The analyses also indicated that phosphorus was already present in excess. Thus, only nitrogen needed to be supplied. Using the available nitrogen concentration measured in the soil analyses, the amount of nitrogen necessary to fulfill the C:N:P ratio of 100:4:1 was determined. Ammonium nitrate (NH_4NO_3) was used to provide two forms of available nitrogen. Also, the nitrate provided an alternative electron acceptor to the anaerobic microorganisms in anoxic pockets in the aquifer. The total amount of ammonium nitrate to be delivered in well A was calculated to be 1609g, which amounted to 31g of NH_4NO_3 per week over a period of one year. The NH_4NO_3 was supplied in four liters of solution of 0.5% NaCl, which was the salinity of the well water, as determined by refractometry. The goal of the nutrient additions was to maintain low levels of available nitrogen in the well, so as not to cause toxicity and to prevent significant losses due to movement of water out of area A by the tidal influences. In the beginning of the experiment the nutrients were added weekly, but this became less frequent when NH_4^+-N and NO_3^--N levels in the well water increased to concentrations greater than 10 mg/L.

2.2. Soil Sampling

Soil cores were collected four times throughout a one-year period. The first two samplings were accomplished by Geolab, Honolulu, HI., using a truck equipped with a Geoprobe auger. The equipment used in the first two samplings provided soil cores one inch in diameter. We attempted to collect the soil cores at two feet intervals starting at four feet below the surface (asphalt) and ending at ten feet. We were unable to collect complete core samples (4 to 10 feet) on several occasions due to mechanical difficulties in drilling for the deeper samples with the Geoprobe. For this reason a different company was contracted for the last two samplings. TEG Hawai'i, Honolulu, HI., provided a more powerful auger, the

Strataprobe. They were able to provide complete samples, as well as larger cores (two inches in diameter). Each sampling was designated by the day number. Day 0 (June 23, '99) was the first sampling prior to the start of any treatments, subsequent samplings were Day 117 (Oct. 18, '99), 299 (April 17, '00), and Day 390 (July 17, '00). Within the 10' x 10' area around each monitoring well (A, B, and C) the soil samples were collected. On each sampling day six randomly selected borings from each area were sampled. The cores were collected in poly-acetate sleeves, which were capped with Teflon sheets and kept under ice while transporting back to the lab. At the lab, the samples were separated in zones according to Fig. 6, removed from each core, and transferred into acetone-rinsed glass jars for mixing and characterization. The soil samples were stored at 4°C prior to hydrocarbon and microbial analyses, and the samples for nutrient analyses were stored frozen.

2.3. Monitoring-Well Water Sampling

Water from the monitoring wells was collected for two purposes: for microbial enumerations, and for measurements of dissolved oxygen (DO), temperature, pH, NH_4^+-N and NO_3^--N measurements. The water samples for the microbial enumerations were collected before the start of the treatments, and once between two soil samplings. Sampling for the other measurements occurred more often, approximately twice a month. Water samples were collected at high tide, at an approximate depth of six to seven feet below the ground surface. The water was collected with a bailer, then transferred to Whirlpak™ bags and kept on ice while transporting back to the lab. A portion of the water samples was frozen at -20°C for subsequent NH_4^+-N and NO_3^--N analysis.

2.3.1. Dissolved Oxygen (DO), Temperature, and pH Measurements

The water was collected with the bailer, then the temperature and dissolved oxygen measurements were taken with a YSI DO probe in the field, immediately after collection. The pH measurements were carried out in the lab. Samples were allowed to warm to room temperature then the pH was determined using a pH electrode (Orion).

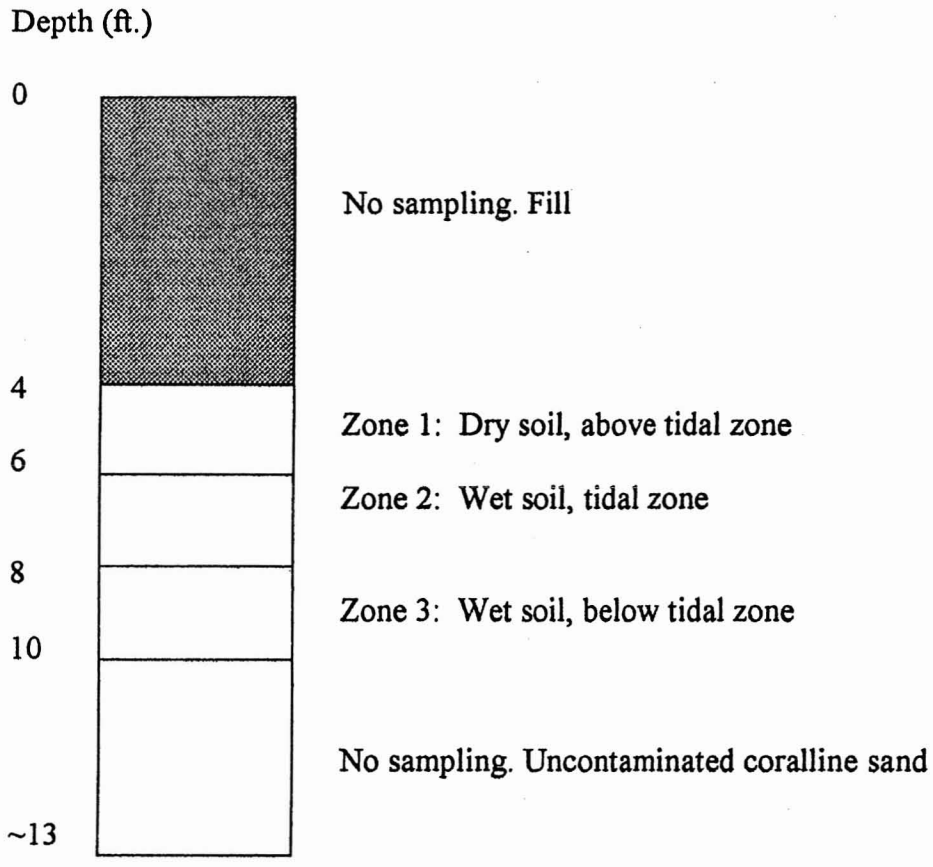


Fig. 6. Schematic diagram of a soil core and zone determination

2.3.2. Ammonium-N Measurements

Ammonium-N determinations were made using the NH_4^+-N Low Range 0-2.5 mg/L, Salicylate Method kit from the HACH Co., (Ames, IA). Standards of NH_4^+-N (NH_4Cl) were prepared in 0.5% NaCl solution for the calibration curve preparation. The water samples were thawed and filtered through 0.4 μm pore size nuclepore membranes to remove particulates, the pH of each sample was then adjusted to 7.0 using 0.1N HCl. Two ml of the sample was added to 3 ml of Amver™ diluent contained in a tube provided by the manufacturer and mixed. To each tube, one ammonia salicylate pillow and one ammonia cyanurate pillow was added using a small funnel and the tube was shaken for thirty seconds. The green color was allowed to develop for 20 minutes and the absorbance was measured in a spectrophotometer at 655 nm. The concentration was determined by reference to the standard curve.

2.3.3. Nitrate-N Measurements

Nitrate-N measurements were made using the NO_3^--N Medium Range (0-5 mg/L) Nitraver5™ kit from the HACH Co., (Ames, IA). Standards of NO_3^--N (KNO_3) were prepared in 0.5% NaCl solution for the calibration curve preparation. The water samples were thawed and filtered (as in section 2.3.2) to remove particulates, 10 ml was then transferred to straight tubes topped with rubber stoppers. One Nitraver5™ pillow was added to each sample and shaken for exactly one minute. The light brown color was allowed to develop for 5 minutes, and the absorbance was measured in a spectrophotometer at 400 nm. The concentration was determined by reference to the standard curve.

2.4. Enumeration of Soil and Water Microorganisms

For both soil and water samples microbial enumerations of total heterotrophic bacteria, phenanthrene-degrading bacteria, diesel-degrading microorganism, and pristane-degrading microorganisms were carried out. All plates were incubated at 26°C, which approximated the temperature measured in the well water.

2.4.1. Total Heterotrophic Bacteria

Total soil bacteria numbers were determined by total heterotrophic plate counts. The total viable heterotrophic bacteria were enumerated by spread plating on R2A agar plates (Standard Methods for the Examination of Water and Wastewater, 1986) containing 0.5% NaCl. The medium contained (per liter) 0.5 g of yeast extract, 0.5 g of proteose peptone No. 3, 0.5 g of casamino acids, 0.5 g of dextrose, 0.5 g of soluble starch, 0.3 g of sodium pyruvate, 0.3 g of dipotassium phosphate, 0.05 g of magnesium sulfate, and 15.0 g of agar. The pH of the medium was adjusted to 7.5 by adding 0.1N NaOH before autoclaving.

2.4.2. Phenanthrene-Degrading Bacteria

Phenanthrene-degrading bacteria were enumerated on mineral medium double-layer agar plates using a modification of the method of Bogardt and Hemmingsen (1992). The mineral medium consisted of (per liter) 50 ml standard salt solution (5 g K_2HPO_4, 2.5g $MgSO_4$, 2.5 g NaCl, 0.05 g $Fe_2(SO_4)_3$, 0.05 g $MnSO_4$ and 1000 ml water; pH adjusted to 7.0 - 7.2), 1 ml micronutrient solution (0.05 g K_2MoO_4, 0.05 g $NaBO_4$, 0.05 g $FeCl_3$, 0.05 g $Co(NO_3)_2$ 6 H_2O, 0.05 g $CdSO_4$, 0.05 g $CuSO_4$, 0.05 g $ZnSO_4$, 0.05 g $MnSO_4$, and 1000 ml distilled H_2O), 5 g ammonium nitrate, 0.14 g calcium chloride, 1 L filtered water, and 15 g Noble Agar. The medium was adjusted to contain 0.5% NaCl, and adjusted to pH 7.4 with 0.1N NaOH. After autoclaving 0.1 ml/L of sterile Hemmingsen's vitamin solution was added to the cooled medium. The vitamin solution consisted of (per 100 ml of distilled water) 2 mg biotin, 2 mg folic acid, 5 mg thiamine HCl, 5 mg D-calcium pantothenate, 5 mg vitamin B_{12}, 5 mg riboflavin, 20 mg niacin, 3 mg pyridoxal HCl, and 2 mg *para*-aminobenzoic acid. Phenanthrene was dissolved in acetone to yield a concentration of 11.3 mg/ml. For each plate, 0.2 ml of the phenanthrene solution was added to a tube of 6 ml of molten mineral medium (Noble agar 1.5%) kept in a water bath at 58°C, vortexed, and poured onto the bottom layer of mineral medium agar. The acetone was allowed to volatilize at 30°C for 24 hours, then 0.1 ml of a soil suspension was spread on the surface of the plates, and incubated for two weeks.

Colonies of phenanthrene-degrading bacteria were distinguished by the presence of clear halo in the turbid phenanthrene layer.

2.4.3. Diesel and Pristane Degraders

Most-Probable Numbers (MPN) of microorganisms capable of growing on #2 diesel fuel (Unocal *76*) and pristane (Aldrich, Milwaukee, WI.)were determined using 24-well tissue culture plates (Falcon). Each well received 2 ml of Bushnell-Haas (B-H) mineral medium supplemented with micronutrients, as in section 2.4.2, and 0.5% NaCl. The B-H medium consists of (per liter) 0.2 g magnesium sulfate, 0.02 g calcium chloride, 1 g monopotassium phosphate, 1 g dipotassium phosphate, 1 g ammonium nitrate, and 0.05 g ferric chloride (Difco Manual). The micronutrient solution was autoclaved separately and was added after autoclaving the B-H medium. The pH was adjusted to pH 7.5 with a predetermined amount of sterile 0.1 N NaOH. Five wells per dilution and 10-fold soil dilutions were used. Each well was inoculated with 0.5 ml of a soil dilution, and a 5 µl drop of sterile hydrocarbon (diesel or pristane) was added to the surface of the liquid in each well using a sterile 23-gauge hypodermic needle and syringe. Uninoculated controls and controls devoid of hydrocarbons were also prepared. Positive wells, based on visible microbial growth or turbidity, were scored after one-month incubation and the MPN were calculated using the MPN tables of Alexander (1982).

2.4.4. Sulfate-Reducing Bacteria (SRB)

MPN enumerations of SRB were performed on composite samples collected from each area (A, B, and C), and each zone (2 and 3). One ml of each soil dilution from cores of each area and zone were pooled in a sterile tube (i.e., six samples from area A, zone 2 at 10^{-2} dilution). These composite samples were used to inoculate tubes containing 5 ml of Bacto-Sulfate API Broth supplemented with 0.2% Agar + 0.5% NaCl. The pH was adjusted to 7.5. Five tubes for each dilution were inoculated with 0.5 ml of the diluted soil samples. The Bacto-Sulfate API Broth medium contained (per liter) 1 g yeast extract, 0.1 g ascorbic acid, 5.2 g sodium lactate, 0.2 g magnesium sulfate , 0.01 g dipotassium phosphate, 0.1 g ferrous

ammonium sulfate, 5 g sodium chloride (salinity at the site), 1 L distilled water, and 0.2% DIFCO agar. The tubes were incubated in an anaerobic chamber for three weeks. Tubes that contained a black precipitate were scored positive. The precipitate is ferrous sulfide (Fe_2S), which is indicative of sulfate reduction to hydrogen sulfide. The iron supplied in the media combines with sulfide to form the ferrous sulfide precipitate. This method does not enumerate all of the SRB and is limited to those that can utilize lactate as a carbon source.

2.5. Measurement of Hydrocarbon Content in Soil

2.5.1. Determination of Soil Oven-Dry (OD) Weight

For increased recoveries of hydrocarbons extracted from the wet samples, the samples were air-dried, prior to extraction, for approximately twelve hours until the sample was free-flowing. In order to normalize the hydrocarbon concentrations of the soil, the soil oven-dry weight of the air-dried soil was determined as follows. Approximately ten grams of air-dried soil from each sample was placed into previously weighed aluminum cups. The weight of the cups filled with soil were measured and the cups were placed into a 105°C oven for at least twenty-four hours. The cups were then removed from the oven and allowed to cool in a desiccator. After cooling, the weight of the oven-dried soil was determined. An amount of air-dried soil equivalent to the desired amount of oven-dried soil was used for hydrocarbon extraction and quantification.

2.5.2. Total Petroleum Hydrocarbons (TPH) Determination

TPH were determined by gravimetry and gas chromatography (GC). Gravimetry (Sorkhoh, 1995) utilized an amount of air-dried soil, equivalent to twenty grams of oven-dried soil, mixed with and equal amount of anhydrous Na_2SO_4 in a separatory funnel. Glass wool was placed in the outlet of the funnel to retain particulate matter. Forty ml of hexane was added to cover the soil and was left in contact with the soil for 30 minutes. The hexane extract was eluted and 10 ml of hexane was added and directly eluted. Between 20-30 ml of the extract was evaporated in an aluminum cup. The weight of the residue was determined after

hexane volatilization (2 hours) and the concentration of TPH in soil was computed. The detection limit was 0.5 mg. The TPH concentrations measured by gravimetry were used to estimate dilution factors for GC quantification and surrogate spiking levels.

The GC-FID (gas chromatograph equipped with a flame ionization detector) analysis used the modified EPA methods 3550 for sonication extraction and 8015B for nonhalogenated organics analysis using GC-FID. The extraction method used an amount of air-dried soil equivalent to ten grams of oven-dried soil. The soil was placed in a forty ml Teflon-capped Quorpak™ vial and 1 ml of the surrogate compound docosane (C_{22}) in methylene chloride was added. Twenty ml of methylene chloride extractant was added. The soil suspension was vortexed briefly, then placed in a bath sonicator (50/60 Hz, 117 Volts, Branson-Smithkline Co.) for 30 minutes. The samples were vortexed again which allowed the soil to settle so a clear supernatant fluid was obtained. The supernatant was pipetted off into a two-funnel series containing anhydrous Na_2SO_4. After passing the extract, the Na_2SO_4 was rinsed with methylene chloride to remove any residual extract. Twenty ml of methylene chloride was added to the soil and the sample was re-extracted following the same procedure. The extracts were pooled in an erlenmeyer flask and filtered using a 0.45 mm nylon Acrodisc® (Gelman Sciences, MI) into another forty ml Quorpak™ vial.

After extraction the samples were concentrated in a Rotovap evaporator to ten ml volumes. The purpose of the evaporation step was to increase the detectability of samples with low concentrations of diesel fuel. Not all of the samples contained low concentrations, but evaporating the samples also makes calculations using the final 10 ml volume easier. Dilutions were performed for some samples when needed. The extracts were then passed on a J&W Scientific DB-5 (30 m x 0.32 mm I.D., 0.25 mm) capillary column (Folsom, CA.) connected to a Hewlett-Packard 5890A GC-FID with the following conditions: splitless injection for the first 2 minutes, initial temperature 40°C, hold for 1 minute, program 12°C/minute, final temperature 320°C, hold for 15 minutes, detector temperature 325°C, injector temperature 295°C. Docosane, pristane, and phytane peaks were identified based on

retention times that matched with alkane and isoprenoid standards (Restek Corp., Bellefonte, PA.). Normally, the quantification of compounds by GC uses peak area integration provided by the Hewlett-Packard 3396A Integrator. This area integration, however, does not include the region termed the unresolved complex mixture (UCM), or "hump", observed in petroleum hydrocarbon fingerprints. To include this region, the baseline was extended by hand and the entire chromatogram from a retention time of five minutes to nineteen minutes was cut out. This retention time range corresponded to the retention time range of C_{10}-C_{21}, which was the range of hydrocarbons in the weathered diesel fuel product at the site. The chromatogram cutting was weighed on an analytical balance. Diesel standard (Unocal *76*) chromatograms were cut and measured with the same method, and used to generate a standard curve (40 mg/L to 125 mg/L). TPH was quantified based on the curve. The surrogate compound, docosane, was quantified using the area integration. This compound was not present in the sample naturally, and its peak eluted beyond the "hump". Because of this the docosane peak reached the baseline to give accurate area measurements. Docosane standards (range) were passed on the GC to generate the standard curve (2 mg/L to 10 mg/L) for quantification. Recovery percentages were determined based on the concentration measured divided by the initial concentration added (expected concentration). The isoprenoid compounds, pristane and phytane, were measured by peak height. Peak height was used because these compounds eluted in the middle of the "hump", so for ease in measurement the heights were used instead of area or weight. The top of each peak was measured to the extended baseline. Standard solutions (Restek Corp., Bellefonte, PA.) containing both compounds were passed on the GC-FID to generate the standard curve (0.25 mg/L to 3 mg/L) for quantification.

2.5.3. Polycyclic Aromatic Hydrocarbons (PAH)

PAH concentrations were determined by the RaPID Assay colorimetric immunodetection kit (Strategic Diagnostics, Inc., Newtown, PA). Extraction of the PAH from air-dry soil samples was done in methanol. The extract was diluted and reacted with a monoclonal antibody raised to phenanthrene conjugated to a chromogenic compound. The

antibody has some crossreactivity with other PAH: fluoranthene, benzo[a]pyrene, pyrene, chrysene, anthracene, indeno(1,2,3-c,d)pyrene, benzo[a]anthracene, fluorene, benzo[b]fluroanthene, and benzo[k]fluoranthene. Extraction and quantification by colorimetry were performed according to the manufacturer's instructions. The limit of detection in soil is 70 ng/g.

2.6. Quality Controls (QC) for Hydrocarbon Extraction and GC Determination Procedure

Various samples served as quality control measurements for the hydrocarbon extraction and GC determination methods. The QC samples and functional descriptions are listed in Table 1. Duplicates of each QC sample were spiked with various Unocal *76* diesel standards in methylene chloride, extracted, and quantified as mentioned above in section 2.5.2.

Table 1. Quality control for the TPH determination by GC

QC sample name	Description	Function
Unocal *76* diesel fuel in methylene chloride	10 ml of methylene chloride spiked with 5000 µg/ml of diesel	recoverable diesel fuel without matrix effects
Silica sand + Unocal *76* diesel fuel	10 g of silica sand spiked with 5000 µg/ml of diesel	recoverable diesel fuel with a non-sorbing matrix
Site coralline sand + Unocal *76* diesel fuel + water	10 g of uncontaminated sand collected from the site, spiked with 6000 µg/ml of diesel, and wetted to simulate saturated conditions in the subsurface	recoverable diesel fuel in a similar matrix, under moisture conditions similar to those in the subsurface of the site
Unocal *76* diesel fuel check standards	diesel standards in methylene chloride 100 µg/ml	passed on the GC every 12th sample to monitor drift

2.7. TPH Recovery Experiment Using Uncontaminated Site Soil

2.7.1. Soil Preparation

Soil samples from previous samplings at the site, determined to be uncontaminated by GC-FID, were pooled and mixed thoroughly. This soil mixture was air-dried, with periodic mixing for approximately twelve hours. The oven-dried weight of the air dried soil was then

determined following the same procedure as in section 2.5.1. The equivalent of 10 g of oven-dry soil was placed into teflon-lined Quorpak™ vials for spiking with Unocal *76* diesel fuel.

2.7.2. Quantification of TPH in Site Soil Spiked with Unocal *76* Diesel Fuel

The soil in the Quorpak™ vials was spiked with five different concentrations of diesel standards prepared with Unocal *76* diesel fuel in methylene chloride. Duplicate samples were spiked with one ml of the diesel standard solutions at concentrations of 50 µg/ml, 500 µg/ml, 1000 µg/ml, 3000 µg/ml, and 5000 µg/ml. A total of ten samples were spiked and mixed thoroughly. These samples were extracted and the TPH were quantified following the method described in section 2.5.2. Recovery percentages were calculated based on the measured concentration divided by the concentration of the initial spike (expected concentration).

2.8. Characterization and Identification of Hydrocarbon-Degrading Bacterial Isolates From the Site

2.8.1. Characterization and Growth of Indigenous Bacteria on Various Hydrocarbons

Presumptive hydrocarbon-degrading bacteria were isolated from various media used for enumeration. Colonies growing on R2A medium (total heterotrophic bacteria), bacteria isolated from positive diesel fuel and pristane MPN wells, and phenanthrene degrading bacterial colonies were chosen for growth tests on various hydrocarbons. Each isolate was characterized based on culture characteristics and colony morphology on half-strength BUGM medium + 0.5% NaCl (Biolog Univeral Growth Medium, Biolog, Hayward, CA.), and gram reaction. Broth cultures of each isolate were prepared in the half-strength BUGM + 0.5% NaCl and incubated at 26°C for 24 to 48 hours. After incubation the broth cultures were transferred to sterile centrifuge tubes and centrifuged at 5,000 rpm for 10 minutes. The cells were washed by discarding the liquid supernatant and resuspending the pellet in 20 ml of 0.5% sterile saline solution. The cell resuspesion was re-centrifuged at the same settings mentioned above, the washing step was repeated to obtain a medium-free suspension of cells. After the second wash the cells were resuspended in 2.5 ml of 0.5% sterile saline. This dense suspension of cells was used to test for the degradation of phenanthrene and pyrene by spot

plating. For these, 20 µl of the suspension was pipetted, using a sterile Eppendorf pipette, onto double layer mineral media agar plates containing phenanthrene and pyrene separately. Degradation of the phenanthrene and pyrene was determined by the presence of a halo around the spot of inoculation in the turbid top layer.

The isolates were also tested for other hydrocarbon degradation including Unocal *76* diesel fuel, hexadecane (C_{16}), pristane, and mineral oil (white oil #9, Hawaii Chemical Co., Honolulu, HI). To the dense suspension of cells mentioned above, 5-15 ml of sterile 0.5% saline solution was added to obtain a cell suspension of approximately 10^7 cells/ml. Using this diluted cell suspension 0.5 ml was used to inoculate duplicate tubes containing 5 ml of Bushnell-Haas (B-H) medium + 0.5% NaCl. To these tubes 5 µl of each sterile hydrocarbon was added to the surface using a sterile syringe fitted with a 21-gauge needle. A set of controls was also prepared for each batch of isolates tested. These included inoculated B-H medium without adding the hydrocarbons, and uninoculated B-H medium with the added hydrocarbons. These were to test whether the cells could grow (on dead cells) in the medium without a carbon source, and to test whether the hydrocarbons were sterile. The tubes were incubated at 26°C and growth on the hydrocarbons was visually determined and recorded periodically for a one-month period.

2.8.2. Identification of Hydrocarbon-Degrading Bacteria by the Biolog System

The Automated Microbial Identification System produced by Biolog Inc.(Hayward, CA.), is based on a colorimetric assay to detect the utilization of 95 substrates by a microorganism.

Isolates from the soil and well water samples were tested for degradation activity on various hydrocarbon sources. The isolates that were positive for degradation of any hydrocarbons were identified using the Biolog(system. This system bases identification on metabolic profiles of 95 different carbon sources supplied in a 96-well microtiter plate. When the organism utilizes carbon substrate, a dye-compound is reduced and the well appears purple. The similarity percentage is determined by comparison between the pattern of

positive wells in the sample and the metabolic profiles of known bacteria in the Biolog database. This database, however, does not contain the metabolic profiles of all bacteria.

Pure cultures of Gram-positive and Gram-negative isolates were grown in one-half strength BUGM broth or grown on one-half strength BUGM plates for 24 hours. A suspension of cells in sterile 0.5% saline was prepared by centrifuging and washing the broth cultures as described above, or by rolling a sterile cotton swab over the plate culture. The cell suspension was adjusted to the turbidity suggested by Biolog Inc. Each well of the 96-well Biolog plate was inoculated with 150 µl of the cell suspension. After 24 and 48 hours of incubation the plates were automatically scored positive or negative by colorimetry in a Biolog microplate reader. Some isolates were read after longer periods (i.e., 96 hours) because of their slow-growing nature. The results were then automatically compared to those in the Biolog database of substrate utilization patterns of known bacteria using Biolog version 3.50.

2.8.3. Phylogenetic Affiliations of Hydrocarbon-Degrading Bacteria

In addition to identification by the Biolog system, the hydrocarbon degrading isolates were identified by 16S ribosomal DNA (rDNA) homologies. An alternative to the Biolog method of identification is the molecular approach of using rRNA or rDNA to determine genetic relatedness. One method of identifying rRNA genes (rDNA) is to extract DNA from environmental samples or pure cultures using PCR amplification, cloning, and sequencing techniques (Dojka et al., 1998).

Genomic DNA was extracted from each pure culture using the G NOME DNA kit (Bio 101, Vista, CA). Total genomic DNA (100-200 ng μl^{-1}) was added to a polymerase chain reaction (PCR) mixture in a final volume of 50 µl per reaction, with 0.5 µl *Pfu* DNA polymerase reaction buffer (Stratagene, La Jolla, CA.), 0.15 mM of each of the deoxynucleoside triphosphates (dNTP), 0.4 µM of 16S rRNA bacterial specific primers 27F, 519F, and 1492R (Suzuki and Giovannoni, 1996), and 2.5 units of *Pfu* DNA polymerase (Stragene, La Jolla, CA.). DNA was amplified in a thermal cycler (Perkin Elmer Gene Amp System 2400) under the following conditions: initial DNA denaturation at 94°C (hot start)

for 3 minutes followed by 30 cycles of 94°C for 45 seconds, 55°C for 1 minute, and 72°C for 90 seconds. The final extension step was carried out at 72°C for 7 minutes, followed by cooling to 4°C (Hugenholtz et al., 1998). The PCR product was visualized on a 1% agarose gel (Agarose LE, Promega, Madison, WI.) containing 0.5 mg ml^{-1} ethidium bromide and a 1000 base pair DNA marker (Lambda, Promega, Madison, WI.) to confirm amplification of the 16S rDNA fragments.

The PCR amplicon was purified using the Qiaquick PCR purification system (Qiagen, Valencia, CA) and amplified in a Beckman CEQ™ dye terminator sequencing PCR (Beckman Instruments, Inc., Fullerton, CA) with the primers described above. DNA amplified in the sequencing PCR was precipitated with sodium acetate, EDTA (Sigma, St. Louis, MO.) and glycogen (Beckman), and hydrated in an ethanol/water series. The pellet was dried in a rotary vacuum dryer, resuspended in 40 µl sterile deionized formamide, and stored frozen (-20°C) until analyzed in a Beckman CEQ™ 2000 DNA Analysis System according to the manufacturer's instructions. The DNA sequencer was calibrated with DNA standards supplied by the manufacturer, and sequence quality was checked manually with CEQ sequencing software. The phylogenetic affiliation of pure strains was determined through comparisons of their 16S rRNA base sequences with those in BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997).

Chapter 3
RESULTS

3.1. Contaminant Fingerprint

Gas chromatography (GC) analysis of the hydrocarbons in the soil samples indicated that the diesel fuel contaminant was highly weathered. The GC profile did not have the same shape or peak pattern as the fresh diesel standard (Figure 7 and 8). It was determined by GC-Mass Spectrometry (MS) that the two most prominent peaks were the branched alkanes pristane and phytane. There were no peaks that matched the retention times of the diesel range (C_{10}-C_{25}) *n*-alkane standard (Restek, Bellefonte, PA.), so it was evident that the contaminant had undergone significant weathering.

3.2. Monitoring-Well Water Analyses

Well water analyses included visual characterization and measurements of pH, dissolved oxygen, temperature, NH_4^+-N and NO_3^--N. As the water was collected, observations were made of its color, turbidity, odor, and the presence of free product.

3.2.1. Well Water pH and Temperature

At the start of the well water observations (May 28, '99), all wells had a pH range between 7.5 and 8.0. These values fluctuated during the study (Fig. 9). From approximately day 170 (Dec., '99), the pH in wells A and B was consistently higher than in well C and the Ocean well. The pH in well C and the Ocean well fluctuated between pH 7.5 and 7.8.

Throughout the study, well water temperatures ranged from 27.5°C-32°C (Fig. 10). Temperature fluctuations appeared to follow a seasonal pattern, being higher during the summer (29°C-32°C), and cooler during the winter (28°C-30°C). The amplitude of the temperature fluctuation was the greatest range in the Ocean well (28°C-32°C). In general there was a temperature gradient between the Ocean well and well A, which was the farthest from the ocean (Ocean well > well C > well B > well A).

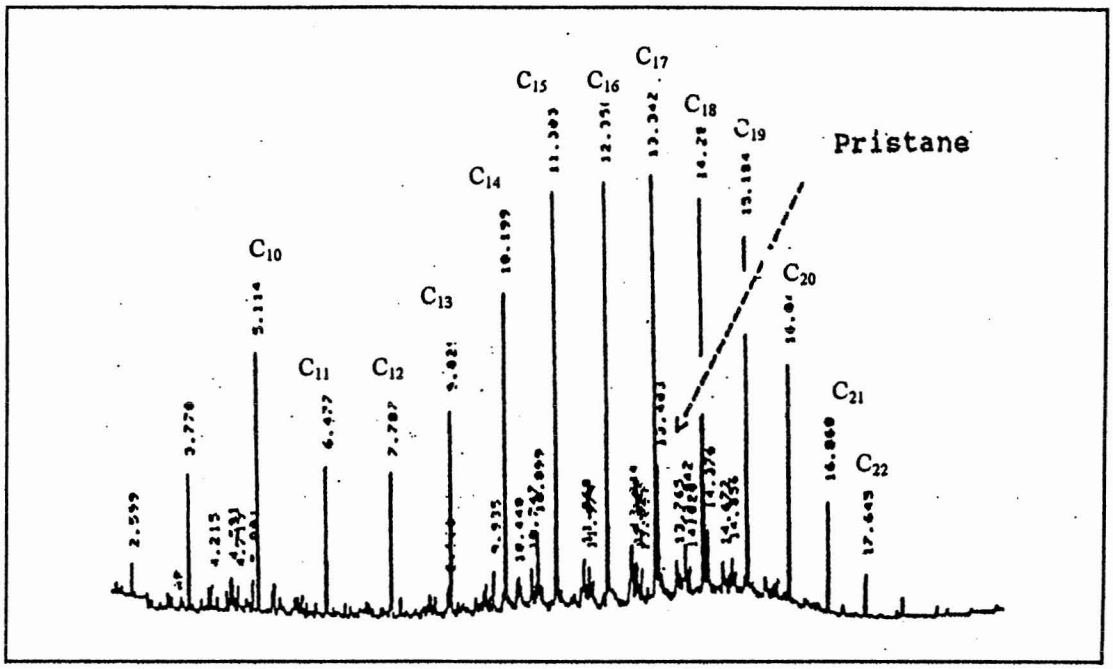


Fig.7. Gas chromatogram fingerprint of a Unocal *76* diesel fuel standard

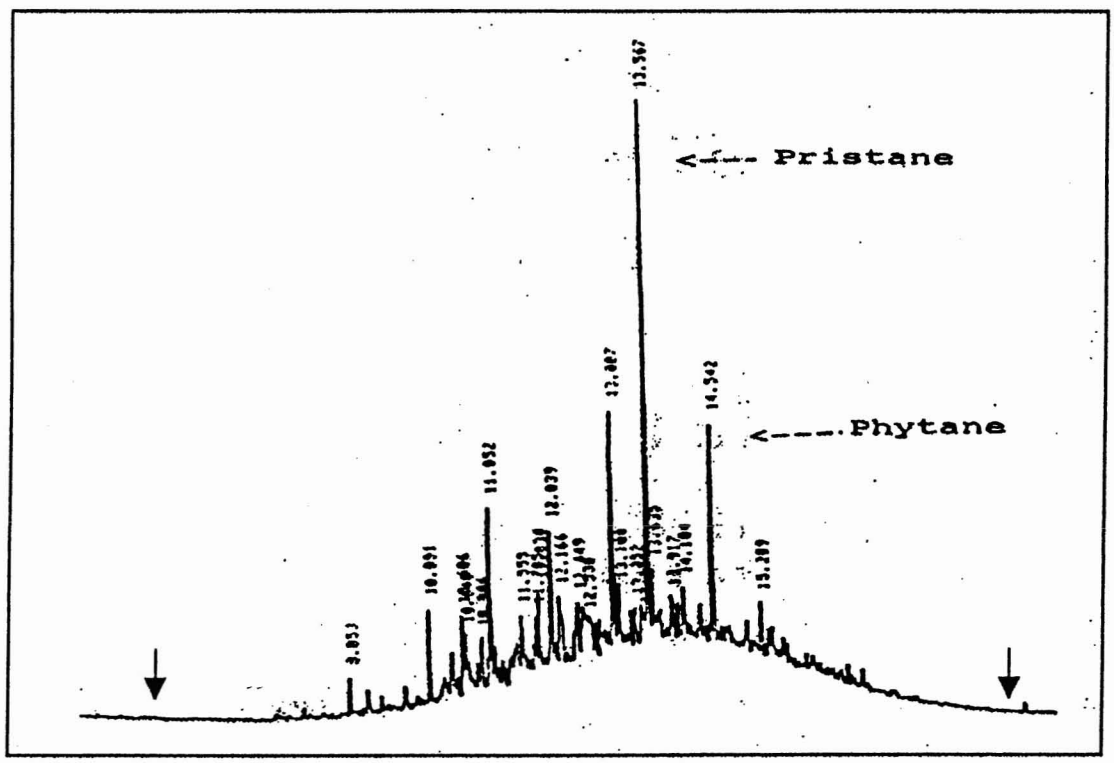


Fig.8. Gas chromatogram fingerprint of weathered diesel fuel in a soil sample from the site

(↓ arrows indicate the integration points for quantitation)

