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

**A Review of Technical Literature
1988 - 1991**

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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 supplement 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

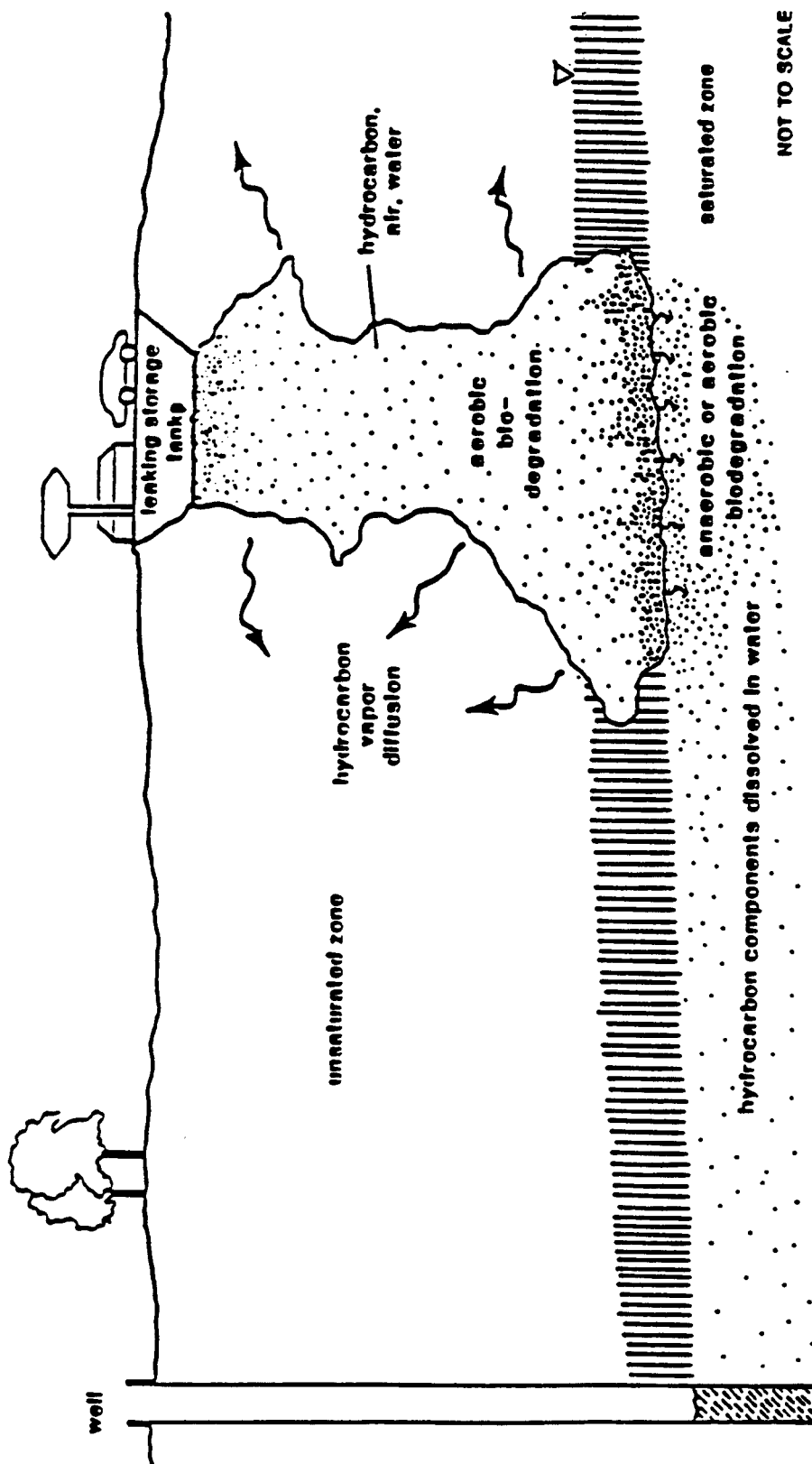


Figure 1. Transport and Biodegradation of Spilled Petroleum Hydrocarbons in the Subsurface under Natural Conditions

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 bioremediation 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* bioremediation 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* volatilization; *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

