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I am submitting herewith a dissertation written by Raymond D. Stapleton, Jr. entitled "Natural Attenuation of Petroleum Hydrocarbons in Groundwater." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology.

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NATURAL ATTENUATION OF

PETROLEUM HYDROCARBONS IN GROUNDWATER

A Dissertation
Presented for the
Doctor of Philosophy
Degree

The University of Tennessee, Knoxville

Raymond D. Stapleton, Jr.
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ABSTRACT

A multidisciplinary field research experiment was conducted at Columbus AFB, Mississippi, in order to develop a better mechanistic understanding of the various processes involved in the natural attenuation of petroleum hydrocarbons. This experiment was termed the Natural Attenuation (NAT) Study. The NAT Study was conceived to evaluate the efficacy of linking microbiological evidence generated from laboratory assays of core samples obtained form the subsurface with measurements collected under field conditions.

Initial studies were performed to evaluate the microbial potential for natural attenuation at Columbus AFB. Mineralization assays utilizing ^{14}C-labeled substrates indicated indigenous organism possessed the capacity to biodegrade aromatic hydrocarbons without any nutrient augmentation or environmental manipulation. Molecular analysis using an array of DNA probes developed for this study further indicated the near ubiquitous distribution of aromatic degrading genotypes at the research site.

Microbiological analyses performed after 278 days reflected the impact of hydrocarbons in the indigenous microbial community, with mineralization of naphthalene reaching levels of 60% in 24 hours and hydrocarbon degrading genotypes reaching levels of almost 50% of the total microbial community. After 462 days, microbial mineralization and hydrocarbon degrading populations were at lower levels.

Groundwater measurements indicated that migration of the contaminant hydrocarbons had been retarded at the field scale by microbial processes. The NAT Study provided the ability to link field measurements of natural attenuation with laboratory evidence from examining the indigenous microbial community.

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NOMENCLATURE AND ABBREVIATIONS

AFB	Air Force Base
ANOVA	Analysis of Variance
AODC	Acridine Orange Direct Counts
ATP	Adenosine Triphosphate
bmin	24 hour benzene mineralization assay
ben	Benzene
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
^{14}C	Radio-isotope Form of Carbon
CAFB	Columbus Air Force Base
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CFU	Colony Forming Units
DAPI	4,6-diamidino-2-phenylindole
dec	Decane
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DTT	Dithiothreitol
eben	Ethylbenzene
elev	Elevation

Made-1	First Macrodispersion Experiment at Columbus AFB
Made-2	Second Macrodispersion Experiment at Columbus AFB
naph	Naphthalene
nmin	24 hour naphthalene mineralization assay
NAT Study	Natural Attenuation Study
^{32}P	Radio-isotope Form of Phosphorus
PAH	Polycyclic Aromatic Hydrocarbons
PCB	Polychlorinated Biphenyl
PCP	Pentachlorophenol
PCR	Polymerase Chain Reaction
PFLA	Phospholipid Fatty Acid
pmin	24 hour phenanthrene mineralization assay
ppb	parts per billion (defined as μg / L fluid or μg / kg soil)
ppm	parts per million (defined as mg / L fluid or mg / kg soil)
pxyl	*p*-Xylene
RCRA	Resource Conservation and Recovery Act
rDNA	Ribosomal Deoxyribonucleic Acid
SPSS	Statistical Package for the Social Sciences
SRB	Sulfate Reducing Bacteria
tmin	24 hour toluene mineralization assay
TCE	Trichloroethylene

temp	Temperature
tol	Toluene
TVC	Total Viable Counts

Chapter 1

INTRODUCTION

Background

Petroleum hydrocarbon mixtures such as gasoline, diesel fuel, kerosene, and jet fuel are comprised of up to 200 different organic compounds (Fetter, 1993). Due to the substantial volume required by today's industrialized societies, petroleum products must be refined, transported, and stored in many different places around the world. When dealing with the large volume of petroleum in use around the world, it is inevitable that transportation accidents, human error, poor handling methods, and equipment failure will introduce hydrocarbons into the biosphere. Another potential source of contamination is stationary refining and distribution facilities. Chronic pollution of the local ecosystems by these facilities occurs by the discharge of waste effluent containing hydrocarbons and repetitive small spills. While the aforementioned examples contribute to petroleum contamination of the environment, a majority of hydrocarbon pollution is the result of leaking underground storage tanks (Bowlen and Kosson, 1995). Petroleum hydrocarbons released into the biosphere have the potential to infiltrate the topsoil and redistribute through the unsaturated layer then collect on top of the water table,

impacting potable water supplies. These events have a tremendous impact on the subsurface microbial ecology of the underlying aquifer.

Petroleum is a naturally occurring mixture of organic compounds produced by the incomplete decomposition of biomass over a geologically long period of time (Freedman, 1989). Of the complex mixture of organic compounds in petroleum, hydrocarbons are the quantitatively most important constituents (Freedman, 1989). Due to the period of time required to naturally produce petroleum, it is considered to be a nonrenewable resource. Studying the effects of contamination on groundwater ecosystems has come into focus only recently. Unlike marine and freshwater environments there is no wildlife to protect per se in a ground water ecosystem, only microorganisms (Madsen and Ghiorse, 1993). However, contamination of groundwater does have a direct impact on human health and in many cases, financial well-being (Madsen and Ghiorse, 1993). As analytical technology increases, so does public awareness of contaminated groundwater. This has forced the petroleum industry and federal government agencies to increase research efforts designed to gain a better understanding of groundwater ecology as it relates to the fate and transport of contaminants due to increased regulatory measures and public pressures. Increased public awareness and support was the driving force behind legislation, such as the Resource Conservation and Recovery Act (RCRA) and the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), being passed by

the federal government. Government and public support allowed tremendous advancement in understanding the fate of petroleum in the biosphere.

Over the last two decades, scientists and engineers have developed a multitude of methods for the treatment of contaminated surface and subsurface soils. Many factors influence the decision concerning which remediation option is best for any given site. The first factor to be addressed is the potential of ongoing site contamination. Source control may be necessary to prevent further contamination and any pure phase contaminant must be removed. Treatment of contaminated groundwater and/or soils is the next factor to address. The priority of any remediation project is to eliminate the spread of contaminants and treat the contaminated soil/groundwater to a pre-determined, acceptable level.

Bioremediation and Natural Attenuation

Many remediation systems have been designed in attempt to successfully restore contaminated sites. Bioremediation is a method of reclaiming sites that is microbiologically based. The fundamental principle of bioremediation is that the contaminants are destroyed or transformed to less harmful compounds (National Research Council, 1993). Contaminants can potentially serve as sources of energy for indigenous microorganisms. When environmental or contaminant conditions do not favor microbial growth, additional nutrients may be supplied to enhance the necessary biological response. Some sites may provide all essential elements for biodegradation to

occur naturally. In this case, the site under investigation may be a candidate for natural attenuation.

Natural attenuation as a remediation alternative is rapidly gaining interest due to its limited site destruction and contaminant exposure to both workers and local citizens. Physical mechanisms such as advection and dispersion combine with biological transformations to limit transport of contaminants in groundwater. Several factors determine the applicability of natural attenuation. These factors include the cost of alternative methods, biodegradability of contaminants, aquifer conditions, and the potential for impacting a local groundwater supply (Rifai et al., 1995). If conditions are favorable, biological interaction with the contaminants may surpass a steady-state situation where plume expansion ceases and actually begins to decrease in size. Intensive site characterization and an effective monitoring strategy of physical, chemical, and biological processes occurring are required for accurate plume management. If conditions are not favorable, such as the contaminants are poorly degradable, there are low nutrient and electron acceptor levels, or the risk of human exposure is high, a more active approach such as air sparging may be necessary.

Natural attenuation may be chosen as the primary method for remediating a site contaminated with residual concentrations of petroleum hydrocarbons, but is often applied in conjunction with various other processes. When possible, the source of contamination should be removed in order to the limit additional pollution. This

includes recovery of any pure-phase contaminants from the area. Hydrocarbons that have migrated from the source area may then be candidates for natural attenuation.

Several potential problems arise when considering natural attenuation for site remediation. Often, there is a lack of understanding of both the contaminant source and the contaminated area. Ideally, the concentration and composition of the contaminants would be known. While the composition of the contaminant plume may be determined using analytical methods, it is generally very difficult to determine the duration and amount of hydrocarbon discharged into the biosphere. If not already known, it is often difficult to accurately determine the site's geohydrology. Several groundwater monitoring wells may have to be installed in order to determine the direction of groundwater flow. Groundwater samples may also be recovered from these wells for geochemical analysis, but microbiological analyses are most accurate when performed on sediment cores recovered from the site. Subsurface sample recovery is a time-consuming and costly process. Many times, this critical step in the site characterization is skipped and groundwater flow models are used to "predict" plume behavior. Without adequate representation from all factors involved in natural attenuation, these models are often incorrect.

In order to effectively address these concerns, field research efforts need to be conducted. Multidisciplinary studies involving the characterization and monitoring of site geohydrology, microbiology, and geochemistry may provide insight into the intricate interactions occurring during natural attenuation. The ability to database this

information on the fundamental processes demonstrated during these experiments may lead to the development of parameters useful for inclusion in current groundwater models.

Columbus AFB Background

In order for a better mechanistic understanding of natural attenuation to occur, interdisciplinary field scale studies must be performed. One such investigation, termed the Natural Attenuation (NAT) Study, is currently underway at Columbus Air Force Base (AFB), Mississippi. The Columbus AFB Groundwater Research Facility has been the site of several field scale natural gradient tracer studies over the past 10 years. Initial groundwater research at the site during the mid 1980's was primarily concerned with the physical and chemical interactions (dispersion, diffusion, and sorption) that effected the fate and transport of certain target contaminants. The Groundwater Research Facility currently supports over 300 multi-level groundwater sampling wells providing a three dimensional monitoring network of over 6000 potential sampling ports.

In order to successfully conduct field research studies involving the migration of introduced compounds through the subsurface, an intensive physical characterization of the shallow unconfined alluvial aquifer was performed (Boggs et al., 1990; Boggs et al., 1993). Mean porosity was 0.31 ± 0.18 and bulk porosity is 1.77 g/cm^3. Average aquifer thickness was 11m. The water table fluctuates

seasonally, but averages 2-4 m below grade. The aquifer material was determined to consist of poorly-sorted to well-sorted sandy-gravel and gravelly-sand with minor amounts of silt and clay. Sand and gravel components were composed of chert, feldspar, and mica, with the fine-grained materials consisting of quartz, potassium feldspar, muscovite, and the clay minerals kaolinite and illite. Soil particles were commonly coated with iron oxides. The aquitard underlying the aquifer was formed by marine sediments belonging to the Eutaw Formation consisting of clays, silts and fine-grained sands.

Groundwater at Columbus AFB was also intensely studied (Boggs et al., 1993). Total dissolved solids are low, thus making electrical conductance low. Total organic carbon averages 2.3 mg/L. Dominant ions include sodium, silica, and chloride. Average pH is 4.8 with acid neutralizing capacity of the groundwater very low and greatly effected by dissolved carbon dioxide. Dissolved oxygen averages 4.2 mg/L. Average temperature of the groundwater is 18°C.

Spatial variability in aquifer properties such as hydraulic conductivity produce macrodispersion, a process that can affect that fate and transport of contaminants in groundwater systems. Several mathematical models attempting to explain dispersion were derived during the early 1980's (Gelhar and Axness, 1983). To adequately evaluate these models, controlled field experiments providing three-dimensional observations of transport of the nonreactive (conservative) groundwater tracers over large distances were designed. From October 1986 to June 1988, a natural gradient

groundwater tracer test involving a pulse injection of 3 fluorinated benzoic acids and bromide was performed at the Columbus AFB Groundwater Research Facility (Boggs et al., 1992; Boggs and Adams, 1992). The overall goal of the study, termed the Made-1 (first macrodispersion experiment) Study, was to test the validity and practicality of stochastic models which describe the effects of dispersion at field scale. Columbus AFB was chosen because of its high degree of heterogeneity. The site's heterogeneity is reflected in its spatial variabilities in hydraulic conductivities (Boggs et al., 1993; MacIntyre et al., 1997). These spatial variabilities allowed investigators to evaluate macrodispersivity as it is influenced by large-scale flow nonuniformity.

The first natural gradient macrodispersion experiment, termed the Made-1 Study, was conducted from 1986 - 1988. For the Made-1 Study, a pulse injection occurred over 48 hours and consisted of 10 m^3 of groundwater containing 400 mg/L of each benzoic acid, penta-fluorobenzoic acid, tri-fluoromethylbenzoic acid, and ortho-dofluorobenzoic acid, along with 2500 mg/L of calcium bromide. Transport of the tracers was monitored over a 20 month period, with groundwater samples recovered every 5-11 weeks. Evolution of the tracer plume was highly asymmetrical in the longitudinal direction. The main body of the plume remained within the initial 20 m of the site of injection. Mean hydraulic conductivity of this near-field region was determined to be 10^{-3} cm/s. When the leading edge of the plume reached the far field region, characterized by a mean hydraulic conductivity of 10^{-1} cm/s, transport of bromide by advection occurred rapidly in the longitudinal direction.

To further elucidate the affects of dispersion on contaminant fate and transport in groundwater systems, a second macrodispersion experiment (Made-2) was conducted at the Columbus AFB Groundwater Research Facility (Boggs et al., 1993). As a follow up to the Made-1 Study, reactive tracers were chosen instead of conservative tracers. A pulse injection of 9.7 m^3 of organic compounds was injected into the aquifer over a 48 hour time period during June 1990. The compounds chosen for this study included benzene, naphthalene, *p*-xylene, ^{14}C labeled *p*-xylene, and *o*-dichlorobenzene, as well as tritiated water for a conservative tracer. The concentration of each compound added was: benzene, 68.1 mg/L; naphthalene, 7.23 mg/L; *o*-dichlorobenzene, 32.8 mg/L, *p*-xylene, 51.5 mg/L; 2.77 nCi/ml ^{14}C-labeled *p*-xylene, and 55.6 nCi/ml tritium. The dissolved contaminants were monitored in groundwater samples over a 15 month period.

As was seen during the first macrodispersion experiment, the plume resulting from transport of the nonreactive tracer was characterized by a highly asymmetric distribution in the longitudinal direction. The organic compounds were retarded and did not migrate in a manner similar to the tritiated water. Adsorption was shown to affect transport of the organic tracers, but appeared to be a minor process. Since dispersion and sorption could not account for the degree of contaminant disappearance seen during the Made-2 Study, it was proposed that the observed loss of organic compounds was due to microbial activity.

All four organic tracers showed gradual disappearance over 440 days. Six percent of benzene, six percent of naphthalene, and one percent of *p*-xylene remained in the groundwater after 15 months. Thirteen percent of *o*-dichlorobenzene remained at the end of the experiment. Disappearance of the tracers could not be accounted for using advection, sorption, or dispersion models. It was proposed that the fate of the organics was affected by natural biotransformation by indigenous microorganisms. Even though no direct microbiological evidence was collected from the site, indirect geochemical data tends to support this conclusion. Examination of the ^{14}C *p*-xylene revealed greater than 80% of the radiolabel was found as $^{14}CO_2$ and intermediate by-products indicative of aerobic biodegradation. The estimated biological oxygen demand (BOD) was determined to be 1mg/L for biodegradation of the organic tracers at the observed rates. Aquifer heterogeneity allowing the influx of oxygen rich groundwater eliminated the concern of oxygen depletion during biodegradation for this concentration of contaminants.

MacIntyre et al., (1997) further examined the affects of heterogeneity of aquifer properties on solute transport in laboratory analyses. The properties examined were organic carbon content, specific surface area, and hydraulic conductivity. Sorption isotherms of naphthalene on aquifer solids from Columbus AFB were also generated. Correlation between laboratory analyses and field observations during the Made-2 were conducted. Organic carbon content of Columbus aquifer material strongly correlated with the sorption coefficient of naphthalene. However, organic

carbon content varies greatly within the test aquifer, thus producing local heterogeneities of naphthalene sorption. Larger scale heterogeneities seen in the results generated during the Made-2 Study was not reflected in these laboratory experiments. It was postulated that sampling was not conducted sufficiently to correctly predict solute transport at the field scale.

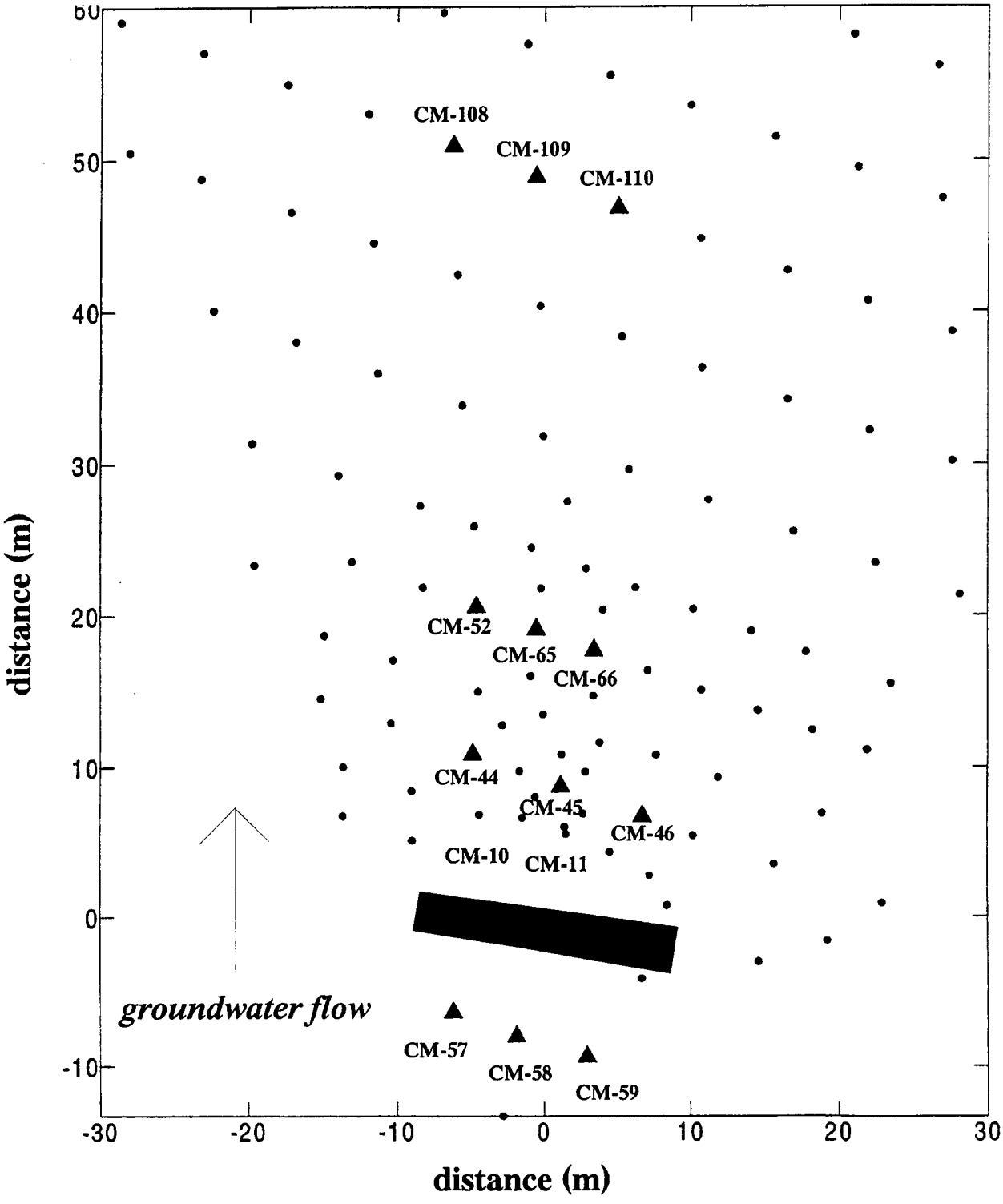
In order to develop a better understanding of the physical, chemical, and biological mechanisms and kinetics of natural biodegradation of contaminant hydrocarbons in groundwater, the Natural Attenuation (NAT) Study was designed for the Columbus AFB Groundwater Research Facility. This field experiment was proposed to further evaluate the fate and transport of hydrocarbons in the subsurface. However, the NAT Study was designed with a much larger concentration of hydrocarbons loaded onto aquifer solids and actually implanted within the test aquifer, as opposed to the pulse injections of the Made experiments. The NAT Study was designed in three major tasks. Task 1 involved an initial characterization of the site's groundwater chemistry and sediment microbiology. Task 2 was development of the contaminated source material and its placement within the aquifer. Task 3 involved monitoring plume evolution over time while measuring geochemical and microbial responses to the individual hydrocarbon plumes as they developed.

The experimental design of the NAT Study was based on field observations recorded during the Made experiments. Disappearance of the tracer organics during the Made-2 suggested that active degradative populations of microorganisms existed

at the site. Subsurface cores recovered from 5 depths in 12 boreholes for a total of 60 sediment samples were used for microbiological and geochemical analyses. Two additional boreholes were added later in the project to elucidate the biological and geochemical processes occurring approximately 1 meter downgradient the contaminant source in the region of high hydrocarbon concentration. Samples were recovered in 30 inch sterile plastic liners placed in steam-cleaned split-spoon sample tubes rinsed with alcohol. The sediment cores were retrieved by augering to the top of the desired sample depth, then hammering the split-spoon sample tube to the appropriate depth. Sediment was immediately bagged on location and stored at 4°C until shipped overnight. The borehole locations were designed to represent areas upgradient the proposed source trench for the NAT Study (as well as the areas of pulse injection for the Made experiments) and downgradient various distances from the site of contaminant release. Figure 1 shows the position of each borehole with respect to the location of the NAT source trench. The three control boreholes were located approximately 10m upgradient from the trench. The second transect of 3 boreholes was approximately 5m downgradient the source. Transect 3 was approximately 15m downgradient and transect 4 was 50m away form the source. Subsequent boreholes were recovered from similar locations.

Instead of injecting aqueous phase organic compounds, a trench to place the source within the aquifer was excavated November 1995. In order to remove material from the saturated zone, the aquifer was de-watered near the proposed trench area,

Figure 1. Plan view map showing the sampling locations at the Columbus AFB Groundwater Research Facility. Closed circles represent locations of multilevel groundwater sampling wells. Closed triangles represent locations of boreholes used for microbiological analyses.


CM-108
CM-109
CM-110
CM-52
CM-65
CM-66
CM-44
CM-45
CM-46
CM-10
CM-11
CM-57
CM-58
CM-59
groundwater flow
distance (m)
distance (m)
60
50
40
30
20
10
0
-10
-30
-20
-10
0
10
20
30

sheet piling drove into the subsurface, and aquifer solids removed. The $30m^3$ trench was back-filled using an artificially contaminated mixture of coarse, medium and fine grained sands having a higher hydraulic conductivity than the native aquifer material. The contaminated source was prepared by dissolving benzene, toluene, ethylbenzene, *p*-xylene, and naphthalene in decane, then mixing the hydrocarbon mixture with sand. The amount of each hydrocarbon in the source was determined to be (in mg/kg): benzene, 8; toluene, 1100; ethylbenzene, 1200; *p*-xylene, 1200; naphthalene, 1200; and decane, 12,000. After the source trench was filled, the remaining subsurface void was filled using the original subsurface material and the de-watering stopped. Time zero corresponded to removal of the sheet-piling during the last week of November 1995.

Chapter 2

OBJECTIVES

The purpose of this dissertation was to determine whether molecular based methods of monitoring microorganisms could be used to quantify shifts and dynamics in microbial populations during natural attenuation at Columbus AFB. An array of DNA probes was designed to add insight into changes occurring in aromatic hydrocarbon degrading populations in response to contaminant exposure. This knowledge could be used to evaluate microbial populations at similar contaminated sites. Such capabilities may potentially generate microbiological parameters important to groundwater models that predict natural attenuation or biodegradation. Specific objectives and goals are:

I. To characterize the indigenous microbial community at Columbus AFB prior to contaminant exposure using molecular based methods for degradative population structure and mineralization assays using ^{14}C-labeled compounds for determining metabolic activity.

II. To characterize and quantitate population shifts of the indigenous microbial community in response to hydrocarbon exposure using molecular based methods for degradative population structure and mineralization assays using ^{14}C-labeled compounds.

III. To quantify the dynamic response of the hydrocarbon degrading populations in relation to hydrogeological transport and fate of chemical contaminants during natural attenuation.

Chapter 3

LITERATURE REVIEW

Bioremediation

Several investigators have examined the ability of microorganisms indigenous to soil to actively metabolize aromatic compounds, including benzene, toluene, xylenes and naphthalene (Waksman, 1932). Other investigators found certain microbes are able to metabolize complex organic substrates such as petroleum hydrocarbons (Van Der Linden and Thijsse; 1965). R.L. Raymond and his co-workers (1960; 1961; 1967) found several soil isolates from the genus *Nocardia* possessed the capacity to co-oxidize mono- and dicyclic hydrocarbons. Jones and Edington (1968) provided an ecological survey of microorganisms capable of oxidizing hydrocarbons. They found the highest proportion of hydrocarbon oxidizing bacteria at a depth of 20 cm below ground surface. This corresponded to the area with the highest accumulation of organic, lipid, and hydrocarbon-type material. A very important idea reported by Jones and Edington involved the discussion of a particular type of microorganism as a function of the whole population. This idea was also used by Stapleton et al., (1997) to describe molecular data generated by the normalization of

aromatic hydrocarbon genotypes on total population estimates based on highly conserved 16S rDNA sequences.

During the early 1970's, investigators began applying information gained through laboratory experiments with microorganisms capable of biodegrading petroleum hydrocarbons to environments contaminated with gasoline. McKee et al., (1972) documented a fuel spill at Forest Lawn, CA. This represented the most extensive report on the physical removal of hydrocarbons by bacteria. No attempt was made to stimulate bacterial growth in order to enhance microbial degradation of the pollutants. Jamison et al., (1975) described a gasoline leak in the area of Ambler, PA that contaminated the township water supply. After exhaustive attempts to remove the contaminant, approximately 50% of the gasoline still remained in the system. Microbial degradation was investigated as a possible means to eliminate the residual gasoline. Initial laboratory studies proved indigenous microbes possessed the ability to utilize components of the gasoline as a sole carbon source, but maximum degradation was limited by low concentrations of nitrogen, phosphorus, and oxygen. Microbial growth in the contaminant zone was stimulated by the addition of a nutrient fertilizer and air, but the elevated population numbers were not sustained. R.L. Raymond and his co-workers at Suntech received a patent in 1974 entitled "Reclamation of contaminated groundwater" for this work. Oxygen was shown to be a key factor in accelerating bacterial degradation of gasoline.

Walker et al., (1975) evaluated petroleum degrading bacteria in Baltimore harbor water and sediment polluted by oil. Contaminated water samples showed a greater variety of bacterial species capable of degrading petroleum hydrocarbons than sediment. Greater degradation of petroleum hydrocarbons, as measured by gas-liquid chromatography, was also seen in water samples than sediment. Both water and sediment contained the bacteria *Pseudomonas* and *Acinetobacter* sp.

Raymond et al., (1976) investigated oil degradation in field soils from widely separated geographical locations. Six oils ranging from used crankcase oils from cars, to home heating oil number 2 were applied to 14 field plots covering a period of 1 year. One-half the plots of each location were fertilized. Oil degradation, monitored by gas chromatography and silica gel fractionation, showed the more polar compounds degraded more slowly. Significant increases in hydrocarbon utilizing bacteria were seen based on growth on pure hydrocarbons or the applied oils as sole carbon source. No significant increases in oil-degrading fungi were determined.

Heterotrophic microbiology of an uncontaminated stream and its associated groundwater in Marmot Basin, Alberta, Canada, was determined by Ladd et al., (1982). Total microbial abundance and biodegradation of naturally occurring labile and refractory organic compounds was monitored. Enumeration of bacteria using spread plates was 2-3 orders of magnitude lower than total biomass estimates based on acridine orange direct counts (AODC). This study represents the first biodegradation experiment of ^{14}C-labeled lignin by groundwater and stream bacteria.

In 1983, papers presented at a symposium on the microbiology of subsurface environment and groundwater contamination were published in Developments in Industrial Microbiology. Hirsch and Rades-Rohkohl (1983) described the diversity of microorganisms present in groundwater samples from Germany. Microbial diversity was determined by microscopy, enrichments and colony isolations. The study determined that the groundwater bacteria were oligotrophic and thus grew best on media dilute with carbon substrate. White et al., (1983) used biochemical methods for determining biomass, community structure and nutritional status in aquifer samples from Florida and Louisiana. They determined there was a 10- to 100-fold decrease in microbial abundance in aquifer material than surface sediments. Ghiorse and Balkwill (1983) assessed the microbiology of subsurface sediments from Louisiana using microscopic methods based on acridine orange direct counts and morphology. They found the sediments to support 1 to 10^6 bacteria per gram sediment, with the indigenous microbial community possessing diverse morphology. Biotransformation of a variety of synthetic organic compounds including chlorinated ethanes and benzene, as well as aromatics such as toluene, was investigated in subsurface sediments from Oklahoma and Louisiana (Wilson et al., 1983). No transformation of the chlorinated ethanes was seen from either site, and chlorobenzene was degraded in only the Oklahoma sample. Aromatic compounds were degraded slowly in samples from both sites. Ehrlich et al., (1983) evaluated the microbial ecology of an anaerobic, creosote contaminated aquifer in Minnesota. Total microbial abundance determined

by acridine orange direct counts ranged from 10^6-10^9 total organisms per gram sediment. Samples from the contaminated area of the aquifer showed methane formation from the degradation of phenolic compounds, suggesting methanogenesis was a key microbial process.

Dagley (1984) reviewed the importance of microbial biochemistry in bioremediation and its importance in maintaining the global carbon cycle. Microorganisms not only transform aromatic compounds initially thought to persist in the environment, they successfully send these compounds through a cascade of enzymes that manipulate the molecules into Kreb's cycle intermediates. This manipulation allows the organism to derive energetic benefits from biodegradation.

Several studies during the mid 1980's addressed the issue of metabolic diversity and activity of heterotrophic bacteria in subsurface samples. Microbial degradation of polyaromatic hydrocarbons and pentachlorophenol (PCP) was assessed at an abandoned wood-treatment facility in Texas (Lee et al., 1984). Total microbial population values ranged from 10^3 - 10^5 viable cells per gram sediment using dilute agar media to 10^5 - 10^7 cells per gram sediment using AODC. The results of this study showed subsurface organisms were capable of degrading the PAH compounds anthracene, dibenzofuran, flourene, naphthalene, as well as the chlorinated aromatic compound PCP. Ventullo and Larson (1985) collected groundwater samples from the United States and Canada for microbiological analyses. Microbial enumerations varied based on technique used. Total viable organisms determined by dilute agar

media spread plates were ranged from 10^2 - 10^3 cells per ml. AODC estimated microbial abundance at 10^4 - 10^6 cells per ml. Using the most probable number technique, 10^3 - 10^4 cells per ml were determined. Biodegradative potential was determined using ^{14}C-labeled substrate assays. Ward (1985) determined the microbial activity on both aerobic and anaerobic subsurface samples recovered from a septic tile field. Using $^{14}CO_2$ evolution from labeled parent compounds, it was determined that the subsurface samples possessed lower activities than surface samples. However, the subsurface samples retained the capacity to mineralize natural compounds such as acetic acid, and synthetic compounds, such as methyl parathion and 2,4-dichlorophenoxyacetic acid (2,4-D), under both aerobic and denitrifying conditions.

Lee and Ward (1985) reviewed the concept of harnessing biological processes for restoring contaminated aquifers. Increased information on contaminated subsurface environments showed considerable variation in nutrient and terminal electron acceptor requirements. Investigators began experimenting with several methods of oxygen addition, including peroxide and ozone, to stimulate microbial activity.

Natural Attenuation

Certain environments provide natural conditions for natural biodegradation of petroleum hydrocarbons to occur. Indigenous microorganisms possess the capacity to degrade the contaminants, dissolved oxygen is maintained at adequate levels

(generally above 2 ppm (mg / L), and other necessary nutrients such as nitrogen and phosphorus are present. When this is the case, natural attenuation, also termed intrinsic remediation, *in situ* bioremediation or natural bioremediation, becomes a feasible option in removing contaminants such as petroleum hydrocarbons. Natural attenuation can be a cost-effect means to protect groundwater resources that have inadvertantly become contaminated with hydrocarbons. For natural attenuation to become a universally accepted means of site remediation, a mechanistic understanding of the response of the indigenous subsurface microbial community must be developed. Few field studies involving natural attenuation have been conducted, with none of these studies involving molecular monitoring of specific microbial populations.

