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In Situ and On-Site Biodegradation of Refined and Fuel Oils:

A Review of Technical Literature

1988 - 1991

American
Petroleum
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PREPARED BY:

RONALD J. BAKER AND ARTHUR L. BAEHR
DREXEL UNIVERSITY
PHILADELPHIA, PENNSYLVANIA

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API STAFF CONTACTS

Bruce Bauman, Ph.D., Health and Environmental Sciences Department

MEMBERS OF THE GW-28 PROJECT TEAM
SOIL & GROUNDWATER TASK FORCE

Timothy E. Buscheck, Chevron Research & Technology Company
Cindi Cozens-Roberts, Amoco Research Center
Stephanie Fiorenza, Amoco Production Company
Robert L. Huddleston, Conoco Inc.
Patrick C. Madden, Exxon Research & Engineering Company
Sara McMillen, Exxon Production Research Company
Kirk O'Reilly, Chevron Research & Technology Company
Joseph P. Salanitro, Ph.D., Shell Development Company

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1
INTRODUCTION

1.1 Background and Organization

This literature review covers research activity published between 1988 and 1991 in the area of on-site and *in situ* bioremediation of petroleum hydrocarbons. It is intended to be a suplement to a previous comprehensive review of the literature up to 1988 (**Riser-Roberts, 1992**), published by both the U.S. Navy and subsequently in book format by a commercial publisher. That report was intended to serve as a reference base for the utilization of bioremediation at Navy sites. It is a detailed review of the following topics: composition of petroleum products, basic microbiology, microbial transformation of hydrocarbons, and the enhancement of biodegradation rates. This API report will describe limited relevant background material as necessary to orient the reader, but will emphasize the findings of recent literature published since the completion of the Navy's review.

The focus of this report is on current field and laboratory research related to petroleum hydrocarbon biodegradation. Although the subject under review is lubricating and fuel-oil biodegradation, to restrict the discussion to these product types would be unnecessarily limiting. Much research related to biodegradation of crude oil and solvents is directly applicable to the subject at hand. Similarly, in addition to reviewing all available literature describing on-site and *in situ* petroleum product biodegradation, a body of literature addressing microbial activity in environments contaminated by petroleum hydrocarbons has been reviewed.

This literature review is divided into seven chapters. Chapter 1 introduces the subjects of on-site and *in situ* bioremediation, and describes recently published literature reviews on subjects related to petroleum hydrocarbon biodegradation. Chapter 2 focuses on the microorganisms involved in petroleum hydrocarbon biodegradation. Chapter 3 reviews literature on naturally occurring biodegradation of petroleum products. Chapters 4 and 5 describe on-site and *in situ* bioremediation, respectively. Chapter 6 presents recent work in fate and transport modeling that can be applied to petroleum hydrocarbon contamination in groundwater. Chapter 7 offers some conclusions and recommendations.

Ideally, each article and book reviewed would be described contiguously in the most appropriate chapter and subchapter, and each chapter and subchapter would contain only literature consistent with its title. However, many articles include information about more than one subject, e.g., *in situ* and on-site biodegradation, or aerobic and anaerobic degradation. Therefore, priorities of organization had to be established. It was decided that articles would be reviewed and discussed in their entirety in the most appropriate place, at the expense of having some material not presented in other relevant chapters.

1.2 On-site vs. *In Situ* Bioremediation

The principal difference between on-site and *in situ* treatment is that *in situ* bioremediation does not involve disturbing the site by soil excavation or removing water from aquifers for treatment. Water recycling schemes fall under the *in situ* definition. **Molnaa and Grubbs (1989)** cite advantages of on-site remediation as better control of environmental conditions (oxygen, temperature, moisture, etc.), better control of treatment substance application, and shorter treatment time requirements. **Figure 1** is a conceptual view showing biodegradation zones in a subsurface contamination site. Degradation may occur in the vadose zone overlying a contaminant source, at the water table, and/or in the saturated zone. In any case, biodegradation can only take place in the aqueous phase, since that is the only phase which supports microbial growth. Hence, in the unsaturated zone, biodegradation takes place in the interstitial pore water. Aerobic biodegradation may occur in the portion of the unsaturated and saturated zones having sufficient molecular oxygen. If an anoxic zone is present, a variety of electron acceptors may be involved in biodegradation, including nitrate, ferric iron, sulfate and carbon dioxide. Methanogenesis may occur in the anoxic zone in the absence of oxygen-containing electron acceptors. *In situ* remediation involves the enhancement of any of these biodegradation processes in the unsaturated or saturated zone without excavating the contaminated material or removing the water. Advantages of *in situ* bioremediation include generally lower cost, since excavation is not required and space requirements are less. **Molnaa and Grubbs (1989)** divide biological treatment into two categories, depending upon the origin of the microbes used: in biostimulation indigenous organisms are used, and in bioaugmentation previously acclimated or bioengineered organisms are introduced at the site. *In situ* bioremediation is relatively new. The

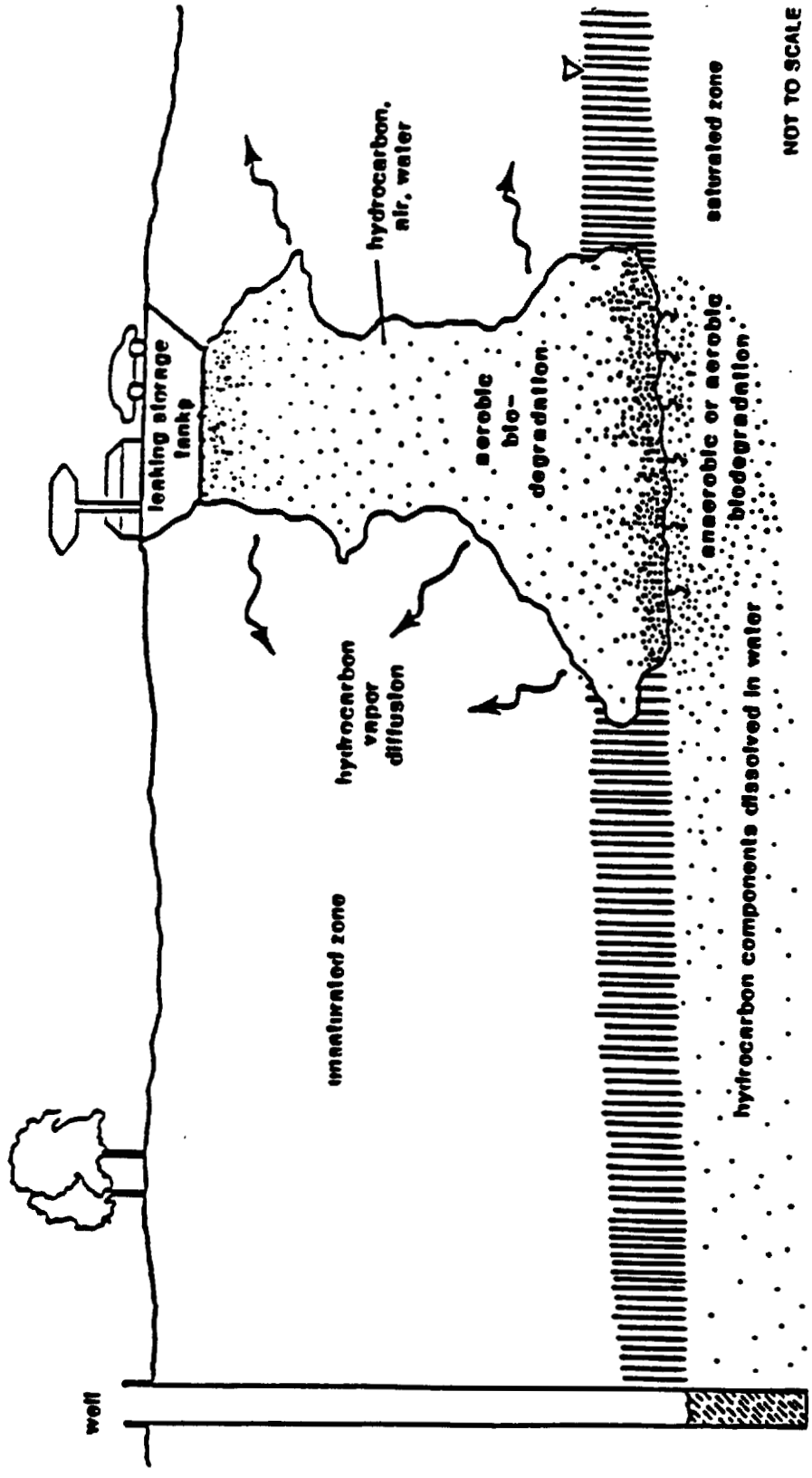

Figure 1. Transport and Biodegradation of Spilled Petroleum Hydrocarbons in the Subsurface under Natural Conditions

technology initially gained wide recognition from published reports of bioremediation of an underground gasoline spill in Southeastern Pennsylvania **(Raymond *et al.* 1975)**. Several proprietary *in situ* processes have since been patented (e.g. **Raymond, 1974; Jhaveri *et al.* 1983)**.

1.3 Related Literature Reviews

Several reviews of hydrocarbon biodegradation literature have been published since 1987. A literature review of biorestoration of aquifers contaminated with organic compounds was published by **Lee *et al.* (1988)**, and covered all aspects of microbially-mediated remediation of environmental contaminants. Their literature reviewed is divided into three sections: *in situ* remediation, withdrawal and treatment, and hydrological considerations and mathematically modeling. **Battersby (1990)** reviewed the literature related to biodegradation kinetics in the aquatic environment. Rate expressions are described, and relevant literature is used to show how to choose the most appropriate kinetic model for a set of biodegradation data. Biodegradation in soil is also reviewed. **Alexander and Scow (1989)** reviewed the subject of biodegradation kinetics in soil, using a textbook-style presentation. Kinetic models are developed for growing and nongrowing organisms, and for Monod and first-order kinetics. Diffusion and adsorption effects are covered, and the special case of fungal metabolism kinetics is described.

Thomas and Ward (1989) discussed *in situ* biorestoration of organic contaminants in the subsurface as part of a five-article series on remedial actions and technologies. Other articles in the series dealt with field instrumentation for assessing hazardous waste sites; advantages and limitations of pump-and-treat technology; technologies for treating aqueous streams, sludges and solids; and waste minimization. The article by **Thomas and Ward (1989)** discusses the need for subsurface characterization prior to implementing *in situ* bioremediation, and the site-specific nature of the technology. Examples of pilot-scale and field investigations are presented, including the use of endogenous and applied microorganisms.

A summary description and evaluation of 13 remedial methods for soil and groundwater cleanup was prepared by **Preslo *et al.*, (1989)** for the electric utility industry. The review is

divided into two main sections, covering *in situ* technologies and non-*in situ* technologies. In addition to physical and chemical remediation technology, biodegradation (*in situ*, land treatment and bioreactor technologies) are described. Economic and environmental feasibility are considered for each remediation method.

Leahy and Colwell (1991) reviewed literature on microbial degradation of hydrocarbons in the environment. Physical and chemical characteristics of petroleum hydrocarbon molecules which control biodegradation rates were discussed. The physical state (separate phase product, emulsion or dissolved) and concentration are of primary importance in determining degradation rates. The effects of temperature, oxygen concentration, nutrients, salinity, pressure and pH are discussed. The microbial species shown to degrade hydrocarbons are reviewed. Bacteria are thought to be much more important than fungi in marine hydrocarbon degradation, but the relative importance of these two groups in soil and freshwater hydrocarbon biodegradation is not yet known. Literature on microbial adaptation and microbe seeding to increase degradation rates was reviewed.

A general discussion of remediation options for hydrocarbon-contaminated groundwater was presented by **Thomas and Stover (1989)**. Air stripping, steam stripping, activated carbon adsorption, biodegradation, membrane processes, electrodialysis and ion exchange processes are discussed. Conditions under which each process is potentially suitable are presented. **Thayer (1991)** wrote a general discussion of bioremediation. He described the regulatory climate, which is the driving force behind most contaminant remediation. He divided bioremediation into three broad categories: land treatment, bioreactors, and *in situ* treatment. Each is described, and examples are given. **Barker and Mayfield (1988)** divided their descriptive review of aromatic hydrocarbon biodegradation into four categories, depending upon the characteristic oxidant used (O_2, NO_3^-, SO_4^{2-} or CO_2). Biological processes using each of these oxidants are described, and examples of aromatic biodegradation are given. They cited degradation rates from recent literature and their own work. They concluded that monoaromatic hydrocarbons can be biodegraded in all groundwater environments. **Dragun (1988)** wrote a general discussion of petroleum-degrading microbial populations in soil, and described how degradation is effected by

soil factors and chemical structure of contaminant components. Genera of hydrocarbon-degrading bacteria and fungi are listed. Microbial transformation reactions are tabulated. Biodegradable organic molecular fractions (e.g. aldehydes, esters, etc.) are also listed. The author points out the tools for predicting biodegradation rates are absent or primitive, and this will be an active research area in the future. **Dragun (1989)** presented an overview of recovery and treatment technologies for petroleum products in soil and groundwater. Natural degradation, land treatment, composting and *in situ* biodegradation were the microbiological technologies discussed. **Bauman (1989)** stated current issues in management of motor fuel contaminated sites. Current soil cleanup standards and accuracy problems inherent in currently practiced analytical methods, were reviewed. The relationship between cleanup objectives, cost, and relative risk to human health and the environment is addressed. **Raymond *et al.* (1990)** presented an overview of *in situ* bioremediation of petroleum hydrocarbons in the unsaturated and saturated zones. Case studies are given, and relative costs of remediation options are discussed. **Fournier (1988)** wrote a descriptive history and introduction to *in situ* bioremediation from the perspective of the pulp and paper industry. Essential preliminary site evaluation steps, and commonly practiced remediation strategies are presented.

A review of iron and manganese reducing organisms was published by **Lovely *et al.* (1991)**. A complete discussion of the various electron acceptors known to contribute to degradation of organic matter in the environment is included. Types of organisms involved (Fe and Mn reducers that are fermentative, sulfur-oxidizing, hydrogen-oxidizing, organic acid oxidizing and aromatic compound oxidizing) were reviewed. The effects of anaerobic organisms in mobilizing and immobilizing metals in soil were discussed.

Government agencies, e.g. state or federal transportation departments, are often required to remediate hydrocarbon-contaminated sites in the course of completing highways or other public projects. **Orokunle (1990)** prepared a report for the Georgia Department of Transportation in which state of the art remediation methods for organic contaminated soil are described. Advantages and limitations of excavation and disposal; utilization in asphalt manufacturing; *in situ*

soil washing with surfactant solutions; *in situ* volatization; *in situ* vitrification; and *in situ* biodegradation of contaminated soil were discussed and tabulated.

Mobility and transport of petroleum-derived hydrocarbons was reviewed by **Ptacek and coworkers (1987).** Mechanisms that control the fate of benzene, toluene and xylenes (BTX) and other petroleum hydrocarbons are described. A case study is used to demonstrate retardation of BTX by sorption, and to show that BTX compounds can be mobile in groundwater.

CHAPTER 2
PETROLEUM HYDROCARBON MICROBIOLOGY

2.1 Genetics and Metabolic Pathways

Biodegradation of petroleum hydrocarbons requires specialized, microbially-produced enzymes. Production of these enzymes is genetically controlled, and biodegradation pathways are determined by the genetic makeup of the microorganisms involved. This chapter presents some recent microbiological genetics research and recent information about the organisms responsible for petroleum hydrocarbon biodegradation. The waste treatment industry has utilized microbes for degradation of organic substances for several decades **(Metcalf and Eddy, Inc., 1979)**. Domestic and industrial wastewater treatment plants commonly use aerobic degradation (e.g. activated sludge) and anaerobic degradation (e.g. anaerobic digestion of sludge). Information about the responsible organisms, methods of determining rates of degradation and biomass accumulation, and energy requirements have been worked out. However, *in situ* and on-site biodegradation reaction rates are more difficult to measure, particularly in underground contaminant remediation, and the wide variety of subsurface organisms have not all been identified and characterized. Although progress is being made toward measuring and modeling rates of degradation, this is still a relatively new research area.

Biodegradation of hydrocarbons is a multistep process involving a series of enzymes. Examples of degradation pathways for benzene and toluene are shown in **Figure 2**. Genetic control of degradation metabolism and microbe viability are the two principal areas of concern. The organisms must be equipped to metabolize one or more problem contaminants and be viable in the *in situ* or on-site environment. If engineered microbes are introduced into a natural system, such as an aquifer, it may be desirable to genetically "program" them to be viable for a limited time, to avoid unlimited proliferation of the engineered genetic material into the natural microbial gene pool. As recombinant DNA research is relatively new, there is little material available on using engineered organisms for bioremediation at this time.

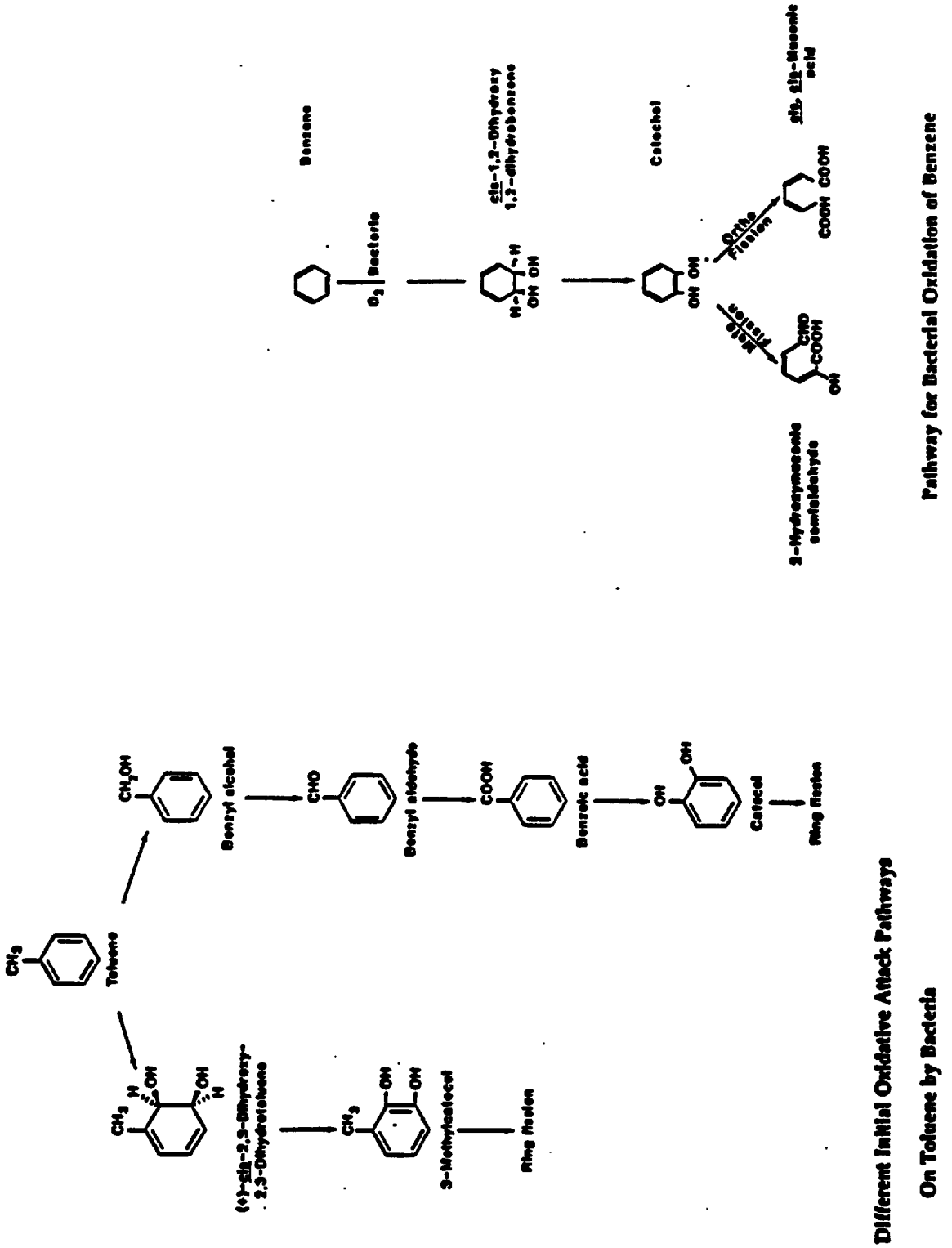

Figure 2. Bacteria Oxidation Pathways for Toluene and Benzene

Burlage *et al.* (1989) reviewed literature pertaining to the TOL (pWWO) catabolic plasmid. Plasmids, as defined by **Crosa and Falkow (1981)**, are autonomously replicating extrachromosomal DNA within bacterial cells. They are not essential to the survival of the organism, but may enable it to adapt to a wider variety of conditions. The TOL plasmid encodes the enzymes that initiate degradation of toluene, *m*- and *p*- xylene and related compounds. This plasmid occurs in *Pseudomonas* and *Alcaligenes* bacteria species. A complete sequence of enzymes and intermediates has been determined since the plasmid was first described by **Williams and Murray (1974)**. In toluene degradation, the methyl group is oxidized to carboxyl, then removed as benzoate is converted to catechol, at which point the ring is broken, and a series of intermediates leads to the formation of pyruvate and acetaldehyde, which are easily metabolizable compounds. The literature review of **Burlage *et al.* (1989)** describes the genetic composition of TOL plasmids, and lists the enzymes involved.

An alternative toluene catabolism pathway was discovered by **Shields *et al.* (1989)**. The strain G4 organism isolated from a waste treatment lagoon (not otherwise identified) can grow on toluene, phenol, and *o*- and *m*-cresol. However, it cannot convert indole to indigo, as can the enzyme toluene dioxygenase. Therefore, a different toluene degrading enzyme system (and pathway) was suspected. A strain of G4 that cannot grow on toluene was generated by mutagenesis. This strain (G4 102) can partially degrade toluene to *o*-cresol, then to 3-methylcatechol. This suggests that for G4, toluene is degraded via hydroxylation at the ortho position, followed by a second hydroxylation at the meta position. This pathway is different from the dioxygenase pathway usually described for toluene degradation, but similar to that often described for phenol and cresol degradation.

Frequency of plasmid DNA occurrence has been correlated with the presence of bioavailable petroleum hydrocarbons **(Day *et al.*, 1988)**, suggesting that plasmid DNA is responsible for production of many or possibly all of the enzymes needed to metabolize petroleum hydrocarbons. **Leahy *et al.* (1990)** found that the microbial population in a marine sediment (Campeche Bank, Gulf of Mexico) relatively free from oil contamination was unable to metabolize petroleum hydrocarbons, and had low incidences of plasmid DNA.

Carney and Leary (1989) found that the bacteria strain *Pseudomonas putida* R5-3 can alter their plasmid DNA content when the sole-source hydrocarbon substrate is changed. When cultures grown on *p*-methylbenzoate were transferred to *m*-xylene or toluene, a 95 kilobase plasmid was replaced by a 50 or 60 kilobase plasmid. Further evaluation showed that a fragment of the original 95 kilobase plasmid had been broken off and integrated into the chromosomal DNA of the cell. This fragment appears to contain DNA homologous to the meta-fission pathway genes, and is probably essential to *m*-xylene and toluene degradation in this organism. When a culture is returned to *p*-methylbenzoate from toluene or *m*-xylene the 95-kilobase plasmid is restored, indicating that the metabolic shift is reversible.

White and Wilson (1989) described quantitative methods for measuring components of bacterial membranes. These analyses give estimates of biomass, community structure, nutritional status and impacts of contaminants. Neutral, glyco- and phospholipids are quantified. Nutritional deprivation results in production of poly beta-hydroxyalkanoate (PHA), and balanced growth in the presence of adequate nutrients results in the formation of phospholipid ester-linked fatty acids (PLFA). The PHA/PFLA ratio indicates the nutritional status of the population.

The addition of inducer compounds to a microbial environment can lead to expression of a metabolic pathway not previously utilized by the indigenous microbes. Once induced, an organism can metabolize a wider range of substrates. Salicylate was added to bacterial cultures in an effort to induce the degradation of naphthalene **(Ogunseitan *et al.*, 1991)**. Salicylate induces the expression of operons for the degradation of naphthalene and related compounds in certain strains of bacteria. Soil samples taken from a PAH-contaminated site in Venice, CA were found to contain several strains of naphthalene-degrading bacteria. Over a 30-day incubation period, endogenous bacteria cultures were given 0, 16 or 160 µg/g salicylate, and some were also inoculated with a naphthalene-degrading strain of *Pseudomonas putida*. In general, 160 µg/g of salicylate stimulated the genetic operon for naphthalene degradation in both endogenous and *P. putida* bacteria, but 16 µg/g salicylate had little or no effect. The authors suggest that inducer compounds could be applied to contaminated soil to stimulate the

biodegradation of specific contaminants as an alternative to introducing engineered or cultured organisms.

2.2 Naturally Occurring Microorganisms

The ability of naturally occurring microorganisms to biodegrade petroleum hydrocarbons is discussed in this section. Types of microorganisms discovered and isolated, microbial counting procedures, and hydrocarbon degrading capabilities of microorganisms are discussed.

Table 1 lists some microorganisms that can degrade petroleum hydrocarbons. In many studies the degrading organisms are not identified, as the researchers are primarily interested in degradation rates. Typically, total cells, viable cells or hydrocarbon degrading organisms are counted, but not taxonomically identified. Where the organisms are identified, *Pseudomonas* species were found most often.

TABLE 1. Hydrocarbon-Degrading Microorganisms

microorganism	substrate	reference
Corynebacterium isolated from Traverse City, MI jet fuel spill	jet fuel hydrocarbons	Francy, *et al.* 1991
Pseudomonas (several naturally occurring species)	diesel-contaminated soil	Brookner, *et al.* 1988
Pseudomonas putida (several strains	alkylbenzenes, benzoates	Carney and Leary, 1989
Pseudomonas putida (several strains, *P. aeruginosa*, *P. fluorescens*, *P. desmolyticum*, *P. rhodochrous*, *P. mildenbergii*, *Aeromonas sp.*, *Moraxella sp.*, *Beijerinckia sp.*, *Flavobacterium sp.*, *Achromobacter sp.*, *Nocardia sp.*, *Corynebacterium renale*	aromatic hydrocarbons	Atlas, 1984 (Table 3.1)

TABLE 1. Hydrocarbon-Degrading Microorganisms (Continued)

microorganism	substrate	reference
Pseudomonas pudita *Moraxella sp.*	benzene	Ribbons and Eaton, 1982 (review article)
P. pudita *Achromobacter sp.* *Nocardia corallina* *P. desmolytica S449B1* *P. convexa S107B1* *P. puyida biotype A, PaWI*	alkylbenzenes	
Archromobacter *Acinetobacter* *Alcaligenes* *Arthrobacter* *Bacillus* *Brevibqcterium* *Chromobacterium* *Corynebacterium* *Cytophaga* *Erwinia* *Flavobacterium* *Micrococcus* *Mycobacterium* *Nocardia* *Proteus* *Pseudomonas* *Sarcina*	hydrocarbons	Dragun, 1988 (review article)
Serratia *Spirillum* *Streptomyces* *Vibrio* *Xanthomonas* 31 fungi genera	hydrocarbons	Dragun, 1988 (review article)
Acinetobacter calcoaceticus RAG-1	hydrocarbons	Fogel *et al.*, 1989
Pseudomonas Stutzeri *P. Diminuta* *Acinetobacter lwoffii* *Nocardia rugosa* *P. cepacia* *Alcaligenes faecalis*	BTX compounds	McLaughlin, 1991

TABLE 1. Hydrocarbon-Degrading Microorganisms (Cont.)

microorganism	substrate	reference
Pseudomonas sp.	alkanes (C_{18}-C_{36})	Miller and Bartha (1989)
Pseudomonas putida *P. fluorescens*	naphthalene	Ogunseitan *et al.* 1991
Acinetobacter calcoaceticus	asphalt	Pendrys 1989
Pseudomonas aeruginosa *P. putida* *P. stutzeri* *P. maltophillia* *P. alcaligines* *Norcardia sp.* *Alcaligenes denitrificans* *Micrococcus*	gasoline	Ridgeway *et al.* 1990
Pseudomonas putida	toluene	Robinson *et al.* 1989

Table 2 provides summary information for 11 laboratory and field studies of BTEX biodegradation. Most studies show fairly rapid degradation of BTEX under aerobic conditions. Results under anaerobic conditions are more variable and may indicate that site-specific conditions may play a styronger role than is true for aerobic reactions.