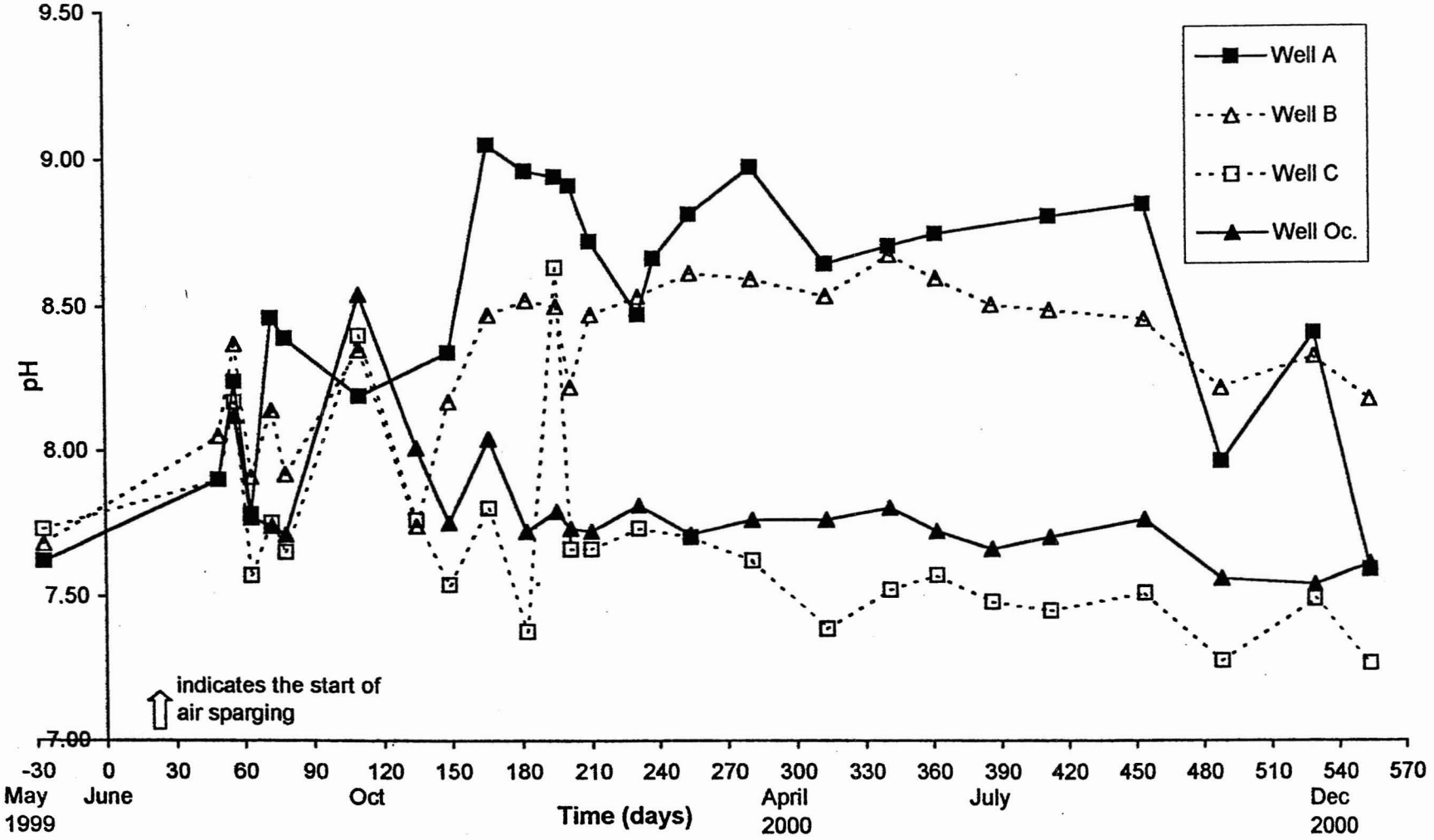


Fig. 9. Monitoring well-water pH fluctuations during the course of the experiment (5/28/99 to 12/28/00)

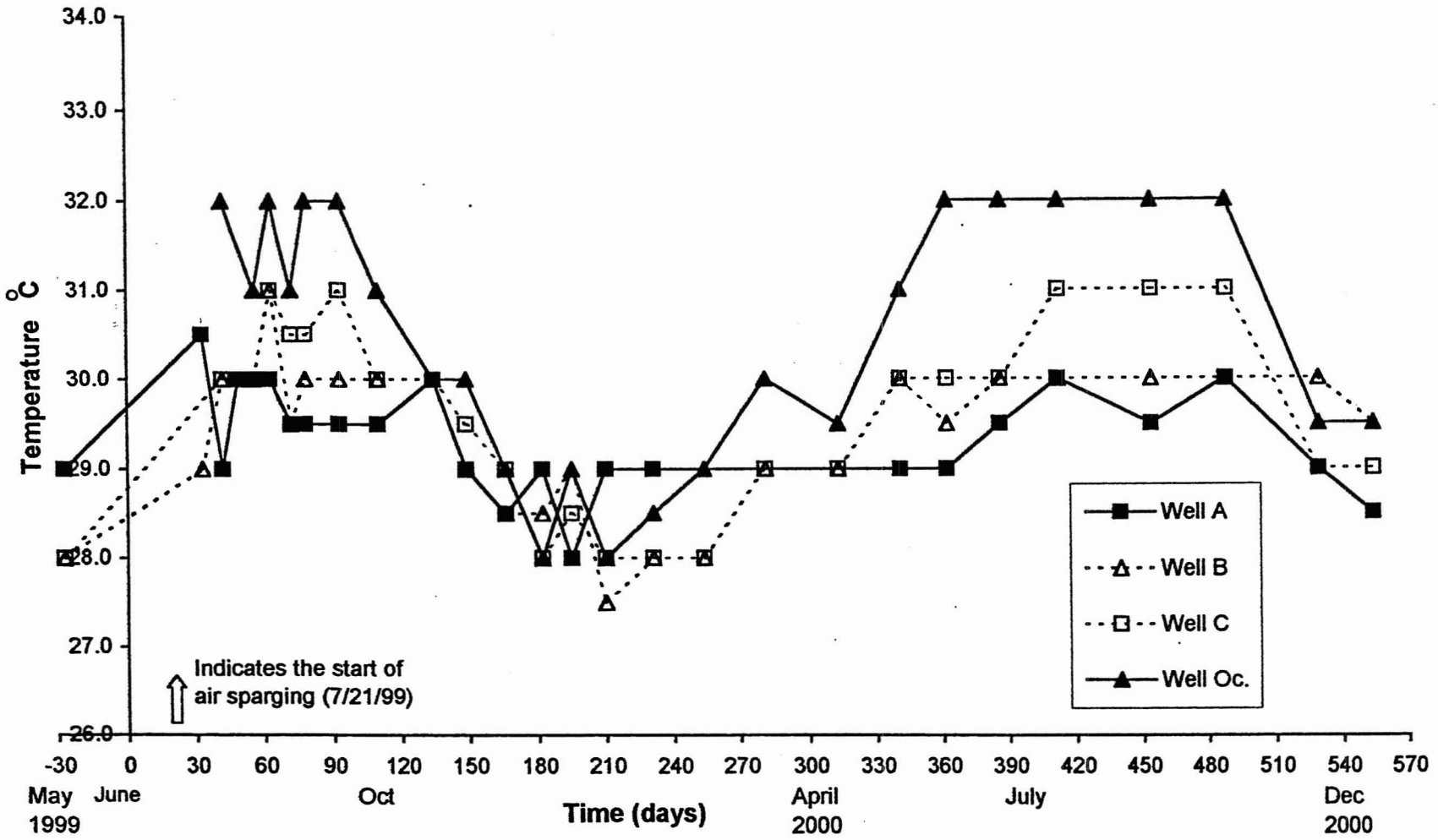


Fig. 10. Monitoring well-water temperature fluctuations during the course of the experiment (5/28/99 to 12/28/00)

3.2.2. Observations of Free Product

In terms of appearance, water collected from each well did not change noticeably throughout the experiment, except for that in well A. Water from well B and the Ocean well was consistently clear, odorless, and devoid of free product. Water from well C was always turbid, with a milky-gray appearance; there was also a faint odor of hydrogen sulfide. The appearance of water from well A changed dramatically during the study (Fig. 11). When first collected (May 28, '99), water from well A contained a small amount of free product, and smelled of diesel fuel. During subsequent samplings, free product was either present or absent. When present, the free product was accompanied by a stringy, brown material that appeared to be degraded diesel. The latter material was less oily and viscous, with a faint odor of diesel fuel. Until day 150 (Dec. '99), when contaminated, well A contained small amounts of free product and/or the stringy, degraded product. After day 150, however, the free product was much more prominent. On day 210 (February '00), there was a dramatic increase in the amount of oily free product in the well water; the bailer used for collection was virtually full of free product. This product was dark brown with a strong odor of diesel fuel. No stringy, degraded product was present. The four samplings after day 210 revealed the same high contamination by heavy free product in the well water. The amount of free product gradually decreased after day 281 (end of March '00) and by day 341 (May '00) none was seen in the well water. From day 341 the well water sample was slightly turbid, yellowish in color, with brown, stringy degraded product, and a faint odor of diesel fuel, or was devoid of the contaminant. During later samplings, on days 529 and 554 (both in Dec. '00) greater amounts of stringy, degraded product of a darker appearance were present. This material was oilier, and had a slightly stronger diesel fuel odor. On the most recent sampling, day 597 (Feb. '01), a large amount of the dark free product was observed in well A, the bailer used for water collection was full of this free product.

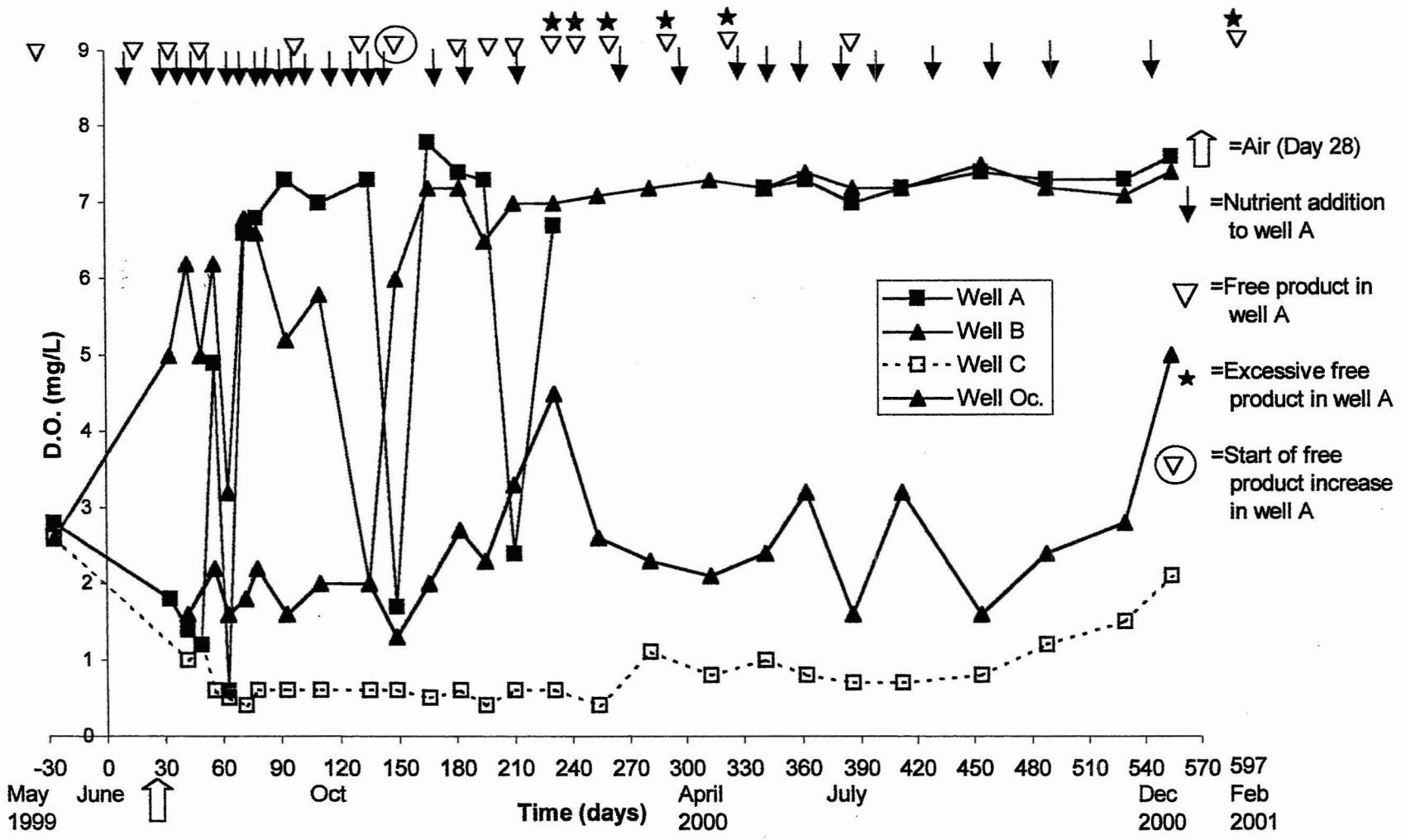


Fig. 11. Fluctuations of free product and dissolved oxygen in monitoring well-water during the course of the experiment (5/28/99 to 12/28/00)

3.2.3. Dissolved Oxygen (DO) Measurements

The first sampling (May 28, '99) indicated that the concentration of dissolved oxygen was low (near 2.8 mg/L) and similar in wells A, B, and C (Fig. 11). After the start of aeration of wells A and B (July 21, '99) the DO levels in both wells approached saturation (7.4 mg/L) at the salinity of the well water (0.5%). The DO concentration in well A did not increase immediately; however, approximately one month after the start of the aeration, it was at levels near saturation. The DO concentrations in wells A and B were maintained near saturation throughout the experiment, except where technical problems were encountered with the air compressor, or when increased contamination was observed in well A. Decreases due to technical problems are evident in Figure 11, as the first large decrease in wells A and B at day 60 (Aug. '99), and the second large decrease in well B near day 135 (Jan. '00). The other large decreases in DO levels in well A occurred when this well contained more free product, indicated as the second and third large decreases around days 150 (Nov. '99) and 210 (Jan. '00). No determinations of DO in well A could be made between days 250 (Feb. '00) and 350 (June '00) due to the presence of free product in the water. DO concentrations in well A were back to near saturation when no free product was observed in this well, around day 360 (June '00). This DO concentration was maintained until the last observation at day 554 (Dec '00).

In non-contaminated well C low levels of DO (<2 mg/L) were observed throughout the experiment. These low levels indicated near anoxic conditions in this well. In the Ocean well DO concentrations fluctuated from 1 to 5 mg/L, but for the most part were below 3 mg/L.

3.2.4. Nitrate-N and Ammonium-N Measurements

Prior to the start of the nutrient additions to well A (June '99) the concentrations of nitrate-N (NO_3^--N) and ammonium-N (NH_4^+-N) in the water of monitoring wells A, B, and C were low (approximately 2 mg/L) (Fig. 12, 13). Because of these low inherent nitrogen concentrations and the uncertainty of the diluting effect by the tidal action, the nutrient solution was supplied frequently (weekly) with the intention of maintaining low levels of

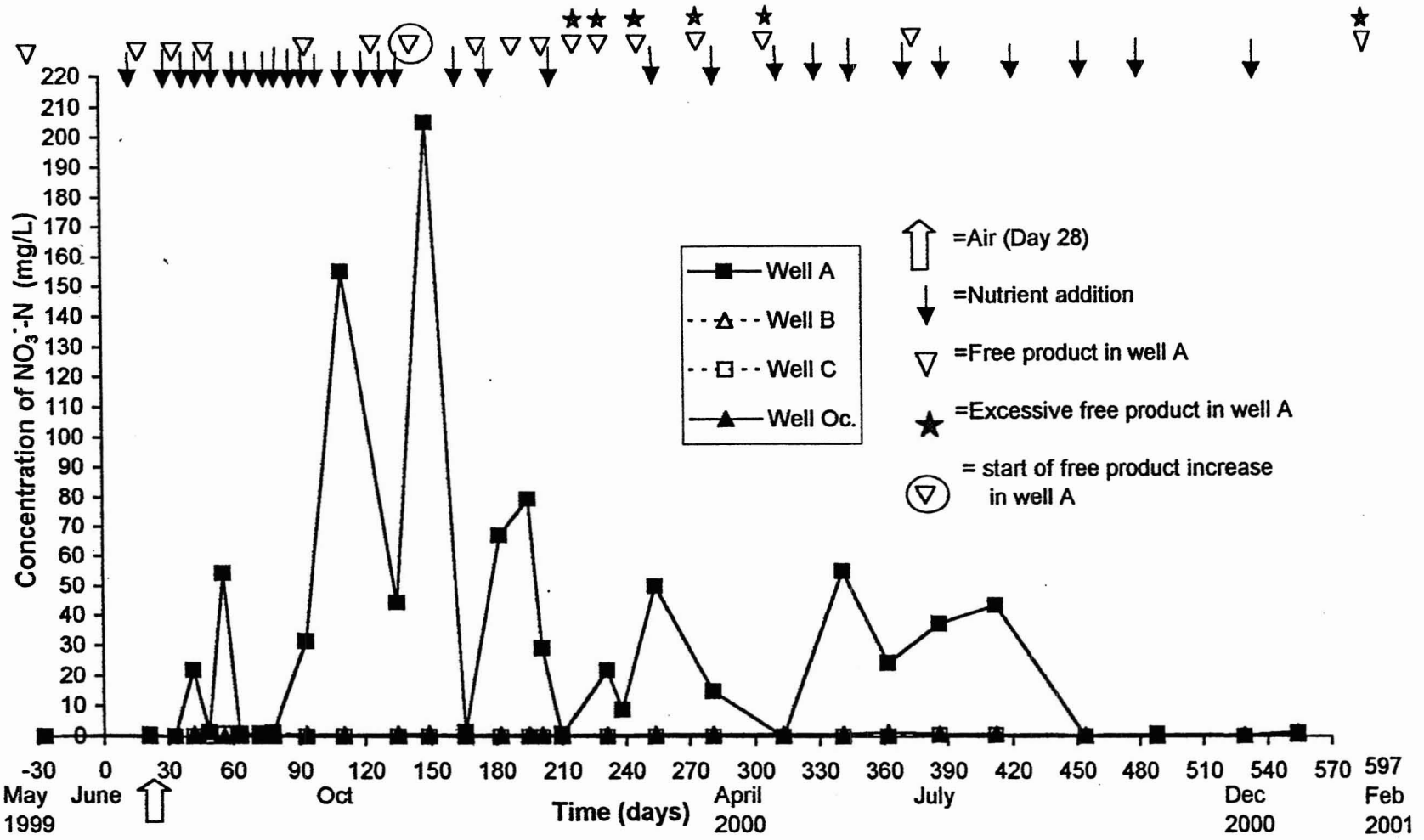


Fig. 12. Fluctuations of nitrate-N concentrations in monitoring-well water during the course of the experiment (5/28/99 to 12/28/00)

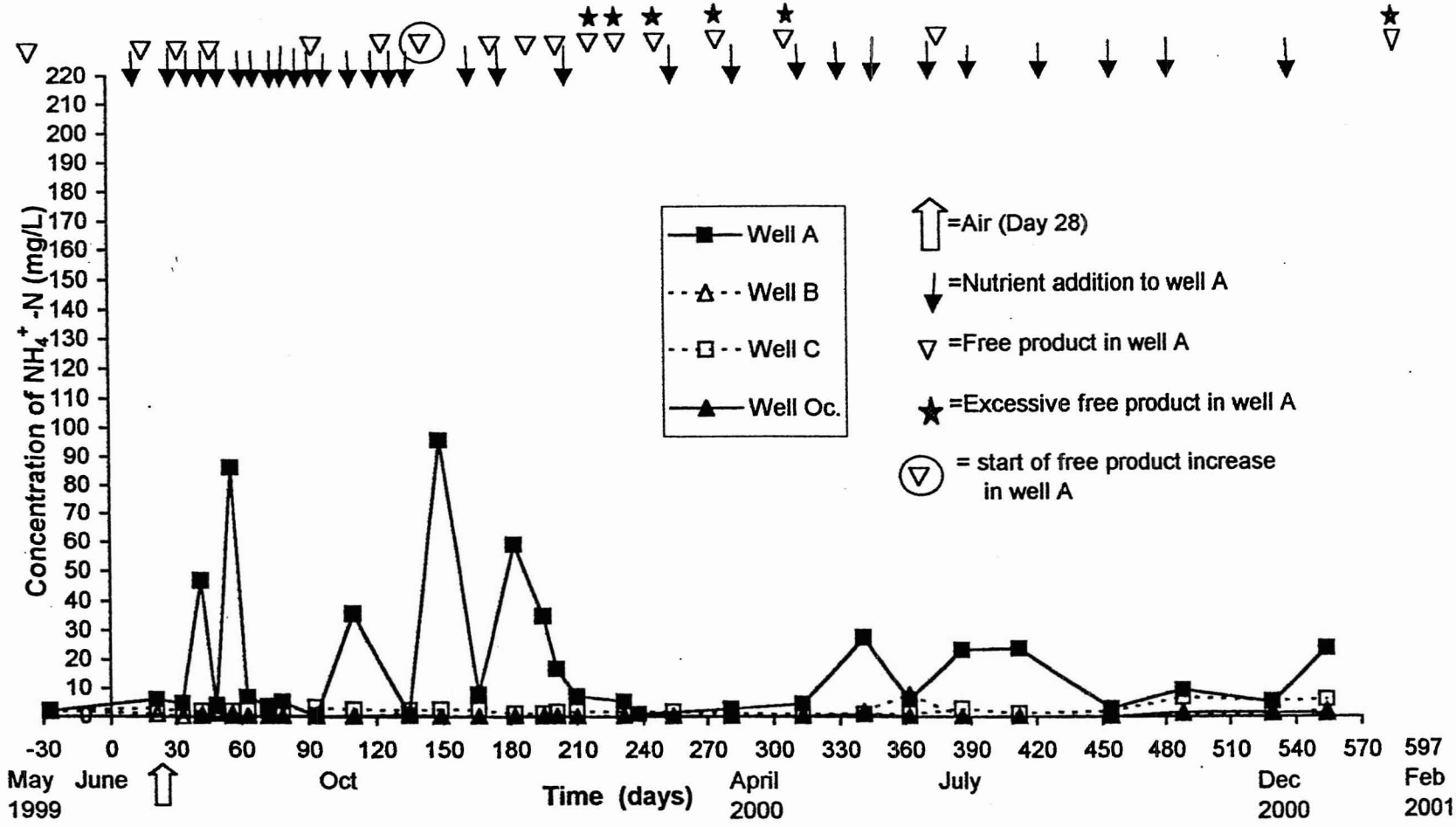


Fig. 13. Fluctuations of ammonium-N concentrations in monitoring-well water during the course of the experiment (5/28/99 to 12/28/00)

available nitrogen between 5 and 10 mg/L in the well water. The nutrient additions are indicated as black arrows at the top of Figures 12 and 13. For the first two months after the start of the nutrient additions to well A water, the concentrations of both NH_4^+-N and NO_3^--N significantly increased immediately after the nutrient addition, but rapidly decreased to the desired level (sometimes within three days). Because a large build-up of NO_3^--N occurred between days 100 and 150 (Fig. 12) and a lesser one of NH_4^+-N (Fig. 13) occurred during the same period, addition of the nutrients were done less frequently after that time (Fig. 12, 13). Based on Fig. 14, in general, higher concentrations of nitrate-N than ammonium-N were present in well A water throughout the experiment and the levels of these two forms of nitrogen fluctuated in a similar manner.

During the major recontamination of well A, which started near day 150 and ended approximately around day 290 (April '00), the levels of NH_4^+-N were rapidly depleted after the addition of nutrients (day 170 and during the period between days 180 and 240). The levels of NO_3^--N followed a similar pattern but remained higher than those of NH_4^+-N during the period of heaviest contamination (day 210 to 240).

The concentrations of nitrate-N and ammonium-N in the other wells were very low throughout the experiment. Concentrations above 5 mg/L were rarely measured.

3.2.5. Well Water Microbial Enumerations

3.2.5.1. Total Heterotrophic Bacteria

Heterotrophic bacteria numbered approximately 10^6 cells per ml of water in wells A, B, and C (Fig. 15) on the first day of this study prior to the start of air sparging (day 28) and nutrient addition to well A (day 19). Throughout the experiment higher numbers of heterotrophic bacteria were present in well A than in wells B, C and the Ocean well. Total heterotrophic bacteria increased in number when a high level of free product started to be seen in well A water (day 210). Averaging well water enumerations, well A contained approximately 10^7 cells per ml of water, while wells B and C averaged approximately 10^5 cells per ml. Heterotrophic bacteria counts in the Ocean well averaged just below 10^4 cells per

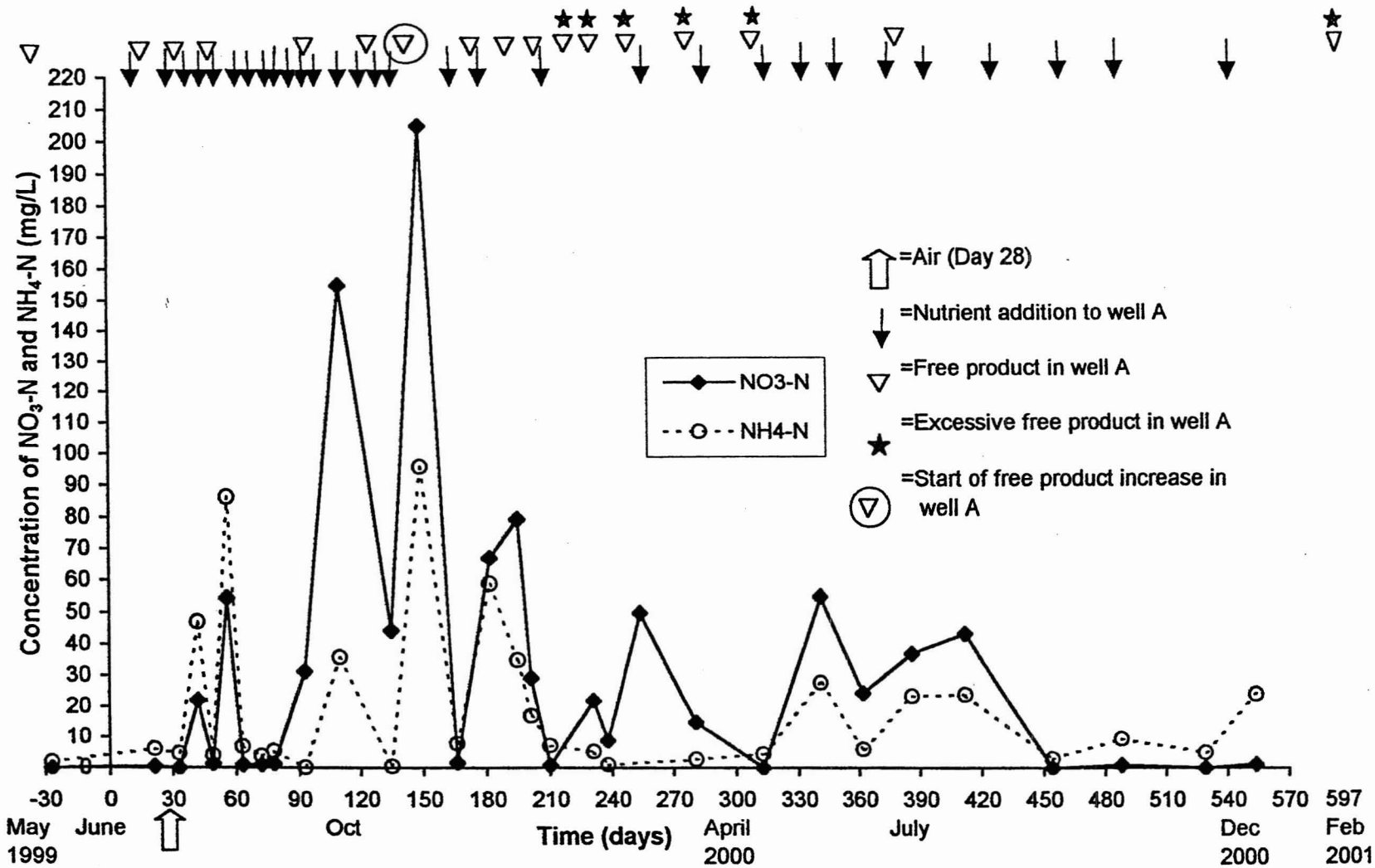


Fig. 14. Fluctuation of nitrate-N and ammonium-N concentrations in monitoring-well A water during the course of the experiment (5/28/99 to 12/28/00)

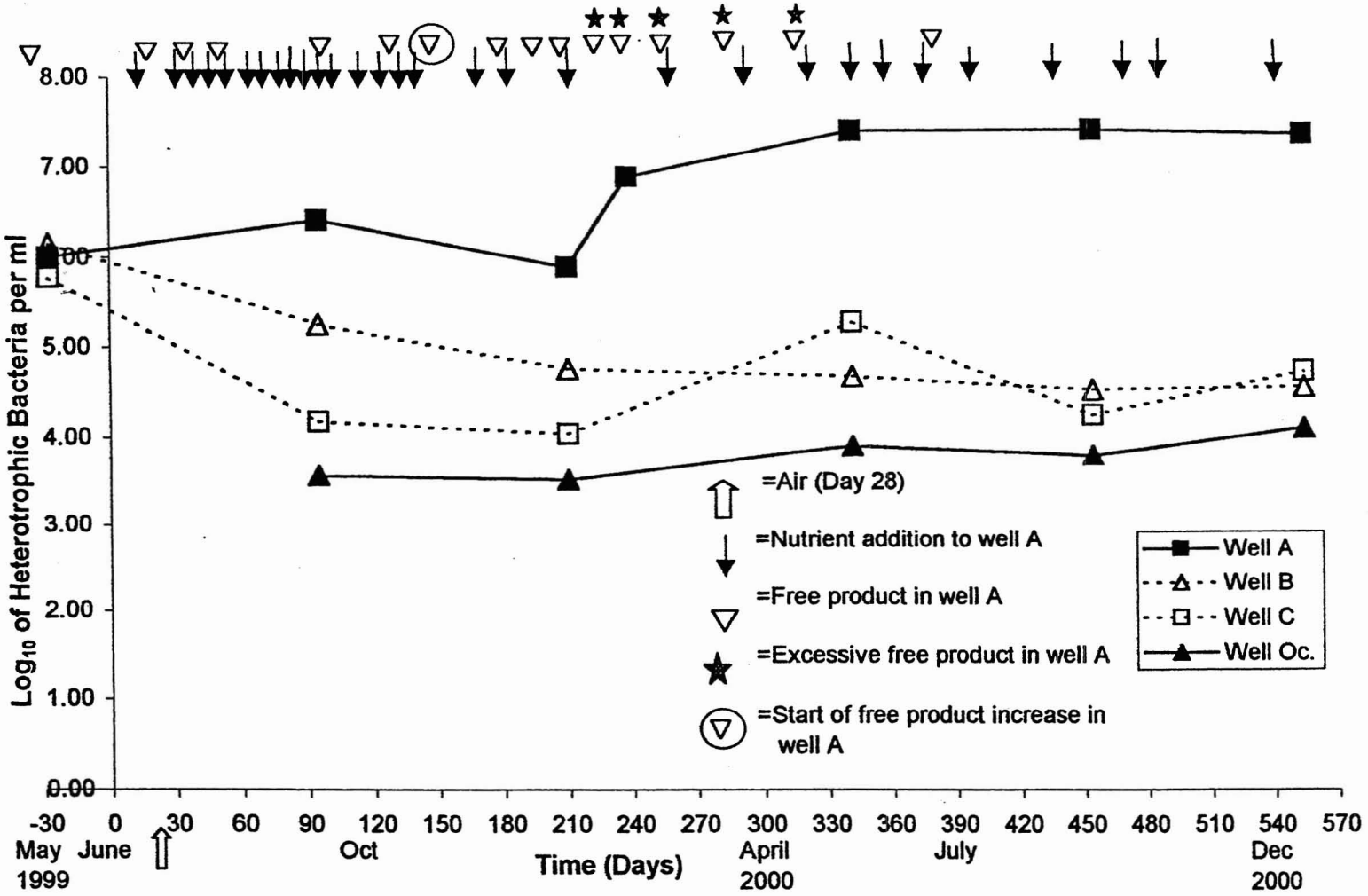


Fig. 15. Enumeration of total heterotrophic bacteria in the monitoring- well water during the course of the experiment (5/28/99 to 12/28/00)

ml. Total heterotrophic bacteria numbers were consistently 1-2 orders of magnitude higher in well A than in the other wells.

3.2.5.2 Phenanthrene-Degrading Bacteria

At the time of the initial water sampling wells A, B, and C contained similar numbers of phenanthrene degrading bacteria (Fig. 16), approximately 10^4 cells per ml of water. This represents 1% of the total heterotrophic bacteria (Fig. 15). In well A the number of phenanthrene degrading bacteria increased by almost two orders of magnitude at the time of the major recontamination (approximately day 240) and remained high until day 450. After that time the number decreased when the contaminant was no longer present in the well. When the growth of the phenanthrene degrading bacteria was stimulated by the presence of free product in well A (day 238), the number of phenanthrene-degrading bacteria represented 6% of the total heterotrophic bacteria. After the initial sampling the phenanthrene degrading bacteria in the uncontaminated wells (B,C, and the Ocean well) were undetectable or near the limit for accurate counts (300 cells/ml) by this technique.

3.2.5.3. Diesel-Degrading Microorganisms

At the initial sampling, the number of diesel degraders in well A was approximately two orders of magnitude lower than the number in wells B and C (Fig. 17). The numbers of diesel degrading bacteria in well A increased drastically with the appearance of large amounts of free product in this well. These numbers decreased rapidly after the disappearance of most of the free product. In wells B and C there was a continual decline in the number of diesel degraders over time. The counts stabilized near 10^3 cells/ml. In the Ocean well, the numbers were virtually constant at levels near the detection limit. On average, however, differences between diesel degrader numbers in wells A, B, C, and the Ocean well were negligible.