Barker et al., (1987) produced the first field experiment labeling biodegradation of introduced BTX (benzene, toluene, and xylenes) as natural attenuation. The experiment was performed in 1984-1985 at Canada Forces' Base, Borden, Ontario to examine the processes controlling the fate and persistence of specific components of gasoline in a sandy aquifer. Initial laboratory experiments were conducted in order to assess the biodegradability of BTX under several ranges of BTX and dissolved oxygen, including no oxygen or anaerobic conditions. A total of 1800 L of groundwater spiked to a final concentration of 7.6 mg / L total BTX was introduced into the study aquifer below the water table. Changes in BTX and dissolved oxygen were used to determine aerobic biodegradation and anaerobic biodegradation was monitored by phenolic and simple organic acids. The dominant

control over biodegradation was the availability of dissolved oxygen. Longitudinal dispersion was found to be a much more dominant process affecting contaminant transport than lateral dispersion.

The natural bioremediation of a different class of common organic contaminants was described by Klecka et al., (1990). Low levels of phenolic and polycyclic compounds from a charcoal manufacturing waste site were detected in nearby groundwater. However, approximately 100 m downgradient the waste site, the organic contaminants had been reduced to below detection levels. Dissolved oxygen had been depleted at the waste site due presumably to aerobic biodegradation. Computer models could not account for the observed attenuation in terms of known physical characteristics of the aquifer. Laboratory microcosm experiments using ^{14}C-radiolabeled compounds showed mixtures of the dominant organic compounds (phenols, methyl-substituted phenols, and naphthalene) could be degraded by microorganisms naturally occurring in sediment samples recovered from the site.

Proving *in situ* biodegradation of organic pollutants in subsurface environments is often a difficult process . Madsen et al., (1991) discuss the limitations and concerns that arise during this process. Central to proving *in situ* biodegradation is mass loss of pollutants and the causative agents of this process are microbes. Madsen et al., (1991) suggest certain approaches to determine the pattern and distribution of microbial activity indicative to biodegradation. Sediment samples, not groundwater samples, were gathered at consistent depths in boreholes located in areas

that spanned a gradient of contaminant concentration at a buried coal tar site. Microbial adaptation to the organic pollutants in contaminated areas but not pristine (control) areas was shown using ^{14}C-radiolabel compound mineralization assays. Increased numbers in total microbial populations were seen in regions of elevated contaminant concentrations. Furthermore, elevated levels of protozoan grazers indirectly reflected increased bacterial populations values.

Natural attenuation of benzene at concentrations ranging from 3 to 27 mg / L was shown at a manufacturing facility located on the eastern seaboard of the U.S. Laboratory mineralization experiments using ^{14}C-radiolabeled benzene showed 50% disappearance of the parent compound in 4 - 14 days (Davis et al., 1994). While laboratory experiments also showed benzene could be degraded anaerobically, dissolved oxygen concentrations in the groundwater samples remained at levels conducive to aerobic biodegradation. Computer analyses showed benzene transport was influenced by advection and adsorption, but could not account for the observed biological attenuation.

As current perspectives concerning natural attenuation evolve, certain aspects of the technology have clearly been demonstrated. Most importantly, for natural attenuation to remain a viable option in the restoration of contaminated subsurface environments, multidisciplinary teams must be enlisted to supervise site remediation (Hart, 1996). Natural attenuation requires careful assessment of the site's physical, chemical, and biological characteristics. Oxygen and nutrients must be constantly

replenished in order for microorganisms to, not necessarily totally eliminate the contaminants, but contain them and reduce the concentration to an acceptable level. Also, common concerns about the technology must be addressed, including the bioavailability of the contaminants and the length of time required for contaminant concentration reduction to acceptable levels.

Subsurface Microbiology

For several years, the subsurface was considered void of microorganisms (Waksman, 1932). It was not until the early 1970's that investigators began exploring the possibility of microorganisms existing below the soil horizon. The driving force behind these investigations revolved around the fate of anthropogenic contaminants released either purposely or inadvertently into the subsurface.

Early studies generally involved enumerating microorganisms from either sediment or groundwater samples using spread plate analyses. It was quickly determined that these experiments measured only the easily culturable organisms, which led to gross under-estimations up to 1000 fold of total microbial abundance (Ammann et al., 1995). Cellular stains, such as acridine orange (AO) and DAPI, allowed investigators to estimate total microbial abundance by direct microscopic counts, without having to cultivate any organisms.

After successfully enumerating total heterotrophic organisms in the subsurface, the next question to be addressed was one of microbial activity. Subsurface and

groundwater environments are known to be oligotrophic in nature. Nutrients known to limit microbial growth in these systems include nitrogen, phosphorus and carbon. To determine potential microbial interactions with anthropogenic chemicals, activity assays using ^{14}C-labeled compounds were developed. Evolution of $^{14}CO_2$ was used to determine the complete mineralization of target substrates.

After computer databases were expanded and several common biochemical pathways elucidated, molecular probes were developed to monitor specific genotypes. It became possible to evaluate the abundance and distribution of particular genotypes in any given sample. Gene probes based on highly conserved DNA sequences were established for functional groups of organisms such as mercury resistant organisms and nitrogen fixing bacteria, as well as catabolic genotypes such as naphthalene and toluene oxidizing bacteria.

Enumerating Microorganisms in the Subsurface

During the mid-1970's, several investigators began reporting on the presence and abundance of microorganisms in groundwater systems. McNabb and Dunlap (1974) presented an extensive review on subsurface biological activity in relation to groundwater pollution. They recognized very early on that the subsurface should be treated as a heterogeneous habitat for microorganisms that encompasses a wide range of environmental conditions. Several factors including temperature, sediment texture, organic content, oxygen levels, redox conditions and pH fluctuate from site to site,

thus the associated microbiology will also fluctuate. Basic ecological ideas including selection of the fittest individuals was also discussed. Willis et al., (1975) presented data on microbial enumerations from deep saline aquifers. Using samples retrieved from depths ranging from 1,000 to 1,500 feet below ground surface, they determined there to be an average of 60 to 141 bacteria per liter of groundwater.

Wilson et al., (1983) determined the microbial abundance on several subsurface core samples from Lula, Oklahoma using plate counts and acridine-orange direct counts (AODC). They found plate counts to be at least 10-fold less than direct counts. Microbial abundance was estimated to range from 10^3 to 10^6 cfu / gram sediment determined by spread plate analysis and 10^6 to 10^7 bacteria / gram sediment by microscopic analysis. They also found much greater variability in plate counts when compared to direct counts, suggesting many organisms did not grow on the DSEA medium used to determine colony forming units.

Webster et al., (1985) determined microbial cell numbers in subsurface samples from Oklahoma and Texas. They found 10^6 to 10^7 bacteria / gram sediment using AODC. ATP was also used as an indicator of biomass in the samples. Interestingly, they found there was much less variability in the amount of ATP recovered when using large sample sizes (100 grams) verses using small sample sizes (0.5 grams). Federle et al., (1986) determined the microbial biomass, activity and community structure in unsaturated, subsurface soils. Using phospholipid fatty acid analysis (PLFA), they determined biomass decreased with depth through the soil

horizon. Stetzenbach et al., (1986) isolated various bacteria from well water and determined total microbial abundance. They found total heterotrophic microorganisms ranged from 118 to 310 cfu / milliliter of groundwater.

Fredrickson et al., (1988) analyzed subsurface sediment for heterotrophic aerobic bacteria as well as anaerobic organisms. Heterotrophic bacteria were determined to be greater than 10^6 cfu / gram sediment for 60% of the samples. Nitrifying, sulfur-oxidizing, and hydrogen oxidizing bacteria were also cultured from the samples. Sediment texture was found to be a major factor influencing microbial abundance and distribution. Sinclair and Ghiorse (1989) reported 10^7 cfu / gram sediment of heterotrophic organisms for the same sediments. Sinclair et al., (1990) conducted a survey of microbial populations in aquifer sediments from Kansas. Bacterial counts ranged from 10^1 to 10^8 cfu per gram sediment, with direct microscopic counts ranging from 10^6 to 10^8 bacteria / gram sediment. Surface soils were shown to have 10 to 1000-fold more bacteria than corresponding aquifer sediments, as determined by AODC.

Johnson and Wood (1992) reported on the microbial potential of sandy aquifer material in the London Basin. Aerobic heterotrophic microorganisms ranged up to 10^6 cfu / gram sediment with anaerobic organisms ranging up to 10^5 cfu / gram sediment. Barbaro et al., (1994) investigated the relationships between aquifer properties and microbial populations. Viable cell counts ranged from 10^1 to 10^6 cfu / gram sediment.

Cell counts were found to correlate with dissolved oxygen and inversely correlate with depth. Hydraulic conductivity correlated with cell counts in one sample tested.

The distribution of bacteria in different size fractions of aquifer sediment was reported by Albrechtsen (1994). All aquifer samples had a high amount of coarser particles, 92 to 100% of the viable bacteria were found in the 1.2 to 100 μm sized fraction. Total microbial abundance averaged 10^6 cfu / gram sediment.

Microbial Activity in the Subsurface

The ability to label common contaminant compounds with ^{14}C atoms provided a readily usable laboratory assay to monitor biodegradation of target substrates by microorganisms. The final products of aerobic respiration are CO_2 and H_2O. Microbial degradation of ^{14}C-labeled substrates by microorganisms releases $^{14}CO_2$ which can be recovered and evaluated by liquid scintillation counting. The percent mineralization of the parent compound is calculated by dividing the amount of $^{14}CO_2$ evolved by the amount added and multiplying by 100%.

Pioneering work on microbial mineralization of organic material in aquatic ecosystems initiated in the 1960's. Using the uptake of radioactive compounds by natural populations of plankton, Parsons and Strickland (1962) developed a method for evaluating the heterotrophic potential in seawater. Additional work on the uptake of organic solutes in aquatic systems was performed by Wright and Hobbie (1965). Unlike Parsons and Strickland, Wright and Hobbie used a range of ^{14}C-labeled

substrate concentrations to monitor uptake the uptake of organic compounds such as glucose.

Harrison et al., (1971) developed a method for measuring mineralization of organic solutes in lake sediments. Using ^{14}C-labeled glucose and acetate, they monitored $^{14}CO_2$ evolution over time and calculated several parameters for substrate mineralization by natural populations, including total uptake and degradation kinetics. Caparello and LaRock (1975) used the method of Harrison et al., (1971) to develop a radioisotope assay for quantification of hexadecane biodegradation. A series of 50 ml Erlenmeyer flasks containing 10 ml buffer and 2 cm^3 harbor sediment was innoculated with ^{14}C-lableled hexadecane. $^{14}CO_2$ that was evolved by microbial metabolism was collected on filter paper and monitored by liquid scintillation counting. McKinley et al., (1983) improved efficiencies by adding wetting agents and methanolic sodium hydroxide to the filter paper used to collect $^{14}CO_2$.

In attempt to reproduce the often oligotrophic environment of many aquatic ecosystems, Jannasch (1967) investigated the growth responses of several species of marine bacteria to limiting concentrations of carbon substrates. This led Jannasch to describe the potential for a threshold level of carbon substrate necessary for maintaining bacterial activity. Shehata and Marr (1971) investigated similar conditions on *Eschericia coli*. They found that at high nutrient concentrations, microbial growth was independent of nutrient levels. However, at sub-optimal concentrations, growth rate is a function of nutrient concentrations.

Several reports appeared during the late 1970's and 1980's attempting to link this early work by Jannasch to biodegradation studies. Initial studies indicated laboratory biodegradation assays involving chemical concentrations greater than that found in nature may not correctly reflect the potential for biodegradation of the substrate in natural ecosystems (Boethling and Alexander, 1979). It was also found that biodegradation in natural systems may be limited by low substrate concentrations (Boethling and Alexander, 1979). Huang et al., (1981) showed that biodegradation of test chemicals under natural conditions may be limited by protozoan predation on bacteria responsible for mineralizing the substrate. Another limitation of mineralization of organic chemicals in natural systems was found to be clay particles (Subba-Rao and Alexander, 1982). Additional studies on the effect of a minimal threshold level of substrate concentration on biodegradation were conducted by Subba-Rao et al., (1982). They found that degradation kinetics and carbon assimilation were different at trace levels of substrate compared with higher concentrations of organic chemicals. This topic is still hotly debated.

As literature accumulated on the biodegradation of both naturally occurring and anthropogenic compounds, it became apparent that an acclimation period was often required prior to the onset of microbial activity. Microbial adaptation is a process that refers to an increase in the rate of biotransformation of a substance resulting form pre-exposure to that substance (Wilson et al., 1985). Adaptation may

be the result of increased growth of active organisms, induction of biochemical pathways, or genetic mutation.

Microbial transformation of polycyclic aromatic hydrocarbons (PAHs) found in petroleum was studied at an oil tank farm in Knoxville, TN (Herbes and Schwall, 1978). Transformation of ^{14}C-labeled naphthalene, anthracene, benz[a]anthracene, and benz[a]pyrene were examined in both pristine and petroleum contaminated river sediments. Naphthalene and anthracene turnover was much greater acclimated communities in contaminated samples as compared to pristine sediments. However, the study suggests higher molecular weight PAH compounds composed of 4- and 5-ring structures may persist in sediments even at locations of chronic exposure.

The effects of prior exposure to xenobiotics on biodegradation rates in estuarine and freshwater sediment was investigated by Spain et al., (1980). Sediment samples were tested for microbial adaptation to methyl parathion and *p*-nitrophenol by monitoring $^{14}CO_2$ evolution over time. Results of this study indicated that the ability of microbial populations to adapt depends on the presence of microbial species capable of biodegrading the contaminants of interest. Adaptation was seen in river sediment samples, but not in salt marsh cores samples. Spain and van Veld (1983) determined the effects of contaminant concentration, exposure time, inoculum, and chemical structure on microbial populations in freshwater river, estuarine, and marine sediments. Activity was assessed using $^{14}CO_2$ evolution from ^{14}C-labeled *p*-nitrophenol, *p*-cresol, 2,4-D, and trifluralin. No correlation was seen between

mineralization rate and total biomass. Adaptation was proportional to test concentration and not concentration used for pre-exposure, however a threshold concentration of 10 ppb was required for adaptation. Freshwater sediments adapted faster to organic compounds after pre-exposure, but estuarine and marine sediments did not.

Wilson et al., (1985) explored microbial adaptation to PAH compounds at a wood-treatment facility in Texas. Microbial depletion of organic substrates was measured in microcosms using gas chromatography. Degradation occurred in contaminated samples but not in pristine samples. The process of adaptation produced rates of biotransformation 1-2 orders of magnitude higher in contaminated samples as compared with samples having no prior exposure. Lewis et al., (1986) used bacteria and periphyton isolates from rivers to show adaptation lag periods for microbial utilization of *p*-cresol at low concentrations may reflect concentrations of available nitrogen and phosphorus. Wiggins et al., (1987) suggest the acclimation period preceding mineralization of organic chemicals in lake water and sewage was due to the time necessary for the multiplication of an initially small population of microorganisms capable of utilizing the chemicals as growth substrates. They also show this acclimation period may be extended by limiting the supply of inorganic nutrients or predation by protozoa. Thornton-Manning et al., (1987) found nitrogen and phosphorus addition did not stimulate mineralization of phenol in surface soils, but did result in faster and more extensive mineralizations in subsurface samples.

Temperature appeared to affect mineralization rates as a function of soil types. Fleming et al., (1993) showed naphthalene mineralization in samples from manufacturted gas plant sites correlated positively with *nahA* transcript levels, *nahA* gene frequency, and soil naphthalene concentrations.

Microbial adaptation to xenobiotic compounds in aquifer material was investigated intensively during the late 1980's. Federle (1987) suggests microbial adaptation to organic chemicals in the subsurface is due to prior acclimation to natural organic compounds infiltrating with water from the soil surface. Aelion et al., (1987) found three levels of response by microorganisms after prior exposure to contaminants. No adaptation was seen for chlorobenzene or 1,2,4-trichlorobenzene. A linear increase in mineralization over time was seen for the compounds *m*-cresol and *m*-aminophenol. Phenol, *p*-chlorophenol, and ethylene dibromide required no acclimation period for mineralization. Microbial enumerations demonstrated that acclimation is accompanied by an increase in the number of microorganisms capable of growth on the target compound. Further work showed that the addition of multiple inorganic nutrients resulted in greater mineralization activity than the addition of a single nutrient, and alternate carbon sources such as glucose, were inhibited degradation of the xenobiotic substrates (Swindoll et al., 1988a). Swindoll et al., (1988b) demonstrated subsurface microbial communities present in pristine aquifer sediments could possess the ability to biodegrade a wide variety of both natural and man-made compounds at several concentrations. Aelion et al., (1989) showed that a

significantly smaller percent of the added substrate was incorporated into cellular material and more respired as CO_2 in adapted communities than un-adapted communities. This result was later confirmed by Sharabi and Bartha (1993).

Knaebel et al., (1994) showed the mineral and organic constituents of soils effected microbial mineralization of surfactants. Surfactants pre-adsorbed to sand or kaolinite did not differ in there mineralization kinetics from samples with the surfactants added in the aqueous phase. However, surfactants pre-adsorbed to illite, montmorillonite, or humic acids showed greater lag periods than samples with surfactants added as aqueous solutions.

Molecular Microbiology in Environmental Samples

The ability to monitor changes in microbial populations reflecting contaminant exposure has in advanced with the development of molecular based techniques. Traditional microbiological methods were very time intensive and uncomprehensive for piecing together microbial populations present at any given site or determining response to perturbation. Intensive laboratory techniques including dilution series spread plating, enrichment cultures based on a particular substrate, and general microscopy were initially used to analyze environmental samples. These samples were limited by the ability to culture specific organisms from environmental samples. Thus, when dealing with culture based analysis of environmental samples, it is feasible that 99% of the

microorganisms are misrepresented (Ammann, et al., 1996, Torsvik et al., 1990; Ogram and Sayler, 1988).

Culture bias was eliminated with the development of nucleic acid based diagnotical procedures for monitoring microbes in the environment (Torsvik and Goksoyr, 1978; Torsvik 1980; Ogram et al., 1987; Holben, et al., 1988). Initial experiments designed to recover nucleic acids from the environment revolved around the ability to differentially separate microbial cells from soil particles by fractional centrifugations (Faegri et al., 1977). The cells were enzymatically lysed and the DNA recovered. Contaminating soil organic material was purified away using ion exchange / size exclusion chromatography. While this technique greatly advanced the ability of microbial ecologists, it still suffered from preferential recovery of certain microorganisms. Not all microorganisms were readily separated from soil particles. A major advance in this technique involved the ability to lyse microbial cells in situ (Ogram et al., 1987). This technique eliminated the bias associated with preferential recovery of organisms from soil particles. Cell lysis was initiated by heat treating a sediment sample slurried with sodium phosphate buffer and sodium dodecyl sulfate. After incubation for one hour with occasional mixing of the sample by hand, cell lysis was completed by bead-mill homogenization. Direct counts performed prior to heat treatment and after bead-mill homogenization revealed greater than 90% cell lysis. Nucleic acids were recovered by subsequent washing of the soil. The extracted nucleic

acids were concentrated and solvent purified. Again, additional purification steps utilizing ion chromatography were needed to remove undesirable organic contaminants.

Once nucleic acids could be easily recovered from the environment, investigators worked to develop techniques of manipulating the DNA for analysis of the native microbial community. Early biodiversity experiments performed on environmental nucleic acids involved reassociation kinetic studies (Torsvik et al., 1990; Ogram and Sayler, 1988). Another approach to microbial community patterns involved the use of DNA probes designed for detection of a specific target sequence expected to be present in the sample (Ogram et al., 1987). Nucleic acids recovered from an environmental sample could be vacuum blotted onto nylon membrane and fixed to the membrane by heat. DNA:DNA hybridization could be performed on the DNA samples using radiolabeled (^{32}P) gene probes.

Microbial diagnostics using specific DNA probes has gained rapid acceptance and interest over the past decade. Nucleic acid probes had previously been used to examine spread plates for the presence of particular genotypes using the colony hybridization technique (Sayler et al., 1985). Early targets of gene probes included chlorobiphenyl (Pettigrew and Sayler, 1986) and naphthalene degrading strains (Sayler et al., 1985), as well as specific physiological groups including nitrogen-fixing bacteria (Masterson et al., 1985). While colony hybridization served as a powerful technique useful in the detection of culturable organisms, it limited by the same culture-bias problems previously described for traditional microbiological analyses. The idea of

detecting specific microbial genotypes using DNA:DNA hybridization was easily adapted to nucleic acids extracted from the environment. Since this time, gene probes have been extensively used in microbial ecology and biodegradation research.

DNA hybridization is currently a powerful diagnostic tool for monitoring microorganisms in diverse environmental samples including soil, sediment, activated sludge, surface water, and groundwater. Increased microbiological and biochemical knowledge has provided the ability to characterize, monitor, or assess the microbiological potential of any given site. Functional groups of anaerobic microorganisms such as methanogens, acetogens, sulfate-reducing bacteria, and nitrate reducing can be analyzed. Also, microbial genotypes commonly associated with the biodegradation of many anthropogenic contaminants, such as 2,4-dichlorophenoxyacetic acid (2,4-D), toluene, naphthalene, and polychlorinated biphenyl, can also be detected. Wide arrays of gene probes have been developed to allow investigation of various functional groups present in the indigenous microbial community. Table 1 shows the gene probes currently in use at the Center for Environmental Biotechnology.

Application of molecular biology to studies in microbial ecology has steadily increased since the mid-1980's. Increased information on microbial community structure and function has been enhanced using molecular based diagnostic procedures. Early molecular studies in microbial ecology centered on the use of colony blotting and probing. This method employed traditional microbiological

Table 1. DNA probes used for assessing microbial communities at CEB / UT.

probe	reference organism	Target	reference
alkB	*P. oleovorans*	alkane hydroxylase	Kok et al., 1989
codh	*M. thermophila*	carbon monoxide dehydrogenase	Jablonski and Ferry, 1992
fthfs	*C. thermoaceticum*	formyltetrahydrofolate synthetase	Lovell and Hui, 1991
lux	*V. fischeri MJ-1*	luciferase enzyme	Baldwin et al., 1989
mcr	*M. barkeri*	methyl coenzyme M reductase	Hales et al., 1996
mdh	*M. organophilum*	methanol dehydrogenase	Machlin and Hanson, 1988
nahA	*P. putida* G7	naphthalene dioxygenase	Simon et al., 1993
nahG	*P. putida* G7	salicylate hydroxylase	You et al., 1991
nahH	*P. putida* G7	catechol-2,3-dioxygenase	Ghosal et al., 1987
nahR	*P. putida* G7	NAH7 regulatory protein	You et al., 1988
smmo	*M. trichosporium Ob3b*	soluble methane monooxygenase	Tsein and Hanson, 1992
todC1C2	*P. putida* F1	toluene dioxygenase	Zylstra and Gibson, 1989
tomA	*B. cepacia* G4	toluene monooxygenase	Shields et al., 1989
xylA	*P. putida*	xylene monooxygenase	Suzuki et al., 1991
23S rDNA	*P. fluorescens*	23S large subunit ribosomal DNA	Festl et al., 1986
16S rDNA	*E. coli*	16S small subunit ribosomal DNA	Stahl et al., 1988
Lambda	Lambda phage	internal standard for DNA extraction	Sanger et al., 1982

spread plating techniques which were then followed by transferring the colonies to a nylon membrane (Grunstein and Hogness, 1975). The colonies were then lysed on the membrane support and then the DNA was denatured and fixed directly to the membrane. These membranes were then probes with gene probes to detect specific genotypes of microorganisms.

The application of DNA:DNA hybridization techniques for determining populations possessing specific gene sequences in environmental samples was first reported on by Sayler et al., (1985). DNA colony hybridizations using the catabolic plasmids TOL and NAH7 as probes were used to enumerate populations on degradative microorganisms in microcosm sediment samples exposed to synthetic oil. Population estimates based on selective growth on toluene or naphthalene as a sole

carbon substrate overestimated the degradative population values compared with using colony hybridization. It was thought non-specific growth on contaminating carbon sources in the media led to the over-estimates.

Barkay et al., (1985) used colony hybridization analysis to detect mercury resistance genes in gram-negative bacteria. All of the gram-negative bacteria used in this study possessed DNA sequences homologous to the *mer* gene probe, whereas no such homologies were detected in the gram-positive strains. The *mer* gene probe was later used to monitor the genotypic adaptation of bacterial communities in sediment to mercury stress (Barkay and Olson, 1986).

Pettigrew and Sayler (1986) used the colony hybridization procedure to isolate bacteria capable of 4-chlorobiphenyl. Eight pure cultures possessing 4-chlorobiphenyl genes were isolated from freshwater sediment. Each strain that produced a positive hybridization signal with a DNA probe for 4-chlorobiphenyl was subsequently shown to degrade 4-chlorobiphenyl to 4-chlorobenzoate.

DNA probes targeting *Pseudomonas aeruginosa*-infecting bacteriophages have been used to screen natural samples of lake water, sediment, soil, and sewage (Ogunseitan et al., 1992). This study suggested up to 40% of *P. aeruginosa* in nature contain DNA sequences homologous to bacteriophage genomes.

Colony hybridization procedures have also been used to detect bacteria possessing genotypes commonly associated with soils and sediments contaminated with polychlorinated biphenyl compounds. Walia et al., (1990) compared the presence

of PCB-degrading genotypes present in garden soil to those in soils contaminated with toxic organic wastes. They found that less than 1% of the garden soil bacteria possessed genotypes commonly associated with PCB degradation, whereas > 80% of the bacteria present in contaminated soils hybridized with the *chpABCD* gene probe. Colony hybridization analysis of a New England soil showed no detectable PCB degrading genes present prior to enrichment with 2-chlorobiphenyl (Layton et al., 1994). After 2-3 weeks of enrichment with 2-chlorobiphenyl, organisms possessing genes for PCB degradation were detected.

DNA probes were used in colony hybridization analysis of microorganisms present in Alaskan sediments contaminated with petroleum hydrocarbons (Stotsky et al., 1994). Gene probes designed for detection of aliphatic (*alkB*) and aromatic (*xylE*) hydrocarbon degrading bacteria were used. Bacteria possessing the *xylE* gene made up the highest proportion of bacteria present prior to enrichment with petroleum hydrocarbons. Addition of naphthalene to the sediments greatly increased the amount of *xylE* genotypes, but did not influence the *alkB* genotypes. Enrichment of the sediment with crude oil increased the frequency of *alkB* genotypes.

A major problem associated with the colony hybridization technique is the inability to culture up to 99% of the bacteria in any given sample (Ammann et al., 1995; Torsvik et al., 1990; Ogram and Sayler, 1988). The ability to extract DNA from soils and sediments evolved from the need to eliminate the biases of traditional techniques. Directly extracting DNA from environmental matrices produces a more

representative sample of the indigenous microbial community and adds insight into specific genotypes present or absent within that sample. The extracted DNA can be hybridized with specific gene probes of interest to produce quantitative data.

Two general methods are currently used for extracting and purifying DNA from soil. The basic difference between the two techniques is that one method relies on the removal of bacterial cells from the particulate matter and the other method lyses the cells directly in the presence of these particulate materials.

The technique of recovering DNA from soil bacteria was reported on by Faegri et al., (1977), Torsvik and Goksøyr (1978) and by Torsvik (1980). This protocol employs the homogenization of each sample followed by fractional centrifugations to separate bacterial cells from soil particles. Once the cells have been separated from the environmental matrix, they are lysed and the DNA recovered. A subsequent modification of the same technique showed the only 33 to 34% of the cells are recovered (Holben et al., 1988).

Ogram et al., (1987) developed a new method of isolating bacteria DNA from soils and sediments based on the direct lysis of cells in there native matrix. This method lysed cells *in situ* using an initial heat-detergent (SDS) incubation step followed by ballistic disintegration of the cell envelope using bead-mill homogenization. It was determined that greater than 90% of the native microorganisms were lysed using this protocol. Furthermore, this technique provided

DNA of sufficient quantity and quality for use in DNA:DNA hybridization studies with a gene probe for nitrogen-fixing bacteria.

Since the development of this technique, several investigators have attempted to create shortcuts in the isolation and purification of DNA from the environment (Lovell and Piceno, 1994; Jacobsen and Rasmussen, 1992; Tsai and Olsen, 1991). A comparison of methods for DNA extraction from stream sediments was recently published (Leff et al., 1995). It was found that the Ogram method generated the highest DNA yield per sample.

Regardless of the technique chosen for DNA isolation, the particular probe or probes chosen for the study determine the usefulness of the technique (Ogram and Sayler, 1988). It is critical the probe specificity is known and proper positive and negative controls accompany hybridization experiments. Eliminating the use of entire plasmids as probes and generating probes by polymerase chain reaction (PCR) for short, highly specific target sequences of DNA help to eliminate this. Molecular microbial ecology as a whole has greatly advanced with the advent of the polymerase chain reaction (Mullis and Faloona, 1987). As in colony hybridization, extracting DNA from environmental samples allows investigators to detect specific genotypes present in the microbial community.

Several genotypes have been defined within soil and sediment microbial communities using gene probes targeting specific functional groups of organisms.

Nitrogen fixers, sulfate reducing bacteria, methanotrophs, acetogens and nitrate reducing organisms have all been detected using DNA hybridization.

Barkay et al., (1989) hybridized probes targeting genes encoding mercury resistance in bacteria with whole-community DNA extracted from fresh and saline water samples. Four probes targeting gene sequences encoding mercury resistance in both gram positive and gram negative bacteria were used to determine the response of the indigenous microbial community to mercury pollution. The resistance level, determined by *mer* gene abundance, to mercury was shown to increase 3.5 fold in microbial communities exposed to the pollutant. In similar work, Rochelle et al., (1991) measured the distribution of DNA sequences encoding mercury resistance in water and sediment isolates. Using three different gene probes, a wide distribution of mercury resistance genes was found in organisms both selected for and unselected for mercury resistance. A range of genetic diversity was found using these three probes, suggesting the use of multiple detection techniques and gene probes should be used for such studies.

Several investigators have developed DNA probes for the gene that encodes nitrogenase, the enzyme responsible for reducing di-nitrogen to ammonia. DNA sequence homology in the *nifH* subunit of the multisubunit nitrogenase protein has allowed investigators to develop gene probes used to detect most diazotrophs in the environment. Masterson et al., (1985) showed *nif* and *nod* sequences are conserved in strains of both *Rhizobium japonicum* and *Bradyrhizobium japonicum* using

hybridization patterns with DNA probes, regardless of whether the genes were chromosomally or plasmid encoded. Sadowsky et al., (1987) screened 20 field isolates of *Bradyrhizobium japonicum* using nif and nod probes in order to distinguish specific relationships between serotypes and nodulation patterns of soybeans. They found that hybridization patterns with a *nif* gene probe indicated a relationship between serotype, nodulation class, and physical organization of the genome. Conservation of the *nif* sequence was not carried over in the adjacent DNA sequences resulting in the different banding patterns seen in the Southern hybridization analysis.

Ogram et al., (1987) used a DNA probe targeting the genes encoding nitrogenase for detecting bacteria capable of fixing nitrogen in lake sediments. This probe was later used to screen microbial isolates from deep subsurface sediments recovered from the Savannah River Site in Aiken, SC (Bowman et al., 1993). Ben-Porath and Zehr (1994) characterized *nifH* and 16S rDNA gene sequences in cyanobacteria. They determined the *nifH* DNA sequences, along with the corresponding 16S rDNA sequences, varied sufficiently to distinguish heterocystous and nonheterocystous cyanobacteria. Kloos et al., (1995) used gene probes for denitrification, nitrogen fixation and nitrification to screen *Hyphomicrobium* in a sewage treatment plant. Several isolates of *Hyphomicrobium* produced positive signals during Southern hybridization analysis with gene probes for denitrification and nitrogen fixation, but no positive signals were seen with a DNA probe for nitrification.

This study showed that methylotrophic prosthecate bacteria of a sewage treatment plant can occupy several ecological niches.

Methanotrophs, bacteria capable of utilizing methane as a growth substrate, have been shown to be involved in the co-oxidation of trichloroethylene (TCE) in contaminated environments (Bowman et al., 1993). Tsien and Hanson (1992) developed a gene probe targeting the soluble methane monooxygenase component B for identification of methanotrophs in environmental samples. Seven of 12, all Type II methanotrophs, isolates produced positive hybridization signals with the *smmo* gene probe. The single Type X organism tested did not produce a positive hybridization signal with the DNA probe. The methanol dehydrogenase enzyme is used by methanotrophs and methylotrophs in the oxidation of methanol. Advances in molecular and biochemical knowledge have shown that *smmo* genes are not universal to all methanotrophs. McDonald and Murrell (1997) used this information to develop a functional gene probe for methanotrophs and methylotrophs based on the *mxaF*, methanol dehyrogenase, gene sequence.

Bacterial denitrification is a respiratory process in which nitrate and nitrite are reduced to gaseous end products. DNA probes targeting bacterial populations capable of denitrification were successfully designed and used in denitrifying bacteria from enrichments, bioreactors, and aquifer microcosms (Smith and Tiedje, 1992). Gene probes have also been used to determine the occurrence and distribution of

denitrifying *Hyphomicrobium* species in a sewage treatment facility (Kloos et al., 1995).