Table 2. Biodegradation Rates of BTEX Compounds

Ref.	Oxygen	Compound(s)	Degradation Rate	Conditions
1.	aerobic	BTEX mixture	100% degradation in 100 days	microcosms, soil slurry,
	anaerobic	BTEX mixture	0% degradation in 100 days	O_2, H_2O_2, or anaerobic
2.	aerobic	BTX Mixture	100% gone in 78 days	microcosms and field
	anaerobic	BTX Mixture	0% degradation in 78 days	study, 1.1-2.4 mg/L BTX
3.	aerobic	Benzene	10-100mg/L: 2.5-4.5 day halflife	microcosms, 500-50,000
	anaerobic	benzene	no degradation observed	ppb benzene

Table 2. Biodegradation Rates of BTEX Compounds (Continued)

Ref.	Oxygen	Compound(s)	Degradation Rate	Conditions
4.	aerobic	benzene	50% degradation in 48 hours 8% degradation in 22 hours with spill site microbes	microcosms, inoculated Toluene
5.	aerobic	benzene benzene	faster in presence of tol or xy slower in presence of pyrrol	microcosms, 0.1-0.2 mg/L mixed hydrocarbons
6.	anaerobic	BTX mixture	some degradation observed	field study, CO_2, CH_4, organic acids monitored
7.	denitrif.	toluene benzene xylenes	90-100% degraded in 1-3 mo. not degraded not degraded	microcosm, materials from 7 locations with hydrocarbons
8.	denitrif. aerobic	BTX BTX	47-95% degraded in 54 days 35-95% degraded in 54 days	microcosms, denitrifying cond., 13-34mg/L each BTX
9.	denitrif. O_2 added	tol, m-xy tol, m-xy	rapid degradation denitrification, no degradation	column study, NO_3^-, NO_2^-, NO_2 reduction
10.	denitrif. aerobic O_2, NO_3	BTX BTX BTX	80-95% degraded in 62 days 80-99% degraded in 62 days 80-100% degraded in 62 days	microcosms, aerobic and denitrifying
11.	aerobic anoxic	benzene benzene benzene benzene	14.5% degraded in 28 days 47.08% degraded in 28 days 2.43% degraded in 28 days 16.49% degraded in 28 days	microcosms, sandy aridisol microcosms, riparian soil microcosms, sandy aridisol microcosms, riparian soil

References: 1. Payne and Floyd, 1990; 2. Barker and Major, 1987; 3. Kemblowski, 1987; 4. Karlson and Frankenberger, 1989; 5. Arvin *et al.*, 1989; 6. Cozzarelli *et al.*, 1989; 7. Evans, *et al.*, 1991; 8. Gersberg, *et al.*, 1989; 9. Kuhn, *et al.*, 1988; 10. Major, *et al.*, 1988; 11. Watwood *et al.*, 1991.

Metabolic adaptation of a microbial population to a petroleum hydrocarbon substrate is often required for populations not previously exposed to the substrate. **Aamand *et al.* (1989)** determined the adaptation periods, or lag times of bacteria with different degrees of previous exposure to test substrates. They listed four possible explanations for lag times:

1. Time needed for induction of substrate-specific enzymes in individual organisms;
2. Exchange of genetic material mediated by plasmids;
3. Genetic changes leading to new metabolic capabilities; and
4. Growth of the segment of the microbial population already able to utilize the substrate.

Groundwater from contaminated and uncontaminated areas of a leaking fuel tank site (Gassehaven, Denmark), and from the site of a two-year-old gasoline leak were used. An aqueous solution of 2 mg/L each of toluene, *o*-xylene, 1,3,5-trimethylbenzene, naphthalene, 1-methylnaphthalene, biphenyl, 2-ethylnaphthalene, and 1,4-dimethylnaphthalene was used to prepare microcosms.

In Experiment 1 groundwater samples were diluted 1:10 with distilled water in 5.5 liter bottles, which were continuously stirred and kept at 12°C. Lag times and times for complete removal of hydrocarbons was determined for unpolluted, slightly polluted and heavily polluted water samples. Results were as follows:

lag times of individual compounds (days)

	fuel oil			gasoline
compound	**heavily polluted**	**slightly polluted**	**unpolluted**	**heavily polluted**
naphthalene	1.2	1.9	12	1.4
2-ethylnaphthalene	1.3	2.3	16	1.9
biphenyl	1.4	2.4	48	0.6
1-methylnaphthalene	2.0	2.7	14	3.0
toluene	n.d	3.7	56	0.7
o-xylene	2.4	2.4	14	2.0
1,3,5-trimethylbenzene	4.0	9.3	53	6.7
1,4-dimethylnaphthalene	5.9	9.0	44	5.2

Results were similar for a second experiment, where equal numbers of bacteria (as determined by acridine orange direct count) from each area of the site were used, rather than equal volumes of groundwater, as in Experiment 1. It is apparent from the data that greater exposure to the substrate resulted in shorter lag times for nearly all hydrocarbon components. Lag times for the gasoline-exposed populations were similar to those of the fuel oil-exposed populations. This work demonstrated a common finding: that unacclimated populations require longer adaptation periods than previously acclimated populations before biodegradation of petroleum hydrocarbons can proceed.

Lag times were also measured in a sorption and aerobic biodegradation study of benzene, *o*-xylene and trichloroethylene (TCE) in sandy loam soil samples by **English and Loehr (1990)**. The soil was relatively high in organic content (3.25%). 40-mL VOA vials were used as microcosms, and aerobic conditions were maintained. The microcosms were unsaturated, and the 10 g of soil contained about 16% moisture by mass. Lag times of about one week were required before benzene biodegradation occurred. *o*-xylene degradation had a lag time of about two weeks. **English and Loehr (1990)** attributed the lag time to microbial adaptation. Zero-order degradation rate constants continued to increase throughout the 16-day study, and the authors believe this is either from further metabolic adaptation of individual organisms or from growth in the hydrocarbon degrading population. No aerobic TCE degradation was observed over 7 weeks.

Garcia-Valdes *et al.* (1988) isolated over 100 strains of bacteria, mostly belonging the genus *Pseudomonas*, that are able to utilize naphthalene as the sole carbon and energy source. Isolates were derived from Mediterranean Sea sediment near Barcelona, Spain. The area has a significant history of aromatic hydrocarbon contamination. The microbes isolated differed metabolically and morphologically from previously described members of the *alcaligenes* or *pseudomonas* groups found in fresh water. This suggests that, at least for the site studied, marine hydrocarbon degraders are not closely related to aquifer hydrocarbon degraders.

Ridgeway *et al.* (1990) isolated 309 gasoline-degrading bacteria from well water and soil core material from a site contaminated by unleaded gasoline. The source of the contamination was a leaking underground storage tank in a shallow coastal aquifer in Southern California. Bacterial isolates were obtained by spread-plating dilutions of groundwater and soil samples on mineral media agar, and providing gasoline vapor as the carbon source. Isolated were then tested for the ability to grow aerobically on each of 15 gasoline hydrocarbons. Isolates that had the ability to grow on the same hydrocarbon(s) were assigned to the same catabolic group, and 111 catabolic groups made up the 297 isolates analyzed. Substituted monoaromatics were favored as growth substrates by most isolates. All 15 hydrocarbons treated were degraded by at least one isolate. In decreasing order of frequency, most isolates degraded 3, 2, 5, or 4 different hydrocarbons. A few isolates degraded only one hydrocarbon. Even fewer degraded more than eight. The hydrocarbons most often degraded in decreasing order were toluene, *p*-xylene, ethylbenzene, trimethylbenzene and benzene. Alkanes, cycloalkanes and alkenes were degraded by fewer catabolic groups. Many of the isolates were identified, and most common bacteria identified were *Pseudomonas aeruginosa*, *Pseudomonas Putida*, and *Micrococcus*. This study shows that there is metabolic and genetic heterogeneity among hydrocarbon-degrading bacteria present in subsurface petroleum-product spills, and many different organisms may be required to completely degrade a complex hydrocarbon mixture such as gasoline.

The stratification of anoxic BTEX-degrading bacteria was determined at three petroleum-contaminated sites by **Mikesell *et al.* (1991)**. Total colony forming units (CFU) were counted under three conditions: aerobic with organic substrate (tryptone, dextrose and yeast extract), anaerobic with BTEX vapors, and aerobic with BTEX vapors. Rates of BTEX compound disappearance were monitored in saturated anaerobic microcosms prepared with supernatant from soil slurries. Nitrate reductase (an enzyme involved in hydrocarbon biodegradation via a denitrification pathway) was also measured in microcosm water. This work was done with pristine and BTEX-contaminated soil from each site, and at four to six different depths at each site. Natural site remediation via denitrification was evidenced by correlations between high TOC, low DO and low nitrate values in contaminated areas as compared to pristine areas at each site.

A nonparametric scoring system was devised to compare BTEX degrading potential among microcosms, and losses of BTEX were compared to losses in sterile control microcosms. At each site maximum degradation rates were observed in soil from a zone two or more feet below the water table. At each site high CFU counts were associated with microcosms having the greatest rates of BTEX degradation. In two of the three sites, denitrification potential was associated with the depths having high numbers of anoxic BTEX degraders. Aerobic CFU (total and BTEX degraders) were evenly distributed with depth at all three sites, ranging from 4×10^5 to 3×10^6 CFU/mL enrichment culture, and were not correlated with BTEX degradation rates.

Dean-Ross (1989) counted the viable and total bacteria in fill material at various depths up to 20 feet at a hazardous waste land fill. The site had received construction debris, oils and solvents. Toluene was detected at three of five well locations at concentrations up to 80,000 ppm. Total viable bacteria counts were higher on-site than in the off-site control location. Numbers were generally 10^6-10^8 CFU. However, within the contaminated area exposure to a particular contaminant was not associated with an increased populations able to degrade that contaminant. The two locations having phenol as a contaminant had low populations of phenol degraders, and one location which had no phenol had high populations of phenol degraders. It is not clear whether this observation is a reflection of the inaccuracy associated with currently practiced specific hydrocarbon degrader counting methods, or if it truly shows an anomaly at this site (i.e. that exposure to phenol did not result in increased populations of phenol-degrading microorganisms). This study illustrates the need to develop more definitive tests for counting specific-substance-degrading organisms.

Frederickson *et al.* (1991) isolated a deep-subsurface organism that can grow on toluene, naphthalene, *p*-cresol and all isomers of xylene. The bacteria, designated F199, was found in core samples from the Department of Energy (DOE) Savannah River Site (Allendale, S.C.). F199 is aerobic, gram-positive, catalase-positive, and oxidase-negative. Naphthalene and toluene degradation were inducible, and naphthalene mineralization was induced by the presence of toluene. However, toluene mineralization was not induced by the presence of naphthalene. Taxonomic identification of the organism has not been successful, but the author believes it

belongs to the CMN (*Corynebacterium, Mycobacteria, Nocardia* and *Rhodococcus*) grouping. Although aerobic, F199 requires microaerobic conditions to grow on aromatics. The isolation of this organism demonstrates that there are indigenous deep-subsurface bacteria capable of degrading petroleum product hydrocarbons.

Jensen (1989) noted that the two approaches used in most biodegradation studies to date consider either bacterial abundance and characterization, or biological removal of specific organic substrates. Few studies have attempted to relate biodegradation of organics to active biomass, and this information is valuable for fate modeling in which microbial degradation is considered. Jensen evaluated the relationships between bacterial numbers and microbial degradation of hydrocarbons in petroleum-contaminated and uncontaminated groundwater. Well water samples from a gasoline contamination-site, a fuel oil site and a site near several petroleum installations were used. Acridine orange direct counts and plate counts using PTYG agar (peptone, tryptose, yeast extract and glucose dissolved in agar) were made. ATP (adenosine triphosphate, a biological energy-yielding compound) was quantified in groundwater samples, and heterotrophic activity (ability of microbial populations to grow on various substrates) was measured individually for acetate, glucose and naphthalene. Heterotrophic bacteria production was estimated by measuring thymidine incorporation into biomass.

Acridine orange counts ranged from 0.3×10^7 to 1.6×10^7 cells/mL, and were about three orders of magnitude greater than plate counts. ATP concentrations varied from 0.27 to 2.48 ng/mL. Using the ratio of 16-6 ng ATP/cell derived from marine and soil studies, ATP measurements provide biomass estimates intermediate between acridine orange counts and plate counts. For naphthalene, correlation coefficients between maximum naphthalene utilization rate and four biomass estimation methods were:

ATP measurement: r=0.95
Acridine orange: r=-0.52
Full-strength PTYG agar plate count: r=0.99
1:10 diluted PTYG agar plate count: r=0.96

These results indicated that acridine orange direct count is a poor predictor of this population's ability to degrade naphthalene, and both plate-counting techniques and ATP measurement correlate well with naphthalene degradation rates. Naphthalene utilization rates were determined in 12-hour incubation studies. Utilization was only observed in samples from contaminated sites, indicating that microbes at those sites and not at uncontaminated sites have metabolisms adapted to naphthalene utilization. Note that a 12-hour incubation period is insufficient for previously unexposed organisms to adapt their metabolisms for naphthalene degradation.

Three microbial quantification methods were compared during *in situ* bioremediation of a small gasoline spill **(Litchfield *et al.*, 1988)**. At four observation wells near the spill, acridine orange direct count (AODC), nutrient agar plating and ATP concentrations were monitored during the bioremediation effort, which involved adding nutrients and hydrogen peroxide to the groundwater. Only the plating technique appeared to reflect the microbial population of the groundwater. ATP was not found to be a good predictor of microbial biomass. AODC did not correlate with any plate count or ATP data. The authors concluded that of the three methods evaluated, the plate count technique is probably the best indicator of biological activity during *in situ* bioreclamation of a petroleum hydrocarbon spill.

Madsen *et al.* (1991) studied microbial mineralization of naphthalene and phenanthrene in laboratory microcosms. Sediment samples were taken from a coal tar burial site and adjacent pristine area. Viable counts of organisms were consistently higher closest to the contaminant source at the site. Microscopic counts of total bacteria showed declining numbers with depth, but little difference between contaminated and pristine samples. Actinomycetes were found in two-thirds of the unsaturated and water table samples, but not in saturated samples. Low numbers of fungi were found throughout the site, and were not associated with depth or PAH contamination. High numbers of protozoa were associated with high levels of PAH mineralization, implying that the protozoa were feeding on the bacteria.

Song and Bartha (1990) investigated microbial numbers and activity in soil contaminated by a jet fuel spill. In laboratory experiments, samples of loam taken from a jet fuel spill (Bayway Refinery, N.J., Exxon USA) were exposed to jet fuel. Two hydrocarbon concentration levels (50 mg/g soil and 135 mg/g soil) were used, and all work was done at 27°C. Aerobic surface and oxygen-limited (but not anaerobic) subsurface conditions were used. Direct counts of total viable bacteria, viable counts of aerobic heterotrophs and counts of hydrocarbon-utilizing microorganisms were made periodically over the 16-week experiment.

TABLE 3. Comparison of Results for Three Methods of Quantifying Microbes from Soil Samples Taken from a Gasoline Spill Site over 16 Weeks

DIRECT COUNT RESULTS (g SOIL^{-1})

Surface:
no jet fuel: no trend, 1 x 10^8 to 5 x 18^8 (week 16)
with jet fuel: 1 x 10^8 to 1 x 10^{10} (week 4) to 8 x 10^8 (week 16)

Subsurface:
no jet fuel: 1 x 10^8 to 5 x 10^8 (week 1), stayed constant
with jet fuel: 1 x 10^8 to 5 x 10^8 (week 1), stayed constant

AEROBIC HETEROTROPH COUNT RESULTS (g SOIL^{-1})

Surface:
no jet fuel: 3 x 10^8 to 2.2 x 10^7 (week 4), stayed constant
50 mg jet fuel g soil^{-1}: 3 x 10^8 to 8 x 10^9 (week 4) to 7 x 10^7 (week 16)
135 mg jet fuel g soil^{-1}: 3 x 10^8 to 8 x 10^9 (week 4) to 8 x 10^7 (week 16)

Subsurface:
no jet fuel: 3 x 10^8 to 2.2 x 10^7 (week 4), stayed constant
50 mg jet fuel g soil^{-1}: 3 x 10^8 to 1 x 10^8 (week 1) to 3 x 10^8 (week 2), slight decrease thereafter
135 mg jet fuel g soil^{-1}: 3 x 10^8 to 8 x 10^9 (week 4) to 3 x 10^8 (week 16)

HYDROCARBON DEGRADER COUNT RESULTS (g SOIL^{-1})

Surface:
no jet fuel: 3 x 10^8 to 2.2 x 10^7 (week 4), stayed constant
50 mg jet fuel g soil^{-1}: 3 x 10^8 to 8 x 10^9 (week 4) to 7 x 10^7 (week 16)
135 mg jet fuel g soil^{-1}: 3 x 10^8 to 8 x 10^9 (week 4) to 8 x 10^7 (week 16)

Subsurface:
no jet fuel: 4 x 10^4 to 10^5 (week 4) to 5 x 10^3 (week 16)
50 mg jet fuel g soil^{-1}: 4 x 10^4 to 3 x 10^8 (week 2) to 3 x 10^7 (week 16)
135 mg jet fuel g soil^{-1}: 4 x 10^4 to 3 x 10^8 (week 2) to 7 x 10^9 (week 4) to 3 x 10^7 (week 16).

Results (Table 3) showed that, under experimental conditions used, microbial counts increased when jet fuel was applied to the soil, no matter which of the three counting methods was used. Most of the increase in numbers appears to be viable hydrocarbon degraders. Numbers tended to decrease after reaching a maximum in about 4 weeks. Sub-surface samples showed less significant increases in microbial numbers than surface samples. Length of fungal hyphae followed trends similar to those observed in bacterial number. Close agreement between direct count and total viable count was achieved by using fluorescein diacetate stain, which only stains viable cells, prior to epifluorescence microscopic direct counting. Microbial counts were generally higher with bioremediation (liming to pH 7.5 and adding ammonium nitrate and dipotassium phosphate).

Payne and Floyd (1990) evaluated the potential of indigenous microbes to degrade petroleum and chlorinated hydrocarbons at an abandoned waste site in a U.S. Air Force base (undisclosed location). Acridine orange direct count indicated 7.6×10^6 to 1.68×10^8 cells/g wet weight, and viable cell count ranged from 1.0×10^2 to 7.1×10^6 CFU/g. Soil samples were taken from six borings adjacent to boreholes where high concentrations of chlorinated and nonchlorinated hydrocarbons had been found.

Aerobic and anaerobic microcosms were run using a 200 mL of 10% soil slurry of groundwater from the site and soil. Aerobic microcosms were oxygenated with either pure oxygen or H_2O_2. Additions of 100 mg/L H_2O_2 were added on day 0, day 20, and day 40. Essential mineral nutrients were supplied. Aerobic microcosms were sacrificed on days 1, 24, 49 and 100. Anaerobic microcosms were run identically, but without an O_2 source, and 500 mg/L was provided as a primary substrate, and 500 mg/L sodium sulfate was added as an O_2 scavenger. Anaerobic microcosms were sacrificed on days 1, 25, 50 and 100.

Aromatic, aliphatic and polar compounds were among the species analyzed by a series of extractions using solvent extraction and GC/FID analysis. Nearly all aromatics and aliphatics were completely removed by day 100 in O_2 and H_2O_2 microcosms. No decrease in hydrocarbons was observed in anaerobic microcosms.

Murray *et al.* (1989) determined, from their work and other published results, that *in situ* denitrification rate is more highly dependent upon a carbon source and oxygen concentration than nitrate concentration. This is especially true of mixed denitrifyer cultures.

2.3 Cultured Isolates of Microorganisms

Microorganisms can be artificially cultured and selectively adapted to metabolize contaminants. Once the microbial population is acclimated to a particular substrate, e.g. BTEX compounds, it can be used for on-site or *in situ* bioremediation. However, the ability of an organism to degrade the contaminant of concern does not ensure its ability to survive in the environment being treated, be it *in situ* in soil or water or an on-site reactor. Such factors as limited microbe mobility, sensitivity to the chemical environment or pH, predation from other organisms, substrate being in the wrong form (e.g. separate phase or adsorbed, not dissolved), and temperature fluctuations can render an introduced organism nonviable.

Limited solubility of petroleum hydrocarbons can be rate-limiting for hydrocarbon biodegradation. Emulsification of hydrocarbons by microbiological excretions can enhance bioavailability of hydrocarbons and increase biodegradation rates. Microbial biosurfactant production was investigated by **Francy *et al.* (1991)**. Heterotrophic bacteria were isolated from the site of an aviation fuel spill (Traverse City, MI) and from an unleaded gasoline spill site (Seal Beach and San Diego, CA). Biosurfactant production was evaluated for the two groups of isolates. Because the microbes themselves act as surfactants due to the presence of lipophilic and hydrophilic cell wall and cell membrane components, it was necessary to test for cell-free biosurfactants. Therefore, emulsification capacity of cell suspensions and cell-free supernatants were compared. Emulsification capacity was determined visually by vortexing a test tube containing either cell suspensions or cell-free supernatants and an overlying layer of hydrocarbons. After two hours the emulsion quality was assigned a number between 0 and 4, where 0 = complete phase separation, 4 = complete emulsification of the hydrocarbon layer.

Pure cultures were obtained from the jet fuel site by serially diluting and streak-plating aqueous soil extracts. Each isolate is therefore descendent from a single cell and is therefore

a pure culture. Seventy isolates were tested for emulsifying capability: 22, 26 and 28 from biostimulated, uncontaminated, and contaminated but not biostimulated areas, respectively. An additional 19 isolates were obtained from the two California sites.

Generally, uncontaminated areas of the site produced isolates with little emulsifying capacity, contaminated areas yielded isolates evenly distributed along the emulsification range of 0-4, and the distribution of isolates from the biostimulated area was sharply skewed toward high emulsification capability. This suggested that microbial populations exposed to the aviation fuel and/or to biostimulating nutrients can emulsify the aviation fuel. These observations were supported by the results of a Mann Whitney U test, a nonparametric analogue to the t-test.

Lovely and Lonergan (1990) investigate the anaerobic oxidation of toluene and other aromatic hydrocarbons by a pure culture of GS-15, an iron-reducing organism. GS-15 was grown anaerobically in the presence of Fe^{3+} and either toluene, phenol or p-cresol for 7 weeks. Radio-labeled hydrocarbons were used (^{14}C on the ring). Growth of GS-15 coincided with Fe^{3+} reduction when toluene was the sole electron donor, and there was more Fe^{3+} reduction with higher toluene concentration. Complete stoichiometric oxidation of toluene to CO_2 was observed, based on a 36:1 Fe^{2+}:CO_2 production. Magnetite was formed by the reduction of Fe^{3+}, which has also been observed around hydrocarbon seeps, and has been attributed to hydrocarbon bioreduction coupled with Fe^{3+} reduction.

2.4 Bioengineered Microorganisms

Although biotechnology may eventually lead to the development of microorganisms that rapidly degrade petroleum, the use of DNA-recombinant organisms for on-site or *in situ* remediation of petroleum hydrocarbons has not yet appeared in the literature. Even if capable organisms were available today, great caution would be required in their use. If allowed to propagate, genetically engineered organisms can alter the natural wild strains in an ecosystem. Safeguards must be built in to contain such organisms in order to preserve the genetic ecological balance at sites undergoing remediation and beyond.

Contreras *et al.* (1991) investigated a bacterial "suicide system" in which a bacterial strain is genetically programmed to be non-viable except in the presence of a substance termed an "effector compound". The effector disengages the suicide system. The effector would have to be introduced along with the bioengineered microbes in order for the microbes to be viable and degrade hydrocarbons. Their limited life span would depend upon continued application of the effector.

Bej *et al.* (1988) designed a model suicide vector by constructing a plasmid containing the *Hok* gene, which codes for a polypeptide (Hok) which is lethal to the organism. The intent of this type of cell-killing system is to prevent movement of an engineered gene into the indigenous microbial population. The Hok polypeptide causes loss of cell membrane potential, leading to cell death. In this work, *E. coli* was given the *hok* gene, spliced to its *lac* promoter gene. Expression of the *hoc* gene, resulting in the formation of Hok polypeptide, is induced by the inducer IPTG. **Bej *et al.* (1988)** were able to induce this suicide vector system both *in vitro* and in soil microcosms. They did not, however, achieve complete loss of viability upon induction. They also demonstrated that this suicide vector can be overridden by interfering compounds, carbenicillin in this case.

The fate of recombinant microorganisms (GEMs) in the aquatic environment was studied by **Awong *et al.* (1990)**. Microcosms with membrane diffusion chambers was used. Recombinant strains of *Escherichia coli* and *Pseudomonas putida*, and wild-type strains as reference standards, were exposed to environmental variables within the microcosms. The *E. coli* strain was given a gene for mercury resistance and the genes for degradation of 2,4-dichlorophenoxyacetate. The *P. putida* was engineered to have resistance to three antibiotics. Rates of population decline increased as a function of temperature for both *P. putida* and *E. coli*. Rates were similar for wild and GEM *P. putida* at 15°C, but wild-type *E. coli* declined significantly faster than the *E. coli* GEM. At 25°C the wild-type *P. putida* declined faster than the GEM, and rates of decline were similar for the wild and GEM strains of *E. coli*. At 30°C, the wild-types of both species declined faster than the GEMs. For both species, the use of non-sterile lake water had a greater adverse effect on population than sterile lake water. The herbicide Hydrothol-191

was more toxic to the wild strain of *P. putida* than to the GEM. The point of this study was to show that deliberate changes in microbial genetic make-up to achieve a particular purpose can alter an organisms ability to survive in an ecosystem in unexpected and possibly undesirable ways.

2.5 Transport of Microorganisms in Groundwater

Transport of bacteria in groundwater, which has long been a public health concern, is also important in *in situ* biodegradation of organic contaminants. Organisms that are attached to soil can provide a biofiltering effect as groundwater moves through the soil. Conversely, organisms that are transported in the aqueous phase can travel and multiply within a contaminant plume. Some organisms can also move independently from the groundwater flow via their own motility. Relative rates of transport between organisms and contaminants can determine the length of time that contaminants are in contact with the microorganisms. These relative rates of organism, contaminant and water transport strongly effect biodegradation rates of hydrocarbons in groundwater.

Cunningham *et al.* (1988) discussed factors controlling the transport of microorganisms in groundwater. They contrasted biofilms, which are stationary, to mobile, suspended microorganisms. Advection, diffusion (described in this case by the Stokes-Einstein equation), and microbial growth and decay are mentioned as transport mechanisms. The effect of biofilm thickness on aquifer permeability is discussed. Filtration by porous media, which effectively converts suspended microbes into biofilm, is also mentioned.

Harvey *et al.* (1989) measured migration rates of chloride, bromide, fluorescent carboxylated microspheres and fluorescent-labeled bacteria through a sandy aquifer on Cape Cod, MA. Bromide was used as a conservative tracer. A forced-gradient experiment was run by pumping water from a supply well into an injection well. The injection water received pulse additions of bacteria (0.2, 0.7 and 1.2 um diameter), surface-charged microspheres (0.23, 0.53. 0.91 and 1.35 um diameter), uncharged microspheres (0.6 um diameter), and chloride or bromide tracer. Two multi-level samplers 1.7 and 3.2 m downgradient intercepted the injected water for

analysis by specific ion electrode (bromide and chloride) and microscopic counting (microspheres and marked bacteria). Transport was evaluated in terms of maximum concentration, relative breakthrough, attenuation and retardation.

The samples collected from closer to the source showed no significant difference in migration rates of bacteria and bromide, but microspheres moved faster and were more strongly attenuated than either bacteria or bromide. In a natural gradient test, bacteria, microspheres and chloride were injected downgradient of a treated sewage infiltration bed. Microspheres migration rates were a function of size with the largest spheres moving fastest. Uncharged microspheres moved faster than those of similar size with surface charge, and carboxylated surface charge retarded migration to a greater extent than a lesser-charged polyacrolein surface.