3.2.5.4. Pristane-Degrading Microorganisms

The number of pristane degraders (Fig. 18) in each well was low and virtually constant throughout the experiment. The numbers averaged 10^3 cells/ml in wells A and B, and 10^2

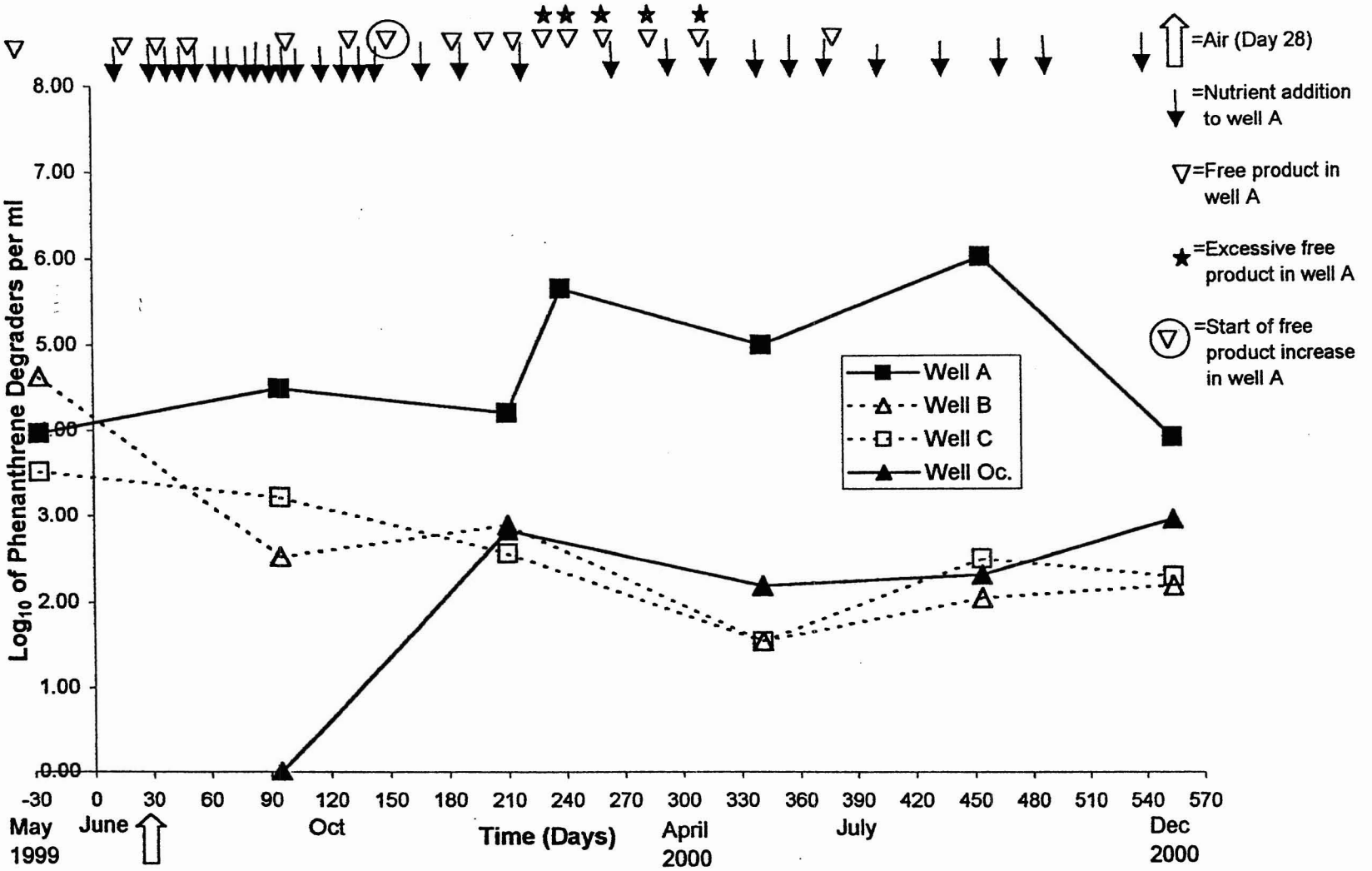


Fig. 16. Enumeration of phenanthrene degrading bacteria in the monitoring-well water during the course of the experiment (5/28/99 to 12/28/00)

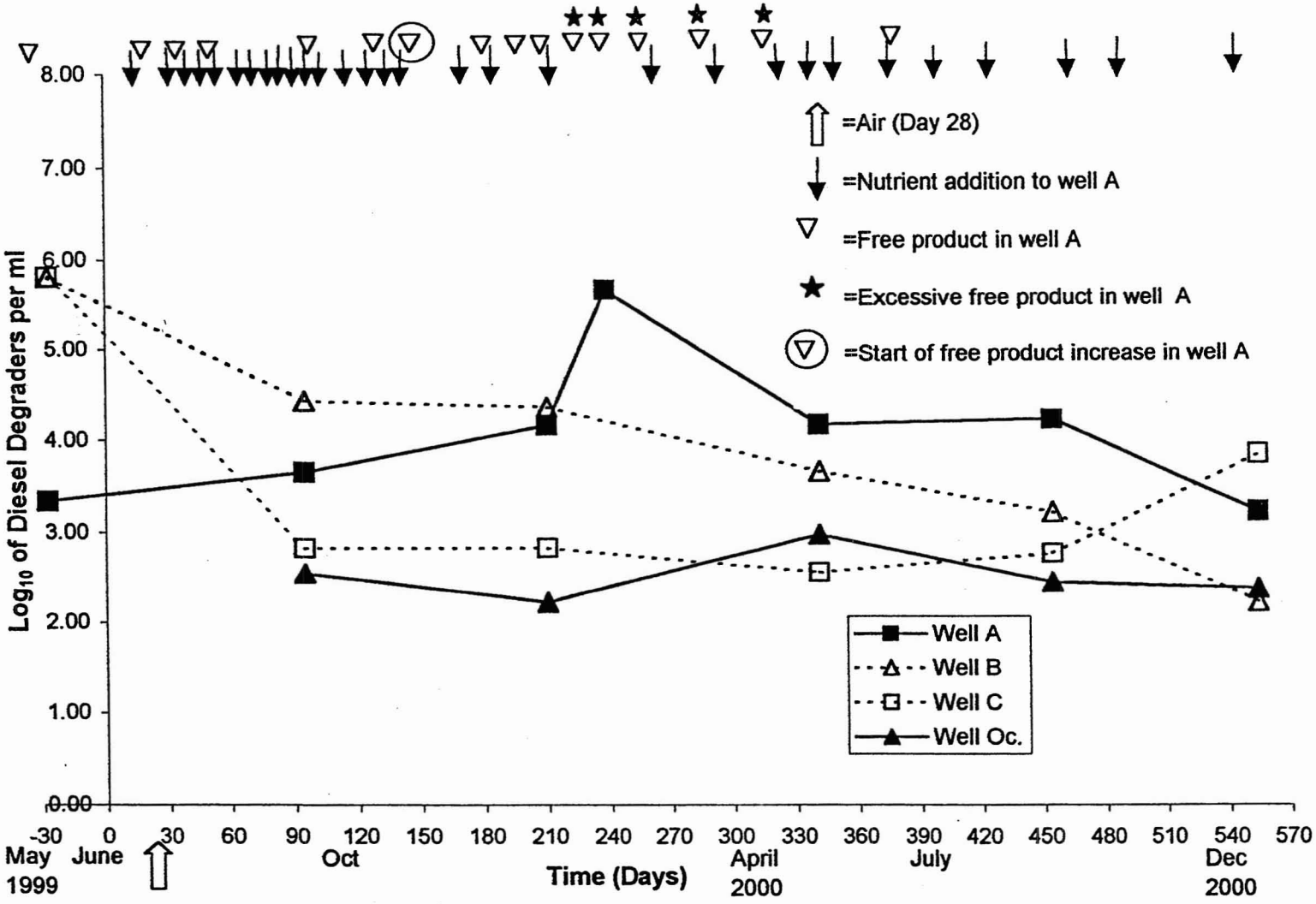


Fig. 17. Enumeration of diesel degrading bacteria in the monitoring- well water during the course of the experiment (5/28/99 to 12/28/00)

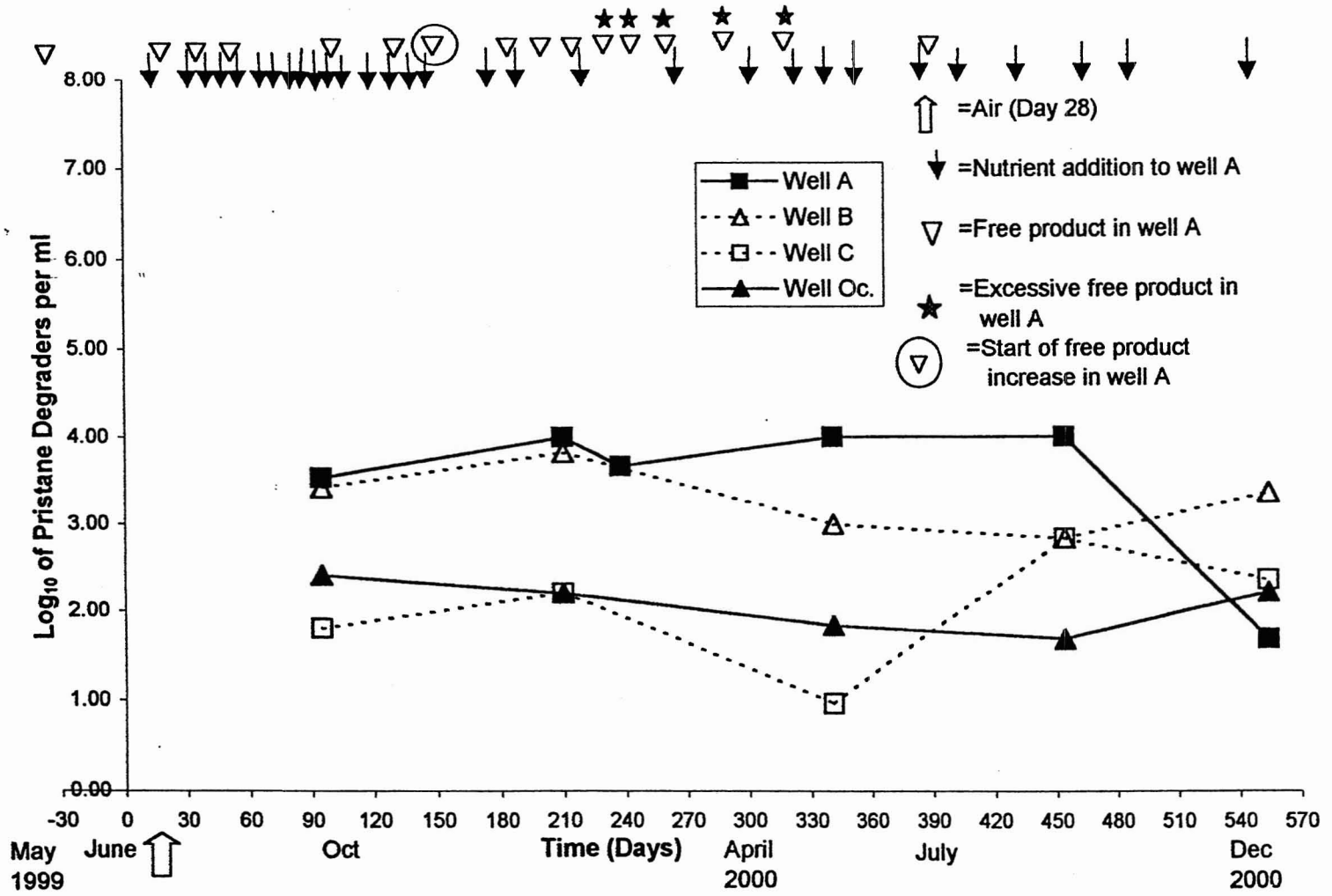


Fig. 18. Enumeration of pristane degrading bacteria in the monitoring- well water during the course of the experiment (5/28/99 to 12/28/00)

cells/ml of water in well C and the Ocean well. The pristane degrader numbers in well A were not affected by the recontamination in well A (day 240).

3.3. Soil Hydrocarbon Analyses

Based on the available maps (Fig. 4 and 5b) showing free product floating over the water in existing monitoring wells, and our findings reported below (section 3.3.1), our hypothesis was that the contaminant was probably most heavily smeared in the tidal zone (zone 2) which extended approximately from six to eight feet below the surface. Therefore, our analyses were done in more detail in zone 2 than in the zone below the tidal zone (zone 3).

3.3.1. Subsurface Soil Characteristics

The subsurface-soil cores collected revealed layers of soil with different textures (Fig. 6). Just below the asphalt was a dark red, clayey road fill material, which was discarded. The next layer extended approximately from four to six feet below the surface, was above the tidal zone, termed zone 1. This layer consisted of dry coralline sand that was not contaminated. Below zone 1, was the tidal zone, or zone 2, which extended approximately from six to eight feet below the surface. This zone was heterogeneous and composed of sand, coral chunks, and clay in various proportions. This zone was wet and subjected to fluctuations in the water table due to diurnal tidal changes. Contamination, in the form of free product pockets was visible in this zone, which, in general, smelled strongly of diesel fuel. Below the tidal zone was zone 3, approximately eight to ten feet below the surface, which consisted of a dense, black colored, silty material. This zone was saturated with water and very uniform throughout the study area. Contamination was heterogeneous in zone 3. Directly under zone 3 was a coarse coral rock layer, which was white colored and devoid of contamination.

3.3.2. Quality Control (QC) Samples in Gas Chromatography Analysis of TPH

Quality control procedures are necessary to evaluate the method performance and GC system operation. Each laboratory must demonstrate proficiency by generating data with

acceptable accuracy and precision. For this experiment, quality control procedures found in the Environmental Protection Agency (EPA) Test Methods for Evaluating Solid Waste SW-846, Method 8015B (Nonhalogenated Organics Using the GC/FID), and Chapter One (Quality Control) were performed. The results for the QC procedures are listed in Table 2. The percent recoveries were calculated based on the diesel fuel spike concentrations (expected concentrations), and are averages of the duplicate samples.

Table 2. Typical recovery percentages for QC samples

QC sample name	Expected concentration (mg/L or mg/kg)	Concentration (mg/L or mg/kg)	Percent recovery
Unocal *76* diesel fuel in methylene chloride	5000	6072.71	122%
Silica sand + Unocal *76* diesel fuel	5000	6605.40	132%
Site sand + Unocal *76* diesel fuel + water	6000	5166.28	86%
Unocal *76* diesel fuel check standards:	100 3 samples	1) 138.99 2) 137.082 3) 139.34	1) 139% 2) 137% 3) 139%

3.3.3. Recovery of Unocal *76* Diesel Fuel from Spiked Site Soil and of the Docosane Surrogate

The purpose of this experiment was to evaluate the performance of the extraction and GC determination methods for measuring diesel fuel TPH. The performance of these methods are affected by different matrices, so a suitable matrix is one similar to that being analyzed. A composite sample of uncontaminated soil from the site was used for the determination of diesel fuel TPH extractable and measurable at various concentrations. Recovery percentages (Table 3) are based on the initial amount of diesel fuel added (expected concentration), and are averages of duplicate samples.

Table 3. Recovery percentages for Unocal *76* diesel fuel from spiked soil

Sample	Expected Concentration (mg/kg)	Measured Concentration (mg/kg)	Percent Recovery
Comp[a] 1	50	45.19	90.38%
Comp 2	500	432.87	86.58%
Comp 3	1000	841.78	84.18%
Comp 4	3000	2519.23	83.98%
Comp 5	5000	3727.27	74.5%
Average Recovery			83.9%

[a]Comp indicates a composite of all texture types and moisture levels of the soil present at the Gray Line site (includes soil from zones 1, 2, and 3).

Docosane (C_{22}) was used as the surrogate compound to determine the efficiency of the methylene chloride sonication extraction and GC-FID analytical method for measuring TPH. Samples were spiked with known amounts of a solution of docosane in methylene chloride just before extraction. An example of the docosane recovery data for a sampling batch is shown in Table 4. The average recovery obtained from the surrogate compound was 83.57%, which was similar to the average recovery of diesel fuel from the composite soil mentioned above.

3.3.4. Soil Petroleum Hydrocarbon Concentrations in the Tidal Zone (Zone 2)

Insignificant concentrations of TPH and PAH were found in soil samples of area C in both zone 2 and zone 3 during the course of the experiment. Also, area C was not sampled on day 117 due to mechanical difficulties. Therefore, area C could not be used as a no-action control to study intrinsic bioremediation of the contaminant and the data collected from area C soil were not taken into consideration in the analysis of the results. Raw data pertaining to area C can be found in Appendix 1.

Table 4. Representative recovery percentages of the surrogate compound (docosane)

Samples[a]	Expected Conc (mg/kg)	Measured Conc (mg/kg)	% Recovery
A21	80	65.23	81.54
A22	80	60.53	75.66
A23	80	62.55	78.18
A24	200	116.55	58.28
A25	20	15.09	75.46
A26	40	32.98	82.46
A31	10	10.14	101.38
A32	10	8.17	81.72
A33	40	35.78	89.45
A34	10	9.05	90.52
A35	120	110.73	92.28
A36	20	17.23	86.14
B21	80	69.68	87.10
B22	40	40.06	100.16
B23	7	5.19	74.14
B24	10	8.96	89.62
B25	20	15.80	79.01
B26	80	74.46	93.07
B31	20	17.84	89.19
B32	20	18.05	90.26
B33	7	6.10	87.18
B34	40	36.52	91.29
B35	10	10.00	99.97
B36	40	37.89	94.71
C21	7	5.19	74.11
C22	7	6.06	86.58
C23	7	5.33	76.20
C24	7	5.97	85.35
C25	7	5.86	83.71
C26	7	4.16	59.36
C31	7	6.23	89.06
C32	7	5.95	84.99
C33	7	5.53	78.96
C34	7	6.18	88.32
C35	7	5.68	81.09
C36	7	3.64	51.96
			83.57 Average Recovery

[a]Sample code: letters (A,B, or C) indicate which square area, the first number indicates the zone (2 or 3), and the second number indicates the core number (1 to 6).

All soil hydrocarbon analyses were carried out on area A, which received air and nutrients, and was shown in section 3.2 to have undergone recontamination from February to May 2000, and area B, which received air only, and did not show signs of recontamination.

At all sampling times TPH measurements for both areas A and B varied greatly over the sampling area. Figures 19 and 20 depict the heterogeneous TPH concentrations in the tidal zone (zone 2) within the 10' by 10' area around each well on each of the four sampling days. There was clearly no spatial pattern of contamination and cores only a few feet away differed greatly in TPH concentrations.

The means of TPH concentrations in areas A and B over time are compared in Fig. 21. A two-way ANOVA was used to compare the means (n = 6) of TPH concentrations (responses) using the sampling day and areas as factors (Table 5). These statistical tests were used to determine mean differences as a result of the sampling day and/or area. With these tests, the main effects of the two factors can be distinguished from each other, and the occurrence of an interaction between the two main effects can be determined.

Table 5. Two-way analysis of variance for TPH concentration in zone 2

Source	Degrees of Freedom	Sum of Squares	Mean Square	F statistic	P-value
Day factor	3	4016888	1338963	2.00	0.129
Area factor	1	1969680	1969680	2.94	0.094*
Interaction	3	2370128	790043	1.18	0.329
Error	40	26772243	669306		
Total	47	35128939			

*significant P-values with $\alpha = 0.1$

The *P*-values in table 5 indicate that for TPH measurements in zone 2 there was a significant difference between areas A and B at the 0.1 level. This is supported by the plot of TPH concentrations (Fig. 21) where there is divergence of the lines for the means of areas A and B. The plot seems to indicate an interaction, where the two lines cross near day 0. However, the interaction is not statistically significant, probably because of the small sample size (n = 6). There is no significant effect of sampling day at $\alpha = 0.1$ (Table 5). The two-way

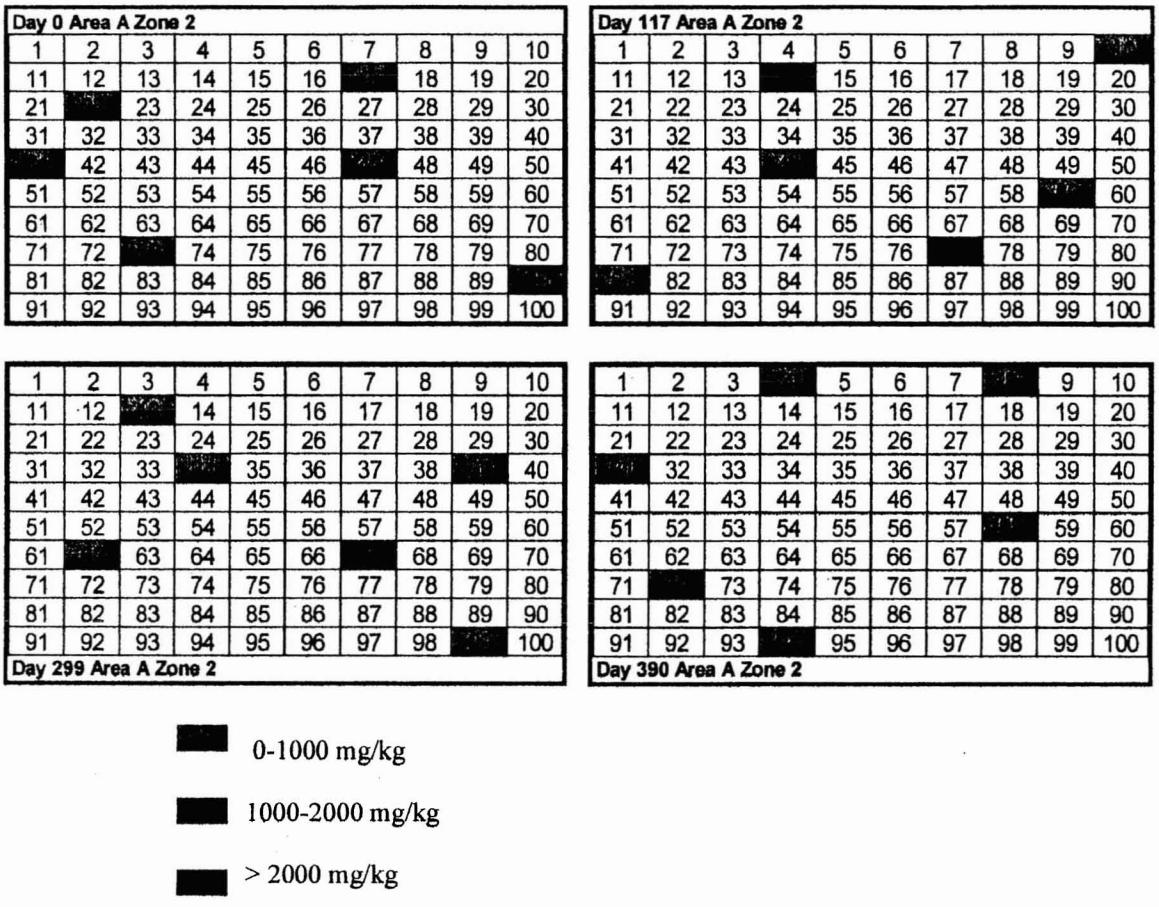


Fig. 19. Heterogeneity of TPH concentrations (as measured by gas chromatography) in the 6 soil cores randomly chosen for sampling in area A, zone 2, on each of the four sampling days. The 100-square grid represents 100 possible sampling points.

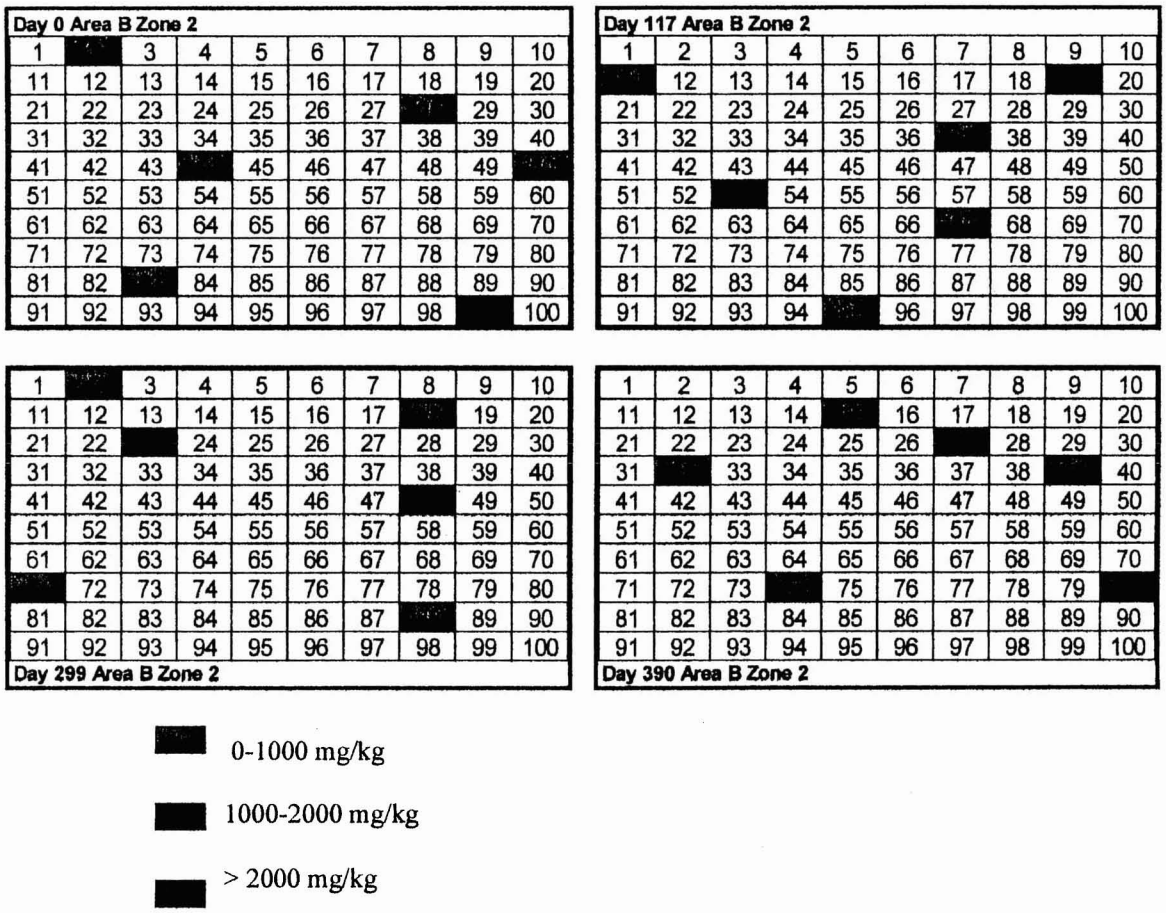


Fig. 20. Heterogeneity of TPH concentrations (as measured by gas chromatography) in the 6 soil cores randomly chosen for sampling in area B, zone 2, on each of the four sampling days. The 100-square grid represents 100 possible sampling points.

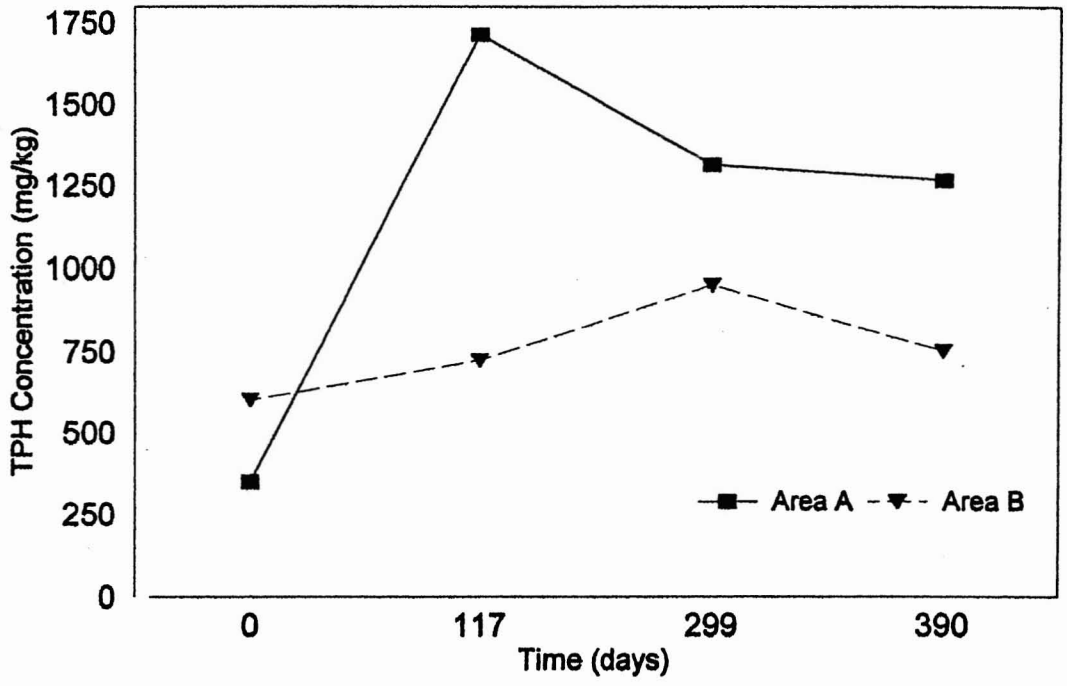


Fig. 21. Effect of area and sampling time on TPH concentration in the soil of the tidal zone (zone 2). Each point represents the mean of the concentrations in six cores.

ANOVA (Table 5) and the plot of TPH means (Fig. 21) indicate that area A had significantly different TPH concentration means than area B, except on day 0.

The means of PAH concentrations, as determined by immunoassay, in areas A and B of zone 2 over time are depicted in Fig. 22. A two-way ANOVA of PAH concentration was used to analyze the data (Table 6).

The *P*-values for PAH measurements indicate that the day effect causes significant difference ($\alpha = 0.1$) between the means of A and B. The plot in Figure 22 shows that the means for area A and area B do not differ significantly, except on day 117.

Table 6. Two-way analysis of variance for PAH concentration in zone 2

Source	Degrees of Freedom	Sum of Squares	Mean Square	F- statistic	P-value
Day factor	3	143703	47901	3.53	0.023*
Area factor	1	22502	22502	1.66	0.205
Interaction	3	27682	9227	0.68	0.569
Error	40	542579	13564		
Total	47	736466			

*significant P-value with $\alpha = 0.1$

The differences in the pattern of TPH concentrations (Fig. 21) and PAH concentrations (Fig. 22) indicated that the PAH percentages of TPH was not constant in the weathered product. These percentages varied greatly, from 5 to 26%, among all the samples in both areas A and B (data not shown). The average PAH percentages of TPH, however, was the same for both areas A and B, at 14.5% and 14.7% respectively. These average percentages in the weathered product are higher than the percentage found in fresh diesel fuel #2 (2% by the same immunoassay; Chua-Chiaco, 1998).

3.3.5. Microbial Enumerations in the Tidal Zone (Zone 2)

3.3.5.1. Total Heterotrophic Bacteria

Enumerations of the total heterotrophic bacteria in zone 2 are shown in Fig. 23. In area B, the numbers were low (approximately 10^4 cells/g OD soil) and remained virtually

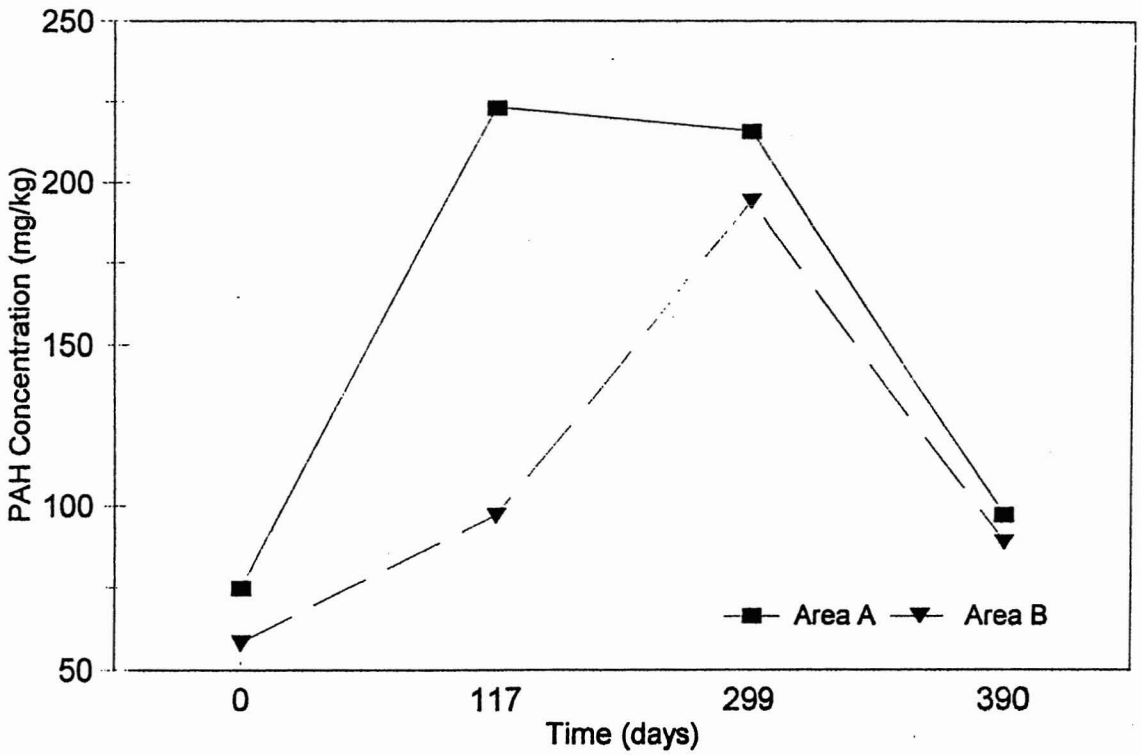


Fig. 22. Effect of area and sampling time on PAH concentration in the soil of the tidal zone (zone 2). Each point represents the mean of the concentrations in six cores.

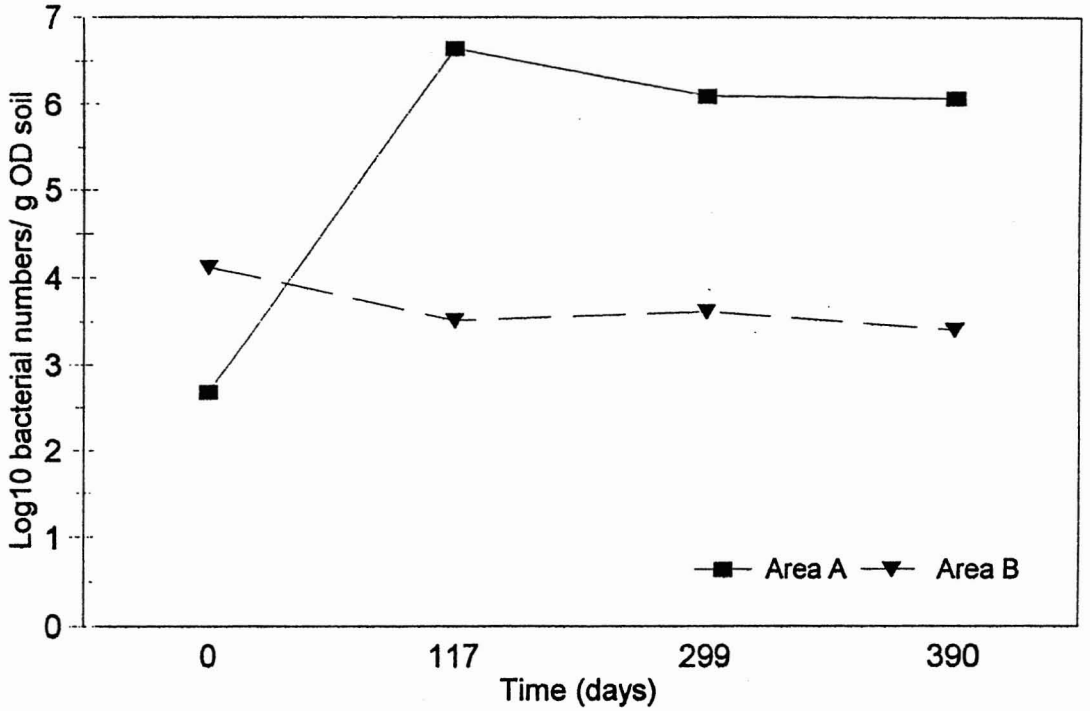


Fig. 23. Effect of area and sampling time on total heterotrophic bacteria enumerations (n = 6) in the soil of the tidal zone (zone 2).

unchanged throughout the experiment. The numbers in area A, however, increased by two orders of magnitude at day 117 and remained at that level until the end of the experiment. A two-way ANOVA analysis (Table 7) indicated a significant interaction ($\alpha = 0.1$) between the

Table 7. Two-way analysis of variance for microbial enumerations in soil from zone 2

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-statistic	P-value
Total Heterotrophic Bacteria					
Day factor	3	9.250	3.083	5.47	0.003*
Area factor	1	48.481	48.481	85.98	0.000*
Interaction	3	20.811	6.937	12.30	0.000*
Error	40	22.554	0.564		
Total	47	101.096			
Phenanthrene Degrading Bacteria					
Day factor	3	10.46	3.49	1.96	0.136
Area factor	1	75.68	75.68	42.43	0.000*
Interaction	3	29.51	9.84	5.52	0.003*
Error	40	71.34	1.78		
Total	47	186.98			
Diesel Fuel Degrading Microorganisms					
Day factor	3	3.232	1.077	1.84	0.156
Area factor	1	25.259	25.259	43.03	0.000*
Interaction	3	14.209	4.736	8.07	0.000*
Error	40	23.483	0.587		
Total	47	66.183			
Pristane Degrading Microorganisms					
Day factor	3	5.410	1.803	1.97	0.134
Area factor	1	7.403	7.403	8.07	0.007*
Interaction	3	5.257	1.752	1.91	0.143
Error	40	36.672	0.917		
Total	47	54.741			

*significant P-value with $\alpha = 0.1$

time of sampling and sampling area. This was due to the fact that on day 0 (before the nutrients and air additions) the heterotrophic bacteria counts were similar in areas A and B. After treatments of areas A and B were initiated, there was a significant difference in heterotrophic bacteria numbers between the two areas until the end of the experiment. This can be seen by comparing the means of areas A and B at each sampling time (Table 8). These comparisons calculate a t-statistic for determining the difference between two sample means, which is in this case the difference between area A means and area B means for each sampling day.

