The Biodegradation Potential of Methanol, Benzene, and m-Xylene in a Saturated Subsurface Environment

by

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(ABSTRACT)

The increased use of alcohols as gasoline additives, and possible substitutes, has prompted the investigation of the fate of gasoline/alcohol mixtures in the environment. In situ bioremediation is one technique that can successfully be applied to remove ground water contaminants particularly in situations where the adsorptive capacity of the soil plays a major role. Frequently, enhanced in situ bioremediation techniques rely on indigenous microorganisms to degrade ground water contaminants; this technique may sometimes include the addition of acclimated bacteria.

In this study, soil microcosms were constructed in order to simulate the conditions found in a saturated aerobic aquifer. The biodegradation potential of methanol, benzene, and m-xylene was investigated. Uncontaminated soil from the surface, 12, 16.5, and 18 foot depths was utilized to observe the differences in microbial responses throughout the soil profile. The biodegradation potential of the indigenous microbiota was determined and compared to that of benzene acclimated bacteria, for all the compounds in the mixture. To observe the impact that chemical and physical soil characteristics may have

on microbial responses, soils from each depth were classified on the basis of their particle size, moisture content and pH.

Substantial methanol, benzene, and m-xylene biodegradation by the indigenous microorganisms occurred in all subsurface soils. While methanol was readily biodegradable over concentrations ranging from about 80 mg/L to about 200 mg/L, benzene inhibited methanol biodegradation at about 125 mg/L in all soil depths. The addition of benzene acclimated bacteria considerably increased the biodegradation rates of all compounds in the mixture. Such increases in biodegradation rates may be attributed to the activities of both groups, the indigenous microorganisms and the benzene acclimated bacteria. The results obtained by this study suggest that biodegradation of methanol, benzene, and m-xylene can readily occur in a saturated aerobic subsurface environment. The physical and chemical properties of a ground water aquifer seem to have a marked effect on microbial responses, and consequently on the biodegradation potential of water contaminants.

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CHAPTER I

INTRODUCTION

Gasoline hydrocarbons originating from leaking underground storage tanks and pipelines or accidental spills represent a major threat to ground water supplies (Ridgway et al., 1990). Ground water is widely used in the United States for drinking purposes; it is estimated that more than 40% of the population relies on this source, often with only disinfection as treatment (McCarty et al., 1981). About three fourths of the major cities in the United States and as much as 95% of rural populations rely on ground water for drinking purposes (Canter et al., 1990). Even though ground water has traditionally been considered a pristine source, evidence indicates that contamination of ground water, especially with petroleum, is a widespread problem.

After a gasoline spill, the contaminants will percolate through the soil matrix and will partition themselves in the three phases that constitute the soil environment, namely the air, water and soil phases. If a large quantity of gasoline is spilled, a fourth phase consisting of free product can exist above the water table (Hathaway and Andrews, 1990). When a significant quantity of gasoline accumulates in the area of the water table, ground water contamination occurs as a result of dissolution of the compounds into the water phase (Hathaway and Andrews, 1990). The water soluble fraction of gasoline then becomes of extreme importance since ground water movement is the main mechanism by

which contamination is spread (Aamand et al., 1989; Wilson and Brown, 1989).

To understand the fate of organic compounds in the environment, an accurate assessment of their susceptibility to biodegradation is needed. Biodegradation, the biological transformation of an organic compound to another form, often results in the transformation of a substance into simpler, less toxic forms, ultimately leading to the formation of carbon dioxide, water, and inorganic forms as end products. In this sense, biodegradation often plays a beneficial role in the clean up process of petroleum contaminated aquifers.

Increased concern over the environment and the potential of oil shortage has resulted in the exploitation of alternative fuels and the increased use of alcohols as gasoline additives. This had led to the study of the effect that gasoline/alcohol mixtures may have on the subsurface environment. The biodegradation of petroleum hydrocarbons in aerobic environments is well documented and while most studies concentrate on the influence of physical, chemical, and biological factors on the biodegradation of individual compounds, little is known about mixed substrate interactions and their corresponding effects on soil microorganisms. In a substrate mixture, an individual substrate may interfere with the utilization of another. Biodegradation may take place in a sequential pattern, in which the substrate supporting the highest growth is utilized first, or it may be inhibited altogether by high concentrations of a particular compound.

While some studies have documented the degradation of benzene, toluene, ethylbenzene, and xylene mixtures, (usually referred to as BTEX), under aerobic conditions (Lodaya et al., 1991; Chiang et al., 1989; Karlson and Frankenberger, 1989; McCormick, 1991), few concentrate on the effects that alcohols may have on the degradation of these highly toxic compounds. Alcohols are of interest because they are often added to gasoline to boost octane ratings and because of air pollution concerns. Eighty five percent methanol solutions have been proposed as gasoline substitutes (Gandhidasan et al., 1991).

Karlson and Frankenberger (1989) studied the biodegradation potential of benzene and toluene in petroleum contaminated ground water. They found that considerable biodegradation by the indigenous microorganisms could be attained, and that biodegradation was highly dependent on oxygen and nitrogen availability. The study also found that the addition of an enrichment culture of hydrocarbon oxidizers considerably enhanced biodegradation when oxygen and nitrogen were not limiting factors. The initial benzene and toluene concentrations utilized in this study were about 450 and 550 μg/L, respectively.

Batch studies conducted by Kim (1987) showed that a benzene concentration of 200 mg/L was toxic to soil microorganisms; in this study benzene was the sole substrate. Microcosm studies conducted by McCormick (1991) on the degradation of BTEX under aerobic conditions indicated that m-xylene hindered biodegradation at concentrations of

about 60 mg/L. McCormick also reported sequential substrate utilization in soils with a high clay content; benzene was the primary substrate and m-xylene the secondary substrate. Goldsmith (1985) conducted studies on the degradation of a mixture of BTX and methanol utilizing soil microcosms. His study indicated that methanol can readily be utilized by subsurface microorganisms. Degradation of the BTX fraction was also observed. Goldsmith reported that the addition of benzene, toluene and xylene did not adversely affect the degradation of methanol. For these mixed substrate studies, the initial methanol concentration was in the 100mg/L range, and the total BTX concentration in the 12mg/L range.

The biodegradation potential of contaminants in the subsurface often depends on physical and chemical factors affecting the microbial population of a particular location. Such factors are site specific and often include soil characteristics such as solution pH, nutrient and oxygen availability, organic matter and moisture content, and soil particle size. Biodegradation is also dependent on the nature of the contaminant mixture, and on whether the site has been previously exposed to the contaminants. Much of what is known about BTEX biodegradation in the subsurface comes from studies involving ground water samples. Few studies concentrate on the effects that soil may have on biodegradation rates. Evidence indicates that a considerably large fraction of the microbial population of an aquifer is associated with solid surfaces (Arvin et al., 1988; Harvey et al., 1984). In addition, factors such as the adsorption of the contaminants to soil particles and nutrient availability may play a significant role in biodegradation.

Ground water samples may not adequately address these parameters. Furthermore, since biodegradation is a site specific process, the use of soil microcosms to closely mimic natural environmental conditions in the subsurface environment may be one of the best methods for determining biodegradation rates.

In order to determine the biodegradation potential of gasoline/alcohol mixtures in the subsurface, this study utilized soil microcosms containing indigenous microorganisms and benzene acclimated bacteria dosed with methanol, benzene and m-xylene. Since the concentration of BTEX compounds is one factor that may govern their susceptibility to biodegradation, a wide range of benzene and m-xylene concentrations was utilized in this study, including Kim's (1987) and McCormick's (1991) reported inhibitory levels. Since the microbial communities of a particular soil may be highly dependent on soil characteristics, soil collected from the surface, 12, 16.5 and 18 foot depths was utilized in an effort to determine variations in biodegradation rates throughout the soil profile. The specific objectives of this study were:

1. To obtain the biodegradation rates of methanol, benzene, and m-xylene by indigenous microorganisms of different soil depths.

2. To determine the effect of methanol on the degradation of benzene and m-xylene in subsurface soils.

3. To determine possible toxicity thresholds to which the indigenous microbial communities present at different depths throughout the soil profile may be susceptible.

4. To determine the effect that the addition of benzene acclimated bacteria may have on biodegradation rates.

CHAPTER II

LITERATURE REVIEW

2.1 Gasoline composition and characteristics

Gasoline is a mixture of refined petroleum hydrocarbons including more than 200 different compounds (Ridgway et al., 1990). Tables 1 and 2 show the typical composition of gasoline and the physical/chemical properties of selected gasoline constituents respectively. The major components are aromatics, branched and cycloalkanes (McDuffie, 1982).

The aromatic components of gasoline, in particular, benzene and xylene, are of interest because of their relatively high percentage in gasoline, high water solubilities and toxic effects (Barker et al., 1987). Because they have a relatively high aqueous solubility and corresponding low partition coefficients, these compounds have a greater potential to be mobilized into ground water supplies. These characteristics may increase not only the rate of ground water contamination of an aquifer but also the extent of the contaminated area (Hathaway and Andrews, 1990).

Benzene, a known human carcinogen, is believed to be more toxic than any other constituent of gasoline; it comprises up to 5% of gasoline (Goldstein, 1983; Hanckock, 1985). The xylenes may comprise up to 20% of gasoline (Hanckock, 1985). Because of

their toxicity and long term health effects, their maximum permissible concentrations in drinking water are federally regulated, under the 1986 Safe Drinking Water Act. As of January, 1991, the maximum contaminant level for benzene was 0.005 mg/L, and for total xylene (ortho, meta and para) 10 mg/L.

TABLE I: Gasoline Composition

Hydrocarbons	Typical Composition (Percentage)
Alkanes	52.6
Monocycloalkanes	34.6
Dicycloalkanes	5.2
Alkylbenzenes	
Benzene	up to 5.0
Toluene	up to 20.0
Xylenes	up to 20.0
Indanes and Tetralins	0.9
Naphthalenes	0.3

(After McDuffie, 1982)

TABLE II: Physical/Chemical Properties of Selected Gasoline Constituents

Compound	Molecular Weight	Vapor Pressure (@ 25 °C, mm Hg)	Aqueous Solubility (@ 25 °C, mg/L)	Henry's Law Constant (Atm/mM)	Partition Coefficient (log Kow)
Aromatics					
Benzene	78	95	1780	7.0	2.11
Toluene	92	29	535	6.7	2.69
O-xylene	106		175		
M-xylene	106		200		
P-xylene	106		200		3.15
Ethylbenzene	106	7	152	6.6	3.15
Propylbenzene	120		60		
Naphthalene	128		32	10.0	3.36
Biphenyl	154		7.5		4.09
Trimethylbenzene	120				3.7
Alkanes					
Butane	58		79		
Pentane	72		2		
Hexane	86				
Cyclohexane	84		130		
Heptane	100		50		
Octane	114		16		
Iso-octane	114				

(After McDuffie, 1982)

2.2 Methanol as a gasoline additive

Alcohols are the prime candidates for supplementing or even replacing gasoline. Nowadays methanol may comprise as much as 5% of gasoline (Novak et al., 1985, citing Colby et al., 1979). Methanol is completely soluble in water and in many polar organic liquids (Eden, 1985). In general, the solubility of gasoline in water is in the range of 20 to 80 mg/L, averaging 50 mg/L (McKee et al., 1972). Since methanol is completely miscible in water, it might contaminate a larger region of the aquifer. Because methanol cannot be detected by taste and odor as readily as some other gasoline constituents, its concentration might reach levels high enough to cause adverse human health effects. Ingestion or prolonged exposure to methanol can lead to death (Gandhidasan et al., 1991). In general, though, the most prominent health hazard associated with methanol is blindness (Wade et al.,1960). Because of its infinite solubility in water and its possible increase in use as a gasoline additive, methanol as well as benzene and m-xylene are of particular interest to this study.

2.3 Ground water Remediation

The pump and treat technique is the most popular ground water cleanup method. The mechanisms of ground water flow through wells are understood and easily implemented. Once on the surface, hydrocarbon contaminated ground water recovered by pumping wells is subjected to conventional treatment systems (Canter et al.,1990). However, this technology alone is not effective in areas of highly impermeable soils, extremely shallow formations, or for the removal of contaminants present in the vadose

zone or that have adsorbed to soil particles (Canter et al., 1990; Nyer and Skladany, 1989). Therefore, for aquifer contamination problems in which the soil absorptive capacity plays a major role, and where removal of trace organics is often the main concern, other forms of remediation such as in situ biological treatment might present a better alternative.

In situ treatment technologies achieve treatment of the contaminants within the subsurface environment, either chemically or biologically. Most chemical methods of in situ treatment are not as efficient as their biological counterpart in that they usually immobilize the contaminants not removing them. Furthermore, it is only used in cases where the extent and the levels of contamination are defined, and where the type of pollutant is known (Canter et al., 1990).

In situ biological treatment is based on the same principles as traditional biological waste water treatment. Under the proper environmental conditions and nutrient availability, microorganisms are capable of utilizing the contaminants as their substrate or food source, transforming them into simpler organic forms and eventually mineralizing them to carbon dioxide and water (Grady, 1985; Canter et al., 1990). In situ bioremediation affects both adsorbed and dissolved contaminants.

In situ biodegradation can be accomplished through enhancement of the microbial environment and/or though the acclimation and addition of microorganisms to the

subsurface. In enhanced biodegradation the subsurface environment is modified to attain the appropriate conditions under which the desired biological pathways can take place. This is accomplished through the addition of an adequate electron acceptor and nutrients. In acclimated biodegradation, microorganisms that were cultured and acclimated to the specific environmental conditions and contaminants of interest, are introduced into the subsurface environment as seed for the cleanup process (Canter et al.,1990).

2.4 The Soil Environment

In situ biological treatment usually requires the modification of the soil environment for the enhancement of microbial activity. Optimal degradation occurs when the soil environment has the appropriate temperature, pH, oxygen, moisture content, and inorganic and organic nutrients (Bossert and Bartha, 1984).

Hydrocarbon degradation can occur over a wide range of soil temperatures, with the highest biodegradation rates occurring between 30 and 40° C (Bossert and Bartha, 1984). Nevertheless, biodegradation has been reported to occur at temperatures as low as -1.1 °C, as long as the soil solution does not freeze. Hydrocarbon degradation is very dependant on the nature of the oil mix. Some studies have reported that at low temperatures, low molecular weight hydrocarbons which may be toxic to microorganisms, volatilize at a slower rate delaying the onset of biodegradation (Atlas, 1981). Higher temperatures do not always mean higher degradation rates. Some studies have reported that at temperatures of 37° C, higher metabolic rates are counterbalanced by increased

membrane toxicity of some hydrocarbons (Bossert and Bartha, 1984). In general, however, higher metabolic rates corresponding to a decrease in lag period duration and an increase in biodegradation rates have been reported as incubation temperatures increase from 5 to 30°C (Hickman and Novak, 1989).

Often temperature is not a limiting factor in hydrocarbon degradation, except as it relates to factors affecting the physical state of the pollutant or the availability of the soil solution in a liquid state. For the most part, hydrocarbon degradation can occur at the low temperatures in the area of 5° C, expected to be found in environments likely to become contaminated by oil spills (Atlas, 1981).

Most soil bacteria grow best at neutral to alkaline conditions and have a low tolerance for acidic conditions [-pH less than or equal to 5-] (Focht, 1988). In acidic soils, most hydrocarbon degradation is carried out by fungi. However, biodegradation rates under these conditions are lower than under neutral or slightly alkaline environments where degradation is carried out by both bacteria and fungi (Bossert and Bartha, 1984). In general, maximum degradation rates for organic compounds have been reported to be in the pH range of 6.0 to 8.0 (Atlas and Bartha, 1986). McCormick reported maximum combined degradation rates for BTEX in the pH range of 5.4 to 6.6 (McCormick, 1991).

A major problem in the treatment of petroleum spills in soil systems is the low availability of nitrogen and phosphorus (Focht, 1988). The concentration of these

inorganic elements is a limiting factor in the degradation of petroleum hydrocarbons in that a proper C:N:P ratio is required for microbial activity (Atlas, 1981). When hydrocarbons are introduced in the environment, the concentration of available organic carbon increases and alters the C:N:P ratio. Therefore, the addition of nitrogen and phosphorus in an attempt to establish the appropriate balance between organic carbon and inorganic nutrients is crucial in the stimulation of oil degradation (Bossert and Bartha, 1984).