The authors believe that the movement of bacteria through porous media can be described chromatographically. Bacteria are excluded from the smaller pores, and therefore have a more direct path than solutes, e.g. bromide. This is analogous to size exclusion chromatography. Micropores appeared to have greater interaction with sediment particles than bacteria cells. The phenomena of retardation and attenuation did not seem to be related. Since bacterial growth (reproduction) would counter the effects of attenuation, growth rate would effect how far indigenous bacteria could be transported. None of the microspheres tested appeared to be useful as bacterial tracers, because their transport behavior differs substantially from that of bacteria.

Transport of genetically engineered microorganisms should be considered when assessing the risk associated with releasing such organisms into the environment for *in situ* bioremediation. **Trevors *et al.* (1990)** studied the transport and survival of a genetically engineered *Pseudomonas fluorescens* C5t through vertical microcosms flushed with water. One loamy sand and one loam were used, and 70 g moist soil was used in each 2.5 cm diameter x 13.4 cm long syringe tube. Large microcosms were made by packing 750 g of soil into plastic columns 5 cm diameter x 34 cm long. Portions (7 g for small microcosms, 75 g for large microcosms) were inoculated with 10^8 viable cells per gram of soil, and packed into the tops of the microcosms. Various flow rates

of water through the columns were achieved by varying water application rates at the tops of the columns. Percolation effluent water was collected, and C5t organisms were counted using an immunofluorescence technique. Significant, but not complete, breakthrough of organisms was seen in both soils regardless of percolation flow rate, but little to no transport was noted in columns not receiving percolation water. Wheat plants were grown on top of some columns, but the presence of plants (and associated roots) did not appear to alter transport rates for C5t cells, except that some minor cell transport in non-percolated columns was associated with the presence of roots. This study suggests that vertical water movement may be necessary to optimize distribution of organisms introduced into the subsurface for *in situ* bioremediation.

CHAPTER 3
NATURALLY OCCURRING BIODEGRADATION OF PETROLEUM PRODUCTS

Petroleum hydrocarbon biodegradation has been observed under a variety of aerobic and anaerobic conditions. This chapter presents recent work in which natural degradation was studied, and the effects of environmental conditions on degradation rates were quantified. Biodegradation rates of hydrocarbons are highly dependent upon solute transport phenomena in subsurface unsaturated and saturated zones. Hydrogeologic conditions (e.g. unconsolidated marine sediment vs. karst topography) effect transport and distribution of hydrocarbons **(Compton 1988)**, and therefore effects biodegradation. In some cases, naturally occurring biodegradation of contaminants may occur at a rapid rate, so that no remediation steps are necessary. In such cases, a no-action scenario (also termed passive remediation) may be the most practical and cost-effective remediation strategy.

Hydrogeological conditions cannot always foretell contaminant migration rates from a site, as observed by **Spruill (1990)** at an abandoned refinery in Arkansas City, Kansas. The soil types at the site are well-drained sandy loams with hydraulic conductivities of 10^{-2} to 10^{-5} cm/sec. However, hydrocarbons and other organic and inorganic contaminants are not moving off-site. Adsorption of contaminants by naturally occurring organic material is cited as a possible explanation. Although not specifically mentioned by the author, biodegradation might be a factor in limiting the transport of organic contaminants.

Liquid-to-gas mass transfer limitations can be a rate-limiting factor for biodegradation. **Pauss *et al.* (1990)** determined by theoretical modeling and experimentation that the aqueous solubility and mass transfer coefficient of a gas determine whether the gas partitions according to Henry's law, or is thermodynamically overconcentrated in either the liquid or gas phase. Generally, the more soluble gases (CO_2, NH_3, and H_2S) are less prone to overconcentration, whereas less soluble gases (N_2, H_2, O_2 and CH_4) are more likely to overconcentrate in the aqueous phase with respect to Henry's law. Three types of lab-scale anaerobic reactors were used to confirm the mass transfer modeling results. In a completely stirred reactor with thorough

mixing of the liquid phase, large hydrogen and methane overconcentrations were observed in the liquid phase, reflecting low mass transfer values ($k_L a$) between phases. A sludge bed reactor also had high overconcentration values for hydrogen and methane. Similar results were noted for an upflow sludge-bed filter reactor (UBF reactor). From the reactor results and theoretical considerations presented in the paper, it was determined that highly soluble gases would not be effected by low $k_L a$ values, but poorly soluble gases would be overconcentrated in the phase from which they originated. This could reduce the rate of reoxygenation and decrease rates of aerobic biodegradation. In anoxic environments the excess accumulation of H_2 can be inhibitory to some biological processes. Accumulation of CO_2 and H_2S can decrease the pH of water and lead to decreased biodegradation.

Biodegradation rates can be obtained by quantifying CO_2 generated by microbial respiration of the substrate(s) of interest. Radiolabeled substrates are often used, and a mass balance is calculated by measuring the radiolabeled carbon in biomass, CO_2 and residual substrate. This approach is used for both aerobic and anaerobic biodegradation rate determination. **Watwood *et al.* (1991)** developed a method of capturing and measuring CO_2 generated during biodegradation in calcareous soils, which generate large amounts of abiotic CO_2 when acidified. A soil sample is placed in a flask or other suitable container containing microbially active soil, water, and radiolabeled organic substrate(s). The container must have gas-filled headspace for the accumulation of CO_2. After a pre-determined period of incubation, the soil is acidified, volatizing the dissolved CO_2 into the headspace. The headspace contents are then nitrogen-purged through a tube filled with activated carbon (to remove any volatile ^{14}C-containing substrate), and bubbled into a container of 2 N NaOH to capture the CO_2 for scintillation counting. This method was used successfully to quantify aerobic and anaerobic degradation rates of benzene in two soils from Albuquerque, NM:

soil type	aeration status	% benzene biodegradation (4 weeks)
Sandia (sandy aridisol)	aerobic	14.85
	anaerobic	2.43
Bosque (riparian soil)	aerobic	47.08
	anaerobic	16.49

This paper presented a method of quantifying biodegradation rates based on CO_2 production, and demonstrated the variability encountered in biodegradation rates of benzene in different soils, under aerobic and anaerobic conditions. Abiotic CO_2 does not interfere, as long as a sufficiently large volume of NaOH is used in the trap to ensure that all the CO_2 is captured.

3.1 Aerobic Biodegradation

Magazu and Carberry (1989) examined conditions under which biodegradation of five petroleum products occurs. Soil samples from five petroleum-contaminated sites were each incubated under six sets of conditions while hydrocarbon degradation, as measured by decreasing chemical oxygen demand (COD), was monitored. Total viable microbes were determined by plate counting. One-gram samples were placed in 500 mL stirred reactors filled with water and other materials creating the following six different conditions:

1. Distilled water only
2. Nutrients
3. Nutrients and oxygen
4. Nutrients, oxygen and microbial supplementation (1.0 mL)
5. Nutrients, oxygen and microbial supplementation (2.0 mL)
6. Nutrients, oxygen and microbial supplementation (5.0 mL)

The nature of the microbial supplementation was not described.

For most conditions tested, hydrocarbon utilization rates were linear over a 15-day period. Virtually no degradation occurred in samples that did not receive oxygen. In cases where degradation occurred, the rate was greater with more microbial supplementation and was correlated with the amount of microbial supplement added.

Harder and Hopner (1991) evaluated the effect of soil moisture content and nutrients on the rate of n-hexadecane biodegradation. n-Hexadecane was added to three soil types at a concentration of 7 mg/g dry soil. The soil was inoculated with soil from a gasoline spill area that was subsequently exposed to n-hexadecane. Nitrate and phosphate were added as nutrients. A 38% slurry was prepared and pH was adjusted to 7.2. Water was adjusted by heating to 35°C over silica gel as a desiccant under sub-ambient pressure. Eight samples with different moisture

contents ranging between 2% and 35% were thus prepared. Samples were incubated in a Warburg vessel equipped with CO_2 adsorbers for 3 days. Oxygen consumption and CO_2 production were monitored.

For the first soil tested (a German loess), oxygen consumption per gram of soil was greater with increasing moisture content, and the effect was most apparent at soil-moisture levels greater than 25%. In the other two soils tested (Israeli Bet Dagan and Gilat) the degradation rate increase reached a maximum at 5% soil-moisture, and further increase in soil-moisture did not increase the biodegradation rate of n-hexadecane. n-Hexadecane degradation rates were calculated from oxygen utilization rates and CO_2 production rates. However, direct measurements of n-hexadecane were not made. The calculations assumed complete mineralization of n-hexadecane. This work shows the relationship between soil moisture content without the confounding variable of limiting oxygen availability. The authors believe that at lower moisture contents, microbial mobility is limited, and that desiccated organisms are less metabolically active.

Barker *et al.* (1987) conducted field and lab experiments to monitor natural attenuation of aromatic hydrocarbons in a shallow sand aquifer. Microcosm experiments used microbial populations and groundwater from their site in Base Borden, Ontario, Canada. Aerobic and anoxic conditions were used, and concentrations of BTX compounds were 0.4-15 mg/L. After lag phases lasting 2-10 days, benzene, toluene and *o*- and *m*-xylene were aerobically degraded to below detection limits within 78 days. The measured rates have little meaning, since degradation rates were limited by rate of oxygen leakage into the microcosms. This was indicated by:

1. Apparent zero-order kinetics,
2. Similar degradation rates for all hydrocarbon species, and
3. Lack of sufficient dissolved oxygen in the microcosms to degrade the large amounts of hydrocarbons present.

It was later confirmed **(Barker *et al.* 1989)** that biodegradation rate was limited by the rate of oxygen leaking into the microcosms.

Anaerobic degradation of benzene, toluene and o-xylene was not detectable. Nitrate addition was found to enhance biotransformation in the Borden sand used in this study.

In the field study, 1800 L of groundwater was spiked with 1.1-2.4 mg/L of BTX compounds and chloride and 1 mg/L oxygen, and injected into the uncontaminated aquifer through a single well 1.4-2.0 m below the water table. Attenuation of the hydrocarbons and chloride was monitored for four months. The chloride acted as a conservative tracer and provided information about advection and dispersion of the plume. All hydrocarbon components moved and spread at slower rates than the chloride, indicating sorptive retardation. Total chloride mass in the plume remained constant through day 108 when chloride measurement was halted. Total mass of each hydrocarbon decreased over time. Mass loss rate appeared to be zero order, and rates were similar to microcosm mass loss rates. The authors caution against inferring rate order from the field data, because reported hydrocarbon concentrations are vertically integrated, and degradation rates may be different at different depths. Oxygen availability was cited as a rate-limiting parameter in attenuation. Hydrocarbon persistence was associated with low dissolved oxygen concentrations. Benzene was the most persistent hydrocarbon. All hydrocarbons (initial total concentration = 7.5 ppm) were essentially removed in 14 months.

Biodegradation of BTEX compounds in groundwater as it infiltrates through a soil column was studied by **Allen *et al.* (1987)** at Canadian Forces Base, Borden, Ontario. An undisturbed column of soil was separated from the surrounding environment by pile-driving a 3 ft OD steel casing down to the water table. Water containing 10-35 mg/L levels of BTEX compounds was applied to the soil surface and allowed to percolate down to the water table. At various depths, soil gas and water samples were taken and analyzed for BTEX and oxygen. Four time-series experiments were run. Water application was continuous throughout each experiment at a rate of 1.37 cm/hr. 30-50% of the applied concentrations of BTEX was lost to volatization before reaching the soil surface, and this was taken into account when BTEX attenuation rates were determined.

Results of the first experiment (designated IA) showed that BTEX compounds broke through the unsaturated zone and emerged at the water table after about 75 hours, but as the microbial population became acclimated, the concentrations of BTEX in water reaching the water table gradually attenuated. By 200 hours no BTEX was breaking through to the water table. The

second experiment (IB) was run identically to the first, and showed no BTEX breakthrough to the water table, indicating that the microbial population was well acclimated to the BTEX substrates. In the third experiment (II) BTEX concentrations in the source water were increased to about 35 mg/L after about 100 hours. BTEX breakthrough was noted in the groundwater, and breakthrough concentrations increased corresponding to the feedwater concentration increase at 100 hours. Oxygen concentrations decreased with depth, but never reached metabolic limiting levels. In the fourth experiment (III) BTEX concentrations of 15-20 mg/L were used, and no breakthrough was noted.

Results of IA are explained as purely physical transport and sorption until microbial acclimation occurred, after which time BTEX compounds were aerobically degraded to below detectable levels at the water table. The acclimation hypothesis is supported by lack of BTEX breakthrough during the identical experiment IB, and an increase in viable organisms noted in the upper levels of soil. Although BTEX concentrations were decreased during infiltration in experiment II, some breakthrough occurred. No explanation for this was offered, though the observed BTEX breakthrough is likely the result of increasing source water concentrations from 10 to 35 mg/L. Experiment III results, where no breakthrough occurred, were consistent with Experiments I and IA.

The effects of high concentration of JP-4 jet fuel on the *in situ* microbial community was investigated by **Aelion and Bradley (1991)**. 80,000 gallons of fuel had been spilled from an above-ground storage tank, and a sandy, shallow aquifer was contaminated with up to 4000 mg petroleum hydrocarbons per kg of dry sediment. The objective of the study was to evaluate the potential of microorganisms in the sediment to biodegrade petroleum hydrocarbons. Saturated microcosms were prepared by placing 3 g saturated-zone sediment into 20-mL vials, then filling the vials with aqueous mineral and/or organic nutrient solutions, leaving no headspace. CO_2 production was monitored while the microcosms were incubated at room temperature. Organic substrates were ^{14}C labeled glucose, benzene or toluene. Carbon mass balances were conducted by measuring ^{14}C concentrations in the biomass, sediment and solution phases. Unsaturated microcosms were prepared by measuring 50 mL sediment and 2 mL of autoclaved

water (with or without inorganic nutrients) into 125-mL serum vials, leaving significant headspace. O_2 and volatile hydrocarbon consumption and CO_2 production were monitored using gas chromatographic analysis of headspace.

Respiration rate in glucose-amended saturated microcosms reached a maximum of 9% per day after 3 days. Addition of 0.1 mM NO_3^- or NH_4^+ did not significantly change respiration rates. Addition of 1.7 mM NO_3^- decreased CO_2 production, but increased the amount of ^{14}C-labeled carbon incorporated into cellular material. Over 26 days, total glucose metabolized in NO_3-amended microcosms was twice that of unamended microcosms. Respiration of ^{14}C-labeled benzene and toluene was negligible in microcosms with or without 3 mM NO_3^- over three months.

In unsaturated microcosms, significant CO_2 production was only observed when NO_3^- was added. Addition of PO_43^+ and NO_3^- was not more effective in stimulating respiration than NO_3^- alone. Benzene and toluene were utilized in microcosms containing sediment from the jet-fuel contaminated area of the site, but *n*-hexane was metabolized at a higher rate. This study confirmed the presence of a microbial population capable of growing and utilizing some organic compounds present in JP-4 jet fuel in highly contaminated soil. Aerobic activity of the site appears to be limited by the availability of nitrogen.

Chlorinated hydrocarbons are common groundwater contaminants. **Wilson *et al.* (1988)** studied the ability of aquifer microorganisms to aerobically cometabolize trichloroethylene and other chlorinated hydrocarbons while metabolizing methane as carbon and energy sources. Columns 5 cm i.d. x 150 cm long were packed to a depth of 140 cm with a fine sandy soil. Flow-through experiments involved pumping influent water containing chlorinated and con-chlorinated organics through the columns and measuring effluent composition. Conditions were aerobic, and methane was provided to stimulate methanotrophic bacteria. At a 21 cm/day hydraulic loading rate, 19 to >98% removal of 12 chlorinated organics was reported. This is one of many studies (beyond the scope of this Technology Review) that describes cometabolism of chlorinated organics as nonchlorinated hydrocarbons are utilized by bacteria (e.g., **Lanzarone and McCarty, 1990; Wackett and Gibson, 1988**). As many contaminated sites have both chlorinated and

unchlorinated hydrocarbons, and aerobic as well as anaerobic conditions, this area of research shows promise for multiple-contaminant *in situ* and on-site bioremediation processes.

Bauer and Capone (1988) studied microbially-mediated mineralization of aromatic hydrocarbons in sediment obtained from a salt marsh on Long Island. Their objective was to determine the effect of previous exposure to one polyaromatic hydrocarbon (PAH) on the degradation of another, and relative rates of several PAH's present as a mixture in marsh sediment. The sediment used has no known previous exposure to PAH's. Conditions were aerobic, and test compounds were anthracene and naphthalene. Sediment samples were pre-exposed to either glucose, anthracene, benzene, naphthalene or phenanthrene for either 7 or 14 days. Rates of mineralization of the two test compounds were then determined in each sediment sample, using 3 replicates in each case. None of the pre-exposure treatments inhibited mineralization of anthracene or naphthalene, glucose had no measurable effect, and benzene pre-exposure had a minor but statistically significant effect in stimulating anthracene mineralization. It is noteworthy that anthracene pre-exposure had no effect on anthracene mineralization, as compared to a control sediment sample which was not pre-exposed to anthracene. Naphthalene and benzene pre-exposure both stimulated naphthalene mineralization. This work showed that cross-acclimation among different PAHs can occur in marine sediment bacteria, and that degradation of each individual compound may proceed independently of the other components once cross-acclimation has taken place.

Mihelcic and Luthy (1988a and 1988b) compared degradation rates of naphthol, naphthalene and acetonaphthene under aerobic, denitrifying and anaerobic conditions. 50 mL centrifuge tubes were filled with soil slurry samples made from a grassland subhumid soil from Cass County, N.D. Information about previous exposure to the test PAH compounds was not provided. Under aerobic conditions, all three compounds were completely degraded within 10 days. Initial concentration were 9, 7 and 1 mg/L for naphthol, naphthalene and acetonaphthene, respectively. Naphthol was most rapidly utilized, and was not detectable after 3 days. The authors believe that the hydroxyl substitution causes naphthol to be more biologically reactive than its parent compound, naphthalene.

Pendrys (1989) isolated aerobic bacteria capable of degrading asphalt, which is the unrefined residue of crude oil fractional distillation. Composition of asphalt varies with crude stock and production methods. Asphalt cement-20 (commonly used in road construction in temperate climates) from R.H. Smith Co., Branchville, MD was used in this study. Cultures of asphalt-degrading organisms were obtained from the edge of an asphalt road at the Naval Surface Warfare Center, Silver Springs, MD. Seven bacterial species were identified: *Pseudomonas mendocina, Alcaligenes calcoaceticus, A. denitrificans subsp. xylosoxydans, Flavobacterium sp., Flavimonas oryxihabitans, Pseudomonas aeruginosa*, and *P. cepacia. A. calcoaceticus* excretes an emulsifier which makes some asphalt compounds bioavailable.

After 3 weeks of incubation, asphalt surfaces became brittle and flaky, and growth of a mixed asphalt-degrading culture ceased. Remelting the asphalt caused renewed bacterial growth, indicating that the biodegradation takes place on the surface. Melted asphalt was chromatographically separated into four fractions: saturates, naphthene aromatics, polar aromatics and asphaltenes. Saturates supported the most rapid bacterial growth, followed by naphthene aromatics. Polar aromatics and asphaltenes could support a bacterial population at a low level (4×10^4 CFU/mL), but could not induce growth. Some isolates grew better in mixed cultures than in pure culture. The author suggested that those species may rely on others to produce biosurfactants to make the asphalt compounds bioavailable.

Shiaris (1989) investigated seasonal effects on aerobic biodegradation of low molecular weight PAHs in sediment from a polluted estuary (Boston Harbor, MA). Sediment from three sampling sites with varying degrees of naphthalene, phenanthrene and benzo[a]pyrene contamination were used. Sediment samples were diluted 10:1 with harbor water sampled from the water columns overlying the sediment sampling sites. Resulting slurries were supplemented with ^{14}C-labeled PAHs. Mass balances were determined for the PAHs during incubation experiments by measuring resulting radioactive species, which included residual parent compounds, CO_2, polar compounds and non-extractable, cell-bound materials. Temperatures were maintained at Boston Harbor sediment ambient values during the experiments. PAH concentrations in the harbor were assumed to be at steady state, and that the rate of PAH input

is equal to the rate of biodegradation. Therefore, the initial measured degradation rate was assumed to be the rate in effect in the harbor, and change in reaction rate as a function of concentration was not considered separately at each site. The effect of PAH concentration on degradation rate was studied by comparing the rates at the three sites which differed significantly in initial PAH concentration.

Biotransformation rates were found to be a function of PAH concentration, with higher concentrations leading to higher transformation rates for all three compounds. Generally, benzo[a]pyrene degradation rates were lower than those of naphthalene or phenanthrene, which had similar degradation rates. Seasonal patterns of total PAH biodegradation varied from site to site, but over a year it was evident that more rapid degradation was associated with warmer weather. Salinity was statistically found to effect transformation rates, but sometimes positively and other times negatively, with no clear pattern.

Literature values for PAH turnover time (the time required to remove all PAH at the measured removal rate) were reviewed and found to be highly variable. Differences were explained in part by experimental and analytical inconsistency. Naphthalene turnover values of 0.3-800 days were tabulated. Turnover rate comparisons between PAHs showed more consistency in the literature. Increase in molecular weight or number of aromatic rings generally meant an increase in turnover time. Also, turnover times tend to be shorter in sediments more highly contaminated with hydrocarbons.

Madsen *et al.* (1991) used samples of soil from a PAH-contaminated coaltar burial site in the eastern US to investigate microbial acclimation to PAHs as their carbon and energy source. 125 mL flasks containing 4 g of soil were filled with water containing dissolved naphthalene and phenanthrene. Soil samples were taken from unsaturated, water table, shallow saturated and deep saturated zones. These microcosms, prepared with soil from one upgradient contaminated, one downgradient and one adjacent pristine areas, were monitored for PAH degradation. Mineralization of PAHs was detected only in samples taken from within the plume. Naphthalene and phenanthrene were mineralized in samples from all four depths in the upgradient samples

(closest to the source). In the downgradient microcosms, naphthalene was degraded at all depths except the deep saturated zone, and phenanthrene was only degraded in water table samples. Water table soil samples were the most active in PAH mineralization in all cases.

Before hydrocarbons can be metabolized they must first be transported through the cell membrane into the cell. In order to determine whether transport limitation accounts for the xenobiotic nature of high molecular weight hydrophobic alkanes, **Miller and Bartha (1989)** aerobically cultured a pseudomonas isolate on C-18 and C-36 alkanes (octadecane and hexatriacontane) with and without liposome encapsulation. Liposome encapsulation surrounds hydrophobic hydrocarbons with a hydrophilic coating and renders them easily transportable through cell membranes. Cell mass growth rates with encapsulated alkanes as the sole carbon source were similar to those with succinate as the carbon source. Nonencapsulated hexatriacontane yielded virtually no cell growth, and nonencapsulated octadecane yielded growth rates 1 to 2 orders of magnitude lower than encapsulated octadecane. Incubation temperatures above and below the melting points of both alkanes were used in order to observe differences in degradation rates for liquid and solid hydrocarbons. Although cell mass production rates declined with temperature, the effect seemed to be related to temperature-controlled microbial kinetics, and not the phase (liquid or solid) of the hydrocarbon substrate.

Cell membrane transport limitations were shown to limit biodegradation rates of the two hydrocarbons tested, and the authors suggested that other solubilizing strategies could be used to increase biodegradation rates. Detergents and solubilizing agents were suggested.

Foght *et al.* (1989) evaluated the effect of emulsan on biodegradation of crude oil by pure and mixed bacterial cultures. Emulsan is an extracellular heteropolysaccharide produced by Acinetobacter calcoaceticus. Presumably, emulsan helps solubilize hydrophobic crude oil hydrocarbons, enabling them to cross cell membranes more readily and enter bacterial cells. Prudhoe Bay crude oil, containing 23% paraffin (vol/vol) and 25% aromatics (vol/vol) was used. Generally, emulsan decreased biodegradation of hydrocarbons, particularly saturated alkanes. Some stimulation of biodegradation was observed for aromatics in some pure cultures. It was

speculated that emulsan-coated hydrocarbons are not able to have direct contact with internal cell enzymes, and therefore degradation cannot be initiated. This study shows that merely solubilizing hydrocarbons does not necessarily render them more biodegradable, and in some cases biodegradability is inhibited.

Song and Bartha (1990) demonstrated that jet fuel hydrocarbons can be rapidly degraded when applied to aerated surface soil previously exposed to jet fuel. In 12 weeks, 135 and 50 mg g^{-1} jet fuel was reduced to 10 and 0 mg g^{-1}, respectively.

Song *et al.* (1990) compared bioremediation potentials of five fuel oils. They found that environmental persistence increases in the following order: jet fuel > heating oil > diesel oil. Increasing temperatures decreased half-life of persistence. Soil type (sand, loam or clay) had little effect on biodegradation potential.

As part of a gasoline spill monitoring program, **Kemblowski *et al.* (1987)** evaluated the aerobic biodegradation potential of benzene. Of the 15,000 gallon tank spill in Indian River County, Florida, 5000 gallons had been recovered from a recovery well. Samples from monitoring wells indicated that naturally occurring biodegradation of hydrocarbons was attenuating the dissolved contaminant plume to levels below detection limits before the effected groundwater reached surface discharge areas or drinking water wells. Microcosms consisting of test tubes filled with groundwater-and-soil slurries were prepared using site materials. The microcosms were periodically analyzed for benzene over a 70-day period. Another set of microcosms were spiked with ^{14}C benzene, and biodegradation was monitored by quantifying dissolved $^{14}CO_2$.

Half-lives for benzene at 10, 50 and 500 ppb were 2.5-4.5 days. At 5000 ppb the half-life was 5-28 days. No nutrients were added. Initial dissolved oxygen (D.O.) was 8-9 ppm. Virtually no degradation was noted in microcosms containing 50,000 ppb benzene. A threshold concentration of about 0.5 ppm oxygen was determined from the $^{14}CO_2$ microcosms, which were prepared with a range of D.O. concentrations from 0.1 to 6.7 ppm. Little benzene degradation was noted at D.O. levels below 0.5 ppm.

Karlson and Frankenberger (1989) evaluated the aerobic biodegradability of benzene and toluene in gasoline-contaminated groundwater. They used water from a monitoring well at a depth of 12-15 ft in Los Angeles, CA. The water contained 6.2 mg/L gasoline hydrocarbons and 710 colony-forming units per mL of gasoline-degrading organisms. An additional bacterial population, isolated from a gasoline-contaminated site and known to utilize gasoline as a sole carbon source, was used as an inoculum to accelerate degradation rates. 50 mL samples were allowed to incubate with shaking at 23°C. Toluene concentrations decreased from 477 μg/L to below California action levels (100 μg/L) in 23 hours. Benzene degraded more slowly, decreasing from 480 to 218 μg/L in 48 hours.

Arvin *et al.* (1989) investigated the benzene-degrading ability of bacterial cultures acclimated to degrading individual and mixtures of petroleum hydrocarbons. Toluene, o-xylene, naphthalene, 1,4-dimethylnaphthalene, phenol and pyrrole were the hydrocarbons used. 250-mL bottles were completely filled with water containing essential minerals, oxygen, and 0.1 to 0.2 mg/L of one or more hydrocarbon. Residual hydrocarbon concentration, and biomass production (as measured by ATP concentration) were measured after 5 and 11 days. Two bacterial inocula were used. One was acclimated to degrading only aromatic hydrocarbons. The other was also exposed to organics containing nitrogen, sulfur and oxygen. Benzene degradation rates were lower in inocula containing compounds with nitrogen, sulfur and oxygen in their molecular structure. Benzene degradation rates were high when xylene (isomer not specified) or toluene were present, and were inhibited by the presence of pyrrol. When toluene and xylene were both present, benzene degradation rates were lower than when only one of the substituted benzenes was present. Dicyclic and tricyclic aromatic compounds did not stimulate benzene degradation, indicating that metabolic pathways used to degrade these larger aromatics are not used for benzene degradation. The inhibitory effect of pyrrol suggests that pyrrol may inhibit degradation of creosote waste. No correlation was found between biomass produced and benzene degradation rate.