Table 8. T-statistic calculations for the difference between two sample means

Total Heterotrophic Bacteria area A vs. area B means					
	Day	**t-statistic**	**P-value**		
	0	0.53	>0.5		
	117	7.25	<0.001*		
	299	5.71	<0.001*		
	390	6.13	<0.001*		
Phenanthrene Degrading Microorganisms area A vs. area B means					
	0	0.22	>0.5		
	117	5.23	<0.001*		
	299	2.6	<0.001*		
	390	4.98	<0.001*		
Diesel Degrading Microorganisms area A vs. area B means					
	0	0.11	>0.5		
	117	2.89	~0.0066*		
	299	3.14	~0.004*		
	390	7.05	<0.001*		

*significant P-values with $\alpha = 0.03$

3.3.5.2. Phenanthrene-Degrading Bacteria

Fig. 24 displays the counts of the phenanthrene degrading bacteria in zone 2 during the course of the experiment. In the soil of zone 2, the phenanthrene-degrading bacteria occurred at very low levels in area A and B on day 0 prior to treatment implementation. In area B the counts never increased after treatment with air, but progressively declined. Phenanthrene-degrading bacteria enumerations at these low levels are not reliable for statistical analysis because the number of colonies per plate is very small (1-2/plate at 10^{-2} dilution). No enumerations could be carried out at the 10^{-1} dilution due to the interference of soil particles

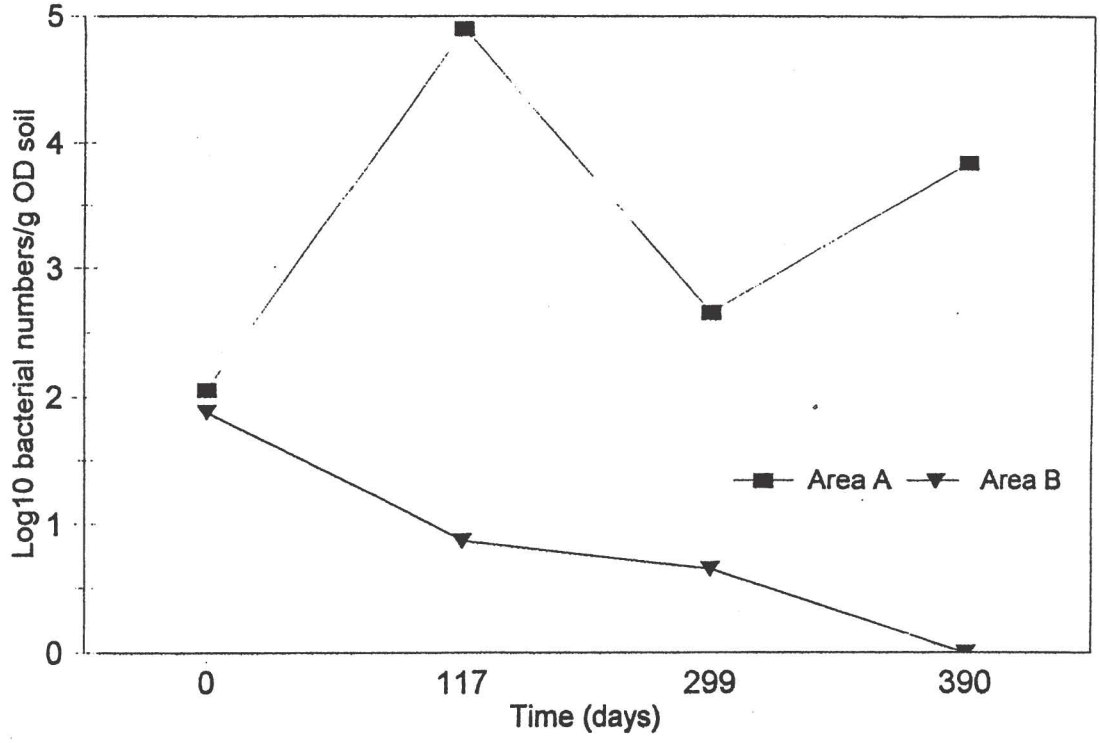


Fig. 24. Effect of area and sampling time on phenanthrene-degrading bacteria enumerations (n = 6) in the soil of the tidal zone (zone 2).

on the plate with the detection of haloes around the colonies. A great variability between replicates ensued. In area A the phenanthrene-degrading bacteria counts increased by three orders of magnitude on day 117, then decreased close to the limit for statistically reliable counts for this technique (approximately 10^4 cells/g OD soil). A two-way ANOVA was performed on the data (Table 7) and significant interaction between time and the area of sampling was found. Mean comparisons were done between phenanthrene-degrading bacteria numbers in areas A and B for each sampling time (Table 8), which indicated that the interaction was due to similar numbers on day 0. After day 0, however, the number of phenanthrene-degrading bacteria were significantly different between the two areas. Thus, area A had higher numbers of phenanthrene-degrading bacteria than area B after day 0.

3.3.5.3. Diesel-Fuel Degraders

The enumerations of diesel-fuel degrading microorganisms in the soil of zone 2 are shown in Fig. 25. On day 0, prior to treatment implementation, approximately 100 diesel-fuel degraders/g OD soil were found in both areas A and B. Thereafter, the numbers in area B soil remained constant until day 299, then decreased to less than 10 microorganisms/g OD soil. On the other hand, the counts in area A soil increased by at least one order of magnitude after day 0 and remained at that level throughout the experiment. The two-way ANOVA of these data (Table 7) indicates a significant interaction between time of sampling and sampling area due to the occurrence of similar counts in both areas A and B on day 0. Comparisons of the numbers in areas A and B on individual sampling days (Table 8) indicated that after the treatment began the numbers in area A were significantly higher than the numbers in area B from day 117 until the end of the experiment

3.3.5.4. Pristane-Degrading Microorganisms

The numbers of pristane-degrading microorganisms were very low in zone 2 soil of both areas A and B (Fig. 26). The two-way ANOVA (Table 7) indicated no interaction between the time of sampling and the sampling area, but a significant main effect of the area of

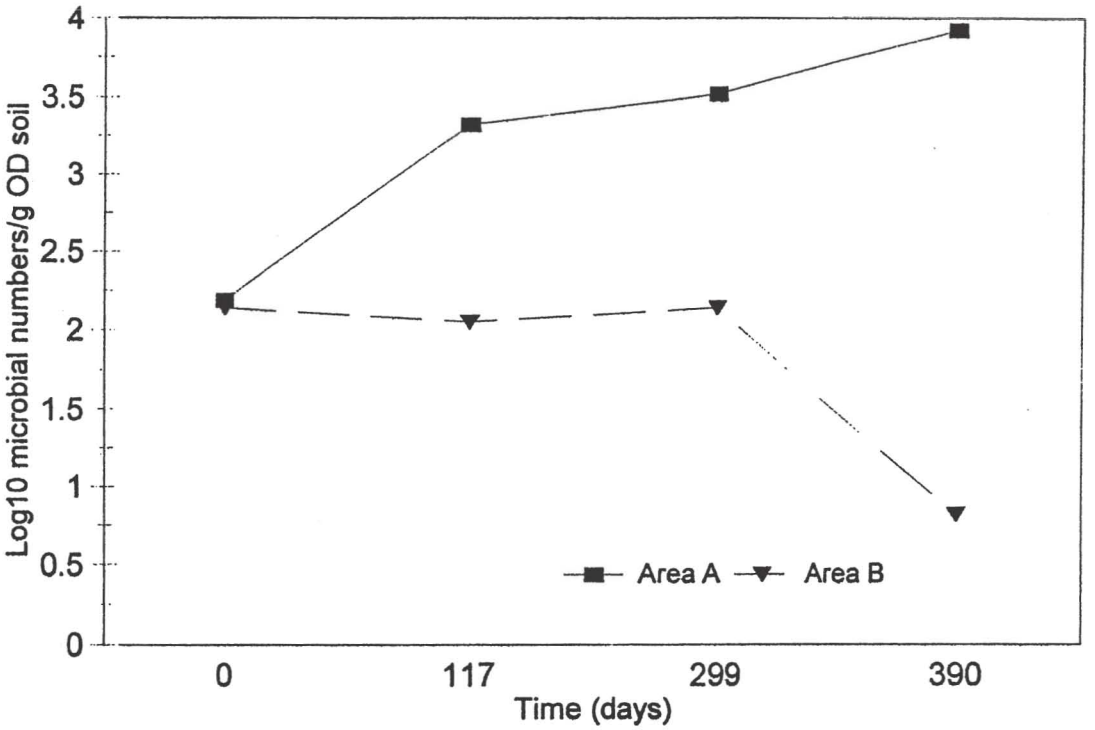


Fig. 25. Effect of area and sampling time on diesel- fuel degrading microorganism enumerations (n = 6) in the soil of the tidal zone (zone2).

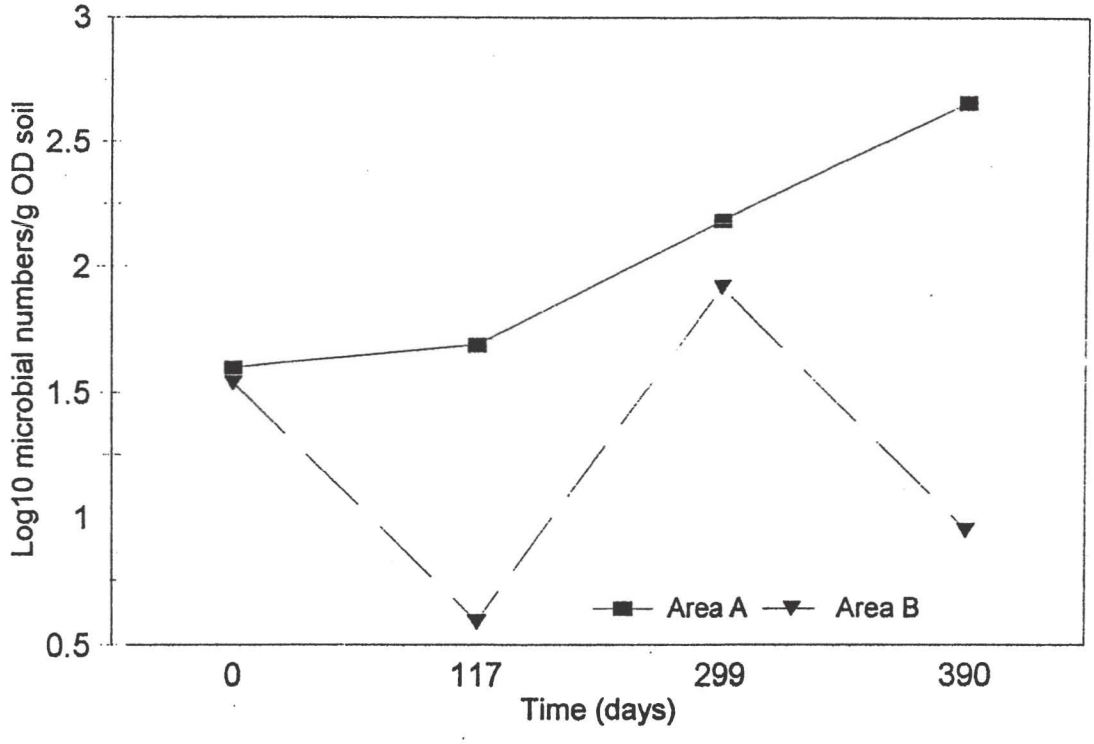


Fig. 26. Effect of area and sampling time on pristane-degrading microorganism enumerations (n = 6) in the soil of the tidal zone (zone 2).

sampling. This indicated that after implementation of treatments (after day 0), the pristane degraders were significantly higher in area A than in area B.

3.3.5.5 Sulfate-Reducing Bacteria

The sulfate-reducing bacteria (SRB) were enumerated in each area in composite samples (6 pooled cores), as explained in section 2.4.4. The numbers of SRB in all areas were low throughout the experiment, and did not decrease significantly in areas A and B after the addition of air on day 28, except for area A on day 299 (Fig. 27).

3.3.6. Soil Nutrient Analyses in the Tidal Zone (Zone 2)

Analyses of the nutrients in the composite samples of six cores from zone 2 soil are shown in Appendix 2A. These analyses, performed by UH-Manoa Agricultural Diagnostic Service Center, appear biased and may not be reliable. Data for all areas (A, B, and C) are high or low, depending on the day of the experiment (or analysis). Nutrient levels in areas A and B were analyzed to determine whether lower microbial counts in zone 2 of area B, compared to the counts in zone 2 of area A, were due to a shortage of nutrients in area B. The data in Appendix 2A do not indicate that area B was deprived of nutrients, since similar values were found in area A.

To determine whether the nutrients added to well A moved to the periphery of square A, nutrient analyses were performed on individual soil cores in each area (Appendix 2B). One core was close to the central monitoring well, where the nutrients were added, and the other core was chosen far from the central monitoring well. The results showed no differences in the levels of NH_4^+-N and NO_3^--N between the two cores, with respect to location. Thus, the nutrient analyses did not provide information on the migration of the nutrients added to well A through the soil of area A.

3.3.7. Soil Petroleum Hydrocarbon Concentration in the Sub-Tidal Zone (Zone 3)

The distribution of TPH was very heterogenous in zone 3, plots of individual core data for areas A and B (Figures 28 and 29) show varying concentrations. Generally, the concentrations in both areas A and B were low (below 1000 mg/kg), but one core in area A

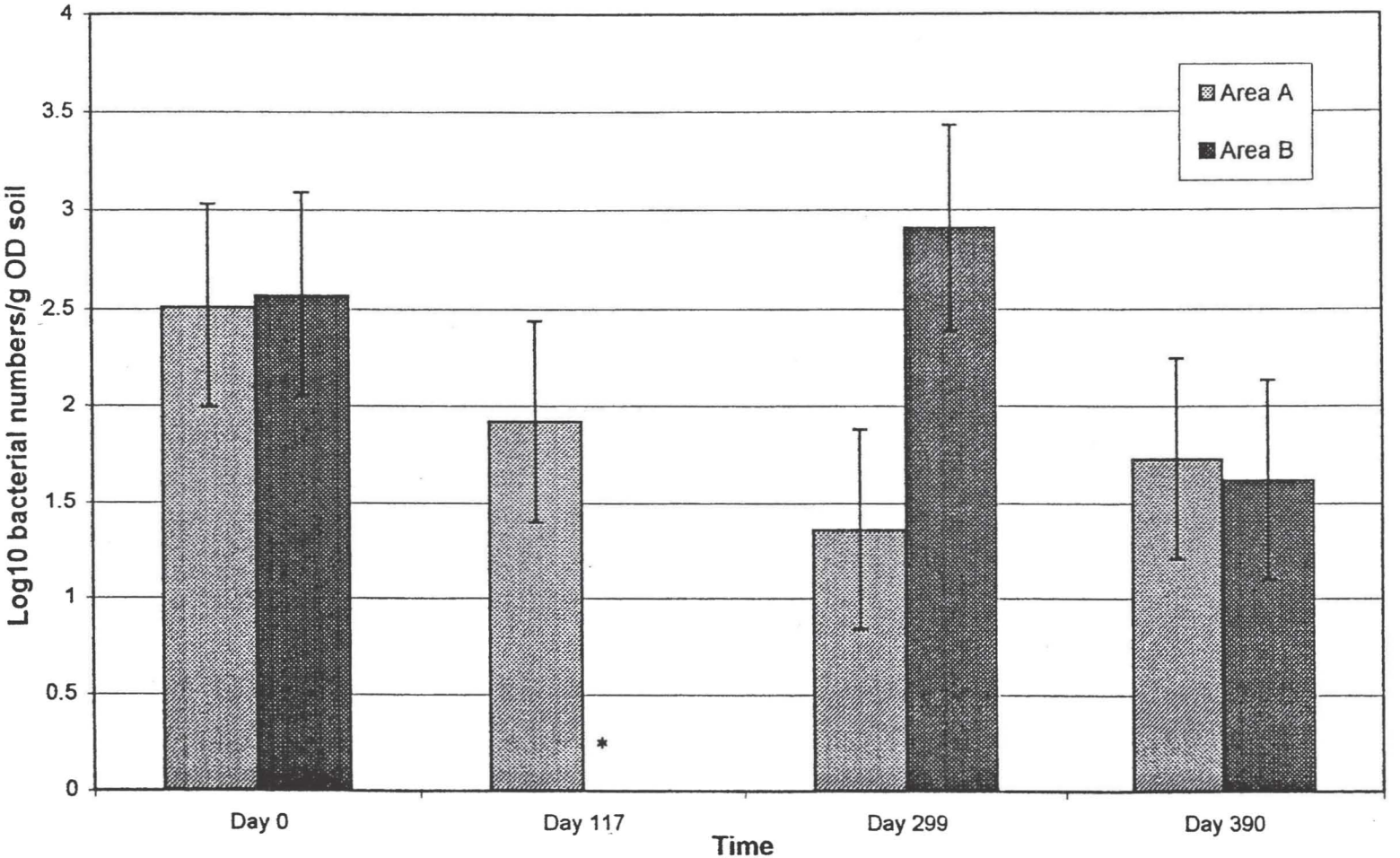


Fig.27. Enumerations of sulfate-reducing bacteria by the MPN technique in soil from zone 2. The data represent counts in a composite sample obtained by pooling equal amounts of soil from six cores/area. Error bars are the 95% confidence intervals. *No data are available for area B on day 117

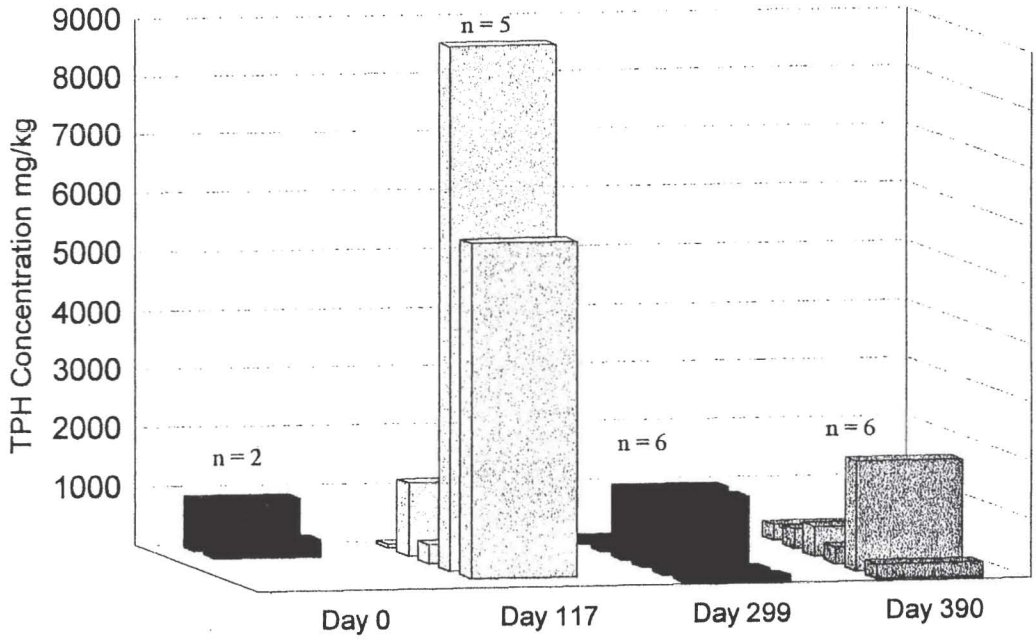


Fig. 28. Heterogeneity in the levels of TPH in the subtidal zone (zone 3) in area A.

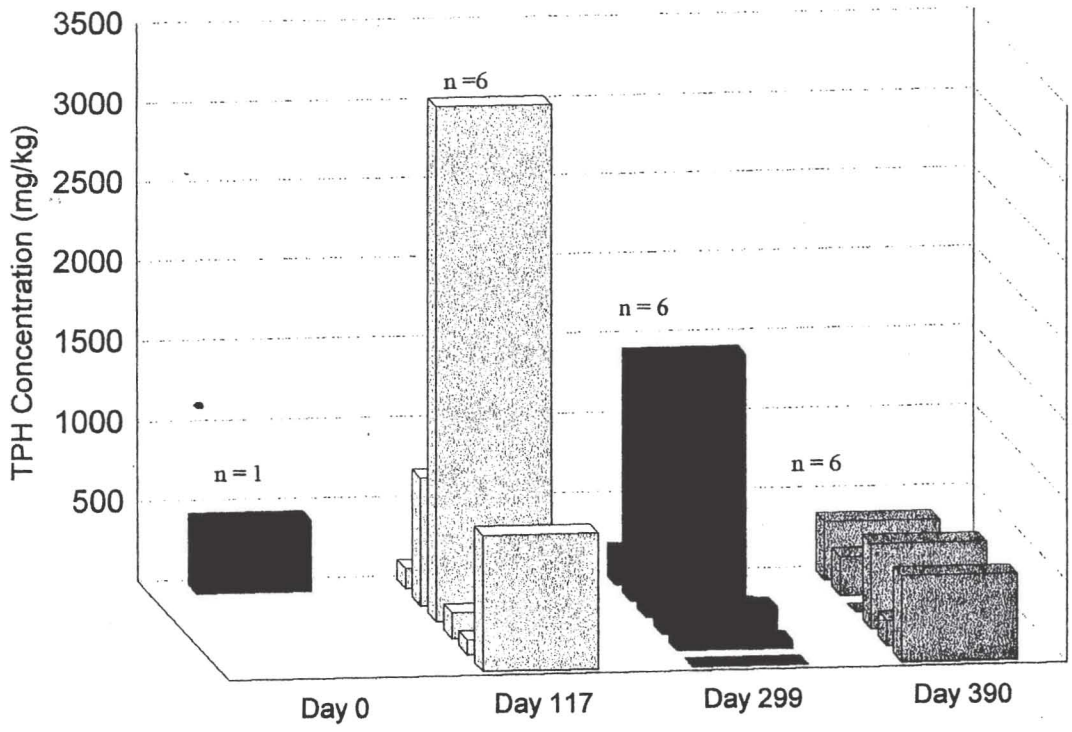


Fig. 29. Heterogeneity in the levels of TPH in the subtidal zone (zone 3) in area B.

showed a high level of contamination (>5000 mg/kg). The number of cores collected was not equal, except for the last two sampling days. This was due to technical problems associated with the machine auger on the first two sampling days. Due to the incomplete data set for zone 3, no statistical analysis was performed on these data. PAH concentrations were not determined in zone 3 soil samples.

3.3.8. Microbial Enumerations in the Subtidal Zone 3

3.3.8.1 Total Heterotrophic Bacteria

The numbers of total heterotrophic bacteria below the tidal zone in both areas A and B were very similar (Fig. 30 and 31). Most samples contained less than 10^4 cells/g of OD soil. The numbers were similar for each sampling day, indicating no differences with time.

3.3.8.2. Phenanthrene-Degrading Bacteria

The numbers of phenanthrene-degrading bacteria were low or undetectable in both areas A and B in zone 3 (Fig. 32 and 33). The data showed variability between samples, but in general the numbers were very low, mostly less than 10^3 cells/g of OD soil. Again, there were no large differences with time.

3.3.8.3. Diesel-Degrading Microorganisms

The numbers of diesel-degrading microorganisms were all under 3.5 log units in both areas A and B (Fig. 34 and 35). These microorganisms were detected in all samples collected except one at day 390 in area B. Time did not affect the counts.

3.3.8.4. Pristane-Degrading Microorganisms

Pristane-degrading microorganisms occurred in very low numbers in zone 3 (Fig. 36 and 37). Most counts were below 2.5 log units in both areas A and B throughout the course of the experiment.

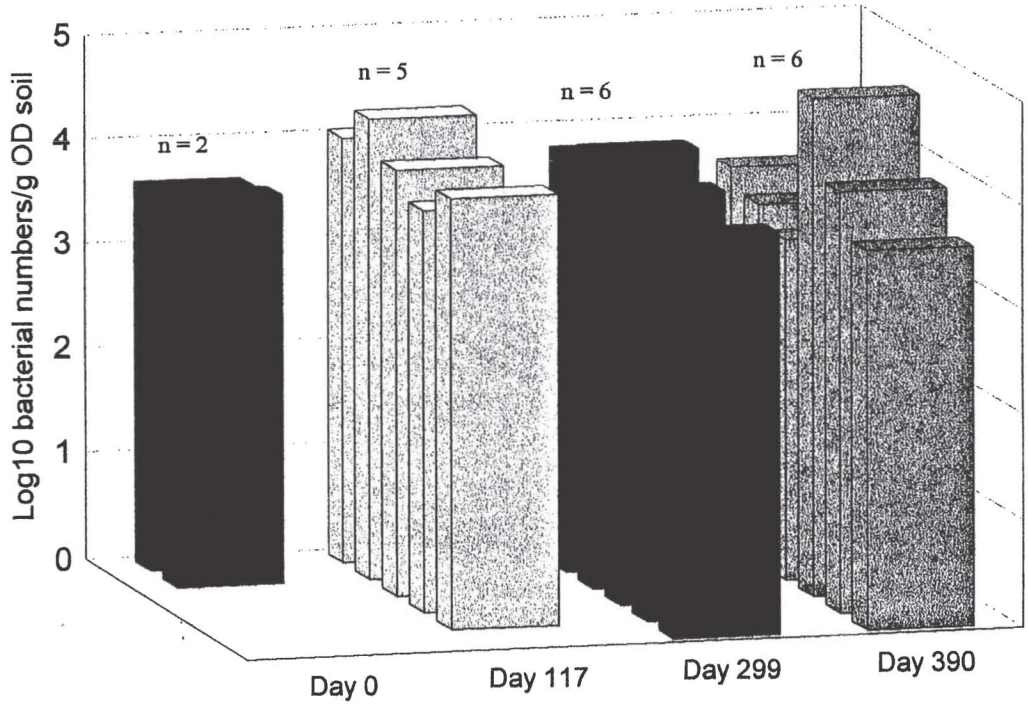


Fig. 30. Enumerations of total heterotrophic bacteria in individual soil cores in zone 3, area A during the course of the experiment.

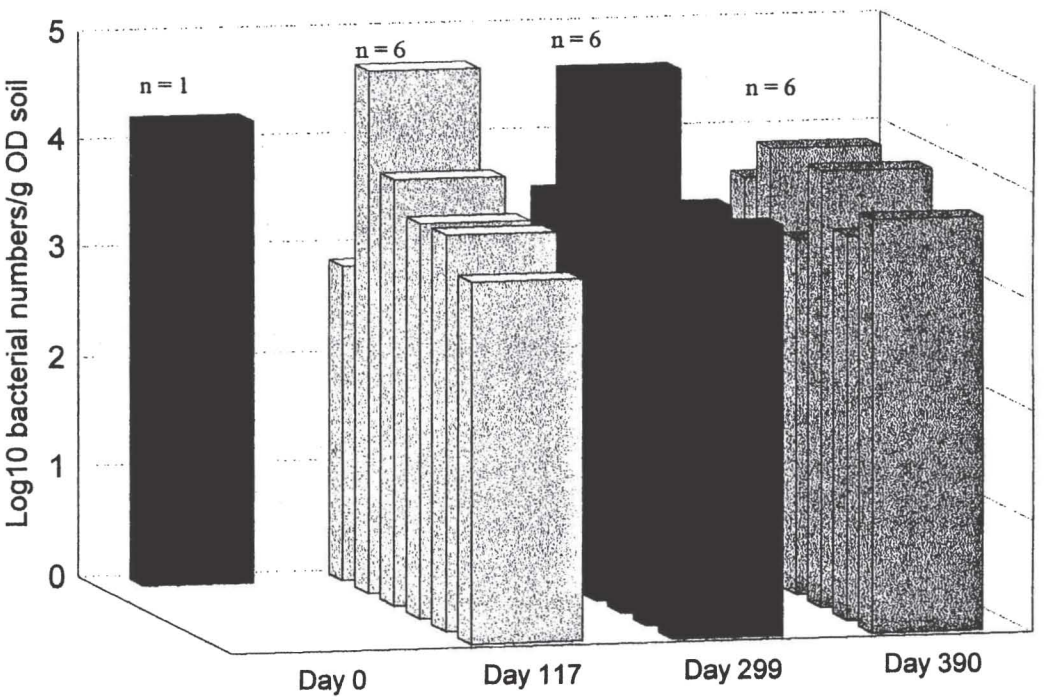


Fig. 31. Enumerations of total heterotrophic bacteria in individual soil cores in zone 3, area B during the course of the experiment.

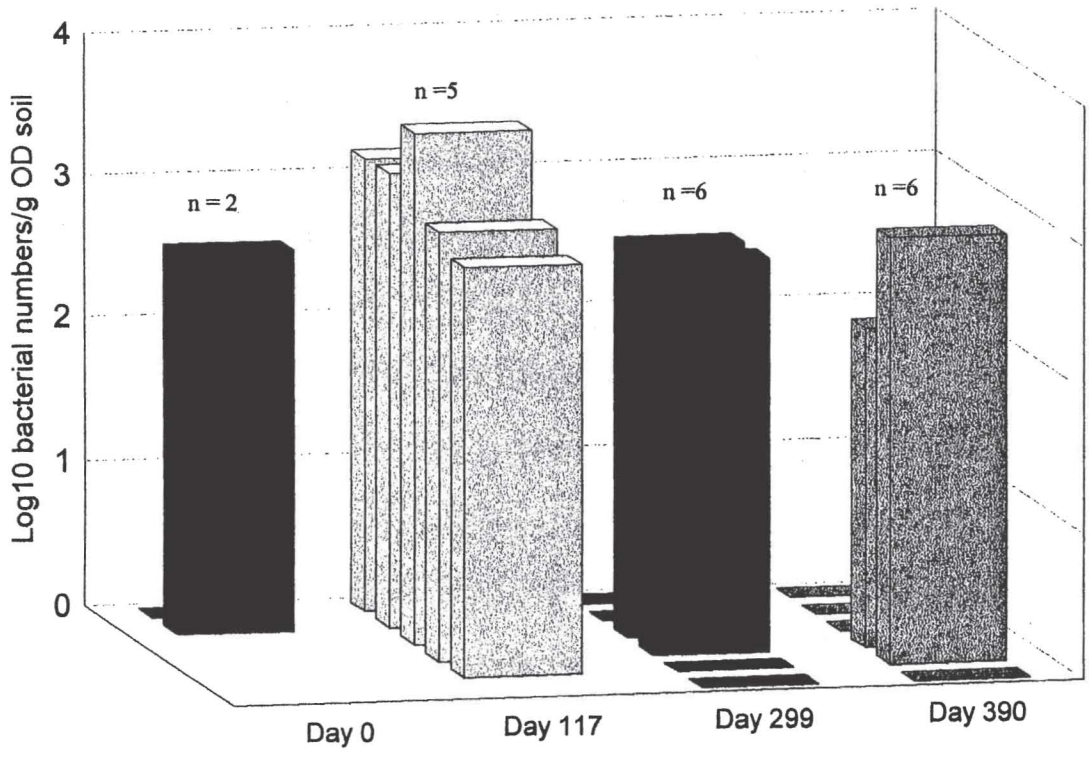


Fig. 32. Enumerations of phenanthrene-degrading bacteria in individual soil cores in zone 3, area A during the course of the experiment.

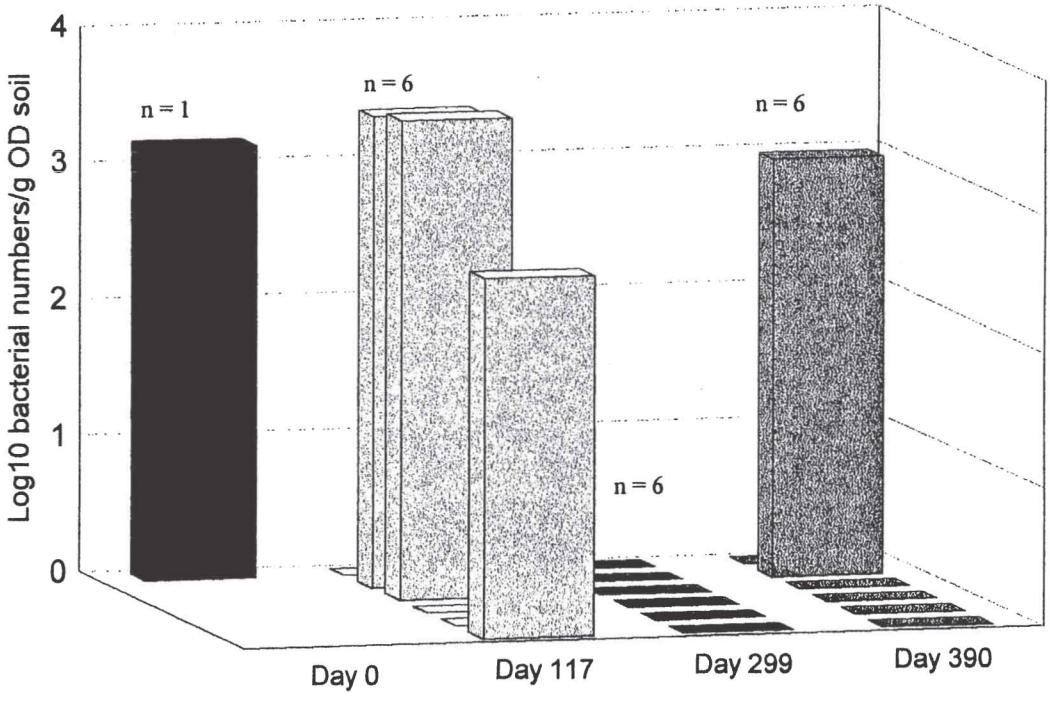


Fig. 33. Enumerations of phenanthrene-degrading bacteria in individual soil cores in zone 3, area B during the course of the experiment.