Sulfate reducing bacteria (SRB) play a key role in anaerobic biogeochemical processes occurring in nature. Molecular probes developed for SRB eliminates lengthy and tedious culture techniques traditionally required for detecting these organisms. SRB hydrogenase genes are thought to play a key role in biologically mediated metal corrosion. Voordouw et al., (1990) used DNA probes to determined the distribution of hydrogenase genes in SRB from an oil field. The genes encoding the [NiFe] hydrogenase were found to be ubiquitously distributed in members of the genus *Desulfovibrio* in both isolates and whole community bacteria DNA from oil field samples, but not other SRB. It was additionally suggested that other metabolic activity tests be used to support the molecular data in order not to overlook SRB not detected using the DNA probe. In 1993, Voordouw published a new method for detecting SRB based on reverse sample genome probing. In this analysis, chromosomal DNA obtained from isolates are spotted on a master filter which can then be hybridized with labeled, whole community DNA isolated from an environmental sample. Ideally, this approach produces a reproducible fingerprint of the SRB population present in a given sample.

Acetogens produce acetate from carbon dioxide and hydrogen using the unique the acetyl coenzyme A pathway. Even though acetate is a common substrate in many anaerobic environments, quantifying the distribution of acetogens has been difficult.

Lovell and Hui (1991) developed a functional group-specific DNA probe, based on the formyltetrahydrofolate synthetase enzyme sequence, to assist in the study of natural populations of acetogenic bacteria. Several acetogenic strains, as well as whole bacterial community extracted form horse manure, produced positive signals when hybridized with the *fthfs* gene probe.

Anthropogenic contamination of the environment has increased interest in developing molecular probes for groups of bacteria capable of biodegradation. Biological interactions greatly affect the fate and persistence of chemicals in environments such as farm soils, lake sediments, and aquifer sediments. Over the past decade, DNA probes for genotypes commonly associated with the biodegradation of 2,4-D, toluene, and naphthalene. Brockman (1995) presents a thorough review of nucleic acid based methods for monitoring the performance of *in situ* bioremediation.

Persistence of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in the environment has increased interest in its affect on indigenous soil microorganisms. Holben et al., (1992) developed a gene probe for the analysis of microbial populations in soils with a history of herbicide treatment. Using DNA probes targeting the genes encoding 2,4-D degradation located on the large catabolic plasmid pJP4, dramatic increases in the number of degradative organisms were seen. Significant increases in both *tfdA* and *tfdB* genes were seen in soils with and without prior exposure to the herbicide after laboratory enrichment studies. Neilson et al., (1992) used developed a gene probe for the *tfdB* gene on plasmid pJP4. This gene probe was used in

hybridization analysis of polymerase chain reaction products for detecting a 2,4-D degrading bacteria, *Alcaligenes eutrophus* JMP134, that had been added to soils. Ka et al., (1994) used *ftd* gene probes to determine the genetic and phenotypic diversity of 2,4-D degrading bacteria isolated from soil. Hybridization analysis showed 75% of the isolates possessed plasmids encoding 2,4-D degradation, while 25% possessed chromosomally encoded pathways. Further comparison of the isolates revealed that organisms similar at the plasmid level were not necessarily similar at the organismal level, suggesting the possibility that genetic exchange had occurred. Further analysis of 2,4-D degrading bacteria showed that a mosaic of catabolic genes has evolved for biodegradation of the herbicide. Fulthorpe et al., (1995) hybridized DNA from isolates from diverse geographic locations with *tfdA*, *tfdB*, and *tfdC* gene probes. They found many isolates did not hybridize with one or more of the tested DNA probes. The data produced from this study provides additional support for the idea of interspecies transfer of a variety of degradative genes involved in the evolution of 2,4-D metabolizing bacteria. Kamagata et al., (1997) evaluated 2,4-D degrading bacteria present in several pristine soils from very diverse geographic locations, ranging from Hawaii to Saskatchewan. Only one of the 8 isolates used in this study produced positive hybridization signals with a *ftdA* gene probe, suggesting the catabolic genes present in 2,4-D degrading bacteria present in environments with no history of herbicide exposure differ significantly from those sites with chronic exposure.

Several investigations have been conducted monitoring the *nahA* genotype in environmental samples suffering from polyaromatic hydrocarbon (PAH) contamination. Sanseverino et al., (1993) used a *nahA* DNA probe to quantitate shifts in microbial populations in soils from manufactured gas plants and a wood-treatment facility. They found *nahA* gene frequency and naphthalene mineralization correlated with naphthalene concentration in the samples. This study was followed by Fleming et al., (1993) showing *nahA* messenger RNA transcripts correlated with naphthalene concentration. Herrick et al., (1993) used PCR amplification of *nahA* genes and 16S rDNA genes to determine microbial diversity and prevalence at a site contaminated with coal-tar waste. A follow up study by Herrick et al., (1997) investigated natural horizontal transfer of the naphthalene dioxygenase between bacteria native to a coal tar-contaminated field site. While the predominant allele was closely related to the archetypal allele, DNA sequences varied as much as 7.9%. Hodson et al., (1995) developed an *in situ* PCR method for detecting *nahA* genotypes in complex mixtures of organisms. Pellizari et al., (1996) evaluated the ability of several isolates from tropical soils to degrade polychlorinated biphenyls and naphthalene. Southern hybridization analysis showed the *bph* and *nah* DNA probes hybridized with separate bands, suggesting that multiple dioxygenases were present.

Several biochemical pathways involved in contaminant biodegradation funnel into catechol dioxygenase enzymes that show striking DNA sequence homologies (Ghosal and Gunsalus, 1987). Joshi and Walia (1996) used PCR amplification of

catechol-2,3-dioxygenase sequences to determine the distribution of hydrocarbon degrading bacteria in groundwater. PCR products were analyzed by Southern hybridizations using a DNA probe for the catechol dioxygenase. Chandler and Brockman (1996) used a most probable number PCR procedure to estimate biodegradative gene numbers at a jet-fuel contaminated site. Genes encoding naphthalene dioxygenase and catechol dioxygenase were detected in significant portions of the microbial community, while the gene encoding alkane hydroxylase was not.

Chapter 4

MATERIALS and METHODS

Site Description

The study site is a shallow unconfined alluvial aquifer averaging approximately 11 m in thickness located on the northeast corner of Columbus AFB, MS, (Boggs et al., 1990; Boggs and Adams, 1992). Alluvial deposits range from sand (fine-granular) to gravelly-sand (coarse-granular) material (Boggs et al., 1990; Boggs and Adams, 1992). The water table level fluctuates with the seasons, but averages 61 m above sea level (approximately 4 m below ground surface). Clays, silts, and fine sands create the aquitard at 50 m above sea level (approximately 15 m below ground surface). Over 300 multilevel groundwater sampling wells provide more than 6000 individual sampling points across the study site. Detailed aquifer characteristics have been previously published (Boggs et al., 1990; Boggs and Adams, 1992; Boggs et al., 1993, MacIntyre et al., 1997; Stauffer et al., 1997), with general information listed in Table 2.

Source Emplacement

The contaminant source trench was installed November 1995. After installation of sheet-pile to sequester the proposed trench area, local de-watering of the

Table 2. Summary of Columbus AFB groundwater and aquifer characteristics[a].

	Mean
pH	4.8
Eh, mV	543
temperature, °C	17.9
total organic carbon, mg/l	2.3
carbon dioxide, mg/l	73
sulfate, mg/l	2.0
iron, μg/l	15
nitrate-nitrite, mg/l	1.5
dissolved oxygen, mg/l	4.2
bulk density, g/cm^3	1.77
particle density, g/cm^3	2.57
porosity	0.31
horizontal hydraulic conductivity, cm/s	0.02
vertical hydraulic conductivity, cm/s	0.0028

[a] Data adapted from Boggs et al, 1990 and Boggs et al, 1993.

Columbus AFB aquifer occurred from November 1 until November 11. A total of approximately 1,040 m^3 (275,000 gal.) of groundwater was removed during this period. Effluent was transported by tanker trucks for disposal in the CAFB sewer system. From November 4 through November 9, excavation of the trench area was performed. Soil recovered from the unsaturated zone was stockpiled on plastic sheeting and kept separate from soil excavated from below the water table.

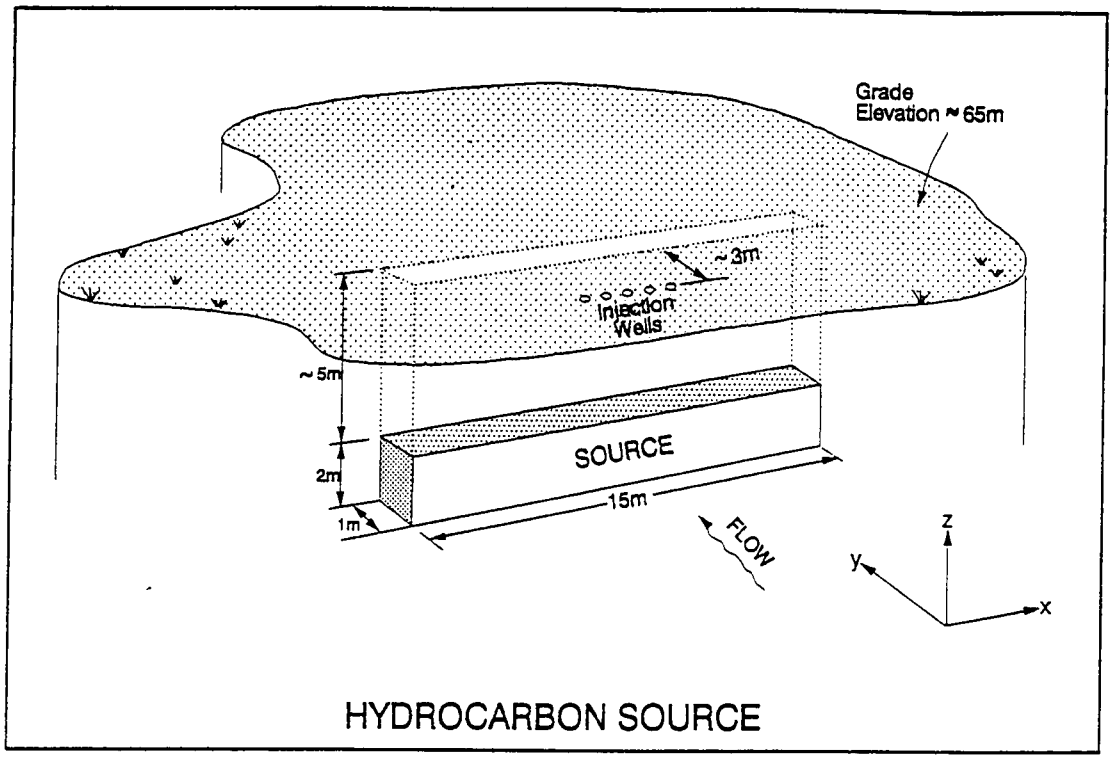
The hydrocarbon source material was mixed with sand and placed in the excavated trench area over a period of seven hours on November 10. Two cement mixers were used to mix the hydrocarbons and sand, along with aqueous bromide solutions prior to filling the trench.

Upon completion of the source placement, the trench was surveyed. The trench was back-filled and de-watering of the aquifer was terminated. Sheet-pile removal was not completed until December 1, due to contractor delays. The NAT Study officially commenced with sheet-pile removal on December 1, 1995. A cartoon of the source trench is seen in Figure 2.

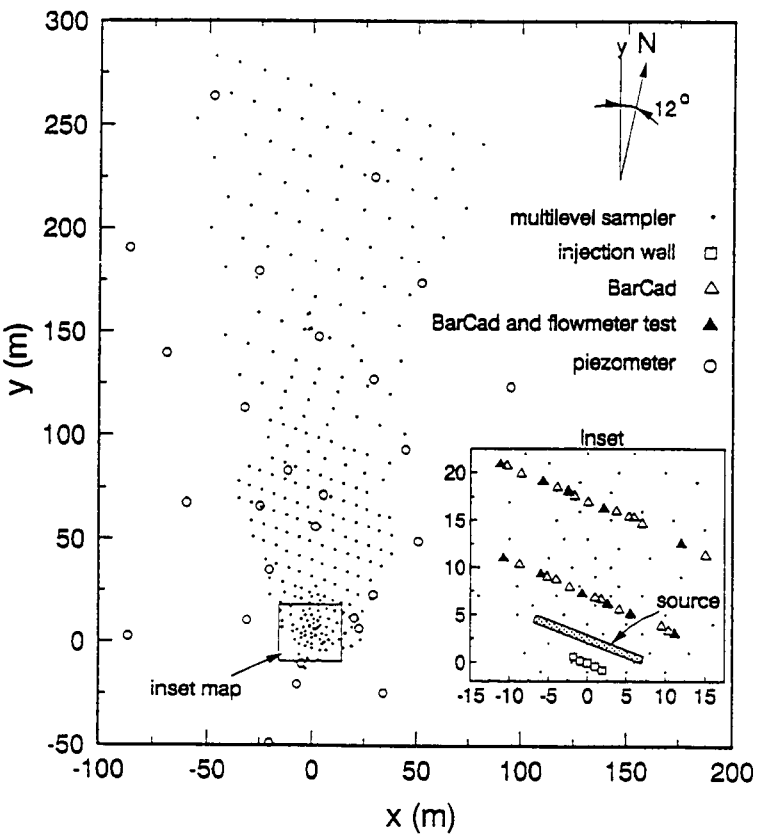
Sample Collection

Using aseptic technique, 60-80 sample cores were recovered from 5 preset depths of 12 boreholes. Borehole locations were chosen based on proximity to groundwater monitoring wells (Figure 1). Sample depths were converted to m above sea level (asl) in order to be defined in three dimensional space within the aquifer. Thus, Columbus AFB sediment sample depths were defined as elevations of 61, 60, 59, 58, and 57 meters above sea level, corresponding to 4, 5, 6, 7, and 8 m below ground surface respectively. For sample gathering, a truck mounted drill was used to auger to the top of the desired depth, then a split-barrel sample tube containing sterile plastic liners was hammered to the appropriate depth using a truck mounted hydraulic hammer. The split-barrel sample tube was steam-cleaned between samples and then rinsed with an alcohol solution (70% isopropanol). Once successfully recovered from the subsurface, sample cores were immediately processed at the Columbus AFB mobile laboratory. Each sediment core was homogenized and divided into sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) for overnight shipping on ice. Total

Figure 2. Cartoon representation of the NAT Study source trench at Columbus AFB. The contaminant source was created by excavating aquifer solids and replacing them with a coarse sand mixture loaded with hydrocarbons.



HYDROCARBON SOURCE



viable counts and mineralization assays were initiated immediately on the freshly recovered samples. Samples to undergo molecular analysis were maintained at -80°C. Sediments used in this study were not sieved or processed in any fashion in order to reflect the microbial community of this coarse-granular aquifer in the most accurate manner (Peterson and Klug, 1994). Source samples were recovered using a GeoProbe hydraulic penetrometer.

Total Viable Counts

Total viable counts (TVC) for aerobic heterotrophic microorganisms were determined in triplicate by adding 1 gm of sediment to 9 ml of sterile 0.1 % (w/v) sodium pyrophosphate dilution buffer (pH 7.0) (Balkwill et al., 1977), then vortexing vigorously for 30 seconds on, 30 seconds off, and 30 seconds on. Appropriate dilutions were plated from each series in duplicate on 1/10th strength YEPG (YEPG = g/L dH_20: yeast extract, 0.2; polypeptone, 2.0; D-glucose, 1.0; ammonia nitrate, 0.2; agar, 17.0; pH 7.0) (Sayler et al., 1985) and incubated at 25°C for one week before determining colony forming units (cfu). It was determined to be very important to check sterility of dilution buffer prior to use by plating 100 µl on YEPG agar and incubating overnight at 25°C.

Direct Microscopic Counts

Direct microscopic counts were performed using a modified method of Porter and Fieg (1980). Sediment used for direct counts was preserved by adding 1 gram sediment to 9 ml 2.5% formaldehyde in triplicate and storing in the dark at 4°C. Samples were stained with 40 µl 0.25 M sodium pyrophosphate and 10 µl (2 mg / ml) DAPI (4,6-diamidino-2-phenylindole) solution to one ml of preserved sample in a sterile microcentrifuge tube. After mixing thoroughly, the samples were placed in a sonicating water bath for 30 minutes, then stored in the dark at 4°C and stained overnight. Samples were then filtered through 0.2 µm black polycarbonate methane filters (Costar, Cambridge, MA), with the filters then being placed onto a glass slide and covered with a cover slip. Cells were enumerated in 15-20 fields per slide under 1000X oil immersion on a Nikon Optiphot HFX-II microscope (Garden City, NY).

Colony Hybridization

Colony hybridizations (Grunstein and Hogness, 1975) were performed as described by Sayler et al., (1985). Colonies were transferred to 0.45µm mesh nylon membranes (ICN Biomedical, Costa Mesa, CA) and lysed by addition of 0.5 N NaOH. DNA was fixed to the membranes by baking at 80°C for 1 hour. Filters were rinsed in 2X SSC (g/L dH_2O: sodium chloride, 17.53; sodium citrate, 8.82), then baked another hour at 80°C. Prehybridization was performed in an orbital rotary shaking water bath

at 65°C overnight in hybridization slime (0.5M NaH_2PO_4, 1mM EDTA, 7% [w/v] sodium dodecyl sulfate, 1 L dH_2O; pH to 7.2 with sodium hydroxide pellets) (Church and Gilbert, 1984) and then the appropriate catabolic gene probe was added using aerosol resistant tips (Molecular Bio-Products, Inc.). Hybridization with the ^{32}P-labeled catabolic gene probe was allowed to go overnight. Excess probe was removed by three 30 minute washes with high stringency wash buffer (g/L dH_2O: NaCl,0.59; Tris-Base, 2.42; EDTA, 0.37; SDS, 5.00; pH 7.5).

Mineralization Assays

Mineralization assays utilizing ^{14}C-labeled benzene, toluene, naphthalene, and phenanthrene (specific activities [mCi/mmol]: benzene, 58.2; toluene, 60.0; naphthalene, 49.8; phenanthrene, 13.3; Sigma; St. Louis, MO) were performed using the procedure described previously by Parsons and Strickland (1962) and Wright and Hobbie (1965) and modified by Sanseverino et al. (1993). Briefly, 2 g of sample were slurried in a 40 ml EPA certified vial (Eagle Picher, Miami, OK) with 1 ml sterile dH_20. An 8 ml vial (Fischer Scientific, Pittsburg, PA) containing 0.5 ml of a 0.5 M NaOH solution was inserted into the 40 ml vial as a $^{14}CO_2$ trap. Approximately 50,000 dpm of ^{14}C-labeled substrate was added to each sample and the vial sealed with a Teflon lined septum and screw cap. The vials were incubated at 25°C at 100 rpm on a rotary shaker. Microbial activity was stopped by the addition of 1.0 ml 2 N H_2SO_4

followed by additional shaking for 2 hours at 100 rpm. Solution from the NaOH trap was removed and added to 10 ml Ready Safe Scintillation Cocktail (Beckman Instrumentation, Inc., Fullerton, CA), along with 1 ml of dH_2O. Liquid scintillation counting was used to measure $^{14}CO_2$ evolution. Multiple time point assays (1,3,5,7, and 14 days) were initiated in order to evaluate contaminant degradation over time. Negative (killed) controls were prepared by addition of 1.0 ml 2 N H_2SO_4 prior to addition of the labeled substrate. Background values produced by the killed controls were generally under 1000 dpm and subtracted from experimental assays.

DNA Extraction from Aquifer Sediment

Using the method of Ogram et al. (1987), DNA extractions were performed on 50 g samples. Direct lysis of the samples was initiated by adding 125 ml 0.12 M Na_2HPO_4 (pH 8.0) and 25 ml 5% SDS (sodium dodecyl sulfate) solution, then incubating in a rotary water bath at 70°C for 1 hour, with periodic mixing by hand inversion. Lysis was completed in a Bead-Beater (Bio Spec, Bartlesville, OK) with 25 g of 0.1 mm diameter glass beads by blending for 2.5 minutes on, 0.5 minute off, 2.5 minutes on. The samples were successively alkaline extracted by centrifugation at 5500 x g for 25 minutes at 10°C twice using 0.12 M Na_2HPO_4 (pH 8.0), with each supernatant collected and pooled with the previous supernatant. Additional particulate matter was removed by a final 30 minute centrifugation at 11,500 x g. Pooled supernatants were concentrated by an overnight precipitation at -20°C by

adding 0.1 volume 2 M sodium acetate (pH 5.2) and equal volume isopropanol. After precipitation the samples were centrifuged at 4°C for 30 minutes at 11,500 x g and the supernatants discarded. The resulting pellets were dried under vacuum, then resuspended in 20 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.5). Exhaustive dialysis against TE buffer using Spectropore® dialysis tubing (MW cutoff 6-8000; Spectrum Medical Industries, Inc., Los Angeles, CA) was performed in order to reduce excessive salt concentrations. Samples were then subjected to one extraction with Tris saturated phenol (pH 8.0), followed by one chloroform/isoamyl alcohol (24:1) extraction. Recovered aqueous phases were precipitated overnight at -20°C by addition of 0.1 volume 2 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. The tubes were centrifuged and the resulting pellets dried under vacuum. The final pellets were suspended in 1 ml sterile TE buffer, then stored at -20°C until used.

Enzymatic Digestion of Samples with DNase and RNase

For DNA hydrolysis, samples were treated with 10 mM magnesium chloride / 1 mM DTT (Dithiothreitol) and 20 Units RNase-free, DNase I (Boehringer Mannheim, Indianapolis, IN) for at least one hour at 37°C prior to vacuum blotting. For RNA hydrolysis, samples were treated with 1 μg DNase-free, RNase (Boehringer Mannheim, Indianapoils, IN) for 30 minutes at 37°C prior to vacuum blotting. Samples receiving both treatments were initially treated with RNase as described, then

10 mM magnesium chloride / 1 mM DTT added along with 20 Units DNase for 1 hour at 37°C.

Lysis Efficiency

Cellular lysis efficiency was monitored on select samples by performing direct counts prior to beginning the extraction process and immediately after alkaline extraction of free DNA. One gram samples were weighed out in triplicate and each placed in 9 ml 2.5% formaldehyde then stored at 4°C in the dark. Direct counts were performed as previously described. Lysis efficiency was determined by dividing the number of organisms per gram sediment prior to lysis by the number after lysis and multiplying by 100%.

Recovery Efficiency

DNA recovery efficiency was determined on all samples (Applegate et al., 1995). After the bead-beat ballistic disintegration step, an exogenous fragment of lambda DNA was added as an internal standard. A 500 bp fragment representing bases 7131 to 7630 on the lambda map (Sanger et al., 1982) was amplified using control primers and template in a Perkin Elmer PCR Reagent Kit (Roche Molecular Systems, Inc., Branchburg, NJ), run on a 1.0% agarose gel to check for proper band size and cloned into the TA Cloning® vector (Invitrogen, San Diego, CA) using the manufacturer's protocols. Plasmid clones were screened using the rapid-boiling mini-

preparation method (Holmes and Qugley, 1981), restriction digested with *Eco*RI (Gibco BRL, Gaithersburg, MD), and subjected to gel electrophoresis to check for the correct insert. A large scale plasmid preparation (Promega, 1992) was then performed and the amount of DNA determined using a Beckman DU 70 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). A working solution of 100 ng / 100 µl was prepared. Prior to use as an internal standard, the plasmid was linearized with *Hind*III (Gibco BRL, Gaithersburg, MD) producing a single DNA fragment with a total size of 4.4 kb (0.5 kb lambda insert plus 3.9 kb vector).

Preparation of DNA Standards for Gene Probing

Standards used for vacuum blotting were prepared by PCR amplification of the desired gene fragment from chromosomal or plasmid preparations from appropriate reference strains. PCR products were run on 1.0% agarose gels to visualize the DNA bands and cloned into the TA Cloning® vector (Invitrogen, San Diego, CA) following the manufacturer's instructions. Plasmid clones were screened using the rapid boiling mini-prep method (Holmes and Quigley, 1981), restriction digested with *Eco*RI (Gibco BRL, Gaithersburg, MD) and run on a 1.0% agarose gel to check for the correct insert. Large scale plasmid preparations (Promega, 1992) were performed and the amount of DNA recovered was determined using a Beckman DU 70 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Working solutions of 2 ml were prepared for each amount represented in the standard curve and maintained

at -20°C. DNA concentrations per 100 µl of 100ng, 10ng, 1ng, 0.1ng, 0.01ng, 30ng, 3ng, 0.3ng, and 0.03ng were prepared for standard curves.

Mini-Preparations of Bacterial Plasmid DNA

A rapid boiling mini-preparation of bacterial plasmids based on the Holmes and Quigley (1981) protocol was used to screen DNA clones. After suspending a loop full of the bacteria in 40 µl STET buffer (50 mM EDTA, 50 mM Tris, 5% [w/v] Triton 100X, 8% [w/v] sucrose; pH 8.0) in a sterile microcentrifuge tube, 4 µl of lysozyme (10 mg / ml in 50 mM Tris, 20% [w/v] sucrose; pH 8.0) was added. The sample was vortexed, boiled for 1 minute, then centrifuged at 14,000 x g for 10 minutes at 25°C. The resulting pellet was removed using a sterile toothpick and the supernatant is precipitated at -20°C for at least 1 hour by adding 200 µl 0.3 M potassium acetate and 2 volumes ice cold ethanol. Plasmid DNA was collected by centrifugation at 14,000 x g for 15 minutes at 4°C. After decanting the supernatant, the pellet was dried under vacuum. Dried plasmid DNA was suspended in 22 µl sterile dH_2O, 2.5 µl appropriate digestion (enzyme) buffer, and 0.5 µl enzyme (*Eco*R1), and allowed to digest for 1-2 hours at 37°C. The digested DNA was loaded onto a 1.0% agarose mini-gel and separated by electrophoresis. DNA bands were stained with ethidium bromide and visualized under ultra-violet light.

Midi-Preparations of Bacterial Plasmid DNA

Bacteria possessing plasmids containing inserts for DNA sequencing were grown overnight in 125 ml LB (10 g tryptone, 5 gram yeast extract, 10 gram sodium chloride, 1 liter dH_2O; pH to 7.5) plus antibiotics at 37°C. Cells were harvested by centrifugation at 6,000 x g for 10 minutes at 20°C. The resulting pellet was suspended in 3 ml cold lysis buffer (50 mM Tris, 1 mM EDTA, 25% [w/v] sucrose; pH 8.0) and transferred to a sterile 30 ml centrifuge tube where 6 ml of freshly prepared 0.2 N NaOH, 1% [w/v] was added. The sample was mixed by hand inversion (not by vortexing), then 3.75 ml 5 M potassium acetate was added. Again, the sample was mixed by hand inversion, then centrifuged for 30 minutes at 14,000 x g at 20°C. The supernatant was filtered through cheesecloth into a fresh 30 ml centrifuge tube and an equal volume of isopropanol was added to precipitate nucleic acids. The sample was pelleted by centrifugation for 30 minutes at 14,000 x g at 10°C. The supernatant was decanted and the resulting pellet dried under vacuum. RNA was hydrolyzed by suspending the sample in 500 µl sterile dH_2O and 5 µl DNAse-free, RNAse (Boehringer Mannheim; Indianapolis, IN), then incubating at 37°C for 1 hour. The sample was then transferred to a sterile microcentrifuge tube and extracted with an equal volume of phenol chloroform, followed by a chloroform extraction. The recovered aqueous phase was precipitated with equal volume isopropanol at room temperature and centrifuged at 14,000 x g for 10 minutes at room temperature. The

final pellet was dried under vacuum and suspended in 200 µl sterile TE buffer for storage at -20°C.

Large-Preparations of Bacterial Plasmid DNA

Large scale preparations of bacterial plasmids were performed using the Promega protocol (Promega Technical Bulletin, 1992). Bacterial cells were grown overnight in LB media with antibiotic selection at 37°C. Cells were collected by centrifugation at 6,000 x g for 15 minutes at 4°C. The resulting pellet of cells was suspended in 24 ml cold lysis buffer (25 mM Tris, 10 mM EDTA, 15% [w/v] sucrose, 2 mg / ml lysozyme; pH 8.0) by pipetting with a sterile transfer pipette and incubated in an ice-water bath for 20 minutes. Forty-eight ml of freshly prepared 0.2 N sodium hydroxide, 1% [w/v] SDS was added and the sample mixed thoroughly by hand inversions, then incubated in an ice-water bath for 20 minutes. Following centrifugation at 14,000 x g for 20 minutes at 4°C , the supernatant was filtered through cheesecloth and extracted with an equal volume of phenol:cloroform (1:1). After mixing thoroughly by hand inversions, the sample was centrifuged at 14,000 x g for 10 minutes at 4°C. The aqueous phase was again extracted with an equal volume phenol:chloroform as above, followed by an extraction with chloroform: isoamyl alcohol (24:1). The aqueous phase was precipitated with 2 volumes ethanol at -20°C for at least 2 hours. The precipitated sample was recovered by centrifugation at 14,000 x g for 30 minutes at 4°C and dried under vacuum. The final pellet was

suspended in 8 ml sterile TE buffer. The sample was prepared for plasmid purification by ethidium bromide / cesium chloride ultracentrifugation by adding 8 gms cesium chloride and 0.8 ml ethidium bromide solution (10 mg / ml). After loading sample into ultracentrifugation tube and properly balanced, the sample ran overnight at 55,000 rpm. Plasmid DNA bands were recovered form the centrifugation tubes using an 18 gauge needle and 3 ml sterile syringe. The DNA was transferred to a sterile 15 ml polycarbonate tube and the ethidium bromide removed by several extractions with equal volume of water saturated sec-butanol. The sample was brought up to 10 ml with sterile TE buffer and precipitatied overnight at -20°C with 0.1 volume 3 M sodium acetate and 2 volumes ethanol. Plasmid DNA was recovered by centrifugation at 14,000 x g for 30 minutes at 4°C. The supernatant was discarded and the final pellet dried under vacuum, then suspended in 1 ml sterile TE buffer and stored at -20°C.

DNA:DNA Hybridization

Nucleic acid extracts were probed using the catabolic gene probes designed for the NAT Study (Table 3). Nucleic acids were vacuum blotted onto 0.22 μm mesh Biotrans nylon membrane (ICN Biomedical, Inc., Costa Mesa, CA) using a Slot-Blot Apparatus (Biorad, Hercules, CA), baked at 80°C for 1 hour, rinsed in 2X SSC (g / L dH_2O: sodium chloride, 17.53; sodium citrate, 8.82; pH 7.0), then baked for another hour at 80°C. Blots hybridized with catabolic gene probes were pre-hybridized in

hybridization solution (0.5 M NaH_2PO_4, 1 mM EDTA, 7% [w/v] SDS, 1 L dH_2O; pH to 7.2 with sodium hydroxide pellets) 12-18 hours at 65°C in a rotary water bath (Church and Gilbert, 1984). Hybridization experiments were performed at the same temperature with a single-stranded PCR generated ^{32}P-labeled probe (see below) for 12-18 hours. Blots hybridized with the16S rDNA Universal oligonucleotide (Stahl et al., 1988) were pre-hybridized at 42°C 12-18 hours, then hybridized with an end-labeled Universal oligonucleotide probe for 12-18 hours. Following hybridization, membranes were washed 3 times for twenty minutes each under high-stringency conditions (g / L dH_2O: NaCl,0.59; Tris-Base, 2.42; EDTA, 0.37; SDS, 5.00; pH 7.5) at the same temperature as hybridization occurred, allowed to air dry, and placed with x-ray film at -80°C to visualize positive signals.

DNA Probe Preparation

Single-stranded catabolic gene probes were generated by (asymmetric) polymerase chain reaction using a Perkin Elmer PCR kit (Roche Molecular Systems, Inc., Branchburg, NJ). The PCR recipe for single-stranded probe reactions included 1X PCR buffer provided in the kit (50 mM KCl, 1.5 mM $MgCl_2$), 200 μM each nucleotide, 2.5 Units *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD), 5 - 10 ng of one primer, approximately 1 ng double-stranded DNA template and ^{32}P-labeled α-dCTP (ICN Radiochemicals, Costa Mesa, CA; specific activity, 800 Ci/mmol) in place of the unlabeled dCTP. Step cycles for the probe reactions are as follows:

lambda, *alkB*, *nahA* and *nahH/xylE* amplified at 94°C for 30 seconds, 42°C for 30 seconds, and 72°C for 2 minutes; *todC1C2* and *xylA* amplified at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 3 minutes. All amplifications were performed for 38 cycles. PCR generated probes were purified using NucTrap® Push Columns (Stratagene, La Jolla, CA). At least 10^5 cpm / μl (total > 10^7 cpm added), as determined by scintillation counting of 5 μl of purified probe in 10 ml Beckman Ready Safe scintillation cocktail (Beckman Instruments, Inc., Fullerton, CA), was added to the pre-hybridized membranes. Two hundred nanograms 16S Universal rDNA oligonucleotide (Stahl et al., 1988) probe was end-labeled at 37°C for 30-60 minutes with γ-ATP-^{32}P (ICN Radiochemicals, Costa Mesa, CA; specific activity, 7000 Ci/mmol) using T4 Polynucleotide Kinase (Gibco BRL, Gaithersburg, MD) and a 5' DNA terminus labeling system (Gibco BRL, Gaithersburg, MD). End-labeled oligonucleotides were also purified using NucTrap® push columns (Stratagene, La Jolla, CA) and quantified by scintillation counting prior to hybridization.