Oxygen availability is essential for significant biodegradation activity to occur. Anaerobic biodegradation of hydrocarbons is much slower than aerobic biodegradation and it is believed to occur at relatively negligible rates in nature (Focht, 1988; Bossert and Bartha, 1984; Atlas, 1981). Metabolism of intermediate products of aerobic biodegradation is known to occur under anaerobic conditions, using sulfate, nitrate, carbonate or even other organic compounds as electron acceptors (Bossert and Bartha, 1984). The availability of oxygen in the soil environment depends, among other things, on the total amount of oxygen filled pore space and on the size of the pores. Large amounts of oxygen filled pore space and large pores render an ample supply of oxygen and a better chance for diffusion. Oxygen diffusion decreases as the soil pore size decreases. Diffusion of oxygen into the soil solution is also affected by the elevation of the water table; water saturation decreases the amount of oxygen that can be replaced by diffusion. It has been reported that saturation in the 50 to 80% range is optimal for aerobic degradation (Bossert and Bartha, 1984).

2.5 Effect of depth on microbial activity

In most cases, surface soils exhibit the greatest microbial activities because of their higher organic and nutrient content, increased aeration, and increased exposure to biodegradable organic matter. In subsurface soils, however, nutrients and oxygen are not as readily available; microbial populations may have never been exposed to petroleum contamination and may require prolonged acclimation periods (Thorn and Ventullo, 1988). Studies indicate that subsurface bacteria are extremely small, generally accustomed to low levels of nutrients, and able to degrade some pollutants commonly detected in ground water (Kuhn et al., 1985). Since surface soils receive a continuous supply of organic materials, their existing bacterial populations may be adapted to degrade compounds that are relatively easy to metabolize, allowing more recalcitrant compounds to infiltrate to the subsurface. As a result, subsurface microorganisms may be more acclimated to these more recalcitrant compounds (Hickman and Novak, 1989).

2.6 Responses of the microbial community to hydrocarbon pollution

The abundance of organic compounds and relatively high availability of attachment surfaces favor microbial growth and diversity, rendering soils as the richest microbial habitat (Bossert and Bartha, 1984). Typical total microbial counts of fertile soils have been reported to be in the range of 10^7 - 10^9/g of soil, while typical counts of hydrocarbon degraders in uncontaminated environments are in the range 10^5 - 10^6 (Bossert and Bartha, 1984).

In unpolluted environments, hydrocarbon degraders generally make up 0.1% of the total microbial population; in oil polluted environments, they can make up to 100% of the community. In general, the population levels of hydrocarbon degraders and their proportions with respect to the total microbiota appear to be a valid index of environmental exposure to hydrocarbons (Atlas, 1981). The introduction of petroleum hydrocarbons into a pristine environment promotes growth of that fraction of the population able to utilize the new substrate. A study conducted by Aamand et al. (1989) on the degradation of petroleum hydrocarbons in heavily polluted and in slightly polluted aquifers found significant differences in the duration of lag periods for the degradation of hydrocarbons including toluene and o-xylene. The study found that shorter lag periods in heavily polluted ground water corresponded not only to higher initial bacterial numbers but also to a higher percentage of the bacterial population being able to degrade petroleum hydrocarbons as a result of previous exposure (Aamand et al., 1989). Petroleum addition reduces aeration and disturbs the carbon to inorganic nutrient ratio needed for growth though, and while substrate concentration must be high enough to allow the necessary enzymes to be induced, toxicity effects may occur.

Physical-chemical characteristics of a petroleum hydrocarbon mixture such as volatilization, sorption and solubility must also be considered in biodegradation studies. Percolation of low molecular weight hydrocarbons such as BTEX and methanol decreases their volatilization, as microbial activity takes place, the rate of biodegradation rather than volatilization becomes the main mechanism by which removal can be accomplished.

Most organic chemicals tend to sorb onto soil particles. Because of the unfavorable partitioning between the soil and the water phases, only a small fraction of the contaminants may be in solution (McCarty, 1988). Studies conducted by Robinson et al. (1989) on the availability of sorbed toluene for biodegradation suggest that biodegradation rates are limited by slow desorption. These studies found that the majority of sorbed toluene (>90%) desorbed rapidly into the water while the remaining fraction desorbed very slowly. The acclimated microbes used in this study, Pseudomonas putida (biotype B), were able to degrade the fraction of sorbed toluene that desorbed rapidly, and was readily available to microbial attack, at a fast rate. The remaining sorbed fraction, on the other hand, remained undegraded until desorption started to occur at a much slower rate. Slow desorption rates resulted in limited availability of toluene to microbial metabolism which in turn limited biodegradation (Robinson et al., 1989).

2.7 Biotransformation processes

In general, microbial counts and activity increase when oil contamination increases. Petroleum degradation is mainly accounted for by bacteria and fungi. Soil bacteria are characterized by a broad metabolic diversity which facilitates their ability to utilize a large variety of hydrocarbons. Enzyme specificity is widely recognized, but this specificity is with respect to the type of reaction they mediate. Enzymes are less specific with respect to the substrate itself (Grady, 1985). Therefore, it is not unusual for enzymes to act on analogues of a recalcitrant substrate provided that the active sites of the compounds do not differ greatly. In this case metabolism appears to be gratuitous or

fortuitous in that enzymes synthetized for one compound end up acting on the catalysis of another substrate.

Many microorganisms are capable of acting on one or more steps of a catabolic pathway but are not able to further utilize the product for growth and energy (Focht, 1988). The degradation of one compound in the obligate presence of another organic compound that serves as the primary substrate is known as cometabolism. A petroleum hydrocarbon mixture provides an ideal environment for cometabolism to occur because of the large number of potentially degradable substrates present within the oil. Cometabolism enhances the biodegradation of mixed petroleum hydrocarbons by allowing compounds which otherwise would not be degraded to undergo enzyme attack by microorganisms grown on other hydrocarbons within the petroleum mixture.

2.8 Aerobic Respiration

Mineralization of petroleum hydrocarbons in soil environments is normally highly dependent on molecular oxygen (Atlas, 1981). Aerobic respiration is an oxidation-reduction process in which molecular oxygen serves as the terminal electron acceptor. Because of its high reduction potential, the maximum amount of available energy is released from catabolism of a growth substrate when oxygen is the electron acceptor. To a lesser extent, molecular oxygen also acts as a reactant in the degradation of some compounds. In the degradation of aromatic hydrocarbons oxygen may be incorporated into the organic compounds through oxygenase enzymes. Once oxygen is incorporated,

oxidative catabolism of these simpler forms may proceed (Brock and Madigan, 1991).

2.9 Hydrogen peroxide as an oxygen source

Generally, oxygen is the limiting factor in the bioremediation of petroleum hydrocarbon contamination of aquifers. Because oxygen has a low aqueous solubility, hydrogen peroxide is frequently used as an oxygen supply in saturated subsurface environments. Hydrogen peroxide has the advantage of being miscible in water. It reacts to form water and molecular oxygen as follows (Hinchee et al., 1991):

$$H_2O_2 \rightarrow H_2O + \frac{1}{2} O_2$$

The use of hydrogen peroxide has its disadvantages however. It is toxic to microorganisms at high concentrations and it is unstable (Spain et al., 1989; Pardieck et al., 1990). Studies show that hydrogen peroxide decomposes at a fast rate in ground water and soil. If the rate of oxygen formation by peroxide decomposition exceeds the rate of oxygen utilization, oxygen in gaseous form will escape the solution and will no longer be available to microorganisms (Hinchee et al., 1991).

Many compounds present in ground water and soils act as catalysts in the decomposition of hydrogen peroxide. These catalysts can be inorganic, namely aqueous species of iron and copper or organic, and enzymatic catalysts such as peroxidase or catalase. Catalase is by far the strongest catalyst in the decomposition of hydrogen peroxide (Aggarwal et al., 1991). It is present in practically every living aerobic cells

(Lodaya et al., 1991).

Because hydrogen peroxide decomposes at a fast rate in the subsurface environment, the use of additives that will retard decomposition may be required for enhanced in situ biodegradation. There are several organic and inorganic substances known to stabilize hydrogen peroxide. Phosphate is the most common additive used as a stabilizer (Aggarwal et al., 1991). However, soluble phosphate may become insoluble in the presence of calcium commonly found in ground waters and precipitate out of solution; phosphate precipitation may reduce the aquifer's hydraulic conductivity. In addition, phosphate only deactivates inorganic catalysts and does not act on catalase. Since catalase activity plays such an important role in the decomposition of hydrogen peroxide, phosphate addition alone may not be the most adequate form of stabilization (Aggarwal et al., 1991; Hinchee et al., 1991).

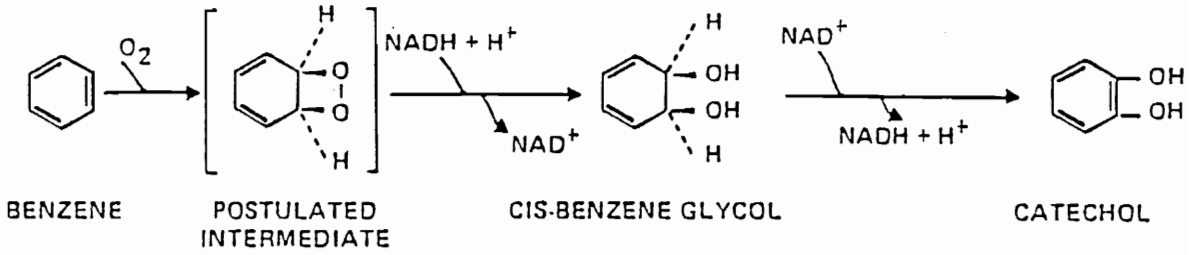
Catalases are inhibited by a diversity of inorganic and organic compounds, including fluoride, borate, sulfide, acetate, citrate, hydroquinnon, and catechol among others (Aggarwal et al., 1991). Since catechol is an intermediate metabolite in the degradation of BTEX, inhibition of catalytic activity by catechol resulting in reduced hydrogen peroxide decomposition may increase oxygen availability.

2.10 Degradation pathways of benzene and m-xylene

The general degradation pathways of BTEX under aerobic conditions have been documented extensively (Gibson et al., 1968, '73, '74; Davey and Gibson, 1974; Dagley, 1964; Nozaka and Kusunose, 1968). Bacteria of the genus Pseudomonas have been the best studied. The ability of bacteria to catalyze oxidations using molecular oxygen enables them to initiate a series of reactions that lead to the common catabolic pathways.

Metabolism of benzene is initiated through the introduction of oxygen by monooxygenases or dioxygenases into the aromatic nucleus, leading to the formation of catechol (Gibson et al., 1968). M-xylene follows a similar degradation path prior to ring fission, leading to the formation of 3-methyl catechol (Hou, 1982). Figure 1 shows the degradation pathways for benzene and m-xylene.

Ring cleavage of catechol or methyl-substituted catechol can be accomplished via the ortho pathway which involves cleavage of the bond between the carbon atoms bearing the hydroxyl group or via the meta pathway which involves cleavage of the bond between a carbon atom with a hydroxyl group and an adjacent carbon atom not bearing a hydroxyl group (Cerniglia, 1984).



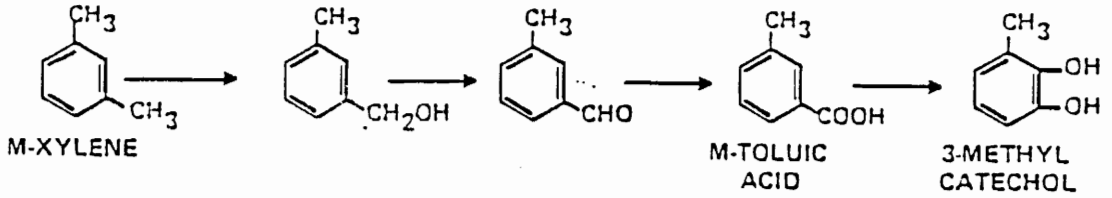


Figure 1. Aerobic Degradation Pathways of Benzene and m-Xylene.

2.11 Degradation pathway of methanol

One-carbon compound oxidizing microbes are ubiquitous to almost any aerobic environment (Vestal, 1984). Since C_1 compounds contain no carbon-carbon bonds, organisms that can grow only utilizing one-carbon compounds must completely generate their own carbon-carbon bonds. As opposed to autotrophs, methylotrophs require a carbon compound more reduced than CO_2 as substrate. Some methylotrophs are able to utilize other organic compounds in addition to methanol (Brock and Madigan, 1991).

The degradation pathway of methanol is shown in Figure 2. Methanol is oxidized to form formaldehyde. Formaldehyde can then be used in an assimilatory fashion to synthetize cell material or it can continue to be oxidized to CO_2 in a dissimilatory way with the production of reducing power for energy generation (Vestal, 1984).

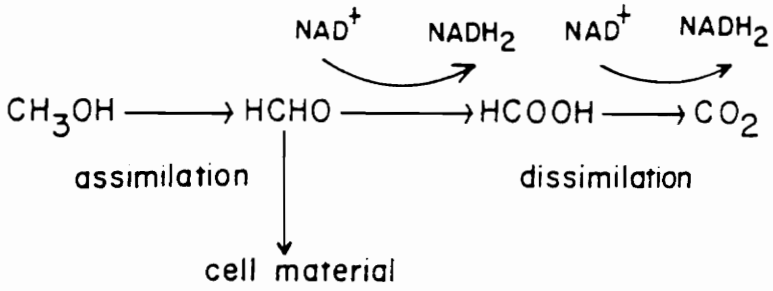


Figure 2. Aerobic Degradation Pathway of Methanol.

CHAPTER III

METHODS AND MATERIALS

3.1 Experimental Approach

The biodegradation potential and rates of degradation for benzene, m-xylene, and methanol mixtures was evaluated though microcosm studies. These compounds were selected because of their relatively high water solubilities with respect to other gasoline compounds which increased their potential for ground water contamination, and thus, their potential impact on human health. The increased use of methanol as a gasoline additive calls for attention to the study of the biodegradation potential of gasoline/alcohol mixtures because methanol laden ground water may mobilize benzene and m-xylene or alter their biodegradability.

This investigation consisted of two major phases. Phase I of this study used microcosms which contained only indigenous microorganisms. Phase II included microcosms containing both indigenous microorganisms and benzene degraders. Each set of microcosms contained soil from 4 different depths: surface, 12, 16.5, and 18 feet. The study included controls containing autoclaved soil to account for abiotic losses such as volatilization and sorption. Control and viable microcosms were dosed with the same spiked nutrient solution. A wide range of concentrations was covered in both phases I and II to evaluate the possible inhibitory effects of one compound over the degradation

of another.

3.2 General Methods

All material that came in contact with the soil was sterilized either by autoclaving for 30 minutes at 120°C and 15 psi of pressure or by alcohol flame sterilization. All glassware was acid washed for 24 hours in a 10% HCl bath.

3.3 Sample Collection

Soil was collected from a dairy farm located on Virginia Polytechnic Institute and State University property. The sample site was located on pasture land receiving little run off from adjacent forested land, and had no known history of petroleum contamination. Soil samples were taken from the surface, 12, 16.5, and 18 foot depths using a hand auger. Weathered bedrock was encountered at 18 feet. The water table was not reached prior to this and all samples were unsaturated. Each sample was then removed from the hand auger with sterilized spatulas and directly transferred to sterilized mason jars fitted with teflon caps. The jars were immediately transported to the laboratory and placed in an incubator at 10° C.

3.3 Soil Moisture and pH measurements

The soil moisture content was determined by method 21-2.2.2 from "Methods of Soil Analysis". The samples were weighed and dried in an oven at 105°C until they reached a constant weight. The samples were then placed in a desiccator until cooled,

and weighed again. The soil moisture content was attributed to be the amount of mass lost in the process, obtained by the following formula:

$$\%M = \frac{\text{Weight of wet soil} - \text{Weight of dry soil}}{\text{Weight of dry soil}} 100$$

Soil pH measurements were obtained by method 12-7.6 from "Methods of Soil Analysis". Five grams of soil were mixed with 5 milliliters of distilled water for 5 seconds, and allowed to settle for 10 minutes. The hydrogen ion concentration of the soil solution was measured using a pH meter.