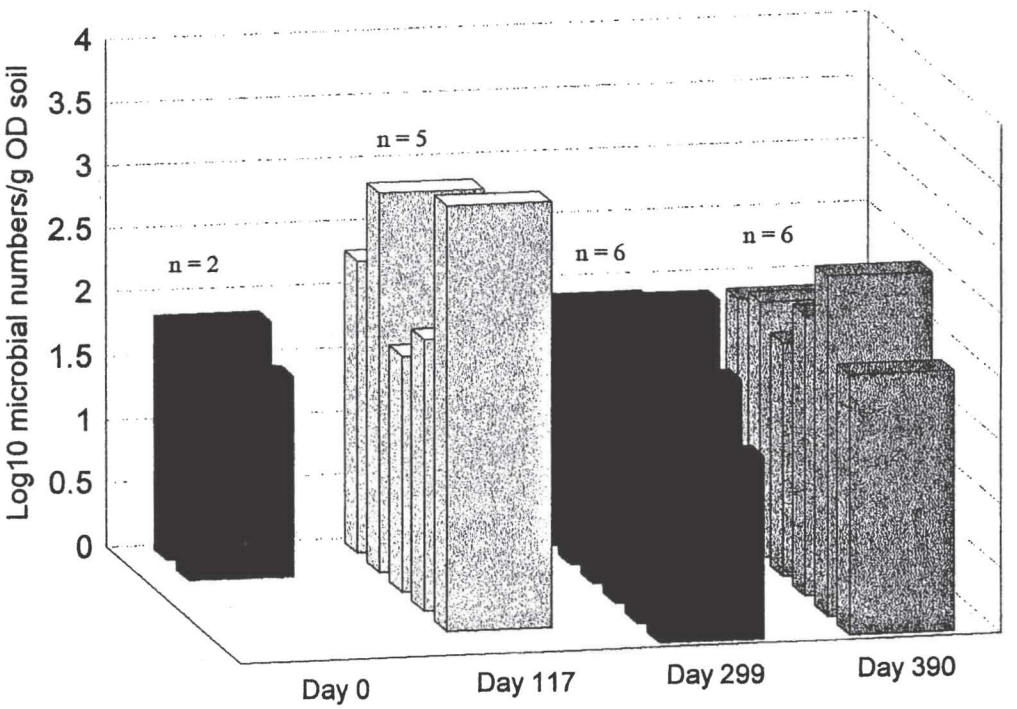


Fig. 34. Enumerations of diesel-degrading microorganisms in individual soil cores in zone 3, area A during the course of the experiment.

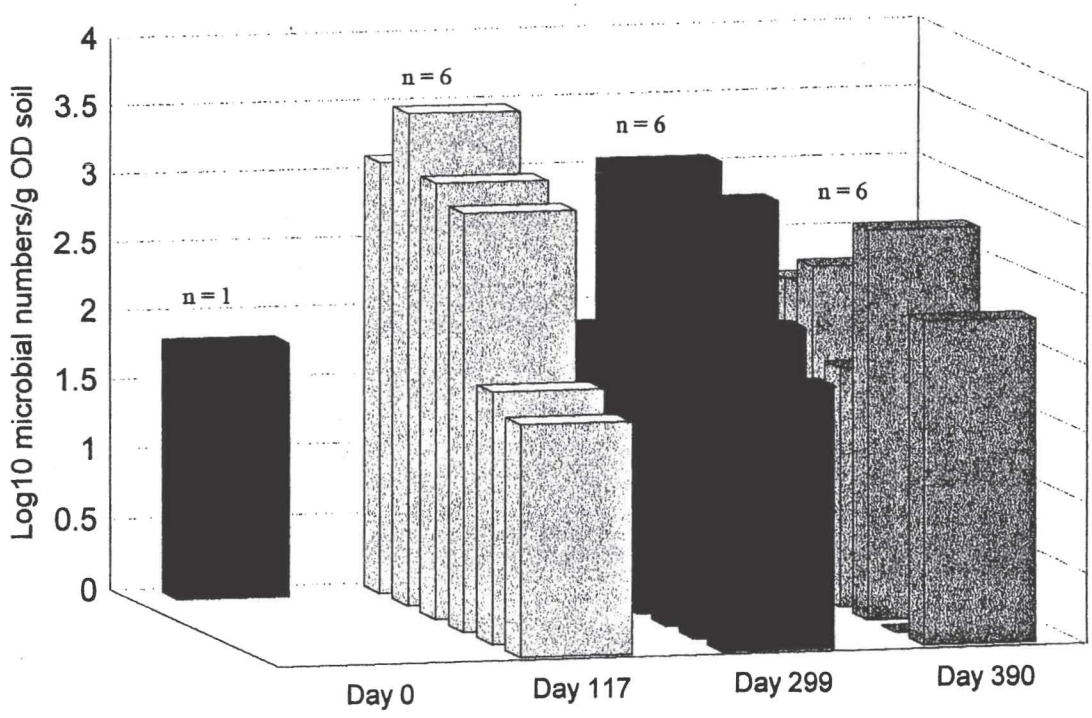


Fig. 35. Enumerations of diesel-degrading microorganisms in individual soil cores in zone 3, area B during the course of the experiment.

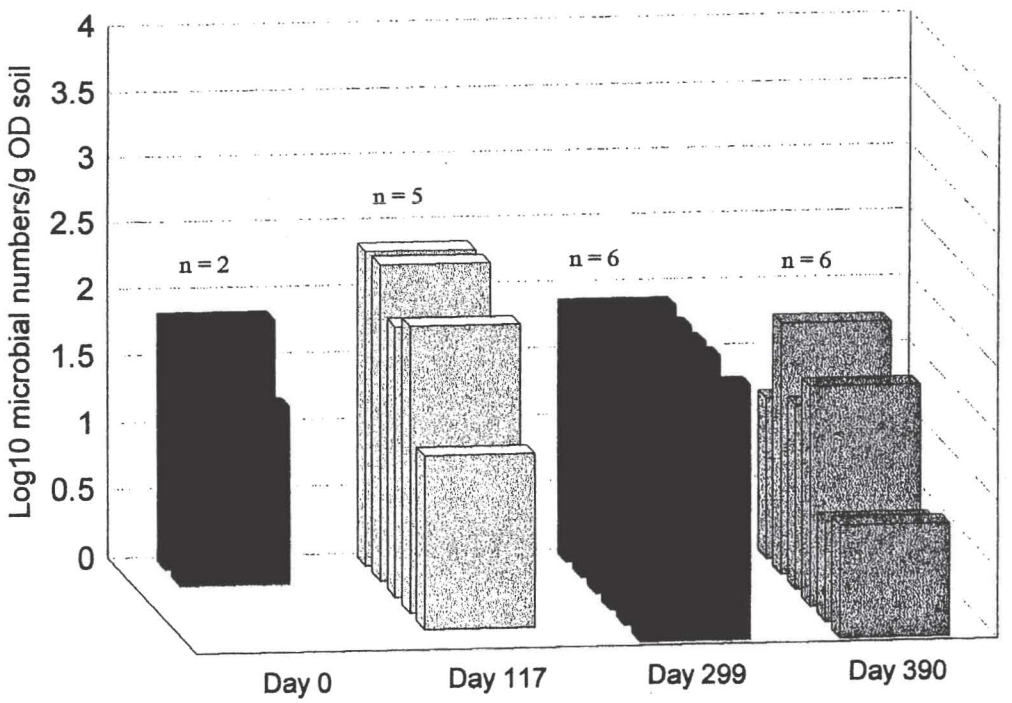


Fig. 36. Enumerations of pristane-degrading microorganisms in individual soil cores in zone 3, area A during the course of the experiment.

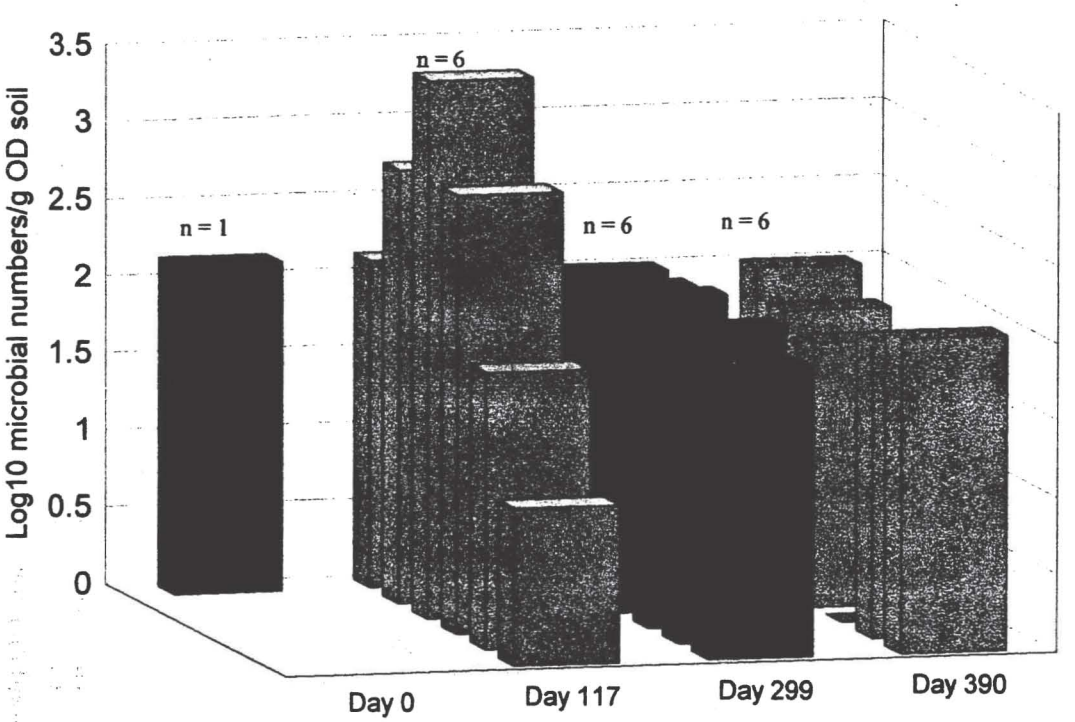


Fig. 37. Enumerations of pristane-degrading microorganisms in individual soil cores in zone 3, area B during the course of the experiment.

3.3.8.5. Sulfate-Reducing Bacteria in Zone 3

The SRB numbers in zone 3 were very low and even lower than those of zone 2 (Fig. 38). From the data collected, the SRB numbers in area B were relatively consistent throughout the experiment, however, the numbers in area A varied with time.

3.3.9. Biolog Identification of Select Bacterial Isolates from the Site

The hydrocarbon-degrading isolates from the well water and soil were identified by the Biolog system (Table 9). The bases of identification was on the pattern of substrate

Table 9. Isolate identification using the Biolog system and 16S rDNA sequence homologies

Name	Isolate origin	Gram stain	Growth on	BIOLOG ID	% Similarity	16S rDNA sequence homology	Homology[a] %
WWy1	well water 2/17/00	Neg	phenanthrene	*Sphingomonas paucimobilis*	76.6	Sphingomonas *sp.*	514/539 (95.36)[b]
PA1	well A water 5/26/00	Neg	phenanthrene	*Pseudomonas stutzeri*	63.8	*Pseudomonas stutzeri*	498/507 (98.2%)[b]
PA2	well A water 5/26/00	Neg	phenanthrene	No growth	ND	*Hyphomonas polymorpha*	1285/1290 (99.61)
PA3	well A water 5/26/00	Neg	phenanthrene	ND	ND	*Sphingomonas paucimobilis*	551/559 (98.6%)[b]
A23 Die	area A, zone 2 soil day 299	Neg	phenanthrene	*Pseudomonas stutzeri*	83.5	*Pseudomonas stutuzeri*	560/594 (94.28)[b]
BWD1	well B water 6/26/00	Neg	phenanthrene	*Sphingomonas paucimobilis*	45.9	*Sphingomonas* sp.	673/709 (94.92)[b]
A23 Pris	area A, zone 2 soil day 390	Pos	hexadecane, diesel, pristane, and mineral oil	*Rhodococcus rhodochrous*	56.8	ND	ND
B22 Pris	area A, zone 2 soil day 299	Neg	hexadecane and diesel	ND	ND	*Acinetobacter junii* X81664	1092/1095 (99.73)

[a]homology percentages are determined by the number of base similarities (ratio)
[b]unedited sequences that have not been quality controlled, but based on the homology percentages give an indication of phylogenetic affiliation.
Not determined (ND)

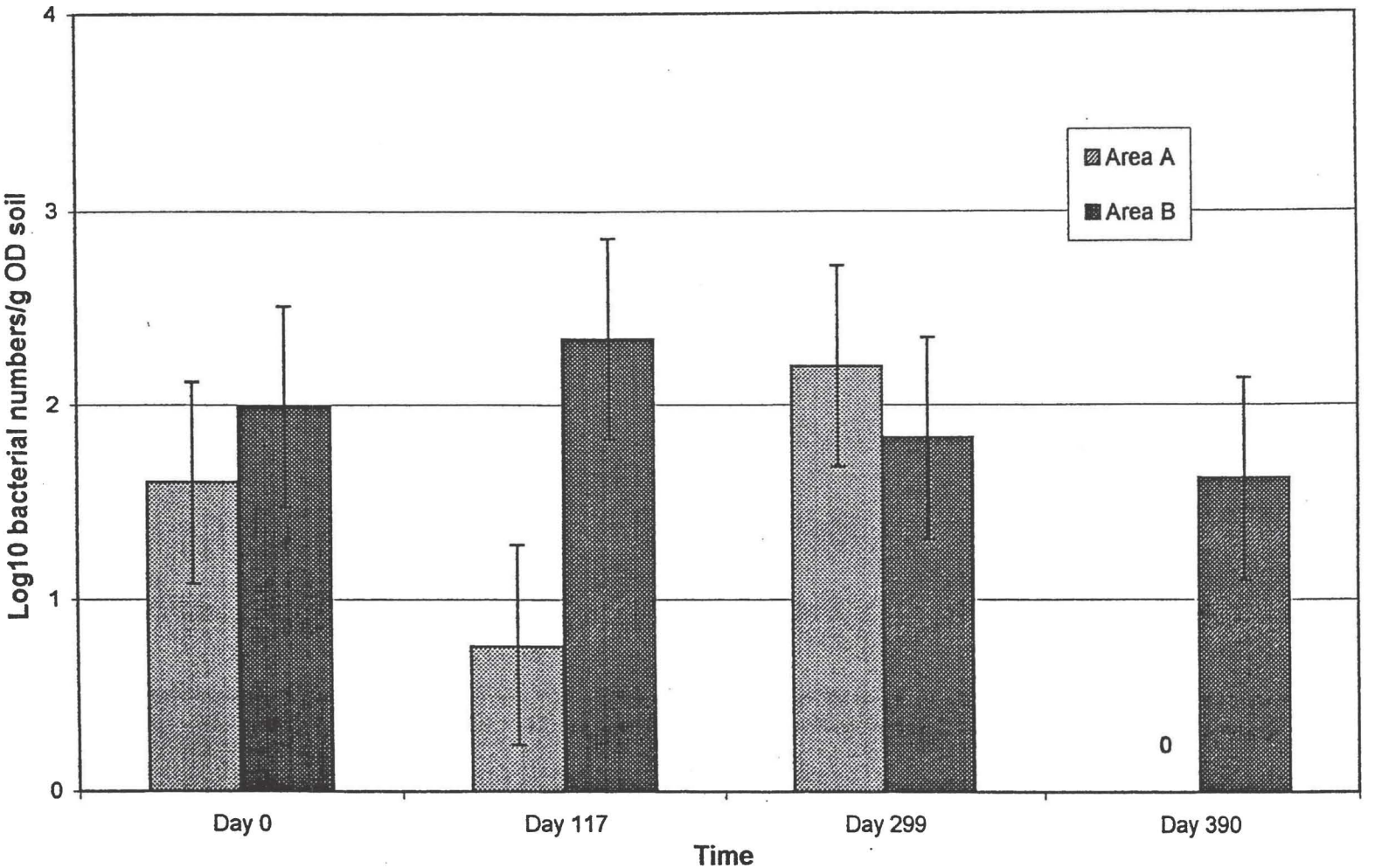


Fig. 38. Sulfate-Reducing bacteria numbers in composite soil samples of the subtidal zone 3.
Error bars are the 95% confidence intervals.
No SRB were detected in area A on day 390

utilization of 95 different carbon sources. The isolates identified belonged to the genera *Sphingomonas, Pseudomonas,* and *Rhodococcus.* Other isolates, not identified, were either not tested or did not grow on the carbon substrates.

3.3.10. Sequence Homologies of Isolates Identified by 16S rDNA Analysis

In addition to identification by the Biolog system, the hydrocarbon degrading isolates were identified by 16S ribosomal DNA (rDNA) homologies. Identification using rRNA or rDNA to determine genetic relatedness is applicable because a detailed evolutionary tree for prokaryotes has been developed based on analyses of 16S rRNA (Woese, 1987). The results of the identification based on 16S rDNA sequence homologies are shown in Table 9. The genera identified by sequence homologies were *Sphingomonas, Psuedomonas, Hyphomonas, and Acinetobacter.* Comparing the methods of identification, there was good agreement between the Biolog system and the 16S rDNA sequence homology.

Chapter 4
DISCUSSION

4.1. Contaminant Fingerprint

Weathering is a complex process involving several environmental processes such as volatilization, biodegradation, biotransformation, and dissolution (Millner et al., 1994). In this case, the weathering of the diesel fuel contaminant (Fig. 7 and 8) was due to significant biodegradation of the *n*-alkanes. Evidence for this is in fact that branched alkanes and *n*-alkanes have similar water solubilities and octanol water partitioning coefficients (Bregnard et al., 1998). Thus, the absence of *n*-alkanes in the weathered product has to be due to biological activity and not to transport or sorption processes. Also, the unresolved complex mixture (UCM) or "hump" is reported to be especially pronounced for biodegraded petroleum (Gough and Rowland, 1990). In the fingerprint (Fig. 7 and 8), the UCM is prominent and is different from that of the fresh diesel fuel standard. Also, it is well known that the *n*-alkanes are the most easily degraded hydrocarbon compounds (Olson et al., 1999). Thus, the weathering pattern of the diesel fuel at the site provides evidence that prior to our experiment, intrinsic bioremediation of the product had occurred. Consequently, to the extensive loss of the *n*-alkanes the branched alkanes, pristane and phytane, became the dominant resolved peaks. The presence of these peaks, along with the UCM hump indicates that the diesel fuel contaminant had significantly changed chemically and physically since its release into the subsurface.

4.2. Monitoring-Well Water

4.2.1. Well-Water pH

The high pH values measured (Fig. 9) are not unusual for a site adjacent to the ocean. The subsurface is characterized by coral rock, which contains high levels of calcium carbonate ($CaCO_3$), thus alkaline conditions prevail in the subsurface. Wells A and B had higher pH values than well C and the Ocean well, except on few occasions. The higher pH in these wells

can be explained by two possibilities, increased mixing, or microbial activity. In wells A and B there may be turbulence of the ground water and increased mixing caused by the air sparging. The mixing will often increase contact between the soil and the ground water (Brown and Jasiulewicz, 1992). In an area such as this where calcium carbonate deposition is high, the increased ground water mixing is likely to raise the pH. The pH may also be higher because of microbial activity. It is reported that alkalinity is a qualitative indicator of biodegradation, and that an increase in alkalinity in the area of the dissolved plume is indicative of ongoing intrinsic remediation (Curtis and Lammey, 1998). In well-buffered alkaline to neutral environments rich in calcium, carbon dioxide from aerobic or anaerobic microbial oxidations precipitates, at least in part, in the form of $CaCO_3$. The processes of ammonification, nitrate reduction, and sulfate reduction increase alkalinity and under appropriate conditions can contribute to $CaCO_3$ precipitation (Atlas and Bartha, 1993). The soil chemical analysis data (Appendix 2A) shows that there are high levels of calcium in the soil, ranging from 5000 to 8000 mg/kg. These high levels of calcium occurring at the site may combine with the carbon dioxide resulting from hydrocarbon transformation to precipitate $CaCO_3$. Also, in anaerobic sites in the subsurface there may be nitrate reduction and/or sulfate reduction occurring that would add to the alkalinity.

4.2.2. Well-Water Temperature

The temperature fluctuations in the wells appeared to follow a seasonal pattern (range being 27.5°C-32°C), but were small (Fig. 10). With these temperatures mesophilic conditions for bacterial growth existed at this site. Most soil microorganisms grow within the mesophilic range (Eweis et al., 1998), and optimum temperatures for biodegradation of petroleum products were found to be within the mesophilic range (Margesin and Schinner, 1997). The Ocean well showed the most fluctuation and the highest temperatures, due to its location very close to the shore and its shallowness (2-3 feet to the water table). It was under the most influence of the tide and probably was subjected to the heating and cooling of the asphalt surrounding it.

4.2.3. Well-Water Free-Product Observations

Based on the results of the visual observations of each well, there was a significant recontamination of well A water (Fig. 11). The fluctuations of the free product amount observed in well A seemed to indicate a transient contamination condition of the well water. It is reported that petroleum hydrocarbon contamination can exist in the subsurface as three condensed phases: mobile free product (separated phase), residually saturated soil (adsorbed phase), and contaminated ground water (dissolved phase). The distribution of the contaminant into these different phases is a function of dynamic transport, and of the physical and chemical properties of the various phases. Movement of ground water is the primary long-term mechanism for spreading contamination once the free product layer has achieved flow equilibrium (Brown and Crosbie, 1994). Hydrogeologic factors, such as seasonal fluctuations in ground water levels and changes in ground water flow directions, have been reported to affect the efficiency of bioremediation (Chapelle, 1999). At this site the free product movement observed in well A can probably be attributed to changes in ground-water flow direction. The visual observations indicated that an annual seasonal pattern of the fluctuations of the free product amount in well A probably exists. Based on the observations that were made on the first movement of free product in the well water observed from Dec. '99 to June '00, and part of the second movement observed from Dec. '00 to Feb. '01, the free product movement may be explained by the following pattern.

The start of the free product movement into well A occurs in late December. The peak of the influx occurs sometime during February, and the excessive free product remains until April. Gradually the free product moves out of well A, until it is no longer visible sometime around May.

The other wells (B, C, and Ocean) did not appear to be affected by the changes in ground water flow. All observations for these wells were consistent, and there was no free product in any of them throughout the experiment. Either these wells are not in an area highly influenced by ground water changes, or the free product contaminant is only located near well

A, so as to only infiltrate well A. The latter is a more likely reason, since the UST's were previously located close to well A (Fig. 2 map) and it had been reported (Rooks, 1995) that some product entered the subsurface immediately adjacent to the tanks.

4.2.4. Well-Water Dissolved-Oxygen (DO) Measurements

The measurements of DO levels in the well water not only allowed us to monitor the effectiveness of the aeration in wells A and B, but it also provided direct and indirect evidence for a bioremediation effect in well A. The initial DO concentration was just less than 3 mg/L for all wells, indicating low concentrations of available oxygen in all wells (Fig. 11). The application of the air treatment had two purposes: to enhance biodegradation and possibly to increase volatilization of susceptible compounds. After the start of aeration treatment in wells A and B the DO levels increased and reached near saturation levels (7.4 mg/L) in well water at 0.5% salinity. There was a lag in the increase of DO in well A, but approximately one month after the start of the aeration the DO levels began to increase greatly. This lag can be explained by an initial decrease in oxygen transfer efficiency, which is an inherent feature of air injection systems (Johnson et al., 1997). This transfer efficiency is explained as air channels that grow away from the injection point. As these air channels stop growing, the branches are depleted of their oxygen as it is transferred into the ground water or consumed by biotic and abiotic reactions. The net result is a decrease in oxygen mass transfer into the ground water until a steady-state is reached. Once there is an equilibrium established between the oxygen transfer efficiency and oxygen consumption, the levels of DO will increase, as we have seen in well A.

In well A the DO measurements also provide us with direct evidence of bioremediation According to Brown et al. (1994), and Johnson et al. (1997), the consumption of oxygen, or the decrease in DO levels are direct evidence of increased microbial activity. The second (day 150) and third (day 210) large decreases in DO levels in well A occurred when increased free product was observed. These decreases in DO indicate that the bacteria responded to the increased amount of contaminant, i.e., the increased amount of carbon supplied by the free

product. During the period where the five observations of heavy free product in the well were made (days 225 to 345), the DO levels could not be measured due to possible probe damage. It can be speculated, however, that the DO levels would have been lower than previously (<7 mg/L), due to microbial activity. Once the free product was no longer observed in the well, the DO levels returned to near saturation (7.4 mg/L) and were maintained until the end of the D.O. observations (day 554).

Well C, which was a non-contaminated, non-treated control, maintained very low levels of DO throughout the experiment. The DO concentration in this well was consistently less than 2 mg/L, indicating that near anoxic conditions prevailed. The Ocean-well DO levels fluctuated from 1 to 5 mg/L, but for the most part remained at a level lower than 3 mg/L. Again, fluctuations in this well probably reflect its location close the shoreline, under the most influence of the tide.

4.2.5. Ammonium-N and Nitrate-N Measurements in Well Water

The goal of maintaining low concentrations of available nitrogen in well A was attained for the most part (Fig. 14). On brief occasions both ammonium-N and nitrate-N levels were both very low, but otherwise there was a consistent supply of nitrogen in well A. In general, the concentrations of ammonium-N were lower than the concentrations of nitrate-N. This can be attributed to the fact that assimilation of ammonium ions into amino acids or other nitrogen-containing biochemicals by microorganisms is preferred to assimilatory nitrate reduction. Numerous microorganisms are known to assimilate ammonium ions relatively rapidly into organic nitrogen. In addition, ammonia inhibits assimilatory nitrate enzyme systems (Atlas and Bartha, 1993). The nitrate disappearance can probably be attributed to dissimilatory nitrate reductase, or denitrification. Dissimilatory nitrate reductase is not inhibited by ammonia, and has been found to be important in some sediments. Denitrification occurs under strict anaerobic conditions or under reduced-oxygen conditions, and may even occur in aerobic environments if there are anoxic microhabitats. It is a more environmentally

significant process than dissimilatory nitrate reduction to ammonia for the removal of nitrate and nitrite ions. However, the latter has been reported in sediments (Atlas and Bartha, 1993).

Another possibility for the losses of ammonium-N and nitrate-N could be the movement of ground water out of well A with the tidal fluctuations. This is less plausible because it would be expected that nitrate would show a more rapid disappearance than ammonium. Clay minerals and organic matter in soil possess sites of negative electrical surface charges, which attract positively charged ions, such as NH_4^+, and repel negatively charged ions such as, NO_3^- (Eweis et al., 1998). Thus, the nitrate would leach more rapidly as a result of the ground water movements, and there would be lower nitrate-N concentrations than ammonium-N concentrations. The data (Fig. 14) show, however, that throughout most of the experiment, the ammonium-N concentrations were slightly less than those of the nitrate-N. If the decreases in both were due to movement out of the well, the nitrate-N concentrations would be expected to be significantly less than the ammonium-N concentrations.

The measurements of nitrate-N and ammonium-N were both negligible in the water from wells B, C, and the Ocean well. These low values indicated that the nutrient solution added to well A was not moving toward the other wells located down-gradient of the presumed ground water flow (Fig. 5b).

4.2.6. Well-Water Enumerations

4.2.6.1. Total Heterotrophic Bacteria

Microorganisms capable of utilizing hydrocarbons are widespread in nature and can occur in soils as well as aquatic environments. The numbers of hydrocarbon degrading microorganisms that occur in nature are found to be related to the degree of hydrocarbon pollution (Blakebrough, 1977; Higgins and Gilbert, 1977). The first water sampling indicated that all monitoring wells (A, B, and C) had similar levels of heterotrophic bacteria, approximately 10^6 cells per ml of water (Fig. 15). At the start of the experiment the chemical conditions were similar in all three wells, so that they sustained approximately the same

number of microorganisms. Throughout the experiment the number of total heterotrophic bacteria increased in well A, especially at the time of recontamination. The number of total heterotrophic bacteria in wells B, C and the Ocean well, however, did not increase. The increase in heterotrophic bacteria observed in well A coincides with the recontamination, or the increase in available carbon in well A. Higgins and Gilbert (1977) found the number of hydrocarbon degraders in water samples from polluted sites to be two orders of magnitude higher than in non-polluted sites. Higher numbers were found where pollution was greater. Other studies have also reported that influx of oil into the environment increases the number of hydrocarbon-degrading microorganisms (cited by Higgins and Gilbert, 1977). Since both wells A and B were aerated, and there was no observed recontamination in well B, the increase in total heterotrophic bacteria cannot be attributed solely to the nutrient additions to well A. However, since higher numbers of total heterotrophic bacteria were maintained in well A, unlike the decrease in the other wells, it may be possible that the nutrients were stimulating the bacteria to utilize the available carbon, even if it was in low concentrations. No increase in numbers were seen at the start of the nutrient additions (day 28) because of low available carbon concentrations. Adding nitrogen will not increase the numbers if there is no carbon. The recontamination event supplied more available carbon to well A, and that plus the nutrients may have cause the increase in numbers during that time. The bacteria needed to have an increase in both the carbon and nitrogen source to support growth.

4.2.6.2. Phenanthrene Degrading Bacteria

The initial water sampling showed that all wells (A, B, and C) had similar levels of phenanthrene degrading bacteria (Fig. 16). During the time of the major recontamination (day 238), the levels of phenanthrene degrading bacteria increased in well A, but not in the other wells. Phenanthrene-degrading bacteria accounted for 1% of the total number of heterotrophic bacteria in well A on the first water sampling (day -27), and for 6% on day 238. This increase in the percentage of hydrocarbon degrading bacteria is considered to be indicative of bioremediation (Riser-Roberts, 1998). Since the number of phenanthrene-degrading bacteria

increased in well A only at the time of the recontamination, this increase can be attributed to more contamination in well A. The nutrient additions to well A may not be the cause of the increase in phenanthrene-degrading bacteria numbers at day 238, because the nutrients were present in well A at all times throughout the experiment. Again, however, the added carbon from the recontamination event plus the nutrients may have caused the increase in phenanthrene-degrading bacteria in well A, because the numbers were maintained from the start and increase when the recontamination occurs.

4.2.6.3. Diesel Degrading Microorganisms

The numbers of diesel degraders in all wells were low compared to the number of total heterotrophic bacteria, generally less than 10^5 cells per ml of water. The MPN technique allows growth of fungi as well as of bacteria. The majority of the microorganisms, however, probably belong to the bacteria group. The reason for the low numbers of diesel degraders is probably the chemical nature of the weathered diesel fuel product. Based on the GC fingerprint of the weathered product (Fig. 8), there were no remaining *n*-alkanes, only isoprenoid alkanes and compounds of the unresolved complex mixture (UCM). The microorganisms in the well water may be better adapted to these types of compounds, rather than to those that are found in the fresh diesel fuel used in the MPN assay. A similar result was obtained by Bregnard et al. (1996) in microcosm studies that compared weathered diesel fuel and *n*-alkane bioremediation. In this study, there was an increase in the number of diesel degraders in well A during the time of recontamination (Fig. 17). This suggests that there is some degradable portion of the free product pollutant that is recontaminating well A, which the microorganisms can utilize as a carbon source. The degradable portion of the contaminant probably stimulates hydrocarbon-degrading microorganisms in the well water. This stimulation is reflected in the increase in the diesel-degrader numbers (Fig. 8).

4.2.6.4. Pristane Degraders

The number of pristane degraders in the well water was low throughout the experiment (Fig. 18). Although the contaminant was highly weathered and contained branched

hydrocarbons, pristane is known to be very difficult to degrade (Olson et al., 1999; Balba et al., 1998). Reasons for such difficult degradation include extensive branching, and relatively low concentrations. These characteristics make pristane less bioavailable and degradable, and as a result, more persistent in the environment. Also, co-oxidation appears to be significant in the degradation of some hydrocarbons, so studies of growth on individual hydrocarbons are not a good indication of microbial responses to hydrocarbon mixtures, such as diesel fuel (Blakebrough, 1977).

4.3. Hydrocarbon and Microbial Analyses of the Contaminated Subsurface Soil

4.3.1. Hydrocarbon Recovery in Gas Chromatography

The results of the quality control experiment (Table 2) and the recovery of spiked diesel fuel (Table 3) and docosane experiments (Table 4) indicated that the laboratory results were reliable and accurate. These experiments were done to demonstrate that the entire analytical method was accurate. This means that the extraction of the product from soil and its measurements by the GC method were reproducible and the instrument was working properly. Mean recoveries obtained with the commercial diesel fuel (Table 3) and with the surrogate compound, docosane (Table 4), were similar, 83.9% and 83.57% respectively. Since the soil type of the site was very different from the standard matrices (Table 2) used for quality control purposes, an uncontaminated composite soil from the site was used for the determination of these recoveries. This demonstrated that the surrogate compound was a reliable indicator of recovery, and that TPH recoveries were within the acceptable range (70-130%) for evaluating method performance (EPA Method 8000B).

4.3.2. Results for Soil in the Tidal Zone (Zone 2)

The two-way ANOVA test for TPH means (Table 5) indicated that the main effect of area accounted for the differences among the means of areas A and B. Mean TPH measurements (Fig. 21) were higher in area A after the first sampling (day 0). Higher TPH concentrations measured in area A after day 0 seem to reflect the recontamination period

observed in the water of well A (Fig. 11). The TPH concentrations in the soil of zone 2 increased during the recontamination period, and decreased on day 390, when this period was over and no free product was observed in the well water. In general, the mean TPH concentrations in area B soil were less than 1000 mg/kg for each of the four samplings. Occasionally, a single core showed higher TPH concentrations, which is probably due to sampling near pockets of the diesel fuel free product within the soil. These contaminant pockets can be created by stratigraphic trapping, which occurs in the subsurface caused by variations in hydraulic conductivity (Chapelle, 1999). Therefore, although no free product was observed in the water of monitoring well B, there is still some residual contaminant that may exist within parts of the subsurface soil.

The mean PAH concentrations in area A were not significantly different from the means in area B, except on day 117 (Fig. 22). This single significant difference (day 117) may reflect the start of the recontamination in well A. Higher TPH concentrations would be expected to correspond to higher PAH values. Thus, with more contaminant in area A, an increase in both TPH and PAH concentrations would be expected. The mean PAH concentrations in area A, however, decreased after day 117, and were similar to concentrations found in area B for the last two samplings (days 299 and 390). This follows the trend during the later period of the recontamination of well A, where the diesel fuel free product slowly starts to move out of the well after day 313 (Fig. 11).

There was an inconsistency in the PAH concentrations of the soil on day 299, where the PAH concentrations in both areas A and B were similarly high. It was expected that the PAH concentration would be significantly higher in area A when the recontamination was high, however, concentrations of the contaminant in the well water may not be reflected in the same manner in the concentration of PAHs in the soil. Hydrophobic molecules, such as PAHs, are known to bind to organic carbon in soil by sorption (Weissenfels et al., 1992). Soil at this site contains little organic carbon, less than 2% (Appendix 2A), so sorption might be limited once the binding sites are occupied. The remaining non-sorbed portions of the

contaminant can subsequently move freely in the ground water, and some will even be diluted. Another reason for not measuring higher PAH concentrations in the soil of area A on day 299 is that more time may be required for the contaminant in the water to migrate onto the organic soil material, or into micropores that can form in sandy soils, which also bind hydrocarbons (Loser, et al., 1999). It is known that the longer a contaminant is in contact with soil, the greater the sorption will be onto the organic material (Weissenfels et al., 1992). Since this site is subjected to daily ground water fluctuations, it is highly likely that concentrations of PAHs in the soil may differ from that in the water, due to the movements of the ground water and soil washing. These processes may not allow enough time for the contaminant to adsorb tightly. Also, the mean PAH concentration for area B may have been high because of a single core with high TPH and PAH concentrations. As mentioned previously, there were pockets of free product within the soil that could have caused these high values in one sample, which would raise the mean value.