Quantitation of DNA

The amount of DNA per sample was determined in nanograms by inserting sample intensities into the standard curve regression equation produced from values obtained by computer aided analysis of autoradiogram images (SigmaGel, Jandel Scientific, San Rafael, CA). Cell numbers were estimated using the equation described in (Applegate et al., 1995), without adjusting cell number estimates for

recovery efficiency. Percent community values were generated using the equation described by Stapleton et al. (1997).

Statistical Analysis and Database Development

A interactive database program containing microbiological and geochemical parameters, as well as hydrocarbon profiles, was created by Mike McGee at the Norris Engineering Laboratory of the Tennessee Valley Authority. This database allowed for development of a numerical matrix of data collected by several investigators involved in the NAT Study. Statistical analysis of the NAT Study data set was performed using *Statistical Package for the Social Sciences (SPSS)*. The analyses used for this study included descriptive statistics, correlation analysis, and regression analysis. All data analyses were performed on a Gateway P5-133MHz personal computer with a Pentium processor.

Chapter 5

RESULTS

Assessing the Potential for Natural Attenuation at Columbus AFB

Establishing Nucleic Acid Analyses for Subsurface Microbial Ecology

Two important concerns that must be addressed when assaying nucleic acids from environmental matrices are cellular lysis efficiency of indigenous organisms and the recovery of DNA from soil. Preliminary investigations were conducted in triplicate on two representative sediment samples (Table 4). Using the particle size distribution listed in Table 4, one sediment sample was defined as fine-granular and the other sample was defined as coarse-granular. Grain size distribution for the fine-granular sample was 97.4% sand, 2.1% silt, and 0.5% clay. The coarse-granular

Table 4. Particle size distribution of samples used in Columbus AFB variability study.

	fine-granular	coarse-granular[a]
sand	97.4 %	98.3 %
silt	2.1 %	1.3 %
clay	0.5 %	0.4 %
pH	5.98	6.20

[a] The coarse granular sample was determined to be 40% gravel (by weight) using a 60mesh sieve (>250μm).

sample was determined to consist of 40% gravel by weight (defined as particles > 250 μm), with a remaining particle distribution of 98.3% sand, 1.3% silt, and 0.4% clay.

To check cellular lysis efficiency, DAPI direct counts were performed on samples prior to and immediately following the cell lysis step of the DNA extraction technique. Lysis efficiency, determined in triplicate on the fine-granular and coarse-granular study samples, was greater than 86% in each. This falls within the range of current studies showing 26-92% (Zhou et al., 1996) and 81-89% (Lovell and Piceno, 1994). To further support the use of nucleic acid analysis for this study, an internal standard was developed using an exogenous Lambda DNA fragment to provide a means for monitoring DNA recovery during the extraction technique. No detectable levels of Lambda DNA were found in samples extracted and probed without adding the internal standard. Average DNA recovery determined by internal standard recovery for the 60 samples used for assessing the microbial potential for natural attenuation was 78 ± 15%.

Specificity of catabolic gene probes used in this study is listed in Table 5. The gene probes *alkB*, *nahA* and *todC1C2* hybridized only with the appropriate reference bacteiral strains, *Pseudomonas oleovorans*, *Pseudomonas putida* G7 and *Pseudomonas putida* F1 respectively. Many degradative pathways funnel through catechol-2,3-dioxygenase enzymes. The *nahH / xylE* gene probe, which targets the catechol-2,3-dioxygenase enzyme, hybridized with 3 of the 4 reference organisms. While cross hybridization of the probe with the reference strains mt-2 and G7 was

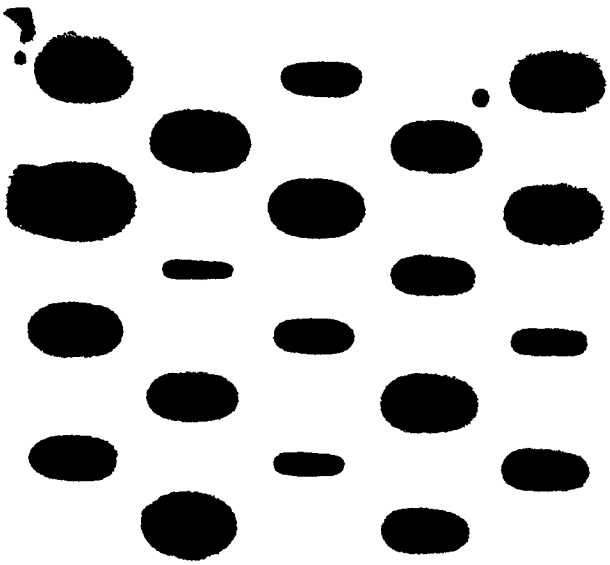
Table 5. Probe specificity check using colony hybridization of the reference organisms

probe	*P. oleovorans*	*P. putida* F1	*P. putida* G7	*P putida* mt-2
alkB	+	-	-	-
nahA	-	-	+	-
nahH / xylE	+	-	+	+
todC1C2	-	+	-	-
xylA	+	-	-	+

expected, the positive hybridization signal seen with *P. oleovorans* has no precedence in the literature. The *xylA* gene probe showed cross-reactivity with both of the reference organisms *P. putida* mt-2 and *P. oleovorans* (Suzuki et al., 1991).

Specificity of the 16S rDNA Universal oligonucleotide is shown in Figure 3. The nucleic acid samples on the membrane in Figure 3 were loaded as the same amount of total nucleic acids (appr. 2.5 μg). Of the various DNA samples loaded on to the membrane representing the three major kingdoms Archea, Bacteria and Eukarya and including both aerobic and anaerobic bacteria, only 3 organisms showed significantly different hybridization signals. These organisms were the iron-reducing organism, *Geobacter metallireducens*; a yeast isolated from acidic soil in a coal pile drainange basin, SRS3; and *Burkholderia cepacia* G4. This could be due to a variation in the number of rDNA genes on the chromosome, chromosome size, or potential discrepancies in rDNA sequences.

A



B

	1	2	3	4	5
A	*Methanosarcina thermophilis*		*Mthylosinus trichosporum* OB3b		*Pseudomonas putida* mt-2
B		*Desulfovibrio desulfuricans*		SRS1	
C	*Clostridium magnum*		*Bacillus subtilis*		*Pseudomonas oleovorans*
D		*Geobacter metallireducens*		SRS2	
E	*Clostridium thermoautotrophum*		*Euglena* sp.		*Burkholderia cepacia*
F		*Pseudomonas fluorescens*		*Pseudomonas putida* F1	
G	*Halobacterium* sp.		SRS3		*Pseudomonas pickettii*
H		*Eschericia coli*		*Pseudomonas putida* G7	

Figure 3. Specificity of the16S rDNA Universal oligonucleotide. Figure 3A shows a slot blot hybridization of the various DNA's listed in Figure 3B. Approximately 2.5 μg of total DNA was loaded into each well.

Table 6. Comparison between samples using the 16S rDNA Universal oligonucleotide.

	fine granular	coarse granular
extracellular	19.77 ± 7.88	11.62 ± 8.26
intracellular	20.91 ± 2.04	21.92 ± 3.18
whole community	28.89 ± 2.25	19.60 ± 1.15
RNased whole community	32.90 ± 5.96	25.71 ± 1.78

Two prominent concerns for using the 16S rDNA Universal oligonucleotide were addressed. One concern is the influence of extracellular DNA on enumerating microbial populations in natural ecosystems. Extracellular DNA was recovered in quantities sufficient for DNA hybridization with the Universal oligonucleotide (Table 6). However, great care must be given to the interpretation of these results. The method used for extracellular DNA recovery was very similar to the DNA extraction method of Torsvik and Goksoyr (1978), in which microorganisms were first washed from soil particles, then the DNA was purified. Thus, while extracellular DNA can be extracted from environmental samples, the method of recovery must be chosen very carefully and the results analyzed cautiously. This experiment appears to overestimate the influence of extracellular DNA. The second concern is the influence of ribosomal RNA that may co-extract with chromosomal DNA from indigenous soil microbes. Table 6 shows there is little difference in values for whole-community

DNA probed with the Universal oligonucleotide verses whole-community DNA that had been treated with RNase and probed with the Universal oligonucleotide.

Evaluating Sample Heterogeneity

Previous groundwater investigations at Columbus AFB have demonstrated the inherent physical heterogeneity characteristic of the test aquifer. Laboratory studies were designed to address the effects of the aquifer's physical heterogeneity on the molecular techniques chosen to assess the microbial community present within the study area. Since only one 50 gram sample was to be extracted for DNA, preliminary experiments were performed on two sediment samples (one fine-granular and one coarse-granular) in triplicate. Table 7 shows a comparison of fine- verses coarse-granular sediment on the native microorganisms, as well as variability within a single 50 gram sample, using a molecular based approach. Recovery of the internal standard for DNA extraction was approximately 50% for both samples. Recovery of both *nahA* and *nahH* gene sequences from within a single 50 gram fine-granular sample and a 50 gram coarse-granular sample were minimal. Except for recovery of *nahA* gene sequences from the fine granular sample, the standard deviation for 16S rDNA, *nahA*, and *nahH* genes was at least 10-fold less than the calculated average for each sample. As can be seen, the standard deviation is minimal and the variability in data obtained using a single, large nucleic acid extraction falls well within reasonable values.

Table 7. Variability within samples using gene probe data values.

probe	fine granular	coarse granular
Lambda (recovery of internal DNA standard)	50.65 ± 1.11 %	48.31 ± 4.14 %
ng 16S Universal Oligo	32.90 ± 5.96	25.71 ± 1.78
ng *nahA*	0.32 ± 0.21	1.77 ± 0.29
ng *nahH*	2.23 ± 0.35	1.85 ± 0.03

Enumeration of Microorganisms in the Subsurface

Techniques for enumerating the total biomass of microorganisms from environmental samples were compared on one fine-granular sample and one coarse-granular sample. Total viable counts on dilute agar medium, direct microscopic counts based on DAPI cell staining, and DNA:DNA hybridization using the Universal 16S rDNA oligonucleotide (Stahl et al., 1988) were used to enumerate total microbial abundance (Figure 4). Spread plate analysis for total viable bacteria yielded microbial abundance values of 2.09×10^5 ($\pm 6.60 \times 10^4$) colony forming units (cfu) / gm sediment for the fine-granular sample and 1.55×10^6 ($\pm 3.99 \times 10^5$) cfu / gm for the coarse-granular sample. Direct microscopic counts performed on the fine-granular sample revealed 5.95×10^7 ($\pm 3.16 \times 10^6$) cells / gm sediment, with 6.29×10^7 ($\pm 2.48 \times 10^6$) cells / gm sediment for the coarse-granular sample. The highest total biomass estimate was generated using the Universal 16S rDNA gene probe. Total cell numbers based on DNA:DNA hybridization with the Universal 16S rDNA oligonucleotide in

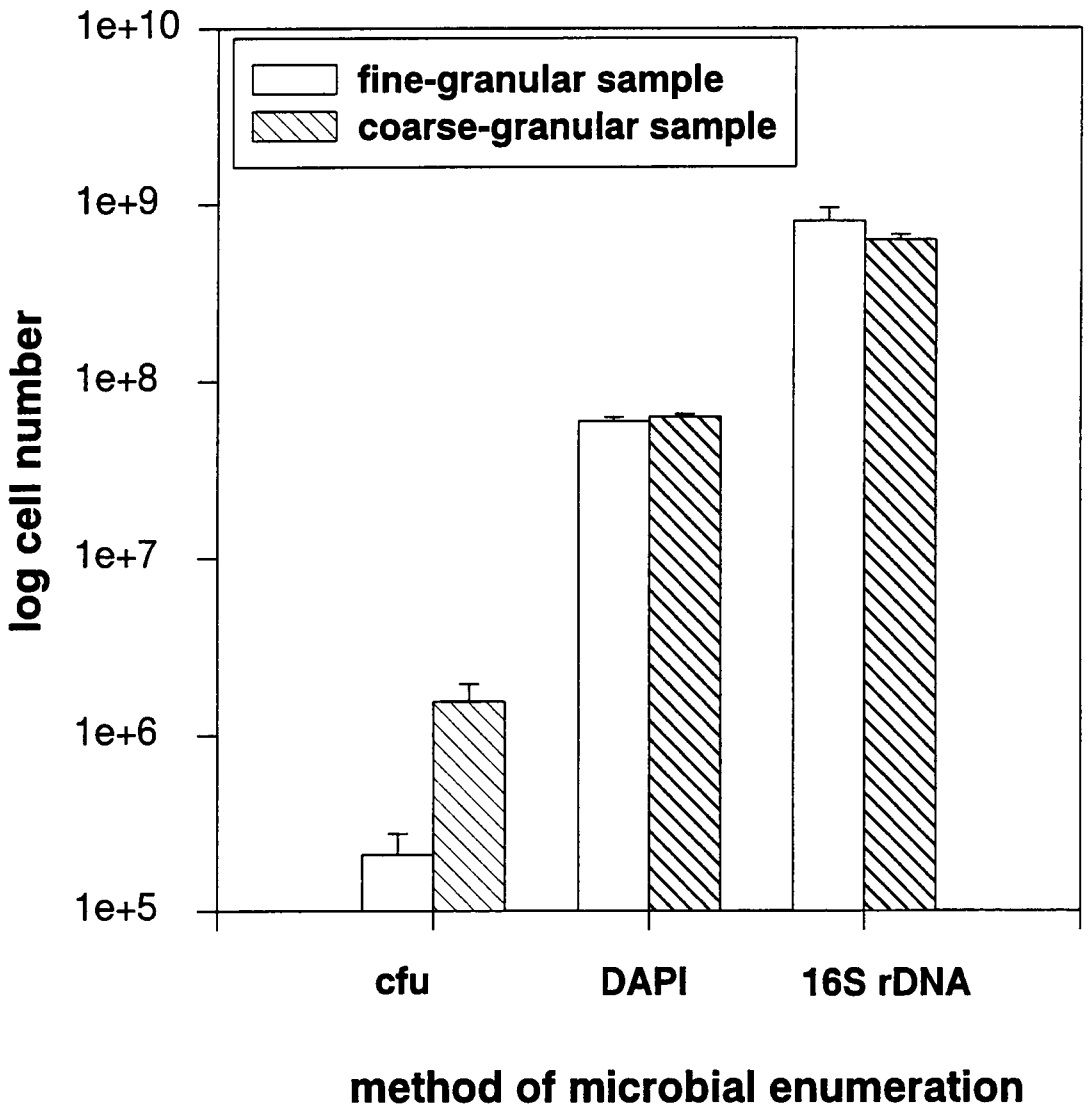


Figure 4. Comparison of microbial enumeration techniques on Columbus AFB aquifer samples. Each value represents the average of triplicate samples. The coarse-granular sample consisted of 40% gravel sized particles.

cell numbers from the coarse-granular sample were 6.27×10^8 ($\pm 4.33 \times 10^7$) per gram sediment. Total viable counts were determined on each sample recovered from Columbus AFB. The abundance of culturable microorganisms ranged from 10^3 to 10^6 cfu/gm. Molecular probing produced total microbial values higher than total viable counts from spread plates, with population estimates ranging from 10^7 to 10^8 organisms per gram sediment (Figure 5).

Biodegradation of ^{14}C-labeled Substrates

Biodegradation of ^{14}C-labeled organic substrates to $^{14}CO_2$ was used to determine the potential for microbial catabolic activity at Columbus AFB. Multiple time point degradation assays performed on samples revealed that each target compound was mineralized to varying degrees over time. The data in Figure 6 represent averages of multiple time point degradation analyses from individual cores within 4 boreholes (CM-58, CM-45, CM-65, and CM-109) representing a longitudinal transect of the study site (Figure 1). After 5-7 days, 40% of each substrate, except benzene, was mineralized. Benzene degradation occurred to a lesser extent than the other compounds, but gradually increased to 20-60% mineralization after 14 days (Figure 6A). Acclimation to toluene occurred between 3 and 5 days, with maximum $^{14}CO_2$ evolution ranging from 60-90% after 2 weeks (Figure 6B). Onset of naphthalene degradation was very rapid, occurring within 1 to 3 days (Figure 6C). Maximum naphthalene mineralization was reached after 14 days with $^{14}CO_2$ evolution

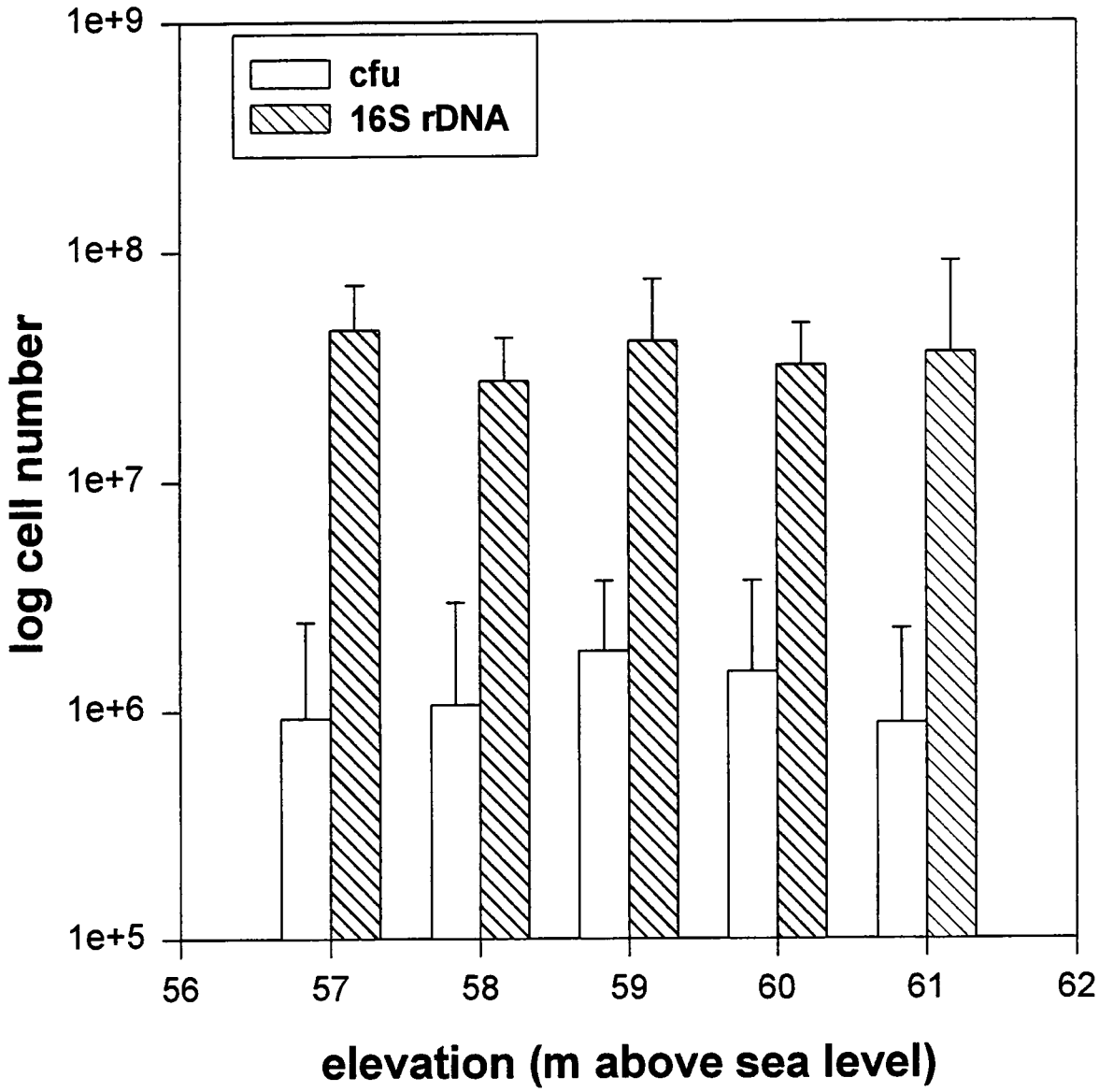
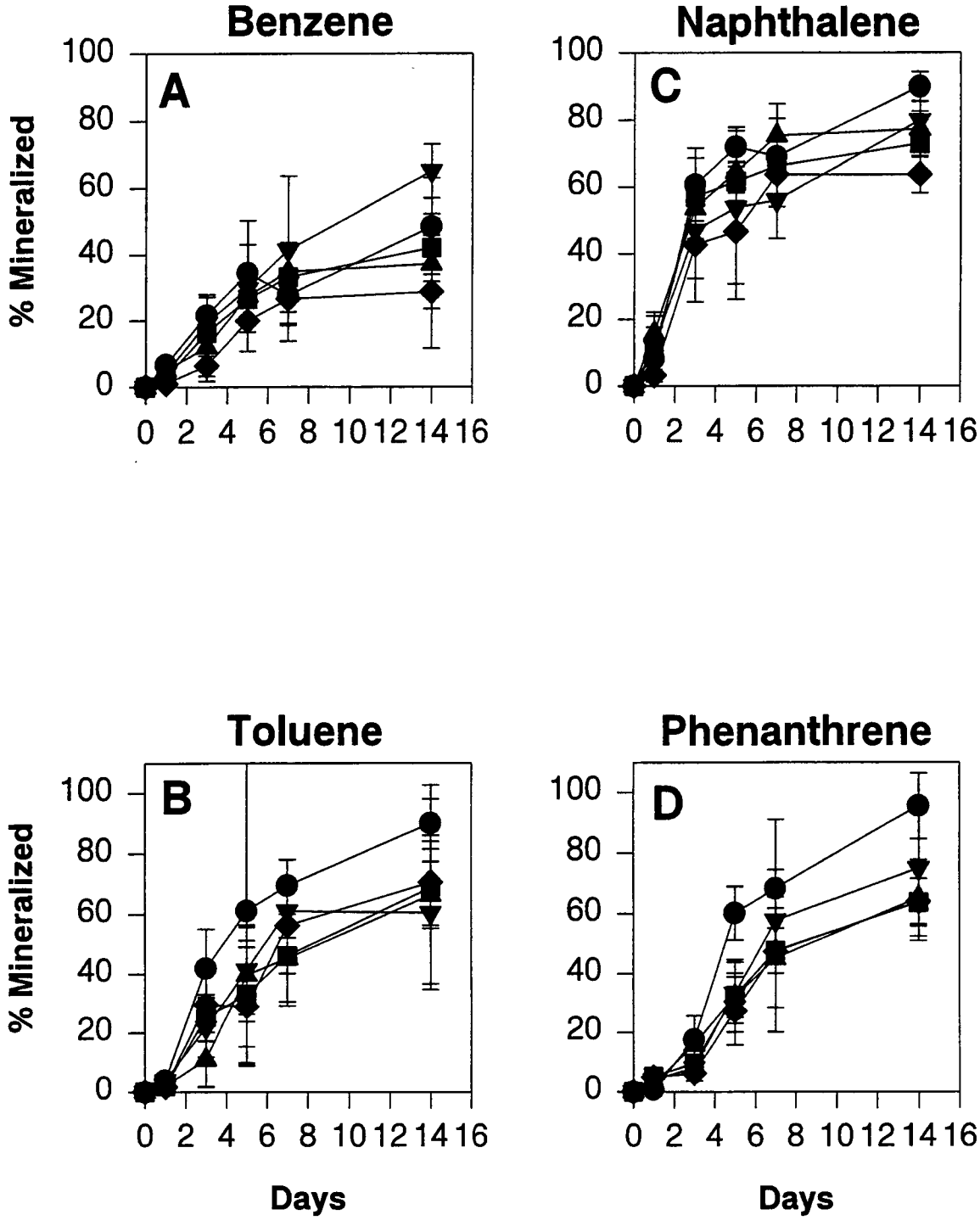


Figure 5. Microbial enumeration comparison between cfu from spread plate analysis and DNA probing for Columbus AFB aquifer samples from 1/96. The data in this graph represents averages from the same elevation above sea level for 12 separate boreholes.

Figure 6. Percent of each target substrate mineralized in Columbus AFB aquifer samples. Each point represents the average over the same elevation above sea level (asl) of 4 separate borholes. Closed circles represent samples from 61 meters asl. Closed squares represent samples from 60 meters asl. Closed triangles represent samples from 59 meters asl. Inverted closed triangles represent samples from 58 meters asl. Closed diamonds represent samples from 57 meters asl.


Benzene
A
Naphthalene
C
Toluene
B
Phenanthrene
D
% Mineralized
% Mineralized
Days
Days
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16

ranging from 60-90%. Microbial acclimation to phenanthrene occurred after 3 to 5 days, with maximum mineralization reaching 60-90% after 14 days (Figure 6D).

Nucleic Acid Analysis

DNA extracted from Columbus AFB samples was of sufficient quality and quantity to be used for DNA:DNA hybridization. Molecular diagnostics based on DNA:DNA hybridization with specific gene probes indicated that catabolic genotypes were present throughout the samples used for degradation studies (Table 8). These samples correspond to the samples used for the time course mineralization analyses. Hydrocarbon degrading genotypes were ubiquitously distributed along vertical and longitudinal profiles of the research area. The detection limit for each probe was 0.01 ng. Converted to cell numbers, the detection limit was 2.43×10^6 for 16S rDNA and *alkB*, 1.83×10^6 for *nahA*, *nahH*, and *todC1C2*, and 9.13×10^5 for *xylA*. Detection limits were dropped to 0.01 ng later in the study by increasing probe strength. CM-45 supports the highest average biomass as determined by 16S rDNA Universal oligonucleotide analysis. CM-58 supports the lowest total biomass, as well as the lowest amount of hydrocarbon degrading gene sequences. *xylA* was consistently the genotype found in the lowest abundance in each borehole. Genotypes possessing the naphthalene dioxygenase enzyme were consistently higher in the samples recovered from 61 meters asl.

Table 8. Distribution of degradative genotypes at Columbus AFB during January 1996.

borehole	m above sea level	total biomass	*alkB*	*nahA*	*nahH*	*tod*	*xylA*
CM-58	61	632 ng 1.54×10^9	5.01 ng 6.11×10^7	4.95 ng 8.99×10^7	3.61 ng 6.56×10^7	2.53 ng 4.59×10^7	1.18 ng 2.14×10^7
	60	616 ng 1.50×10^9	6.55 ng 7.98×10^7	1.78 ng 3.23×10^7	4.79 ng 8.72×10^7	4.43 ng 8.06×10^7	1.86 ng 3.38×10^7
	59	268 ng 6.53×10^8	3.65 ng 4.45×10^7	1.04 ng 1.89×10^7	2.69 ng 4.90×10^7	2.51 ng 4.55×10^7	1.04 ng 1.89×10^7
	58	313 ng 7.62×10^8	bdl bdl	1.67 ng 3.03×10^7	2.89 ng 5.25×10^7	2.39 ng 4.34×10^7	0.73 ng 1.33×10^7
	57	128 ng 1.28×10^8	4.32 ng 5.27×10^7	1.40 ng 2.55×10^7	2.71 ng 4.93×10^7	2.80 ng 5.08×10^7	0.75 ng 1.35×10^7
CM-45	61	310 ng 7.56×10^8	0.90 ng 1.10×10^7	9.31 ng 1.69×10^8	0.84 ng 1.53×10^7	bdl bdl	0.61 ng 1.11×10^7
	60	822 ng 2.00×10^9	4.55 ng 5.55×10^7	5.40 ng 9.81×10^7	4.24 ng 7.70×10^7	3.71 ng 6.74×10^7	1.24 ng 2.25×10^7
	59	457 ng 1.11×10^9	4.84 ng 5.91×10^7	3.48 ng 6.33×10^7	3.42 ng 6.21×10^7	2.97 ng 5.40×10^7	1.01 ng 1.84×10^7
	58	807 ng 1.97×10^9	4.88 ng 5.95×10^7	3.19 ng 5.79×10^7	4.24 ng 7.71×10^7	4.32 ng 7.85×10^7	1.15 ng 2.09×10^7
	57	955 ng 2.33×10^9	1.75 ng 2.13×10^7	2.58 ng 4.69×10^7	3.67 ng 6.68×10^7	3.48 ng 6.32×10^7	0.71 ng 1.29×10^7

Table 8. Distribution of degradative genotypes at Columbus AFB during January 1996 (continued).

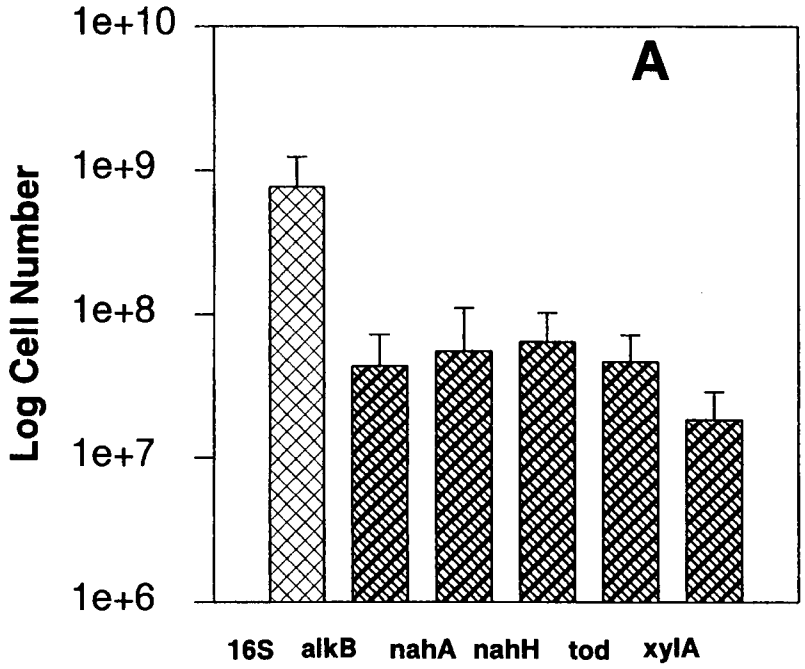
borehole	m above sea level	total biomass	*alkB*	*nahA*	*nahH*	*tod*	*xylA*
CM-65	61	433 ng	3.01 ng	4.82 ng	2.74 ng	1.87 ng	1.15 ng
		1.06×10^9	3.67×10^7	8.77×10^7	4.99×10^7	3.39×10^7	2.09×10^7
	60	269 ng	3.68 ng	2.38 ng	3.46 ng	2.62 ng	0.87 ng
		6.55×10^8	4.49×10^7	4.33×10^7	6.28×10^7	4.76×10^7	1.58×10^7
	59	280 ng	3.61 ng	1.01 ng	2.95 ng	1.83 ng	0.81 ng
		6.83×10^8	4.40×10^7	1.83×10^7	5.36×10^7	3.33×10^7	1.48×10^7
	58	175 ng	3.83 ng	3.13 ng	3.32 ng	3.42 ng	1.20 ng
		4.27×10^8	4.67×10^7	5.69×10^7	6.04×10^7	6.22×10^7	2.17×10^7
	57	363 ng	3.01 ng	2.56 ng	2.05 ng	3.03 ng	0.59 ng
		8.85×10^8	3.67×10^7	4.65×10^7	3.72×10^7	5.51×10^7	1.07×10^7
CM-109	61	433 ng	0.24 ng	7.04 ng	0.89 ng	bdl	bdl
		1.06×10^9	2.96×10^6	1.28×10^8	1.61×10^7	bdl	bdl
	60	269 ng	4.48 ng	2.47 ng	2.93 ng	2.60 ng	0.67 ng
		6.55×10^8	5.47×10^7	4.49×10^7	5.33×10^7	4.73×10^7	1.21×10^7
	59	280 ng	3.35 ng	1.80 ng	2.76 ng	2.09 ng	0.86 ng
		6.83×10^8	4.08×10^7	3.28×10^7	5.01×10^7	3.79×10^7	1.56×10^7
	58	175 ng	3.37 ng	2.11 ng	2.64 ng	2.03 ng	0.53 ng
		4.27×10^8	4.11×10^7	3.84×10^7	4.80×10^7	3.68×10^7	9.64×10^6
	57	363 ng	2.31 ng	2.19 ng	2.70 ng	2.07 ng	0.38 ng
		8.85×10^8	2.81 e 10^7	3.97×10^7	4.90×10^7	3.77×10^7	6.97×10^6
detection limits		0.1 ng	0.1 ng	0.1 ng	0.1 ng	0.1 ng	0.1 ng
		2.43×10^6	2.43×10^6	1.83×10^6	1.83×10^6	1.83×10^6	9.13×10^5

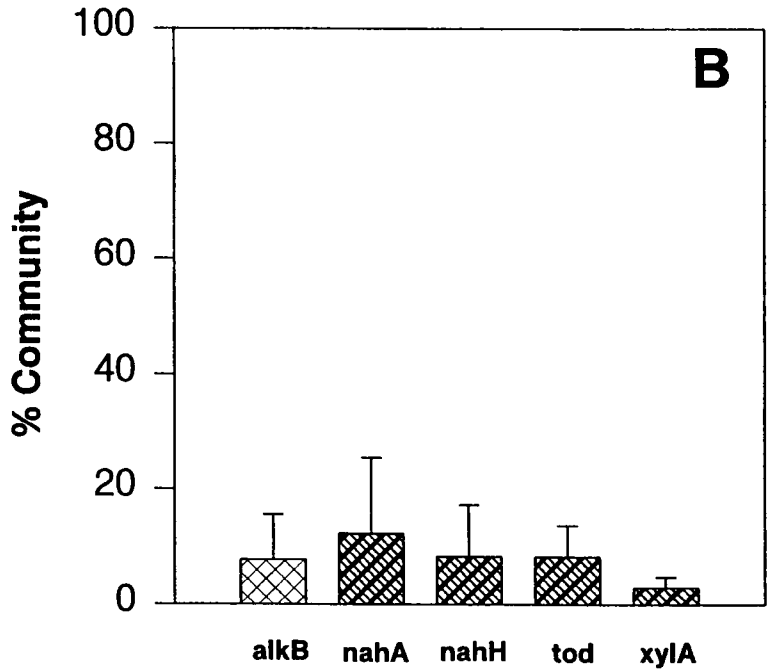
bdl refers to samples producing positive hybridization signals, but outside the known standards used during the study.