Arvin *et al.* (1988) compared the biodegradability of several petroleum hydrocarbon components in groundwater. Experiments were run aerobically in 1.3 L bioreactors, with and

without 10 g of soil added per liter of groundwater. Groundwater samples were diluted 10:1 with mineral solution made from deionized water and essential inorganic nutrients. Samples were taken from four sites contaminated by petroleum products, one with gasoline, one with heavy fuel and two with fuel oil. Initial hydrocarbon concentrations of 100 to 2000 μg/L were used. Times required to reduce hydrocarbon components to 1 μg/L were determined for each set of conditions (site, pollution level, with or without soil added).

Time required to reduce hydrocarbons from about 1500 μg/L to 1 μg/L was largely dependent upon the degree of oil pollution in the groundwater used. For example, unpolluted, slightly polluted and heavily polluted water from a site designated Gassenhaven had degradation times of 78, 17 and 10 days, respectively. These figures are for the sum of toluene, xylenes, naphthalenes and biphenyl present. Reduction to 1 μg/L of the same set of compounds from groundwater from the site designated Horsholm required 9 days. In the least-heavily contaminated groundwater, addition of 10 g of soil from the site had no effect on degradation rates. For more heavily contaminated groundwater, adding 10 g of soil accelerated the degradation by about a factor of 2. This showed that only soil close to the oil spill had a significant active biomass, at least in terms of petroleum hydrocarbon degradation. The amounts of bacteria initially present in the groundwater was about equal to the number introduced by the addition of 10 g soil (10^7-10^8 cells/L). Therefore, the attached bacteria at the lesser-polluted sites appear to be much less effective in hydrocarbon degradation than the free-living bacteria in the groundwater. Conversely, in more-polluted sites, attached bacteria appeared to be about equal to free-living bacteria in terms of hydrocarbon degradation.

This work suggests that free-living bacteria can degrade significant amounts of petroleum hydrocarbons, and in some cases fixed bacteria are less effective. The authors observe that free-living bacteria can migrate with a contaminant plume, therefore having a greater exposure time than fixed bacteria, which may have insufficient contact time for acclimation and/or degradation before the plume moves away.

Bioavailability of toluene sorbed onto soil was studied by **Robinson *et al.* (1990)**. It was found that toluene sorption is initially rapid, followed by slow sorption persisting beyond 150 days for the soil used, which was mostly silt and sand. Aqueous desorption rates followed a similar pattern, with initial rapid desorption followed by a long period of slow desorption. Microcosms were prepared using 6 x 100 mm screw-cap test tubes with 6 g of dry, sterilized soil. The remaining volume of each tube was filed completely with water, leaving no headspace, and H_2O_2 and a microbial inoculant (*Pseudomonas putida*) were added.

Dissolved toluene was biodegraded to below detection levels within 2 days. Sorbed toluene was degraded as it desorbed, and the toluene concentration in solution remained below detection limits after Day 2. When the microbial population was killed with a lethal dose of H_2O_2, additional desorbing toluene remained in solution, and the toluene concentration increased. This study showed that sorbed toluene was much less bioavailable that dissolved, and possibly entirely unavailable. The rate of sorbed toluene degradation is therefore limited by the rate of desorption.

Nitrification of ammonia under aerobic conditions can result in some loss of nitrogen to the atmosphere in the form of N_2O and NO. **Tortoso and Hutchinson (1990)** measured the N_2O and NO production rate relative to the NO_2^- and NO_3^- production rate. With glucose as a substrate, they found that NO and N_2O represented 1.7 and 0.02%, respectively, of the amount of ammonia nitrified. This would not represent a significant loss of nitrogen if ammonia or other reduced forms of nitrogen were used as *in situ* or on-site amendments for enhancing biodegradation.

3.2 Anaerobic Biodegradation

Anaerobic degradation of petroleum hydrocarbons in groundwater can be indicated by the presence of methane and organic acids. **Cozzarelli *et al.* (1989)** observed sharp decreases in monoaromatic hydrocarbons downgradient from a crude oil spill in Bemidji, MN. The highest concentrations of CH_4, CO_2 and organic acids were in the anaerobic zone. During a four-year period following the spill, concentrations of methane increased by a factor greater than 25, and conditions became increasing reducing with time **(Baedecker *et al.*, 1989)**. The authors

concluded that anaerobic degradation of aromatic hydrocarbons is an important geochemical process at the Bemidji site.

Mihelcic and Luthy (1988a) were able to anaerobically degrade naphthol but not naphthalene or acenaphthene under anaerobic conditions in laboratory microcosms. Naphthol degraded at a slower rate anaerobically than aerobically, requiring 15 days to decrease from 9 mg/L to nondetectable. Under denitrifying conditions, all three PAH compounds were degraded, but at rates lower than those observed under aerobic conditions. All detectable naphthol was removed in less than 16 days, and the other two compounds were nondetectable after 45 days. Degradation of unsubstituted PAH compounds had not been previously reported, and this study suggests that bioremediation of PAH and alkyl-substituted benzenes is possible without the presence of molecular oxygen if denitrifying conditions are present.

Mihelcic and Luthy (1988b) further investigated biodegradation of naphthalene and acenaphthene under denitrifying conditions. Using the same experimental procedures described in **Mihelcic and Luthy (1988a)**, they found that background soil organic material can compete for nitrate, and that biodegradation of the two study compounds depended upon nitrate availability. Observed acclimation periods that preceded significant biodegradation were about 3 weeks for background organics and two weeks to one month for the PAH compounds. The acclimation periods were attributed to denitrifier population increases rather than metabolic acclimation or mutation.

Evans *et al.* (1991) studied the anaerobic degradation of toluene, *p*-xylene and *o*-xylene under denitrifying conditions. Soil or sediment from seven sources was mixed with nutrient mineral solution and incubated in microcosms. Soil and sediment sources were:

East River boat marina sediment;
Secondary anaerobic digester sludge;
Soil from a lagoon receiving naptha cracking waste;
BTX-contaminated water after activated carbon treatment ;
Soil from a partially treated gasoline spill site;
Soil from an untreated gasoline spill site; and
Soil from a petroleum distillate spill site.

Microcosms were incubated at 30°C, and pH was adjusted to 7.5. Gas composition in the headspace was monitored. In all cases, 90-100% of the toluene was degraded in 1-3 months. Substrate loss was accompanied by loss of nitrate and an increase in nitrogen gas. Benzene, *p*-xylene and *m*-xylene were not degraded. Partially degradation of *o*-xylene was noted, and degradation was more rapid in the presence of toluene.

A mass balance of species in the untreated gasoline spill site showed that CO_2 production accounted for only 57% of the toluene lost, and the rest was assumed to have been utilized in cell synthesis. The combined degradation-synthesis equation used to describe the observed mass balance was:

$$C_7H_8 + 4.8\ NO_3^- + 0.6\ NH_3^+ \text{---> } 4\ CO_2 + 0.6\ C_5H_7O_2N + 2.4\ N_2 + 5.2\ H_2O$$

Without toluene present, *m*-xylene was degraded in the untreated gasoline spill soil microcosm. After 1 week 50% of the *m*-xylene was gone, and 100% was degraded within 2 weeks. As in the case of toluene, 57% of the *m*-xylene consumed was mineralized to CO_2, and the rest was assumed to be utilized for cell synthesis.

Gersberg *et al.* (1989) used gasoline-contaminated water from a site in San Diego, CA to measure rates of BTEX degradation under denitrifying conditions. 120 mL bottles were completely filled with contaminated groundwater, which had DO levels of less than 1 mg/L. Nitrate, phosphate and a solution of trace minerals were added, and the microcosms were incubated at room temperature. Some microcosms received oxygen in the form of H_2O_2, some received only nitrate as the terminal electron acceptor, and some (control) received neither H_2O_2 nor nitrate. Initial levels of about 13, 34 and 15 µg/L for benzene, toluene and total xylenes, respectively were reduced by 80%, 95% and 47% in 54 days. Removal rates were similar under aerobic conditions with nutrients added (87%, 95% and 35% for benzene, toluene and total xylenes, respectively), but rates of removal were low (numbers not given) under aerobic conditions without nutrients. This indicated that either nitrogen or phosphorous is low enough in the groundwater to be rate-limiting.

Kuhn *et al.* (1988) investigated the biodegradation of *m*-xylene and toluene in water containing mineral salts. The water was flowed through glass columns filled with porous media (30% aquifer material, 70% expanded slate grain). Rapid degradation of the target compounds was measured anaerobically when nitrate was present in excess. When oxygen was introduced, degradation and denitrification ceased. When nitrate was replaced with nitrite, degradation decreased by about 50%, but replacement of nitrate with nitrous oxide caused no decrease in degradation. The authors suggest that nitrous oxide may be useful for bioremediation of aromatics, since it is highly soluble in water and is effective as an electron acceptor in anaerobic degradation as shown here.

In order to test the specificity of microorganisms adapted to *m*-xylene degradation, other aromatics were added to the column influent solution. Toluene was completely degraded, 3-ethyltoluene was partially removed, and three others (benzene, ethylbenzene and *o*-xylene) were slightly reduced. Propylbenzene, *p*-xylene and three naphthalene compounds were not reduced. This shows that the metabolic pathways used to anaerobically degrade *m*-xylene in these experiments is highly compound-specific. The authors attempted to shed light on the nature of the metabolism based on its selectivity hypothetical intermediate compounds. They found that *m*- and *p*-cresol but not *o*-cresol were degraded in the columns, and substituent aldehydes and acids of toluene and *m*-xylene were degraded, but not the alcohols. This shows that oxidative attack on the ring or on the methyl groups could be the initial step in degradation of these compounds by the nitrate reducers present in this study. The authors caution that pathway studies must analyze for the presence of intermediates and products in order to be conclusive.

Anaerobic biodegradation of toluene and *m*-xylene with sulfate as the terminal electron acceptor was demonstrated in gasoline-contaminated marine silty sandy aquifer material by **Edwards *et al.* (1992)**. 250-mL glass bottles were used as microcosms. Each was given 100 g of aquifer material and 100 mL of an inorganic mineral nutrient solution, and incubated in an anaerobic glove box. Approximately 5 mg/L of benzene, toluene, ethylbenzene, *p*-xylene, and *o*-xylene were added to each microcosm. Toluene was the first compound to degrade, followed by *p*-xylene, then *o*-xylene. These three compounds were reduced by 80% in 40, 72 and 104

days, respectively. After 270 days no degradation of either benzene or ethylbenzene was detected. Sulfate was confirmed as the electron acceptor because no methane was produced, sulfate concentrations decreased, sulfate deposits formed, and degradation slowed as sulfate was depleted and resumed when more sulfate was added. Complete mineralization of up to 90% of the initial toluene and xylene to CO_2 was confirmed by using ^{14}C-labeled toluene and xylene. Some production of the recalcitrant byproducts benzylsuccinic acid and benzylfumaric acid were observed **(Beller *et al.* 1992)**. This study showed that sulfate-reducing bacteria can degrade selected hydrocarbons while leaving others unaffected. In a separate study with the same aquifer material, **Edwards and Gbric-Galic (1992)** showed that benzene can also be anaerobically degraded as a sole carbon source by sulfate-reducing organisms if the microcosms does not contain other degradable hydrocarbons, such as toluene or xylenes.

Major *et al.* (1988) conducted a microcosm study on biodegradation of BTX compounds under denitrifying conditions. Soil from a shallow, sandy aquifer (Canada Forces Base, Borden, Ontario) was used. The soil had no detectable BTX compounds, but had been exposed to BTX from a previous field injection test. Microcosms consisted of vials containing 25 g of soil and 46 mL sterilized groundwater from the site, had no headspace, and were incubated anaerobically. Temperature was maintained at 10°C for six weeks prior to introduction of BTX in an attempt to simulate oligotrophic aquifer conditions and to select against surface microbes that may have been introduced during sampling and microcosm preparation. 3 mg/L each of benzene, toluene, *o*-xylene and *m*-xylene was added to 20 microcosms. Other (control) microcosms received no BTX. BTX microcosms were run with various electron acceptors: none, nitrate, oxygen, or nitrate and oxygen. After 62 days the following results were obtained:

Percent of BTX remaining after 62 days incubation

compound	sterile control	no electron acceptor	nitrate	oxygen	nitrate and oxygen
benzene	79	66	5	1	0
toluene	86	65	2	0	0
o-xylene	80	73	15	15	19
m-xylene	80	59	12	11	8

Losses from sterile microcosms were attributed to sorption. Benzene degradation with nitrate was zero-order. When microcosms were run with limiting concentrations of nitrate, the BTX losses were stoichiometric with respect to the available nitrate. This gave further evidence that denitrification was responsible for BTX degradation. For conclusive proof of denitrification, acetylene was used to block the biological conversion of N_2O to N_2. Accumulation of N_2O corresponded to disappearance of BTX in the microcosms, and this positively confirmed the biodegradation of BTX compounds under denitrifying conditions.

Hutchins *et al.* (1991) conducted a lab-scale study on the potential of denitrification for biorestoration of an aquifer contaminated with JP-4 jet fuel. This study is in support of an on-going field investigation of biorestoration of a jet fuel spill at Traverse City, MI. Anaerobic microcosms were prepared with aquifer core material, nitrate, essential minerals, and either individual hydrocarbons or a mixture of benzene, toluene, *o*-,*m*- or *p*-xylene, ethyl benzene, or 1,2,4-trimethylbenzene. Degradation rates in uncontaminated core material were compared to rates in jet-fuel contaminated core material. In uncontaminated microcosms, toluene was rapidly removed with no lag period. A 30-day lag period preceded degradation of xylenes, ethylbenzene and 1,2,4-trimethylbenzene. o-Xylene degradation occurred slowly. First-order rate constants for degradation of each compound were determined. In contrast, a 60-day lag period preceded degradation of toluene, *m*- and *p*-xylene and 1,2,4-trimethylbenzene in contaminated-soil microcosms. Biodegradation rates in these microcosms were 3-7 times lower than in the uncontaminated cores. This study demonstrated that indigenous microbes at this site can degrade monoaromatic hydrocarbons with the possible exception of benzene under denitrifying conditions. Degradation rates were more rapid and lag times shorter in uncontaminated samples than in jet fuel contaminated samples. The authors suggest that in contaminated samples, microbes were degrading indigenous organic carbon in preference to monoaromatics, and they do not rule out the possibility of microbial toxicity from JP-4 or metabolic byproducts.

Biodegradation of naphthalene and other PAH compounds in groundwater is thought to be coupled to desorption from soil **(Mihelcic and Luthy, 1989, 1991)**. Degradation rates were measured under denitrifying conditions in 10:1 water:soil slurries. Acclimated soil containing

about 10^5 denitrifiers per gram of soil was used. Experiments were run in 50-mL glass centrifuge tubes, to which 1-5 g soil, aqueous mineral solution, and naphthalene were added. The degradation rate was found to be consistent with a coupled desorption, Michaelis-Menton kinetic model with the following rate constants:

soil g/50 mL H_2O	Kmax mg/L day	Ks mg/L	naphthalene mg/L (aqueous)	Nitrate mg/L (aqueous)
1.1104	0.20	0.33	2.0	134
5.2789	0.47	0.40	2.0	135
2.0000	0.75	1.00	3.1	35
2.0000	0.39	0.20	5.6	37
2.0000	0.35	0.36	4.0	50
2.0000	0.83	0.54	3.6	37

The rate of naphthalene degradation was independent of nitrate concentration within the range used, and degradation rate was proportional to the soil-to-water ratio. Sorption of naphthalene was reversible, and was rapid compared to the rate of degradation.

Anaerobic biodegradation of crude oil in the subsurface can lead to progressively more reducing conditions. **Chang *et al.* (1987) and Baedecker *et al.* (1989)** found that manganese reduction, followed by iron reduction and methanogenesis were important mechanisms of crude oil decomposition at a spill in Bemidji, MN.

CHAPTER 4
ON-SITE BIOREMEDIATION

This chapter covers recent literature on on-site bioremediation, where the site was disturbed in order to contact the petroleum hydrocarbon contaminants with oxygen or other electron acceptors, nutrients, and/or microorganisms. Biological wastewater techniques such as activated sludge reactors, trickling filters, stabilization basins and rotating disc filters can be used for on-site bioremediation of water removed from an aquifer. Composting and land treatment can be used to bioremediate hydrocarbon-contaminated soil and sediment. The chapter is divided into aerobic studies and anaerobic on-site biodegradation studies. *In situ* bioremediation, in which the site is not excavated or otherwise mechanically disturbed other than inducing fluid flow, is covered in Chapter 5.

4.1 Aerobic On-site Bioremediation

A pilot-scale rotating disk biooxidation process was evaluated for removal of benzene, toluene and xylenes on a pilot scale, and compared to an air stripping process **(Castaldi and Andrechak, 1988)**. Groundwater from a petroleum hydrocarbon (type unspecified) spill, containing 38 mg/L toluene and lesser amounts of benzene and xylenes was used. Insoluble hydrocarbons were removed by gravity separation, then the water was split between biooxidation and air stripping treatment trains. Effluents were treated with granular activated carbon and discharged to an industrial wastewater treatment system.

The biodisk consisted of a trough containing a 5.2 ft diameter vertical rotating disk, which was 40% submerged at all times in the water being treated. Three biodisks were run in series. Hydraulic retention times of 1.5-4.5 hours were used. The system was initially inoculated with hydrocarbon-degrading bacteria from an industrial wastewater treatment plant. BTX compounds were below detection limits (EPA Method 602) in system effluent at a flow rate of 1.1 gallons per day per square foot of disk area (gpd/ft^2), and near detection limits at 2.1 and 3.2 gpd/ft^2. BTX losses did not appear to be from volatization, based on low concentrations in the headspace air

above the biodisks compared to concentrations of BTX observed in headspace above abiotic biodisks. BOD removal depended upon hydraulic residence time, and was 51-92%.

The kinetics of biological removal was modeled using the Kincannon and Stover model, which relates rate of constituent utilization to flow rate, volatile solids (biomass) concentration and constituent loading rate. Maximum rate and half-reaction constants (similar to Monod constants) were determined. The system did not attain a substrate-limiting mass loading rate for any constituent. The biodisk system was more effective than the air stripping system.

A proprietary system for biologically removing low concentrations (less than 50 mg/L) of BTX and other petroleum hydrocarbons from contaminated groundwater was described by **Galaska *et al.* (1990)**. A cylindrical tank is filled with plastic media (not described), which develops a biofilm when an aqueous solution of biodegradable organic is recirculated through the system. A low-concentration waste stream is then run through the reactor in plug-flow mode. Trace organic contaminants, such as BTX compounds at low ppm levels are cometabolized as starved microbes utilize all available substrates, principally other microbes. Two case studies are described:

1. Groundwater contaminated by BTX compounds from a service station leak in West Virginia. Treated effluent BTX concentrations were about 0.1 mg/L, representing 99.3% removal, and were consistently below the state regulatory limit of 0.15 mg/L total BTX.
2. A similar site in Michigan was treated with a similar system, but regulatory requirements specified BTX limits of 0.02 mg/L in groundwater. Treated effluent BTX concentrations were consistently less than 0.005 mg/L.

Galaska *et al.* (1990) designed a bio-airtower for removing volatile organics from soil venting off-gas. It operates like an air stripping tower, but in reverse: contaminants are dissolved into the water as it contacts the off-gas. The microbial population in the water then degrades the petroleum hydrocarbons.

The use of biofilters for biodegradation of volatile hydrocarbons in the vapor phase was reviewed by **Kosky and Neff (1988).** This technology involves venting hydrocarbon-contaminated gases through perforated pipes into microbially-active soil or composted organic material. The bioactive media should be moist (70-90% moisture by weight), and gas retention time should be greater than 15 seconds. The authors reviewed literature describing several successful systems and described case studies. Biofilters for gas treatment appear to be a viable option for treating air-stripping and soil venting off gases.

Wang *et al.* (1990) observed the fate of PAHs in diesel oil during simulated on-site bioremediation procedures. 90 x 90 cm lysimeters were filled with 35 cm of a sandy loam, and 2.3 mL of diesel oil per cm^2 surface area was applied surficially, resulting in an initial diesel oil contamination level of 60 mg/g of soil. The lysimeters were placed outdoors at the Rutgers University, New Brunswick, NJ campus. The area was sheltered from precipitation, and water was applied at a rate of 2.6 mL/cm^2 on a weekly basis. Some samples were bioremediated (tilled weekly, limed, and fertilized with nitrogen and phosphorus to yield a C:N:P ratio of 1000:5:1).

After two weeks, both the untreated and treated soil displayed reduction in the lower-molecular-weight hydrocarbons. All PAH compounds were substantially reduced in the treated soil, but not in the untreated soil. After 12 weeks with bioremediation all PAH compounds were reduced to near or below detection levels, whereas higher molecular weight PAHs persisted in the untreated soil. Although the contributions for the three remediation procedures (liming, fertilizing and tilling) were not evaluated separately, the study showed that a combination of these procedures can be effective in accelerating bioremediation of diesel oil hydrocarbons, including PAHs.

Yare (1991) conducted two pilot-scale on-site bioremediation demonstrations using hydrocarbon-contaminated soil from an abandoned petrochemical reprocessing site near Houston, TX. Soil in an unlined feed storage pit and clay used to backfill and close the pit contain high concentrations (several thousand µg/kg) of volatile and semi-volatile organics. Chlorinated and non-chlorinated organics and polynuclear aromatic hydrocarbons (PNAs) were present.

Solid-phase bioremediation was evaluated by constructing an high density polyethylene (HDPE) lined enclosure 22 m x 43 m, filling the enclosure with 150 m^3 contaminated soil, and amending the soil with aqueous nutrient and microbial inoculation preparations. The treatment bed was rototilled daily. A portion of the treatment bed was treated with nutrients to achieve a 50:2:1 ratio of carbon:nitrogen:phosphorous. An overhead spray system was used for applying an ammonium phosphate solution.

The second pilot procedure evaluated slurry-phase bioremediation. Slurries were prepared by mixing contaminated soil with water to yield a 30% solids mixture. Two 380 L agitated tanks were filled with slurries made from samples from various locations in the site. These samples differed in types and concentrations of contaminants. Compressed air was injected into the bottoms of the bioreactors to keep dissolved oxygen above 2.0 mg/L. pH was maintained between 5.5-8.0, and cooling was necessary to keep temperatures less than 41°C. Nutrients were added to give initial concentration ratios of 100:5:1 carbon:nitrogen:phosphorous. Bioremediation was monitored over a 10-day period.

Volatization and biodegradation were assumed to be responsible for attenuation of organics in the soil. Half-lives for volatile organics were 1.2 days and 7.6 days for solid-phase and slurry-phase processes, respectively. Both processes achieved greater than 99% removal of volatiles during the treatment period, and in both cases residual concentrations were less than the regulatory maximum of 5960 µg/kg for individual volatile compounds. Semivolatile removal was less efficient for both processes. In solid-phase remediation total PNAs and phenanthrene were removed by 91% and 87%, respectively, with half-lives of 27 and 32 days over the 94-day treatment period. Slurry-phase removals were 62% and 58%, with half-lives of 7.1 and 8.0 days for total PNAs and phenanthrene, respectively. Total organic carbon (TOC) removal was 19% in the solid-phase system and 22% for slurry-phase remediation, with half-lives of 310 and 22 day, respectively. TOC removal as an indicator of bioremediation efficiency can be misleading. For example, **Portier *et al.* (1990)** achieved 97% TOC removal from an oil-and-mud sludge in liquids/solids contact reactors. This figure cannot be compared directly to the 19-22% removals reported by **Yare (1991)**, as soil and contaminants were probably different.

For both demonstrations, the highest microbial activity was associated with the highest decrease in phenanthrene. ATP measurements further indicated that much of the organic removal was biologically mediated, as higher ATP levels were also associated with higher organic removal.

Stegmann *et al.* (1991) observed that the windrow method of composting for on-site bioremediation is the most common biological soil treatment method used in Germany, but that closed bioreactors are being used with increasing frequency. They point out that closed reactor treatment is appropriate when:

1. Emission control is needed;
2. Some organics are difficult to degrade;
3. Soil to be treated is high in clay content; and
4. Biological processes must be enhanced.

These authors advocate the use of laboratory-scale tests using jar testing, respirometers and small-scale bioreactors to determine oxygen requirements, temperature control parameters and optimum ratios of soil to compost. They developed a respirometer technique to evaluate biodegradation of diesel fuel in a sandy soil high in humic content. In the respirometer, a soil sample is exposed to a constant supply of oxygen, and oxygen consumption is monitored. Biological activity is calculated from oxygen uptake. They also used jar tests, in which biologically-active soil contaminated with petroleum hydrocarbons is placed in an air-tight jar for a period of time. Initial and final concentrations of chemical species of interest are determined, and compositional changes are used to determine degradation rates.

Rittmann and Johnson (1989) compared aerobic biodegradation rates of used lubricating oil under various conditions:

1. Soil plots vs. slurry bioreactors;
2. With and without application of an oil dispersant; and
3. With and without addition of acclimated microorganisms.

300-350 grams (dry weight) of moist silty clay loam soil placed in a 32 oz. cup was used as an experimental plot. Used motor oil was present at either 2% or 5%, oil-degrading bacteria were

added at 0, 7.2 x 10^7, and 5 x 10^8 organisms/g dry weight of soil. The oil dispersant Corexit 7664 was used on half the test plots.

Slurry reactors consisted of 2-L Erlenmeyer flasks containing 750 mL of a mineral medium, 94 g dry weight of soil, and 14 g of used motor oil. The flasks were continuously mixed on a shaker table. Biodegradation rates were generally an order of magnitude faster in the slurry reactors than in the test plots. The dispersant had no statistically significant effect on degradation rates in the test plots, but slightly increased rates in the slurry reactors. The addition of large amounts of acclimated microbes increased degradation rates in both environments. Examples of measured zero-order rate constants are:

soil or slurry	initial oil concentration	added inoculum	initial or long-term	degradation rate g oil/kg soil/day.
soil	2%	0	initial	5.4
			long-term	0.39
soil	5%	0	initial	8.5
			long-term	1.2
soil	2%	7.2 x 10^7/g	initial	4.3
			long-term	11.0
soil	5%	7.2 x 10^7/g	initial	1.2
			long-term	7.9
slurry	15%	0	initial	27.3
			long-term	4.4
slurry	15%	3 x 10^8/g	initial	61.4
			long-term	2.6
slurry	15%	3 x 10^8/g	initial	46.5
			long-term	3.3

Barnhart and Myers (1989) conducted pilot-scale testing of on-site bioremedition of oil tar residue at a former oil gasification-site. BTX, grease and PAHs are present in soil at the site. A clay-lined pit was filled with 4800 cubic meters of contaminated soil for the pilot test. A nitrogen source (type not specified) was added, and 1200 gallons bacterial suspensions were applied approximately four days per week.

Over an 8-week period contaminant levels decreased as follows:

contaminant	initial conc. (ppm)	final conc. (ppm)	% reduction
BTX	3.6	1.0	73
oil, grease	1538	990	36
PAH	335	151	45

Two- and three-ring PAHs decreased by 92% over 8 weeks. Four-ring compounds decreased by 80%, and five-ring compounds by 65%. Rate of biodegradation for PAHs was shown to be a function of ring number at this site.

On-site treatment of air stripper off-gas is required when concentrations of hydrocarbons or other volatile organics exceed safe or regulated levels. **Douglas *et al.* (1991)** evaluated the use of biofilm reactors for treating off-gases. Eight commercially available packing materials were compared. Six vertical PVC pipe columns 20.3 cm diameter x 2.1 m length were used. 1.5 m packing material was supported by pea gravel. Humidified air containing hydrocarbons was pressure-blown through each column at ≤ 0.1 m^3/min flow rate. Once packed, activated sludge liquor and groundwater containing hydrocarbon-degrading organisms were used to seed the columns.