The fact that the PAH concentration in the weathered product was 14.5% of the TPH concentration, compared to 2% measured in fresh diesel (Chua-Chiaco, 1998), indicated that the diesel fuel contaminant had undergone significant biodegradation. The remaining product is a residual concentration that consists of branched and aromatic hydrocarbons. In bioremediation studies, residual concentrations often remain, commonly consisting of non-degraded branched and aromatic compounds that are difficult for microbes to degrade (Cornelissen et al., 1998; Bregnard et al., 1996). The remaining residual concentration may be due to its form as a non-aqueous phase liquid (NAPL). Once released, petroleum products exist as NAPLs, but with time some will transfer to the dissolved and sorbed phases. NAPLs affect the concentration of contaminant available for the microorganisms to degrade, by sequestering a large fraction of a hydrophobic pollutant away from the aqueous phase. This reduces the biodegradability of that compound (Eroymson, 1995). In addition to sequestering by the NAPL, the soil may affect degradation, since PAHs sorbed to soil also may not be

available to the microorganisms. This would increase the persistence of these residual PAH concentrations (Weissenfels et al., 1992; Cornelissen et al., 1998).

The microbial enumerations also reflect the pattern of recontamination in area A soil. The number of total heterotrophic bacteria increased by 2.5 orders of magnitude after day 0 in area A soil (Fig. 23). This increase can be attributed to an increase in available carbon from the recontamination of area A. The same is seen for the number of phenanthrene-degrading bacteria and of diesel and pristane degraders (Fig. 24, 25, and 26). There were significant differences between areas A and B after day 0, with the numbers in area A increasing. The increases in numbers observed in area A did not occur in area B, which seems to reflect the higher concentrations of TPH measured in area A (Fig. 21). These data are logical because the size of a microbial community in a soil sample (indicated by total bacterial numbers) should reflect the relative size of the metabolically active microbial community (Long et al., 1995). In this case there was an increase in total bacterial numbers, and in the numbers of hydrocarbon degraders. The fact that the numbers of total bacteria and specific hydrocarbon degraders were higher in area A than in area B is indicative of contaminant-adapted communities in area A, that were responding to the increase in organic carbon and energy from the diesel fuel free product.

The presence of sulfate reducing bacteria was expected in the microbial community of these diesel fuel contaminated subsurface samples. Sulfate is one of the most abundant electron acceptors in marine environments (Coates et al., 1997), and various sulfur compounds are present in diesel fuel that could fuel oxidative or reductive sulfur transformations. A wide variety of microorganisms are known to degrade hydrocarbon contaminants under highly reduced, anaerobic conditions, linked to sulfate reduction (Coates et al, 1997). In zone 2 the SRB numbers (Fig. 27) were low (2.5 log units) in both areas A and B. The counts of SRB were not significantly reduced after starting air sparging except for area A at day 299. Air sparging causes increased mixing of the aerated water with the deeper

anaerobic portions of the well and is expected to make the conditions less favorable for the SRB. However, anaerobic microsites may exist in aerated soil (Atlas and Bartha, 1993).

Higher contamination levels in area A only reflects the proximity of area A to where the bulk of the free product exists. According to the map of the previous location of the diesel fuel USTs (Fig. 2), well A is closest to this area where the leak occurred. The tidal influence probably causes smearing of the product throughout zone 2, due to the vertical movement of the water table (Johnson, 1994), and most of the product migration is probably due to ground water flow. This migration may occur on a daily basis and may be greater during certain periods of the year. A study on tidal effects of ground water levels indicates that the most influential and persistent variables affecting tides and ground water levels are the daily and semi-daily tidal fluctuations (Marquis and Smith, 1993). The same study also showed that there was a reversal in the direction of the net flow of ground water within only a 25-hour period. Thus, at any given time one single observation of ground water level or flow direction cannot be taken as accurate measurements of such parameters, because it is known that the ground water gradient and flow direction may vary with time.

According to the weathered diesel-fuel contaminant fingerprint (Fig. 8), it was determined that significant intrinsic degradation of the *n*-alkanes had occurred at the site. The remaining contamination is considered to be the residual concentration, which remains undegraded in the soil even when biodegradation conditions are optimal. The residual concentration is due to mainly the NAPL form of the contaminant (Efroymson and Alexander, 1995) and the soil characteristics and contaminant ageing (Nocentini et al., 2000). The diesel NAPL contaminant may sequester a large fraction of the hydrophobic pollutant away from the aqueous phase, which causes the concentration of the contaminant in water to fall below the threshold for biodegradation (Efroymson and Alexander, 1995). Although the soil at this site does not contain a large amount of clay material nor high amounts of organic matter, the residual hydrocarbons may be bound adsorptively and not be available for degradation. In a study by Loser et al., (1999), it was found that sandy soils can bind

hydrocarbons because of micropores which enlarge the soil surface area. These micropores were characterized as pores and fissures which increased the soil particle surface on a molecular level. After a period of rapid degradation, there was a dramatic decline followed by a complete stop in degradation, even when residual diesel fuel contamination was still present. The conditions at the Gray Line site reflect such processes. The diesel fuel contaminant is highly weathered, in a NAPL form, and is probably adsorbed onto the sandy matrix tightly. Thus, the residual concentrations of diesel fuel may not be stimulating the microorganisms. This is supported by the low initial values of TPH in the soil of areas A and B and in the water of wells A and B, and also by the small initial microbial populations in the soil of zone 2. After Day 0 the TPH concentrations and microbial numbers in area A increased presumably because of the recontamination, which probably provided some degradable components that the microorganisms could utilize. In area B, these increases in microbial populations did not occur because there was no recontamination. The lower values of hydrocarbon measurements and microbial enumerations in area B probably reflect the residual diesel fuel concentrations that either remained unavailable to the microorganisms throughout the experiment or were not degradable at an observable rate.

4.3.3. Results for Soil in the Subtidal Zone (Zone 3)

The TPH concentrations measured in zone 3 show heterogeneity among the cores sampled (Figs. 27 & 28). Generally, the concentrations in area A and B were less than 1,000 mg/kg. There was one exception where a concentration near 9,000 mg/kg was measured. However, this may or may not be an accurate measurement of the TPH concentration in zone 3. There is a possibility that this high concentration and two concentrations of 5,000 and 3,000 mg/kg, respectively, measured in two other samples may be attributed to the sampling method using the auger. As the auger is pushed deeper into the soil, it is conceivable that some free product might fall into the lower zone. As mentioned previously, most of the free product was observed in the tidal zone (zone 2), but since it is a free liquid it can move readily within the poly-acetate sleeves used for collecting the soil. On the other hand, these

more concentrated samples may be due to free product that traveled down through cracks between coral pebbles. Free product is known to follow pores and discontinuities in the subsurface (Riser-Roberts, 1998) and the gravel in some cores showed evidence of contamination and smelled of diesel fuel. If these few, more contaminated, samples are not taken into consideration, the data show that zone 3 is less contaminated than zone 2 in both areas A and B.

The zone 3 soil is saturated with water at all times, and the soil particles are fine and densely packed (mostly sand and silt). Because of this the porosity does not seem to be conducive to liquid flow. This would make it impermeable to liquid hydrocarbons or free product within the subsurface. According to the data and observations gathered throughout the experiment, it appears that there is some vertical migration of the free product in zone 2, but that migration is limited when the free product reaches the depths of zone 3. When a nonaqueous-phase liquid (NAPL) reaches the vicinity of the water table, its downward movement is slowed and it starts to spread laterally (Johnson, 1994). Hence, most of the diesel fuel contaminant is in the tidal zone, and may move laterally with directional changes of ground water flow.

In zone 3 the numbers of total heterotrophic bacteria and hydrocarbon degraders were very low. These low numbers may reflect the saturated conditions of this zone and/or the low levels of contamination. Since all enumerations were carried out aerobically, only the facultatively anaerobic bacteria were enumerated. The soil and contaminant levels in zone 3 were rather homogeneous and low in general, so the bacterial numbers were similarly low for all the cores sampled (Fig. 30-37). Other studies of microbial communities in contaminated and uncontaminated subsurface soils also find smaller total microbial populations with increasing depth (Long et al., 1994; Kampfer et al., 1991).

The sulfate-reducing bacteria (SRB) numbers in zone 3 (Fig. 38), were also less than those in zone 2. In zone 3 there was less contamination than in zone 2, and so there would be less carbon available to the SRB. In zone 3 the numbers in area B were relatively constant

throughout the experiment, but those in area A varied more. These variations observed in area A may be due to environmental changes caused by ground water and contaminant fluctuations, since these processes do not occur similarly in all areas.

4.4. Identification and Characterization of Hydrocarbon-Degrading Bacterial Isolates

Hydrocarbon-degrading bacteria isolated from the soil and well water showed little diversity, as only four different genera were identified. There are a few reasons for the apparent lack of diversity of the bacterial strains identified. First, it is well known that only a very small percentage of bacteria are culturable due to restrictions by the medium used and by the abundance of microorganisms in the sample. It is usually accepted that only 0.1 to 1% (or less) of all bacteria are isolated and cultured in a given sample (Kampfer et al., 1991; Bowman and Sayler, 1996). Second, the diesel fuel contaminant has significantly changed from its original state, and now contains more difficult-to-degrade compounds. These compounds may only be degraded by a select group of bacteria that are able to grow on them by secreting some type of emulsifier or by some mechanism of cometabolism. Some bacteria known to produce emulsifiers are *Pseudomonas, Acinetobacter,* and *Rhodococcus* (Harayama et al., 1999). Three of the isolates from the Gray Line site (Table 9) belong to those three genera. Lastly, the diversity of the culturable bacteria may be low because of the prevailing conditions in the subsurface before the implementation of the bioremediation treatments. The subsurface was largely anaerobic and nitrogen limited before the start of the experiment. These conditions may have selected for anaerobic and/or facultatively anaerobic bacteria, some of which would not be cultured by the methods used in this experiment. Enumerations of sulfate-reducing bacteria were made, but no attempts were made to isolate and identify these bacteria.

An additional analysis of the DNA in soil by molecular methods may reveal a more diverse bacterial community. One method would be the identification of rRNA genes in DNA extracted directly from the environment, typically by polymerase chain reaction (PCR)

amplification, cloning, and sequencing. A number of studies on molecular microbial diversity of soil have been reported, and only recently has there been a similar analysis of a contaminated aquifer undergoing intrinsic bioremediation (Dojka, et al., 1998). Due to financial and time constraints, an analysis like this could not be implemented for this experiment. Such analysis at the Gray Line site would be very beneficial for studying the dynamics of a complex microbial community in the subsurface.

4.5. General Conclusions

Bioremediation has been used to treat petroleum-hydrocarbon contaminated ground water systems for nearly three decades. The technologies evolved in ways that were clearly anticipated by early investigators, and in ways not foreseen. Even with recent technologies, Chapelle (1999) reports technical problems associated with effectively delivering nutrients and electron acceptors to contaminated ground water and aquifers. Most of these technical problems are directly related to hydrogeologic complexities and heterogeneity of ground water systems.

In this study, it was determined that the diesel fuel contaminant was highly weathered due to the occurrence of instrinsic degradation of the *n*-alkanes before the start of our bioremediation treatment.

Area A was significantly recontaminated. During this recontamination event, which may occur annually, microbial growth and oxygen demand increased. This may indicate that some fractions of the weathered diesel fuel are susceptible to biodegradation. Since hydrocarbon concentrations in the well water and soil of area A increased during recontamination, direct proof of statistically significant hydrocarbon biodegradation enhancement in the subsurface remains elusive. Instead, logically linked indirect evidence, such as increased bacterial numbers and decreased dissolved oxygen concentrations in area A, provide indication of *in situ* biodegradation.

Effects of air and nutrient treatments in area A could not be evaluated because of the recontamination. Although the hydrocarbon concentrations increased, they did not exceed the State of Hawaii, Department of Health 5,000 mg/kg action levels for TPH-diesel in soil. This does not infer, however, that TPH-diesel concentrations in other areas of the site do not exceed the action level.

The diesel fuel contaminant is at a residual concentration that is difficult for the microorganisms to degrade. It is not known if this concentration can be decreased, since studies report that the hydrocarbon content cannot be reduced to zero when bioremediation treatment is applied, and as a rule about 10 to 30% of the initial contamination amount remains (Margesin and Schinner, 1997). However, bacteria isolated from the site degraded various hydrocarbons. This indicates that microorganisms in the subsurface may have the potential to degrade the residual diesel fuel contaminant. Since a residual contaminant persists, there is an opportunity for advanced bioremediation studies. In advanced studies, traditional techniques, such as nutrient and air treatments, are replaced by more progressive methods. Such studies may include surfactant additions to enhance bioavailability or cometabolism, or the introduction of specialized bacterial strains proven to degrade specific compounds or produce their own bioemulsifiers to speed degradation (Alexander, 1999; Riser-Roberts, 1998).

APPENDIX

Appendix 1. Raw data of individual core analyses for each sampling day

Day 0 Data

Core	TPH (mg/kg)	PAH (mg/kg)	Pristane (mg/kg)	Phytane (mg/kg)	R2A*	PHE*	DIE*	PRIS*	SRB* (avg)
A17-2	581.25	174.80	10.69	7.37	3.59	3.12	2.26	2.40	
A22-2	0.00	41.30	4.30	3.10	4.15	3.21	2.95	2.00	
A41-2	0.00	1.20	0.00	0.00	3.00	0.00	1.60	1.85	
A47-2	890.12	98.10	12.90	9.28	4.10	2.99	1.96	1.79	
A73-2	9.87	3.50	1.59	1.55	3.81	0.00	2.30	0.00	
A90-2	616.44	129.80	10.27	7.12	4.18	2.95	2.09	1.58	2.51
B4-2	490.93	98.00	8.55	6.95	4.65	2.31	2.60	1.99	
B28-2	67.41	9.80	2.51	2.18	3.95	0.00	1.81	0.00	
B44-2	29.21	6.20	1.77	1.67	3.98	3.40	2.60	2.71	
B50-2	389.90	63.50	8.28	6.10	3.98	2.93	2.08	0.89	
B83-2	152.61	39.20	1.98	3.06	3.92	0.00	1.84	1.85	
B99-2	2470.64	131.80	21.05	16.98	3.74	2.63	1.93	1.79	2.57
C4-2	0.00	0.00	0.00	0.00	3.86	0.00	2.41	1.27	
C32-2	0.00	0.58	0.00	0.00	4.76	0.00	4.12	2.33	
C39-2	0.00	1.08	0.00	0.00	4.17	2.71	2.47	2.84	
C67-2	55.75	0.00	2.27	2.02	4.45	2.98	2.82	2.60	
C83-2	88.05	0.63	2.03	1.86	4.45	0.00	2.71	1.82	
C98-2	37.71	3.70	1.88	1.77	4.33	2.71	4.60	3.63	3.58
A47-3	860.67		13.20	9.20	3.61	0.00	1.84	1.84	
A90-3	302.21		4.30	4.07	3.68	2.64	1.57	1.31	1.60
B4-3	444.60		11.54	8.26	4.22	3.17	1.81	2.13	1.99
C4-3	0.00		0.00	0.00	4.65	3.53	1.61	1.69	
C39-3	0.00		0.00	0.00	4.63	3.15	2.70	1.89	
C67-3	0.00		2.27	2.02	3.56	0.00	2.14	2.02	
C83-3	12.71		1.59	0.00	4.60	2.67	2.70	2.30	
C98-3	75.81		2.60	2.30	3.32	0.00	1.48	1.76	2.36

*indicate Log10 values of bacterial and microbial enumerations

D117 Data

Core	TPH (mg/kg)	PAH (mg/kg)	Pristane (mg/kg)	Phytane (mg/kg)	R2A*	PHE*	DIE*	PRIS*	SRB* (avg)
A10-2	1913.75	321.30	33.48	22.49	7.08	4.56	3.28	1.04	
A14-2	2052.30	286.00	37.89	24.79	7.46	5.66	2.76	1.29	
A44-2	751.40	266.00	14.94	9.85	5.49	4.20	3.51	1.29	
A59-2	1521.41	96.00	24.37	17.30	7.41	5.76	2.79	0.00	
A77-2	2629.08	141.00	46.83	30.61	5.28	4.55	3.27	2.74	
A81-2	1434.15	230.00	27.12	18.02	7.20	4.69	4.32	3.78	1.92
B11-2	0.00	0.63	0.00	0.00	2.90	0.00	0.73	0.73	
B19-2	1152.08	130.20	21.75	15.04	3.97	0.00	3.11	2.79	
B37-2	684.32	102.00	12.94	9.17	3.40	0.00	2.78	0.00	
B53-2	1702.92	242.00	30.28	20.23	3.41	2.61	3.29	0.00	
B67-2	698.41	99.00	13.18	9.06	3.89	2.61	1.74	0.00	
B95-2	101.68	10.00	1.29	1.32	3.51	0.00	0.65	0.00	ND
A10-3	57.04		0.99	0.96	4.04	3.15	2.29	2.34	
A44-3	1234.60		24.91	17.21	4.38	3.16	2.98	2.36	
A59-3	318.93		6.05	4.21	4.04	3.56	1.85	2.01	
A77-3	8952.08		143.92	94.91	3.81	2.97	2.13	2.13	
A81-3	5718.71		94.06	61.74	4.08	2.84	3.33	1.27	0.76
B11-3	125.47		2.79	2.27	2.85	0.00	3.11	2.12	
B19-3	795.59		15.61	11.16	4.78	3.45	3.56	2.81	
B37-3	3229.42		51.22	33.99	3.88	3.51	3.14	3.49	
B53-3	157.60		3.34	2.45	3.59	0.00	3.01	2.85	
B67-3	91.99		1.78	1.50	3.60	0.00	1.80	1.80	
B95-3	842.27		13.88	10.17	3.28	2.63	1.65	1.02	2.34

*indicate Log10 values of bacterial and microbial enumerations
ND not determined

Day 299 Data

Core	TPH (mg/kg)	PAH (mg/kg)	Pristane (mg/kg)	Phytane (mg/kg)	R2A*	PHE*	DIE*	PRIS*	SRB* (avg)
A13-2	1211.38	234.6	47.25	35.05	5.57	0.00	2.94	2.57	
A34-2	1515.84	340	49.40	34.46	6.78	4.08	3.93	2.53	
A39-2	369.70	27	10.90	9.53	7.18	4.09	4.28	2.73	
A62-2	49.52	6.7	2.47	2.08	6.37	3.82	3.73	1.41	
A67-2	3234.46	303	106.48	75.94	6.95	3.93	3.45	2.76	
A99-2	1496.88	386	52.90	38.44	3.72	0.00	2.78	1.08	1.36
B2-2	596.94	141.4	21.67	15.78	3.89	0	2.83	1.83	
B18-2	55.51	122	3.27	2.55	2.97	0	1.95	1.95	
B23-2	3091.48	586	115.00	79.93	3.65	0	2.93	2.76	
B48-2	1833.78	303	70.13	48.02	4.17	3.89	2.26	2.26	
B71-2	0	0	0.00	0.00	3.21	0	1.60	1.28	
B88-2	121.80	13.3	5.52	4.67	3.84	0	1.28	1.43	2.91
C5-2	0	0	0.00	0.00	3.50	0	2.26	1.48	
C19-2	0	0.5	0.00	0.00	3.42	0	2.04	1.88	
C23-2	0	0.84	0.00	0.00	3.73	0	2.41	2.26	
C57-2	0	0.73	0.00	0.00	3.44	0	2.81	1.72	
C63-2	0	0.22	0.00	0.00	2.81	0	1.88	2.00	
C96-2	55.30	5.05	1.70	1.82	3.83	0	1.60	1.49	2.26
A13-3	105.49		3.95	3.01	3.37	0.00	1.89	1.89	
A34-3	145.52		5.07	3.61	3.96	0.00	2.00	1.86	
A39-3	1213.53		32.44	22.76	4.14	2.72	2.18	1.86	
A62-3	1176.97		36.21	25.68	3.88	2.72	2.34	1.87	
A67-3	97.48		3.00	2.28	2.94	0.00	1.88	1.57	
A99-3	111.21		3.54	2.61	3.80	0.00	1.41	1.88	2.2
B2-3	205.72		6.15	4.47	3.48	0	1.86	2.00	
B18-3	1529.12		53.98	37.38	4.73	0	3.15	1.99	
B23-3	56.27		2.04	1.55	3.40	0	1.87	2.04	
B48-3	151.30		5.25	3.74	3.67	0	3.04	1.88	
B71-3	44.87		1.30	0.00	3.39	0	2.20	2.04	
B88-3	0		0.00	0.00	3.73	0	1.88	1.88	1.83
C5-3	0		0.00	0.00	4.45	0	1.98	1.83	
C19-3	0		0.00	0.00	3.89	0	2.00	1.85	
C23-3	0		0.00	0.00	3.56	0	1.87	2.04	
C57-3	0		0.00	0.00	4.41	0	2.18	2.00	
C63-3	0		0.00	0.00	3.29	0	1.85	1.85	
C96-3	0		0.00	0.00	3.44	0	2.20	1.86	2.18

*indicate Log10 values of bacterial and microbial enumerations

Day 390 Data

Core	TPH (mg/kg)	PAH (mg/kg)	Pristane (mg/kg)	Phytane (mg/kg)	R2A*	PHE*	DIE*	PRIS*	SRB* (avg)
A4-2	1673.07	108.00	24.14	16.77	6.64	4.18	4.76	3.43	
A8-2	1434.26	114.00	26.52	16.91	6.11	4.31	4.75	3.91	
A31-2	1183.56	88.40	22.34	14.78	6.46	4.27	3.76	3.28	
A58-2	1660.83	175.00	22.34	16.11	5.81	3.32	3.28	1.28	
A72-2	415.38	20.00	8.84	6.00	7.11	4.35	4.91	2.43	
A94-2	1225.52	79.00	19.52	12.52	4.28	2.61	2.08	1.63	1.73
B15-2	916.08	149.00	20.10	12.78	2.87	0.00	0.00	0.70	
B27-2	1783.56	114.50	33.92	22.49	3.40	0.00	1.73	0.00	
B32-2	0.00	0.20	0.00	0.00	3.04	0.00	0.72	1.08	
B39-2	243.35	15.00	6.24	4.14	3.96	0.00	0.68	0.68	
B74-2	335.48	30.70	4.31	3.74	3.20	0.00	1.78	2.53	
B80-2	1228.67	224.00	24.90	16.11	3.99	0.00	0.00	0.70	1.62
C7-2	0.00	0.50	0.00	0.00	3.71	0.00	1.79	1.54	
C14-2	0.00	0.40	0.00	0.00	3.38	0.00	0.00	0.00	
C26-2	0.00	0.00	0.00	0.00	3.00	0.00	1.77	0.00	
C65-2	0.00	0.20	0.00	0.00	3.67	0.00	2.28	0.00	
C80-2	0.00	0.30	0.00	0.00	4.58	0.00	3.30	0.70	
C91-2	0.00	0.30	0.00	0.00	3.00	0.00	1.76	0.00	2.38
A4-3	267.30		4.64	3.24	3.61	0.00	1.88	1.18	
A8-3	325.87		7.13	4.81	3.41	0.00	2.00	1.86	
A31-3	493.88		10.00	7.13	3.23	0.00	1.83	1.36	
A58-3	280.41		5.79	4.21	4.74	2.21	2.20	1.62	
A72-3	1870.62		41.53	29.07	4.00	2.96	2.66	0.73	
A94-3	255.76		5.38	3.64	3.59	0.00	2.04	0.82	0.00
B15-3	360.48		7.49	5.21	3.58	0.00	2.18	2.00	
B27-3	244.75		5.65	3.94	3.96	3.06	2.36	1.82	
B32-3	0.00		0.00	0.00	3.23	0.00	1.71	1.71	
B39-3	532.34		9.33	5.97	3.98	0.00	2.81	0.00	
B74-3	152.44		3.14	2.28	3.49	0.00	0.00	1.93	
B80-3	536.71		7.65	5.14	3.76	0.00	2.32	2.04	1.62
C37-3	0.00		0.00	0.00	3.38	0.00	2.30	0.00	
C14-3	0.00		0.00	0.00	3.30	0.00	1.68	0.00	
C26-3	0.00		0.00	0.00	3.15	0.00	0.80	0.00	
C65-3	0.00		0.38	0.00	3.34	0.00	1.20	0.00	
C80-3	0.00		0.31	0.00	3.40	0.00	1.45	0.00	
C91-3	0.00		0.00	0.00	3.11	0.00	0.80	0.00	1.86

*Indicate Log10 values of bacterial and microbial enumerations

Appendix 2. A. Results of soil chemical analyses performed on composite samples of six cores from zone 2
Analyses performed by University of Hawaii at Manoa, Agricultural Diagnostic Service Center

Day/Sample	pH	Phos. (P)	Potass. (K)	Calc. (Ca)	Magn. (Mg)	Boron (B)	NO3-N	NH4-N	Nitrogen (N)	O. carbon	EC	SO4-S
		mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	%	%	mmho/cm	mg/kg
Day 0 - A2	8.1	34	716	5366	1538	5.1	0	22	0.07	1.92	5.6	ND*
Day 117 - A2	8.5	12	66	7350	390	0.7	0	2.08	0.02	0.55	0.94	ND
Day 299 - A2	8.3	15	114	8036	512	1.2	11.94	3.34	0.08	0.78	1	200
Day 390 - A2	8.2	9.2	44	7262	354	0.93	0	2.1	0.21	0.56	1.08	189
Day 0 - B2	8.2	41	746	5316	1558	3.8	0	17.54	0.05	1.48	5.3	ND
Day 117 - B2	8.6	16	190	7116	516	0.94	0	2.88	0.02	0.59	0.64	ND
Day 299 - B2	8.6	11	94	7820	424	0.66	1.55	1.61	0.07	0.55	0.65	141
Day 390 - B2	8.4	13	50	7058	292	0.79	0	1.47	0.21	0.52	0.58	77
Day 0 - C2	8.3	39	796	5680	1576	2.7	0	14.53	0.05	1.22	4.4	ND
Day 299 - C2	8.9	10	6.2	8070	312	0.21	0	0	0.07	0.27	0.261	0
Day 390 - C2	8.3	20	158	7278	598	0.97	0	3.11	0.21	0.64	0.55	88

*ND, No data were collected

Appendix 2. B. Results of soil chemical analyses performed on individual cores from zone 2
Analyses performed by University of Hawaii at Manoa, Agricultural Diagnostic Service Center

Day	Core	pH	Phos. (P)	Potass. (K)	Calc. (Ca)	Magn. (Mg)	Boron (B)	NO3-N	NH4-N	Nitrogen (N)	O. carbon
			mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	%	%
Day 0	A47-2 (C)*							0	21.83	0.06	
	A41-2 (F)**							0	24.07	0.08	
	B44-2 (C)							0	22.15	0.08	
	B4-2 (F)							0	19.92	0.08	
	C67-2 (C)							0	18.9	0.06	
	C98-2 (F)							0	23.28	0.08	
Day 299	A67-2 (C)	8.7	12	40	7636	348		0.05	1.3	0.07	
	A99-2 (F)	8.5	19	156	7598	560		0.1	4.75	0.09	
	B48-2 (C)	8.8	8.1	14	7532	266		0.09	0	0.07	
	B2-2 (F)	8.8	7.6	2.4	7788	228		0.05	0	0.07	
	C57-2 (C)	8.8	6.7	8.4	8174	322		0.08	0	0.07	
	C96-2 (F)	8.7	13	14	7874	314		1.3	0	0.07	
Day 390	A58-2 (C)	8.4	13	56	7442	406		0	3.41	0.21	0.76
	A4-2 (F)	8.1	14	100	7224	442		0	4.78	0.21	0.77
	B27-2 (C)	8.8	9.4	20	7220	206		0	0.56	0.21	0.81
	B80-2 (F)	8.6	9.9	32	7102	248		0	0.91	0.21	0.66
	C65-2 (C)	8.4	16	152	7262	536		0	2.92	0.21	0.52
	C91-2 (F)	8.3	17	298	7382	744		0	4.88	0.21	0.61

***(C) = Close to the monitoring well (Injection point for nutrients & oxygen)**
****(F) = Far from the monitoring well (Injection point)**
Individual soil samples from Day 117 were not analyzed due to insufficient amounts of soil collected.

SECTION III

MODELING HYDROCARBON BIODEGRADATION IN TIDAL AQUIFERS

1. Introduction

As explained earlier, indigenous microbial populations have the potential to transform petroleum hydrocarbons to non-noxious products such as water, carbon dioxide, and minerals. These populations must be supplied with enough oxygen and inorganic nutrients (nitrogen, phosphorus, micronutrients) to be able to incorporate the organic carbon into the microbial biomass (Aggarwal et al., 1991; Atlas, 1991). Swindoll et al. (1988) showed that subsurface microbial communities respond better to the addition of multiple inorganic compounds. Therefore it is imperative to assess the fate of oxygen and nutrients as affected not only by bacterial activities but also by water-flow conditions. In addition, environmental variables, such as temperature and water-content distribution, play an important role in the degradation process. Accurate assessment of bacterial activities greatly depends on a knowledge of the spatial distribution of these parameters.

Conditions in nearshore environments can be hydraulically complicated due to tidal influences. Little information is available regarding the response of populations of subsurface microorganisms to alternating periods of soil wetting and drying. The bioremediation of No. 2 diesel fuel in soil columns was more pronounced when periods of dryness alternated with periods of flooding with a solution of nutrients (Widrig and Manning, 1995). In Louisiana salt marsh microcosms, a response to fertilization was observed in the biodegradation of hexadecane and phenanthrene (Jackson and Pardue, 1997). Similarly, in Texas salt marsh microcosms, the biodegradation of crude oil was stimulated by fertilization during the winter but was not affected during the summer (Wright et al., 1997). In contrast to the results of Widrig and Manning (1995), Wright et al.'s (1997) data indicated a higher biodegradation of crude oil when flooding was uninterrupted than when it alternated with drying periods. These diverse responses probably are a reflection of local differences in microbial populations and

environmental conditions, including the nature of the wetting and drying procedure and the oxygenation process. It is reasonable, however, to assume that product degradation due to bacterial activities is optimal at a certain degree of saturation and becomes less efficient at other values.

Cold temperatures affect the physical status of hydrocarbons by increasing the viscosity and decreasing the volatility of short chain alkanes (Atlas and Bartha, 1972). The enzymatic capabilities of some microorganisms decline or even stop under cold conditions. The effectiveness of remediation increases with the increase of temperature up to a point where activities diminish. Bossart and Bartha (1984) reported an optimal temperature of 30 to 40°C. The experiments of Wright et al. (1997) clearly showed the influence of temperature when comparing winter and summer degradation results.

The value of pH in soil is another controlling variable. Most bacteria and fungi prefer a neutral pH, even though fungi can withstand more acidic conditions (Dibble and Bartha, 1979.) In soil, pH can range from 2.5 to 11.0 (Bossert and Bartha, 1984); hence, pH variability is expected to have an important effect on the degradation process.

A large number of models for biodegradation has been documented in the literature. Essaid et al. (1995) compared a large number of models in terms of dimensionality, processes simulated, and kinetic representation. They concluded that most models are concerned with biodegradation processes that include a maximum of two substrates and two electron acceptors. Some models include the fate of nutrients necessary for bacterial growth. To improve on these models, Essaid et al. (1995) developed a two-dimensional model, suitable for field applications, for sequential aerobic and anaerobic biodegradation of volatile and nonvolatile fractions of dissolved organic carbon. The model was tested against both laboratory and field data. Nicol et al. (1994) introduced a detailed approach for one-dimensional modeling of biodegradation of residual hydrocarbons in fully saturated porous media. The model can simulate the degradation of a number of hydrocarbon components, all but one of which are degradable. A specific microbial population is associated with each

degradable component. The model includes nutrient and oxygen transport and a bacterial growth submodel that includes extracellular pseudosolubilization of the product as an intermediate process preceding hydrocarbon consumption by bacteria. The model was applied to a hypothetical soil column for demonstration purposes. However, because of numerical difficulties, this model is not suitable for large-scale field problems.

More recently, Clement (1997) developed a general model, RT3D, that solves the coupled partial differential equations describing reactive-flow and transport of multiple mobile and immobile species in three-dimensional saturated groundwater systems. The model includes an implicit reaction solver that makes the code sufficiently flexible for simulating various types of chemical and microbial reaction kinetics. It supports a number of reaction modules that can be used to simulate different types of reactive contaminants. In addition, the model has a user-defined reaction option that can be used to simulate other types of reactive transport systems. However, the model does not include unsaturated flow or temperature effects.

The objective of this part of the study is to develop a model that is suitable for the assessment of degradation of hydrocarbons in field applications where unsaturated soil conditions and temperature are of importance. The model is as general as that of Essaid et al. (1995) regarding the bacterial submodel, but it differs in including heat transport and water flow and species transport in the unsaturated zone. The model of Essaid et al. employs the method of characteristics in the solution within a finite difference scheme, which necessitates the use of a uniform grid. The current model benefits from the flexibility of the finite element method in handling domain configurations and from the ability to optimize the mesh design by using elements of different sizes. The flow and transport model is based on SUTRA (Voss, 1984), a widely used flow and transport model. Such a model was chosen because of its flexibility in dealing with time-dependent boundary conditions, including tides.

2. The Model

The model solves the convection/dispersion equations for various species within a modified version of SUTRA, each with the appropriate source/sink term. The source/sink terms reflect consumption/generation of species due to bacterial activities. The heat-transport equation is also solved, and temperature values are used to estimate the bacterial-inhibition factors.

Our aim is to introduce an efficient model that is suitable for assessing degradation in relatively large aquifers. Hence, a number of assumptions are adopted to increase the efficiency of the solution. In addition, the absence of a direct means to estimate some parameters can reduce the usefulness of comprehensive models simulating many processes. The assumptions are outlined below.

1. Although SUTRA can handle density variations due to solute or temperature effects, the current formulation neglects such changes, which can put limitations on model use. Ignoring density changes greatly simplifies the modeling effort by reducing the system of equations to a linear system. In principle, the approach can be modified to include density effects, but an iterative procedure should be used within each time step before the bacterial submodel is run. However, overlooking density effects in the current formulation does not prevent accounting for spatial variability of salinity, which can be treated as any other dissolved matter.
2. Details of bioactivities on the microscale (cell scale) is not considered; hence, a macroscopic approach is adopted for biodegradation. The macroscopic approach allows expressing various bacterial-growth processes as averages over a representative control volume.
3. Hydraulic parameters, such as conductivity or porosity, do not change with time, because of bacterial activities or chemical reactions.
4. Microbial transport due to water-flow or growth processes is ignored.

5. Solutes may be subjected to equilibrium adsorption on the porous matrix and to both first-order decay and zero-order production or decay.
6. The Richards (1931) equation for unsaturated flow is assumed valid. The main assumption is that air pressure is constant, and only water, the wetting fluid, is of concern (Brutsaert and El-Kadi, 1984). We are including a bacterial-inhibition equation to account for effects of water-content variation. Hence the influence of unsaturated water content on bacterial activities on the cell level are lumped into the inhibition equation that is assumed valid on the Darcy or macroscopic level. The proposed empirical equation assumes that maximum degradation due to bacterial activities occurs at an optimal degree of water saturation.
7. For the trapped product, such as that at residual saturation, bacteria will facilitate hydrocarbon dissolution before degradation. We assume here that hydrocarbon dissolved in the aqueous phase by bacterial activities is directly metabolized by the bacteria. Nicol et al. (1994) discuss the importance of considering intermediate processes between bacterial dissolution and degradation of the product.