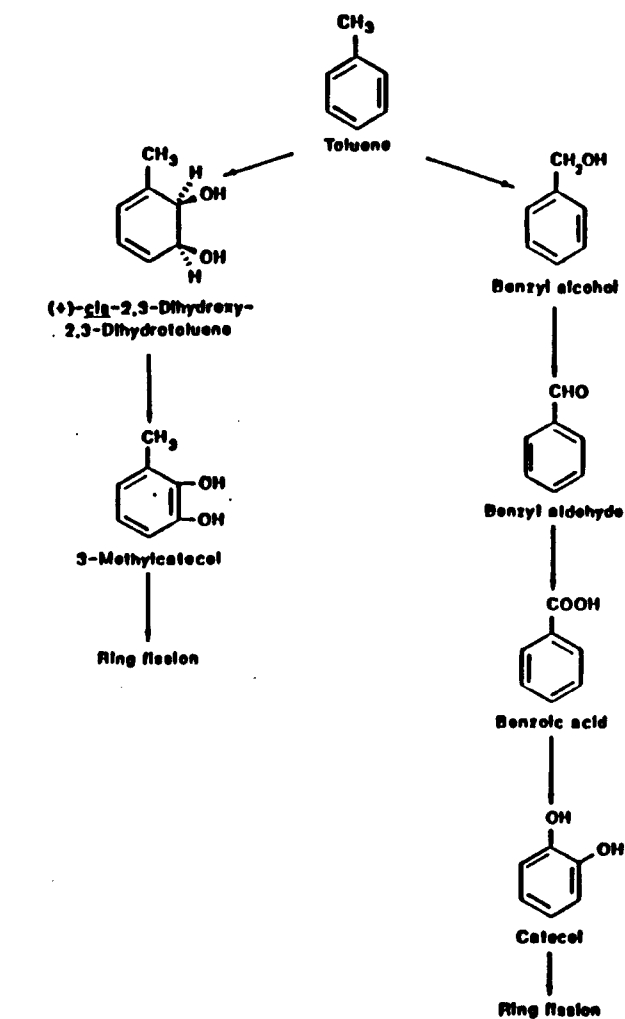
2.1 Genetics and Metabolic Pathways

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.

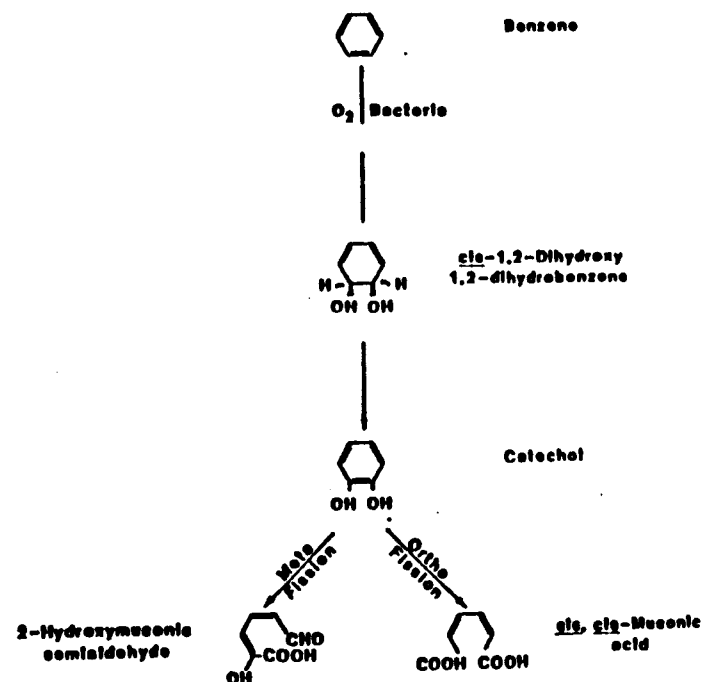
Figure 2.

Bacteria Oxidation Pathways for Toluene and Benzene



Different Initial Oxidative Attack Pathways

On Toluene by Bacteria



Pathway for Bacterial Oxidation of Benzene



Documents

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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

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

TABLE 1. Hydrocarbon-Degrading Microorganisms

(Continued)

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

TABLE 1. Hydrocarbon-Degrading Microorganisms (Cont.)