Hydrocarbon retention due to sorption and solubility was investigated. Theoretical retention times were calculated as:

$$R = R_{tm} (1 + K_d/\beta)$$

Where:

R = retention time

R_{tm} = minimum retention time

K_d = vapor/liquid partition coefficient

β = phase ratio

The corrected partition coefficient (K) is calculated by dividing K_d by β. Values of Kd and K were determined for three packing materials using two hydrocarbons:

packing material	β	toluene K_d	n-heptane K_d	toluene K	n-heptane K
20/40 Ottawa Sand	1.7	3.3	0.010	1.96	0.0059
Peat/$CaCO_3$	2.0	3.33	0.010	1.67	0.0050
Fiberglass	7.2	3.33	0.010	0.46	0.0014

Removal of gasoline, toluene and benzene vapors was measured for different packing materials:

packing material	substrate	flow rate (M^3/min)	concentration (mg/L)	removal (%)
sawdust	gasoline	0.31	59.4	21.6
vermiculite/sludge	gasoline	0.31	52.8	8.5
sawdust/sludge	gasoline	0.31	63.2	5.2
peat	gasoline	0.31	55.5	30.6
sawdust/cow manure	gasoline	0.31	57.9	0
sand	gasoline	0.03	58.3	63.1
sawdust	toluene	0.3	271	11.8
vermiculite/sludge	toluene	0.3	321	45.1
sawdust/sludge	toluene	0.3	191	0
peat	toluene	0.3	304	92.7
sawdust/cow manure	toluene	0.3	237	13.9
sand	toluene	0.06	308	94.3
peat	benzene	2.1	76,400	70.1
sand	benzene	0.9	76,400	81.5

Steady-state was defined as stabilized effluent concentrations, and was reached after 1 to 4 weeks. Sand and peat appeared to be the best media for biodegrading the three substrates tested. Sand, peat with $CaCo_3$, and fiberglass as packings and gasoline as the substrate were used in a second set of experiments. Results are shown below.

Packing Material	Toluene % Removal	Benzene % Removal	Gasoline % Removal	Gasoline (µg/min-M^3)
Ottawa sand	98	95	40	11,225
Ottawa Sand	>99	95	54	15,850
Peat/$CaCO_3$	85	95	21	3,600
Peat/$CaCO_3$	96	97	61	11,545
Fiberglass	90	70	20	14,365
Fiberglass	83	69	-	-

Substrate removal appeared to be related primarily to mass loading, and not to airflow. Maximum degradation rates for benzene and toluene were about 53 to 78 mg/min-M^3. Maximum rates for *o*-xylene were 18-28 mg/min-M^3. The authors believe that low removal rates of total gasoline vapor may indicate that treatment of a vapor stream from soil venting of gasoline may not be feasible. Of the packing media tested, peat buffered with $CaCO_3$ is the lightest, least expensive and among the best packing for biological removal. At 40 mg/min-M^3 contaminant

removal rate, a portable treatment unit would have a maximum mass loading rate of 2,400 mg/min at 93 M^3/min gas flow rate.

van der Hoek *et al.* (1989) ran laboratory-scale bioreactor experiments to examine the feasibility of on-site treatment of soil contaminated with PAHs and BTEX at the site of a former asphalt production plant. The soil contained about 24,000 mg/kg total PAHs and about 700 mg/kg total BTEX. Groundwater at the site had 6100 µg/L total PAHs, 5700 µg/L total BTEX and mg/L concentrations of phenolic compounds. The site is about 1.5 hectare in area and contaminated to a depth exceeding 10 m. Therefore, remediation by excavation was deemed infeasible. Three types of bioreactors were evaluated: a trickling filter; up-flow aerated column bioreactors filled with polyurethane sponge as fixed-film biomass surface (UAC); and rotating disc biological contactors (RBC). On-site experiments used the trickling filter followed by the column; the laboratory experiments used a UAC followed by the RBC. In all cases, contaminated water was treated, not soil. In the laboratory experiments, water (quality unspecified) was percolated through contaminated soil, treated, then recirculated back through the soil in a closed loop arrangement. In the on-site experiments contaminated groundwater was treated in a once-through arrangement. This work simulates pump-and-treat techniques.

In the laboratory experiments the UAC had a volume of 24 L and an airflow rate of 3.4 L/min. The RBC had a total surface area of 15 m^2. The reactors were run parallel and in series. Aqueous flow rates through both reactors were 5.5-6.1 L/h. The UAC on-site was 30 L in volume and had a 2.7 L/min airflow and 60 L/h water flow rate. The trickling filter had a flow rate of 67 L/h.

Removal rates were 94-100% for all PAH and BTEX compounds for the laboratory system, with reactors running separately, parallel or in series. Volatization losses accounted for less than 10% of the BTEX reduction. Adsorption in the RBC accounted for no BTEX losses and 8.1% loss of total PAHs. Therefore, it was concluded that biodegradation is the dominant removal mechanism in the laboratory experiments. On-site system removals were 69-79% for the UAC and 76-80% for the trickling filter. About half of the on-site reduction was attributed to

volatization. Higher hydraulic loading rates were thought to be responsible for the lower degradation rates in the on-site system as compared to the lab system. The authors concluded that pump-and-treat bioremediation is suitable for heavily contaminated groundwater, and removal efficiency is dependant upon hydraulic loading rates; and that pump-and-treat remediation schemes for PAHs are slow, owing to strong adsorption of these compounds onto soil.

Case histories where bioaugmentation (addition of organisms for *in situ* or on-site bioremediation) were described by **Molnaa and Grubbs (1989).** Few details describing remediation procedures were provided. An abandoned oil refinery in Southern California had soil hydrocarbon contamination of 1500 to 30000 ppm. A consortia of organisms was applied, and the site was declared clean within one year. Other bioaugmentation projects described are a petrochemical tank storage area in Carson, CA; a diesel fuel-contaminated plot of land in Sacramento, CA; 1500 yards of diesel-contaminated soil at a truck stop in Sacramento, CA; and an on-going cleanup of 25,000 cubic yards of soil contaminated with lubricating and form oils.

On-site bioremediation of soil contaminated with high concentrations of petroleum processing wastes was studied by **Aprill *et al.* (1990).** In these lab-scale experiments, samples of Kidman sandy loam soil (Typic Haplustoll, Utah) were spiked with either API separator sludge or slop oil emulsion solids, two petrochemical waste products. This study followed the disappearance of PAH compounds, changes in Ames mutagenicity, and decreases in toxicity using the Microtox™ assay over a one-year period. A 12% loading rate was used (wet weight/soil dry weight x 100), and 200 g samples were incubated in 600 mL glass beakers. The soil had not been previously exposed to petroleum hydrocarbons, and the microbial population was considered unacclimated. The soil was mixed periodically to maintain aerobic conditions. Column studies were also run, in which glass columns (5 i.d. x 50 cm long) were filled with 912 g of Kidman sandy loam, 195 g of waste material was added to the top, and down-flow leachate was achieved by applying 8 cm of water head to the top surface of the soil. Fifteen pore volumes of leachate was collected from each column, and Microtox™ analysis was run on 1, 3, 5, 7, 9, 11, 13, and 15 pore volume samples.

Results indicated greater apparent degradation for lower molecular weight PAHs, and less degradation for higher molecular weight PAHs. Specific results are shown below:

parameter	API separator sludge	Slop Oil emulsion solids
initial waste conc. (mg/kg)	14,999	51,120
overall decrease (%) (day 354)	29	43
decrease of specific PAHs (%)		
naphthalene	100	100
fluorene	72	63
phenanthrene	54	43
anthracene	99	100
fluoranthracene	11	38
pyrene	35	43
benzo(a)anthracene	--	27
chrysene decrease	62	60
non-carcinogenic PAHs	71	64
carcinogenic PAHs	24	41

The lower molecular weight, more water soluble, less carcinogenic PAHs were degraded to a greater extent than the higher molecular weight, less water soluble, more carcinogenic PAHs. The authors believe that lower molecular weight PAHs can be utilized by microbes as carbon and energy sources, whereas higher molecular weight PAHs are degraded through co-oxidation processes. They cited earlier studies where degradation of carcinogenic PAHs was correlated with the presence of an oily matrix, but there was no such correlation with non-carcinogenic PAHs. **Aprill *et al.* (1990)** believe that their study demonstrated co-oxidation of carcinogenic PAHs during degradation of biodegradable oil and grease. There was no significant decrease in Microtox™ toxicity for either waste product during the 354-day incubation period.

Land treatment is the controlled treatment of soils and sludges to remove contaminants by spreading the contaminated material over an area of land. The process is described by **Lang and Joyce (1990)**. Most organic contaminant removal during land treatment is by biodegradation. About 100 sites in the US have been used for land treatment of refinery sludges. A common problem, according to **Lang and Joyce (1990)** is overapplication of hydrocarbons, or repeated application without sufficient time for degradation of previous hydrocarbon applications. This has resulted in groundwater and surface water contamination. **Lang and Joyce (1990)** described

land treatment of petroleum contaminated soil at a former oil terminal and tank farm used form 1918-1983 (location not disclosed). The terminal was built on 5-15 feet of loose, highly permeable fill, underlain by silt with low permeability. Total petroleum hydrocarbon levels of greater than 10,000 mg/kg were found at the site, and separate phase oil was observed in some observation wells.

Mechanical mixing and homogenizing of the soil was done after adding sewage sludge and liquid nutrients. Soils are tilled every two months. After one year of operation results are ambiguous but indicate some success in hydrocarbon removal (40-50% removal in some areas) The authors believe that their cleanup goal (not specifically stated) will be achieved after two years of operation.

Lynch and Genes (1989) conducted a pilot-scale full-scale land treatment remediation demonstration at a wood preserving contamination site in Minnesota. In the pilot study three different organic loading rates were tried: 2%, 5% and 10% benzene extractable hydrocarbons. The soil at the site (a fine sand) was contaminated with 1000 to 10,000 ppm total PNAs. Five polyethylene-lined test plots were constructed. pH was maintained at 6.0 to 7.0, carbon:nitrogen ratios were 25:1 to 50:1, and soil moisture was maintained near field capacity.

Total hydrocarbon losses over four months were approximately 40%, with a first-order kinetic constant of 0.004/day. Disappearance of 16 PNA compounds was monitored. Total PNA removals were greater than 62% over four months, and greater removal was consistently achieved for PNAs with fewer rings in their molecular structure. The full-scale system was then constructed by excavating 3-5 feet of contaminated soil from a 125,000 ft^2 area and lining the resulting impoundment with a 100 mil HDPE liner, and installing a leachate collection system. This became the treatment area into which contaminated soil was deposited for composting. Manure was used to supply nitrogen. Contaminated soil was added at a 5% benzene extractable hydrocarbon loading rate. A 3-inch lift of contaminated soil (1200 cubic yards) was mixed with 3 inches of native soil. Daily irrigation was required to maintain soil moisture near field capacity. Results of the pilot test and full-scale operation are shown below:

	average percent removal		average half-live (days)	
parameter	full-scale[a]	test plots[b]	full-scale	test plots
2-ring PAHs	95	93-95	<45	29-33
3-ring PAHs	95	83-85	45	46-49
4 and 5-ring PAHs	72	32-60	115	95-226
total PAHs	90	65-76	65	61-83
BE[c] hydrocarbons	60	35-56	150	106-202

[a] removal efficiency calculated after 193 days of treatment
[b] removal efficiency calculated after 126 days of treatment
[c] benzene extractable

PAHs with fewer rings were degraded more rapidly, and the full-scale effort performed as predicted based on the pilot study.

Laboratory and pilot field studies by **Harmsen (1991)** investigated the optimization of land treatment to remove mineral oil and pentachlorophenol from soil. In a reaction vessel, the rate of oxygen consumption was dependent upon the amount of added nutrients (nitrogen and phosphorus) during a ten-day period. Degradation rates were also increased by tilling the soil every four days. In the field trial, oil-polluted soil was spread in a layer 35 to 40 cm thick over polyethylene. Four treatment conditions were used: gas oil-contaminated soil with and without cultivation, and crude oil-contaminated soil with and without cultivation. Oil concentration in both gas oil plots decreased by 75% over about 90 days, and no additional decrease was apparent over the next two years. No benefit was noted from cultivating other than slightly elevated soil oxygen levels. The crude oil plots showed a similar pattern, with no apparent advantage to cultivation, but some oil increases were noted during the winter months. The increases were thought to be an analytical anomaly - during the winter, high boiling point hydrocarbons not detectable by the gas chromatographic method used are partially degraded, forming gas-chromatographable hydrocarbons, thus increasing the amount of hydrocarbons detected. The author believes that these results are consistent with previous studies, and that hydrocarbon levels of about 500 mg/kg petroleum hydrocarbons are easily achievable using land treatment, but with current land treatment practices levels below 500 mg/kg are difficult to achieve.

A land treatment demonstration project in Florida involving 850 tons of diesel contaminated soil was reported by **Brookner *et al.* (1988)**. The soil was excavated as the result of a leaking underground storage tank, and was transported to the Sarasota County landfill. Three small plots (12' x 10' x 12") and two large plots (50' x 60' x 12" and 50 ' x 160 ' x 12") were formed by spreading the soil. Soil for the small plots was mixed thoroughly before spreading. Three treatment combinations were applied to the five plots:

plot	size	treatment
1	10' x 12'	water only
2	10' x 12'	water and nutrients
3	10' x 12'	water, nutrients and bacteria
4	50' x 60'	water only
5	50' x 160'	water, nutrients and bacteria

Water was pumped from the landfill well (water quality not reported). Nutrients were ammonium nitrate and molasses. The applied bacteria was a proprietary strain of *Pseudomonas*. Test duration was 50 days, during which the plots were watered seven times. Nutrients and bacteria were added in the first two to three weeks of the study. The three smaller plots were tilled six times per week. The large plots were incubated as piles, and were only spread for watering. Results of hydrocarbon remediation appear below:

plot	t=0 ppm O&G[a]	t=3 wks % change	t=6 wks % change
1	3033	66	83
2	3267	77	83
3	3500	80	84
4	402	28	42
5	725	45	64

[a]oil and grease analysis, EPA Method 418.1

Removal rates of oil and grease in the small plots were equal, regardless of the treatment. The authors suspected that the high populations of native organisms, which had long been exposed to the diesel contaminants, assisted the applied organisms. Difficulty was encountered in obtaining representative samples from the large plots, which are heterogeneous with respect to contaminant concentration. Therefore the contaminant removal differences between these two plots may not be significant. The regulatory treatment goal of 5 ppm total petroleum hydrocarbons was not met in any plot. The authors compared costs for rotary kiln incineration,

landfilling and land treatment at the landfill, and concluded that the land treatment option was most cost effective.

Overland flow biological treatment was used to remove hydrocarbons from groundwater contaminated by several thousand gallons of gasoline in Delta, Ontario, Canada **(Devlin *et al.*, 1988)**. Benzene concentrations up to 926 µg/L were reported in several private wells. A purge well was installed where the highest hydrocarbon concentrations were detected. Contaminated water was aerated in sprinkler fountains and divided between overland flow down a grassy field with a 2% slope, and three channels containing either cut hay, *Hydrocharis morus-ranae* (a floating plant) or narrow-leafed rooted plants. All effluents emptied into an artificial marsh, which fed a creek. After seven months of purging, benzene concentrations of all private well were below 10 µg/L. Since considerable water was infiltrating, the shallow groundwater was monitored for volatile organics, which were only occasionally detected at low µg/L levels. About 75% of the dissolved volatile organics were removed by volatization in the aeration sprinklers, the balance was removed by a combination of biodegradation, adsorption and dispersion. No difference was noted in hydrocarbon removing abilities among the vegetation types used.

4.2 Anaerobic On-site Bioremediation

Few studies of anaerobic on-site bioremediation of petroleum hydrocarbons have been published, because hydrocarbons are generally more rapidly degradable aerobically than anaerobically, and supplying oxygen to recovered water or excavated soil is usually simple and inexpensive. Therefore, there is little incentive to design anaerobic on-site biodegradation processes. However, some studies comparing aerobic and anaerobic hydrocarbon degradation rates have been published.

Polycyclic aromatic hydrocarbon biodegradation rates were compared under aerobic, denitrifying and anaerobic conditions by **Mihelcic and Luthy (1988a and 1988b)**. Soil from an undisturbed subhumid grassland site in North Dakota was amended with nutrients and used in 50 mL microcosms made from glass centrifuge tubes. Microcosms were either aerobic, anoxic with nitrate or anoxic without nitrate. Degradation of naphthol, naphthalene and acenaphthalene

was monitored. Aerobically, all three compounds were degraded to below detection limits within 10 days in all microcosms. All three compounds were degraded to below detection limits within 45 days under denitrifying conditions. Only naphthol was degraded anaerobically without nitrate, and disappeared in 15 days. This work indicates that an on-site bioremediation scheme might work for removing anthracene and similar compounds under anoxic conditions, and successful removal is more likely if an alternative electron acceptor (in this case nitrate) is supplied.

Wilson *et al.* (1991) conducted a microcosm study of BTX biodegradation under aerobic and anaerobic conditions. They observed a one order of magnitude decrease in BTX concentration over 8 weeks under anaerobic conditions, and a two order of magnitude decrease in two weeks under aerobic conditions. This again demonstrates the more rapid biodegradation of hydrocarbons aerobically than anaerobically, but that anaerobic degradation should not be ruled out as a remediation strategy.

Lee *et al.* (1991) evaluated the use of anaerobic digesters for degradation of *o*-xylene. First, serum bottle tests were used to determine the concentrations of *o*-xylene that are toxic to an anaerobic digestion population. Thirty mL of a 4:1 fresh:digested, acclimated sludge was incubated at 35°C with concentrations of *o*-xylene from 100 to 5000 mg/L. Gas production from methanogenesis was monitored. The rate of *o*-xylene degradation rate was assumed to be proportional to the gas production rate. Doses over 100 mg/L were found to be inhibitory to the microbial population, with the effect increasing with concentration. Doses over 1000 mg/L were toxic to the microbial population. A second serum bottle test compared the inhibitory effect of *o*-xylene toward acclimated and nonacclimated sludge. 250 mg/L of *o*-xylene significantly stimulated gas production in acclimated sludge, but inhibited it in the unacclimated sludge. This test showed that *o*-xylene at normally inhibitory concentrations can be used as a substrate by acclimated sludge microbes.

Two bench-scale digestors were operated using *o*-xylene concentrations up to 1000 mg/L while two others (controls) received no *o*-xylene. Solids residence time was 15 days and temperature was constant at 35°C. At 1000 mg/L *o*-xylene the digesters failed completely,

indicating toxicity. After a period of recovery, three digesters were given 250, 500 and 750 mg/L *o*-xylene, and gas production in all three was similar to that of the control. After 145 days gas production in the 750 mg/L digesters began decreasing. No significant differences in performance were noted between the control and the two digesters with lesser *o*-xylene concentrations up to 160 days. Removal of *o*-xylene up to 62% was observed in acclimated digesters. This removal was thought to be from degradation rather than from adsorption, since removal was not observed in failed digesters. This work showed that significant removal of *o*-xylene can be achieved in anaerobic sludge digestors if acclimated sludge is used, and if *o*-xylene concentrations do not exceed an in inhibitory threshold, which was between 500 and 750 mg/L in this case.

4.3 Nutrient Addition to Enhance On-site Bioremediation

In order to optimize biodegradation rates, nutrients (usually nitrogen as nitrate or ammonia and phosphate) are often added to the petroleum hydrocarbon contaminated soil or water. The amount, type and method of addition are site- and process-specific. This section presents some recent examples of nutrient addition to enhance on-site bioremediation.

The use of liposomes for delivering nutrients to petroleum-degrading organisms to enhance on-site and *in situ* bioremediation was proposed by **Gatt *et al.* (1991)**. Liposomes are sealed vesicles (sacs) made from phospholipid membranes, and contain water. Mineral nutrients can be dissolved in the water, to be released inside microbial cells when intracellular enzymes disperse the phospholipid membrane. Oil-water interfaces are also affected physically by liposomes. The hydrophobic phospholipid exterior causes liposomes to act as surfactants, reducing the interfacial tension 1,000 to 50,000 fold. This allows trapped oil droplets to escape from soil micropores, coalesce and be transported through the porous media. Additionally, the presence of lyposomes makes oil more bioavailable. **Gatt *et al.* (1991)** found that adding liposomes to a bacterial population acclimated to petroleum degradation can increase bacterial populations by over seven orders of magnitude. Future use of liposomes for *in situ* bioremediation of petroleum-contaminated soil and for cleanup of oil spills is proposed by these authors.

Harder *et al.* (1991) evaluated the effect of adding nutrients to three types of soil in degradation rates of n-hexane in a laboratory bioreactor study. In a Loess soil naturally low in nutrients, addition of only nitrate, ammonia or phosphate slightly increased aerobic n-hexane degradation as measured by respirometer. A much more dramatic increase in respiration was observed with a mixture of phosphate, nitrate, iron, manganese and magnesium. It was found that a ratio of 60 mg nitrate-N and 6 mg phosphate-P would give optimum degradation rates for one gram of n-hexane. This N:P:C ratio agrees with findings of **Gibbs (1975)** for hydrocarbon degradation in seawater.

Enhancement of crude oil bioremediation by nutrient addition was practiced on portions of oil-contaminated beaches as a result of the *Exxon Valdez* spill in Prince William Sound, AK **(Glaser, 1991)**. Four types of fertilizer were tried: slow-release isobutylidene diurea briquettes; oil-encapsulated inorganic nutrients; and a liquid microemulsion; and a liquid water-soluble nutrient solution. Only visual results of the beach test plots were available at time of publication, but they indicated that most plots receiving fertilizer in some form were cleaner than non-fertilized control plots. Preliminary visual data could not determine which fertilizer type was most effective. **Lindstrom *et al.* (1991)** also tried applying fertilizers to accelerate bioremediation of the Exxon Valdez oil spill contaminants from beaches. They found that application of either a water-soluble fertilizer (Customblen 28-8-0) and an oleophilic fertilizer (Inipol EAP22) accelerated biodegradation of crude oil hydrocarbons in some beach ares, but not in others. Increased mineralization of hexadecane and phenanthrene were observed on most plots receiving fertilizer applications. These two studies show that application of commercially available fertilizers can increase the rate of oil-spill bioremediation, but that choice of fertilizer product, application methods and frequency require further research.

Bragg *et al.* (1992) prepared a comprehensive report on the effectiveness of intertidal shoreline bioremediation after the Exxon Valdez oil spill. Initial response efforts included cold- and warm-water washing and manual pickup of oil with rags. On-site fertilizer application was selected as the follow-up technique because it is relatively nonintrusive to wildlife compared to alternatives such as rock washing. No microorganisms were added, therefore the process used

was biostimulation, not bioaugmentation. Oxygen was not thought to be limiting at this site because the sediment is large-grained, and the seawater is constantly reoxygenated by wave action and tidal movement. Therefore, preventing nitrogen and phosphorus limitation was the focus of the remediation effort. A review of available literature showed that application of agricultural fertilizer or Inipol EAP22 (an oleophilic fertilizer, Elf Aquitaine) typically accelerates biodegradation of petroleum hydrocarbons in marine environments by three to ten fold. Microbiological studies showed that the tidal seawater contained about 103 microbes capable of growing on n-hexane per mL of water. Further tests showed that over 90% of Prudhoe Bay crude oil (the type spilled) could be biodegraded by native microbes within 10 days in laboratory microcosms at 15°C.

Saturated seawater column tests evaluated the feasibility of bioremediating subsurface oil. 100 kg of oiled sediment or rock was packed into a column. Water was pumped through to simulate tidal action. Oxygen consumption was monitored at different depths to estimate oil biodegradation rates. Columns were run with and without fertilizers (Customblen, Inipol liquid, and an inorganic fertilizer solution). A sterile control column was also established. Results indicated that both fresh and weathered oil should be amenable to bioremediation. Transport of oxygen and fertilizer nutrients was demonstrated to a depth of 3 ft in sediment from the site.

To quantify the effectiveness of fertilizers in the field study, samples of fertilized and nonfertilized sediment were taken periodically and solvent-extracted. Visual comparisons of solvent extract gas chromatograms showed that the chromatographable alkanes were degraded more rapidly in some fertilized plots than in control plots. By monitoring the concentration of hopane, an essentially nonbiodegradable hydrocarbon as a conservative tracer, mass ratios of biodegradable hydrocarbon species to hopane can indicate any decrease in biodegradable hydrocarbons. Gas chromatographable hydrocarbons and PAH declined logarithmically over time in fertilized plots, but did not decline in nonfertilized control plots. The hopane ratio test should be applicable to other spills.

Once the effectiveness and safety of fertilizers was established, large-scale application was begun starting in winter 1989. Inipol was applied only where surface oil was present, and Customblen was used only on subsurface oil. No fertilizer was applied to water near anadromous streams. Over 90 sites were fertilized, and 73% of these showed increased rates of oil attenuation after fertilizing. The most significant factor associated with the rate of biodegradation was the ratio of total nitrogen concentration to the concentration of oil on the beach material.

CHAPTER 5
IN SITU BIOREMEDIATION

By definition, in contrast to on site bioremediation, the land surface and subsurface are left generally intact during *in situ* bioremediation processes. As characterized by **Alfoldi (1991)**, in *in situ* bioreclamation one cannot select either the location or the space in which the bioreclamation processes take place. In contrast to on-site technologies involving bioreactors or pump-and-treat, *in situ* biotechnologies are applied to a subsurface space, which typically has one or more of the following properties:

1. Non-homogeneity,
2. Open to the atmosphere,
3. Diffuse or arbitrary boundaries, usually difficult to determine,
4. Presence of contaminant in multiple phases,
5. Nonsterile conditions, uncontrollable microbial makeup, or
6. Uncontrollable physical and chemical interactions among substances

Therefore, conditions are generally more difficult to control in *in situ* remediation than in on-site remediation. This tends to make *in situ* remediation schemes more challenging and results less predictable.

Lapinskas (1989) listed the following 11 characteristics of the contaminant(s) and site that must be known or evaluated before *in situ* bioremediation can proceed with reasonably predictable results: contaminant identification, contaminant quantification, contaminant solubility, contaminant biodegradability, soil permeability and transmissivity, nutrient availability, oxygen availability, temperature profile, moisture content, pH profile, and toxicity and inhibition.

Alfoldi (1991) describes geological factors that effect transport of water, contaminants and substances introduced into the subsurface during remediation efforts. The effects of sediment stratification and clay lenses on aquifer hydraulics are discussed. The main point of this paper is that successful *in situ* bioreclamation requires a complete characterization of the hydrogeological conditions of the site before remediation efforts are initiated.

5.1 New Subsurface Sampling Technology

Knowledge of the amount and distribution of petroleum hydrocarbon contaminants is required before *in situ* bioremediation (or any form of remediation) can commence. This requires representative sampling of the site, which is usually accomplished by coring. **Armstrong *et al.* (1988)** developed a core sampling device that yields a more representative core sample from unconsolidated sediment than traditional hollow-stem auger (split-spoon) sampling, especially in the saturated zone where intact cores are difficult to obtain. Basically, a piston is fitted inside the core barrel with air-tight tolerance achieved with neoprene seals. The piston remains stationary with respect to the land surface while the sampler is driven into the ground by impact. This creates a vacuum on the sample as it fills the core barrel liner, and the sample is thus held intact by vacuum as it is retrieved to the surface. Sample heaving during sampling of confined aquifers can also be controlled, as the authors demonstrated at a jet fuel spill site in Ontario, Canada.

An *in situ* core device was described by **Gillham (1989)**. Basically, a stainless steel column with an open bottom and screened top is driven into the ground to a desired depth. Water containing inorganic tracers or organic compounds is introduced at the bottom of the column and withdrawn from the top. *In situ* retardation factors and biodegradation rates can be determined by observing solute breakthrough curves. Gillham used the devices for determining aerobic and anaerobic degradation rates of benzene in the sandy unconfined aquifer at Canada Forces Base, Borden.

5.2 Aerobic *In Situ* Bioremediation

Oxygen demand can be determined for soil samples taken from a contaminated area prior to introducing oxygen to the site by venting or H_2O_2 application. **Powell *et al.* (1988)** compared four methods of oxygen demand determination using 17 contaminated and uncontaminated soil samples from two fuel-contaminated groundwater site. The methods used were chemical oxygen demand (COD); oil and grease analysis by freon extraction and infrared spectrophotometric measurement (O&G); total organic carbon (TOC); and a hydrocarbon gas chromatographic technique referred to as the fuel carbon gas chromatographic method (FC/GC). For the three methods where hydrocarbons were measured (TOC, O&G and FC/GC), the empirical formula of

$CH_{2.16}$ was used to represent hydrocarbons, and a stoichiometric amount of oxygen required to mineralize the hydrocarbon was used to determine oxygen demand.