The most limiting assumptions among those outlined above are probably the neglect of density changes in nearshore environments and the dissolution process preceding the degradation of a trapped product. Future updates can address both of these issues.

2.1. The Model SUTRA

SUTRA (Voss, 1984) is a finite-element simulation model for saturated-unsaturated, fluid-density-dependent groundwater flow. It also solves for energy transport or for a chemically reactive single-species solute transport. The model has been applied in the analysis of nearshore aquifers (e.g., Voss and Souza, 1987; Souza and Voss, 1987). The flow equation is a form of the Richards (1931) equation that includes effects of the unsaturated zone. The hydraulic properties of the soil are represented by the van Genuchten (1978) equations:

$$\Theta = \left[\frac{1}{1+(\alpha|h|)^n}\right]^m \tag{1}$$

$$K = K_s \Theta^{1/2}\left[1-\left(1-\Theta^{1/m}\right)^m\right]^2 \tag{2}$$

$$\Theta = \frac{\theta - \theta_r}{\theta_s - \theta_r} \tag{3}$$

$$m = 1 - \frac{1}{n} \tag{4}$$

Here θ_s is the saturated water content, θ_r is the residual water content, and α and n are fitting parameters.

The modular approach that we have adopted can accommodate other models as well. However, we have chosen SUTRA due to its flexibility in handling time-dependent boundary conditions, including tidal fluctuations. A time-varying condition can be either read in tabulated form from an external file or programmed in a parametric equation. In our model, solute uptake is estimated by the bacterial submodel, saved in an external file, and then read by the transport submodel. Tides are programmed in SUTRA's boundary-condition submodel.

2.2. Bacterial-Growth Model

The formulation adopted here is similar to that developed by Essaid et al. (1995) but differs in including inhibition effects. It will be summarized here for the sake of completeness. Bacterial growth is simulated using the following equation:

$$\frac{dX_k}{dt} = [\mu_k - d_k]X_k \tag{5}$$

where X_k is the biomass concentration of population k [ML^{-3}], $\mu_{k\,1}$ is its specific growth

$[T^{-1}]$, d_k is its specific death/regeneration rate $[T^{-1}]$, and t is time $[T]$. The specific growth rate is estimated from

$$\mu_k = \sum_{m=1}^{M} Y^m v^m \tag{6}$$

where M is the total number of biodegradation processes performed by population k, Y is the cell yield coefficient (M bacteria/M substrate), and v $[T^{-1}]$ is the substrate uptake rate. The latter is estimated from a variation of Monod kinetics, where either multiple or minimum Monod formulation. The minimum Monod formulation can be expressed as

$$v^n = \frac{V_{\max}^n}{I_{nc}} \min[C_i'] \frac{X_k^n}{\sum_{\beta=1}^{n_\beta} I_\beta} \qquad i = 1, 2, \ldots, m \tag{7}$$

where

$$C_i' = \frac{C_i}{K_{Ci} / I_c + C_i} \tag{8}$$

In these equations, i is the solute index, m is the total number of solutes, C_i is the solution concentration $[ML^{-3}]$, K_{Ci} is the saturation parameter for growth limitation regarding species i $[ML^{-3}]$, and $V_{\max}$ is the maximum substrate uptake rate $[T^{-1}]$ for process degradation n. In equations (7) and (8), I_{nc} and I_c are the noncompetitive and competitive inhibition factors, respectively. The variable n_β represents the number of inhibition factors, which can include I_b, I_θ, and I_T (the biomass, water-saturation, and temperature inhibition factors, respectively). Although no simulations are presented here for pH and other factors, such parameters can be included in a similar fashion. For example, the model can estimate the spatial distribution of salinity and correlate this to pH for use in estimating the pH inhibition factor.

Examples of noncompetitive, competitive, and biomass inhibition processes are described by Essaid et al. (1995). In this study, we assume that water content and temperature are other forms of biomass inhibition. Any of these effects on bacterial growth is

assumed to follow a three-parameter modified Gausian distribution form, but other forms can work as well. For example, for water content, the expression reads:

$$I_\theta = a_\theta - (a_\theta - 1)\exp\left[-\frac{(\theta - \theta_m)^2}{2\sigma_\theta^2}\right] \tag{9}$$

where θ is the water content, θ_m is the optimal value of the water content for bacterial growth, σ_θ is the standard deviation of the distribution, and a_θ is the maximum inhibition value. A similar expression is used for temperature, with T, the temperature in degrees, replacing θ in the equation. A value of unity for either I_θ and I_T is obtained at $\theta = \theta_m$ and $T = T_m$, respectively.

Rather than using the minimum value of C_i' in equation (7), the multiple Monod formulation uses a multiplication of the values C_i', with $i = 1, 2, ..., m$. Like the Essaid et al. formulation, the model we developed here is equipped to handle either formulation.

The source/sink term [$ML^{-3}T^{-1}$] for a species is defined by

$$q_i = \sum_{n=1}^{M} \beta_i^n v^n \tag{10}$$

where β_i [M/M^{-1}] is the uptake coefficient of solute i. Following the approach of Essaid et al. (1995), β_i is taken as unity for the primary substrate. For other substrates, it is determined by the stoichiometry of the reaction which is equal to the ratio of the mass of solute i to that of the primary substrate. For example, if for every gram of carbon degraded, 2.6 gm of dissolved oxygen are consumed, then the uptake coefficient for oxygen is 2.6.

The fate of the dissolved hydrocarbon is analyzed by solving the convection/dispersion equation, with the appropriate source/sink term. For a trapped product, the change in concentration is limited to product uptake by the bacteria:

$$\frac{dC_H}{dt} = -q_H \tag{11}$$

where C_H and q_H are, respectively, the hydrocarbon concentration and the uptake coefficient of hydrocarbon as estimated from equation (10). For example, C_H could represent the concentration of the total petroleum hydrocarbon, as measured in the sample, without differentiating between the dissolved or free product.

2.3. Solution method

The solute transport, heat transport, and bacterial growth equations form a nonlinear coupled system of equations. To simplify the solution, especially with the interest in simulating field-oriented situations, we apply a quasi-steady, quasi-linear solution approach as follows.

1. Starting from the initial condition at time t_0, the flow and transport equations are solved at time t (equals $t_0 + \Delta t$), where Δt is the time increment. Initially, that is, at time t_0, zero values are assigned to the source/sink terms for the species transport equations. These terms are updated for each new time step. The bacterial-growth model is applied by solving equations (5) through (11). Equation (5) can be integrated to estimate the biomass concentration as follows:

$$X = X^{\text{old}} \exp\left[(\mu - k_d)\Delta t\right] \tag{12}$$

where X^{old} is the biomass concentration at time t_0. The calculation scheme would include estimating C_i', v, and μ from equations (6) through (8).

2. For the general case, the equation for dissolved hydrocarbon can be solved with the appropriate sink/source term based on the calculations in the above steps. For the special case of trapped hydrocarbon, the concentration can be estimated from equation (11), which is descritized to read:

$$C_H = C_H{}^{\text{old}} \exp[-q_H \Delta t] \tag{13}$$

where $C_H{}^{\text{old}}$ is the hydrocarbon concentration at time t_0.

3. The above steps are repeated, starting with advancing the simulation time by a new time increment. The source/sink terms are updated based on the calculations in the previous steps. To minimize model execution times without sacrificing numerical accuracy, the model has an option to update the source/sink terms at times that are different from those used in solving the transport equations. For example, the updates can be done on an hourly basis, while the transport equations are solved with a time step, Δt, on the order of minutes.

3. Model Verification

3.1. Comparison to Analytical Solutions

The following equation represents the transport for a single solute with linear sorption, and first-order decay:

$$R\frac{\partial C}{\partial t} = D_x \frac{\partial^2 C}{\partial x^2} - V\frac{\partial C}{\partial x} - \lambda C + \gamma \tag{14}$$

where R is the retardation coefficient [dimensionless], D_x is the dispersion coefficient [L^2T^{-1}], V is the seepage velocity [LT^{-1}], λ is the decay parameter (first order) [T^{-1}], and γ is the zero-order production parameter [$ML^{-3}T^{-1}$]. The first-order-decay term is not multiplied by R because it is assumed that the sorpt chemical does not decay. An analytical solution for this equation was derived by Selim and Mansell (1976). SUTRA is equipped to solve this equation and, as is shown later, our test of the model yielded results identical to the analytical results. However, our objective here is to test the linearization procedure that we are adopting in our integrated model and to test the ability to read the source term from an external file. As discussed by Essaid et al. (1995), for $K_{Ci} >> C$ in equation (10), λ will equal V_{max}/K_{Ci}.

The parameters used in this test are listed in Table 1. Fig. 1 compares the analytical and numerical solutions. The terms λC and γ are treated in the numerical solution as sinks or sources that are explicitly estimated in a previous time step. For the case of a first-order decay (Case 1), Fig. 1 shows two numerical results, each with Δt of 100 s for the solution of

Table 1. Parameters for model verification of zero-order-decay case and first-order-decay case (from Essaid et al., 1995)

Parameter	Zero-order decay	First-order decay
L (cm)	200	200
Δx (cm)	2	2
D_x (cm^2/day)	37.5	37.5
V (cm/day)	25	25
C_i (ppm)	0	0
C_0 (ppm)	1	1
$t_{1/2}$ (day)	108	4.5
λ (day^{-1})	7×10^{-09}	0.154
γ (day^{-1})	-0.154	0
R	1	1
V_{max} (day^{-1})	1.54×10^{-2}	1.54×10^{2}
K (ppm)	0.0001	1000
Y	0	0
X_o (ppm)	1	1
t (day)	4	4

the transport equation but with the source term updated every day for one solution and every hour for the other. An excellent match between numerical and analytical solutions can be seen for the latter case. The efficiency of the integrated model is improved by its ability to solve the bacterial-growth submodel at larger intervals than Δt (that is, 1-h intervals compared to 100-s intervals).

Fig. 1 also shows the numerical and analytical solutions for the zero-order-decay case. Excellent agreement can be seen between the results obtained by the explicit approximation,

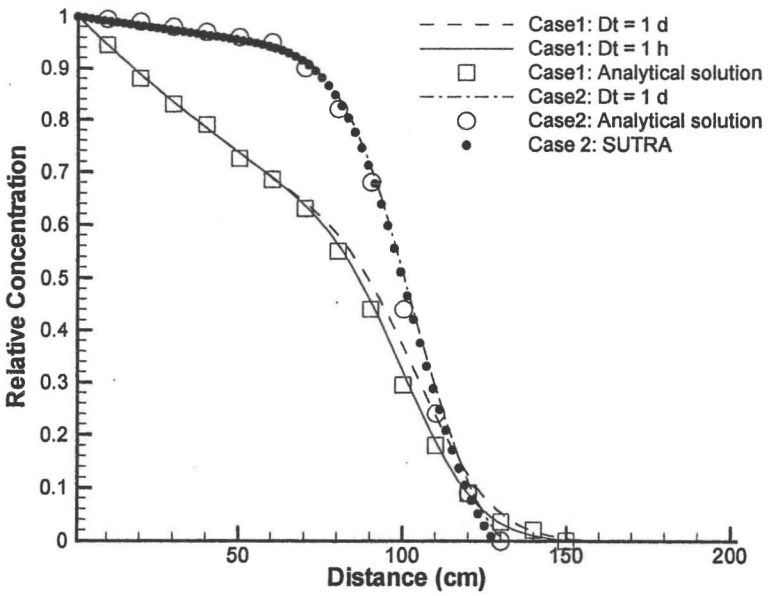


Fig. 1. Comparison of numerical simulations and analytical results. Case 1 represents first-order decay, and Case 2 represents zero-order decay. (See the text for additional explanations.)

by SUTRA, and by the analytical solution. The good match confirms accuracy of the explicit, linearized approach in solving the system of governing equations.

3.2. Aerobic Biodegradation and Transport of Toluene and Benzene

The model was tested against the experimental data of Chen et al. (1992), which was also used to validate the model of Essaid et al. (1995). The experiments dealt with the transport and biodegradation in a continuous-flow, water-saturated soil column. The column was fed a constant composition solution of toluene (20 ppm), benzene (20 ppm), and hydrogen peroxide (132.7 ppm O). Effluent samples were analyzed for toluene and benzene concentrations. We verify our model here against the Essaid et al. model, which uses the same formulation but applies a different solution method for the species-transport equations. We used the model parameters which were independently estimated by Essaid et al. through laboratory tests and literature searches. The respective values for column length, length increment, porosity, seepage velocity, dispersivity, and time increment were given as 0.56 m, 0.01 m, 0.38, 0.33 m d^{-1}, 0.0224 m, and 0.005 d. The simulation was done for 10 d. The rest for the parameters used are listed in Table 2.

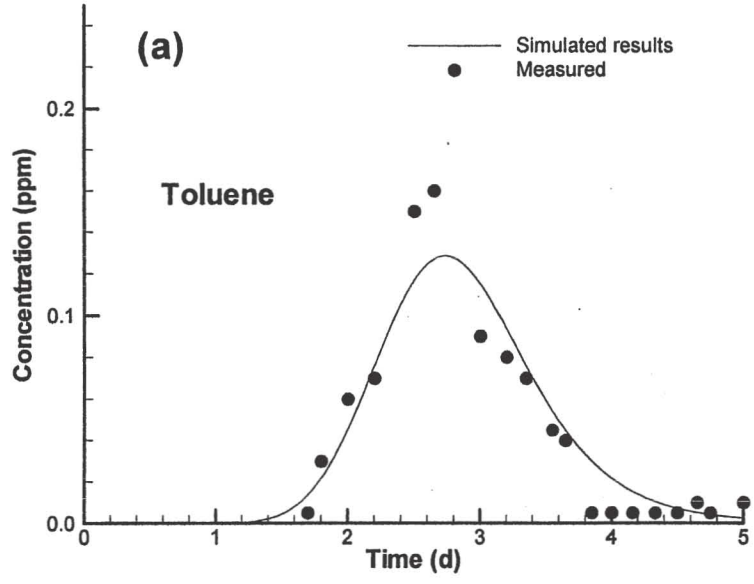
Table 2. Parameters of biodegradation of toluene and benzene (from Essaid et al., 1995)

	V_{max} (day^{-1})	K (ppm)	K_{DO} (ppm)	Y (mg/mg)	β	β_{DO}	X_o (ppm)	d_k (day^{-1})
Toluene	9.9	17.4	0.1	0.5	1.0	2.19	0.82	0.1
Benzene	9.3	12.2	0.1	0.5	1.0	2.15	0.21	0.1

Fig. 2 compares the experimental data and the results of the current model. Good overall match can be seen, especially considering that the model parameters were independently estimated.

4. Cyclic Wetting and Drying (Test Tube Experiment)

A hypothetical homogeneous volume of soil was subjected to cycles of soil moisture changes in a sinusoidal form. In the simulations, there was no water flow dynamics, and the



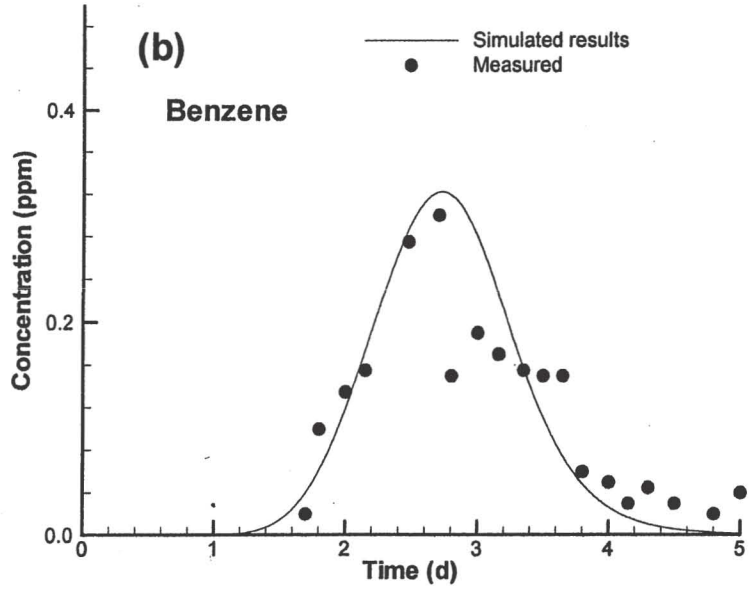


Fig. 2. Comparison of simulated results of the current study and experimental data for (a) toluene concentrations and (b) benzene concentrations.

small size of the sample allowed an immediate and uniform change in the water content without any storage effects. Subjecting soil to cyclic wetting and drying can represent in situ remediation, or landfarming, where the soil is periodically watered and dried. In the following scenarios, drying was caused by water evaporation while the subsequent wetting was done by adding solute-free water. The scenarios differ in the way oxygen is introduced to the solution. O_2 introduced either in a dissolved phase, such as by adding hydrogen proxide, or in a vapor phase through aeration. The scenarios were compared to the case where the sample was subjected to constant saturation under the same starting solute concentrations. The common parameters of these simulations are given in Table 3.

Table 3. Bacterial related parameters of biodegradation of hydrocarbon for the wetting/drying and the example field cases

Parameter	Wetting and drying case	Example field case
V_{max} (day^{-1})	114.6	114.6
K_H (ppm)	17.4	1.74
K_{DO} (ppm)	10.0	200.0
K_N (ppm)	17.4	27.4
K_p (ppm)	17.4	27.4
Y (mg/mg)	5.05	0.5
β_H	1.0	1.0
β_{DO}	2.19	2.19
β_N	5.55	21.9
β_p	5.55	21.9
X_o (ppm)	0.82	0.82
d_k (day^{-1})	0.316	1.157

4.1. Wetting and Drying Scenarios

1. At time zero, the soil sample was mixed with nutrients and O_2, all in dissolved format. No aeration was possible and hence no vapor O_2 existed. No new mass was added to the sample after the first application. Drying was caused by evaporation while wetting was done by adding solute-free water. Drying and wetting should increase and decrease solute concentrations (mass per unit volume of water), respectively. Nutrient and oxygen concentrations were thus assumed to be inversely proportional to water content.
2. At time zero, the sample was mixed with dissolved nutrients and subjected to aeration. With aeration, O_2 availability is dependent on the amount of dry void space. O_2 availability was hence assumed to be directly proportional to air content. Drying and wetting is done in a similar fashion to that in the first scenario where nutrient concentrations depended on water content.

4.2. Simulations

4.2.1. The First: Oxygen is Introduced as a Dissolved Phase

For cyclic wetting and drying, the water content varied between 0.1 and 0.4, with an average value of 0.25. The solution was compared to a case where water content was constant, averaging 0.25. The optimal water content for degradation is commonly assumed to be at 50% to 70% of the soil holding capacity (Pramer and Bartha, 1972.) A value of 0.288 was used here, which is at about 63% of the holding capacity of 0.30. The enhanced degradation was simulated by adding nitrogen, phosphorus, and dissolved oxygen at initial concentrations of 20, 20, and 13 ppm, respectively. The initial hydrocarbon concentration was 2 ppm.

Fig. 3a illustrates the results for bacterial growth and product degradation for the first scenario compared to the constant saturation case. Fig. 3b depicts the time change for O_2 and N concentrations for the same cases. The results in Fig. 3 indicate that degradation is more

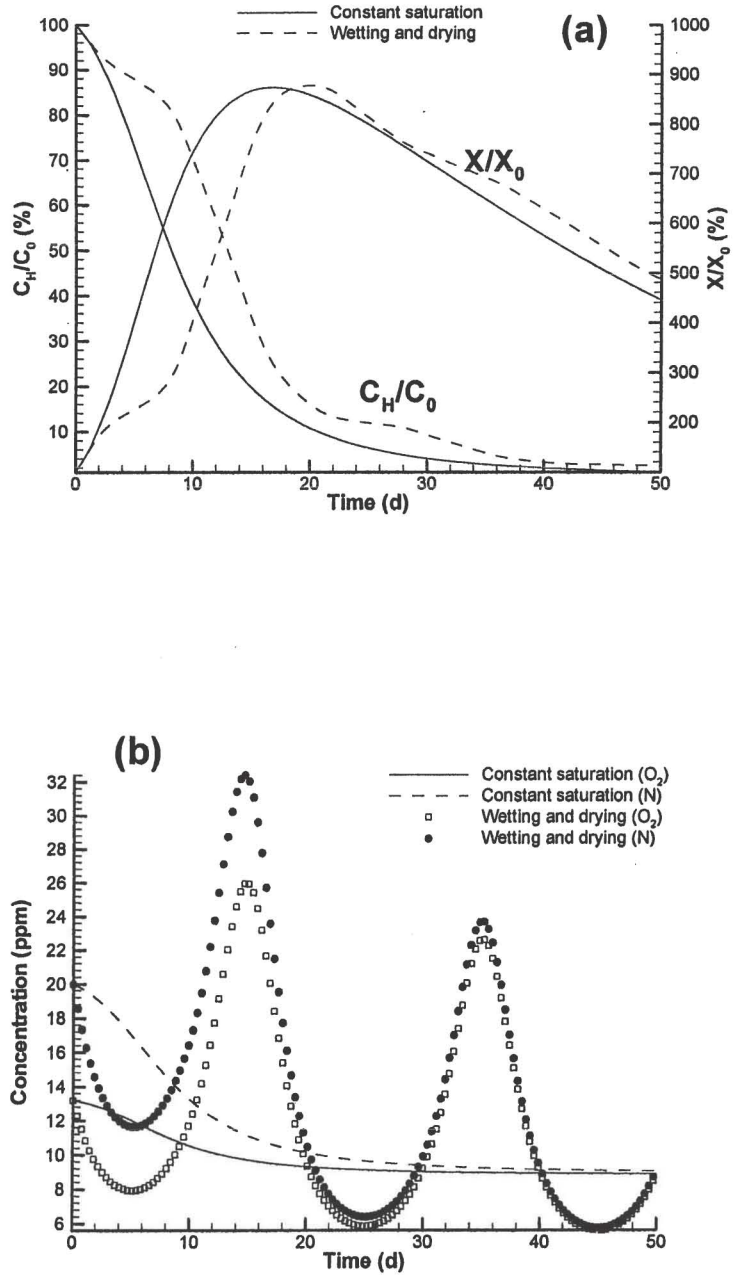


Fig. 3. Comparison of simulations under constant saturation and fluctuating water content: Scenario 1, (a) a biodegradation and biomass concentration and (b) concentrations of O_2 and N.

efficient when water content is constant. Many numerical experiments were completed with different ranges of water-content parameters, and all provided consistent results. Closer results for the cyclic and noncyclic cases were obtained for smaller θ_m, the optimal water content value. However, as studies indicate and as mentioned earlier, this optimal value for the degree of saturation is at 50% to 70% of water holding capacity; hence, it is expected that there should be a significant effect for cyclic wetting and drying. The discrepancy between the wetting and drying case and the constant saturation case increases with a decrease in σ_θ, which affects the range of wetting and drying.

The fact that constant saturation provides more efficient degradation than variable saturation in the first scenario, as shown in Fig. 3a, can be explained by examining equation (9). It is clear that variable saturation will cause a deviation from optimal saturation, except at a limited time range when saturation is near its optimal value. By numerically integrating this equation, the estimated average value of the inhibition factor under a cyclic water content is always larger than that estimated at an average water content. The solute uptake, which can be inferred from Fig. 3b, is lower for the cyclic case at intermediate times because of inhibited bioactivities.

The experimental results of Wright et al. (1997) showed that continuous flooding provides more efficient degradation than alternately flooded treatment; this agrees with the above conclusions. Their explanation included a number of factors; among which was the reduction in O_2 availability, since degradation is often O_2 limited in estuarine sediments. They also used the conclusion of Alexander and Scow (1980), that is, in the absence of water, the association of immiscible organic materials with soils reduces the kinetics of biodegradation.

4.2.2. The Second: Oxygen is introduced in a vapor phase

The second scenario assumed that O_2 was introduced in a vapor phase and thus its availability only depended on the amount of dry void space. In this situation, bacterial activities are enhanced because of increased direct contact with air.

The results of the simulations for these scenarios are seen in Fig. 4. The results are compared to the case of a constant saturation of 40%. The results show clearly that the case of wetting and drying is more efficient in comparison with the case of constant saturation, as shown in Fig. 4a. The higher efficiency is due to the increase in nutrient concentration during the drying process. Note that the results for the constant saturation case would be even less efficient if water saturation is kept near its optimal value, because less dry void space would then be available for aeration. Fig. 4b shows changes in nitrogen and oxygen concentrations for this scenario. The increase and decrease in bacterial activities and the resulting degradation are consistent with the fluctuations in nutrient and oxygen concentrations.

The lower efficiency of degradation under flooding qualitatively agree with the experimental results of Widrig and Manning (1995), who subjected their samples to aeration following periods of flooding. They compared the results to those for samples that were only subjected to flooding.

Although not all variables were considered, we may conclude thus that subjecting the soil to constant water content close to its optimal value is most efficient if O_2 is provided as a dissolved phase. Management aspects of the field or laboratory experiment would be easier because most effort would be directed toward keeping water content closer to its optimal value. However, wetting and drying can have a clear edge if O_2 delivery is only done through aeration or direct contact between air and media. Although no quantitative validation of the model is provided for these cases, the results qualitatively agree with those in the literature (Widrig and Manning, 1995; Wright et al., 1997)

5. Application to a Hypothetical Tidal Aquifer

Enhanced biodegradation of a trapped hydrocarbon was simulated in a tidal aquifer. Hydrocarbon existed at residual saturation and hence no immiscible flow of hydrocarbon occurred. In order to emphasize biodegradation, the simulation ignored product dissolution due to water motion. Therefore, the loss of hydrocarbon mass is only due to bacterial

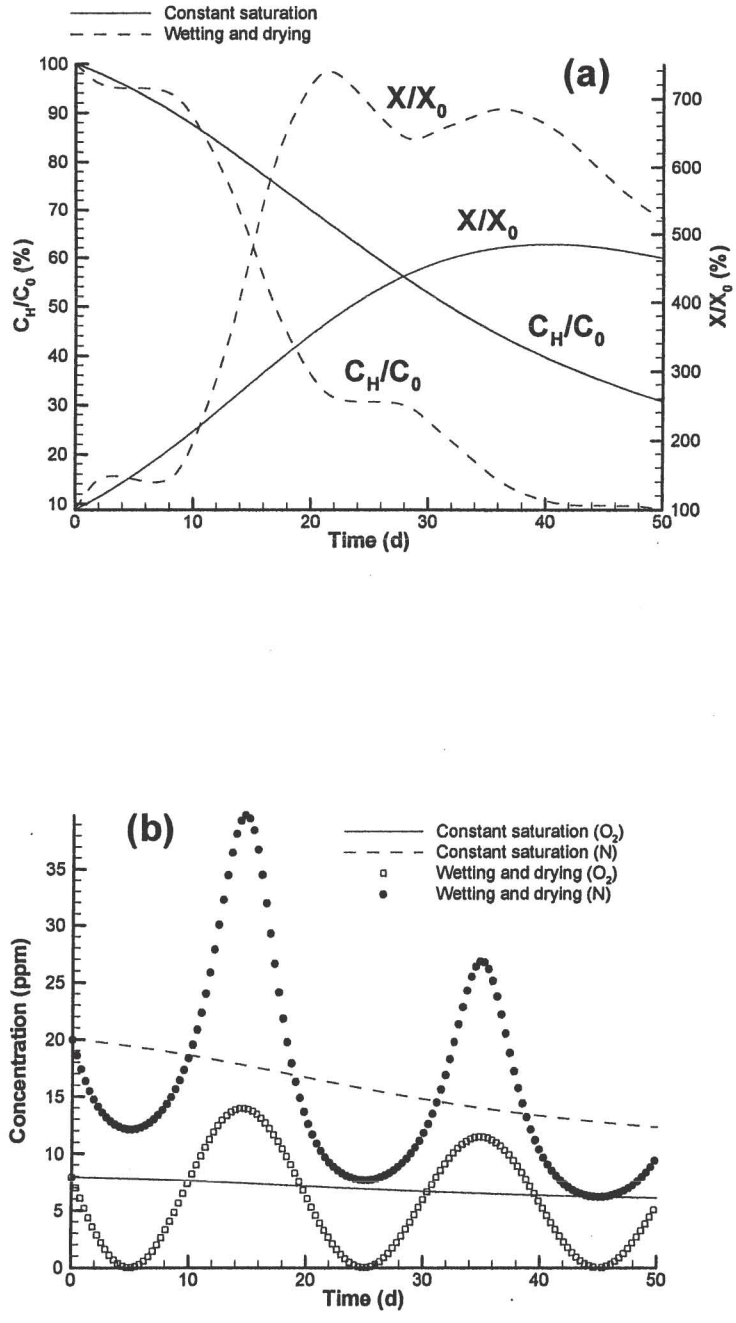


Fig. 4. Comparison of simulations under constant saturation and fluctuating water content: Scenario 2, (a) a biodegradation and biomass concentration and (b) concentrations of O_2 and N.

activities. Note that bacteria will facilitate dissolution as a step toward its utilization. As mentioned earlier, Nicol et al. (1994) stressed the importance of intermediate processes between the dissolution and degradation phases. For simplification purposes, such processes are overlooked in the current formulation. The examples in this section differ from those in the test-tube case by emphasizing the dynamics of water flow and the resulting specious transport. Simulations mainly addressed degradation as influenced by spatially varied water, solute, and temperature conditions.

The aquifer, with dimensions of 40 m by 10 m is shown in Fig. 5. The initial water table is shown with a slope of 0.025 toward the ocean. Both constant head and tides are considered at the ocean side, whereas the water level was unchanged at the left boundary. No-flow conditions are assigned to the top and lower boundaries. A hydrocarbon contaminant is assumed to occupy the outlined area in the central part of the domain (see Fig. 5). The initial concentration of the hydrocarbon was taken as 20.0 ppm. Nitrogen and phosphorus, with a concentration of 20.0 ppm each, were added to the same section. A constant oxygen concentration in dissolved phase of 8 ppm is maintained over the small black rectangle near the center of the Figure. The initial biomass concentration of bacteria was assumed to be 0.82 ppm. Bacterial growth data are listed in Table 3.

The soil and aquifer parameters were taken as follows:

Water-saturated conductivity K_s (equation (2)) = 3.07×10^{-7} m/s

Horizontal and vertical solute dispersivities = 10 and 3 m, respectively

Porosity = 0.38

van Genuchten parameters for unsaturated flow (equation (1)): n = 2, $\alpha = 0.8$ m^{-1}, $\theta_r = 0.1$

Soil and aquifer parameters are consistent with those for silty-clay material (e.g., Fetter, 2001, van Genuchten, 1978). The dispersivity values are within the ranges listed by Gelhar et al. (1992).

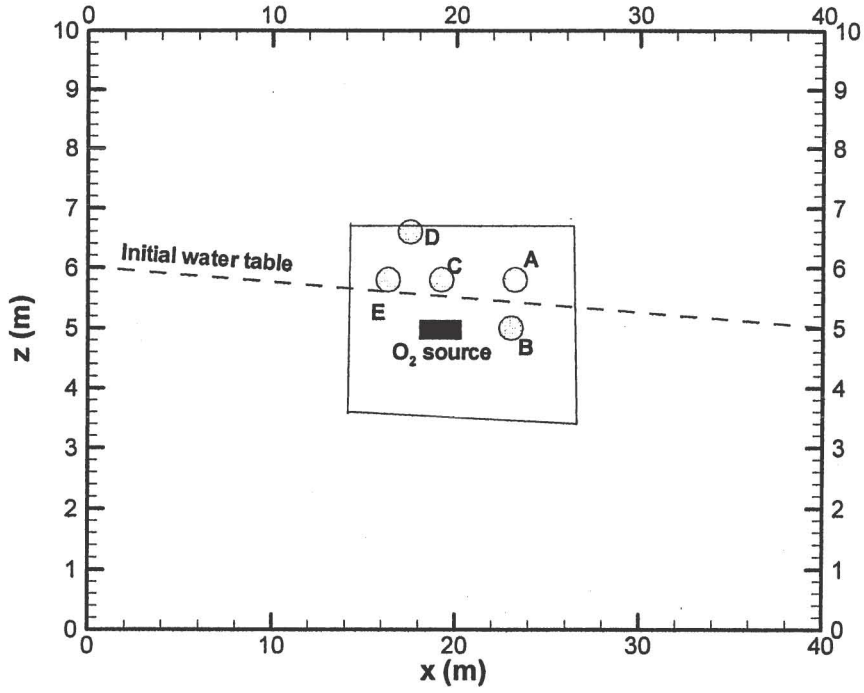


Fig. 5. Cross section of the flow domain, 40 m by 10 m in size. Specified head boundaries are set at the left and right boundaries. Tides can be simulated at the right side. No-flow conditions are assumed at the top and bottom sides. The hydrocarbon is assumed to occupy the outlined area near the center of the domain. The circles represent the monitoring points, and the small black rectangle represents the air-injection area.

5.1. Effects of Tides

Fig. 6 (a, b, and c) illustrates the spatial distribution of oxygen, nitrogen, and hydrocarbon after 120 days of simulation for the case of no tides. Regional flow is derived by the head gradient from left to right, with the water table as shown in Fig. 5. Similar results are shown in Fig. 6 (d, e, and f) for the case with the same regional flow, but with 2.0-m-amplitude tides that were active at the right boundary. No inhibition effects are considered in either case. Note that the hydrocarbon-contaminated area is still bounded by sharp-edged lines since there is no dissolution or immiscible transport of the hydrocarbon. As explained earlier, the only change in hydrocarbon status is due to bacterial uptake.

In Fig. 6, and consistent with Monod kinetics, degradation is more efficient near the air-injection area where concentrations are highest. Tides cause additional mixing for nutrients and O_2, as shown in Fig. 6 (d and e). As a result of O_2 spread, enhanced degradation occurs in the plume in the unsaturated zone (Fig. 6f). Results at monitoring points B, C, and E are shown in Fig. 7. In Fig. 7c, the difference between O_2 concentrations at point B with and without bioactivities reflects O_2 uptake, which is more pronounced at early times under tides. Degradation is enhanced even farther away from the center of the plume due to the additional spread of O_2. Note that such enhancement could be limited to early times in some cases that involve a smaller initial nutrient concentration that can quickly diminish due to tidal effects.

Fig. 8 compares a simulation for a lower initial N concentration of 10 ppm against that of a concentration of 20 ppm, both with tides but no inhibition effects. Bioactivities are reduced, as shown in Fig. 8 (a and b). The degradation is O_2-limited at early times of less than 10 days for the case with an initial concentration of 10 ppm. After that, the process is N limited when the divergence from the higher initial N concentration starts (Fig. 8c.) Fig. 9 depicts the spatial distribution of O_2, N, and hydrocarbon under tidal conditions. By comparing the results in Fig. 9 with those in Fig. 5 (d, e, and f), it is clear that degradation is reduced due to the decline in N concentration.