Cell number estimates for microorganisms possessing the genetic potential to degrade hydrocarbons were generated by DNA:DNA hybridization studies. Total biomass estimates based on the 16S rDNA Universal oligonucleotide averaged 7.67 x $10^8 \pm 4.66 \times 10^8$ cells per gram sediment (Figure 7A). Cell number estimates for populations containing genes that hybridize with the DNA probes *alkB, nahA, nahH, tod*, and *xylA* averaged 4.39, 5.56, 6.44, 4.70, and 1.85 x 10^7 organisms per gram sediment, respectively (Figure 7A). The microbiological parameter "percent community" was adapted from (Jones and Edington, 1968) for molecular diagnostics by normalizing data generated from gene probe analyses with total biomass estimates (Stapleton et al., 1997). The average percent population values were 7.68, 12.19, 8.30, 8.22, and 2.92 for the *alkB, nahA, nahH, todC1C2*, and *xylA* genotypes, respectively (Figure 7B).

Descriptive statistics for the January 1996 data set can be seen in Table 9. The mean values for each gene probe used in the NAT Study ranges from 2.5 to 3.5 ng, except for *xylA*, which has a mean value of 0.94 ng. Pearson correlation coeffecients significant at $\alpha < 0.05$ for the January data set are seen in Table 10. With the exception of *todC1C2*, each of the degradative gene probes correlated with each other. Values obtained from the Universal oligonucleotide correlated with ng *nahA* and ng *todC1C2*. Total viable counts correlated positively with elevation and dissolved oxygen correlated negatively with elevation. pH negatively correlated with dissolved

Figure 7. DNA:DNA hybridization data from Columbus AFB samples recovered during January 1996. Figures 7A and 7B represent the average of 60 total samples for each gene probe. Figure 7A shows cell number estimates and Figure 7B shows the percent of the total population for each hydrocarbon degrading genotype.


A
Log Cell Number
1e+10
1e+9
1e+8
1e+7
1e+6
16S
alkB
nahA
nahH
tod
xylA


B
% Community
100
80
60
40
20
0
alkB
nahA
nahH
tod
xylA

oxygen. Multiple regression analysis was significant for only total viable counts (Table 11), with 14.1% of the variation being explained by elevation.

These results favor the potential for microbiological retardation of petroleum hydrocarbons in the Columbus AFB research aquifer. Furthermore, the methods chosen to evaluate natural attenuation proved to be applicable to this study.

Table 9. Descriptive statistics for NAT Study January 1996 data set.

	N	Minimum	Maximum	Mean	Std. Deviation
ng *alkB*	60	0.04	9.41	3.54	2.34
ng *nahA*	60	0.10	13.77	3.01	3.02
ng *nahH*	60	0.10	9.76	3.48	2.12
ng *todC1C2*	60	0.01	8.90	2.46	1.42
ng *xylA*	60	0.01	2.70	0.94	0.62
ng 16S	60	44.49	954.59	317.61	198.63
TVC	60	2.00E+03	3.10E+06	2.71E+05	5.18E+05
DO	60	0.75	4.60	2.69	1.25
pH	60	5.15	6.20	5.53	0.31
temperature	60	12.00	19.20	15.80	1.78

Table 10. Pearson Correlation Matrix for January 1996 NAT Study data set[a].

	ng *alkB*	ng *nahA*	ng *nahH*	ng *tod*	ng *xylA*	ng 16S	TVC	DO	elev	pH	temp
ng *alkB*	1.000	**0.538**	**0.868**		**0.536**						
ng *nahA*		1.000	**0.495**		**0.361**	**0.343**					
ng *nahH*			1.000		**0.476**						
ng *tod*				1.000	**0.344**	**0.295**					
ng *xylA*					1.000						
ng 16S						1.000					
TVC							1.000		**0.376**		
DO								1.000	**-0.463**	**-0.526**	
elev									1.000		
pH										1.000	**0.319**
temp											1.000

[a] Values are significant at $\alpha < 0.05$.

Table 11. Multiple regression analysis for January 1996 data set.

dependent variable	R^2	probability of a greater f	independent variable	(significance)
TVC	0.141	0.003	elevation	(0.003)

Monitoring Microbiological Changes During Natural Attenuation

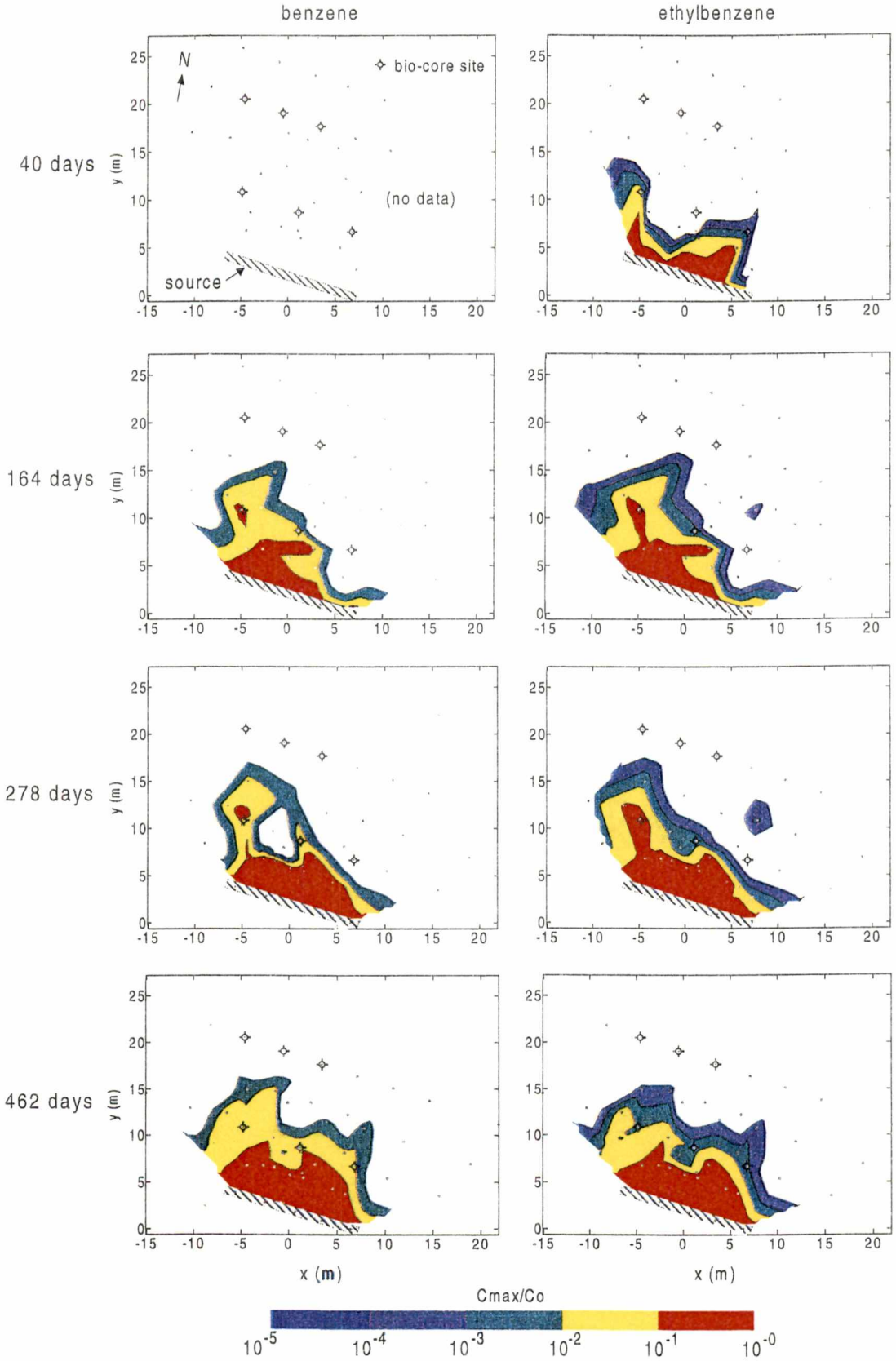
Hydrocarbon Distribution Over Time

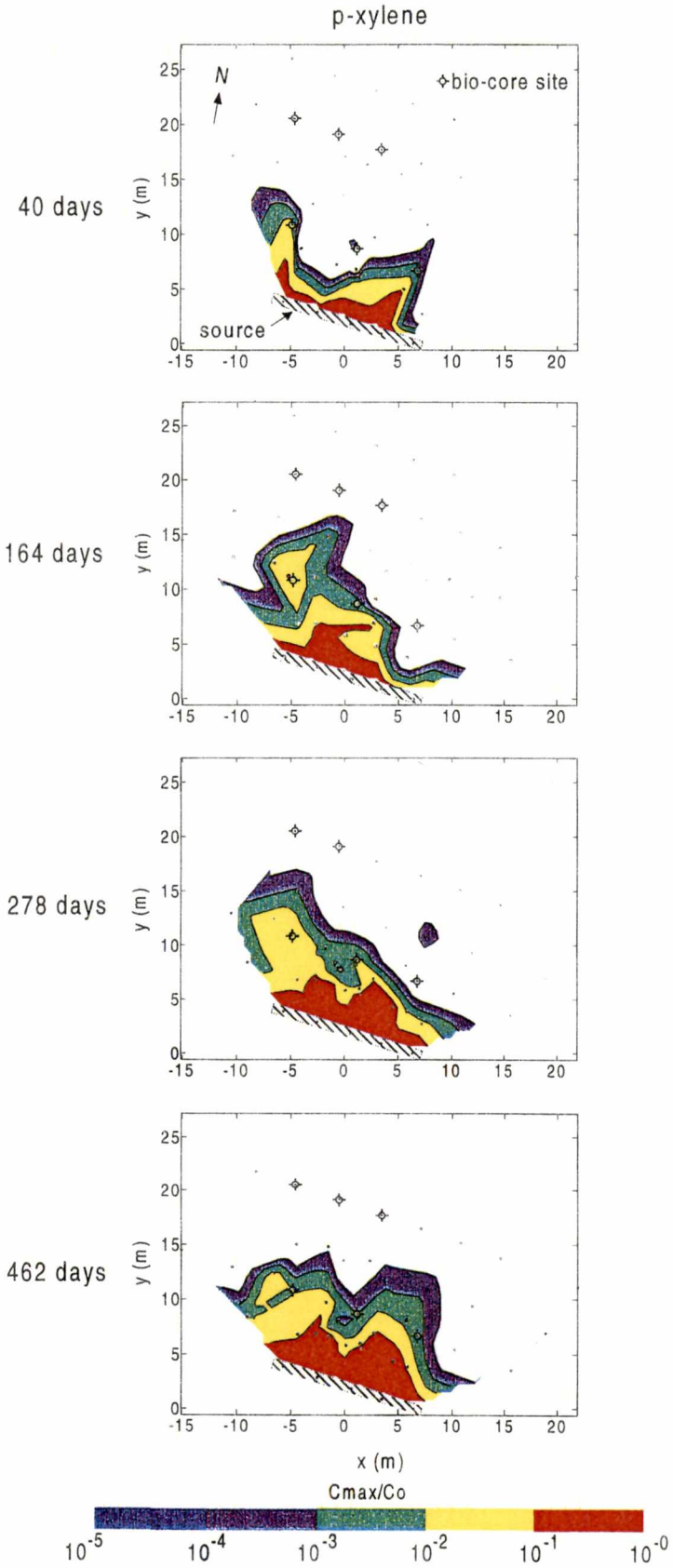
Appreciable concentrations of total hydrocarbons were not detected more than a few meters downgradient of the contaminant source until September 1996. Significant plumes of individual hydrocarbons were readily apparent (Figure 8). The highest concentrations were detected for the compounds ethylbenzene, naphthalene and toluene. Plume evolution did not proceed as expected. Instead of a uniform, mixed body of hydrocarbons developing, individual hydrocarbon plumes expanded primarily off the west end of the source trench during 1996. By March 1997, the plumes had stopped expanding and actually began retracting. This is supported by comparing the hydrocarbon migration to that of the conservative tracer bromide, whose plume had continually expanded. Retardation of the hydrocarbon plumes is also reflected in the microbiological data collected during this period. Figure 9 shows the drop in aqueous concentrations of naphthalene and ethylbenzene in March 1997.

Changes in Microbial Activity

Biodegradation of ^{14}C-labeled substrates revealed measureable changes in the microbial activity associated with subsurface material recovered from Columbus AFB. By September 1996, the hydrocarbon found in the highest mean concentration in the groundwater samples was naphthalene (576 μg / L). Microbial metabolism of naphthalene reflected the distribution of the aromatic hydrocarbon (Figure 10).

Figure 8. Evolution of individual hydrocarbon plumes during the NAT Study. Each hydrocarbon produced a different contaminant plume in the groundwater. (Plots provided by J. Mark Boggs at the Tennessee Valley Authority).


benzene
ethylbenzene
40 days
164 days
278 days
462 days
N
bio-core site
(no data)
source
y (m)
x (m)
Cmax/Co
10-5
10-4
10-3
10-2
10-1
10-0


p-xylene
N
bio-core site
source
40 days
164 days
278 days
462 days
y (m)
x (m)
Cmax/Co
10-5
10-4
10-3
10-2
10-1
10-0

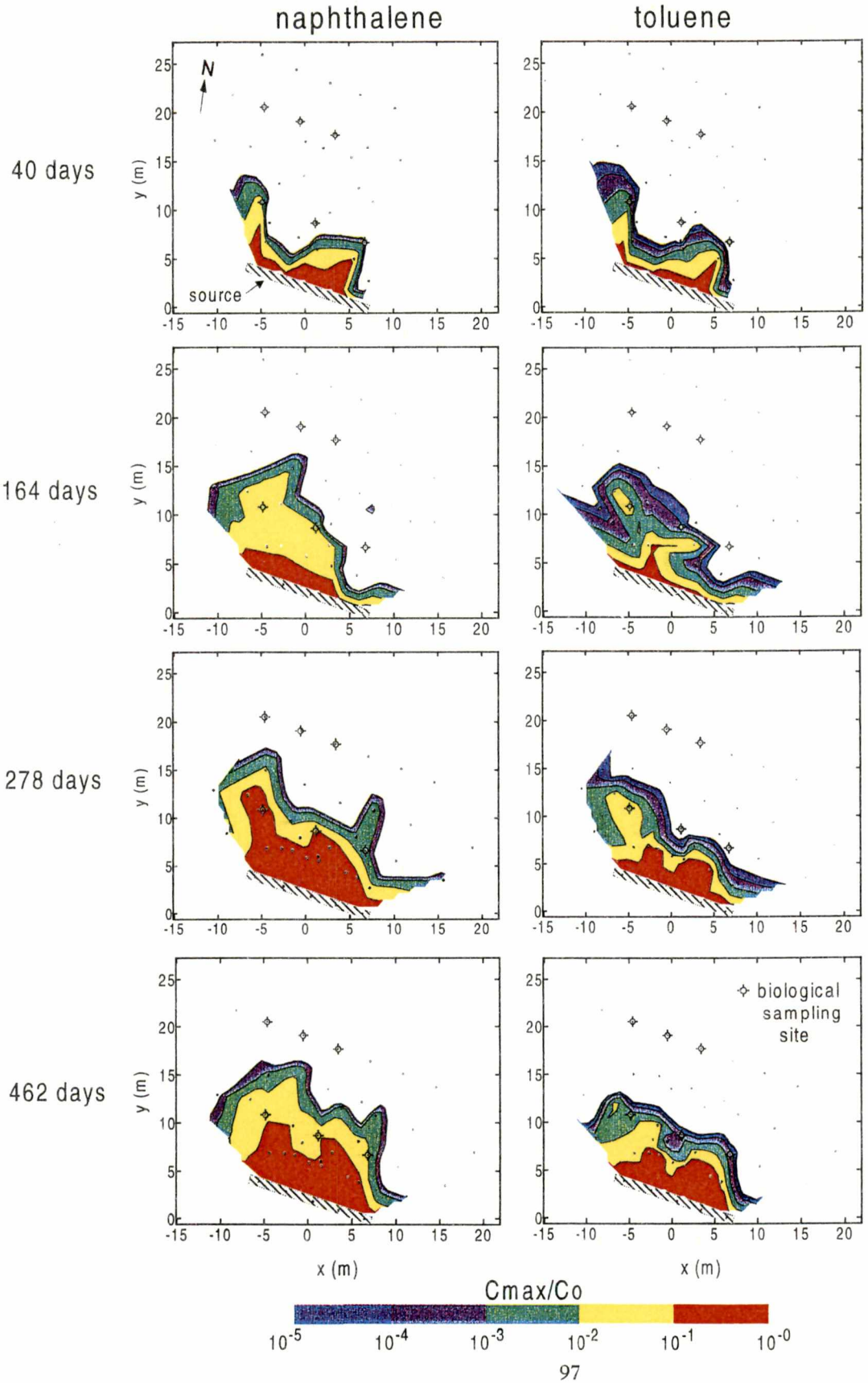

naphthalene
toluene
40 days
164 days
278 days
462 days
N
source
y (m)
x (m)
biological sampling site
Cmax/Co
10-5
10-4
10-3
10-2
10-1
10-0

Figure 9. Aqueous mass concentrations of individual hydrocarbons at Columbus AFB. Note the drop in mass concentrations of naphthalene and ethylbenzene between September 1996 and March 1997. (Plot provided by J. Mark Boggs at the Tennessee Valley Authority).

Aqueous-Phase Hydrocarbon Mass Estimates

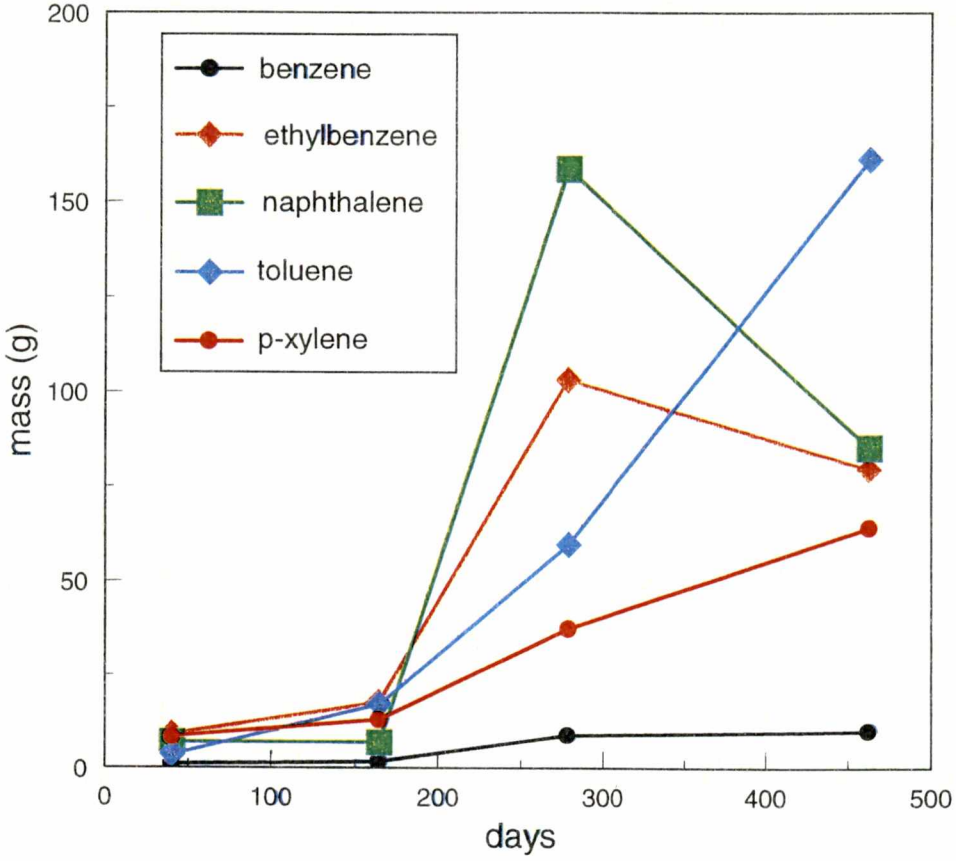
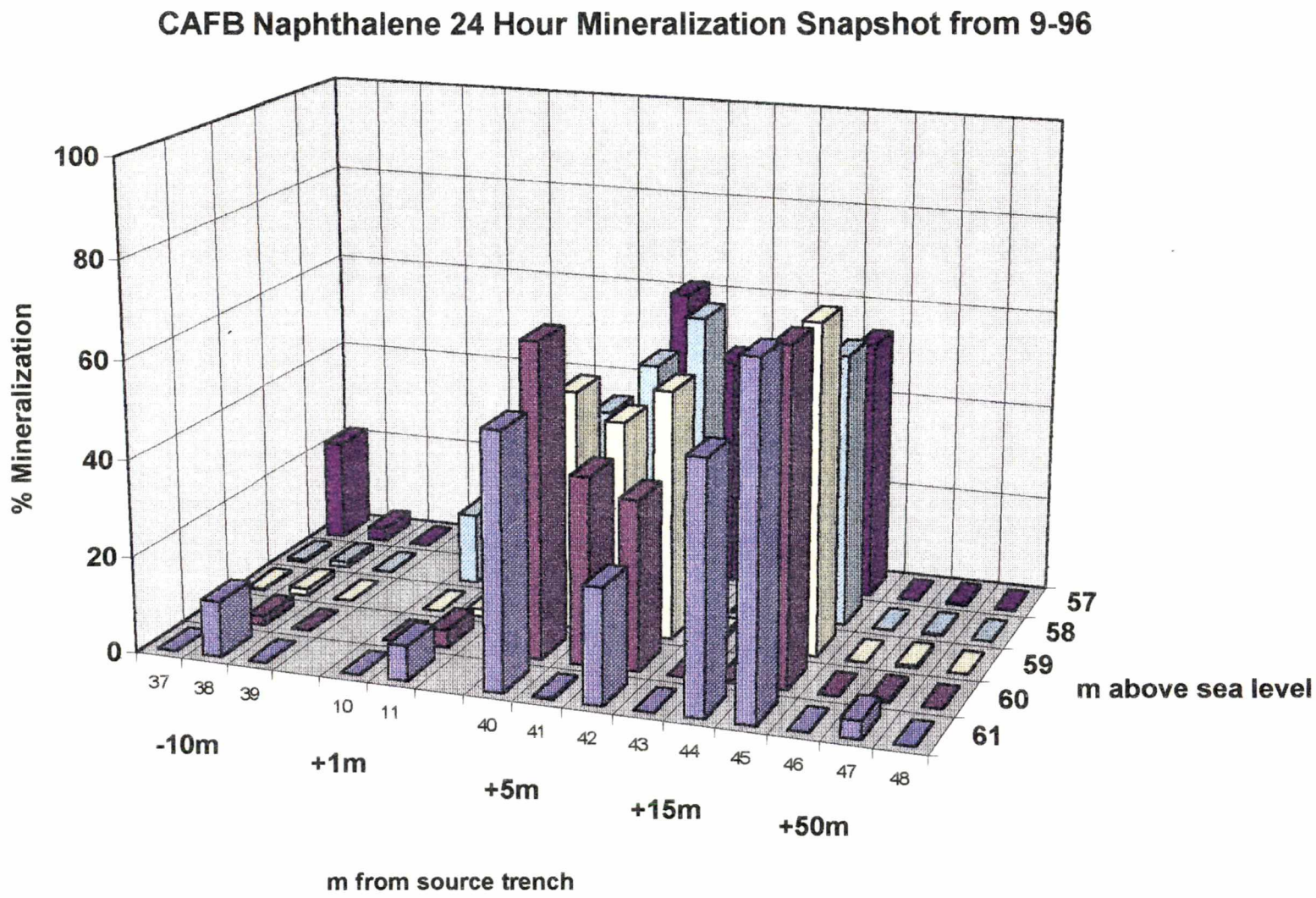


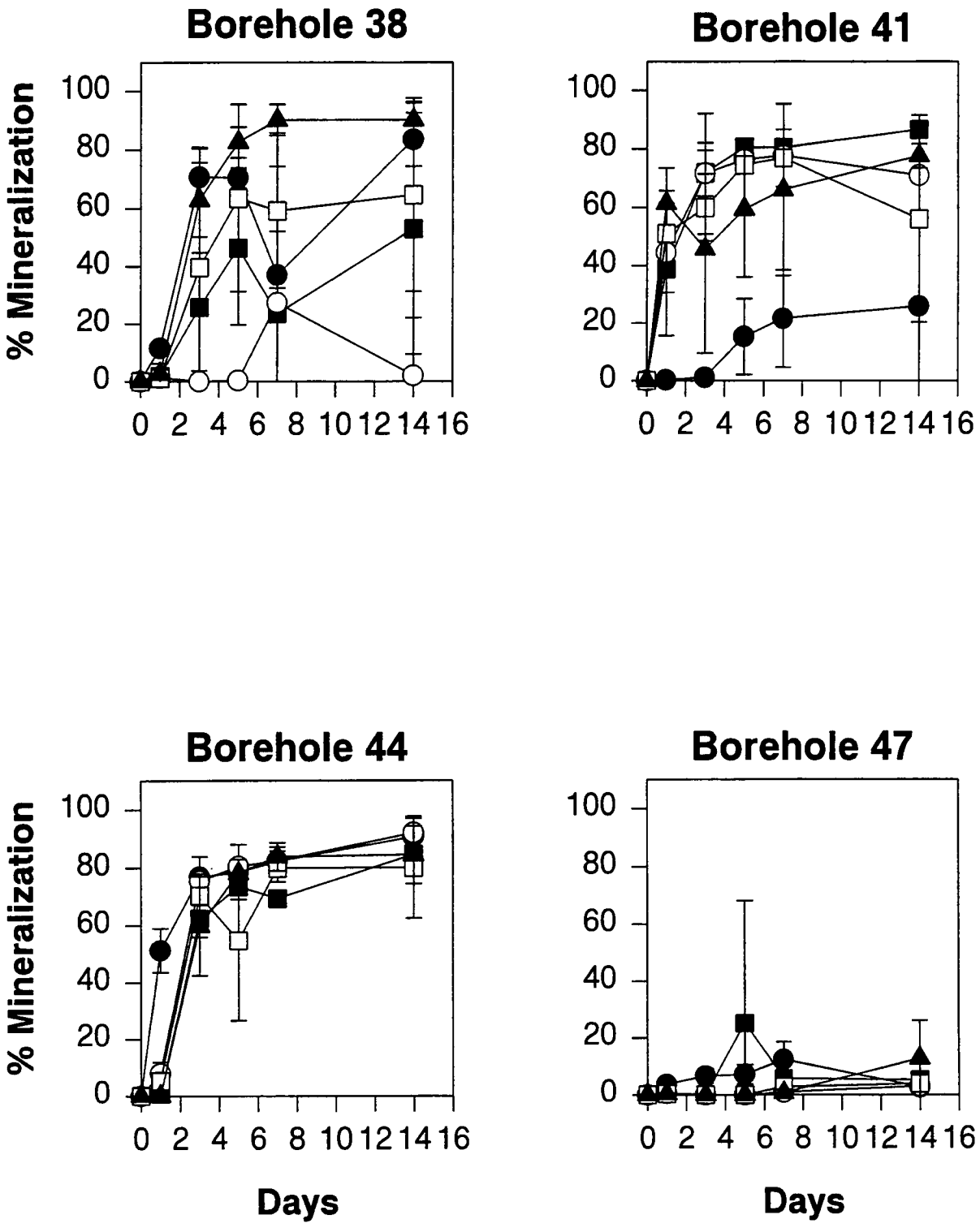
Figure 10. Microbial mineralization of naphthalene in September 1996 samples from Columbus AFB. The assay was performed for 24 hours.


CAFB Naphthalene 24 Hour Mineralization Snapshot from 9-96
% Mineralization
100
80
60
40
20
0
37
38
39
10
11
40
41
42
43
44
45
46
47
48
-10m
+1m
+5m
+15m
+50m
m from source trench
57
58
59
60
61
m above sea level

Depleted microbial activity immediately downgradient the source trench allowed the hydrocarbon plumes to expand approximately 10-15 meters downgradient. Limited degradation in these samples was probably due to decreased levels of dissolved oxygen. Naphthalene mineralization reached levels as high as 60 percent in 24 hours in impacted areas 5 - 15 meters downgradient from the contaminant trench, while mineralization in un-impacted samples was negligible.

Mineralization of naphthalene over time in samples recovered from Columbus AFB during September 1996 is shown in Figure 11. Borehole 38 was upgradient the source trench approximately 10 meters. Naphthalene degradation reached 90% after two weeks, with a great deal of variation between depths. Borehole 47 was located approximately 50 meters downgradient of the source trench and has no detectable contaminants. Mineralization of naphthalene in these samples remained well below 20% over the two week incubation period. Borehole 41 was approximately 5 meters downgradient from the source trench and had levels of naphthalene in the groundwater ranging from 6 to more than 1500 parts per billion (μg / L). The lowest concentration of naphthalene corresponds to the lowest amount of naphthalene mineralization in Borehole 41 sampled at 61 meters asl. Borehole 47 was on the fringe of the hydrocarbon plumes and the native groundwater well oxygenated. Hydrocarbons were not detected in any of these samples. Rapid mineralization in all 5 samples recovered from Borehole 44 was probably due to the plume expanding into this area and already contracting, hydrocarbons being present at trace levels not

Figure 11. Time course mineralization study of naphthalene in samples recovered from Columbus AFB during September 1996. Boreholes 41 and 44 were impacted by hydrocarbons, Boreholes 38 and 47 were not.


Borehole 38
Borehole 41
Borehole 44
Borehole 47
% Mineralization
% Mineralization
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16
Days
Days

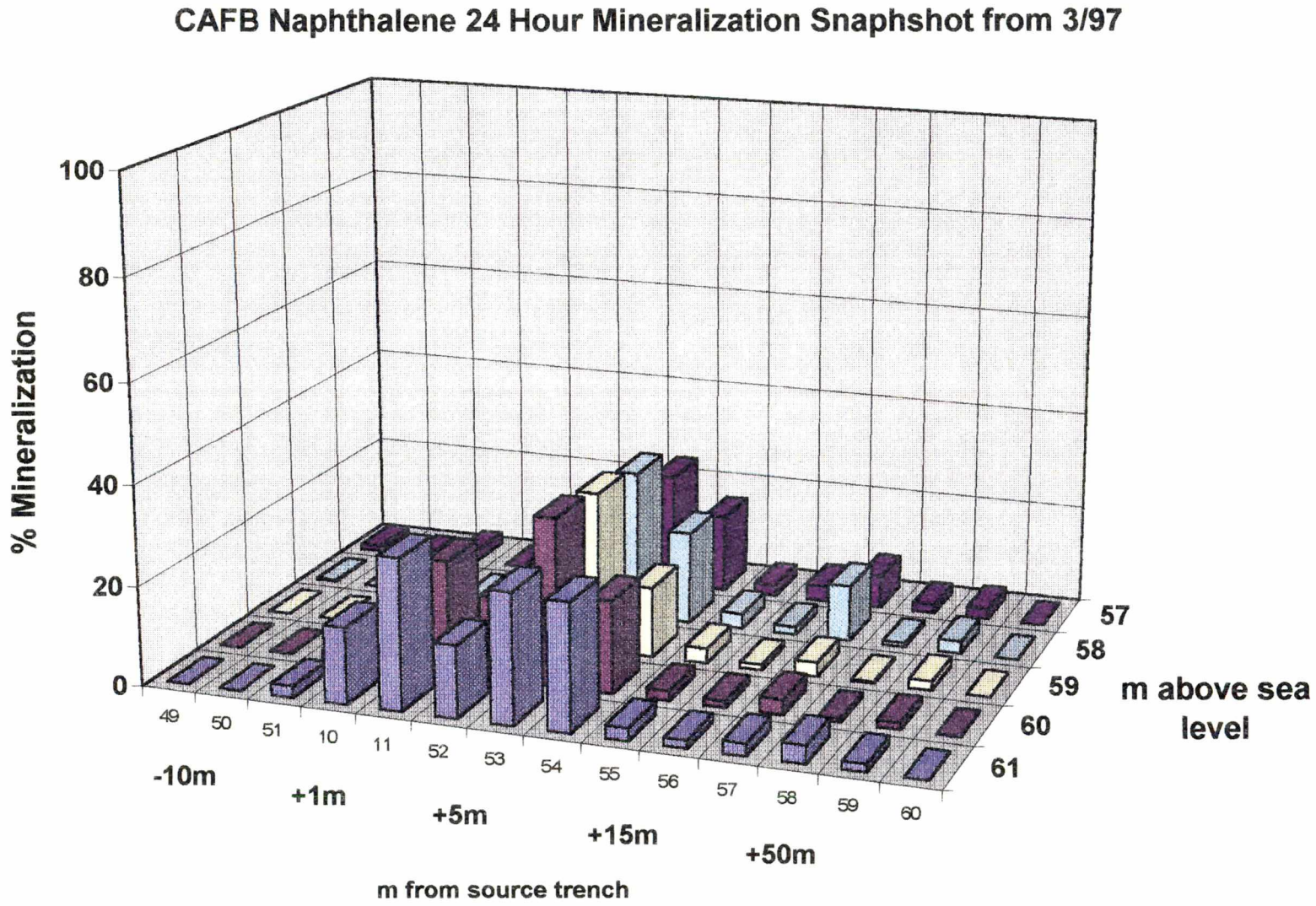
detectable by the chosen methods of analysis, or a response of the microbial community to increased metabolic activity upstream creating favorable conditions for hydrocarbon biodegradation on the plume fringe.