3.5 Microcosm Preparation

Microcosms were prepared using 13 × 100 mm glass screw cap test tubes sealed with a 12 mm teflon cap. Five grams of soil (±0.1 g) were added to each test tube. All microcosms were stored in the dark, at 20°C. Controls were created for all soil depths and substrate concentrations using autoclaved microcosms in an attempt to provide sterile conditions. A portion of the soil collected was set aside for the controls and autoclaved at 120°C and 15 psi of pressure for 30 minutes, 4 times during a 48 hour period. Five grams of autoclaved soil were added to each control test tube. The entire set of control microcosms was then autoclaved again 4 times over a 48 hour period.

Sterile conditions were not attained for some of the control microcosms which exhibited the same patterns of substrate loss as viable microcosms. Failure in achieving complete sterilization of soil is not unusual since soil microorganisms may form cysts or

spores which are highly resistant to heat (Gray and Williams, 1971). Data obtained from successfully autoclaved microcosms were sufficient to establish a pattern of abiotic losses.

3.6 Nutrient Solution Preparation and Microcosm Dosing

A nutrient solution was prepared consisting of 1.9 mg KH_2PO_4, 6.25 mg K_2HPO_4, 0.5 mg $(NH_4)_2HPO_4$, and 0.5 ml salts solution in 1000 ml of sterile distilled water. Due to phosphate buffering, the nutrient solution fixed the pH at about 7.2. The buffered nutrient solution was placed on a magnetic stir plate so that a vortex was formed. Drops of benzene, m-xylene and methanol were then added to the center of the vortex. The bottle containing this solution was immediately sealed and stirred for at least 12 hours to assure proper mixing of the hydrocarbons in the water. Minimal head space was maintained to reduce volatilization. Initial benzene, m-xylene and methanol concentrations varied over a wide range.

The spiked nutrient solution was then pumped to a sterile collapsible teflon bag. Microcosms were dosed from this teflon bag. After partially filling the test tube, the microcosm was closed and mixed on a vortex mixer to assure an even distribution of the nutrient solution and to displace trapped air bubbles. The microcosm was then filled to maximum capacity to minimize head space. Controls were dosed simultaneously with the viable microcosms, with the same spiked nutrient solution. Two drops of the solution containing benzene degraders were added only to those microcosms utilized in phase II of this study. The benzene degraders were isolated and supplied by Sybron Chemical

Co., and stored in the Environmental Engineering Laboratory in shaker flasks containing a nutrient solution identical to that used by Sybron. In this study, the nutrient solutions utilized for dosing of the microcosms and for maintaining the benzene degraders were identical.

3.7 Addition of Hydrogen Peroxide

Hydrogen peroxide was added to every test tube in the study. Each microcosm was dosed with 5µl of a 30% H_2O_2 solution, immediately after dosing with the spiked nutrient solution was completed. This amount yielded 0.03% by volume in the microcosm, a concentration found to be non-toxic by Gullic (1990) and Farmer (1989). In addition, a study conducted by Britton (1985) found that H_2O_2 did not become toxic to bacteria until a concentration of 0.05% by volume was reached. In this study, hydrogen peroxide was added using a 10µl sterilized syringe, placing the tip just below the water surface. Additional amounts were added periodically in 2µl doses.

3.8 Standard Preparation

Two standards were used in this study. The BTEX standard was prepared in Methylene Chloride, since BTEX are completely soluble in this medium. The methanol standard was prepared in distilled water. Since the gas chromatographic response for methanol and BTEX was linear over the range of concentrations utilized (Goldsmith, 1985), a calibration curve was not necessary.

3.9 Analytical Methods

Two microliters of each standard were injected into a Hewlett Packard Model 5880A gas chromatograph with a flame ionization detector (FID). An oven temperature program was utilized to assure proper distribution of output peaks. The oven temperature was held at 100°C for 3 minutes, then increased at a rate of 30°C/minute until it reached 170°C and held there for 20 minutes. The injector port temperature was set at 150°C, and the FID temperature at 225°C. Nitrogen, the carrier gas, flowed at rate of 30 ml/minute through a 6 ft x ⅛ in stainless steel column packed with 0.2% Carbowax 1500 on 80/100 mesh Carbopak-C. A detection limit of 0.1 mg/L was determined by Farmer (1989) and Goldsmith (1985). For the microcosms, 2µl of supernatant were withdrawn and immediately injected into the GC in the same manner described for the standard analysis, with the exception of the initial oven temperature being 150°C instead of 100°C.

CHAPTER IV

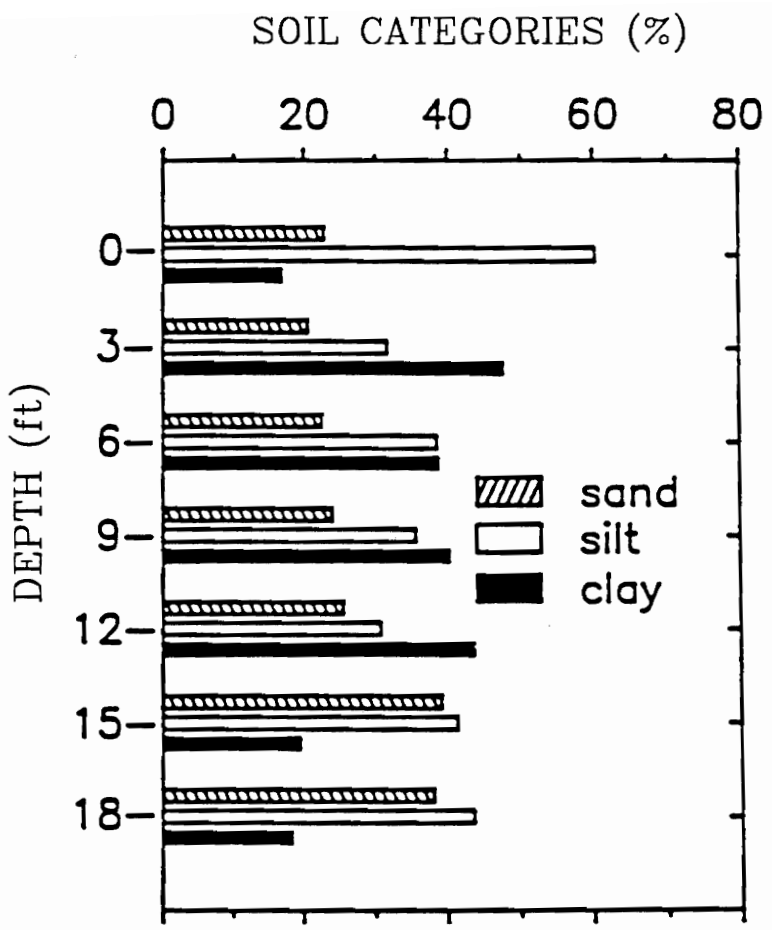
RESULTS AND DISCUSSION

4.1 Soil Characteristics

Since the soil used in this study was collected from sites immediately adjacent to Gullic's sampling site (Gullic, 1990), his soil characterization was assumed to be applicable to this study. The particle size characterization corresponding to the samples collected by Gullic is shown in Figure 3.

Soil samples for this study were collected from the surface, 12, 16.5, and 18 foot depths. The particle size analysis indicates that surface soils had the greatest amount of silt (more than 60%), and comparatively low percentages of sand and clay. The 12 foot soil, on the other hand, was very rich in clay (about 46%), and had relatively low amounts of silt and sand. This soil was similar over the range from 3 to 12 feet so only the 12 foot soil was utilized. The 16.5 and 18 foot soils had about the same proportions of silt and sand, corresponding to about 40% of each, and relatively low amounts of clay.

The moisture content of soils used in this study is shown in Figure 4, and compares well with the analysis conducted by Gullic. Surface soils were very dry, with only about 19% water. However, the moisture content of surface soils is very dependent on the amount of precipitation and thus can vary considerably (Gullic, 1990). The high



(after Gullic, 1990)

Figure 3. Percentages of sand, silt and clay at each soil depth.

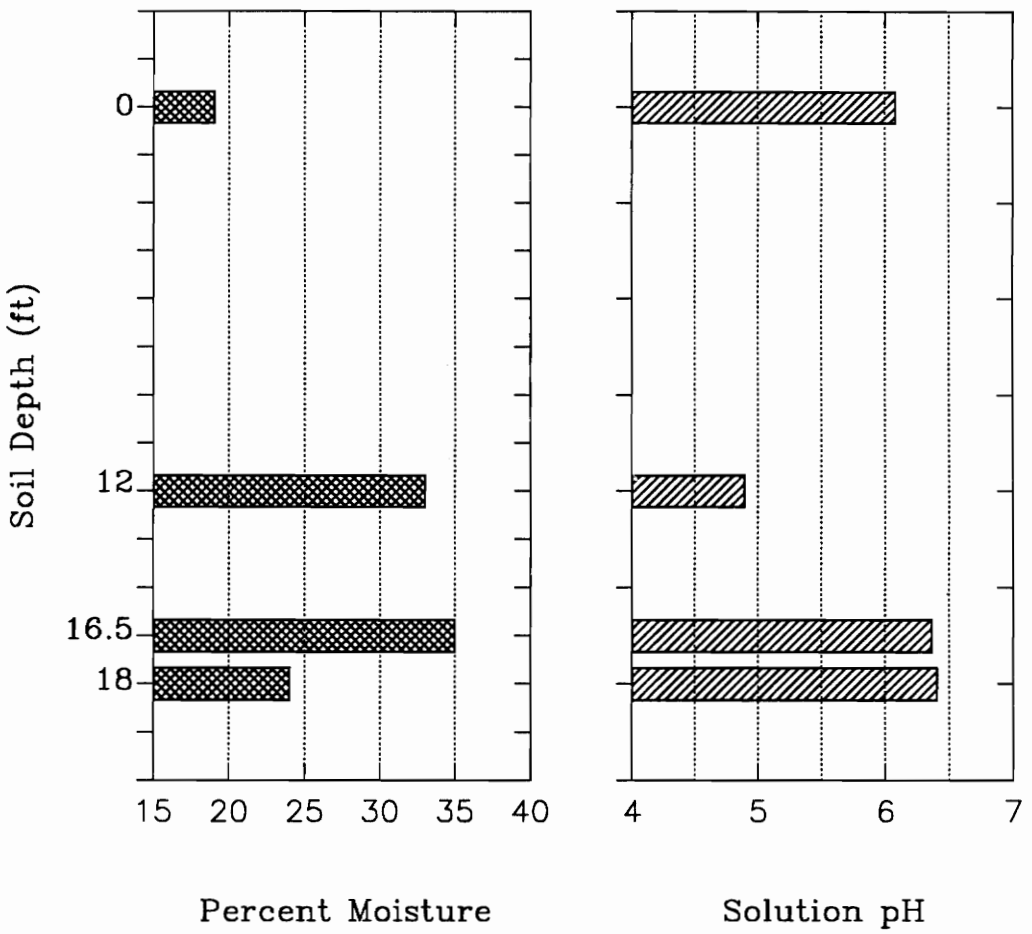


Figure 4. Soil moisture content and natural solution pH with depth.

clay content of the 12 foot soils accounts for their high water content (about 33%). Soils collected from 16.5 feet had the highest moisture content (about 35%), even though they contained relatively low amounts of clay. The 18 foot soil was a sandy-silty soil relatively well drained, and contained only about 24% moisture. Figure 4 also shows the natural pH of the soils utilized in this study. Surface soils had a solution pH of about 6, the high clay content of the 12 foot soil accounted for its low pH (about 4.9), while the 16.5 and 18 foot soils had very similar pH values of about 6.4.

4.2 Abiotic Losses

Initial benzene and m-xylene losses, calculated through the use of autoclaved microcosms, were attributed to abiotic processes such as volatilization and adsorption. The concentrations of benzene and m-xylene decreased significantly in all microcosms over the first 35 days after dosing. These initial losses are shown in Figures 5 and 6, and are typical of the microcosms utilized in this study.

The initial removal of benzene and m-xylene from solution, which ranged from about 20% to 50% of the initial concentration, was attributed to adsorption. Benzene and m-xylene are moderately hydrophobic and tend to adsorb to soil particles. Since m-xylene is less soluble than benzene, it tended to sorb more strongly. Studies conducted on the sorption of BTX compounds found that m-xylene sorbed onto soil more readily than toluene and toluene more readily than benzene (Goldsmith, 1985, citing Rodgers et al., 1980 and Nathwani, et al. 1977). Volatilization occurred while the microcosms were

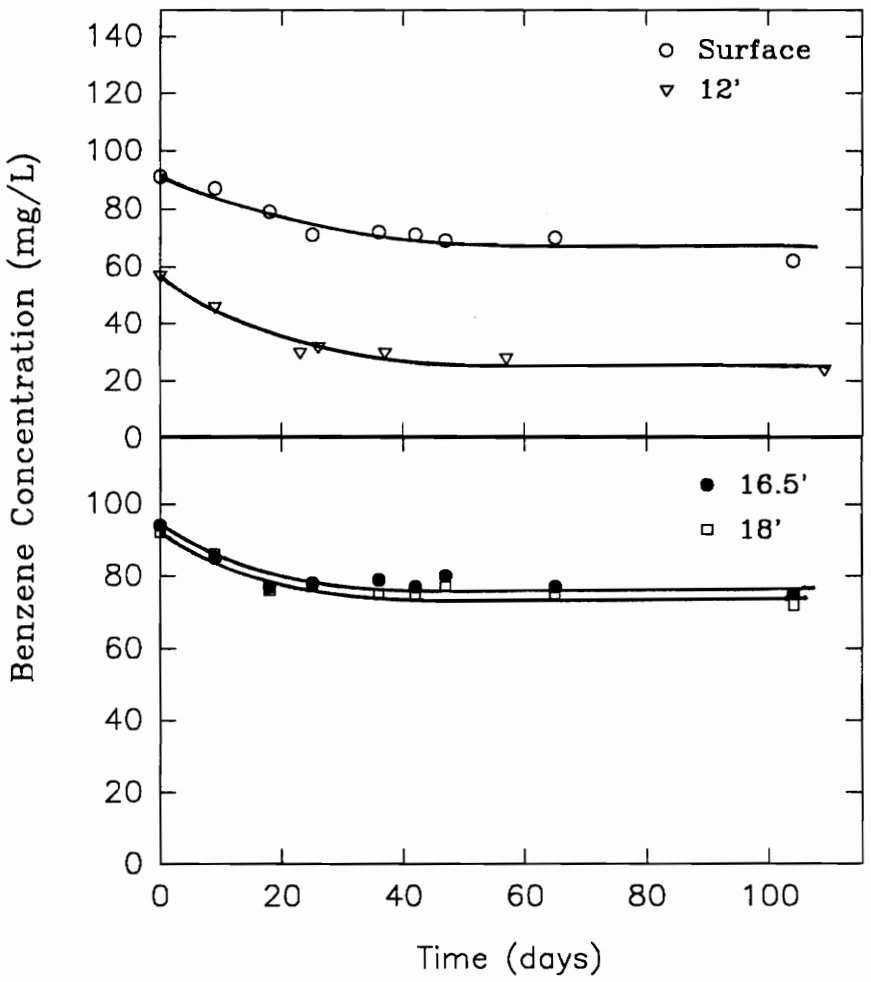


Figure 5. Abiotic losses of benzene in representative microcosms.