The FG/GC method consistently gave the highest oxygen demand values, and the other methods had lower values, always in the same order:

FC/GC > O&G > TOC > COD

Differences were attributed to losses of volatiles from sample preparation and analysis, and the authors believe that the order of volatiles losses is:

COD > TOC > O&G > FC/GC

It was determined that the FC/GC method is most suitable for oxygen demand when a high percentage of hydrocarbons are volatile.

Vapor-phase transport in the unsaturated zone effects the distribution of petroleum hydrocarbons, which in turn effects hydrocarbon biodegradation rates. **Johnson *et al.* (1987)** examined the role of mass exchange between vapor and free-product phases, and between vapor phase and hydrocarbon-contaminated pore water in the unsaturated zone. Advection effects were studied by passing nitrogen through a column of damp sand which was given 2μL of 1:1:50 mixture of benzene, n-propylbenzene and dodecane. The mobile phase residence time was 1.2 minutes. Vapor concentrations in the column effluent were 75-80% of saturation, indicating rapid mass transfer between free product and vapor phases. Persistence of the three compounds in the column was dependent upon their relative vapor pressures: benzene would be removed in 100 pore volumes, n-propyl benzene in 1900 pore volumes and dodecane in 11,200 pore volumes. Diffusion experiments with methane, TCE and chlorobenzene in damp and dry sand were run by capping one end of a column, introducing hydrocarbons at the middle of the column, and observing hydrocarbon breakthrough at the open end. Experimental results were compared to 1-D transport model results. Differences in retardation factors between damp and dry sand were attributed to air-water partitioning of the hydrocarbons. Damp sand (10% of saturation) yielded retardation factors 1.7 and 3.2 for TCE and chlorobenzene, respectively. Calculated

values of retardation factors, based on Henry's law, binary diffusion constants and estimated tortuosity were 1.6 and 3.0 for TCE and chlorobenzene, respectively. This work showed that redistribution of volatile organics by advective or diffusive mass transfer can be deterred predictably by mass exchange between mobile (gas) and stationary (soil moisture) phases in the unsaturated zone. This has implications for *in situ* hydrocarbon removal in the unsaturated zone, since biodegradation rate is nearly always a function of substrate concentration.

Lieberman *et al.* (1989) used on-site and *in situ* bioremediation to remove residual hydrocarbons from a leaking fuel line at Camp Grayling Army Airbase, MI. A four-year program of pump-and-treat remediation followed by granular activated carbon treatment of groundwater failed to significantly reduce hydrocarbon concentrations at the site. Soil contamination of up to 16,000 mg/kg total petroleum hydrocarbons (TPHC) was present in an area 150 ft x 150 ft, 14 ft thick (11,700 yds^3). A plume of groundwater with up to 1046 µg/L BTEX extended 450 ft down-gradient. Recovery wells were installed to prevent further migration of the plume and to capture contaminated water for treatment, nutrient amendment and reinjection, thus establishing a closed-loop remediation system. The contaminated water was treated in two fixed-film bioreactors, then recharged through infiltration galleries. The intent was to stimulate biodegradation in the unsaturated zone by infiltrating bioactive, nutrient-rich, aerated recharge water. After seven months of operation BTEX and TPHC levels in the soil were both reduced by greater than 99%. TPHC in the most highly contaminated groundwater was reduced by 99.6%. The final cost of cleanup was estimated at $30 per cubic yard. This work demonstrated a successful combined on-site, *in situ* aerobic bioremediation strategy to remove petroleum hydrocarbons from soil and groundwater simultaneously.

Nielson-Cerquone *et al.* (1989) also used on-site and *in situ* bioremediation methods in combination at a 100,000-gallon gasoline, diesel and animal fat spill site in Oakland, CA. Underground storage tanks had been removed to facilitate the sale of a site formerly used for food processing and distribution. Free product gasoline up to three feet deep was observed floating on the groundwater, which was nine to ten feet below the ground surface.

Skimming was used to reduce the amount of free produce. Hydrocarbon-degrading microorganisms were injected into the contaminated groundwater. An air-injection system was attached to the product recovery wells to provide oxygen for in-situ bioremediation. 1200 cubic yards of contaminated excavated soil was treated by applying hydrocarbon-degrading organisms and watering, spreading and tilling. Hydrocarbons in two-thirds of the soil were reduced from as high as 20,000 mg/kg to less than 100 mg/kg, the requirement for disposal at a Class III landfill, in five months of treatment. At the time of publication, groundwater extraction and treatment had not yet commenced. The treatment-reinjection system cannot be developed until the extent of the free-product layer is known. The unsaturated zone is being treated by vapor extraction.

Aerobic *in situ* bioremediation may be inhibited by high concentrations of petroleum hydrocarbons. **Watts *et al.* (1989)** measured biodegradation rates of JP-5 jet fuel in sandy soil at a site on the mid-Atlantic seaboard (location not disclosed) at five initial concentrations between 187 and 1565 mg/kg over 120 days. Total viable heterotrophs and petroleum hydrocarbon-degrading organisms were counted using spread-plate techniques. Total petroleum hydrocarbons were below detection limits when initial concentrations were 187 or 487 mg/kg. In both cases total viable bacteria increased during the first 60 days, when hydrocarbon substrate was available, then declined when the hydrocarbon substrate was gone. Hydrocarbon concentrations did not decrease over 120 days when initial concentrations were 825 or 1565 mg/kg, but microbial population showed the same rise and fall pattern seen when lower initial substrate concentrations were used. The authors suspected that low degradation rates were due to limited bioavailability of hydrocarbons at higher concentration since a portion of them would be present as insoluble films and globules. They suggested that a toxic metabolite may be responsible for the decreasing bacterial counts seen consistently after 60 days. Although the specific mechanism is unclear, inhibition of biodegradation of petroleum hydrocarbons at high concentration was shown in this study.

A pilot-scale *in situ* bioremediation study is underway at an undisclosed former chemical manufacturing site **(Newell *et al.* 1990a)**. Benzene, cumene and other organic contaminants are present, and volatile organic concentrations up to 13,700 mg/kg soil have been observed.

Approximately 60,000 cubic yards of sandy fill must be treated to bring contaminant concentrations to below regulatory levels. Treatment options considered were excavation, *in situ* soil rinsing and *in situ* rinsing coupled with bioremediation. Excavation was ruled out since the site is covered by a thick concrete cap and riddled with abandoned pipes and concrete footings. The Bioplume II **(Rifai and Bedient, 1990)** and Oasis **(Newell *et al.* 1990b)** models were used to estimate remediation times prior to initiating pilot work. Three approaches were modeled: soil rinsing alone, rinsing with oxygen addition and rinsing with hydrogen peroxide addition. Modeled results, based on hydraulic conductivity of $k = 10^{-3}$ cm/sec, were:

remediation approach	assumed dissolved oxygen concentration in injection water	estimated remediation time (assuming $k = 10^{-3}$ cm/sec)	estimated relative cost compared to soil rinsing option
rinsing alone	0 mg/L	8 years	1
rinsing, oxygen	30 mg/L	3 years	0.6
rinsing, H_2O_2	100 mg.L	2.5 years	0.6

Based on modeling results, a decision was made to compare rinsing alone to rinsing with pure oxygen addition on a pilot scale. A system of rinsate extraction wells surrounded by water injection wells was installed at the site. One section of the site received oxygen-saturated water with added (unspecified) nutrients, the other section received chlorinated water with high pH (9.5-11).

At the time of publication, only TOC results were available. After 11 weeks, rinsate and site groundwater TOC were reduce by 60-80% for both oxygenated and non-oxygenated areas of the test site. It was concluded that most TOC removal was attributable to rinsing for both areas. Microbial analysis showed high concentrations of total organisms (10^4 to 3 x 10^6 CFU/g) in the oxygenated (biotest) area after the test. Cumene degraders were found in one area of the biotest area at 4 x 10^4 CFU/g. Total microorganism counts up to 10^4 CFU/gm and no cumene degraders were found in the soil-rinsing-only section. Results showed that microbial growth is

being stimulated by the addition of oxygen to the groundwater, and that bioremediation has potential for accelerating the cleanup and reducing costs at this site.

Von Wedel *et al.* (1988) reported preliminary results of *in situ* biodegradation using bioaugmentation with Pseudomonas isolates. Cultures were grown on single BTEX compounds as the sole carbon and energy source, and were then tested for resistance to 13 common antibiotics. Cultures resistant to antibiotics were destroyed, presumably so that microbes utilized for *in situ* bioremediation could be killed if necessary. Antibiotic-sensitive cultures were then expanded to commercial quantities. Blends of pure cultures can degrade complex fuel and lubricant products such as gasoline and motor oil. The mixed cultures are then injected into contaminated groundwater. A petroleum distribution site in California is using this method, which also includes an on-site bioreactor treatment of the contaminated water prior to bioaugmentation and reinjection. Preliminary results of several months of treatment show BTX removal of 33-98%.

Enhanced biodegradation of JP-4 jet fuel by soil aeration, nutrient addition and moisture optimization was investigated by **Hinchee and Arthur (1991)**. Contaminated and uncontaminated soil samples were taken from the spill site at Hill AFB, Utah. Microbial analysis by spread-plating showed that the contaminated area of the site had 10^4-10^6 CFU/g of hydrocarbon degraders through most of the soil profile down to a depth of 20 m. Background (uncontaminated) soil had low hydrocarbon-degrader populations, usually less than 10^4 CFU/g, and mostly occurring within the top 5 m.

Twelve columns (3.8 cm x 30 cm long) were packed with the contaminated soil and dosed with 500 mg/kg of JP-4. Three soil moisture levels were used: 25, 50, and 75% of field capacity (6.1, 12.2, and 18.3% soil moisture). CO_2-free air was passed upward through the vertical columns, and CO_2 was measured in the off-gas. Over 48 days CO_2 production was found to be consistently higher in columns with higher moisture content, and higher in columns receiving mineral nutrients. Sterilized control columns with nutrient addition produced CO_2 at a rate about equal to that of unsterilized, unfertilized columns. The authors assumed that CO_2 from sterilized

column was abiotic, and this contention was reinforced by the absence of viable organisms detected by spread-plating at the end of the experiment.

A pilot-scale field test of aerobic *in situ* bioremediation of aviation fuel was conducted by **Ward *et al.* (1988)**. Nutrients, oxygen and hydrogen peroxide were added to the saturated zone. *In situ* bioremediation is under way on a 30 x 100 ft plot of the Traverse City, MI aviation fuel spill. Major contaminants detected in the groundwater are benzene, toluene, and xylene isomers.

Nutrient requirements of indigenous microorganisms from core samples were tested in a factorial-design microcosm experiment, using with 40 different treatment combinations. Two mL of a soil suspension was added to 50 mL of groundwater, 0.05 mL of Aviation gasoline was added, and the microcosms were incubated for two weeks at 25°C. Cells were then harvested, dried at 37°C and weighed. The relative effects of treatment combinations were evaluated based on biomass accumulated after two weeks of incubation. Significant biomass production required both nitrogen and phosphorus, and iron (as $FeSO_4$) enhanced biomass production. In the pilot system only NH_4Cl, KH_2PO_4 and NA_2HPO_4 were used. The decision to exclude other nutrients was based on their high cost, relative to the small increases in biomass observed.

The BIOPLUME II model was used to make decisions regarding injection-well number and placement. Five injection wells, twelve multi-level cluster wells having four to six screened depths, and nine monitoring wells were installed. The model indicated that 40 gpm flow rate was required to keep the test area aerated during the experiment. Oxygen demand of 5590 mg/kg soil was estimated, based on organic solvent extraction of core samples. The model estimated that complete bioremediation should occur in six months with delivery of 33,890 lbs of oxygen. Liquid oxygen was used to increase dissolved oxygen levels to about 40 ppm initially. After three months H_2O_2 was used, and incrementally increased from 50 to 100 and then to 250 ppm. Oxygen is not breaking through to the down-gradient monitoring wells at shallow, more highly contaminated depths. This indicates oxygen consumption as a result of microbiological activity. Microbial counts using spread-plate techniques yielded approximately the same viable counts (about 10^5 CFU/g of soil) for both nutrient agar and inorganic agar supplemented with aviation fuel

vapors. During the three-month pilot study there was a slight increase, then a slight decrease in viable counts. Hydrocarbon remediation results are not yet available.

Ehrlich *et al.* (1987) determined total numbers of viable microorganisms and numbers of petroleum hydrocarbon degraders in water samples taken from a shallow aquifer contaminated with JP-5 jet fuel. A 1-hectometer area of the U.S. Marine Corps Air Station in Tuscin, Calif. was contaminated when jet fuel was deposited in unlined pits. The contaminated, locally perched, shallow aquifer is separated from the larger regional aquifer by a clayey confining layer, and the contaminants are apparently limited to the shallow aquifer, which discharges into a creek.

Water samples from an uncontaminated area contained about 1300 total cells per mL by Acridine Orange direct count, and less than 1000 of both total viable cells and JP-5 fuel degraders by plate count. In contrast, water samples from contaminated areas contained up to 13,000,000 total cells/mL, 1,200,000 total viable cells/mL and 1,000,000 JP-5 fuel degraders/mL. Nitrogen and oxygen were depleted in contaminated areas. The presence of methane and hydrogen sulfide suggested that anaerobic biodegradation was occurring once oxygen was depleted. The authors suggest that addition of inorganic nitrogen and oxygen may enhance the naturally-occurring biodegradation at this site.

Rainwater *et al.* (1989) are investigating the effect of altering water table levels on biodegradation of diesel fuel in the subsurface at the Kelly Air Force Base in San Antonio, Texas. They believe that oxygenation of the capillary zone can be achieved by sequentially raising and lowering the water table. This would be achieved by either injecting air into the unsaturated zone through injection wells surrounding the contaminated area, thus raising the water table in the contaminated area, or by injecting water into the saturated zone directly below the contaminated area, causing a water mounding effect. The authors favor the air-injection method because it results in oxygenation of the unsaturated zone.

Diesel-degrading organisms were isolated from contaminated sites and are being used in column experiments. Vertical PVC pipes 74-cm tall x 2.5-cm diameter were packed with 40-50

mesh dry sand. Columns were saturated with water containing about 3 x 10^7 cells/mL of diesel-degrading bacteria. Fifteen mL of diesel fuel was introduced at the top of the columns. Degradation was monitored in 9 columns with static water tables and 9 with cyclically altered water tables (15 cm over 48 hours). Initial experiments over three weeks failed to show differences between static and cycled water table conditions, but a 9-week experiment showed greater removal when the water table was cycled. Highest concentrations of diesel residue and microbes were found just above the water capillary zone in all columns. The authors plan to conduct similar experiments of longer duration.

5.2.1 Hydrogen Peroxide Addition

Aqueous hydrogen peroxide (H_2O_2) is sometimes injected into groundwater in order to achieve elevated dissolved oxygen levels. This section reviews literature describing H_2O_2 applications during *in situ* bioremediation.

The U.S. Air Force Engineering and Services Laboratory began a pilot-scale test of enhanced *in situ* biodegradation of JP-4 jet fuel at a spill site on Kelly AFB, TX. **(Downey, 1990)**. A large and adaptable bacteria population had been found at the site during a previous biodegradation study **(Heyse *et al.*, 1986)**. Up to 7 x 10^6 viable cells per g soil were present. Soil at the site was relatively impermeable, and delivery of nutrients and oxygen via injection wells was unsuccessful. Consequently, little biodegradation occurred.

A second site (Elgin AFB, FL) was selected which has sandy soil, a shallow aquifer, and 25000-30000 gallons of JP-4 contamination. Additional information about remediation at this site is given by **Downey *et al.* (1988)**. After most of the free product was removed, soil sampling and analysis determined that over 90% of the fuel was in the unsaturated zone. A soil vapor survey was performed to determine the extent and distribution of residual free product in the subsurface. Forty monitoring wells were installed, and the site was divided into a remediation area and a control area which received no remediation treatment. Laboratory microcosms indicated that the aromatic hydrocarbons could be removed in less than two weeks if adequate oxygen and nutrients were provided. Background DO levels were 0.5-1.0 ppm in the contaminated area.

The nutrient and hydrogen peroxide delivery system consisted of two shallow injection wells, infiltration galleries, and spray irrigation. Water from down gradient was amended with nutrients and hydrogen peroxide, then recharged through the unsaturated zone. After 18 months it was determined that the amount of fuel contamination at the site had not been reduced significantly. The author attributed the lack of remediation to inaccessibility of fuel in the soil micropores to oxygen and nutrients. At the Elgin AFB site, rapid decomposition of H_2O_2 occurred **(Hinchee and Downey, 1988)**, which shows that the use of H_2O_2 may not be economically viable at some sites.

Downey and Elliot (1990) evaluated the performance of several *in situ* soil decontamination technologies applied to sites on Air Force bases. The focus of their evaluation was accessibility of treatment media (liquid or gas) to the fuel contaminants. They suggest that many *in situ* remediation efforts fail because the treatment media never reach the fuel, even though lab and pilot trials were successful. Viscosity, vapor pressure and solubility of the fuel components and soil characteristics are cited as determining factors in fuel accessibility.

Huling *et al.* (1991) examined the feasibility of using hydrogen peroxide as a source of oxygen in bioremediation. Considering the stoichiometry of peroxide decomposition:

$$2\ H_2O_2 \text{ -----> } 2\ H_2O + O$$

it is apparent that 47.1% of decomposing hydrogen peroxide (by weight) is converted to oxygen gas. The authors used a laboratory study and a field evaluation to assess the viability of using hydrogen peroxide for bioremediation. Core samples were taken from the Traverse City, MI aviation fuel spill site and used to fill glass columns 4 cm i.d. x 18 cm long. Aqueous solutions of nutrients and hydrogen peroxide were pumped through the columns at a rate of 45.0 mL/h. Oxygen demand for the three columns influent increased with time, and leveled off at 75 mg/L after about 120 days. Approximately 89% of the influent available oxygen was recovered in the effluent and in gas-trapping tubes, and the balance was assumed to be consumed in abiotic and biological reactions. Oxygen demand values for the three replicate columns were 11.4, 10.4 and 13.5 mg/L. Mass balance calculations determined that 54% of the fuel hydrocarbons originally in the soil was degraded after 140 days. The ratio of oxygen consumed to hydrocarbon degraded

was higher than biodegradation stoichiometry would predict, and the authors attributed this to a combination of endogenous respiration, abiotic oxygen demand and errors in mass balance determinations. Up to 200 mg/L hydrogen peroxide was added to the influent after 140 days, and this concentration was reduced by >89% in all cases in the column effluent. The peroxide degradation was considered to be enzymatic, as evidenced by lack of significant peroxide degradation in an abiotic column using the same core material and influent solutions.

At the Traverse City, MI JP-4 jet fuel spill site water enriched with nutrient and oxygen was injected into five wells. A series of downgradient monitoring wells was also installed. The injection solution contained approximately 380 mg/L ammonium chloride, 190 mg/L disodium phosphate, and 190 mg/L potassium phosphate. Oxygen or hydrogen peroxide concentration was gradually increased in the injection solution to yield from 40 mg/L to 750 mg/L O_2. Available dissolved oxygen in excess of 35 mg/L was observed up to 33 meters from the injection point, indicating that the saturated zone throughout the site was being oxygenated. Unsaturated zone oxygen concentrations in the treated area were generally above atmospheric and ambient background levels, and concentrations as high as 78% O_2 by volume were observed. Hydrocarbon biodegradation rates for the field study were not presented.

Hydrogen peroxide enhancement of JP-5 aviation fuel and diesel fuel biodegradation was investigated by **Flathman *et al.* (1991)**. Columns packed with humus were spiked with up to 44% hydrocarbons, and sand-packed columns were spiked with up to 5.2% hydrocarbons. Additional columns were packed with soil from a temporary staging area for oil-contaminated soil, and were contaminated with JP-5, diesel fuel or waste lubricating oil. A mineral solution with hydrogen peroxide was fed through the columns in an upflow direction. Peroxide influent concentration was incrementally increased until leachate from a sampling port about 1/3 of the way up the column had 500 mg/L hydrogen peroxide. After 69 days (130 days for staging area soil) the columns were vertically sectioned and volatile organics was determined as a function of column depth. Aerobic heterothrophs and petroleum hydrocarbons were also quantified. Analysis of variance was used to test hypotheses about the effect of H_2O_2 on bacterial population density and

enhancement of hydrocarbon biodegradation rates. The effect of H_2O_2 was greater in soil contaminated with JP-5 and diesel oil than in soil contaminated with lubricating oil.

One advantage of hydrogen peroxide over molecular oxygen in enhanced biodegradation is that it can be applied in much higher concentration. However, if biotic or abiotic reactions lead to premature decomposition of H_2O_2 into oxygen and water, much of the oxygen is lost in off-gasing and the advantage of using H_2O_2 is lost. **Lawes (1991)** developed tests to determine the rates of biotic and abiotic decomposition in a given soil. Previous work **(Lawes, 1990)** had shown that exposing soil to 0.1% solutions of H_2O_2 in batch tests can provide information about the ability of a soil to degrade H_2O_2. In those tests, two ratios of 0.1% H_2O_2 solution to soil were tried: high (7.5:1) and low (1:3 or 1:4). The high ratio provided quick results and liquid residual H_2O_2 could be measured directly. The low ratio was a more realistic test, simulating saturated aquifer conditions, but gas collection was required to determine the amount of H_2O_2 degradation because supernatant volume that could be collection was low to nil. In the more recent work **(Lawes, 1991)** additional ratios of H_2O_2 solution:soil in completely mixed and static tests were evaluated. Static tests were run by applying H_2O_2 solution to soil in petri dishes. The objective was to develop a rapid test that would yield decomposition rates similar to those of the original low ratio test described above. A 1.9:1 ratio completely mixed test met this objective, and is recommended by the author.

A test was developed for delineating biotic from abiotic H_2O_2 decomposition. Autoclaving soil reduced or in some cases completely eliminated decomposition, and this was attributed to disabling the responsible enzyme systems. Remaining H_2O_2 decomposing activity of the soil was thought to be mineral in nature. The authors also tried phosphate application to inhibit abiotic decomposition, with the assumption that any remaining decomposition would be biotic. However, this was unsuccessful, because phosphate only partially reduces abiotic decomposition. Thus, the autoclaving method was shown to be superior for determining what portion of a soil's H_2O_2 decomposition activity is biotic.

Borden *et al.* (1989) used field and laboratory studies to study *in situ* biodegradation of polynuclear aromatics with varying concentrations of dissolved oxygen. Principal objectives were to explore the viability of using enhanced bioremediation on the site (oxygen supplementation), determine the minimum residual concentration of PNAs achievable after oxygen addition, and to test a numerical model of *in situ* biotransformation (BIOPLUME). Samples of groundwater from a creosoting site in Conroe, Tx with up to 3.5 mg/L polynuclear aromatics including naphthalene were used in laboratory biotransformation studies. Oxygen concentration was adjusted to either 1.8 or 8.0 mg/L in microcosms filled with contaminated water from the site. Rapid PNA removal occurred for both oxygen levels. Minimum oxygen concentration observed was 0.7 mg/L. Results indicated that DO levels as low as 0.7 mg/L would not inhibit aerobic biodegradation of PNAs in this site. Minimum levels of PNAs achieved were 5 to 30 µg/L for the individual compounds. These residual concentrations persisted even in the high initial DO microcosms, where typically 5 mg/L of DO remained. In field tests, contaminated, anoxic water was pumped from the shallow aquifer into two 55-gallon drums, spiked with chloride, deoxygenated and rapidly recharged. The water was then withdrawn at 50 mL/min. and analyzed for PNAs. The experiment was repeated aerobically by using air to increase DO levels of the injection water to 6.0 mg/L. Oxygen in the recharged groundwater dropped to below detection limits (0.8 ppm) within two days. Total PNAs attenuated slightly more rapidly than the conservative tracer chloride in the anaerobic field test, and much more rapidly during the aerobic test, where total PNA concentration decreased from 936 µg/L to 75 µg/L within 24 hours while chloride only decreased by 15%. The authors conclude that this comparison shows the importance of oxygen in biotransformation of PNAs, and that high rates of biodegradation are possible when both sufficient DO and an acclimated microbial population are present.

Barenschee *et al.* (1991) compared the effectiveness of hydrogen peroxide and nitrate as electron acceptors for *in situ* bioremediation of petroleum hydrocarbons. Columns were filled with washed sand spiked with soil from a contaminated site and 3,800 mg/L diesel fuel hydrocarbons. The 8 cm o.d. x 50 cm long columns contained 4.00 kg of soil, and water with either nitrate or dissolved hydrogen peroxide was pumped through the columns at a rate of 50 mL/h for 345 days.

In the H_2O_2 column, H_2O_2 concentration was increased stepwise from 50 to 250 mg/L, but oxygen breakthrough did not occur until day 230. At that time, CO_2 production decreased from the previous level of 50-70 mg/L in the effluent, pH began rising, and the assumption was made that the majority of contaminants had been degraded. In the nitrate-fed column, nitrate consumption was low (5-10 mg/L) as was CO_2 production (5-10 mg/L in the effluent). Solvent extraction of the soil at the end of the experiment indicated that 68.8% of the hydrocarbons had been biodegraded in the H_2O_2-fed column, compared to 7.8% in the nitrate-fed column. Using a mass-balance approach, ratios of electron acceptor utilized to hydrocarbon degraded (mass/mass) were 5.7 for H_2O_2 and 4.7 for NO_3.

Kinetic data for biodegradation in the H_2O_2-fed column was obtained by using several columns identical except in height. Three kinetic stages were observed:

1. Excess of hydrocarbons, limited oxygen,
2. Limited hydrocarbons, limited oxygen, and
3. Limited hydrocarbons, excess oxygen.

During the oxygen-limited stage, kinetics were zero order with respect to hydrocarbon, and hydrocarbon degradation rate was linear over time and dependent upon oxygen feed rate. 70% of initial hydrocarbon mass was degraded, and dissolved CO_2 measurements showed that it was completely mineralized to CO_2. During the third stage, the reaction was zero-order for oxygen, yet remained linear over time for hydrocarbon degradation. The rate of hydrocarbon degradation had decreased greatly. GC analysis at the end of the experiment showed that the residual hydrocarbons were mostly long chains with low solubility, and the rate of solution appeared to be limiting their rates of biodegradation.

Hydrogen peroxide was used to stimulate bioremediation at a diesel-fuel spill site in San Bernadino County, CA **(Frankenberger *et al.* 1989)**. Up to 1500 mg/kg of hydrocarbons was detected in the soil as a result of a leaking underground storage tank. The numbers of diesel-fuel degrading fungi and yeast were two to three orders of magnitude lower than the numbers of diesel-fuel degrading bacteria (about 10^6 cells/gram of soil), which were about equal to the numbers of glucose-utilizing bacteria.

Eighteen 8-inch I.D. boreholes were drilled to 5-10 feet below the water table, into which 2-inch diameter perforated PVC pipe was installed. The boreholes were sealed by 6 inches of bentonite 5-10 feet below ground surface. The space between the PVC pipe and borehole wall (unlined) was filled with pea gravel. This arrangement allowed for filling the borehole with nutrients by injection of nutrient solutions into the PVC pipe.

A series of nutrient injections were made at two-week intervals over four months. The soil at the site is sandy, and no permeability problems were encountered. The nutrient solution consisted of 250 mg/L NH_4NO_3, 100 mg/L K_2HPO_4, and 100 mg/L H_2O_2 (30%). Over a one-year period hydrocarbons were reduced to less than 1 µg/kg soil, the detection limit for the methods used.

Hydrogen peroxide is used by Cambridge Analytical Associates Bioremediation Systems (CCA) for supplying oxygen to contaminated groundwater and soil during *in situ* bioremediation **(Fogel *et al.* 1989)**. A retail gasoline site in Southern California was remediated using H_2O_2. Groundwater BTEX concentration up to 24 ppm and total petroleum hydrocarbon concentrations (TPH) up to 220 ppm were measured. The sandy-clay aquifer had a hydraulic conductivity of 10^{-6} to 10^{-5} cm/sec. Gasoline-degrading organisms numbered 2×10^4 to 3×10^5 per mL of groundwater. Preliminary microcosm testing showed that 20 ppm of BTEX and trimethylbenzene could be completely mineralized in two days given optimum oxygen and nutrients. At the field site ammonium and phosphate salts and H_2O_2 were recirculated through the contaminated area for 10 months. During the first six months average TPH was 15 ppm. The level fell to 0.1 ppm in the seventh month, and below detection limits thereafter. The number of hydrocarbon-degrading bacteria increased from 2×10^4 to 3×10^5 per mL in the extraction water.