Elevated levels of naphthalene mineralization continued in samples recovered from Columbus AFB during March 1997. Reflecting the reduction in aqueous phase naphthalene concentration (Figure 9), naphthalene mineralization activity was concentrated to samples recovered approximately 5 meters from the source trench (Figure 12). Degradation dropped from 60% seen in impacted samples from September 1996 to 20% in impacted samples from March 1997. Groundwater concentrations of naphthalene also dropped, with the mean of 267 parts per billion (μg / L). Mineralization up to 20% was also seen in samples from approximately 1 meter downgradient the source trench, suggesting favorable conditions were restored in these samples. This postulation is supported by the mean level of groundwater dissolved oxygen increasing from 2 mg / L to almost 3 mg / L. Mineralization of naphthalene was again negligible in un-impacted samples. Multiple time point mineralization assays were not performed on the March 1997 samples.

Changes in Molecular Microbial Ecology

The DNA probe array designed for assessing the potential for natural attenuation was also used to evaluate changes in the indigenous microbial community during the NAT Study at Columbus AFB. Using the array of gene probes, populations

Figure 12. Microbial mineralization of naphthalene in March 1997 samples from Columbus AFB. The assay was performed for 24 hours.


CAFB Naphthalene 24 Hour Mineralization Snaphshot from 3/97
% Mineralization
100
80
60
40
20
0
49
50
51
10
11
52
53
54
55
56
57
58
59
60
-10m
+1m
+5m
+15m
+50m
m from source trench
57
58
59
60
61
m above sea level

of microorganisms capable of degrading the hydrocarbon contaminants were shown to increase and decrease as a percent of the total microbial community during the study. The fluctuation of degradative populations as a percent of the total microbial community follows the activity measurements and changes in aqueous hydrocarbon concentrations.

Samples recovered from the upgradient control area during September 1996 were shown to support each of the degradative genotypes under investigation (Figure 13). All genotypes were present at less than 10% of the total microbial community. This information provides un-impacted sample data for assessing the contamination impact on the indigenous microorganisms at Columbus AFB.

Samples obtained during September 1996 from sites heavily impacted by contaminants approximately 1 meter downgradient the source trench revealed elevated percentages of degradative populations when normalized to the total microbial community (Figure 14). Hydrocarbon concentrations in this area reached over 400 ppb benzene, over 6600 ppb ethylbenzene, over 6800 ppb naphthalene, over 5200 ppb toluene, and over 2100 ppb *p*-xylene. The highest concentration of degradative genotypes corresponded to the hydrocarbons found in the highest concentrations. Microbial populations containing the *nahA* genotype ranged from 10 to 30% of the total community. Microbial populations containing the *todC1C2* genotype ranged from 20 to almost 50% of the total community. Elevated population levels were also seen in the degradative genotypes *alkB* and *nahH*. Samples recovered from the

Figure 13. Hydrocarbon degrading genotypes present in upgradient samples recovered from Columbus AFB during September 1996.

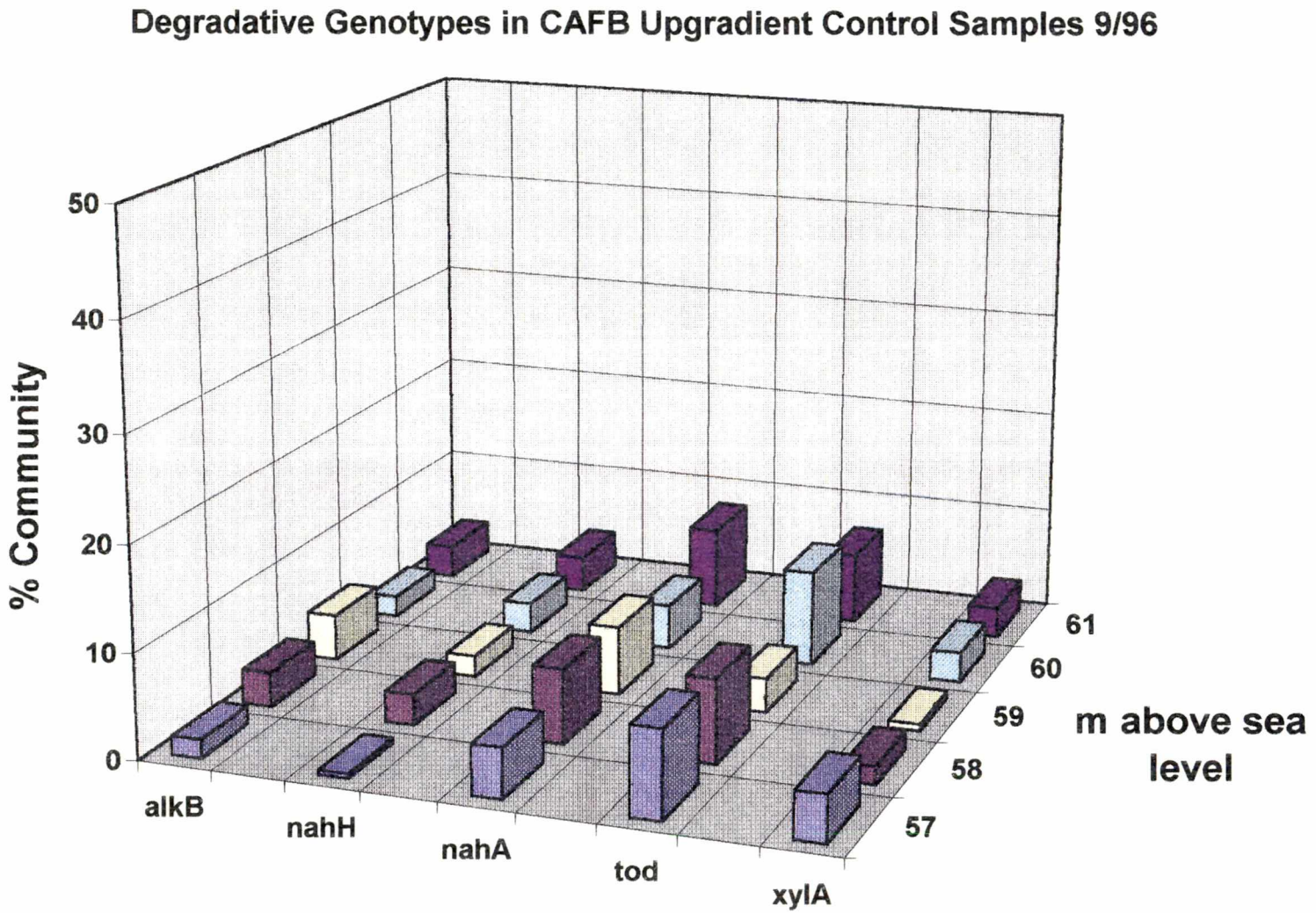
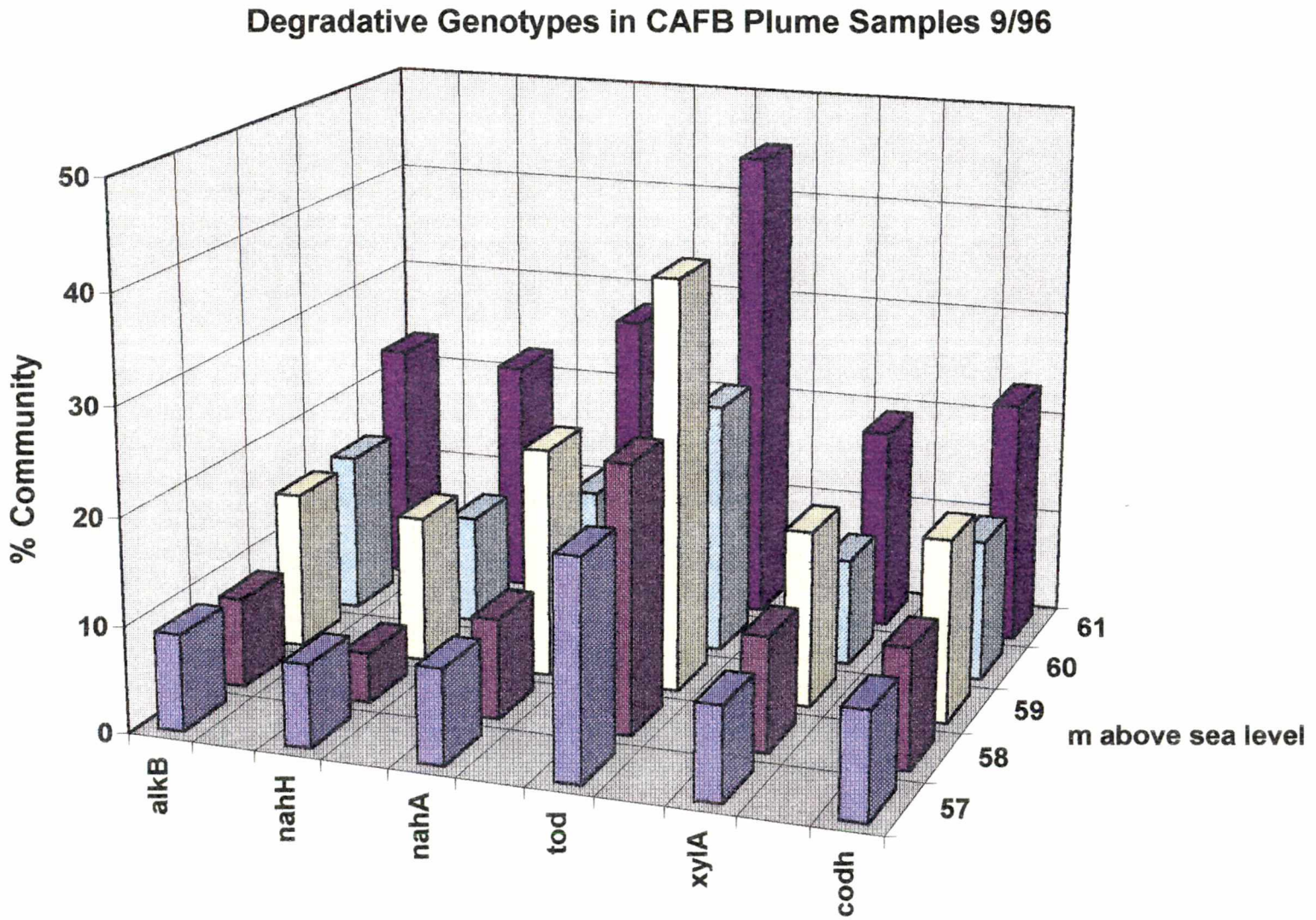

Degradative Genotypes in CAFB Upgradient Control Samples 9/96
% Community
50
40
30
20
10
0
alkB
nahH
nahA
tod
xylA
61
60
59
58
57
m above sea level

Figure 14. Hydrocarbon degrading genotypes present in plume samples recovered from Columbus AFB during September 1996.


Degradative Genotypes in CAFB Plume Samples 9/96
% Community
0
10
20
30
40
50
alkB
nahH
nahA
tod
xylA
codh
57
58
59
60
61
m above sea level

upgradient control area during March 1997 were again shown to support each of the degradative genotypes under investigation (Figure 15). Hydrocarbon degrading genotypes ranged from less than 5 up to 18% of the total microbial community. These data are slightly different than the values seen in the September 1996 samples. Only the genotypes *nahH* and *todC1C2* were found at more than 10% of the total community. The increase in *nahH* genotypes may be due to an increased level plant metabolites such as salicylic acid in the environment.

Samples obtained during March 1997 from sites heavily impacted by contaminants approximately 1 meter downgradient the source trench were still found at elevated levels (Figure 16). The degradative genotypes *nahA* and *xylA* represented approximately 20% of the total community. The genotypes *nahH* and *todC1C2* were greatly reduced in population levels when compared to both the March 1997 upgradient control samples and the September 1996 samples from the plume area. The drop in *todC1C2* genotypes appeared to reflect the drop in mean ethylbenzene concentration instead of the increased mean toluene concentration. This may be due to the fact the reference organism for *todC1C2, Pseudomonas putida* F1, was originally isolated from an ethylbenzene enrichment (Gibson et al., 1968). Moreover, the *xylA* genotypes appear to reflect the increase in mean toluene concentration instead of the mean *p*-xylene concentration because the mean concentration of *p*-xylene remained relatively constant from September 1996 to March 1997. This may be because reference organism *Pseudomonas putida* mt-2 grows on both *p*-xylene and toluene.

Figure 15. Hydrocarbon degrading genotypes present in upgradient samples recovered from Columbus AFB during March 1997.

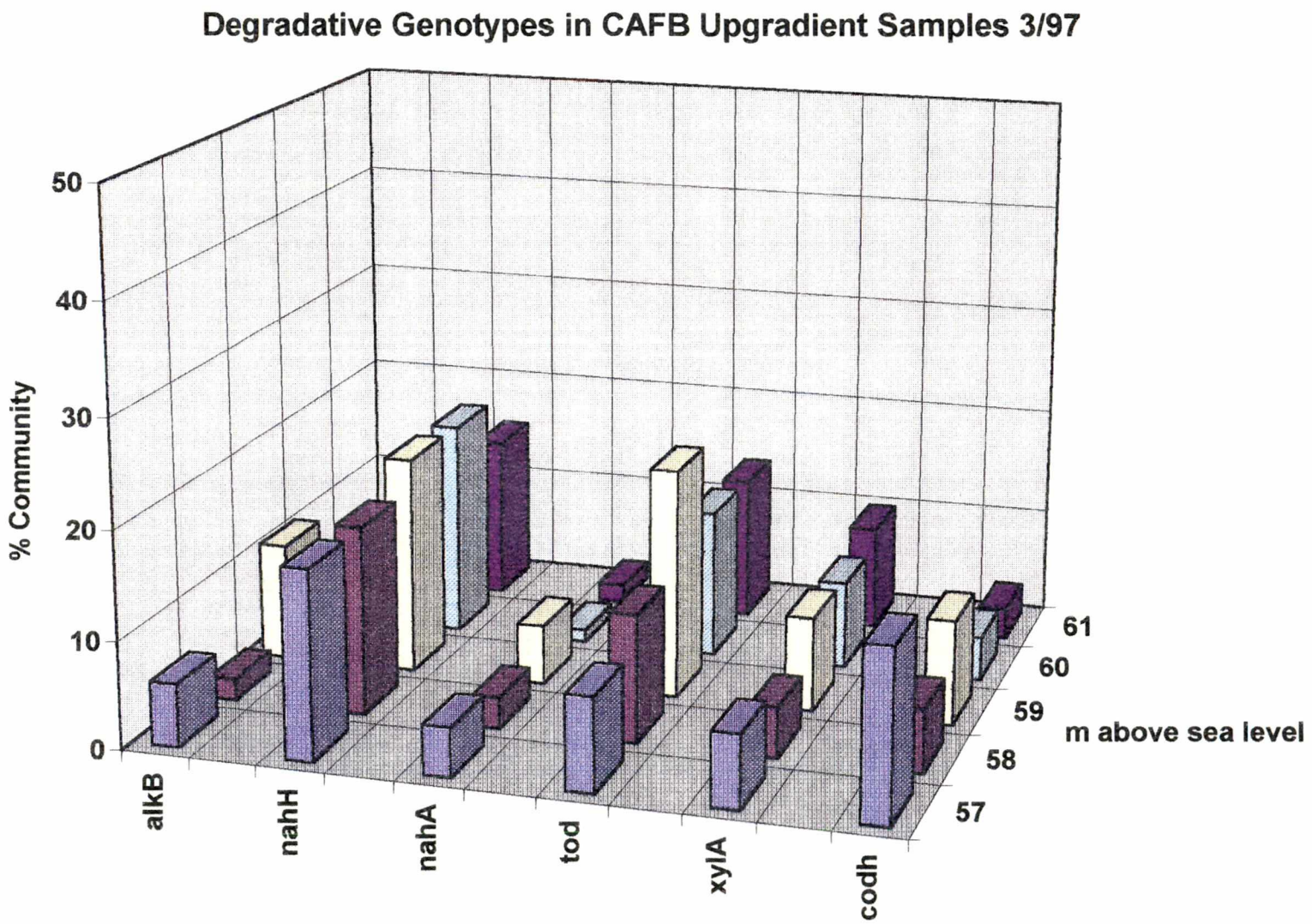
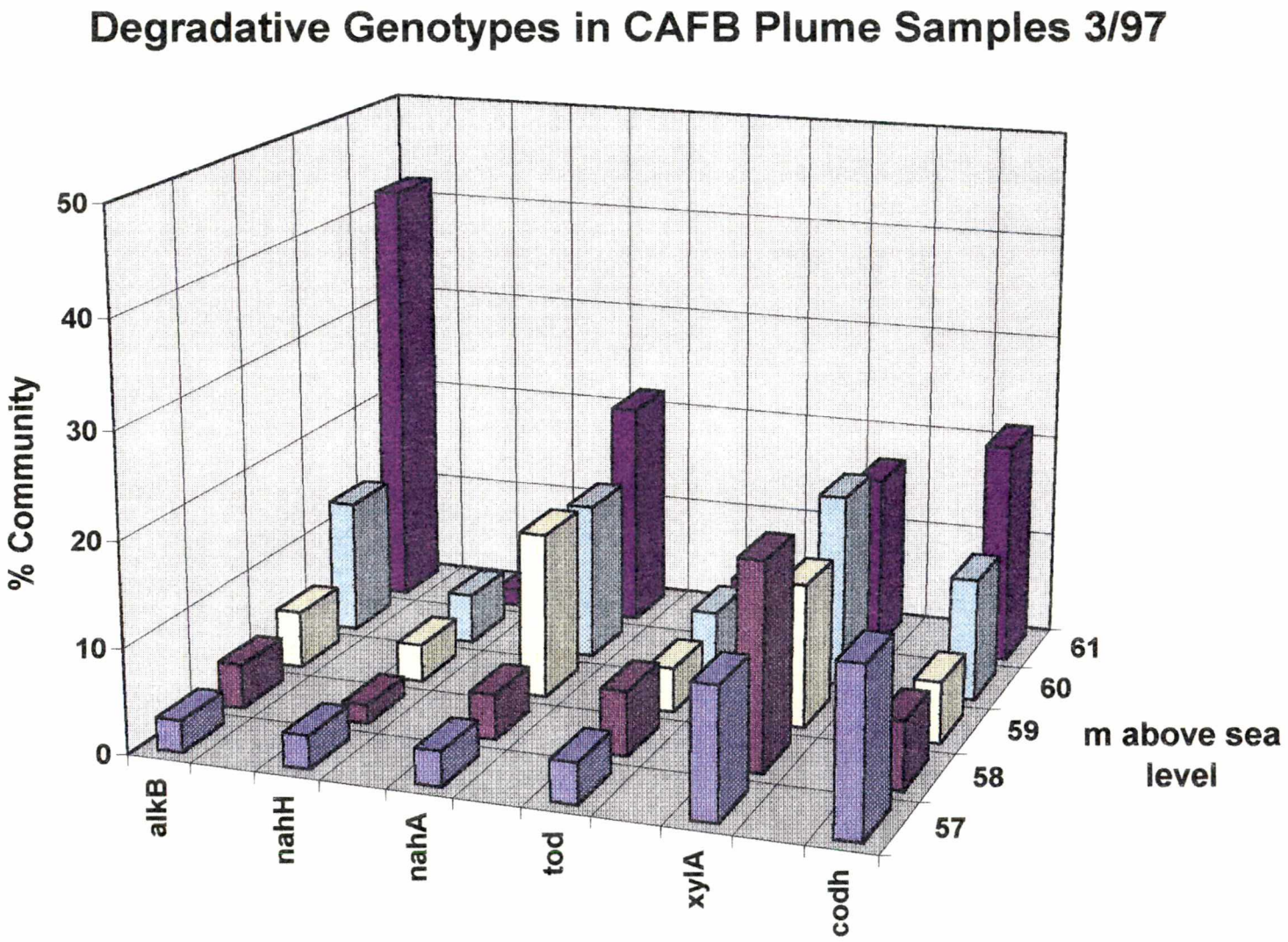

Degradative Genotypes in CAFB Upgradient Samples 3/97
% Community
50
40
30
20
10
0
alkB
nahH
nahA
tod
xylA
codh
61
60
59
58
57
m above sea level

Figure 16. Hydrocarbon degrading genotypes present in plume samples recovered from Columbus AFB during March 1997.


Degradative Genotypes in CAFB Plume Samples 3/97
% Community
0
10
20
30
40
50
alkB
nahH
nahA
tod
xylA
codh
57
58
59
60
61
m above sea level

These results suggest that conditions present during transient fluxes in contaminated environments may lead to complex ecological interactions concerning competition and relative fitness of traits within the microbial community. These interactions may be easily missed when evaluating long-term contaminated sites that have already achieved a new community equilibrium created by fluctuating periods associated with transient changes in available resources and microbial habitats.

Statistical Analysis of Microbial Changes During Natural Attenuation

A summary of the descriptive statistics for the September 1996 NAT Study data set is seen in Table 12. Table 13 provides a similar analysis of the March 1997 NAT Study data set. As can be seen from these two tables, the mean concentrations of contaminant hydrocarbons was less in March 1997 than in September 1996. The mean dissolved oxygen value increased almost 1 mg / L from September 1996 to March 1997. The mean groundwater temperature dropped from 24°C in September 1996 to 18°C in March 1997. The fluctuation of these parameters is also seen in the microbiological data. The mean values for each of the DNA genotypes assayed for except *nahH* dropped between sampling periods. The mean ng 16S rDNA value in March 1997 samples was reduced to one-half the value obtained from the September 1996 samples. A. 1 shows a Pearson correlation matrix for September 1996 samples. For the September 1996 data set, three main groups showed significant correlation. Hydrocarbons showed significant positive correlation with each other as well as pH.

Table 12. Descriptive statistics for September 1996 NAT Study data set.

	N	Minimum	Maximum	Mean	Std. Deviation
ng *alkB*	70	0.16	11.22	3.96	2.20
ng *nahA*	70	0.13	14.79	4.87	3.00
ng *nahH*	70	0.07	8.43	3.22	2.00
ng *todC1C2*	70	0.01	18.99	6.51	4.44
ng *xylA*	70	0.01	8.13	3.21	2.07
ng16S	70	19.29	805.21	296.48	167.80
TVC	70	4.67E+02	5.48E+06	5.30E+05	1.22E+06
bmin	70	0.00	67.50	4.66	11.27
nmin	70	0.00	69.68	15.82	23.62
pmin	70	0.00	15.65	0.85	2.23
tmin	70	0.00	54.78	4.92	9.58
ppb benzene	70	0.00	444.33	32.98	92.19
ppb decane	70	0.00	0.00	0.00	0.00
ppb ethylbenzene	70	0.00	6644.00	439.29	1392.12
ppb naphthalene	70	0.00	6841.00	575.52	1542.44
ppb p-xylene	70	0.00	2160.33	100.21	351.97
ppb toluene	70	0.00	5256.00	179.11	758.61
DO	70	0.17	4.90	2.08	1.06
pH	70	4.12	6.12	5.29	0.25
temp	70	21.00	26.20	24.19	1.15

Table 13. Descriptive statistics for March 1997 NAT Study data set.

	N	Minimum	Maximum	Mean	Std. Deviation
ng *alkB*	70	0.06	16.37	1.89	2.74
ng *nahA*	70	0.01	9.20	1.15	1.42
ng *nahH*	70	0.01	9.12	3.27	2.25
ng *todC1C2*	70	0.06	9.25	3.12	2.65
ng *xylA*	70	0.02	16.27	2.36	2.61
ng 16S	70	8.85	299.97	133.49	75.88
TVC	70	4.00E+02	1.19E+06	7.16E+04	1.74E+05
bmin	70	0.00	15.44	2.04	2.64
nmin	70	0.00	33.01	6.47	9.27
pmin	70	0.00	3.81	0.66	0.89
tmin	70	0.00	12.10	2.14	3.03
ppb benzene	70	0.00	577.50	38.99	110.77
ppb ethylbenzene	70	0.00	4715.50	313.71	987.96
ppb naphthalene	70	0.00	3983.50	266.60	756.94
ppb p-xylene	70	0.00	3024.00	136.14	500.01
ppb toluene	70	0.00	6084.50	253.18	960.20
DO	70	0.20	7.70	2.83	1.82
pH	70	1.14	6.20	5.09	1.11
temperature	70	14.80	23.50	17.66	2.24

Negative correlation was seen between the hydrocarbons and both temperature and well. Except for ng *nahA*, the ng DNA data for the degradative gene probes showed positive correlation between each other and the individual hydrocarbons. Negative correlation was seen between each value for ng *alkB*, *nahA*, and *nahH* and dissolved oxygen. Negative correlation was also seen between the degradative gene probe values and both temperature and well.

A. 2 shows a Pearson correlation matrix for March 1997. Several notable groups formed in this matrix. As with the September 1996 samples, the hydrocarbons positively correlate significantly with each other. For the first time, a negative correlation is seen between the hydrocarbons benzene, ethylbenzene and naphthalene with dissolved oxygen. Each individual hydrocarbon positively correlated with ng *nahA*, but negatively correlated with ng *nahH* and ng *todC1C2*. Elevation was shown to positively correlate with the microbiological parameters ng *alkB*, ng *nahA*, ng 16S and total viable counts (TVC).

Multiple regression analysis helped explain some of the variability in the dependent variables. Table 14 shows the results of multiple regression analysis performed on the September 1996 NAT Study data set. Significant variability ($\alpha <$ 0.05) in the dependent variables ng *alkB*, ng *nahA*, ng *nahH*, ng *todC1C2*, and ng *xylA* by temperature, recovery of the internal standard during DNA extraction, and individual hydrocarbons. Temperature explained 47.0% of the variability in ng *alkB* and 54.0% of the variation in ng *todC1C2*. Temperature and recovery of the internal

Table 14. Multiple regression analysis for September 1996 NAT Study data set.

dependent variable	R^2	probability of a greater f	independent variable	(significance)
ng *alkB*	0.470	0.000	temperature	(0.002)
ng *nahA*	0.324	0.006	ppb benzene	(0.001)
			ppb naphthalene	(0.010)
			ppb toluene	(0.048)
ng *nahH*	0.504	0.000	recovery	(0.000)
			temperature	(0.020)
ng *todC1C2*	0.540	0.000	temperature	(0.000)
ng *xylA*	0.360	0.000	recovery	(0.040)
			temperature	(0.000)

standard added during DNA extraction explained 50.4% of the variability in ng *nahH* and 36.0% of the variability in ng *xylA*. The concentration of benzene, naphthalene, and toluene explained 32.4% of the variability in ng *nahA*. Table 15 shows less variability could be explained in the dependent variables by the independent variables. Elevation explained 28.5% of the variability in ng *nahH*. Dissolved oxygen, elevation, ppb *p*-xylene, and ppb toluene explained 28.2% of the variability in ng 16S. Dissolved oxygen, ppb ethylbenzene, ppb naphthalene, ppb p-xylene, ppb toluene, and TVC explained 59.8% of the variability in the 24 hour naphthalene mineralization assays.

Table 15. Multiple regression analysis for March 1997 NAT Study data set.

dependent variable	R^2	probability of a greater f	independent variable	(significance)
ng *nahA*	0.597	0.000	TVC	(0.000)
ng *nahH*	0.285	0.034	elevation	(0.032)
ng 16S	0.282	0.037	dissolved oxygen	(0.024)
			elevation	(0.024)
			ppb *p*-xylene	(0.047)
			ppb toluene	(0.045)
naphthalene mineralization	0.598	0.000	dissolved oxygen	(0.010)
			ppb ethylbenzene	(0.009)
			ppb naphthalene	(0.003)
			ppb *p*-xylene	(0.000)
			ppb toluene	(0.000)
			TVC	(0.007)

Statistical Analysis for the Entire NAT Study Data Set

Statistical analyses executed on the individual data sets were also performed on the NAT Study data set as a whole in attempt to decipher important interactions. Table 16 shows the minimum, maximum, mean and standard deviation for parameters measured at Columbus AFB. Significant correlation coefficients ($\alpha < 0.05$) are shown in A. 3. For the study, ng DNA values showed positive correlation with each other and, except for ng *nahH*, with the individual hydrocarbons. Total viable counts showed positive correlation with elevation. Individual hydrocarbons showed significant positive correlation with each other and pH, but showed negative correlation with dissolved oxygen concentrations and wells. Evaluation of the

Table 16. Descriptive statistics for NAT study data set.

	N	Minimum	Maximum	Mean	Std. Deviation
ng *alkB*	260	0.01	16.37	2.90	2.66
ng *nahA*	260	0.01	14.79	2.76	2.81
ng *nahH*	260	0.01	9.76	3.37	1.97
ng *todC1C2*	260	0.01	18.99	3.37	3.48
ng *xylA*	260	0.01	16.27	1.96	2.05
ng 16S	260	8.85	954.59	226.92	159.33
TVC	260	4.00E+02	5.48E+06	3.04E+05	7.42E+05
bmin	260	0.00	67.50	7.06	11.43
nmin	260	0.00	78.75	18.76	22.59
pmin	260	0.00	33.26	2.22	4.24
tmin	260	0.00	99.80	11.88	19.25
ppb benzene	260	0.00	577.50	20.38	76.94
ppb decane	260	0.00	4.00	0.05	0.43
ppb ethylbenzene	260	0.00	6644.00	214.53	907.83
ppb naphthalene	260	0.00	6841.00	232.01	918.09
ppb phenanthrene	260	0.00	3024.00	74.71	334.85
ppb toluene	260	0.00	6084.50	122.15	645.25
DO	260	0.17	7.70	2.44	1.39
pH	260	1.14	6.20	5.32	0.64
temperature	260	12.00	26.20	19.14	3.63

simple correlation analysis was performed using partial correlation analysis. Partial correlation analysis was conducted controlling for days, well and elevation (A.4-A.6). Controlling for days altered significant correlation between benzene mineralization and the ng DNA of each the hydrocarbon degrading genotypes. Negative correlation coefficients were added between benzene mineralization and ng *alkB*, *nahA*, and *nahH*, but significant negative correlation values were removed with ng *todC1C2* and *xylA*. Controlling for days also eliminated significant positive correlation between both ng *todC1C2* and *xylA* with ppb *p*-xylene and toluene. Controlling for well removed several significant correlations between individual hydrocarbons and ng DNA values, as well as with dissolved oxygen. Negative correlation coefficients were added for ppb benzene and naphthalene mineralization, but removed for ppb benzene and toluene mineralization. Controlling for elevation provided the least changes. Negative correlation was added between dissolved oxygen and ng *alkB*, but removed between dissolved oxygen and ng *nahA*. Multiple regression analysis helped explain variability in several dependent variables (Table 17). Days helped explain variability in 9 of 11 dependent variables. Temperature helped explain variability in 6 of 11 dependent variables. Wells helped explain variability in 7 of 11 dependent variables. Hydrocarbons helped explain variability in 7 of 11 dependent variables. Toluene and p-xylene may explain variability in the dependent variables because their aqueous concentrations increase throughout the experiment.