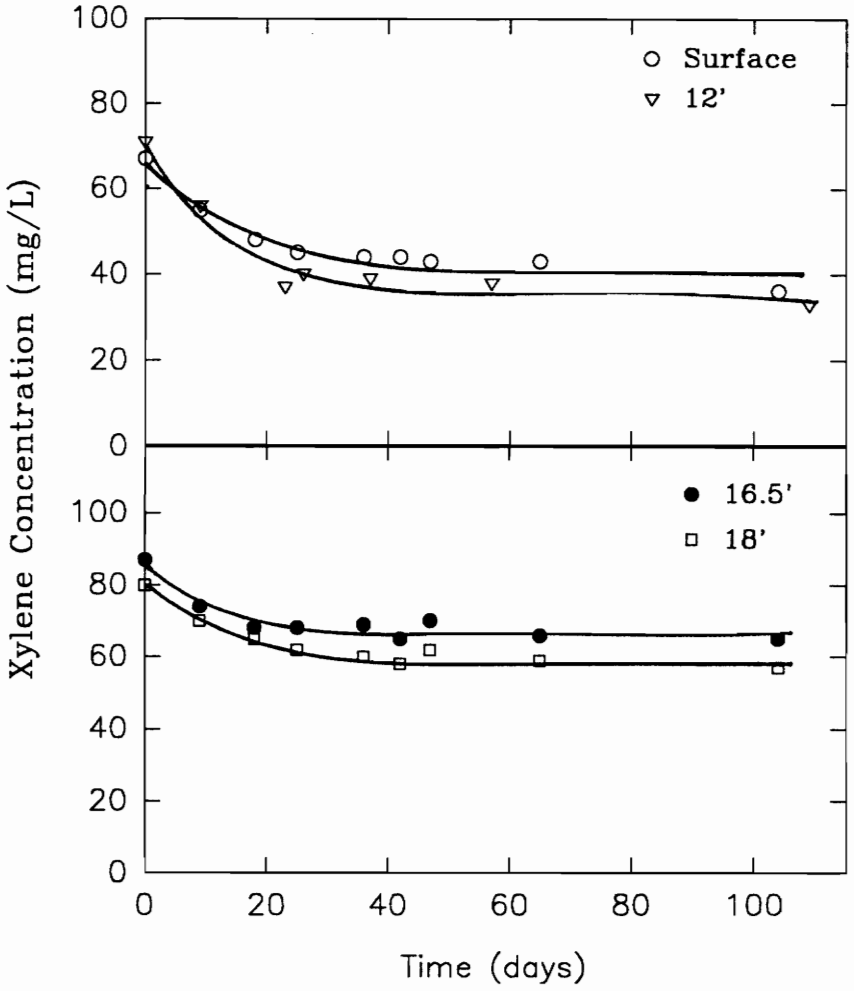


Figure 6. Abiotic losses of m-xylene in representative microcosms.

briefly opened for sampling. However, since the test tubes were uncovered for only a few seconds, volatilization losses were minimal.

Adsorption was also dependent on soil particle size. The 12 foot soil adsorbed considerably more benzene and m-xylene than the other soils, with about 50% of the initial concentration being lost. This can be attributed to the high ionic strength of clayey soils which increased their adsorptive capability (Krone, 1968). Surface soils had the greatest amount of silt and adsorbed considerably less benzene and m-xylene than did 12 foot soils. The 16.5 and 18 foot soils, which had relatively low amounts of clay and contained the greatest amount of sand, exhibited the lowest amounts of benzene and m-xylene adsorption. Abiotic losses for methanol, on the other hand, were not as significant. Since methanol is completely soluble in water, volatilization losses were minimal and adsorption was not obvious. Figure 7 shows typical methanol data obtained from control microcosms.

Studies conducted by Robinson et al. (1989) indicate that toluene adsorption occurs in two stages, an initial rapid adsorption phase followed by a much slower adsorption phase. The study suggests that desorption occurs in a similar fashion, with as much as 90% of the total adsorbed toluene desorbing into the water in a two day period, once the concentration in solution decreased significantly. As a result, greater than 90% of the desorbed toluene becomes readily available to microorganisms for degradation. The remaining sorbed toluene desorbs very slowly, and degradation of toluene becomes

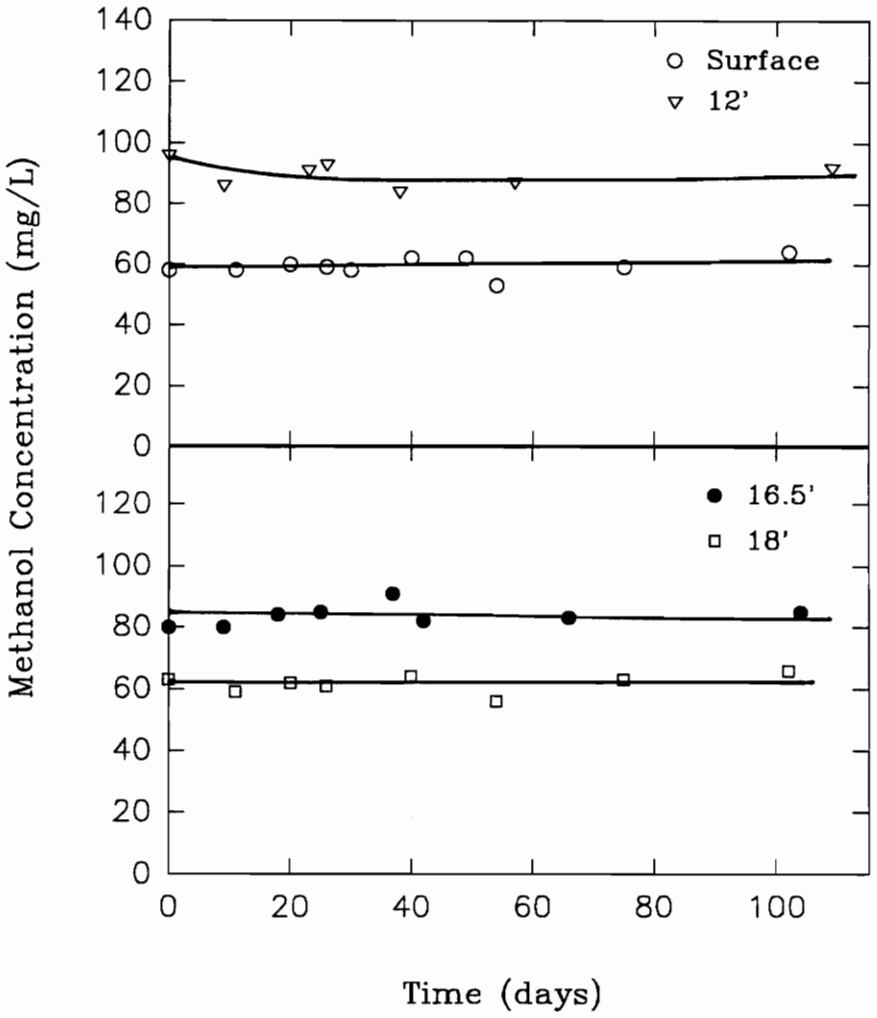


Figure 7. Abiotic losses of methanol in representative microcosms.

limited by desorption.

Since benzene and m-xylene are similar in structure and properties to toluene, it is likely that they follow the same desorption pattern. In viable microcosms, as the aqueous concentrations of benzene, m-xylene, and methanol were reduced by biodegradation, a concentration gradient between the solid and aqueous phases occurs, resulting in desorption of these compounds and thus they become available for biodegradation. Therefore, it is probable that the degradation rates measured in this study for the aqueous fraction of the substrates may represent reductions in both the adsorbed and aqueous phases.

4.3 Acclimation

The observed acclimation periods for the indigenous microbial population are discussed in the following paragraphs. For samples where biodegradation occurred, these microcosms often exhibited a period in which benzene, m-xylene, and methanol degraded very slowly or not at all. This period was followed by a relatively rapid loss of the chemicals, which was attributed to biodegradation. This initial lag period denotes the length of time required for microorganisms to become acclimated to the chemicals to which they are exposed, and often includes:

> (a) The time needed for induction of specific enzymes not present or present at low levels in the population; (b) exchange of genetic material mediated by plasmids; (c) creation of new metabolic capabilities produced by genetic changes or (d) division and growth of the organisms already capable of degrading the new substrate. (Aamand et al., 1989)

Thus acclimation is characterized by a change in the microbial community as a result of prior exposure to the chemicals which increases biotransformation rates.

Figure 8 shows typical degradation patterns for methanol in microcosms. Lag periods up to 20 to 40 days were typical for surface, 16.5 and 18 foot soils. The 12 foot soils exhibited acclimation periods in excess of 60 days. Acclimation periods for benzene and m-xylene, illustrated by Figures 9 and 10, varied considerably for the 12, 16.5 and 18 foot soils. Surface soils for the most part were inactive, showing no measurable benzene and m-xylene biodegradation rates throughout the course of this study. McCormick (1991) who conducted studies on the biodegradation potential of BTEX in soil from the same location, also found very low biodegradation rates in surface soils. Biodegradation at each soil depth is discussed in detail in the following sections.

From Figures 9 and 10 it can be seen that some samples were characterized by very long acclimation periods. This could be the result of a lack of oxygen availability to the microorganisms. As mentioned before, hydrogen peroxide was used in this study as a source of oxygen. It has the advantage of being miscible in water, and reacts to form molecular oxygen. However, hydrogen peroxide has the disadvantage of decomposing at a fast rate in ground water and soil. Consequently, it is possible that the rate of oxygen formation by peroxide decomposition exceeded the rate of oxygen utilization, allowing oxygen in gaseous form to escape the solution and no longer be available to the microorganisms. Since hydrogen peroxide may also be toxic to

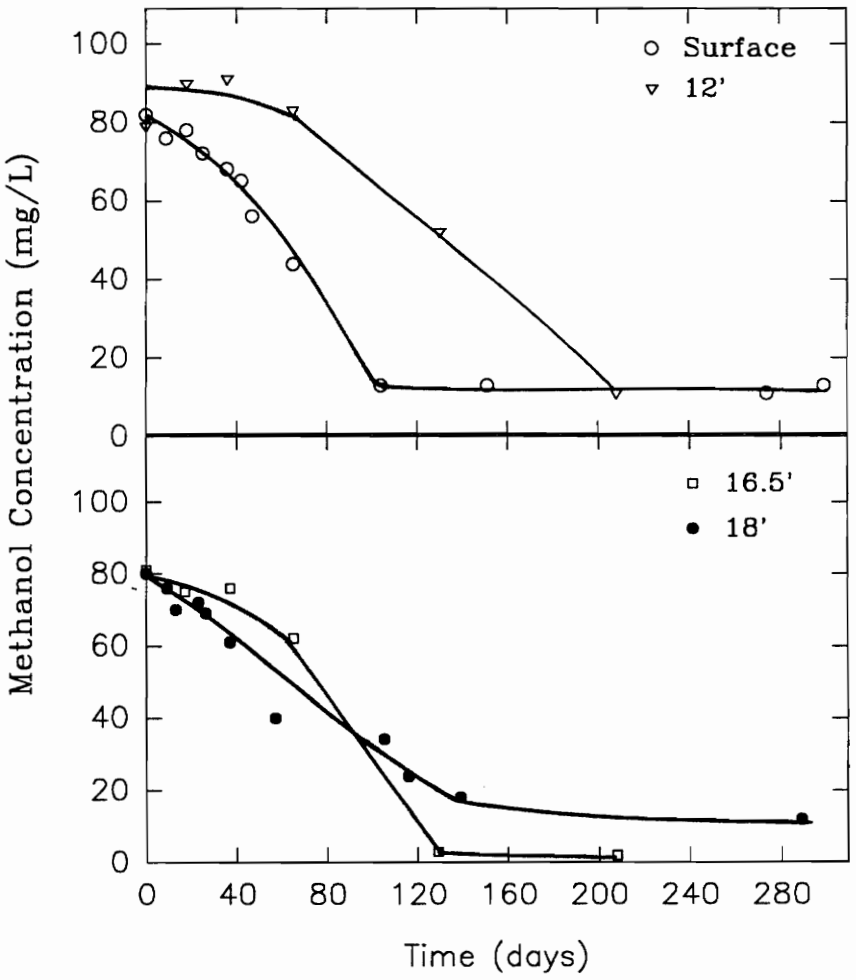


Figure 8. Acclimation periods for methanol in representative microcosms. Indigenous microorganisms.

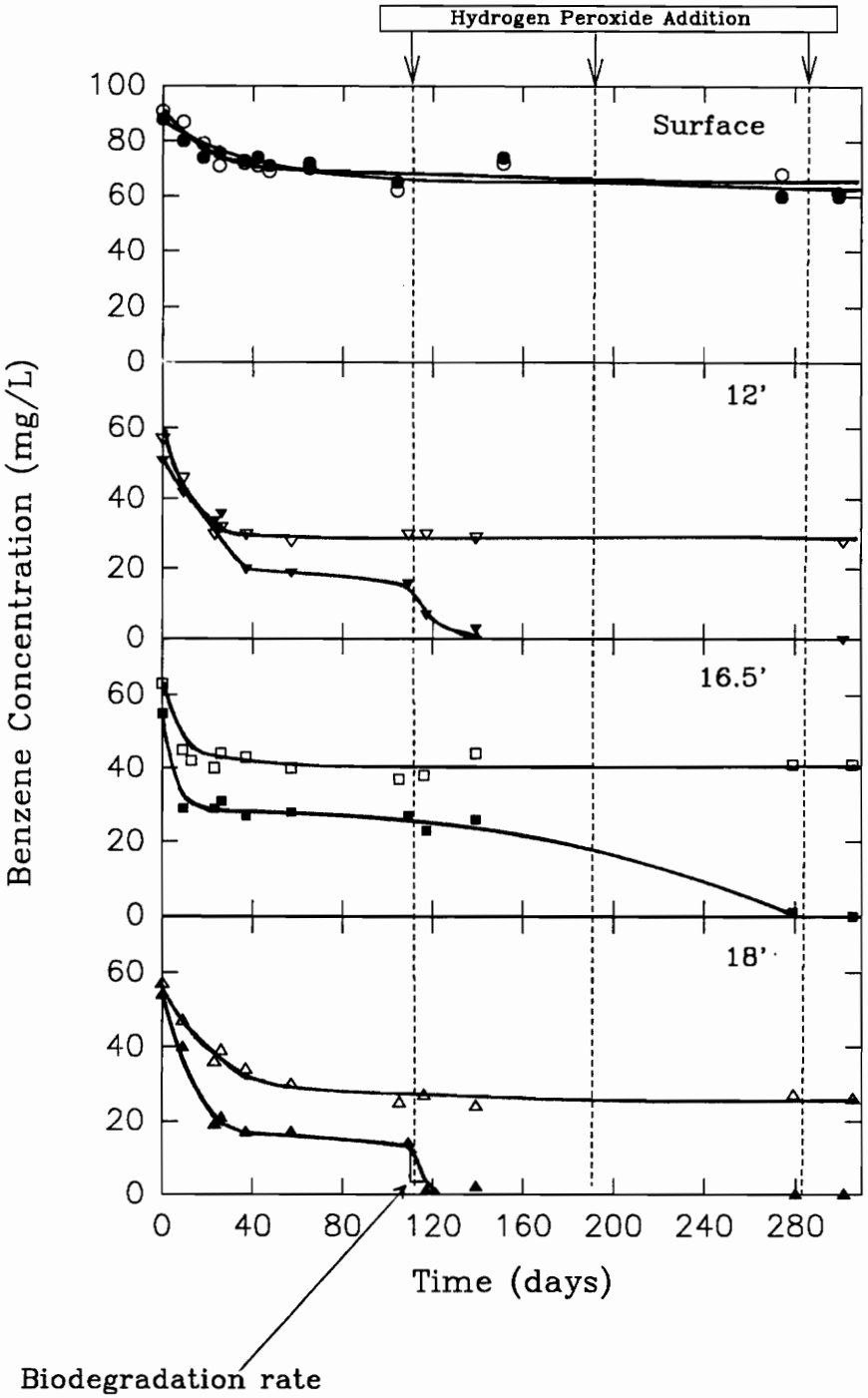


Figure 9. Acclimation periods for benzene in representative microcosms. Hollow symbols represent control microcosms; filled symbols represent viable microcosms; dashed lines represent hydrogen peroxide dosing. Indigenous microorganisms.