Hydrogen peroxide and nutrient addition were used to bioremediate a 1000-gallon leaded regular gasoline spill at Catasaugua, PA **(Yaniga *et al.* 1989)**. A public supply well field 50-100 feet from the leaking storage tank was shut down. After recovering the phase-separated gasoline from the water table by pumping, venting was utilized to volatize hydrocarbons from the unsaturated zone, and *in situ* bioremediation was used to treat the aquifer. The native microbial

population was used (no bioaugmentation). Ammonium and phosphate were added. Remediation measures were terminated when total hydrocarbon concentrations in recovery wells dropped to 1 ppb, and the public water supply well field was placed back into service.

Harper and Williams (1987) attempted to remediate a gasoline spill site in northern Vermont by first recovering phase-separated product from the water table, then installing a soil venting system and a water pump-and-treat systems. However, they estimated that about 10 years would be required to clean up the site. A biofeasibility study indicated that addition of hydrogen peroxide and nutrients to the saturated zone would result in complete remediation in 1.5 years. Methods used to predict remediation times were not reported. The contaminated area consists of 20-25 feet of silty sand underlain by impermeable clay, and an area 200 feet x 250 feet is contaminated at levels above 1 ppm. Phase-separated product was present in some areas.

Hydrocarbon degrading bacteria were found throughout the site, and in high numbers in the most heavily contaminated areas. A 4:1 nitrogen:phosphorus ratio and 10-20 ppm dissolved oxygen was determined to be optimum for hydrocarbon degradation at this site. The delivery system design (not yet built at publication of this paper) includes four water extraction wells, a nutrient/oxygen addition system, and two recharge galleries. Extracted groundwater will be air-stripped, amended with H_2O_2 and nutrients and recharged through the galleries. No remediation results were reported, as the system has not yet been built.

5.2.2 Bioventing

Bioventing is a term applied to a number of related technologies having the common goal of delivering atmospheric oxygen to subsurface contaminants that can be aerobically biodegraded. Bioventing removes volatile compounds, such as BTEX components of gasoline, by a combination of *in-situ* air stripping and aerobic biodegradation.

Hinchee *et al.* (1987) compared the cost effectiveness of soil venting and groundwater extraction and treatment. Their analysis showed that capital and start-up costs for a 10 GPM

extraction system are about four times higher than costs associated with a typical venting system. In terms of operating costs, they point out that pumping air is much less energy-intensive than pumping water. This cost comparison excluded the effect of hydrocarbon degradation enhancement due to increased availability of oxygen. This cost comparison indicates that soil bioventing is a promising, cost-effective alternative to pump-and-treat methods for some sites.

Hinchee *et al.* (1989) described the process and effects of bioventing in general terms. They point out that most of the hydrocarbons at a fuel spill contamination site reside in the unsaturated zone and in the capillary fringe. They also noted that delivering oxygen to saturated portions of the site have met with limited success because fuel residues are occluded in small soil pores, and are inaccessible to bulk groundwater, which caries the oxygen. Soil venting is presented as a treatment alternative, and the authors state that 13 lb of air is required to degrade 1 lb of hydrocarbons. They recommend the use of dewatering wells at the edge of the contamination area to lower the water table, exposing more of the contaminated soil to air flow. Then some combination of vacuum extraction (negative pressure) and air pumping (positive pressure) is used to deliver oxygen to the contaminated unsaturated zone. The use of inorganic nutrients and optimizing moisture levels are also suggested.

In comparing bioventing to aqueous delivery of oxygen to the subsurface, **Hoeppel *et al.* (1991)** noted that 75,000 kg of water saturated with pure oxygen would be needed to degrade 1 kg of fuel hydrocarbons. If hydrogen peroxide was used, 12,000 kg of water would be required. Hydrogen peroxide is also bio-inhibitory at concentrations as low as 100 mg/L. Since air contains over 200,000 ppm oxygen, it is a logical oxygen delivery medium for bioremediation. Air has the additional advantage of diffusing through soil with low water permeability. A table of 10 reported bioventing applications is presented, and future research needs are described.

Optimization of biodegradation during bioventing decreases (or eliminates) the need for off-gas treatment. During a seven-month field trial at a jet fuel spill site in Tyndall AFB, FL, **Miller *et al.* (1990)** evaluated the relative significance of soil moisture content, inorganic nutrients, and temperature to biodegradation during bioventing. Soil moisture in the unsaturated zone were

relatively uniform during the study, and ranged from 6.5-9.8%. Within this range, biodegradation rate was not shown to be a function of soil moisture. Addition of inorganic nutrients also had no effect on degradation rates, and it was concluded that soil at the site in not nutrient-limited. Temperature effected biodegradation rates according to the van't Hoff-Arrhenius equation. Of the BTEX removed during bioventing, Up to 55% was biodegraded over the course of the study. Up to 82% biodegradation removal was achieved by decreasing venting rates to optimize oxygenation while minimizing volatization. Injecting hydrocarbon-contaminated off-gas into soil in uncontaminated areas proved to be an effective bioremediation method. A soil volume ratio of approximately 4:1 uncontaminated:contaminated soil would be required to completely remove BTEX from the soil gas at this site. Oxygen was not biologically rate limiting down to 2-4% in the soil gas. Therefore, biodegradation could be optimized in terms of air flow by maintaining oxygen at these levels. Further oxygen delivery would enhance removal by volatilization, but would not enhance bioremediation.

Herrling *et al.* (1991) investigated the use of the UBV vacuum vaporizer well system (German: Unterdruck-verdampfer-Brunnen, UVB) for bioventing. The UBV system uses a ventilator to produce negative pressure at the top of a vertical well. A vertical pipe within the well connects the atmosphere to a pinhole-plate below the water table, so that operating the ventilator causes fresh air to bubble through water in the well casing. The well is screened in the vicinity of the pinhole-plate so that oxygenated water can move laterally into the aquifer. A second screened area at the bottom of the aquifer allows for water to be supplied to the aeration zone from below, and a pump is sometimes used to aid this upward water movement. Air exiting the ventilator, which may contain volatile organic compounds, is filtered through granular activated carbon, then vented to the atmosphere.

Nutrients can be added to the water in the well casing as needed. Aquifer clogging can occur from bacterial growth and mineral precipitation, so pumping direction can be reversed periodically, which reverses decreases in hydraulic conductivity. The authors claim that this system will address three contaminant conditions: dissolved contaminants, contaminants associated with the porous media that can be mobilized by groundwater, and contaminants that

cannot be mobilized. The sphere of influence of a UVB well is determined by ventilation and water pumping rates and hydrogeological conditions. A method of calculating flow fields and dimensioning UVB installations is presented. The rate of natural groundwater flow is a key parameter in determining the extent and dimensions of UVB spheres of influence.

Hinchee *et al.* (1991) used bioventing to remove JP-4 hydrocarbons from a 100,000 L spill site at Hill AFB near Ogden, UT. Approximately 15,000 m^3 of vadose-zone soil was contaminated to a depth of 15 m with an average of 1500 mg/kg of hydrocarbons. Sixteen 15-m deep vent wells were screened from 10 to 50 feet. Initial hydrocarbon removal rates from venting was so high that the off-gas had to be diluted to below-explosive levels. Off-gas was treated by catalytic incineration. Venting rates were initially 44 m^3/h, and were gradually increased to a final rate of 2500 m^3/h as hydrocarbon concentration of the off-gas declined.

In order to quantify biodegradation rates, the venting operation was shut down three times, and rates of oxygen decline and CO_2 increase were monitored. Biodegradation rates based on CO_2 production only account for the amount of hydrocarbon completely mineralized, so biomass increase and metabolic intermediates were not considered. Oxygen uptake was used as an additional method of estimating biodegradation rates. The authors believe that the oxygen uptake method is more reliable, due to abiotic sources and sinks for CO_2. Between December 18, 1988 and April 1, 1989 2100 to 2200 kg of hydrocarbons (calculated by CO_2 production and oxygen consumption, respectively) was biodegraded. Initial degradation rates accounted for approximately 30% of total hydrocarbons removed, and this declined to a steady-state value of about 15%. Optimizing the biological removal is desirable, because it minimizes venting requirements and off-gas treatment requirements.

Dupont *et al.* (1991) investigated the use of SVE (soil vacuum extraction) to oxygenate subsurface areas contaminated with JP-4 aviation fuel. **Hinchee *et al.* (1991)** had reported that 15 to 25% of hydrocarbon removal at a JP-4 SVE site at Hill AFB, UT was attributable to biodegradation. Dupont and coworkers sought ways to optimize the SVE process for increasing hydrocarbon degradation at this site, where approximately 100,000 L of JP-4 was spilled into the

coarse sand and gravel aquifer. Soil near the leak was contaminated at levels of 1000-20,000 mg/kg. 15 vertical wells and 10 lateral wells were installed for vacuum extraction. 900 pore volumes (7,000,000 m^3) of soil gas were extracted initially to remove the most volatile components. During this time the authors concluded that significant biodegradation was occurring without enhancement, and that nutrient enhancement may increase biodegradation rate. Therefore, a series of laboratory column studies was conducted, and it was found that addition of nutrients and moisture would increase biodegradation rates.

At the Hill site, soil-gas analysis showed that the entire unsaturated contamination zone was aerated at near-atmospheric oxygen (i.e. 21%) at a vacuum extraction rate of 2100 m^3/h. However, to maximize biodegradation while minimizing volatization, 490-970 m^3/h rate was used. At this extraction rate oxygen was not limiting. The biodegradation rate in the field was measured by shutting down the venting system and monitoring changes in soil gas CO_2 and O_2 concentrations over a 10-14 day period. Moisture was increased in the field by surface spray irrigation to a level of 8-12% over the entire contaminated depth. Nutrients were surface-applied and delivered to the contaminated zone via irrigation. Application rate was determined by hydrocarbon concentration, and the carbon:nitrogen:phosphorus ratio 100:10:1 was used. First-order kinetics was assumed for O_2 utilization and CO_2 production.

Reducing the vacuum rate reduced volatization by 90%, but did not reduce biodegradation. Nutrient addition increased biodegradation rates. Oxygen uptake was found to be the preferred parameter for detecting effects of treatments (nutrients, moisture, flow modifications), since CO_2 interacted with the moisture, which was not held constant. With enhanced bioremediation, greater than 80% of the JP-4 hydrocarbons removed were attributable to biodegradation. Modifications in gas flow (reduced flow rate and maximizing flow paths) reduced volatization rates to below regulatory limits of 50 ppm total petroleum hydrocarbons, allowing direct discharge of vented soil gas into the atmosphere.

Miller *et al.* (1990) and **Miller *et al.* (1991)** investigated biodegradation of jet fuel in the unsaturated zone enhanced by soil venting. The objective of their research was to eliminate the

need for expensive off-gas treatment by reducing hydrocarbon emissions through biodegradation. A field investigation was conducted at Tyndall AFB, FL, where sandy soil was contaminated by 30 to 23,000 mg/kg jet fuel hydrocarbons. As **Dupont *et al.* (1991)** had found, an optimum venting rate could be established such that oxygen would not be limiting for the rate of biodegradation, and the off-gas hydrocarbon concentration could be below regulatory limits. Hydrocarbon removal, percent removal attributable to biodegradation, off-gas hydrocarbon concentration, oxygen concentration throughout the unsaturated zone, and expected time to reach remediation goals were all dependent upon the venting rate. As venting rates are reduced, the percentage of hydrocarbon removal due to biodegradation increases, and the percentage due to volatization decreases. But if venting rates are reduced to the extent that oxygen becomes biologically limiting, then both remediation mechanisms are operating at less-than-optimum rates. At the Tyndall Air Force site, two plots were amended with moisture and nutrients and vented at varying rates. The plots were identical except that one received water and nutrients throughout the 29 week venting period and the other had moisture added for only the last 14 weeks and nutrients for the last 7 weeks. Moisture and nutrients made no difference in biodegradation rates, indicating that the site is not nutrient deficient. Temperature was found to effect biodegradation rate, following a van't Hoff-Arrhenius relationship. For this site, optimal air flow conditions (0.5 air void volumes per day) resulted in 82% of hydrocarbon removal attributable to biodegradation, 18% to volatization.

Van Eyk and Vreeken (1991) used a gasoline-contaminated site in Holland to demonstrate that bioventing can achieve Dutch hydrocarbon standards. Maximum allowable mineral soil contamination is 50 mg/kg of dry soil for a 10% organic soil, and 50 µg/L for groundwater. Additionally, BTX compounds have limits of 0.2 µg/L each in groundwater. The site selected has a phreatic aquifer with water table approximately 2 m deep, with a confining layer 12 m deep, and is composed of medium-fine to medium-coarse sand. Preliminary results, based on CO_2 production, indicated that 0.2-0.4 kg/d carbon was being biodegraded during bioventing.

Urlings *et al.* (1991) described two bioventing sites, one a paint factory toluene spill near drinking water extraction wells and one a gasoline spill. The toluene site consists of fine sand

to gravel, with a water table 7 m deep. The soil is contaminated with up to 2200 mg/kg toluene. At this site, addition of moisture and nutrients were ineffective in increasing biodegradation rates.

The site of an abandoned liquid petroleum waste incinerator was evaluated for possible remediation by bioventing with nutrient addition **(Young *et al.*, 1991)**. The incinerator and surrounding open-air storage basins had leaked wastes into a perched aquifer overlying 12 to 15 meters of clay. Laboratory studies were undertaken in which disappearance of PAHs was monitored and isolation and identification of PAH-degrading organisms was attempted. Plexiglass columns (size not reported) were filled with 170 g of the sandy soil characteristic of the site. Air was passed through the columns at 0.79 L/min for 20 days. PAH content was determined for the sand at t=0, 10 and 20 days. Viable organisms were counted using a spread plate technique in which soil extract was provided as the organic substrate.

As a biodegradation experiment, the experimental design met with limited success, since about 80% of the PAH attenuation was attributable to air stripping. Typically, about 50% of the PAH in the soil was removed in 10 days, with little additional removal in the subsequent 10 days. Disappearance of fluorene and about half of the disappearance of anthracene and phenanthrene were biological, however. The addition of nutrients appeared to increase the biodegradation rates of fluorene, anthracene and phenanthrene, but it was difficult to determine the extent of enhancement due to the confounding variable of removal by air stripping.

5.2.3 Carbon Isotope Analysis

Inaccuracy in estimates of aerobic biodegradation rates based on CO_2 production can be introduced by abiotic CO_2 sources and sinks, and by biodegradation of naturally-occurring organics in soil and groundwater. Isotopic carbon analysis can be used to minimize these errors. **Hinchee *et al.* (1991)** measured ^{13}C and ^{12}C of CO_2 generated during bioventing of a JP-4 fuel spill site. $^{13}C/^{12}C$ ratios (R) of CO_2 samples from the site are compared to ratios for a standard. $\delta^{13}C$ is calculated as:

$$\delta^{13}C = [(R_{sample} - R_{std})/R_{std}] \times 1000.$$

$\delta^{13}C$ values are much higher for atmospheric CO_2 and carbonate rock than for CO_2 from petroleum and coal. Therefore, determining the $\delta^{13}C$ value for CO_2 at a hydrocarbon-contamination site can provide evidence for the presence (or absence) of biodegradation, and can indicate the relative CO_2 contributions from biotic and abiotic sources. The $\delta^{13}C$ calculation method was used by **McMahon *et al.* (1990)** in determining that CO_2 produced in South Carolina coastal plains sediment up to 400 m deep was microbially mediated.

Aggarwal and Hinchee (1991) determined the $\delta^{13}C$ for three jet fuel-contaminated sites: Hill AFB, UT, Patuxent River Naval Air Station, MD, and Tyndall AFB, FL. All three sites were aerated with atmospheric air. In all three cases the $\delta^{13}C$ values were consistently lower in soil-gas CO_2 from contaminated areas than from uncontaminated areas:

site	depth (m)	uncontaminated soil $\delta^{13}C$	Contaminated soil $\delta^{13}C$
Tyndall	0.3-1.0	-18.1	-21.1
Hill	3-18	-23.6	-27.4
Patuxent	4.5	-24.4	-27.6

Although $\delta^{13}C$ levels are always higher in fuel-contaminated areas of spill sites than in uncontaminated areas, it is evident that $\delta^{13}C$ values between different sites are not directly comparable. This is due to differences in vegetation, contact with atmospheric CO_2 and background organics composition in the soil between sites. Differences in carbon-fixing pathways are associated with differences in $\delta^{13}C$ values. Temperate climate plants use predominantly a C-3 cycle of carbon fixation which gives $\delta^{13}C$ values of about -25, and tropical and subtropical desert and grassland plants use a C-4 pathway which yields $\delta^{13}C$ values of about -12. Therefore, the important parameter in assessing the extent of petroleum hydrocarbon degradation is the difference between $\delta^{13}C$ values of uncontaminated and contaminated areas of a spill site. In the three sites studied here, differences were consistently about 5 on the $\delta^{13}C$ scale.

The source and movement of CO_2 above a solvent-contamination plume in groundwater was monitored by **Suchomel *et al.* (1990)**. The authors suggested using CO_2 as an indicator of nonvolatile petroleum hydrocarbon contamination, provided that other major sources of CO_2 are

not significant. The major organic contaminant at the site studied (in Phoenix, AZ) was TCE and TCA, which were released by dumping and storage tank leakage between 1963 and 1982. As much as 757,000 L of chlorinated solvents were spilled, resulting in up to 1,000,000 ppb VOCs in the groundwater. Two wells were screened above the water table, and soil gas samples were analyzed for carbon isotopes in the CO_2. $\delta^{13}C$ values for uncontaminated and contaminated areas were compared. Additionally, the ratios of ^{14}C to total C were determined, and results were reported as percent modern carbon (pMC).

CO_2 from uncontaminated areas had $\delta^{13}C$ values of -17.97 to -19.92. pMC levels of 106.6% to 112.3% were observed, indicative of plant root respiration. No distinct vertical trends in carbon isotope composition were noted in the uncontaminated areas. In the contaminated area CO_2 concentration exceeds 15%, and oxygen was present, but as low as 1%. No methane was present, indicating that conditions were aerobic. $\delta^{13}C$ values were -23.27 to -24.70, consistent with values for petroleum products. ^{14}C levels are 7.7-8.6%, extremely low for soil gas. Organic solvents are devoid of ^{14}C due to the age of the petroleum from which they are made. This is because atmospheric ^{14}C is produced by electromagnetic radiation when one proton of a nitrogen atom is replaced by a neutron. This conversion takes place at a constant, low rate, and is balanced by the constant decay of ^{14}C, yielding a constant $^{14}C/^{12}C$ ratio in the atmosphere. However, once incorporated into organisms, the ^{14}C decays but is not replenished. Organic solvents are generally made from petroleum, from which all ^{14}C has long since decayed.

This study demonstrated the applicability of the $\delta^{13}C$ and pMC determinations for detecting the presence of CO_2 derived from biodegradation of petroleum products. It also reinforced the need to obtain background values of $\delta^{13}C$ from uncontaminated areas of the site, since interpretation of the $\delta^{13}C$ data requires background values, which vary greatly from site to site. In this study both $\delta^{13}C$ and ^{14}C measurements indicated that aerobic biodegradation was occurring in areas contaminated by organic solvents.

5.3 *In Situ* Bioremediation under Anoxic Conditions

In situ bioremediation can be achieved in the absence of molecular oxygen, providing that an alternative terminal electron acceptor is naturally available or provided in the remediation process. Such conditions can be termed anoxic, since molecular oxygen is not present. A variety

of electron acceptors can replace molecular oxygen under anoxic conditions. Typically, when a mixture of electron acceptors are available, the microbial community consumes them in order of their oxidation potential, the most oxidized being consumed first. Therefore, a typical sequence of electron acceptor utilization would be O_2, NO_3^-, Mn^{3+}, Fe^{3+}, SO_4^{2-}, CO_2 **(Mikesell *et al.* 1991)**. Other metal ions, including gold and uranium, can serve as terminal electron acceptors **(Lovely *et al.* 1991)** during anaerobic biodegradation.

Biodegradation of an aromatic hydrocarbon mixture under denitrifying conditions was evaluated by **Trizinsky and Bouwer (1990).** Using laboratory-scale batch reactors, degradation rates of benzene, toluene, *m*- and *o*-xylene, phenol, and chlorobenzene were monitored over 72 days with and without acetate as an additional substrate. Nitrate was present in excess, and no other electron acceptor (e.g. O_2 or H_2O_2) was provided. Each aromatic component was present at approximately 2 mg/L. Sequential utilization of substrates was observed. Without acetate, the order of utilization was: *m*-xylene, toluene, phenol, benzene, chlorobenzene, and then *o*-xylene. With acetate present the biodegradation order was: toluene, *m*-xylene, phenol, benzene, *o*-xylene, and then chlorobenzene. The authors believe the difference in substrate preference is due to selection of different bacterial populations when acetate is present as an additional substrate. All aromatics were reduced to below detection limits within 25 days in the presence of acetone, and in 47 days without acetone. No degradation products other than CO_2 were observed. This work shows that nitrate is a potential alternative to oxygen for *in situ* biodegradation of aromatic hydrocarbons.

Hutchins and Wilson (1991) compared JP-4 jet fuel decomposition rates in microcosms to those observed at a field site, under denitrifying conditions. Microcosms consisted of 10 g samples of cores from the Traverse City site in 12 mL serum bottles with 3.5 mL headspace. Aerobic, anoxic nitrate amended, and anoxic conditions were used to degrade a mixture of benzene, toluene, *m*- and *o*-xylene and 1,2,4-trimethylbenzene. Initial concentrations of hydrocarbons were 1 to 4 mg/L for each compound. All compounds were completely degraded within 5 days under aerobic conditions, and zero-order rate constants were >0.3 mg/L/day. Under denitrifying conditions, benzene was not degraded after 100 days, and *o*-xylene degradation was

incomplete (about 90% removed after 20 days, with no further removal up to 100 days). The other four compounds were completely removed after 20 days.

In the field demonstration, a 10 x 10 m section of the Traverse City, MI JP-4 jet fuel spill site is being treated with nitrate. Recirculated groundwater, supplemented with nitrate and nutrients is infiltrated through the hydrocarbon-contaminated zone of the aquifer. Groundwater is then sampled at six depths above, in and below the contaminated zone and monitored for oxygen, nitrate and BTX compounds. The ambient water table, which was near the bottom of the contaminated zone, was raised to above the contaminated zone by infiltration. Nitrate consumption commenced when oxygen concentrations and consumption were low. Over the 120-day experiment approximately 55 kg oxygen and 500 kg nitrate-nitrogen were consumed, coupled with 18 and 450 kg BTEX removal, respectively. Most of the oxygen consumption occurred before the microbial community was acclimated to denitrification (about 20 days). The assumption that denitrifying activity was responsible for the BTX reduction was supported by a decrease in nitrate and increase in nitrite with depth in the infiltration area and increase in denitrifier populations. Mass balances indicated that sufficient nitrate was consumed to account for the reduction in BTX, but oxygen consumption was insignificant compared to BTX reduction.

Zero-order rate constants (mg/L/day) for BTEX biodegradation were determined for laboratory microcosms and for the field demonstration:

zero-order rate constants (mg/L/day)

laboratory compound	laboratory aerobic	field denitrifying	laboratory denitrifying
benzene	>0.5	not degraded	not quantified
toluene	>0.4	0.20	0.32
ethylbenzene	>0.3	0.13	0.15
m-xylene	>0.3	0.14	0.47
o-xylene	0.3	0.13	0.11
1,2,4-tri-methylbenzene	>0.2	0.07	not quantified

Close agreement between microcosm and field rate constants indicates that hydraulic and compositional equilibrium had been reached in the field recycling of groundwater, and that in the field toluene and xylene removal by denitrification was much more significant than other potential removal mechanisms, such as sorption, volatization or abiotic degradation.

Nitrate remediation of groundwater contaminated by gasoline hydrocarbons was investigated by **Berry-Sparks and Barker (1987)**. This field study focused on the anaerobic removal of BTEX compounds via nitrate reduction. Two 2500 L dissolved gasoline plumes were created in a shallow, sandy aquifer at Canada Forces Base, Borden. One was remediated with 2400 L of water containing 44.9 mg/L N as NO_3 and 2000 mg/L chloride. The other plume received 2400 L of unamended groundwater, and was used as a control. Nitrate migrated downgradient faster than BTEX compounds due to retardation of the organics. Nitrate moved at the rate of a conservative tracer (chloride in this case). Previous laboratory experiments indicated that a lag time was required for acclimation to denitrification conditions. Removal of BTEX appeared to be about the same in remediated and unremediated plumes over the 148-day experiment. Some specific compounds (toluene, ethylene benzene and *m*-xylene) were significantly better removed from the remediated plume. No nitrate losses were observed in the remediated plume, indicating that denitrification was insignificant. Apparently most or all of the hydrocarbon degradation took place under aerobic conditions, and the denitrification process was limited by the lack of acclimation time, low concentrations of hydrocarbons when conditions became anoxic, and differential migration of BTEX compounds and nitrate.

Anaerobic *in situ* biodegradation has been reported for halogenated hydrocarbons. In a bench-scale reactor study, pentachlorophenol (PCP) was degraded almost as rapidly anaerobically as aerobically **(Campbell *et al.* 1989)**, and a pilot field test confirmed that a PCP-contaminated site was a viable candidate for anaerobic *in situ* bioremediation in both the saturated and unsaturated zones. A considerable body of literature exists covering anaerobic degradation of halogenated solvents, but this literature is outside the scope of this Review. The subject was recently reviewed by **Chaudhry and Chapalamadugu (1991)**.

5.4 Nutrient Addition to Enhance *In Situ* Bioremediation

Nutrient limitations can result in less-than-optimum *in situ* bioremediation rates. In addition to an electron acceptor, nutrients are often added as aqueous solutions via injection wells during *in situ* bioremediation. Literature reviewed in this section addresses the subject of optimizing bioremediation rates by adding nutrients which are in limiting concentrations in some underground

contaminant sites. It is apparent from recent literature and from the Technology Review by **(Riser-Roberts, 1992)** that inorganic mineral requirements for petroleum hydrocarbon degradation have not yet been clearly established, and may be variable and site-specific. Several laboratory and field investigations have been undertaken and are underway to quantify the inorganic nutrient needs of subsurface microbes. Two problems often encountered are difficulty in delivering nutrients to the contaminated areas, and avoiding inorganic precipitation reactions which can clog pores and greatly reduce the hydraulic conductivity of the subsurface. Furthermore, simply adding inorganic nutrients to a contaminated site will not necessarily increase the remediation process.

Harder *et al.* (1991) added nitrogen (either NO_3^- or NH_4^+) and phosphate to two soils, a German Loess and an Israeli soil referred to as Newe Ya'ar. The soils were coated with n-hexadecane (7.0 to 14.4 mg/g dry soil), and aerobic biodegradation of n-hexadecane was observed in a Warburg apparatus. Oxygen consumption and CO_2 generation were monitored. Results showed that greatly enhanced initial degradation could be achieved with either PO_4^{3+} and either NO_3^- or NH_4^+, and that degradation rates decreased when nitrogen became limiting. When excess nitrogen was available, n-hexadecane continued at a high rate until the substrate became limiting. When more n-hexadecane was added, degradation (as measured by oxygen consumption) resumed. Optimum mass ratios of 60:6:1 for nitrogen:phosphorus:n-hexadecane were determined, and lower nutrient ratios resulted in slower degradation, higher ratios did not increase degradation rates.

Morgan and Watkinson (1990) evaluated the effect of supplying inorganic nutrients to three petroleum hydrocarbon contaminated sites to stimulate biodegradation. Sites A, B and C were contaminated with crude oil, lubricating oil and gasoline, respectively. The soil at each site was sandy, and low in inorganic macronutrient and non-petrogenic organic concentrations. Site A was comprised of mostly high molecular weight polyaromatic and aliphatic compounds, with little vertical penetration of hydrocarbons. An apparent enrichment in polar compounds suggested that biodegradation had been occurring. Site B hydrocarbons consisted of mostly aromatic compounds with some aromatics and polars, and the entire vadose zone was contaminated in

addition to a liquid hydrocarbon phase at the water table. Higher molecular weight compounds predominated near the soil surface, suggesting that biodegradation may have occurred. At site C, BTX compounds were prevalent near the leaking gasoline storage tank, and similarity of composition between gasoline in the tank and hydrocarbons in the spill area implied that little or no meaningful biodegradation had taken place.