Table 17. Multiple regression analysis for NAT Study data set.

dependent variable	R^2	probability of a greater f	independent variable	(significance)
ng *alkB*	0.158	0.000	days	(0.005)
			ppb *p*-xylene	(0.032)
			ppb toluene	(0.014)
			well	(0.035)
ng *nahA*	0.288	0.000	days	(0.000)
			ppb decane	(0.000)
			ppb *p*-xylene	(0.033)
			temperature	(0.000)
			well	(0.001)
ng *nahH*	0.160	0.000	pH	(0.020)
			ppb benzene	(0.006)
			ppb decane	(0.026)
			ppb naphthalene	(0.022)
			temperature	(0.002)
ng *todC1C2*	0.308	0.000	days	(0.013)
			ppb benzene	(0.000)
			ppb ethylbenzene	(0.002)
			ppb naphthalene	(0.038)
			temperature	(0.001)
ng *xylA*	0.247	0.000	days	(0.000)
			ppb benzene	(0.034)
			temperature	(0.005)
			well	(0.000)
ng 16S rDNA	0.190	0.000	days	(0.000)
			temperature	(0.002)
			well	(0.003)
TVC	0.189	0.000	elevation	(0.001)
			temperature	(0.001)
			well	(0.000)

Table 17 (continued). Multiple regression analysis for NAT Study data set.

dependent variable	R^2	probability of a greater f	independent variable	(significance)
benz. 24 hour mineralization	0.118	0.003	days	(0.000)
			ppb *p*-xylene	(0.051)
			ppb toluene	(0.051)
naph. 24 hour mineralization	0.178	0.000	days	(0.000)
			dissolved oxygen	(0.032)
			ppb *p*-xylene	(0.018)
			ppb toluene	(0.028)
			well	(0.019)
phen. 24 hour mineralization	0.174	0.000	days	(0.000)
			elevation	(0.003)
			well	(0.009)
tol. 24 hour mineralization	0.093	0.026	days	(0.000)

Microbiological Changes Within the Source Material

Currently, an important issue that needs to be addressed is the microbial interactions occurring within the source of hydrocarbon contamination. The NAT Study at Columbus AFB provided an opportunity to investigate this topic. As with the actual field site, a microbiological characterization was performed on the source material prior to hydrocarbon contamination. Furthermore, samples were recovered from the source material in conjunction with each field sampling trip. This sampling

design provided data on the transient microbiological changes occurring in the source material.

Initial microbiological characterization of the source material chosen for the NAT Study showed the potential for biodegradation of the petroleum hydrocarbons existed. Time course mineralization experiments showed that indigenous microorganisms possessed the metabolic capacity to degrade toluene, naphthalene, and phenanthrene (Figure 17). No benzene mineralization was seen over a two week period. Mineralization of toluene increased rapidly to 30% between days 3 and 5. However, toluene degradation increased slowly to 45% between days 5 and 14. Degradation of naphthalene lagged behind toluene, with 16% mineralization by day 7 and 25% by day 14. Phenanthrene mineralization did not begin until after day 7. By day 14, phenanthrene mineralization had steadily increased to 40%.

Physical characteristics of the Columbus AFB source material can be seen in Table 18. Coarse, medium and fine grained sand were used for the NAT Study source material. The idea behind this was to create a zone of higher hydraulic conductivity than the surrounding aquifer material in order to facilitate contaminant transport from the source into the research area. The initial concentration of individual hydrocarbons is given in Table 19. Transport of the contaminants from the source material was influenced by natural groundwater principles of advection, dispersion, and diffusion. Contaminant transport from the source was retarded primarily by biological attenuation. Changes in hydrocarbon concentration can be seen in Figure 18.

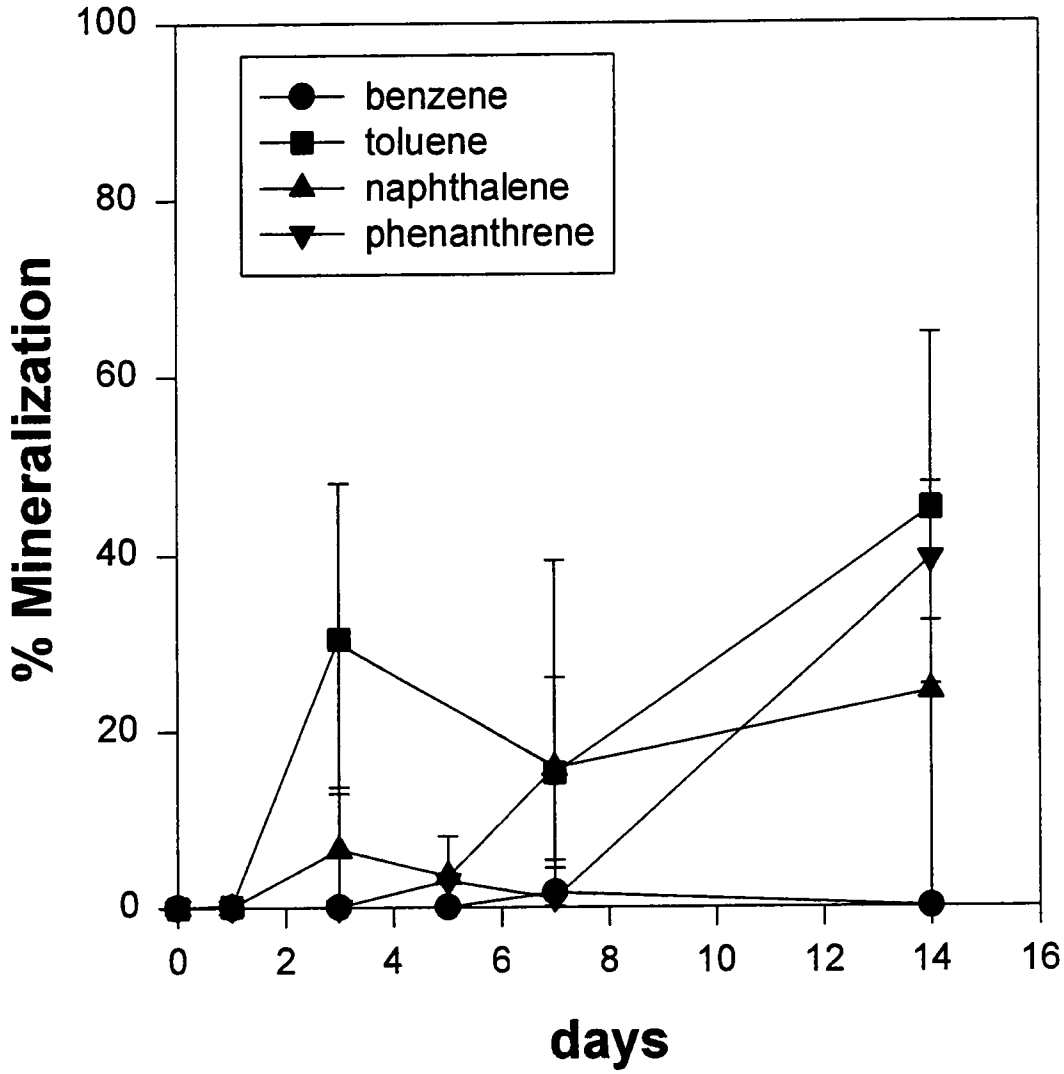


Figure 17. Time course mineralization study on Columbus AFB original source material.

Table 18. Physical characteristics of the NAT Study source material.

parameter	value
grain size	
coarse sand	4% by weight
medium sand	45% by weight
fine sand	40 % by weight
fines	1% by weight
total organic carbon	0.026 by weight
hydraulic conductivity	4×10^{-3} cm/s

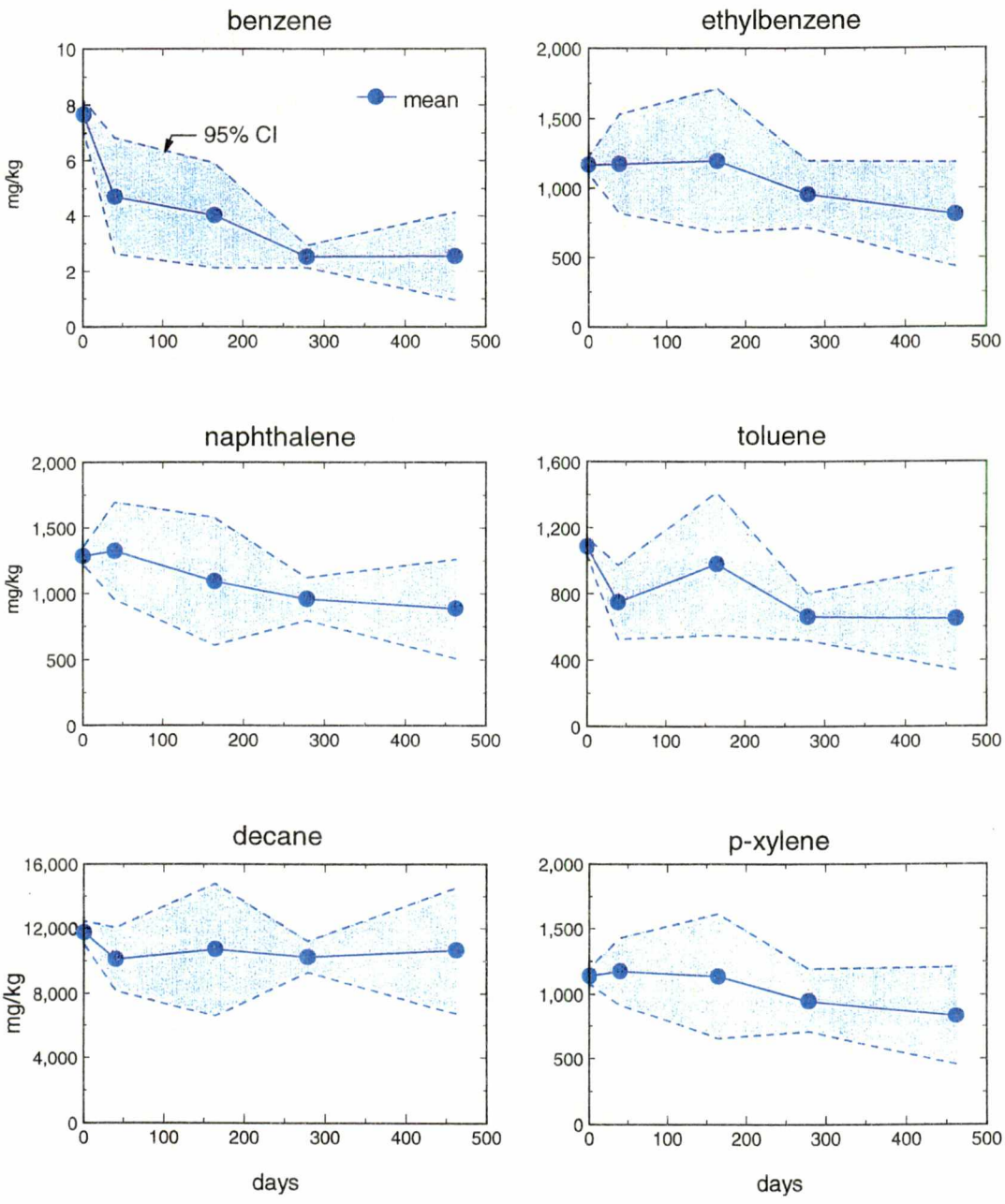
Table 19. Individual hydrocarbon concentrations in the NAT Study contaminant source[a].

	benzene	ethylbenzene	*n*-decane	naphthalene	toluene	*p*-xylene
actual concentration	7.6	1,163	11,713	1,282	1,083	1,134
design concentration	13.0	1,580	18,900	1,580	1,580	1,580

[a] concentrations are given as ppm = mg hydrocarbon / kg soil sample.

Figure 18. Changes in the mass of hydrocarbon present in the NAT Study source material at Columbus AFB over time.

Source Hydrocarbon Concentrations



Changes in the indigenous microbial community reflect hydrocarbon perturbation. Total viable organisms present in the original source material determined by spread plate analysis revealed 3.77 x 10^5 colony forming units (cfu) / gram of sample (Figure 19). After 40 days of hydrocarbon contamination, the culturable microbial population had dropped to 1.28 x 10^4 cfu / gram sample. After 278 days, there were 6.03 x 10^4 cfu / gram sample. Another substantial drop in culturable organisms was seen after 482 days. Total viable counts dropped to 3.23 x 10^3 cfu / gram sample.

Molecular analysis of the Columbus AFB source material before and during the NAT Study revealed the most significant changes within the microbial community. Initial molecular analysis of the source material showed that each degradative genotype targeted by the DNA probe array designed for this study represented under 20 percent of the total microbial community. Examination of individual degradative populations of microorganisms determined by DNA analysis showed different responses of the genotypes targeted for this study (Figure 20). Microorganisms possessing either of the biochemical enzymes naphthalene dioxygenase (*nahA*) or toluene dioxygenase (*todC1C2*) showed peak population levels at day 164, with *nahA* genotypes peaking at 40% and *todC1C2* genotypes peaking at 75% of the total microbial community. Both populations rapidly declined to under 5% of the total community by 482 days. Three genotypes peaked at day 278. Organisms possessing the alkane hydroxylase enzyme reached levels of 45% before dropping to under 5% at

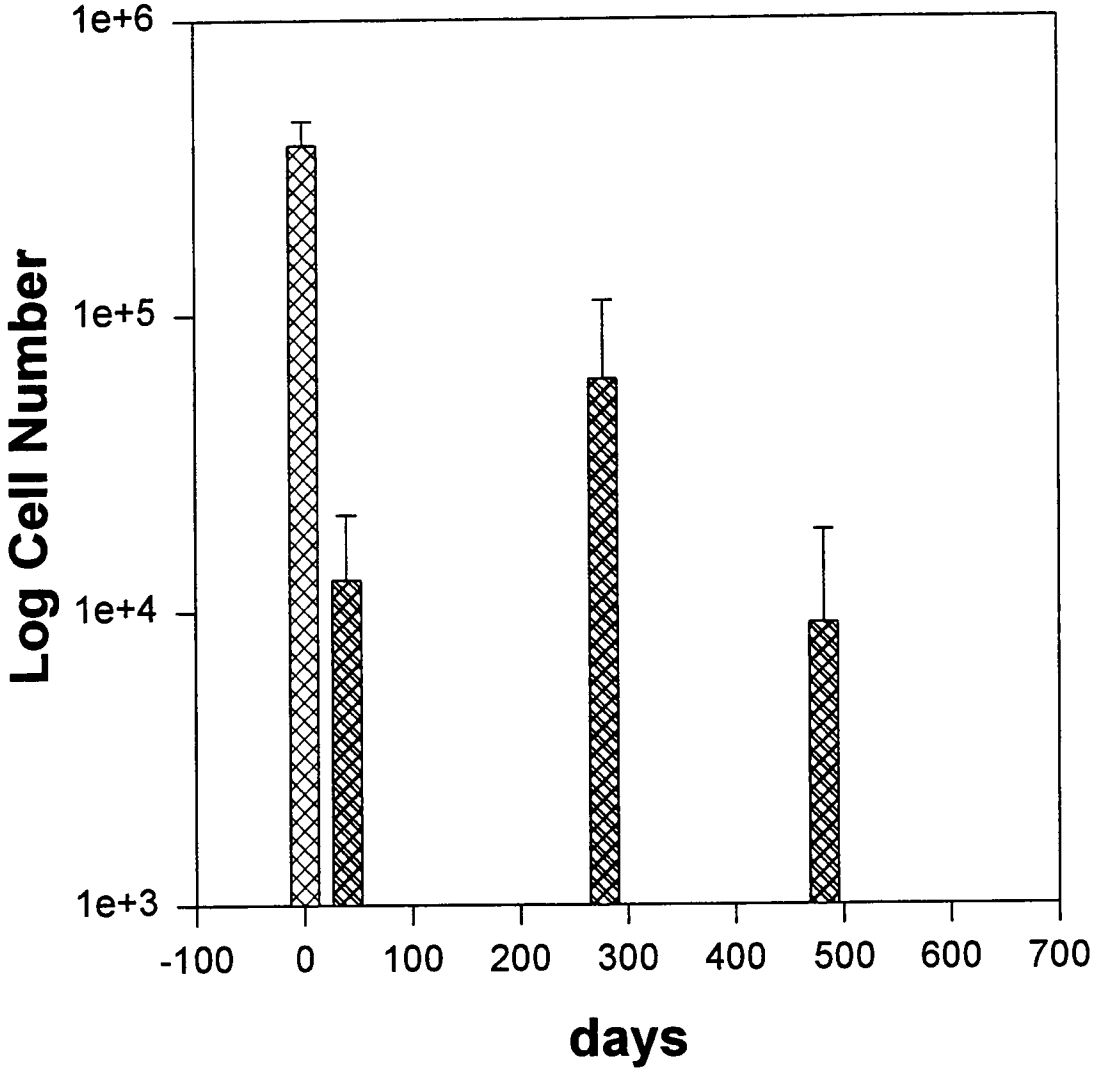
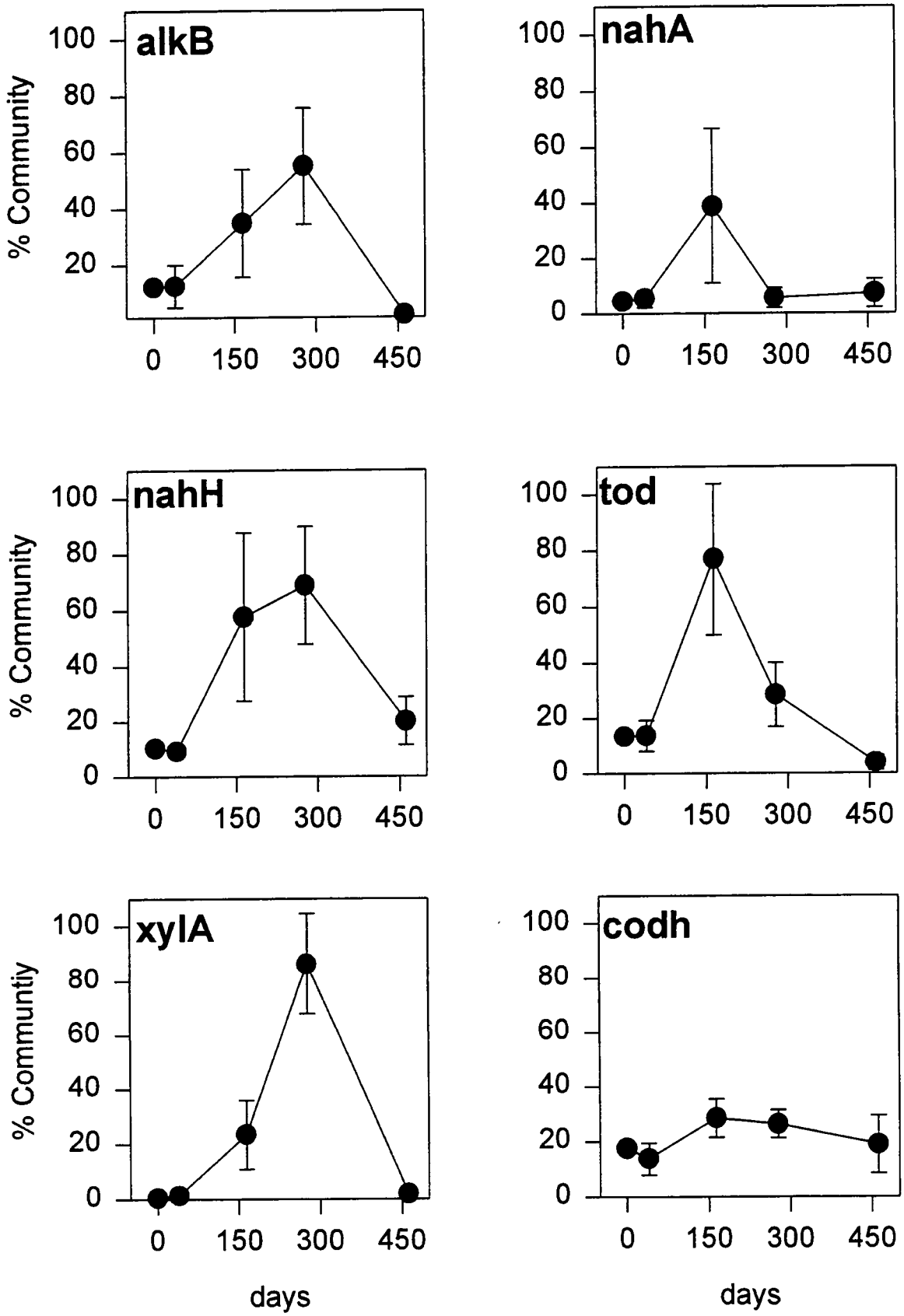


Figure 19. Total viable counts for Columbus AFB source material.

Figure 20. DNA changes in the Columbus AFB source material over time.


alkB
nahA
nahH
tod
xylA
codh
% Community
% Communtiy
100
80
60
40
20
0
0
150
300
450
days

day 482. *nahH* genotypes peaked at 65% by day 278, then dropped off to 20% by day 482. The genotype, *xylA,* increased to 80% of the microbial community prior to dropping off to under 5% after 482 days. The only population to remain constant throughout the study was the methanogens, organisms that hybridize with the gene probe for carbon monoxide dehydrogenase (*codh*). This population remained approximately 20% of the total microbial community for the duration of the study.

Statistical analysis provided insight into differences between the contaminant hydrocarbons and the indigenous microbial community. A descriptive statistic summary of the minimum, maximum, mean and standard deviation is given in Table 20. A correlation matrix for the CAFB source data matrix was generated using a Pearson correlation analysis. At a significance level of $\alpha < 0.05$, three major groups were found to correlate significantly among themselves (Table 21). The first group revealed positive correlation among each of the DNA probes *alkB*, *nahA*, *nahH*, *todC1C2*, and *xylA*, but not the gene probe *codh*. The second group included the contaminant hydrocarbons, benzene, decane, ethylbenzene, naphthalene, *p*-xylene, and toluene. A positive correlation between ng 16S and each hydrocarbon except decane was also included in this group. The final group involved a negative correlation between days and all of the DNA probes. Multiple regression analysis of the independent variables on each dependent variable helped to identify sources of variability within the data matrix (Table 22). Significant variability ($\alpha < 0.05$) in the

Table 20. Descriptive statistics for NAT Study source data set.

	n	minimum	maximum	mean	std. deviation
ng *alkB*	20	0.04	19.06	6.28	6.38
ng *nahA*	20	0.02	13.52	3.45	4.49
ng *nahH*	20	2.75	25.60	8.65	6.90
ng *todC1C2*	20	0.07	30.68	7.83	10.17
ng *xylA*	20	0.13	17.54	6.76	6.56
ng *codh*	20	0.34	8.20	4.32	2.22
ng16S	20	68.38	358.30	183.08	85.21
ppb benzene	20	0.14	5.70	3.04	1.70
ppb decane	20	1300.00	15000.00	10550.00	3370.07
ppb ethylbenzene	20	75.00	1500.00	962.75	417.91
ppb naphthalene	20	110.00	1500.00	982.50	379.33
ppb *p*-xylene	20	80.00	1400.00	961.50	394.57
ppb toluene	20	47.00	1200.00	730.90	337.56

Table 21. Pearson Correlation Matrix for NAT Study source data matrix[a].

	ng *alkB*	ng *nahA*	ng *nahH*	ng *tod*	ng *xylA*	ng *codh*	ng 16S	ben	dec	eben	naph	pxyl	tol	days
ng *alkB*	1.000	**0.870**	**0.920**	**0.890**	**0.902**	**0.449**								**-0.698**
ng *nahA*		1.000	**0.898**	**0.893**	**0.687**									**-0.480**
ng *nahH*			1.000	**0.862**	**0.868**									**-0.476**
ng *tod*				1.000	**0.765**		**0.460**						**0.466**	**-0.631**
ng *xylA*					1.000	**0.607**								**-0.538**
ng *codh*						1.000								**-0.619**
ng 16S							1.000	**0.520**		**0.470**	**0.454**	**0.482**	**0.514**	**-0.458**
ben								1.000	**0.655**	**0.845**	**0.808**	**0.841**	**0.850**	
dec									1.000	**0.835**	**0.900**	**0.863**	**0.809**	
eben										1.000	**0.960**	**0.992**	**0.946**	
naph											1.000	**0.964**	**0.878**	
pxyl												1.000	**0.938**	
tol													1.000	
days														1.000

[a] Values significant at $\alpha < 0.05$.

Table 22. Multiple regression analysis for CAFB source data matrix.

dependent variable	R^2	probability of a greater f	independent variable	(significance)
ng *alkB*	0.854	0.000	ppb benzene	(0.009)
			ppb *p*-xylene	(0.002)
			days	(0.000)
ng *nahA*	0.672	0.027	ppb *p*-xylene	(0.031)
			days	(0.037)
ng *nahH*	0.782	0.003	ppb benzene	(0.016)
			ppb *p*-xylene	(0.001)
			days	(0.010)
ng *todC1C2*	0.854	0.000	ppb *p*-xylene	(0.006)
			ppb toluene	(0.012)
			days	(0.001)
ng *xylA*	0.738	0.009	ppb benzene	(0.019)
			ppb *p*-xylene	(0.017)
			days	(0.015)

dependent variables ng *alkB*, ng *nahA*, ng *nahH*, ng *todC1C2*, and ng *xylA* was influenced by both ppb *p*-xylene and days. The independent variables ppb benzene, ppb *p*-xylene, and days explained 85.4% of the variability in ng *alkB*, while ppb *p*-xylene and days explained 67.2% of the variability in ng *nahA*. 78.2% of the variability in ng *nahH* could be explained by ppb benzene, ppb *p*-xylene, and days, with 85.4% of the variability in *todC1C2* explained by ppb *p*-xylene, ppb toluene, and days. The independent variables ppb benzene, ppb *p*-xylene, and days explained 73.8% of the variability in ng *xylA*.

Chapter 6

DISCUSSION

This dissertation describes the efficacy of linking molecular diagnostic procedures with activity assays utilizing radio-isotopes for the purpose of both assessing the potential of indigenous subsurface organisms for natural attenuation, as well as monitoring changes in microbial community during natural attenuation.

It was determined that favorable microbiological conditions for natural attenuation exist at Columbus AFB. Information generated using traditional enumeration and activity assays was enhanced using data generated by DNA:DNA hybridization studies. An array of molecular probes was successfully developed to evaluate the distribution of genes encoding enzymes commonly associated with the biodegradation of petroleum hydrocarbons. This approach revealed a near-ubiquitous distribution of organisms capable of degrading straight-chain, monoaromatic, and polyaromatic hydrocarbons in subsurface samples. DNA hybridization signals and mineralization of ^{14}C-labeled substrates did not differ between samples recovered from areas having prior exposure to trace levels of organic compounds from the Made-2 study during 1990-1991 and the other samples that had no prior contaminant exposure, suggesting that microbial community of previously exposed sediments had re-

equilibrated after the previous experiment. Mineralization assays showed that the target compounds benzene, toluene, naphthalene, and phenanthrene were rapidly degraded without any nutrient addition. The microbiological data, groundwater and aquifer characteristics, and information gained during the previous Macrodisperion Experiment studies, supports the hypothesis that natural attenuation can occur at Columbus AFB.

Assessing the Potential for Natural Attenuation at Columbus AFB

Previous investigations at Columbus AFB, have demonstrated that mechanisms other than physical and chemical interactions affect the fate and transport of contaminants in groundwater. In order to determine the role of microorganisms in contaminant transport, an extensive sampling network was designed to collect aquifer sediment. Sixty samples from 12 boreholes were recovered to determine the distribution, abundance and activity of microorganisms indigenous to the aquifer. The amount of information generated during the site characterization provided a uniquely detailed data set describing the native microbial community that may potentially be used to help develop computer models.

A method for estimating total microbial abundance using an oligonucleotide probe targeting a highly conserved region of the 16S rDNA gene was developed for this study. This enumeration technique helps eliminate any biases associated with culture-based enumeration methods from environmental samples (Ammann et al.,

1995; Ward et al., 1990). DNA:DNA hybridization experiments allow investigators to enumerate organisms that are not readily isolated from the environment, as well as provide similar total microbial abundance estimates without the problems of background interference commonly associated with direct microscopic counts using cell stains. Most importantly, the Universal 16S rDNA oligonucleotide provides a means to normalize data obtained from DNA:DNA hybridization experiments using probes for genes encoding specific enzymes involved in contaminant degradation. Stahl et al. (1988) used this technique to describe the abundance of particular organisms as a fraction of total population in microbial ecology studies of a bovine rumen. The ability to normalize specific populations as a percentage of the entire microbial community present in any given sample allows direct comparisons to be made between samples taken over time at different study sites. It also helps to eliminate errors in cell number estimates due to variations in DNA recovery during extraction (Stahl et al., 1988).

It is important to establish a method for accurately enumerating changes in microbial populations at each sampling period. During the NAT Study, it was hypothesized that microbial populations measured by DNA:DNA hybridization with the array of gene probes developed for this study will be enriched for over time in response to hydrocarbon perturbation, thus increasing as a percent of the total microbial community. The gene probe array used for the NAT Study (Table 3) was developed by targeting genes commonly associated with the degradation of petroleum

hydrocarbons. Previous work at Columbus AFB showed that mixtures of contaminants appear to develop independent plumes in the groundwater. The array of probes provided a means for tracking the impact of the individual hydrocarbons on indigenous microbial populations.

The array of gene probes chosen for the NAT Study broadly targets the individual hydrocarbons present in the contaminant source mixture. The gene probe *alkB* was developed from the OCT plasmid present in the reference organism *Pseudomonas oleovorans*. The alkane hydroxylase has been shown to be responsible for the formation of primary alcohols from *n*-alkanes containing 6-10 carbon atoms. It is also responsible for the ω-hydroxylation of fatty acids (Grund et al., 1975). The gene probe *nahA* was developed from the NAH7 plasmid present in the reference organism *Pseudomonas putida* G7. The naphthalene dioxygenase is responsible for the addition of molecular oxygen to polyaromatic hydrocarbons naphthalene, phenanthrene, and anthracene (Sanseverino et al., 1993). The gene probe *nahH* is the broadest gene probe used in the array. This probe targets the enzyme, catechol-2,3-dioxygense, which is present in several biochemical pathways other than the pathway for naphthalene degradation on the catabolic plasmid NAH7 from the reference organism *Pseudomonas putida* G7. The gene probe *todC1C2*, from the reference organism *Pseudomonas putida* F1, is chromosomally encoded. The toluene dioxygenase enzyme is responsible for initiating the degradation of benzene, ethylbenzene, catechol, toluene, *p*-xylene, phenol, cresol, and methyl catechol (Gibson

et al., 1968). The gene probe *xylA* was developed from the catabolic plasmid pWWO present in the reference organism *Pseudomonas putida* mt-2. The xylene monooxygenase has been shown to be involved in the degradation of toluene, *p*-xylene, *m*-xylene, *p*-toluate, and *m*-toluate (Burlage et al., 1989).

Differences in microbial abundance determined by spread plate analyses have been well documented for various granular textures of aquifer material (Balkwill, 1989; Fredrickson et al., 1989; Phelps et al., 1989; Fredrickson et al., 1991). At Columbus AFB, differences in microbial abundance between fine-granular or coarse-granular samples depended on the technique chosen (Figure 4). Total population estimates using colony forming units from spread-plate analyses showed close to one order of magnitude greater values from a coarse-granular sample over a sample consisting of fine-granular material. Using this technique alone might lead one to suggest that the differences are related to differences in groundwater flow rates. However, DAPI direct counts and molecular probing using the 16S rDNA Universal oligonucleotide showed no readily discernible differences between samples. Enumeration estimates based on both spread-plate analyses and molecular probing averaged by elevation above sea level for all 12 experimental boreholes showed no discernible trend. Within this shallow aquifer, there is no significant decrease in total microbial density with depth using molecular probing methods. However, total viable counts positively correlated with elevation ($\alpha < 0.05$). Furthermore, multiple

regression analysis showed elevation explained 37.6% of the variability in total viable counts.

Preliminary studies using fine-granular and coarse-granular subsurface sediments were designed to compare methods of enumerating microorganisms. As previously reported, classical microbiological methods underestimated microbial abundance (Ammann et al., 1995; Ward et al., 1990). Traditional microbiological techniques tested (spread plates and DAPI direct counts) underestimated total population values compared with those generated using DNA:DNA hybridization experiments with a 16S rDNA Universal oligonucleotide (Stahl et al., 1988) by 2-3 orders of magnitude (Figure 5) for the two representative aquifer samples. Expanding the comparison to include every sample recovered from the field site showed total viable counts determined by spread plate analysis underestimated total microbial values using molecular methods by 1-2 orders of magnitude. DAPI direct counts were not performed on every sample. Potential concerns revolving around the influence of rRNA on hybridization signal were also analyzed. There was no effect on probe hybridization signal intensity when the extracted DNA was treated with RNase, indicating survival of ribosomal RNA during extraction and hybridization was minimal. RNA rapidly degrades upon alkaline heat-treatment, which is a critical step in preparing DNA for vacuum blotting (Sambrook et al., 1989). Treatment of the samples with DNase successfully eliminated positive signals, indicating probe hybridization was only with DNA. Additionally, variability within samples was

minimal, apparently due to the use of large sample sizes (50 grams) for nucleic acid extraction.