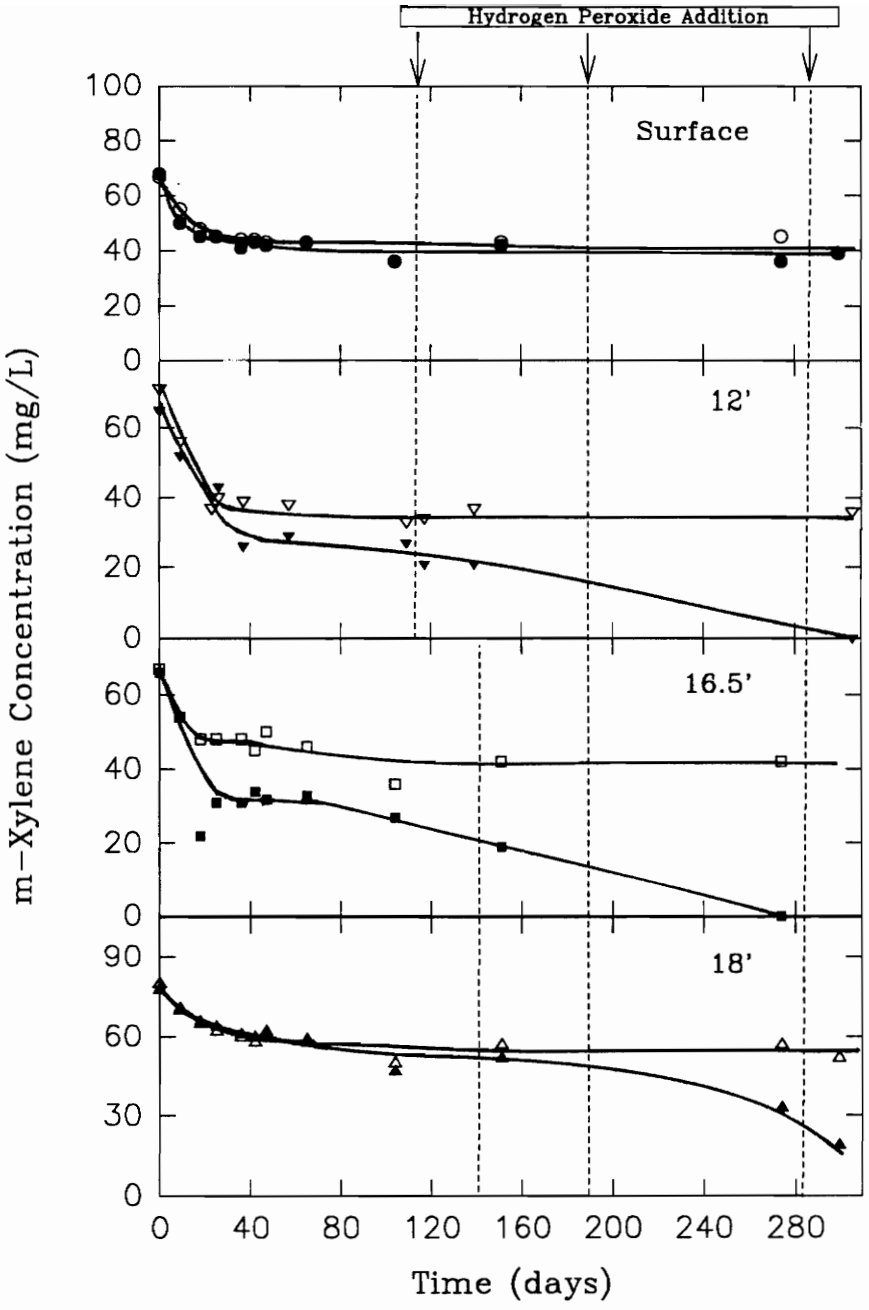


Figure 10. Acclimation periods for m-xylene in representative microcosms. Hollow symbols represent control microcosms; filled symbols represent viable microcosms; dashed lines represent hydrogen peroxide dosing. Indigenous microorganisms.

microorganisms at high concentrations, only small amounts were added to the microcosms on an as needed basis. From the data obtained from some microcosms, especially at the beginning of this research, it can be speculated that some of the long observed acclimation periods were caused by a lack of oxygen. As hydrogen peroxide dosing became more frequent, a decrease in the length of acclimation periods occurred. In that regard, the observed acclimation periods may not be true acclimation periods and are not considered further in this research.

It should be noted that considerable differences were observed in the adsorptive characteristics of soils as well as in the lengths of the acclimation periods, even for soils from the same depth. Data from adsorptive studies conducted by Farmer (1989) on toluene and McCormick (1991) on BTEX biodegradation showed similar results. Variations in the adsorptive characteristics of soils may affect microbial responses within the same depth and thus affect their ability to acclimate to the newly introduced chemicals, reflected by variations in lag periods.

4.4 Biodegradation by indigenous microorganisms (PHASE I)

It was of interest to evaluate the impact of methanol on benzene and m-xylene degradation because of the potential for the addition of methanol to gasoline or its use as a replacement fuel. Therefore, soil microcosms were constructed and dosed with various concentrations of methanol, benzene, and m-xylene. Methanol concentrations of up to 230 mg/L were used to determine its effect on the degradation of benzene and m-xylene. Since batch studies conducted by Kim (1987) reported benzene to be inhibitory to soil microorganisms at about 200 mg/L, an effort was made in this study to cover a wide range of benzene concentrations, including Kim's reported inhibitory level. However, since benzene is moderately hydrophobic, soluble concentrations of only up to 160 mg/L could be attained in the microcosms. Since McCormick (1991) found that m-xylene, at about 58 mg/L, lowered biodegradation rates in some microcosms containing soil from the same location as this study, m-xylene concentrations ranging from 50 mg/L to about 80 mg/L were used.

Biodegradation rates were estimated only after the initial rapid adsorption and lag periods had ceased. Linear approximations were used to estimate maximum degradation rates at points of maximum organic removal, as shown in Figure 9. Table 3 shows average biodegradation rates for each soil depth. Concentration effects of each compound within the mix are examined in detail in the following sections. Edaphic factors such as soil particle size and moisture content which seem to have important implications on microbial responses are also discussed.

TABLE III: Average Biodegradation Rates *
Indigenous microbes (PHASE I)

SET #	Initial Substrate Concentration (mg/L) **	Surface Soil	12' Soil	16.5' Soil	18' Soil
1	M = 200 B = 36 X = 50	2.94 0.82 0.76	1.13 0.13 0.22	2.14 0.27 0.61	1.61 0 0
2	M = 92 B = 40 X = 50	0.91 0 0	0.37 0 0	0.22 0 0.17	0.50 0 0.08
3	M = 85 B = 60 X = 62	0.52 0 0	0.33 0.24 0.13	0.24 0.17 0	0.22 0.63 0
4	M = 80 B = 90 X = 68	0.66 0 0	0.32 0.35 0.25	0.40 0.23 0.21	0.40 0.37 0.69
5	M = 230 B = 130 X = 50	0 0 0	0 0 0	0 0.24 0	0.84 0.57 0.26

* Rates determined at points of maximum organic removal
** M = methanol; B = benzene; X = m-xylene

4.4.1 The effect of methanol on biodegradation rates

Results obtained by this study indicate that the presence of methanol did not adversely affect the biodegradation of benzene and m-xylene. More importantly, the addition of increasing concentrations of methanol seemed to stimulate the biodegradation of not only methanol, but also of benzene and m-xylene, in the surface and 16.5 foot soils. Methanol was readily degraded by indigenous microorganisms in the surface soils. For the most part, benzene and m-xylene were not degraded. These results are in agreement with McCormick's (1991) findings of very low degradation rates for BTEX in surface soils from this location. McCormick reported non existent to very low biodegradation rates of BTEX at this depth and postulated that this phenomenon could be attributed to the ability of surface microbes to metabolize the most readily degradable organic compounds allowing more recalcitrant compounds to be transported to the subsurface (McCormick, 1991; Hickman, 1988). Methanol, with a relatively simple chemical structure and high water solubility, may be more susceptible to microbial attack than benzene and m-xylene. Figures 11 through 14 show the biodegradation rates obtained in phase I of this study.

Methanol is ubiquitous in the environment and can be biodegraded under aerobic and anaerobic conditions. Microcosm studies conducted by White (1986) on the biodegradation potential of methanol as the sole substrate, indicated that complete utilization of concentrations as high as 1000 mg/L occurred in a period less than a year. Goldsmith (1985) conducted microcosm studies on the degradation of methanol, and on

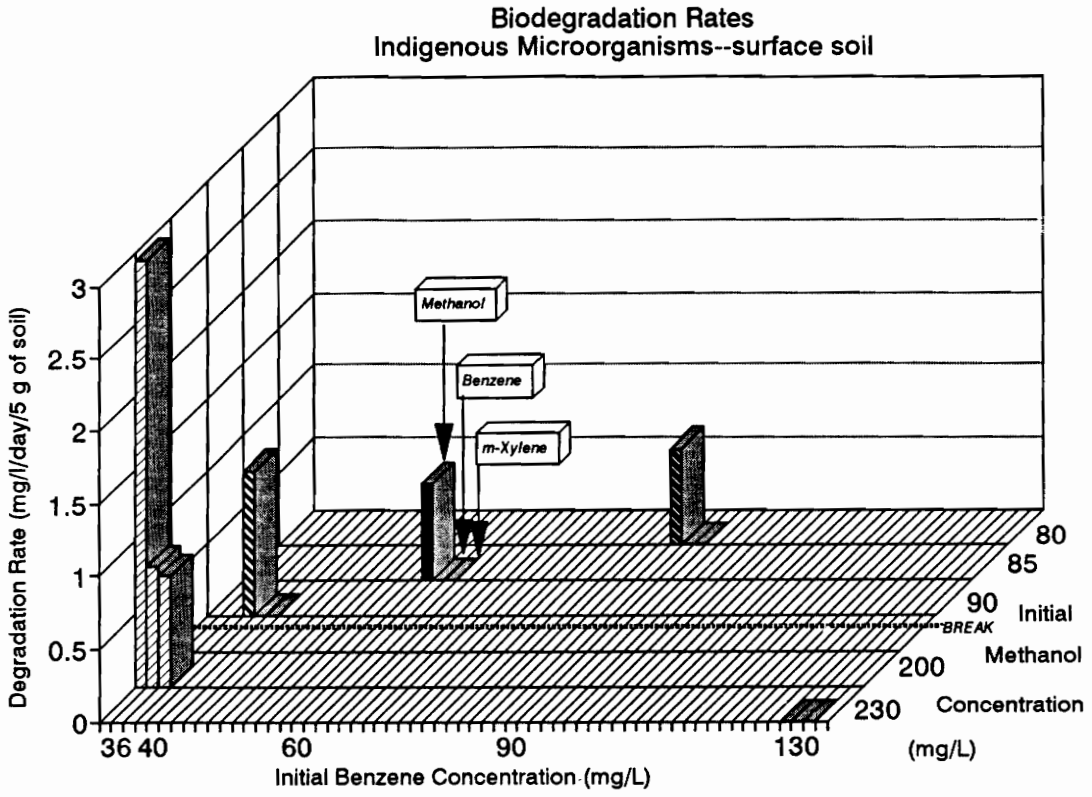


Figure 11. Average biodegradation rates in surface soil with increasing benzene and methanol concentrations. Indigenous microorganisms.

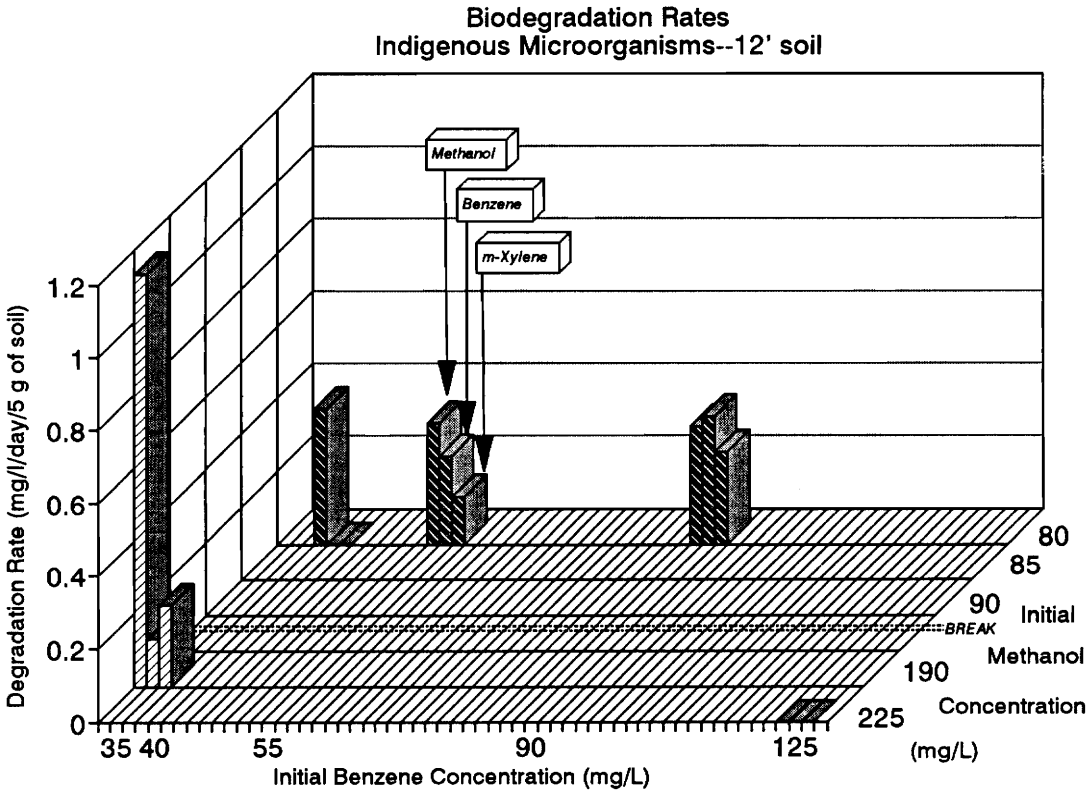


Figure 12. Average biodegradation rates in 12 foot soil with increasing benzene and methanol concentrations. Indigenous microorganisms.

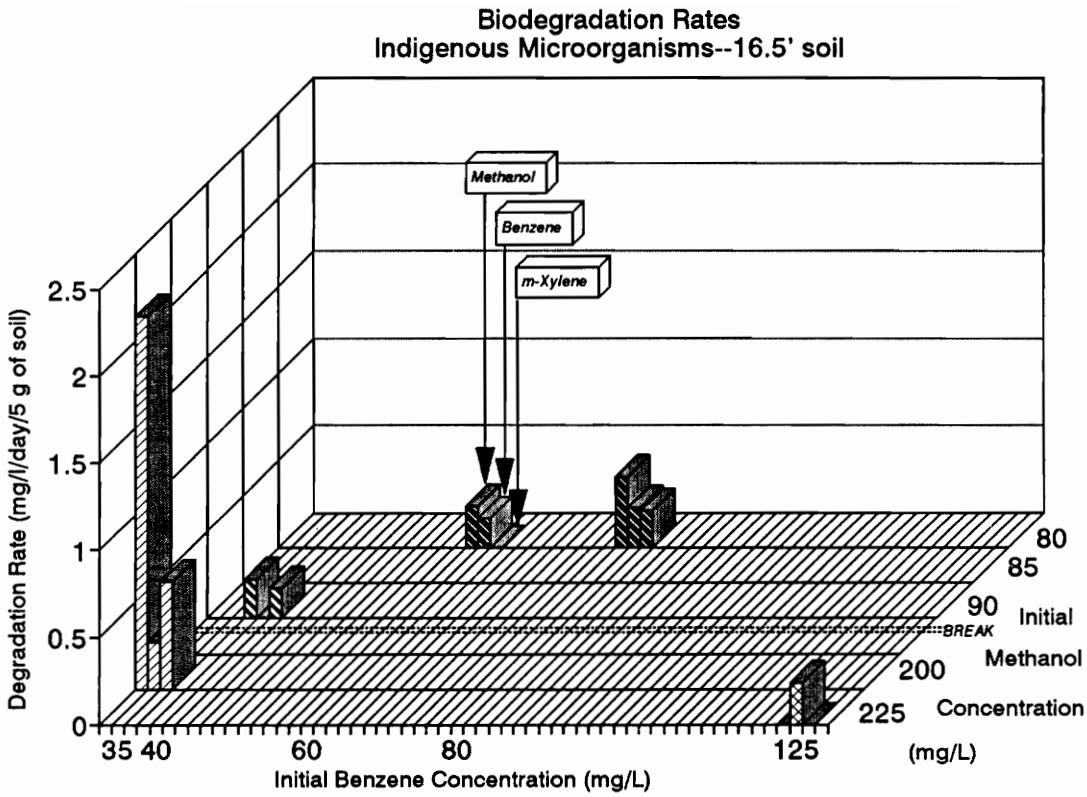


Figure 13. Average biodegradation rates in 16.5 foot soil with increasing benzene and methanol concentrations. Indigenous microorganisms.