Microbial counts of 10^8 and 10^7 total cells per gram of soil were determined by acridine orange direct count (AODC) for sites A and C, respectively. Significant (numbers not reported) numbers of hydrocarbon-degrading organisms were found at all three sites. Degradation rates of 5-gram soil samples were determined in microcosms, in which disappearance of phenanthrene, anthracene, naphthalene and hexadecane was monitored. Untreated samples had degradation rates equal to or greater than rates in samples treated with various combinations of K^+, NO_3^-, NH_4^+, PO_4^{3-}, and urea. Microbial inhibitory effects were also noted when glucose was used as the carbon source. The authors concluded that Site A should be cleaned up by physical methods, Site B would be suitable for biotreatment after removing the liquid hydrocarbon phase, and Site C should be vented before using biotreatment as a polishing stage. This study is significant in that biodegradation was not enhanced as a result of adding inorganic nutrients, and it showed that preliminary laboratory studies are needed to determine whether additional nutrients are needed at enhance degradation at a contaminant site.

Song, *et al.* (1990) investigated the effect of adding nitrogen and phosphorus fertilizers to sand, loam and clay contaminated with various fuel oils. Nutrient levels in untreated soils were not listed, so it is not clear whether rates of biodegradation were limited by availability of nutrients. With fertilizer treatment, half-lives of contaminants were affected as follows:

Unleaded gasoline: little effect
Jet fuel: half-life decreased > 50%
Heating oil: half-life decreased 0-50%
Diesel oil: half-life decreased > 50%
Bunker C (heavy oil): Little or no effect

This study demonstrates the need to investigate the effects of adding supplemental nutrients to contaminated soil on biodegradation rates in small-scale lab studies before proceeding with nutrient enhancement in the field.

Swindoll *et al.*, (1988) evaluated the effectiveness of amending biologically-active subsurface soil samples with inorganic nutrients. Soil samples were collected from 1-2 m below the water table from an uncontaminated aquifer near Lula, OK. Mineralization of toluene and three other compounds by ambient microbes was measured in sealed vials by monitoring CO_2 production. Addition of nitrogen or phosphorus had no significant effect on cell numbers (as determined by acridine orange direct count) or on toluene mineralization. Addition of a mineral salt mixture containing nine essential inorganics stimulated cell production, but not toluene mineralization. The authors suggested that sufficient minerals were available in unamended soil for optimal toluene degradation, and that the amendments stimulated nondegraders. Nitrogen and phosphorus concentrations in the Lula Aquifer had been reported previously, as follows (in milligrams per liter): P_i, 0.12; NH_4^+N,<0.05; and NO_2^- plus NO_3^-N, 0.8.

Aggarwal *et al.* (1991) developed phosphate nutrient solutions that minimize aquifer plugging due to precipitation when used in conjunction with oxygen addition via H_2O_2. Twelve nutrient solutions used for *in situ* bioremediation were evaluated, and phosphate concentrations ranged from 19 mg/L to 52,000 mg/L. Soil pore plugging is caused by an increase in solubility of redox-sensitive elements such as Fe of Mn. This happens in biologically-active organic contamination areas, where microbial activity reduces pH, solubilizing these elements. Addition of oxygen and phosphates then results in precipitation of metal hydroxides and phosphates, causing aquifer plugging. Geochemical models can be used to predict the extent of precipitation upon addition of a nutrient **(U.S.E.P.A. 1988)**. These models determine the over- or undersaturation of an aqueous solution (e.g., groundwater) based on solubility products and ionic concentrations in solution. The U.S.E.P.A. program MINTEQ **(U.S.E.P.A. 1988)** was used to estimate precipitation during an *in situ* bioremediation operation at Eglin Air Force Base, Fla. **(Hinchee *et al.* 1989)**. Modeling indicated that for every weekly application of nutrient solution,

3 cm of soil depth will have its porosity decreased by 50%. Modeled results were consistent with field observations.

Laboratory column tests prescribed by manufacturers of nutrient formulations can underestimate the precipitation potential of their products. Typically, a column is filled with a composite of soil from the site, and deionized water containing the product is recirculated through the column. Phosphate breakthrough rate and permeability decrease are used to evaluate the precipitation potential of the product. However, phosphate precipitation is time-dependent, and there is more time for precipitates to form in the field over months of nutrient application than in quick laboratory column tests. **Aggarwal *et al.* (1991)** suggest that geochemical modeling calculations provide more reliable predictions of phosphate and hydroxide precipitation than short-term column tests.

Orthophosphate in excess of microbial requirements is often added to increase the stability of H_2O_2 in groundwater. The authors suggest adding only the amount of phosphate necessary for optimum biodegradation, although they acknowledge that optimum phosphate requirements in oxygen-limiting aquifer conditions are not well known. They suggest that 10 mg/L orthophosphate may be sufficient for microbial demands and low enough to avoid precipitation problems, but much higher concentrations are needed at the point of injection in order to insure $\geq$10 mg/L phosphate throughout the contaminated area. Polyphosphates are more soluble in solution than orthophosphate, and gradually hydrolyze into orthophosphate, with rate dependent upon pH, ionic composition of the solution, microbial activity and polyphosphate chain length. Thus, polyphosphates can be injected as "slow-release" orthophosphate. Trimetaphosphate was found to adsorb less to soils that other polyphosphates, and is more soluble. In a 450 hour lab-scale aquifer simulation test, hydraulic conductivity did not decrease and phosphate precipitation was not excessive in contrast to trials using orthophosphate.

Davis-Hoover *et al.* (1991) describe the use of hydraulic fracturing to help deliver nutrients throughout a contaminated site. A fluid is pumped into a vertical borehole under high pressure, and at a critical pressure a horizontal fracture is nucleated. A granular material, termed a

proppant, is then injected, carried by a viscous gel. The proppant is usually sand, but can be nutrient or oxygen-releasing granules. The gel breaks down into a thin fluid and is recovered. The proppant remains, holding the fracture open. Field tests of the fracturing process in unsaturated glacial drift produced about 19 fractures, stacked at various depths, roughly parallel to the ground surface. Vertical spacing between fractures is about 15 cm. Fractures had maximum thickness of 1.3-1.4 cm and were filled with sand. The authors propose using the fractures for injecting fluids or delivering solids. Sand-filled fractures would act as preferential flow conduits for delivering dissolved oxygen and nutrients. The proppant could contain time-released, encapsulated nutrients and/or oxygen.

The authors attempted to develop a solid peroxide microencapsulation method to provide time-released oxygen into a fracture. Sodium percarbonate was dispersed into an acetone solution of ethyl cellulose. Microcapsules were formed when the acetone was removed. Comparison between encapsulated and nonencapsulated percarbonate in aqueous batch tests and in a small lab-scale fracture test showed that encapsulating the percarbonate slows down oxygen release measurably, but not sufficiently for long-term (months or years) usefulness in aquifer remediation. The technology appears promising, but needs much more work before it can be used in the field.

Karlson and Frankenberger (1989) attempted to stimulate the rate of aerobic biodegradation of gasoline hydrocarbons (BTX) by adding ammonium nitrate and inoculum from a gasoline-contaminated site known to have organisms that use gasoline as a sole carbon source. The biodegradation rates of toluene and benzene increased above that of the indigenous organisms, but much of the increase appeared to be due to the nitrogen addition. The authors suggest that the need to amend gasoline-contaminated groundwater with nitrogen in order to accelerate bioremediation is common.

Jones and Alexander (1988) found that addition of phosphate, nitrate or sulfate can increase the growth rate of microbes able to degrade specific organics. This implies that population of suitable microbes and their growth rate are possible mechanisms that determine

acclimation rates for organic degradation. Phosphate, nitrate or sulfate addition can decrease the acclimation period for mineralization of low concentrations of *p*-nitrophenol (PNP) in water collected from two lakes near Ithaca, NY. For Beebe Lake water, acclimation time decreased from 13 to 5 days with the addition of phosphate, and to 8 days with nitrate addition. Acclimation times for Cayuga Lake were decreased from >20 days (no mineralization of PNP observed) to 2-8 days, depending upon the initial PNP concentration. Higher initial PNP concentrations resulted in shorter acclimation times.

CHAPTER 6

MATHEMATICAL MODELING OF ON-SITE AND *IN SITU* BIOREMEDIATION

Fate and transport mathematical models can be used to estimate rates of contaminant attenuation by physical and biological processes. They can also be used in designing remediation systems. For example, the number and locations of injection wells for an *in situ* bioremediation project can be modeled when sufficient information about the nature of the subsurface is available. Some models are designed to address a single subject, such as sorption kinetics **(Wu and Gschwend, 1988)**, or solute transport in groundwater **(Corapciouglu and Baehr, 1987; Baehr and Corapciouglu, 1987)**. Most of the models reviewed in this section are concerned with fate and transport of solutes in groundwater, and can model biodegradation rates in addition to physical transport.

Borden *et al.* (1989) used a general mathematical model (BIOPLUME) **(Borden and Bedient, 1986)** to simulate transport and biodegradation of polynuclear aromatic hydrocarbons (PNAs). One-dimensional radial flow away from an injection well was modeled. Change in hydrocarbon, oxygen and microbial concentration over time were modeled by considering injection pumping rate, longitudinal dispersivity, well and aquifer geometry and Monod kinetics. Chloride data was used to calibrate the transport portion of the model. Degradation parameters were obtained from laboratory batch tests and literature values. All model equations and parameter values used are given in the paper. Simulated and measured PNA distributions matched closely for aerobic and anoxic conditions. A minimum degradable hydrocarbon concentration was added to the model, based on observed minimum residual concentrations remain after degradation had ceased in both laboratory and field tests.

Rifai *et al.* (1988) used the BIOPLUME II model (adapted from BIOPLUME) to simulate biodegradation of aviation fuel at the JP-4 jet fuel spill site in Traverse City, MI. In BIOPLUME II the Monod parameters were removed, and the microbial consumption of oxygen and hydrocarbon was considered to be instantaneous. This was based on oxygen transport to the cells being the rate-limiting step for subsurface biodegradation of hydrocarbons. The USGS

Method of Characteristics model **(Konikow and Bredehoeft, 1978)** was modified to implement the BIOPLUME II model. A retardation factor was used to represent slow hydrocarbon movement compared to that of a conservative tracer, and is based on the liquid-solid distribution coefficient for the hydrocarbon. Oxygen and hydrocarbon are tracked in the plume as moving "particles", and the oxygen plume is overlaid on the hydrocarbon plume. Loss of hydrocarbon is calculated from a mass balance between oxygen and hydrocarbon, considering the appropriate reaction stoichiometry, and the rate is determined by oxygen transport. Rate of biodegradation was found to be sensitive to hydraulic conductivity and reaeration rate in model sensitivity tests. Longitudinal and transverse dispersivity had little effect on the biodegraded mass, and porosity had no measurable effect for porosity values of 0.3-0.7.

BIOPLUME II was used to simulate BTX biodegradation at the Traverse City, MI jet fuel site. Sampling of groundwater showed that high BTX concentrations were associated with low DO concentrations (<1 mg/L), and background DO levels of uncontaminated water near the site were 8.0 mg/L. BTX and DO data collected over 21 months was averaged quarterly, and also averaged vertically. Concentration contours show significant loss of BTX mass, and successful confinement of the plume by intercept wells. The authors assumed that the mass loss observed was mostly due to biodegradation. Their assumption was supported by model sensitivity analysis of the dispersion coefficients, and by other work at a similar site **(Chiang, 1987)** indicating insignificant loss of benzene from volatization. The simulated plume matched the observed plume reasonably well. At the beginning of the study (April, 1985), total simulated BTX mass was 1470 kg, compared to 878 kg calculated from measured BTX concentrations in well water samples. Two years later simulated mass was 390 kg, and mass calculated from sample analysis was 77 kg. The authors concluded that BIOPLUME II simulated the plume contours and mass loss accurately, and that differences between simulated and calculated mass loss were attributable to lack of data on the spill source and anaerobic degradation, which was not accounted for in the simulations. Sensitivity analysis of the model is detailed by **Rifai and Bedient (1987).** Hydraulic conductivity, dispersivity (longitudinal and transverse), porosity, reaeration, anaerobic decay, and retardation were analyzed for their effect on simulation results. Mass loss due to

biodegradation was found to be most sensitive to the hydraulic conductivity, the coefficient of reaeration, and the coefficient of anaerobic decay.

In BIOPLUME II simulations of the Traverse City, MI JP-4 jet fuel spill site **(Alder-Schaller and Bedient 1989)** the effects of injecting 0, 3, 20 or 100 mg/L oxygen, in continuous or interrupted mode were evaluated. Oxygen level influenced removal, and simulations showed that all contaminants would be removed within one year with 100 mg/L oxygen continuously or intermittently injected. Approximately 10% of the contaminants would remain after two years of 20 mg/L oxygen injection, and over 20% would remain after two years with no oxygen.

The BIOPLUME modeling concepts were extended by incorporating anaerobic degradation with Michaelis-Menten kinetics, advective and dispersive transport of oxygen and substrate, an option of first-order decay for aerobic or anaerobic conditions, and sorption modeled by linear, Freundlich and Langmuir isotherms **(Srinivasan and Mercer, 1988)**. The model is 1-D, finite-difference, and assumes homogeneous porous media. Some illustrative examples are provided:

1. A batch flushing analysis simulated the permeation of clay underlying a hazardous waste site while the site was being flushed with water.

2. A coal tar contaminant problem focused on subsurface benzene transport from a pond saturated with benzene as a source to a river. Simulations with and without degradation at the source and in the plume were run.

Wheeler *et al.* (1987) developed a groundwater contaminant transport and biodegradation model (BIOPLUS) in which equations for fate and transport of hydrocarbons, oxygen and microorganisms are solved simultaneously. The model was used to assist in designing the bioremediation system at the Traverse, MI JP-4 jet fuel spill site. The method assumes oxygen-limiting conditions, and employs Monod kinetics. Monod expressions for oxygen, hydrocarbon and microbial rates of change are combined with the advection-dispersion equation, and linear instantaneous adsorption is assumed. The resulting system of equations is solved numerically.

Van Eyk and Vreeken (1989) are developing a model to calculate hydrocarbon biodegradation during bioventing. The model assumes oxygen-limiting conditions. Mathematical

details are not provided, but the model is based on a hydrological contaminant transport model, adapted for vapor-phase transport. Instantaneous oxidation of hydrocarbon to CO_2 is assumed. Biodegradation rates can be varied in the model, which then calculates the time required to degrade a specified amount of hydrocarbon. Loss of hydrocarbon due to air stripping is also considered. When finished, the model will also incorporate microbial growth rates and nutrient, hydrocarbon and oxygen consumption.

Birdwell and McConnel (1988) developed an *in situ* bioreclamation mathematical model that incorporated multiphase flow, flux within phases, equilibrium partitioning, and biodegradation. The model is based on 3-D advection-dispersion concepts, with biodegradation considered a sink for hydrocarbons. A modified Monod kinetic expression was used to model biodegradation rates, and kinetic terms for carbon, nitrogen, phosphorus and oxygen are included. Darcy's equation is developed in matrix form for a 3-D Cartesian system to describe the fluid flow. A conservation of mass approach is used to describe mass flux within a single phase, and advection and dispersion in a Cartesian system are described. Raolt's Law, Henry's Law and aqueous solubility are used to describe partitioning of hydrocarbons between phases. The conceptual and mathematical models are presented in this paper.

Schafer and Kinzelbach (1991) used numerical simulations to investigate the effects spatial nonuniformity of pollutant and/or hydraulic conductivity on *in situ* bioremediation. A two-dimensional solute transport model based on advection, dispersion, bacterial growth and interaction between bacteria and dissolved species was developed. Monod kinetics was used. Coupled equations for dissolved oxygen and dissolved organic pollutant are combined with expressions for microbial growth and decay. Linear adsorption isotherms are assumed. Hydraulic conductivity and solute distribution coefficient are thought to be the most sensitive parameters, and are modeled as if they were randomly distributed in space. All other model variables are assumed to be uniformly distributed. Their simulations showed that heterogeneity leads to increase in remediation time. Therefore, remediation time estimates based on homogeneous aquifer conditions may be lower than the actual time required. The authors go as

far as to say that large heterogeneity in aquifer conditions can lead to "expensive failure even if a pollutant is shown to be microbiologically degradable in laboratory column studies".

Widdowson and Aelion (1991) applied a two-dimensional model to the planned bioremediation of a JP-4 fuel spill in Charleston, SC. A shallow water table aquifer contains up to several mg/L dissolved BTX, and up to several thousand mg/kg sorbed BTX. Contaminated groundwater will be withdrawn, 60% will be discharged to the North Charleston sewer district, and 40% will be amended with nitrate and phosphate and recharged through an infiltration gallery. A two-dimensional advective-dispersive transport and biodegradation was developed. A modification of Monod kinetics is used, where biomass growth is dependent upon concentrations of substrate, electron acceptor (oxygen or nitrate) and nutrients. The biodegradation portion of the model is highly mass-transfer (diffusion) dependent, as it is assumed that:

Rate of mass exiting pore fluid = Rate of mass entering biomass = Rate of mass utilized in biomass.

Therefore, the biodegradation rate depends upon transport rates of substrate, electron acceptor and nutrients across a diffusional boundary of finite thickness. This is mathematically similar to biofilm models. On the macroscale, the rate of change of any single component is modeled as the sum of five terms: two-dimensional advection; diffusion in directions longitudinal to and transverse to principal direction of dispersion; rate of mass transport into biomass; and mass entering pore fluid. Microbial population is increased in the model as nutrient concentrations increase. Model simulations predict that oxygen from the infiltration water will be responsible for most of the BTX biodegradation, and that denitrification will play a minor role. Hydrocarbon availability, in particular desorption rate, will limit the rate of bioremediation. Inorganic nutrients may be rate-limiting, but electron acceptor concentration will always be present in excess.

Sleep and Sykes (1991) developed a numerical model that considers three-phase flow and transport (air, organic and water), interphase transport, and biodegradation. Monod kinetics are used to describe utilization of oxygen and substrate and microbial growth. The model allows for forced gas-phase advection, making it unique from other multiphase transport-degradation models for subsurface solute fate. The model was demonstrated by simulating volatization and

degradation of toluene from a hypothetical underground leak. For the conditions used, the rate of toluene attenuation with biodegradation was about twice that without biodegradation. Published toluene degradation kinetics were used in the simulation. Simulations with injected air showed little improvement in rate of degradation, but injection of oxygen did significantly increase the rate. Mass transfer limitations of oxygen dissolving into water appeared to control aerobic degradation rates in these simulations.

In modeling the desorption and degradation of hydrophobic organics adsorbed to soil in the saturated zone, **Mihelcic and Luthy (1991)** assumed that sorbed substrate is not bioavailable. This assumption is supported by the work of **Robinson *et al.* (1990)** on the bioavailability of sorbed toluene. They further assumed that the rate of biodegradation is proportional to the substrate concentration in the bulk aqueous phase and the active cell population, and that desorption is instantaneous compared to biodegradation rate. Two aqueous environments are defined within the saturated pore space: bulk pore water between soil aggregates, and intra-aggregate pore water. Microbes are too large to enter aggregates, so all biodegradation occurs in the bulk pore water. Since dissolved and sorbed concentrations within the aggregates are higher than in the bulk environment, a concentration gradient is established between the interior of the aggregate and the aggregate-bulk pore water interface. Mass transfer between phases is not considered limiting. Therefore, aqueous concentration is controlled by sorbed concentration, diffusion from intra-aggregate pore water to bulk pore water, and biodegradation rate.

The resulting desorption model was coupled with Michaelis-Menten kinetics to yield the following model:

$$\frac{dc}{dt} = \frac{(K_{max}C)}{(K_s+C)(1+Pk_p)]}$$

where:

C = concentration of substrate in the bulk pore water
K_s = half-saturation coefficient
t = time
p = soil-to-water mass ratio
K_{max} = maximum rate of the reaction
K_p = linear sorption partition coefficient

A radial diffusion model was combined with the above expression to yield a coupled solute desorption-degradation model.

Biodegradation of petroleum hydrocarbons in the unsaturated zone was modeled and experimentally verified by **Ostendorf and Kampbell (1989,1991)**. A coupled oxygen and hydrocarbon transport and biodegradation model was constructed, with the following assumptions:

1. Sorption is insignificant;
2. Advection is insignificant; diffusion is dominant transport mechanism;
3. Leaching is insignificant;
4. Contaminant concentrations are non-transient;
5. Microbial population is at steady-state;
6. Soil moisture is vertically uniform;
7. Transport is by gaseous diffusion;
8. Isothermal temperature is assumed;
9. Soil is homogeneous;
10. O_2 is not biologically limiting; and
11. Biodegradation of weathered jet fuel is expressed stoichiometrically.

This implies that as hydrocarbons are degraded they are replaced by diffusion from a source, preserving a constant hydrocarbon concentration everywhere in the contaminated area.

A second model was described, in which the reaction term is described by Michaelis-Menton kinetics, and oxygen is calculated at any given depth based on the hydrocarbon concentration gradient over the entire depth profile. The purpose of the second model is to predict concentration profiles of individual hydrocarbon components as a function of depth.

CHAPTER 7
CONCLUSIONS AND RECOMMENDATIONS

Recent literature on *in situ* and on-site bioremediation of petroleum hydrocarbons has been contributed by authors from several disciplines, including microbiology and several areas of engineering. Academia, the petroleum industry, several federal government agencies and the environmental consulting field are all well represented.

The Technology Review by **(Riser-Roberts, 1992)** included a list of conclusions and recommendations. A logical first step in this conclusion chapter would be to review that list and evaluate the advances in bioremediation technology over the past four years. The fact that this country is faced with an enormous cleanup task due to unsound disposal practices of the past has not changed during the past four years. In fact, more sites are discovered each year. Therefore, the need for new, innovative cleanup technology is greater than ever.

In situ bioremediation was a budding new field four years ago. It was described by **(Riser-Roberts, 1992)** as "not a proven technology." Although still new, *in situ* bioremediation techniques have been responsible for successful full-scale cleanup operations, in that treatment goals were achieved. In other cases contaminant concentrations were reduced biologically, but not to acceptable levels. Other technologies are often chosen over bioremediation because their results are more predictable at this time. For example, **Piotrowski and Yost (1989)** were contracted to remediate a site contaminated by diesel fuel from underground storage tanks. They opted to excavate and landfill 2100 cubic yards of soil and construct a permanent intercept/recovery trench to collect diesel-contaminated perched groundwater. The water is treated by settling, filtration, and granular activated carbon before being discharged into the facility's sewer system. Biodegradation was initially considered as an option, and a suitable microbial population was present on-site, but feasibility testing would have required more time than the site's owner was willing to invest. If bioremediation methods were more proven and as the technologies were more routine and predictable, bioremediation might have been chosen for this site.

Since it is now known that petroleum hydrocarbons are for the most part biodegradable, recent research has focused on bringing together the contaminants, viable acclimated microorganisms and necessary nutrients in a suitable biostimulating environment. In that sense, optimizing *in situ* bioremediation has become more of a hydrological and solute transport problem, and less of a biological problem.

The case histories and laboratory studies of the past four years indicate the need for rapid, efficient screening tests to determine:

1. If a site should be considered for on-site or *in situ* bioremediation;
2. Realistic, achievable cleanup goals for the site, in terms of time and cost;
3. What nutrients need to be supplemented, if any;
4. Whether the indigenous microbiological population will produce rapid contaminant degradation; and
5. What commercially available microbiological strains would accelerate the cleanup?

Such tests should include some combination of mathematical modeling, rapid laboratory bioassays and pilot-scale projects for large sites. In many recent publications, *in situ* bioremediation was attempted on large contaminant sites with limited success, and a small-scale pilot project may have saved much time and money.

The passive remediation option should be considered before any engineered remediation method is installed. If a site can be naturally remediated over a reasonable period of time and the contaminants pose no threat to surrounding water resources, wildlife or man, then a no-action scenario may be warranted. Most engineered *in situ* bioremediation schemes involve adding a supplementary electron acceptor, usually some form of oxygen, but possibly nitrate. Two questions which must be addressed for any site suitable for aerobic bioremediation are:

1. Where is the contaminant (unsaturated zone, saturated zone or both)?
2. In what form will the electron acceptor be delivered?

For unsaturated-zone remediation, air or pure oxygen can be used in a bioventing approach. Bioventing is a rapidly advancing technique. Until recently, the biostimulating effect of introducing oxygen to the contaminated zone was reported as a fringe benefit of soil venting

for physical removal of volatile organic compounds. In more recent literature the biological removal mechanism is emphasized, in part because releasing volatile organics into the atmosphere can have regulatory ramifications. In more than one case history encountered, the bioventing system was designed and operated with the intention of having negligible release of organics into the atmosphere, and rapid site remediation by biodegradation.

Saturated-zone aerobic bioremediation usually requires the delivery of supplementary oxygen to the contaminated area. The two methods most frequently encountered are air sparging and hydrogen peroxide (H_2O_2) addition. The advantage of H_2O_2 is that high concentrations of oxygen (>300 ppm) can be achieved compared to sparging with oxygen (30 ppm) or air (8 ppm). Disadvantages include high cost and much wasted capacity due to abiotic decomposition of H_2O_2. Oxygen requirements should be balanced with time and cost optimization before a choice is made.

When petroleum-contaminated soil excavation is necessary, on-site bioremediation methods can be cost effective. However, no significant technology advances were seen over the past four years. Land treatment and biopiles still appear to be the methods of choice, although some liquid petroleum wastes are treated in bioreactors. Land treatment has advanced somewhat, with improving understanding of the inorganic nutrient needs of microorganisms, particularly with respect to providing a nitrogen source, optimizing the organic loading rate to avoid inhibition or toxicity effects, and the practice of forming vertical piles instead of horizontally spreading the soil over a large area. There appears to be some technology transfer between the practitioners of composting and land treatment.

The high costs associated with excavation makes on-site technology inherently expensive. However, when dealing with excavated soil, on-site processes may be less expensive than landfilling, and in several articles the liability associated with disposal of petroleum wastes in landfills was mentioned. On-site technologies have some advantages over *in situ* processes, the most important being greater control over operating conditions (e.g. oxygen levels, nutrient concentrations and microbial populations).

Genetic engineering of superior hydrocarbon degraders still appears promising, as it was four years ago. However, the ecological risks and legal (and economic) liability are strong deterrents to the introduction of recombinant organisms into the environment. As this technology becomes more widely accepted in other fields (e.g. pharmaceuticals and agriculture) and safety procedures are developed, such as the bacterial suicide vectors, the use of recombinant organisms for bioremediation should become viable. It would first be used in on-site treatment, probably in closed bioreactors, where genetic containment is possible. Eventually, *in situ* bioremediation may benefit from recombinant biotechnology.

The use of biological or artificial surfactants to disperse hydrophobic contaminants and make them more bioavailable has met with mixed results. The surfactants generally have the desired physical result, but biostimulation is generally not achieved, and in some cases inhibition was observed. Another deterrent to using surfactants is that they increase the mobility of hydrophobic contaminants in an aqueous mobile phase, and could lead to further contamination.

Until the Exxon Valdez crude oil spill in Alaska, there had been no field attempts to stimulate oil biodegradation on beaches. From the Valdez experience, there is strong evidence that at least three different fertilizers increase the rate of marine coastal microorganisms to naturally remove oil from beaches. The fact that researchers were experimenting on the oil-soaked beaches of Prince William Sound points out an information gap: oil spill bioremediation technology should have been available, with predictable results, for the personnel attempting to remediate the spill.

The literature reviewed by **(Riser-Roberts, 1992)** and in this review indicates that biodegradation is often the best available mechanism for cleaning up petroleum contaminated sites, but often not the best available technology. As on-site and *in situ* technologies are advanced, they will increasingly displace current options, such as land filling and incineration. The passive bioremediation option is also coming more into favor, and ideally a continuum of level of treatment is possible, from a completely passive approach to an engineered solution using all of the latest technology. The fact that this is paralleled by a cost continuum will provide constant incentive to use the more passive approaches whenever possible.

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