Previous investigations have shown the relevance of monitoring a particular genotype in response to a specific contaminant. Several investigators have monitored microbial genotypes capable of oxidizing naphthalene at coal tar-contaminated sites using a DNA probe targeting the naphthalene dioxygenase gene (Sanseverino et al., 1993; Herrick et al., 1993; Herrick et al., 1997). Sites of chronic polyaromatic hydrocarbon (PAH) exposure, such as manufacturing gas facilities or coal-waste repositories, show elevated levels of *nahA* genotypes. However, there is generally a plethora of compounds present at most contaminated sites. Microbial genotypes capable of degrading contaminants such as BTEX and naphthalene are widely distributed across a range of soils and sediments. The array of DNA probes designed for the Columbus AFB study is unique in that many genotypes commonly associated with the degradation of several petroleum hydrocarbons commonly found in fuels are targeted. Figures 7A and 7B show the average abundance of microbial genotypes as determined by DNA hybridization experiments. Microbial populations possessing the genotypes to degrade petroleum hydrocarbons are represented as a low percent of the total microbial community, with values ranging from a low of 2.92% for *xylA* genotypes to a high of 12.19% for *nahA* genotypes. Using these probes, it will be possible to monitor independent changes in specific microbial populations in response to hydrocarbon perturbation. Additionally, other biochemical pathways do exist to

degrade these hydrocarbons chosen for the NAT Study, this array of gene probes should provide an accurate assessment of the impact of hydrocarbons on this microbial community.

The abundance of particular organisms possessing the genotypic potential for degrading the target substrates chosen for ^{14}C-labeled mineralization assays is seen in Table 8. All samples produced positive signals when hybridized with each of the DNA probes designed for this study. Signal intensities for 4 samples were positive, but fell outside the standard curve and were thus listed as below the detection limits listed in Table 8.

The capacity for indigenous microorganisms to degrade aromatic hydrocarbons was successfully measured using ^{14}C-labeled compounds in laboratory mineralization experiments (Figure 6). Time points ranging from 1 to 14 days were used to determine if an acclimation period was essential for biodegradation of the target compounds (Herbes and Schwall, 1978; Spain and van Veld, 1983). Minimal degradation occurred during the initial 24 hours in all samples tested, suggesting that there was an acclimation period prior to biodegradation. Time course mineralization experiments showed that an acclimation period of approximately 3 days was necessary for degradation to occur. No difference was seen in acclimation periods between the 4 boreholes investigated. Moreover, Figure 6 shows microbial potential for degrading benzene, toluene, naphthalene, and phenanthrene also does not vary with depth. Davis et al. (1994) found a period of 4-14 days for benzene degradation to reach 50% in

samples from a manufacturing facility. It is expected that the acclimation period in mineralization assays will measurable decrease following contamination of the study site with hydrocarbon.

Monitoring Microbial Changes During Natural Attenuation

For natural attenuation to be a viable alternative in the remediation of sites contaminated with petroleum hydrocarbons, contaminant migration, groundwater geochemistry, and subsurface microbiology must be accurately and quantitatively monitored. Successful demonstration of plume truncation, and preferably plume retraction with a reduction in contaminant concentration, must be efficiently linked with changes in the indigenous microbial community. This task was successfully shown during the NAT Study at Columbus AFB.

Figure 8 show the life history of individual hydrocarbon groundwater plumes. Even with the hydrocarbon load buried in the source trench, the plumes did not expand more than 10-15 meters from the source after 278 days into the experiment. After 462 days, the plumes had retracted and were concentrated approximately 5-10 meters down gradient of the contaminant source.

Analysis of changes that occurred in the site' s microbiology and geochemistry lend insight into the evolution of the hydrocarbon plumes. Geochemical information gathered during the NAT Study supported the idea that favorable conditions existed at Columbus AFB for natural attenuation. Microorganisms capable of biodegrading

aromatic compounds, such as BTEX and naphthalene, are ubiquitously distributed in nature (National Research Council, 1993). Background samples collected at Columbus AFB supported this notion and showed the methods chosen for analyzing the microbiology of the site provided quantitative measurements.

Changes in microbial activity and community structure mirror the expansion and retraction cycle exhibited by the contaminant plumes. Early in the experiment, hydrocarbons were very slow to migrate from the source trench. This was because microbial activity both in the source material and in aquifer material approximately 1 meter downgradient was greater than contaminant transport. This provided an initial steady-state in which contaminant migration was limited. However, as microbial activity increased, the abundance of microorganisms capable of growth on the hydrocarbons as a primary substrate and carbon source also increased. This increase in degradative populations of microorganisms, as defined as a percent of the total community, effectively perturbed the steady-state by depleting the concentration of dissolved oxygen more rapidly than it could be replaced. In doing this, the rate of microbial biodegradation of the hydrocarbons became slower than the rate of hydrocarbon transport in the subsurface. This allowed the hydrocarbon plumes to expand further downgradient.

Data obtained from samples recovered from September 1996 show the expansion of the contaminant plumes downgradient 10-15 meters. More importantly, the samples reflect the impact of the newly expanded plumes on the indigenous

microorganisms. Activity assays utilizing ^{14}C-labeled substrates indicated the hydrocarbon generating the greatest impact was naphthalene (Figure 9). Biodegradation increased to almost 60% within 24 hours. This suggests that the native microorganisms no longer required an acclimation period to degrade naphthalene. Molecular diagnostics showed that these very active samples 5-15 meters downgradient the source trench had not yet given way to a shift in community structure as defined by an increase in *nahA* genotypes as a percent of the total microbial community.

Samples approximately 1 meter downgradient the source showed minimal mineralization activity within 24 hours (Figure 9). DNA:DNA hybridization studies reflect a major change in the microbial community had occurred (Figure 13). The degradative genotypes monitored during this study had increased. *nahA* genotypes had increased to 20 to 40% of the total microbial community, compared to less than 10% in the control samples. *todC1C2* genotypes had increased to 20 to almost 50% of the total microbial community in highly impacted samples, compared to under 10% in the un-impacted upgradient samples (Figure 12). Geochemical analysis of the groundwater revealed dissolved oxygen had been depleted to concentrations as low as 0.60 mg / L. This suggests that limitations in dissolved oxygen were suppressing degradation in these samples. Another potential explanation is the possibility of toxicity to the indigenous organisms.

By March 1997, the hydrocarbon plumes had retracted to 5 to 10 meters downgradient the source trench. Microbial mineralization of naphthalene was concentrated in samples recovered form the area 1 to 5 meters downgradient the source (Figure 11). Return of microbial activity in the area 1 meter downgradient the trench corresponds to a somewhat elevated dissolved oxygen concentration. Dissolved oxygen in the local groundwater had risen to about 1.2 to 2.0 mg / L, thus was not at a level that was totally inhibitory of hydrocarbon metabolism in a 24 hour assay. Moreover, molecular analysis showed a decrease in degradative genotypes as compared with the previous samples (Figure 15). This lower abundance of degradative organisms would require lower dissolved oxygen levels. *nahA* genotypes in sediment samples recovered approximately 1 meter downgradient ranged up to 20% of the total microbial community, with *todC1C2* genotypes dropping below 10%. *xylA* genotypes in these samples increased in population levels up to 20% of the total community.

Samples recovered from approximately 5 meters downgradient the source trench during March 1997 mineralized a lower percentage (20%) naphthalene than samples from the same area recovered during September 1996 (>50%). This may be due to dissolved oxygen in the groundwater being somewhat depleted, with concentrations ranging from 0.20 to 2.30 mg / L, or to the lower mean concentration of naphthalene at the site, with levels dropping from 576 μg / L in September 1996 to 267 μg / L in March 1997.

Comparison of the NAT Study with the Made-2 Study

Hydrocarbon transport during the NAT Study did not occur to the extent seen in the previous Made-2 Study. This was primarily due to the manner of contaminant introduction into the aquifer. For the Made-2 Study, approximately 160 mg / L of total dissolved organic compounds were pulsed in to the aquifer in the aqueous phase. This concentration was far less than the total organic concentration used for the NAT Study (Table 19). The NAT Study contaminant source was emplaced in the aquifer with the contaminants dissolved in decane, creating a bolus mass of both pure phase and aqueous phase hydrocarbons in the groundwater. Hydrocarbons distribution into the groundwater was limited by the rate of dissolution from the source trench. Aqueous phase hydrocarbons were transported through the study aquifer by natural groundwater flow. The substantially increased contaminant concentration produced different demands on the microorganisms and geochemistry indigenous to the aquifer than the trace levels of organic compounds used for the Made-2 Study. No direct microbiological data exists from the previous groundwater studies at Columbus AFB to compare with the data obtained during the NAT Study. Indirect evidence is seen in the evolution of $^{14}CO_2$ from the injected ^{14}C-*p*-xylene during the Made-2, which supports the aerobic biodegradation seen in laboratory assays performed during the NAT Study. Dissolved oxygen became somewhat depleted in the injection area

during the Made-2, but did not drop to levels that inhibited aerobic microbiological processes (Boggs et al., 1993).

Introduction of a New Microbial Ecology Parameter for Groundwater Models

Perhaps one of the greatest uses of the data generated from the NAT Study at Columbus AFB is the development of a parameter that emphasizes the importance of microbial ecological interactions with hydrocarbon transport in groundwater. From the microbiological data provided by the University of Tennessee, I would like to introduce the parameter M_e, which incorporates measures of a particular degradative population generated by DNA hybridization, microbial activity as a percent mineralization of a given ^{14}C-labeled substrate, and the concentration of dissolved oxygen. Additional work needs to be performed on this parameter, but an example of its potential importance is given below.

Changes in aqueous naphthalene concentrations during the NAT Study influenced changes in populations of microorganisms capable of biodegradation of naphthalene. For explanation of M_e, data from the September 1996 samples recovered from Columbus AFB are discussed. M_e is defined in the following equation:

$$M_e = \text{ng } \textit{nahA} \text{ DNA} \times \% \text{ mineralization of naphthalene at 24 hours} \times \text{concentration of dissolved oxygen}$$

A comparison of this parameter and the concentration of naphthalene is shown in Figure 21. M_e stays low in samples approximately 1 meter from the source trench due to depleted dissolved oxygen levels decreasing microbial mineralization of naphthalene. Mineralization of naphthalene and concentrations of dissolved oxygen both increase by 5 to 10 meters downgradient the source trench. This is seen by the corresponding increase in M_e. It is important to investigate this concept further.

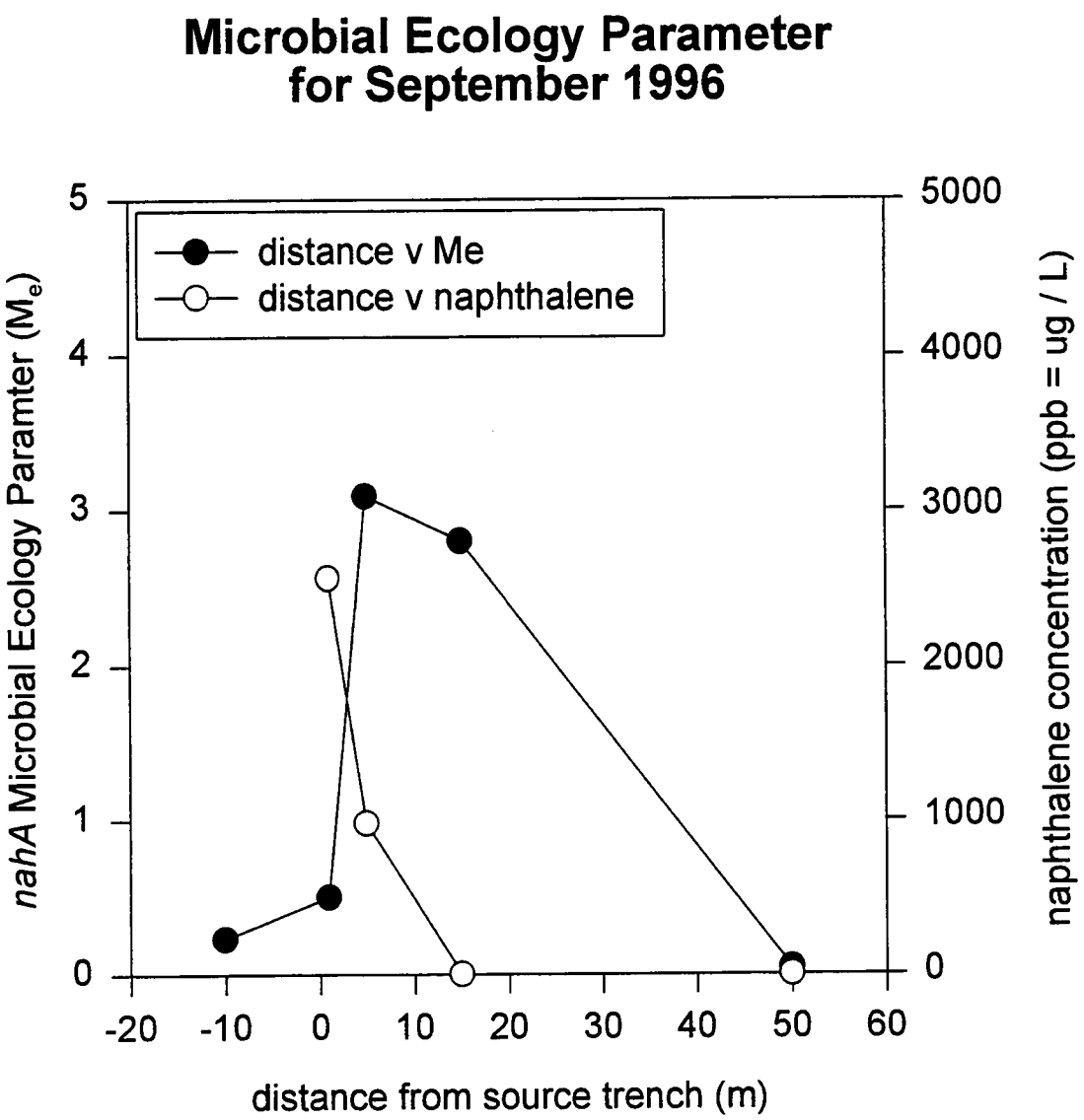


Figure 21. Example plot of the theoretical parameter for inclusion of microbial ecology in groundwater flow models. Values for each data point represent averages form boreholes within a transect.

Chapter 7

CONCLUSIONS

A multidisciplinary field research experiment was conducted at Columbus AFB, Mississippi, in order to develop a better mechanistic understanding of the various processes involved in the natural attenuation of petroleum hydrocarbons. The Natural Attenuation (NAT) Study was initiated December 1, 1995, as a follow to the previous Macrodispersion Experiment (Made) Studies. In particular, the Made-2 Study indicated the potential importance of interactions between contaminants and indigenous microorganisms. While no direct microbiological evidence was collected during the Made-2, indirect evidence linked aerobic biodegradative activity to the retardation and eventual disappearance of a dilute hydrocarbon solution.

The NAT Study was conceived to evaluate the efficacy of linking direct microbiological evidence of biodegradation from laboratory assays with measurements collected from field conditions. Geochemical analysis, including hydrocarbon analysis, cation and anion analysis, pH, temperature, and dissolved oxygen concentrations, was collected for comparison with microbiological data.

Subsurface samples were collected in order to determine the microbial activity level and genetic potential for natural attenuation of petroleum hydrocarbons

commonly associated with jet-fuel. The compounds of most interest for this study were benzene, toluene, ethylbenzene, and *p*-xylene (BTEX), as well as naphthalene and decane.

Initial laboratory analyses showed the microbiological potential existed for natural attenuation of aromatic hydrocarbons in samples obtained form the Columbus AFB study site. Activity assays utilizing ^{14}C-labeled substrates indicated the indigenous microbial community possessed the potential to biodegrade the hydrocarbons, but only after an acclimation period of approximately 3-5 days. Using a biomass estimate produced by DNA hybridization studies with a 16S rDNA Universal oligonucleotide, it was determined that the research area supported 10^8 to 10^9 total organisms per gram soil. Further molecular analysis of the samples was conducted using an array of gene probes designed to monitor specific genotypes commonly associated with the degradation of aromatic hydrocarbons. Degradative populations were shown to be present at 10^7 to 10^8 cells per gram sample. Conversion of this information to a percent of the total community by normalization of the data to the values obtained using the 16S rDNA Universal oligonucleotide indicated that the aromatic hydrocarbon degrading populations measured by the gene probe array were present at levels less than 20%. While this array is not inclusive of every genotype present at a given site, it was designed to provide a more than adequate indication of the impact of petroleum hydrocarbons on the native microorganisms.

During November 1995, a contaminant source trench was created approximately 5 meters below ground surface at the study site at Columbus AFB. December 1 corresponds to T=0 for the NAT Study. After 278 days, significant hydrocarbon plumes had developed in the groundwater approximately 10-15 meters downgradient from the source trench. Microbiological analyses reflected the hydrocarbon impact on the indigenous microbial community. Naphthalene mineralization reached levels of 60% in a 24 hour assay. Molecular analysis revealed aromatic hydrocarbon degrading genotypes monitored by the array of DNA probes were enriched to levels approaching 50% of the total microbial community. After 462 days, both mineralization rates and population levels had decreased as compared to samples from 278 days into the experiment. Analysis of the hydrocarbon plumes suggested that they had not migrated any further downgradient for the source trench and initially appeared to be retracting.

In conclusion, the NAT Study at Columbus AFB provided field observations of natural attenuation of aromatic hydrocarbons. This information was supported by microbiological assays performed in the laboratory. Statistical analysis showed significant correlation of the aromatic hydrocarbon genotypes and the contaminant hydrocarbons monitored during this study, showing that as the hydrocarbon concentrations increased, so did the percent of degradative genotypes as a percent of the total microbial community. Microbial activity assays showed a major increase in hydrocarbon mineralization due to acclimation to elevated levels of contaminants.

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APPENDIX

A.1. Pearson correlation matrix for NAT Study September 1996 data set[a].

	ng *alkB*	ng *nahA*	ng *nahH*	ng *tod*	ng *xylA*	ng 16S	TVC	bmin	nmin	pmin	tmin
ng *alkB*	1.000	**0.290**	**0.319**	**0.647**	**0.491**						
ng *nahA*		1.000			**0.372**	**0.416**		**-0.268**		**-0.239**	**-0.277**
ng *nahH*			1.000	**0.585**	**0.592**						
ng *tod*				1.000	**0.738**						
ng *xylA*					1.000						
ng 16S						1.000	**-0.391**				**-0.255**
TVC							1.000		**-0.255**		
bmin								1.000	**0.655**	**0.321**	**0.757**
nmin									1.000	**0.461**	**0.765**
pmin										1.000	
tmin											1.000
ben											
eben											
naph											
pxyl											
tol											
DO											
elev											
pH											
temp											
well											

[a] Values are significant at the level of $\alpha < 0.05$.

A.1. Pearson correlation matrix for NAT Study September 1996 data set (con't).

	ben	eben	naph	pxyl	tol	DO	elev	pH	temp	well
ng *alkB*	**0.459**	**0.465**	**0.465**	**0.258**		**-0.372**			**-0.515**	**-0.410**
ng *nahA*	**0.291**	**0.237**				**-0.281**			**-0.262**	
ng *nahH*	**0.381**	**0.345**	**0.385**	**0.256**		**-0.277**			**-0.414**	**-0.360**
ng *tod*	**0.517**	**0.510**	**0.486**	**0.405**	**0.370**			**0.287**	**-0.719**	**-0.616**
ng *xylA*	**0.412**	**0.389**	**0.372**	**0.321**	**0.281**				**-0.561**	**-0.390**
ng 16S										**-0.315**
TVC										**0.650**
bmin										
nmin										**-0.269**
pmin								**0.237**		
tmin										
ben	1.000	**0.976**	**0.963**	**0.899**	**0.836**			**0.499**	**-0.552**	
eben		1.000	**0.953**	**0.891**	**0.803**			**0.514**	**-0.551**	**-0.449**
naph			1.000	**0.831**	**0.729**			**0.537**	**-0.497**	**-0.463**
pxyl				1.000	**0.974**			**0.457**	**-0.451**	**-0.382**
tol					1.000			**0.408**	**-0.425**	**-0.339**
DO						1.000	**-0.476**		**0.375**	**0.300**
elev							1.000			
pH								1.000	**-0.340**	
temp									1.000	**0.465**
well										1.000

A. 2. Pearson correlation matrix for March 1997 NAT Study data set[a].

	ng *alkB*	ng *nahA*	ng *nahH*	ng *tod*	ng *xylA*	ng 16S	TVC	bmin	nmin	pmin	tmin
ng *alkB*	1.000				**0.588**						
ng *nahA*		1.000			**0.370**		**0.618**				
ng *nahH*			1.000	**0.666**		**0.616**			**-0.401**	**-0.324**	**-0.295**
ng *tod*				1.000		**0.525**		**-0.296**	**-0.541**		**-0.405**
ng *xylA*					1.000						
ng 16S						1.000				**-0.321**	**-0.414**
TVC							1.000		**0.273**		
bmin								1.000	**0.664**	**0.277**	**0.471**
nmin									1.000		**0.471**
pmin										1.000	
tmin											1.000
ben											
eben											
naph											
pxyl											
tol											
DO											
elev											
pH											
temp											

[a] Values are significant at the level of $\alpha < 0.05$.

A. 2. Pearson correlation matrix for March 1997 NAT Study data set (con't).

	ben	eben	naph	pxyl	tol	DO	elev	pH	temp
ng *alkB*							**0.242**		
ng *nahA*	**0.241**	**0.268**	**0.265**	**0.297**	**0.298**		**0.288**		
ng *nahH*	**-0.405**	**-0.381**	**-0.389**	**-0.318**	**-0.310**			**-0.293**	**-0.311**
ng *tod*	**-0.332**	**-0.296**	**-0.330**	**-0.251**	**-0.244**				**-0.535**
ng *xylA*									
ng 16S							**0.311**		
TVC							**0.357**		
bmin									**0.293**
nmin						**-0.437**		**0.247**	**0.606**
pmin							**-0.361**	**0.251**	
tmin									**0.527**
ben	1.000	**0.981**	**0.992**	**0.845**	**0.802**	**-0.277**			
eben		1.000	**0.982**	**0.901**	**0.866**	**-0.242**			
naph			1.000	**0.876**	**0.835**	**-0.281**			
pxyl				1.000	**0.996**				
tol					1.000				
DO						1.000		**-0.474**	**-0.460**
elev							1.000		
pH								1.000	
temp									1.000

A.3. Pearson Correlation Matrix for NAT study data set[a].

	ng *alkB*	ng *nahA*	ng *nahH*	ng *tod*	ng *xylA*	ng 16S	bmin	nmin	pmin	tmin	TVC	ben
ng *alkB*	1.000	**0.414**	**0.262**	**0.335**	**0.382**	**0.207**				**-0.140**		**0.186**
ng *nahA*		1.000	**0.139**	**0.274**	**0.314**	**0.473**				**-0.130**		**0.148**
ng *nahH*			1.000	**0.316**	**0.134**				**0.122**			
ng *tod*				1.000	**0.480**	**0.229**	**-0.199**	**-0.227**	**-0.182**	**-0.331**		**0.201**
ng *xylA*					1.000		**-0.196**	**-0.169**		**-0.221**		**0.206**
ng 16S						1.000	**-0.143**	**-0.156**		**-0.209**		
bmin							1.000	**0.687**	**0.423**	**0.600**		
nmin								1.000	**0.372**	**0.670**		
pmin									1.000	**0.311**		
tmin										1.000		**-0.123**
TVC											1.000	
ben												1.000
dec												
eben												
naph												
pxyl												
tol												
days												
DO												
elev												
pH												
temp												
well												

[a] Values are significant at $\alpha < 0.05$.

A.3. Pearson Correlation Matrix for NAT study data set (con't).

	dec	eben	naph	pxyl	tol	days	DO	elev	pH	temp	well
ng *alkB*		**0.240**	**0.240**	**0.175**	**0.123**	**-0.163**					**-0.181**
ng *nahA*	**0.202**	**0.183**	**0.217**			**-0.164**	**-0.143**			**0.313**	**-0.225**
ng *nahH*	**0.170**									**-0.157**	
ng *tod*		**0.308**	**0.369**	**0.125**	**0.131**	**0.191**				**0.292**	**-0.142**
ng *xylA*		**0.242**	**0.278**	**0.134**	**0.143**	**0.314**				**0.237**	**-0.271**
ng 16S						**-0.322**					
bmin			**-0.124**			**-0.275**			**0.125**		
nmin						**-0.273**	**-0.217**		**0.148**		
pmin						**-0.298**		**-0.183**	**0.155**	**-0.137**	
tmin						**-0.264**					
TVC								**0.188**		**0.165**	**0.313**
ben		**0.944**	**0.879**	**0.852**	**0.821**	**0.206**	**-0.199**		**0.168**		**-0.436**
dec	1.000					**-0.143**					
eben		1.000	**0.943**	**0.837**	**0.804**	**0.147**	**-0.169**		**0.157**		**-0.403**
naph			1.000	**0.716**	**0.690**	**0.138**	**-0.196**		**0.147**	**0.156**	**-0.389**
pxyl				1.000	**0.967**	**0.144**	**-0.156**		**0.146**		**-0.358**
tol					1.000	**0.156**	**-0.124**		**0.135**		**-0.334**
days						1.000			**-0.256**	**0.235**	**-0.126**
DO							1.000	**-0.268**	**-0.372**	**-0.225**	**0.174**
elev								1.000			
pH									1.000		
temp										1.000	
well											1.000

A.4. Partial Correlation matrix controlling for days[a].

	ng *alkB*	ng *nahA*	ng *nahH*	ng *tod*	ng *xylA*	ng16s	bmin	nmin	pmin	tmin	TVC
ng *alkB*	1	0.398	0.257	0.378	0.463	0.166	-0.156	-0.129		-0.192	
ng *nahA*		1	0.133	0.315	0.39	0.45	-0.171			-0.182	
ng *nahH*			1	0.333	0.159		-0.156	-0.1854			
ng *tod*				1	0.451	0.313			-0.133	-0.296	
ng *xylA*					1	0.17				-0.151	
ng16S						1	-0.255	-0.268		-0.322	
bmin							1	0.662	0.372	0.567	
nmin								1	0.317	0.645	
pmin									1	0.252	
tmin										1	
TVC											1
ben											
dec											
eben											
naph											
pxyl											
tol											
DO											
elev											
pH											
temp											

[a] Values are significant at $\alpha < 0.05$.

A.4. Partial Correlation matrix controlling for days (con't).

	ben	dec	eben	naph	pxyl	tol	DO	elev	pH	temp
ng *alkB*	0.228		0.27	0.269	0.203	0.152				
ng *nahA*	0.188	0.183	0.212	0.246	0.125	0.13	-0.136			0.366
ng *nahH*		0.164							-0.123	-0.149
ng *tod*	0.169		0.288	0.353						0.259
ng *xylA*	0.152		0.209	0.25						0.177
ng16S										0.192
bmin										
nmin							-0.209			
pmin								-0.187		
tmin										
TVC								0.19		0.193
ben	1		0.944	0.878	0.849	0.817	-0.216		0.234	
dec		1								
eben			1	0.942	0.834	0.799	-0.18		0.204	
naph				1	0.71	0.683	-0.207		0.191	0.129
pxyl					1	0.966	-0.166		0.191	
tol						1	-0.135		0.183	
DO							1	-269	-0.37	-0.246
elev								1		
pH									1	
temp										1

A.5. Partial Correlation matrix controlling for wells[a] .

	ng *alkB*	ng *nahA*	ng *nahH*	ng *tod*	ng *xylA*	ng16s	bmin	nmin	pmin	tmin	TVC	
ng *alkB*	**1**	**0.389**	**0.267**	**0.318**	**0.352**	**0.190**				**-0.135**		
ng *nahA*		**1**	**0.145**	**0.251**	**0.27**	**0.461**				**-0.124**		**0.159**
ng *nahH*			**1**	**0.321**	**0.142**				**0.124**			
ng *tod*				**1**	**0.464**	**0.216**	**-0.2**	**-0.246**	**-0.202**	**-0.328**		
ng *xylA*					**1**		**-0.201**	**-0.207**	**-0.137**	**-0.218**		
ng16S						**1**	**-0.143**	**-0.171**		**-0.206**		
bmin							**1**	**0.692**	**0.427**	**0.600**		
nmin								**1**	**0.365**	**0.679**		
pmin									**1**	**0.318**		
tmin										**1**		
TVC											**1**	
ben												
dec												
eben												
naph												
pxyl												
tol												
DO												
pH												
temp												

[a] Values are significant at $\alpha < 0.05$.

A.5. Partial Correlation matrix controlling for wells (con't).

	ben	dec	eben	naph	pxyl	tol	DO	pH	temp
ng *alkB*	**0.121**		**0.185**	**0.188**	**0.12**				
ng *nahA*		**0.192**		**0.145**					**0.319**
ng *nahH*		**0.171**							**-0.157**
ng *tod*	**-0.202**		**0.277**	**0.344**					**0.294**
ng *xylA*			**0.151**	**0.195**					**0.245**
ng16S									
bmin				**-0.129**				**0.125**	
nmin	**-0.135**		**-0.139**			**-0.135**	**-0.203**	**0.148**	
pmin	**-0.128**			**-0.121**				**0.156**	
tmin									
TVC									**0.176**
ben	**1**		**0.933**	**0.856**	**0.828**	**0.797**	**-0.139**	**0.1844**	
dec		**1**							
eben			**1**	**0.932**	**0.811**	**0.776**		**0.17**	
naph				**1**	**0.67**	**0.645**	**-0.142**	**0.158**	
pxyl					**1**	**0.963**		**0.154**	
tol						**1**		**0.023**	
DO							**1**	**-0.377**	**-0.227**
pH								**1**	
temp									**1**

A.6. Partial correlation matrix controlling for elevation[a].

	ng *alkB*	ng *nahA*	ng *nahH*	ng *tod*	ng *xylA*	ng16s	bmin	nmin	pmin	tmin	TVC
ng *alkB*	**1**	**0.412**	**0.26**	**0.335**	**0.3822**	**0.212**					
ng *nahA*		**1**	**0.138**	**0.274**	**0.314**	**0.476**				**-0.128**	
ng *nahH*			**1**	**0.317**	**0.134**				**0.13**		
ng *tod*				**1**	**0.48**	**0.23**	**-0.199**	**-0.227**	**-0.184**	**-0.331**	
ng *xylA*					**1**		**-0.196**	**-0.17**		**-0.221**	
ng16S						**1**	**-0.143**	**-0.156**		**-0.212**	
bmin							**1**	**0.687**	**0.433**	**0.601**	
nmin								**1**	**0.381**	**0.671**	
pmin									**1**	**-0.149**	
tmin										**1**	
TVC											**1**
ben											
dec											
eben											
naph											
pxyl											
tol											
DO											
pH											
temp											

[a] Values are significant at $\alpha < 0.05$.

A.6. Partial correlation matrix controlling for elevation (con't).

	ben	dec	eben	naph	pxyl	tol	DO	pH	temp
ng *alkB*	**0.181**		**0.236**	**0.235**	**0.172**	**0.121**	**-0.137**		
ng *nahA*	**0.145**	**0.199**	**0.18**	**0.214**					**0.316**
ng *nahH*		**0.167**							**-0.155**
ng *tod*	**0.202**		**0.309**	**0.37**	**0.125**	**0.131**			**0.292**
ng *xylA*	**0.205**		**0.242**	**0.278**	**0.133**	**0.143**			**0.238**
ng16S									
bmin				**-0.126**				**0.125**	
nmin							**-0.222**	**0.148**	
pmin								**0.164**	**-0.149**
tmin	**-0.12**								
TVC									**0.177**
ben	**1**		**0.944**	**0.879**	**0.852**	**0.822**	**-0.185**	**0.166**	
dec		**1**							
eben			**1**	**0.943**	**0.837**	**0.804**	**-0.157**	**0.155**	
naph				**1**	**0.716**	**0.67**	**-0.178**	**0.145**	**0.162**
pxyl					**1**	**0.967**	**-0.15**	**0.144**	
tol						**1**		**0.134**	
DO							**1**	**-0.376**	**-0.248**
pH								**1**	
temp									**1**

VITA

Raymond D. Stapleton, Jr. was born in Lynchburg, Virginia on February 24, 1970. He moved to Staunton, Virginia at the age of two. In Staunton, he attended public schools and graduated from Robert E. Lee High School in June, 1988. August 1988, he enrolled at Mary Washington College in Fredricksburg, Virginia, where in May 1992, he received a Bachelor of Science in Biology. For six semesters, he worked as a laboratory technician and performed an independent study research project in the laboratory of Dr. Rosemary Barra, who was an outstanding person and teacher within the scienctific community. While at Mary Washington College, he was the starting shortstop on the men's varsity baseball team for four years, earning several accolades including being named to the NCAA All-South Region team. After working for a year doing neuroscience research at the University of Virginia, he entered the doctoral program in the Department of Ecology and Environmental Biology at the University of Tennessee, Knoxville. While at The University of Tennessee, Knoxville, he worked at the Center for Environmental Biotechnology under the tutelage of Dr. Gary Sayler. The doctoral degree was officially received December 1997. During his stay at The University of Tennessee, Knoxville, he met his wife, the former Wendy Michelle Bradberry, and they were married June 21, 1997.