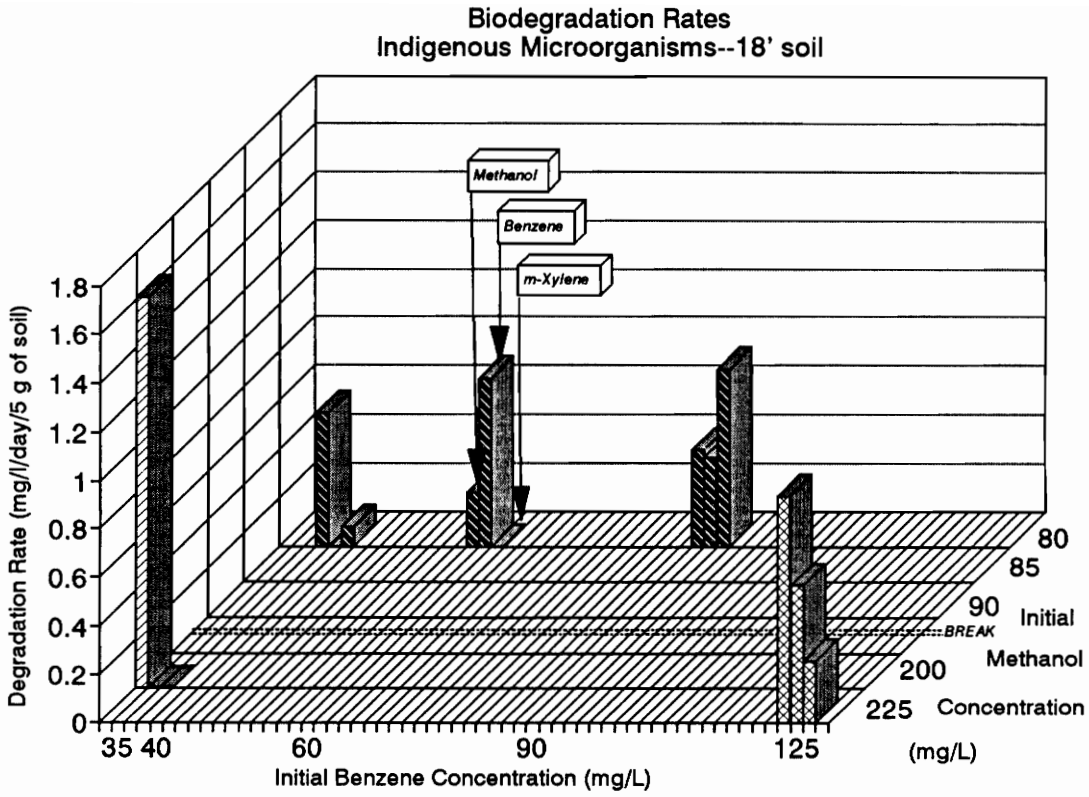


Figure 14. Average biodegradation rates in 18 foot soil with increasing benzene and methanol concentrations. Indigenous microorganisms.

the degradation of methanol and BTX mixtures. He found that methanol was readily biodegraded at concentrations ranging from 80 to 500 mg/L under anoxic conditions. Goldsmith's study also concluded that the degradation of methanol was not inhibited by the addition of a low concentration mix of BTX (total concentration of about 12 mg/L). In fact, for some microcosms, the biodegradation of methanol appeared to be enhanced by BTX addition. Goldsmith suggested that this enhancement effect could be related to the ability of several methylotrophs to oxidize aromatic compounds.

In Figures 11 and 13, biodegradation rates for each compound in the surface and 16.5 foot soils over the range of concentrations utilized in phase I of this study are shown. The data indicate that microcosms containing high initial methanol concentrations and low benzene concentrations exhibited considerably higher degradation rates (benzene seemed to hinder biodegradation at concentrations of about 125 mg/L; this effect is discussed in detail in Section 4.4.3). Comparison of biodegradation rates obtained from microcosms in which the initial benzene concentration was below the level determined to inhibit biodegradation indicates that the methanol concentration was an important factor in the increase of biodegradation rates, since m-xylene did not seem to affect biodegradation over the range of concentrations studied.

The increase in the biodegradation rates of benzene and m-xylene by increasing the concentration of methanol is more evident in the surface soils. As mentioned before, in surface soil, benzene and m-xylene did not readily degrade. In most surface soil

microcosms, methanol was degraded while biodegradation of benzene and m-xylene did not occur. It was not until the initial concentration of methanol was increased to 200 mg/L that significant biodegradation of methanol occurred, but more importantly, benzene and m-xylene were degraded only under these conditions. Microcosm data obtained from surface soils is shown in Figures 15 and 16. Figure 15 represents typical data observed in most microcosms, while Figure 16 shows data obtained at methanol concentrations of about 200 mg/L. Redosing of these microcosms confirmed these results. As expected, higher biodegradation rates were obtained after redosing since the microorganisms were already acclimated to the substrates. The fact that an increase in the initial methanol concentration stimulated the biodegradation of other organic compounds seems to indicate that the dominant microbial population of surface soils is made of organisms able to utilize methanol for energy and growth and simultaneously oxidize benzene and m-xylene, since no sequential substrate utilization was observed in any of the surface soil microcosms containing only indigenous microorganisms. Similarly, biodegradation rates for all compounds in the 16.5 foot soil were also stimulated by higher initial methanol concentrations, but to a lesser degree than in surface soils.

It is known that some bacteria pertaining to the genera Hyphomicrobium and Pseudomonas that will grow on methanol are also able to utilize a large number of non-one-carbon compounds (Brock et al., 1984). Thus it is possible that the addition of methanol to these organisms would increase their numbers and thus enhance biodegradation of other organic compounds due to the lack of substrate specificity

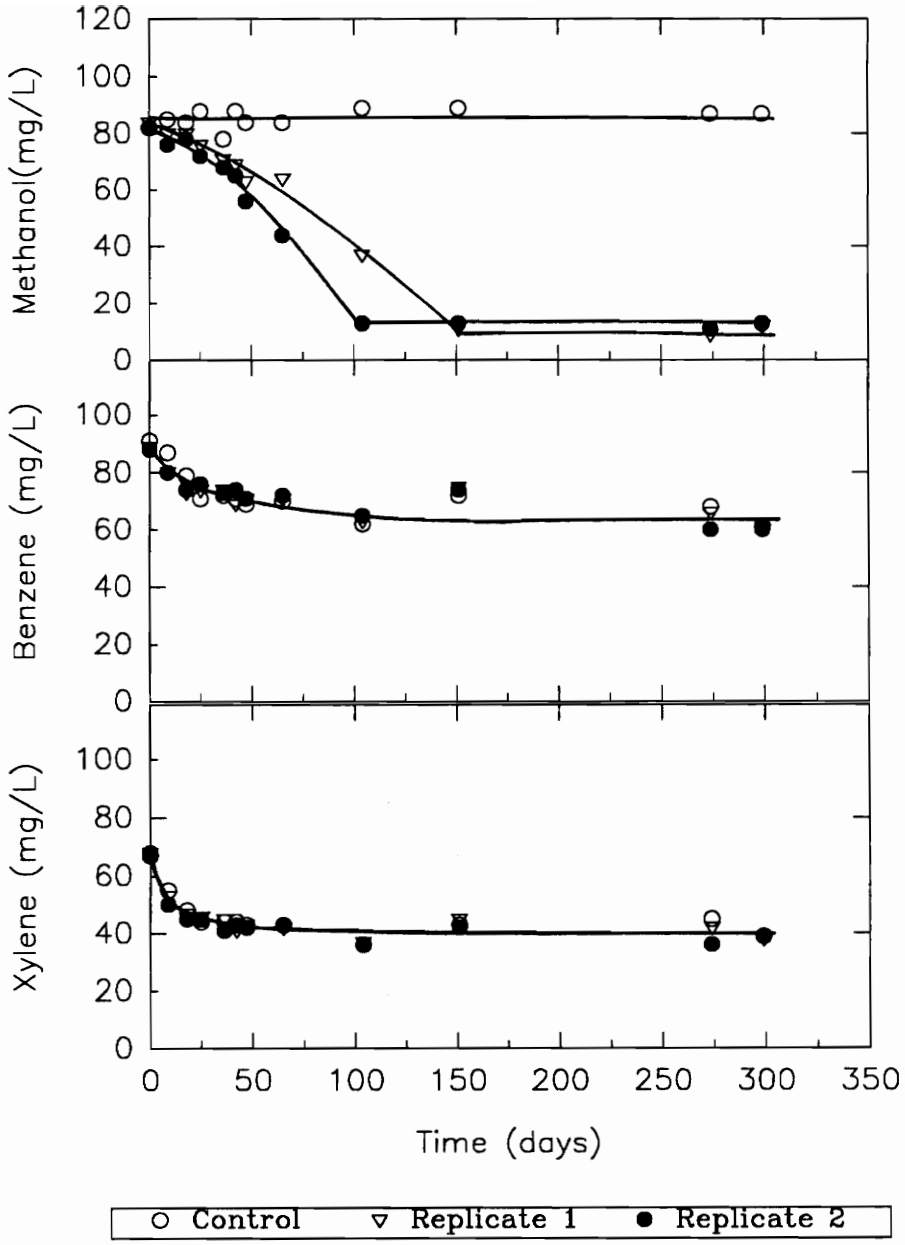


Figure 15. Typical microcosm data for surface soils. Indigenous microorganisms.

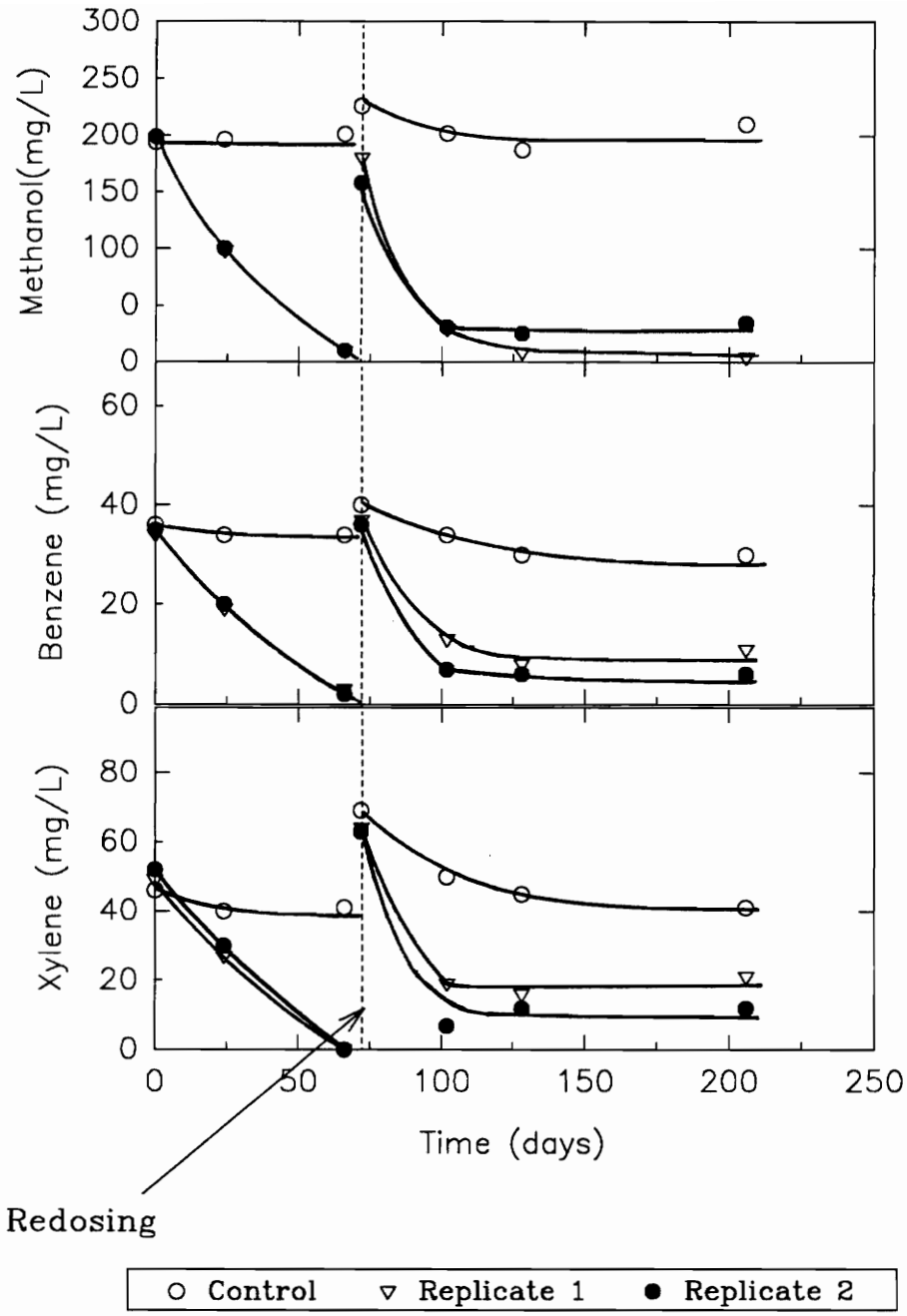


Figure 16. Biodegradation rates of surface soil microcosms dosed with high methanol concentrations. Indigenous microorganisms.

exhibited by some facultative methylotrophs. This mechanism would explain the increase in biodegradation rates for all compounds in surface and 16.5 foot soils at higher methanol concentrations.

As shown by Figures 12 and 14, only the biodegradation rates of methanol increased at higher methanol concentrations in the 12 and 18 foot soils. Higher concentrations of methanol did not seem to adversely affect biodegradation rates of benzene and m-xylene in these soils. Therefore, it was concluded that the addition of higher concentrations of methanol did not adversely affect biodegradation rates of any of the compounds in the mix, and if anything, may be responsible for an increase in biodegradation rates.

4.4.2. Variations in degradation rates within the same depth

Microcosms containing soils from the same depth, and with similar initial concentrations of benzene, m-xylene, and methanol, generally exhibited similar biodegradation responses. However, in some instances, differences in microcosms within the same depth and with similar substrate concentrations occurred. Figures 17 and 18 illustrate this effect. From Figure 17 considerable differences in the biodegradation rates of m-xylene and methanol, between both replicates, can be observed. In Figure 18, while considerable methanol biodegradation occurred in one replicate, minimal degradation occurred in the other microcosm. Variations in microbial responses in microcosms from the same depth are not unusual. McCormick (1991) who conducted studies on the effect

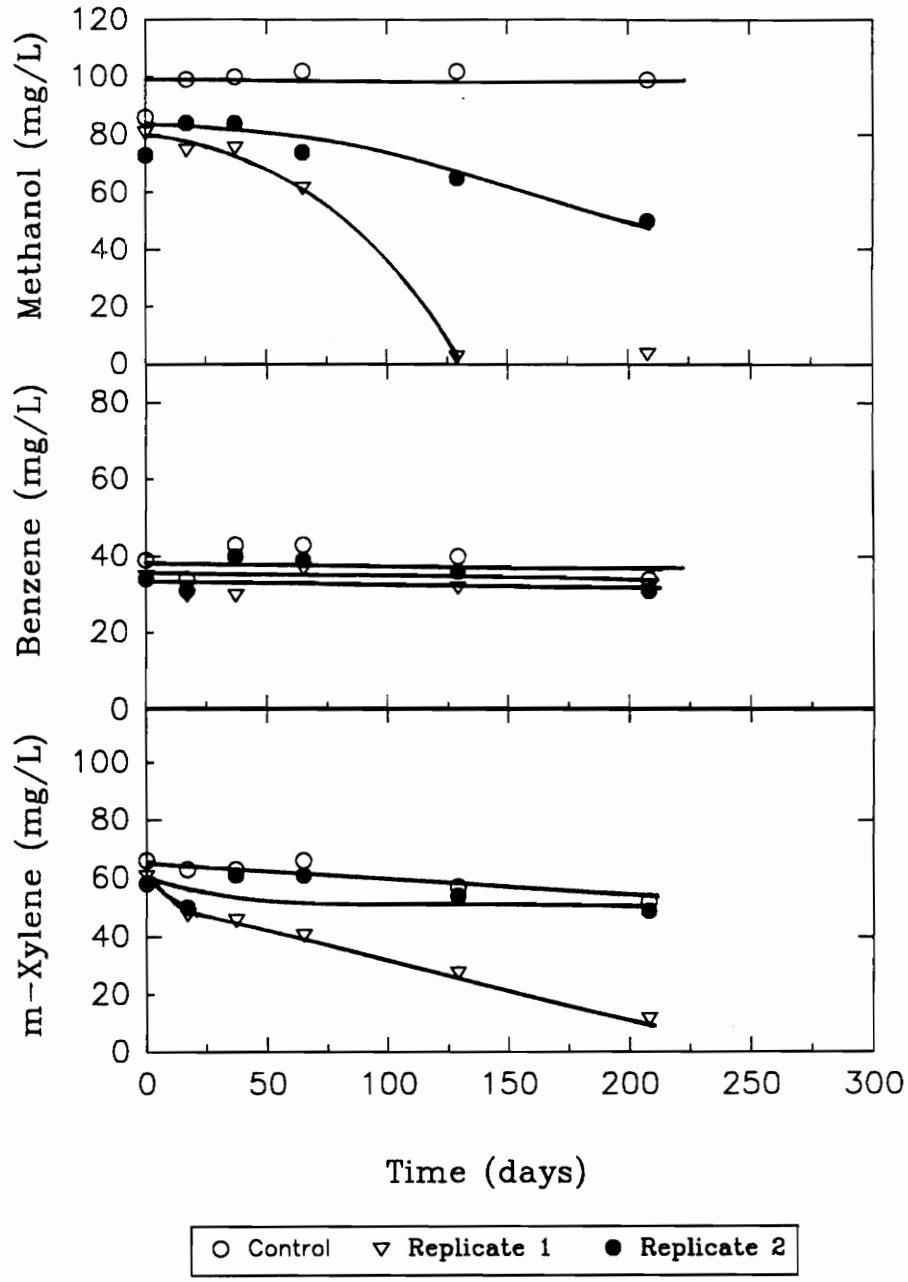


Figure 17. Microcosm data from the 18 foot soil. The initial benzene concentration was about 40 mg/L. Indigenous microorganisms.