<u>microorganism</u>	<u>substrate</u>	<u>reference</u>
<i>Pseudomonas sp.</i>	alkanes (C ₁₈ -C ₃₆)	Miller and Bartha (1989)
<i>Pseudomonas putida</i> <i>P. fluorescens</i>	naphthalene	Ogunseitan <i>et al.</i> 1991
<i>Acinetobacter calcoaceticus</i>	asphalt	Pendrys 1989
<i>Pseudomonas aeruginosa</i> <i>P. putida</i> <i>P. stutzeri</i> <i>P. maltophillicia</i> <i>P. alcaligines</i> <i>Norcardia sp.</i> <i>Alcaligenes denitrificans</i> <i>Micrococcus</i>	gasoline	Ridgeway <i>et al.</i> 1990
<i>Pseudomonas putida</i>	toluene	Robinson <i>et al.</i> 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 stronger 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, O ₂ , H ₂ O ₂ , or anaerobic
	anaerobic	BTEX mixture	0% degradation in 100 days	
2.	aerobic	BTX Mixture	100% gone in 78 days	microcosms and field study, 1.1-2.4 mg/L BTX
	anaerobic	BTX Mixture	0% degradation in 78 days	
3.	aerobic	Benzene	10-100mg/L: 2.5-4.5 day halflife	microcosms, 500-50,000 ppb benzene
	anaerobic	benzene	no degradation observed	

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	benzene benzene	14.5% degraded in 28 days 47.08% degraded in 28 days	microcosms, sandy aridisol microcosms, riparian soil
	anoxic	benzene benzene	2.43% degraded in 28 days 16.49% degraded in 28 days	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:

compound	lag times of individual compounds (days)			
	fuel oil			gasoline
	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
<i>o</i> -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. Isolates 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, 1×10^8 to 5×10^8 (week 16)
with jet fuel: 1×10^8 to 1×10^{10} (week 4) to 8×10^8 (week 16)

Subsurface:

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

AEROBIC HETEROTROPH COUNT RESULTS (g SOIL⁻¹)

Surface:

no jet fuel: 3×10^8 to 2.2×10^7 (week 4), stayed constant
50 mg jet fuel g soil⁻¹: 3×10^8 to 8×10^9 (week 4) to 7×10^7 (week 16)
135 mg jet fuel g soil⁻¹: 3×10^8 to 8×10^9 (week 4) to 8×10^7 (week 16)

Subsurface:

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

HYDROCARBON DEGRADER COUNT RESULTS (g SOIL⁻¹)

Surface:

no jet fuel: 3×10^8 to 2.2×10^7 (week 4), stayed constant
50 mg jet fuel g soil⁻¹: 3×10^8 to 8×10^9 (week 4) to 7×10^7 (week 16)
135 mg jet fuel g soil⁻¹: 3×10^8 to 8×10^9 (week 4) to 8×10^7 (week 16)

Subsurface:

no jet fuel: 4×10^4 to 10^5 (week 4) to 5×10^3 (week 16)
50 mg jet fuel g soil⁻¹: 4×10^4 to 3×10^8 (week 2) to 3×10^7 (week 16)
135 mg jet fuel g soil⁻¹: 4×10^4 to 3×10^8 (week 2) to 7×10^8 (week 4) to 3×10^7 (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 denitrifier 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 $\text{Fe}^{2+}:\text{CO}_2$ 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 *hok* 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 μm diameter), surface-charged microspheres (0.23, 0.53, 0.91 and 1.35 μm diameter), uncharged microspheres (0.6 μm 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, volatilizing 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 CO₂ production, and demonstrated the variability encountered in biodegradation rates of benzene in different soils, under aerobic and anaerobic conditions. Abiotic CO₂ does not interfere, as long as a sufficiently large volume of NaOH is used in the trap to ensure that all the CO₂ 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 CO₂ adsorbers for 3 days. Oxygen consumption and CO₂ 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 CO₂ 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 volatilization before reaching the soil surface, and this was taken into account when BTEX attenuation rates were determined.

Results of the first experiment (designated 1A) 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. CO₂ production was monitored while the microcosms were incubated at room temperature. Organic substrates were ¹⁴C labeled glucose, benzene or toluene. Carbon mass balances were conducted by measuring ¹⁴C 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. O₂ and volatile hydrocarbon consumption and CO₂ 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 NO₃⁻ or NH₄⁺ did not significantly change respiration rates. Addition of 1.7 mM NO₃⁻ decreased CO₂ production, but increased the amount of ¹⁴C-labeled carbon incorporated into cellular material. Over 26 days, total glucose metabolized in NO₃-amended microcosms was twice that of unamended microcosms. Respiration of ¹⁴C-labeled benzene and toluene was negligible in microcosms with or without 3 mM NO₃⁻ over three months.

In unsaturated microcosms, significant CO₂ production was only observed when NO₃⁻ was added. Addition of PO₄3⁺ and NO₃⁻ was not more effective in stimulating respiration than NO₃⁻ 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 acetophenone 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 concentrations were 9, 7 and 1 mg/L for naphthol, naphthalene and acetophenone, 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