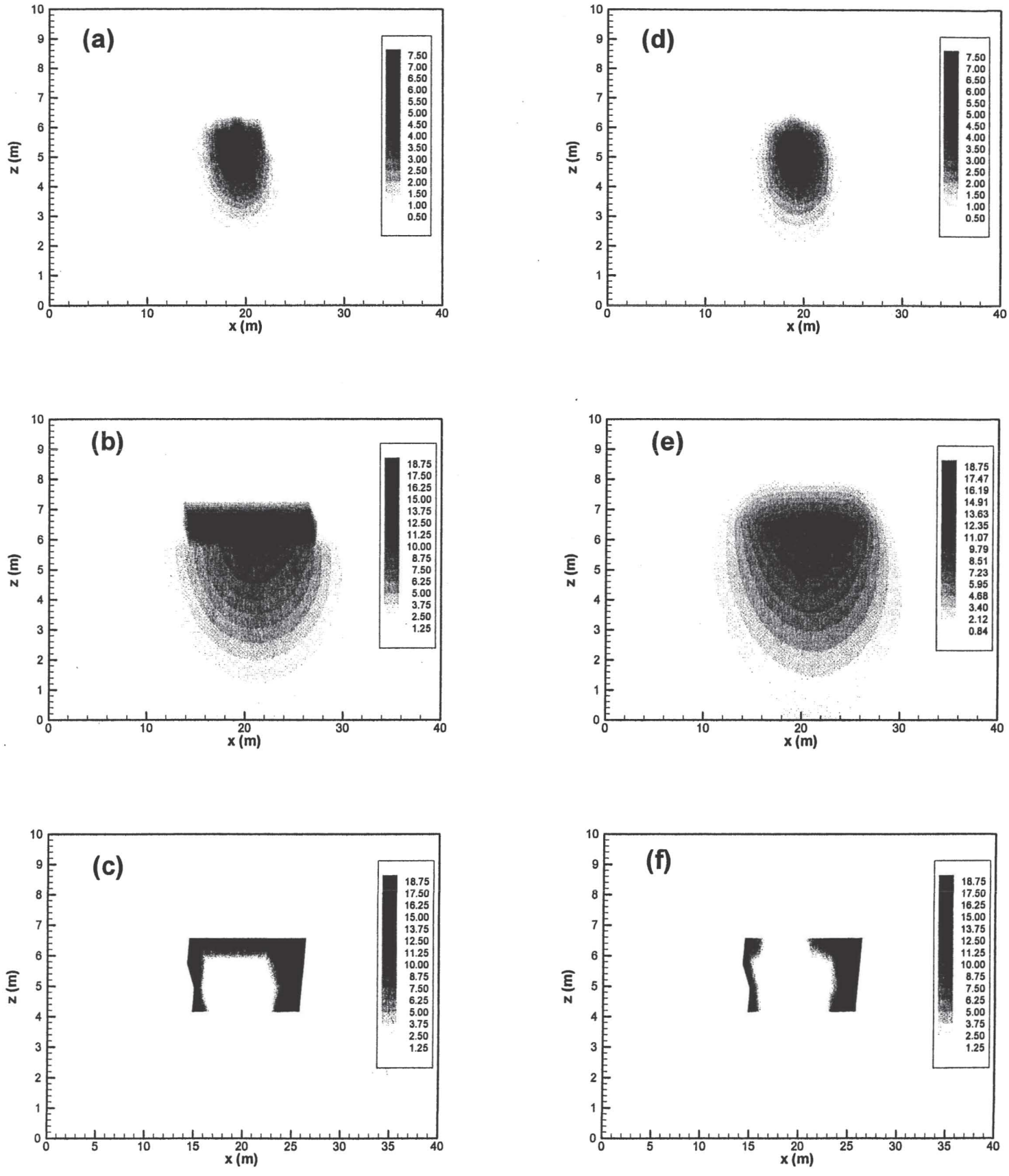
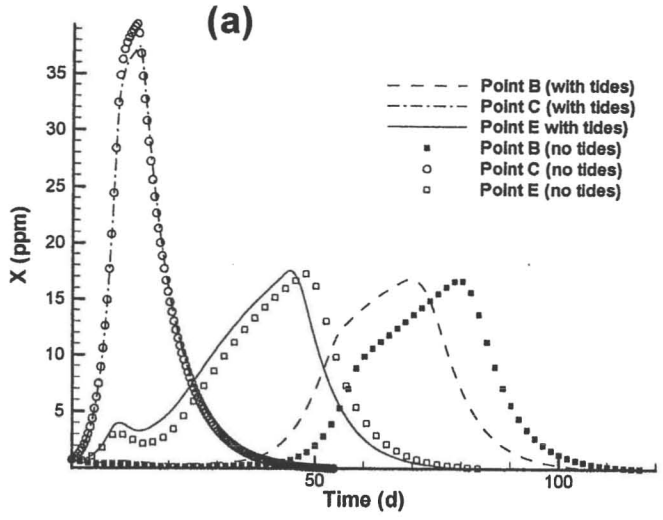
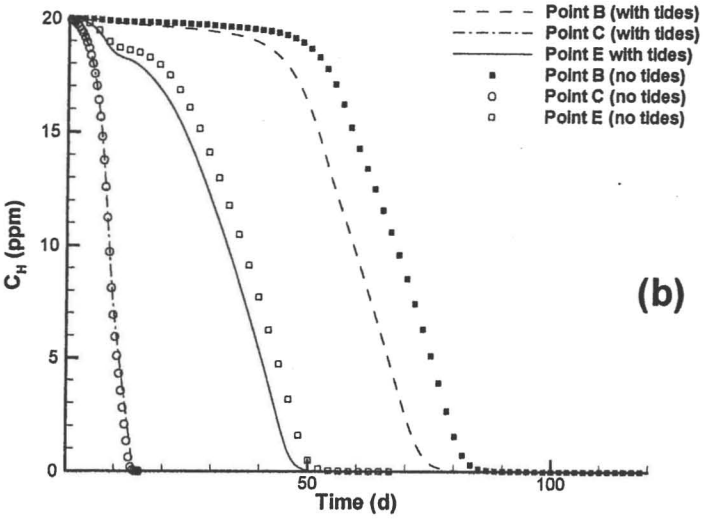


Fig. 6. Spatial distribution of oxygen, nitrogen, and hydrocarbon, all under no-tide conditions (a, b, c, respectively), and under tidal conditions (d, e, and f, respectively). All values are in ppm.





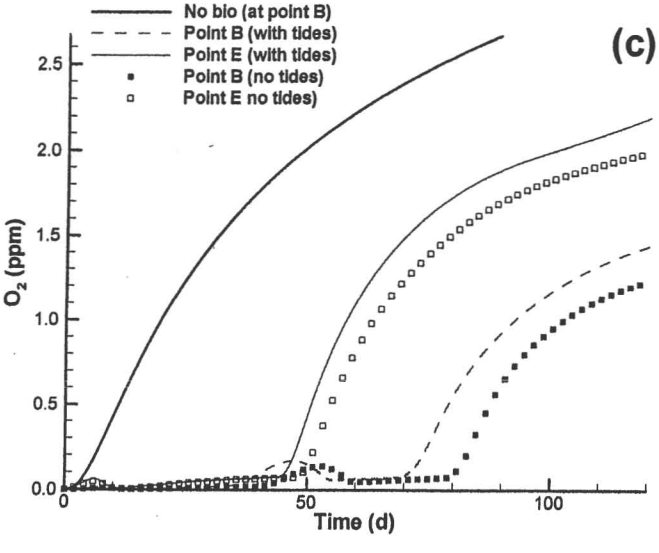
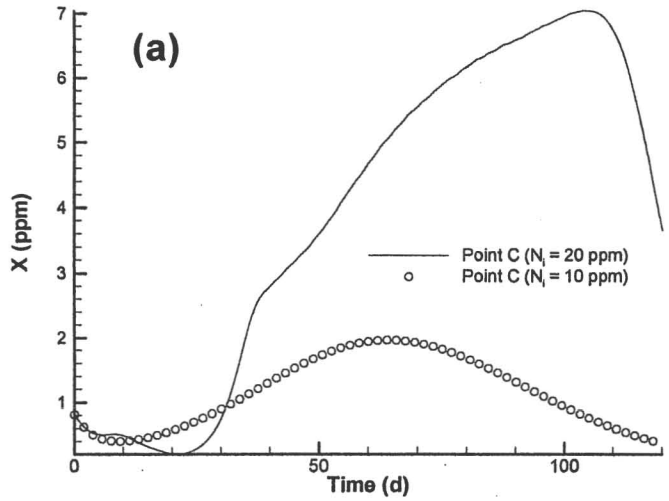
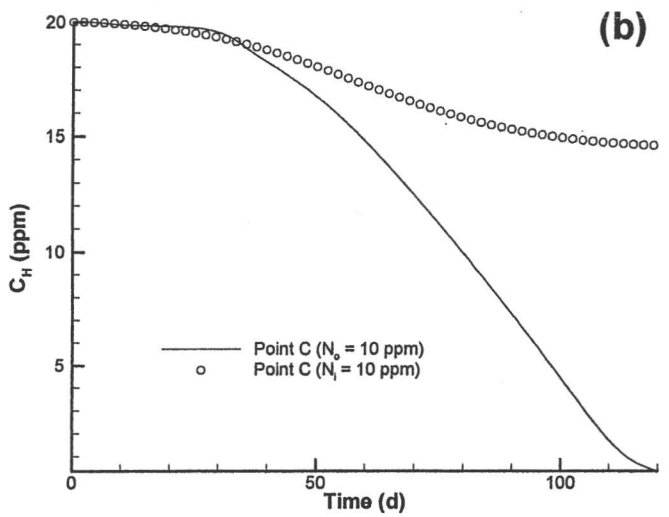


Fig. 7. Monitoring results at points B, C, and E of Fig. 6, with and without tides, for: (a) biomass concentrations, (b) hydrocarbon concentrations, and (c) O_2 concentrations. O_2 concentrations without bioactivities at point B are also illustrated in the last figure.





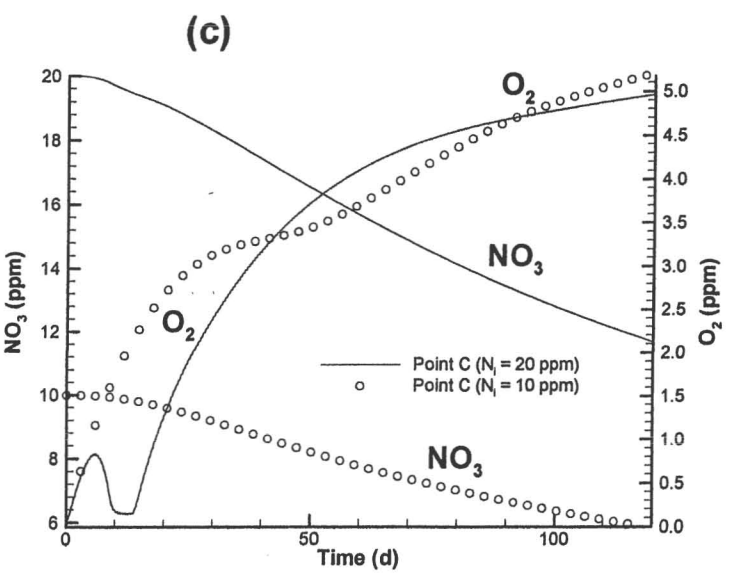


Fig. 8. Monitoring results at point D of Fig. 6, with tides, comparing cases of initial N concentrations of 10 and 20 ppm for (a) biomass concentrations, (b) hydrocarbon concentrations, and (c) O_2 and N concentrations. The last figure illustrates O_2 and N concentrations without bioactivities.

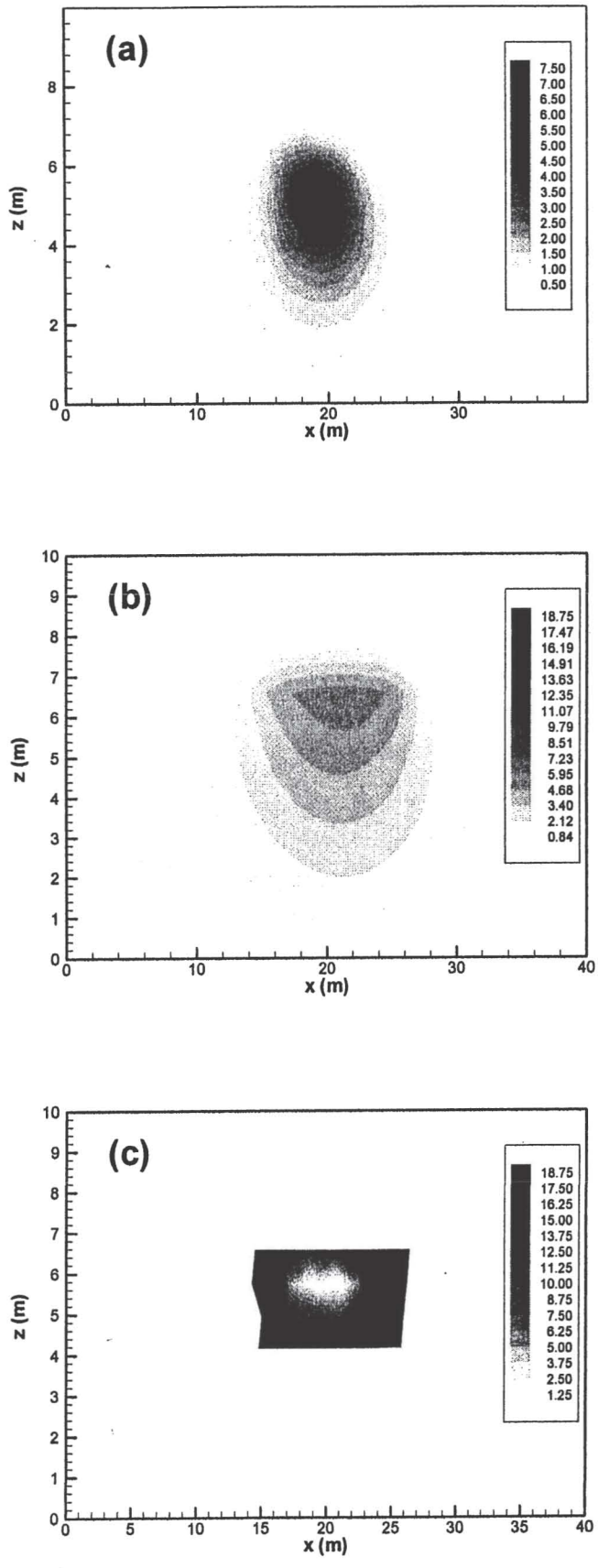


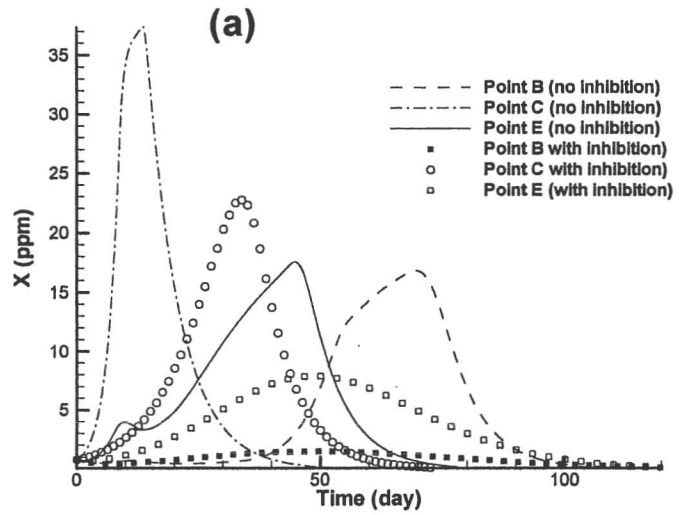
Fig. 9. Spatial distribution of (a) oxygen, (b) nitrogen, and (c) hydrocarbon, all with tides, at an initial N concentration of 10 ppm. All values are in ppm.

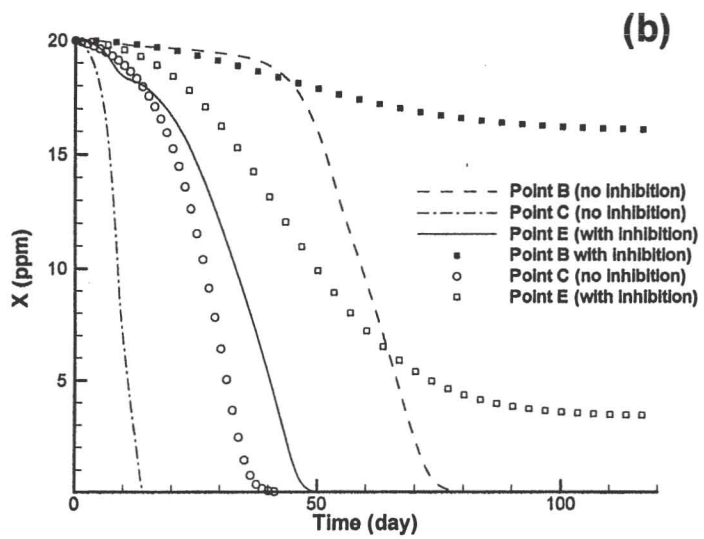
5.2. Effects of Saturation Inhibition

Fig. 10 (a and b) illustrates the biomass and hydrocarbon concentrations at monitoring points B, C, and E for the cases with and without water-saturation inhibition. The change of the inhibition factor as a function of the water content is shown in Fig. 11a. The parameters of the distribution are $\theta_m = 0.228$, $\sigma_\theta = 0.1$, and $a_\theta = 5.0$. Fig. 10 shows the effect of inhibition, as is evident by the reduction of both bacterial growth and hydrocarbon degradation. Fig. 10c shows the change in O_2 concentration at points B and E; it also shows the change in O_2 concentration at point B for the case without bioactivities. Inhibition causes a subdued intake of O_2 compared to the case without inhibition. For this simulations, degradation is oxygen limited, because the simulation started at an O_2 of zero concentration. At point B, the difference in the O_2 concentrations with and without bioactivities reflects the oxygen uptake due to bacterial activities. For the case with saturation inhibition, bacterial activities are reduced and thus the uptake of O_2 is relatively small compared to the case without inhibition [see also equation (3)]. Hence, at early times, O_2, which is continuously supplied at the injection well, is sufficient to maintain a higher concentration and a higher rate of bacterial growth, when compared with the O_2 supply for the case without inhibition. After about 45 days at point E and 70 days at point B, the increase in O_2 concentration is large enough to sustain bacterial growth for cases with and without inhibition. However, saturation inhibition drastically limits the rate of growth, as shown in Fig. 10a. Ultimately, and as should be expected, bacterial growth and product degradation are more efficient without inhibition. However, due to the above description about O_2 intake, bacterial growth and product degradation curves have milder slopes and extend over longer time periods.

5.3. Effects of Heat Inhibition

For these simulations, we used the temperature distribution shown in Fig. 11b, with parameters of the inhibition equation of 30°C, 20°C, and 5, for T_m, σ_T, and a_T, respectively. The case simulated included regional water flow and tides, with no saturation inhibition. The





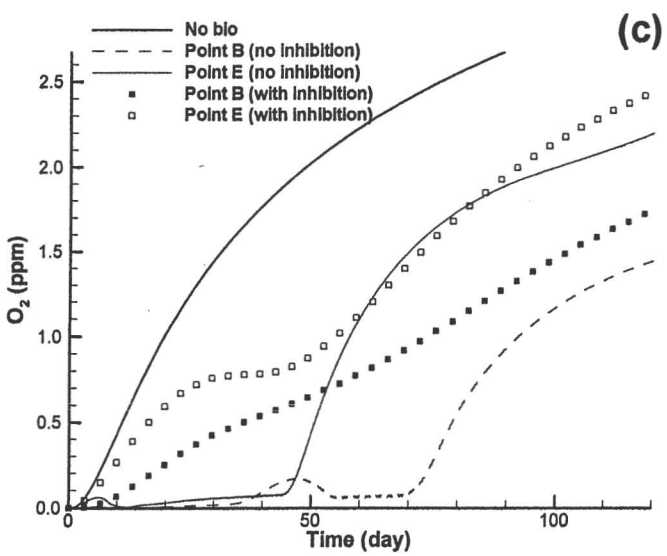
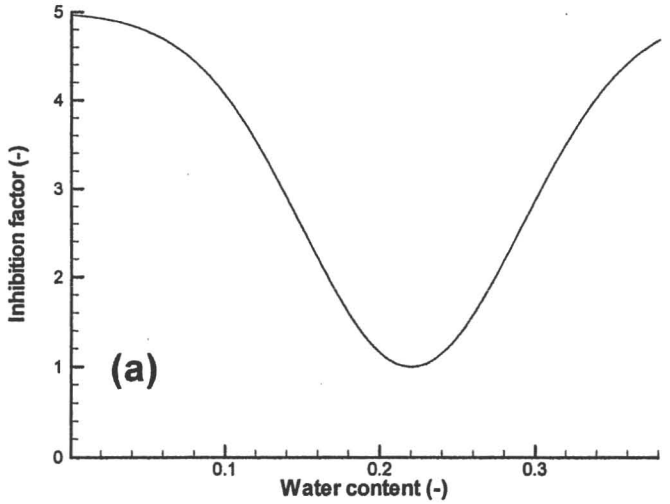


Fig. 10. Monitoring results at points B, C, and E of Fig. 6, with and without saturation inhibition, for (a) biomass concentration, (b) hydrocarbon concentration, and (c) O_2 concentration. O_2 concentrations without bioactivities at point B are also illustrated in the last figure.



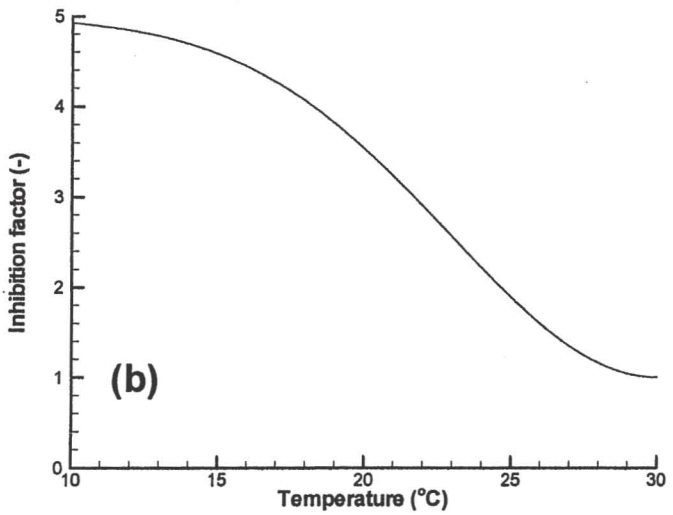


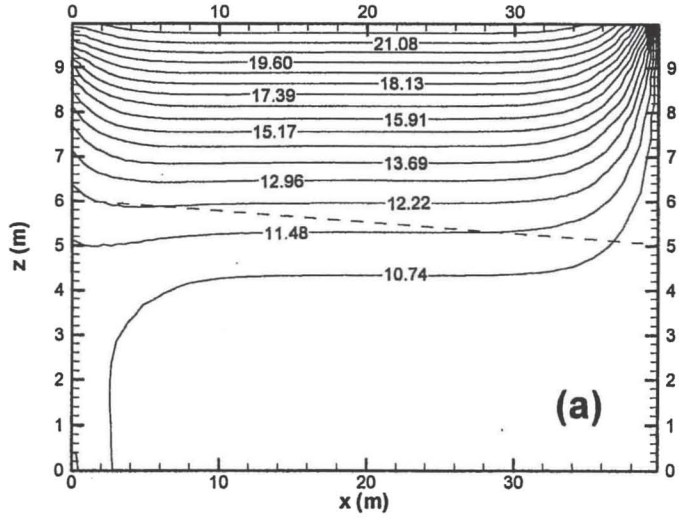
Fig. 11. Inhibition factors: (a) saturation and (b) heat.

boundary conditions for temperature were set at 25°C at the ground surface, 15°C at the left boundary (representing the groundwater ambient temperature), and 10°C at the ocean side. Fig. 12a shows the temperature distribution in the aquifer after 120 days of simulation, and Fig. 12b illustrates the temperature variations at points A, B, and C of Fig. 5. Fig. 13a shows the time change of biomass concentrations, and Fig. 13b illustrates the product concentration at the same points. It is clear that heat inhibition has a significant effect on the degradation process in natural field situations. The effect differs at various points, depending on the location relative to the air-injection points. With inhibition, the biomass concentration rises earlier but with lower rates and slower peaks. This behavior is similar to that explained earlier regarding saturation inhibition. Fig. 13c shows the O_2 concentration at point B. Again, the O_2 deficit reduces the initial product degradation for the case without inhibition; after about 30 days, the O_2 concentration is large enough to ensure fast bioactivities.

6. Summary and Conclusions

This study introduces a model for hydrocarbon biodegradation in field-scale aquifers where unsaturated and heat effects are of concern. The model is flexible in dealing with time-dependent boundary conditions, such as tides in nearshore aquifers. Expressions for inhibition due to saturation and heat effects are proposed. The expressions can be easily adopted to handle pH or solutes (such as salinity) or other factors that are spatially varied and can have inhibition effects. Although empirical in nature, these expressions or similar ones can be useful due to their analytical nature and ease of integration into the biodegradation model. Parameters of these equations should be estimated by fitting the expressions to available data.

The model is based on SUTRA, a popular code for modeling saturated and unsaturated flow and transport. SUTRA was modified to simulate multi-species fate and transport and was combined with a bacterial growth submodel. The hydrocarbon degradation model can simulate biotransformation reactions for any combination of electron donor and acceptor.



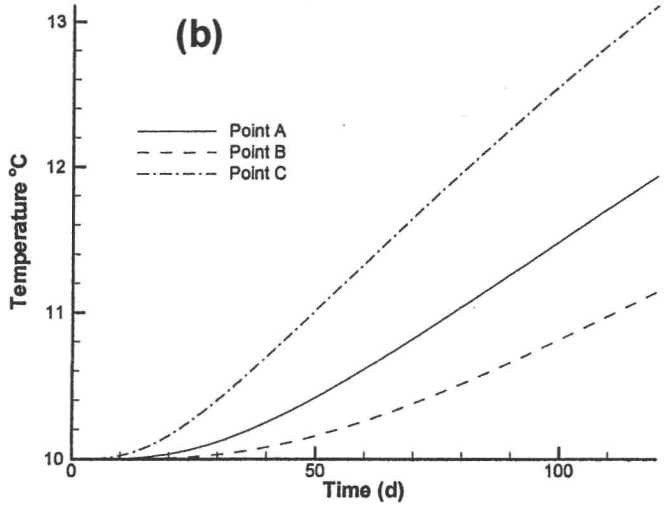


Fig. 12. Results of heat simulations: (a) temperature distribution after 120 days and (b) temperature changes at monitoring points A, B, and C.

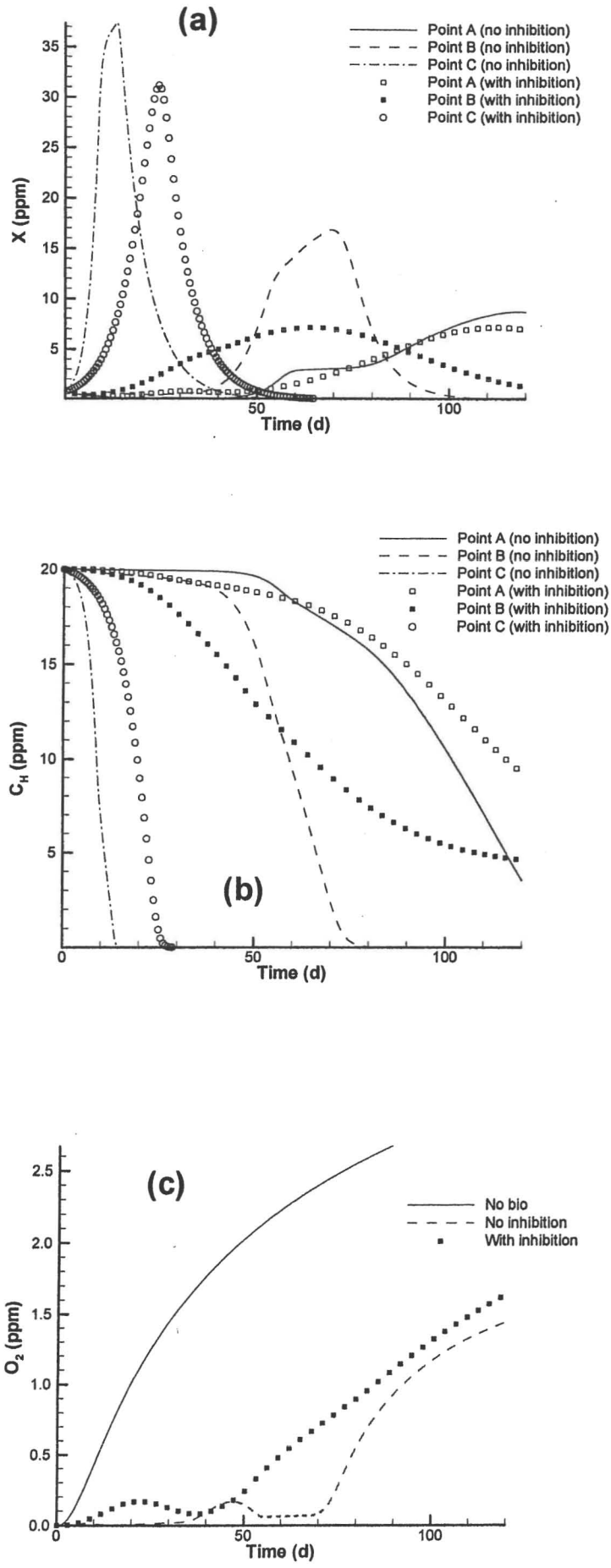


Fig. 13. Monitoring results at points A, B, and C of Fig. 6, with and without heat inhibition, for (a) biomass concentration, (b) hydrocarbon concentration, and (c) O_2 concentration. O_2 concentrations without bioactivities at point B are also illustrated in the last figure.

Although simultaneous multiple degradation processes and microbial populations can be simulated, our emphasis here is on the effect of heat inhibition and saturation inhibition on a single degradation process and population. The model was verified through comparison with analytical solutions for a single solute with linear sorption and with zero-order decay and first-order decay. An excellent match between numerical and analytical solutions was obtained for all cases simulated. The model was also successfully tested against laboratory data that deal with toluene and benzene transport and biodegradation in a continuous-flow, water saturated soil column. Effects of cyclic flooding and drainage of soils were also studied and the results used to explain some of the conflicting conclusions regarding efficiency of degradation. Although the degradation process is expected to be site-specific, the model demonstrated that cyclic flooding and drainage using dissolved O_2 are likely to reduce the efficiency of degradation. Constant water saturation near its optimal value is recommended for best results. However, wetting followed by periods of drying can have positive effects in cases that involve subjecting the soil to aeration as the only method for providing an O_2 supply.

Simulation results of a hypothetical field site examined the effects of cycles of soil wetting and drying associated with tides with and without saturation and heat effects. For the case of O_2-limited degradation, biomass concentration rises earlier but with lower rates and slower peaks. Inhibition causes a subdued intake of O_2. At early times, O_2 is sufficient to maintain a higher concentration and a higher rate of bacterial growth, in contrast to the case without inhibition. After reaching O_2 concentration that is large enough to sustain bacterial growth, inhibition drastically limits the rate of growth. Ultimately, and as should be expected, bacterial growth and product degradation are more efficient without inhibition. Under these conditions, growth and product degradation curves under inhibition have milder slopes and extend over longer time periods.

The study introduces a quantitative approach to assess the effects of fluctuations in water content and temperature and shows that their effects can be significant. The model can

be useful in the design of in situ clean-up projects, either by landfarming or under natural flow conditions. The design can include assessing amounts and frequency of solutes added and the suitability of various wetting and drying scenarios. However, it is imperative to first validate the inhibition expressions proposed in this study, in order to identify parameters of the equations based on experimental measurements.

SECTION IV

ADDITIONAL HYDROLOGICAL ASSESSMENT OF THE SITE

The field work completed in this study showed that the diesel fuel contaminant was highly weathered due to the occurrence of instrinsic degradation of the *n*-alkanes before the start of our bioremediation treatment. However, some fractions of the weathered diesel fuel are susceptible to biodegradation. Increased bacterial numbers and decreased dissolved oxygen concentrations in area A provide indication of *in situ* biodegradation. Recontamination events have prevented comprehensive assessment of biodegradation in the site. Modeling efforts is also hindered by the lack of understanding of specific causes of contamination and its extent at different times. The study also indicated that hydrocarbon concentrations did not exceed the State of Hawaii's Department of Health 5,000 mg/kg action levels for TPH-diesel in soil. This does not infer, however, that TPH-diesel concentrations in other areas of the site do not exceed the action level.

Through discussion with DOH, scope of the work has been modified to include additional site investigation. The objective is to further analyze the effectiveness of in *situ* biodegradation of diesel-fuel contaminated soil. Pending further analysis, natural or enhanced biodegradation could be sufficient remediation tools for this Site. The proposed additional field assessment is needed to confirm such a conclusion. The proposed phase will not include assessing bacterial activities but rather emphasize chemical and physical processes controlling the spread of diesel fuel. The work will help in evaluating the degradation process by constructing a mass budget of the product. URS-Dames & Moore has been retained for managing and performing the additional field work. Their services will be closely coordinated together with the University of Hawaii investigators and State of Hawaii Department of Transportation (DOT) Airports Division.

Scope of Work

The objectives of the new investigative phase will be achieved through the following tasks.

Task 1—The contractor together with the UH and State of Hawaii Department of Transportation (DOT) Airports Division staff will determine the appropriate location of Strataprobe soil borings and sampling depths which should cover the area believed to be contaminated by residual diesel fuel. A maximum of 72 Strataprobe locations will be sampled. The contractor will review existing reports and well logs to identify the most appropriate location and depth of the borings. The assessment will include identifying the approximate location of existing utilities from engineering plans and by performing a magnetometer (utility clearance) survey to estimate the location of electric, gas, and water lines prior to sampling. The work will include creating computer aided design (CAD) drawings of the Site with the planned Strataprobe locations. Available site information and reports will be used to assess geologic and hydrogeologic conditions at the site.

Task 2—Field Investigations

A. Quarterly Strataprobe Sampling—Field monitoring will be done during the four quarters of sampling conducted over a one year period of performance (POP). The sampling schedule will be determined at a later date between the contractor, the funding agency, and the UH team. During each quarterly sampling event, it is anticipated that a maximum of 18 probes will be sampled. The total number of Strataprobe locations may vary and is dependent on the site conditions. The sampling scheme will be similar to that used in the previous phase of the study. It will provide a continuous core for describing the lithology and the collections of samples from the saturated and unsaturated zone.

B. Installation of Micro Wells—A number of micro wells (1" diameter) will be installed in some of the boreholes after finishing the Strataprobe soil sampling. The number of wells will depend on progress made during soil sampling. The location of the micro wells will be decided based on a team discussion. Approximately 24 micro wells will be installed during the

project period. We will periodically measure water table elevation and free product thickness in the accessible wells and compare these levels with those measured in the newly installed micro-wells.

C. Tidal Study—We will conduct a detailed tidal study for an extended period of time (approximately one month) in up to six wells in locations. The objective would be to identify the flow patterns and aquifer properties. Specifically, this study would determine the magnitude of groundwater fluctuations across the Site, the hydraulic gradient, the approximate Darcy flow velocity, and the hydraulic conductivity and specific storage of the shallow unconfined aquifer. To accomplish these objectives, the tidal response at the site will be measured in up to six wells and will be compared to the NOAA tidal data collected at Honolulu Harbor, or nearest location to the site. To adequately cover the site, an array of up to six pressure transducers and data loggers will be selected. The pressure transducers will be used to monitor water table fluctuations over a maximum of 24 tidal cycles throughout a 25-day deployment period. The data loggers will be used to collect and store the data. The data will be downloaded to a personnel computer and for analysis.

The tidal data will be used to evaluate the affect of the subsurface geology on groundwater movement during the rise and fall of the tides.

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