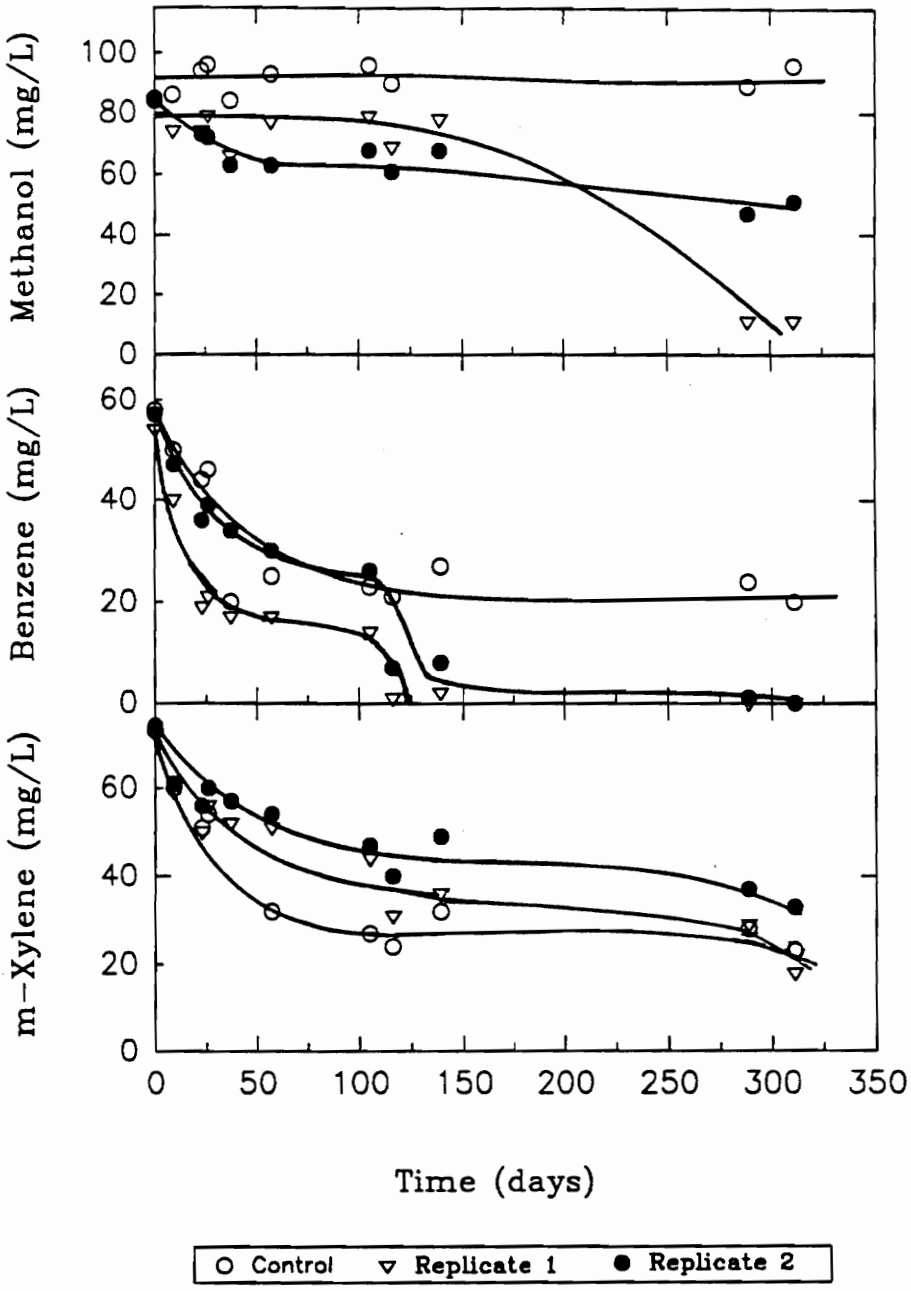


Figure 18. Microcosm data for the 18 foot soil. The initial benzene concentration was about 60 mg/L. Indigenous microorganisms.

of pH on BTEX degradation of soils taken from the same location reported considerable variations in the biodegradation rates of some microcosms containing soil from the same depth and at the same solution pH. As mentioned before, variations in acclimation periods for microcosms within the same depth were observed in several microcosms throughout the length of this study.

Studies conducted by McCormick (1991) on BTEX, and by Farmer (1989) on toluene, reported variations in the adsorptive characteristics of soils within the same depth. Variations in the adsorptive characteristics of soils may be indicative of differences in edaphic factors such as soil particle size, hydraulic conductivity, and moisture and organic contents, which in turn may have a significant impact on microbial populations and activity. Thus, it is possible that even within the same depth, microsites were formed with significantly different characteristics and microbial populations. This heterogeneity would be reflected by variations in acclimation periods and biodegradation rates, even at relatively similar substrate concentrations. Even though variations occurred, it is clear that benzene and m-xylene did not degrade in most surface soil microcosms, and that methanol degraded in all soil depths.

4.4.3 The effect of benzene on the biodegradation of methanol

Of major concern in the bioremediation of gasoline spills is the effect that one compound could have on the degradation of others. In a substrate mixture, biodegradation often takes place in a sequential pattern, in which the substrate supporting the highest

growth rate is preferentially utilized, provided that the initial substrate concentrations are not growth limiting (Harder and Kijkhuizen, 1982). Sequential substrate utilization may sometimes be indicative of inhibitory effects of one substrate on the degradation of another, and it is characterized by utilization of the secondary substrate only after complete biodegradation of the primary substrate occurs. Initial concentrations of an individual component in the mixture may have important implications on the biodegradation rates of not only the compound itself, but of other substrates in the mixture. A compound that does not affect the degradation of another substance at low concentrations may have a significant effect at higher concentrations. While the precise concentration at which the aromatic fraction of gasoline becomes toxic to microorganisms is yet to be found, it is well known that high concentrations of BTX can cause inhibitory effects.

Since the results obtained by this study indicate that methanol was readily biodegradable by indigenous microorganisms in all soil depths, it seemed reasonable to utilize its biodegradation rate as an indicator of microbial activity. In doing so, the effects of benzene and m-xylene could be studied.

M-xylene did not seem to affect the biodegradation of any of the compounds in the mixture, benzene on the other hand, inhibited the degradation of methanol at a concentration of about 125 mg/L in all depths. Figures 11 through 14 illustrate the change in methanol biodegradation rates at high initial methanol concentrations, as the

benzene concentration increased from 35 to 125 mg/L. As it can be seen from Figures 11 and 12, high concentrations of benzene completely inhibited the biodegradation of all compounds in the surface and 12 foot soils. Comparison of the biodegradation rates obtained for the 16.5 foot soil (Figure 13) at high initial methanol concentrations, indicates that benzene inhibited the biodegradation of methanol and m-xylene at 125 mg/L. While methanol degradation occurred in the 18 foot soil microcosms, it appeared that 125 mg/L of benzene lowered its biodegradation rates, as compared to those obtained at low benzene and similar methanol concentrations.

The difference in microbial responses for each depth in microcosms containing 125 mg/L of benzene is shown in Figure 19. Figure 19 also includes biodegradation curves in 18 foot soil microcosms containing low initial benzene concentrations, and about the same methanol concentrations. It is clear that high concentrations of benzene completely inhibited methanol biodegradation in the surface, 12 and 16.5 foot soils, and lowered methanol degradation in the 18 foot soil microcosms. Although methanol biodegradation occurred in the 18 foot soil, it is evident from Figure 19 that biodegradation rates at 125 mg/L of benzene were lower than at 35 mg/L. At low benzene concentrations, methanol biodegradation rates were nearly twice as much as those corresponding to microcosms with similar methanol and m-xylene amounts, but with high benzene concentrations.

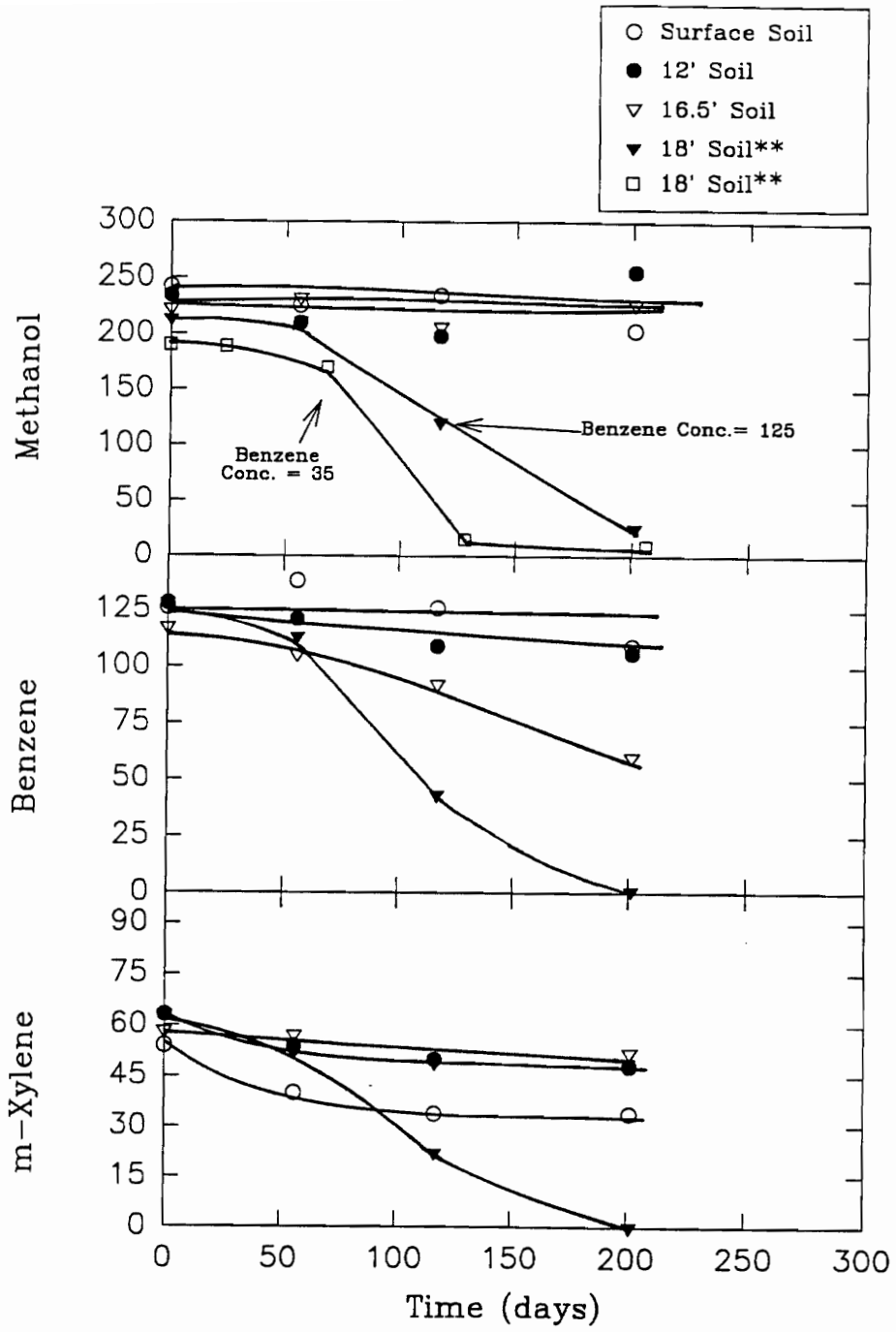


Figure 19. Differences in microbial responses for each depth in microcosms containing initial benzene concentrations of about 125 mg/L. **Comparison of the biodegradation rates obtained in the 18 foot soil microcosms with high and low benzene concentrations. Indigenous microorganisms.

While methanol is readily biodegradable over a wide range of concentrations, more recalcitrant compounds such as benzene and m-xylene may become inhibitory at relatively low concentrations. Microcosm studies conducted by McCormick (1991) showed that m-xylene at concentrations of about 58 mg/L lowered total BTEX degradation rates in 15 and 17 foot soils. Batch studies conducted by Kim (1987) reported toxicity effects of benzene at concentrations of about 200 mg/L. Considering that in a microcosm environment nutrients and oxygen may not be as readily available to microbes as in batch tests and as a result, microbial activity may be more limited, results obtained by this study seem to be well in agreement with Kim's (1987) findings.

4.4.4 Effect of edaphic factors on microbial responses

Soil characteristics may have important implications on the potential for microbial degradation of organic compounds. Parameters such as soil particle size, organic and moisture content, and hydraulic conditions of the soil may affect microbial populations and their potential for biodegradation of certain organic compounds. Differences in microbial responses observed throughout the soil profile may be explained by such parameters.

Biodegradation of benzene and m-xylene did not occur in most surface soil microcosms. It was not until concentrations of methanol were high enough to significantly stimulate biodegradation that biotransformation of benzene and m-xylene was detected. This lack of microbial activity for the degradation of benzene and m-xylene

in surface soils is rather unusual since typically aerobic biodegradation rates are highest in surface horizons (Hickman, 1988). However, the results found by this study are well in agreement with McCormick's (1990) findings of little BTEX biodegradation in surface soils collected from the same location.

Surface soils generally receive an influx of water containing higher concentrations of organic compounds, nutrients and dissolved oxygen than do underlying soil horizons. As a result of increased nutrient and oxygen availability, the microbial population is generally more active than in subsurface soils, and may tend to metabolize more readily degradable compounds, allowing more recalcitrant substances to infiltrate to the subsurface relatively unchanged. This mechanism would explain why methanol was biodegraded in surface soils while benzene and m-xylene were not. Methanol, with a relatively simple chemical structure and high water solubility may be more prone to microbial attack than benzene and m-xylene. Variations in biodegradation rates among soils from the same region and with similar particle size characterizations may occur, in particular for surface horizons. Gullic (1990) found that aerobic biodegradation rates of toluene were highest in surface soils collected from a location adjacent to McCormick's (1991) and this study's sampling site. Surface soils are more directly exposed to localized variations than subsurface soils. The history of the site is of significant importance in assessing the biodegradation potential of an area. The site from which soils were collected for the present study was located in an area where movement of cattle and heavy machinery occasionally occurred. Gullic's sampling site, on the other hand was

located in a more isolated area. Such activities contribute to the compaction of the soil which in turn results in decreased aeration and lower aerobic microbial activity. Therefore, it is possible that the low biodegradation rates observed in surface soils utilized in this study may be attributable to previous compaction of these soils.

Figure 20 shows variations in average maximum biodegradation rates throughout the soil profile. It must be kept in mind that Figure 20 represents average biodegradation rates of the sets studied in phase I, for each soil depth. The apparent degradation rate of benzene and m-xylene in surface soils occurred only at high methanol concentrations and it is not representative of surface soil data in general. Enhancement of biodegradation rates by increasing the concentration of methanol was by far more evident in surface soils, and biodegradation of benzene and m-xylene only occurred under these circumstances.

Mineralization rates of methanol were higher than for benzene and m-xylene, throughout the soil profile, indicating that methanol is a more readily biodegradable substrate. The 12 foot soil with the highest clay content and relatively high moisture content, exhibited the lowest methanol biodegradation rates. In fine particle soils most bacteria are attached. Although their population densities are generally orders of magnitude higher than the free living bacteria, attached bacteria have relatively low activities (Arvin et al., 1988). In addition, the low hydraulic conductivities of clay soils may create an environment in which the flux of water and soluble organics to the attached bacteria is minimal. As a result, these organisms are less likely to come in contact with

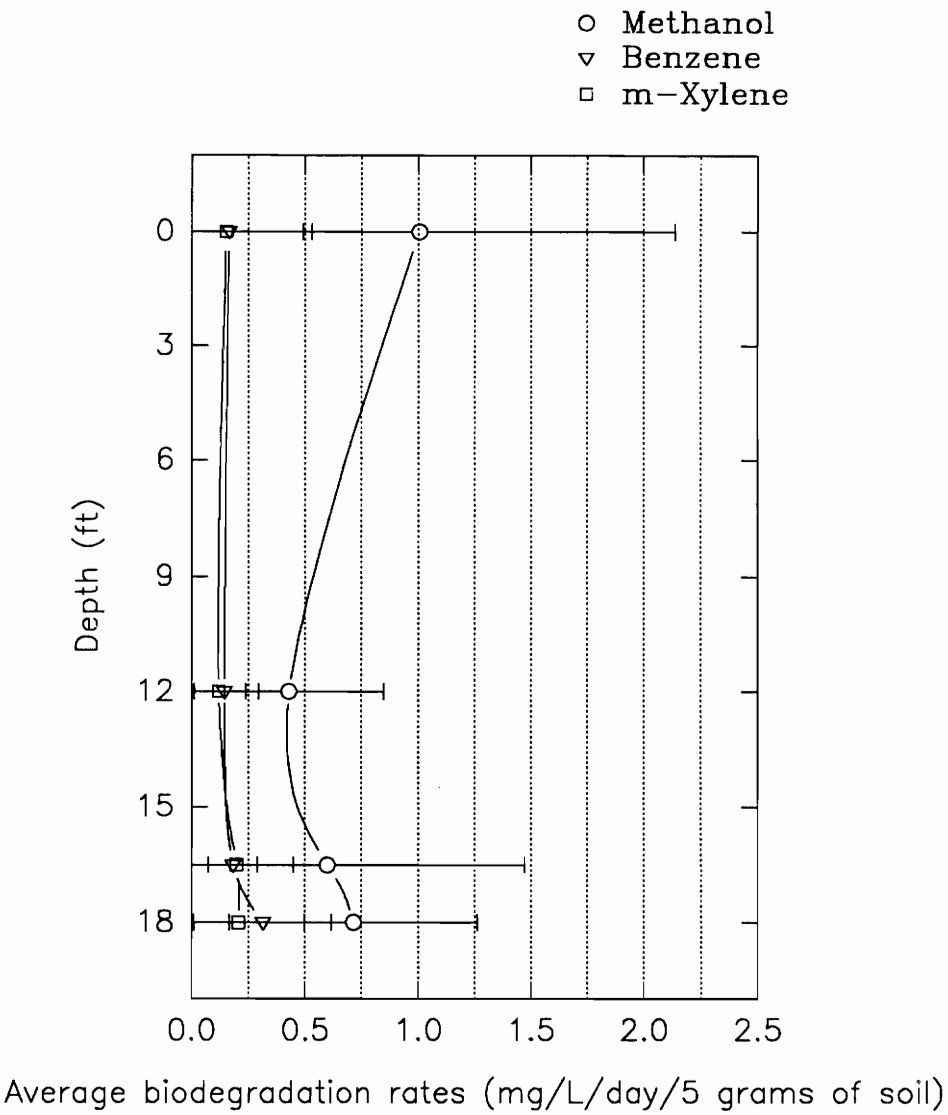


Figure 20. Variations in biodegradation rates with increasing depth. Indigenous microorganisms.

organic substances and thus may require long acclimation periods and may also exhibit slow biodegradation rates. Furthermore, the acidic pH of clay soils may sometimes hinder biodegradation directly or indirectly. Direct inhibition may occur under highly acidic or alkaline conditions in which the microbial cell may be damaged or enzymes may be denatured (Atlas and Bartha, 1986). Extreme pH conditions may also hinder biodegradation indirectly by limiting nutrient availability or by enhancing the effect of inhibitory agents (Cuthbert et al., 1955). Gullic (1990) found that Pseudomonas putida could not degrade toluene when placed in microcosms containing clay soils, with a solution pH of about 4.5 to 5.0. However, when the pH of these microcosms was adjusted to about 7.3, biodegradation of toluene occurred readily. McCormick (1991) also reported low biodegradation rates for BTEX compounds in acidic soils ($pH \leq 5.4$). Even though the soil solution pH of all microcosms in this study was buffered at about 7.2, the actual pH at the end of this study was about 6.8 for surface soils, 6.5 for the 16.5 and 18 foot soils, and 5.8 to 6.0 for the 12 foot soils. Thus, it is possible that the relatively low methanol biodegradation rates exhibited by the 12 foot soil microcosms are a result of microbial inhibition due to lower pH values.

Mineralization rates for benzene and m-xylene in the subsurface environment seemed to increase with increasing soil depths, in particular for benzene in the 18 foot soil. It is possible that benzene with a relatively simple chemical structure is more readily biodegradable than m-xylene. A study conducted by Gibson et al. (1974) found that a strain of Pseudomonas putida could utilize benzene, toluene, and ethylbenzene, as

carbon sources for growth, but that the presence of the two alkyl substituents on the aromatic nucleus of m-xylene hindered the ability of the organisms to utilize the substrate for growth. Furthermore, since benzene is slightly more soluble than m-xylene, it is possible for benzene to be more prone to microbial attack. In general, as the solubility of an organic compound increases, so does the frequency of microbial attack. However, increases in solubility may also be indicative of higher toxicity effects, and the benefits of increased solubility and thus biodegradability, may be counterbalanced by growth inhibition (McGill et al.,1981).

Even though the 16.5 and 18 foot soils had similar particle sizes, differences in the biodegradation potential of these two soils were detected, in particular, the apparent tolerance of the indigenous population of the 18 foot soil to high concentrations of benzene. As it can be seen from Figure 3, a relatively thick layer of clay soil overlaid the 16.5 and 18 foot soils. The low permeability of clays would allow for little seepage to infiltrate from the surface. As a result, fluctuations in the water table may be of significant importance. The 18 foot soil was a sandy soil, with a relatively low moisture content, while the 16.5 foot soil had a high moisture content. The combination of these soil characteristics in the 18 foot soil, may be indicative of a high permeability. In addition, the bedrock just below the 18 foot soil was fractured and well drained. The high permeability of the bedrock would allow for aerobic conditions to predominate, thus allowing for a larger fraction of aerobes to exist at the 18 foot soil depth. It would also allow for an influx of water and soluble organics to reach the 18 foot soil relatively

unchanged. As a result, soils from this depth may be more prone than the 16.5 foot soil to be exposed to a variety of organics. The high permeability of the 18 foot soil would allow for a large portion of its microbial population to be exposed the newly introduced organics, thus allowing microorganisms to acclimate to a variety of compounds. In addition, the high moisture content of the 16.5 foot soil may be indicative of a stronger tendency of this soil to retain water. In water logged soils, aerobic conditions are rare. As water saturation increases oxygen diffusion decreases which in turn results in overall lower biodegradation rates.

4.5 Biodegradation by indigenous microorganisms and benzene degraders (PHASE II)

Phase II of this study included the addition of benzene degrading bacteria isolated and cultured by Sybron Chemical Company to microcosms. The enrichment culture contained several organisms able to utilize benzene as a growth substrate; the dominant species were Pseudomonas putida and Pseudomonas fluorescens E. Microcosms containing a mix of methanol, benzene and m-xylene were prepared in order to determine the effect that the addition of benzene degraders may have on the biodegradation of the three substrates. In order to study the effect that methanol may have on benzene acclimated bacteria, the initial methanol concentration was varied from 0 to about 400 mg/L. The benzene concentration ranged from about 70 mg/L to 160 mg/L. The m-xylene concentration ranged from 40 to 75 mg/L, which was around the levels found to inhibit BTEX biodegradation by McCormick (1991). Microcosms containing only benzene and methanol, and benzene only were also constructed. Biodegradation rates were measured in the same manner described in phase I, and are shown in Table 4. No acclimation periods were detected for benzene and m-xylene, while typical lag periods for methanol were of about 15 days in most of the microcosms.

4.5.1 Effect of the addition of benzene degraders to viable microcosms

Since microcosms studied in phase I consisted of a mix of methanol, benzene, and m-xylene, only those microcosms dosed with the mix in phase II were used to determine the effect of benzene degraders on the overall biodegradation rates. Figure 21 shows the

TABLE IV: Average Biodegradation Rates
Indigenous microbes + Benzene degraders (PHASE II)

SET #	Initial Substrate Concentration (mg/L) **	Surface Soil	12' Soil	16.5' Soil	18' Soil
6	M = 0 B = 112 X = 0	0.56	0.72	2.44	0.56
7	M = 130 B = 75 X = 40	2.04 0.60 0.51	0 0.42 0.30	0.70 0.52 0.20	0.17 0.44 0.12
8	M = 180 B = 160 X = 50	3.29 2.11 0.71	1.09 1.05 0.46	0.67 1.08 0.46	0.74 0 0
9	M = 350 B = 70 X = 56	5.81 0.85 0.82	0 0.49 0.58	2.15 1.42 0.53	0.22 0.34 0.17
10	M = 450 B = 75 X = 0	9.21 0.14	13.68 3.72	7.61 0.24	6.9 0.39

** M = methanol; B = benzene; X = m-xylene

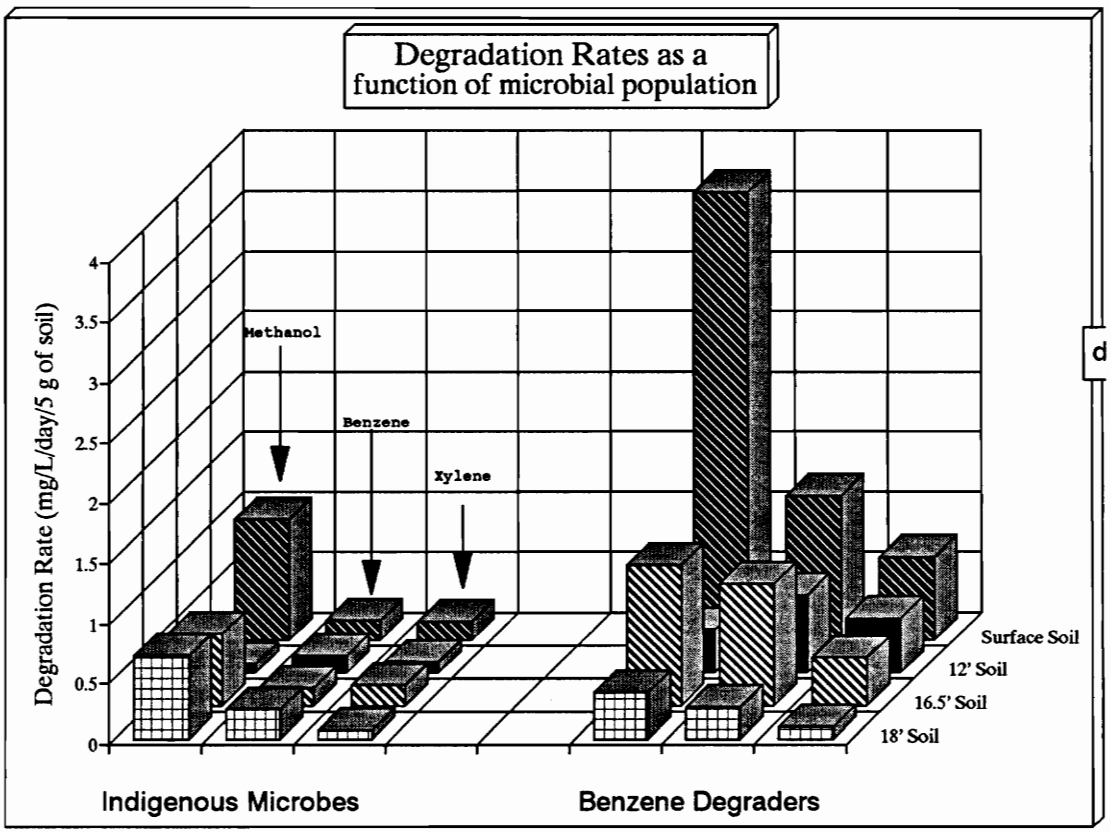


Figure 21. Average biodegradation rates as a function of microbial population. The dominant species for the benzene acclimated bacteria were Pseudomonas putida and Pseudomonas fluorescens E.

average biodegradation rates obtained for each compound and soil depth, for both phases. In order to compare biodegradation rates as a function of microbial population, microcosm data monitored for the same length of time were utilized. Since microcosms dosed with acclimated bacteria were only monitored for about 150 days, it was decided to utilize biodegradation rates of the indigenous microorganisms occurring up to 150 days as well. Microcosms utilized in phase I of this study were monitored for about 300 days.

Inspection of Figure 21 indicates that biodegradation rates of all compounds were significantly higher for surface, 12 and 16.5 foot soils, while biodegradation of benzene and m-xylene were very similar for the 18 foot soil. Variations in microbial responses with depth may be attributable to the apparent inability of benzene degraders to adapt to the environmental conditions of some microcosms, particularly the 18 foot soil microcosms. It is possible that the presence of inhibitors or predator organisms among the indigenous microbiota of the 18 foot soil hindered biodegradation by the benzene acclimated bacteria. Nevertheless, substantial amounts of all the compounds biodegraded in most microcosms as a result of the addition of benzene degraders.

The degradation of m-xylene by benzene degraders is not unexpected since benzene and m-xylene are similar in structure. Thus, the same enzymes utilized to attack benzene may act on m-xylene, resulting in m-xylene utilization by analog enrichment as discussed in Chapter 2. Data from microcosms containing only benzene and methanol seem to indicate that methanol is readily biodegradable by benzene degraders. Sequential

substrate utilization of benzene first and methanol second did not occur in any of these microcosms. For a comparison of these biodegradation rates, refer to Table 4, set #10.

As mentioned before, the organisms supplied by Sybron Chemical Company were not a pure culture, but mainly contained Pseudomonas, which are the most commonly isolated hydrocarbon degrading bacteria from soil (Bossert and Bartha, 1984). It is known that facultative methylotrophs pertaining to the genus Pseudomonas are able to oxidize non-one-carbon compounds as well. These organisms may have been present in the mixture. Therefore, it is reasonable to suggest that the organisms isolated by Sybron Chemical Company, grown on benzene as the sole carbon source, are also able to grow on methanol.

4.5.2 Possible sequential substrate utilization

Sequential substrate utilization of m-xylene first, followed by methanol, occurred in microcosms containing 12 foot soils. Benzene utilization did not seem to be affected by m-xylene, since substantial biodegradation of benzene occurred in the presence of m-xylene. Microcosms in which m-xylene degradation did not occur were also characterized by a lack of methanol utilization, which may be indicative of m-xylene inhibition. Figure 22 shows sequential substrate utilization in 12 foot soil microcosms. Only after complete utilization of m-xylene occurred did methanol utilization begin, suggesting that diauxic growth may be occurring. Diauxic growth is characterized by catabolite repression which occurs when microorganisms able to metabolize the first substrate inhibit the synthesis

and/or tasks of those enzymes needed for metabolism of the second substrate (Harder and Dijkhuizen, 1982). After complete utilization of the first substrate, enzyme inhibition no longer occurs, and after a lag period of growth of variable length, metabolism of the second substrate starts (Harder and Dijkhuizen, 1982). Sequential substrate utilization is not always indicative of diauxic growth; enzymes needed to metabolize the second substrate may be present and uninhibited, yet sequential substrate utilization may still occur. It is not unusual for the substrate permitting a higher growth rate to be utilized first, followed by utilization of the second substrate (Harder and Dijkhuizen, 1982).

Sequential substrate utilization was not typical of surface and 16.5 foot soils microcosms in general. In the 18 foot soil biodegradation of m-xylene was minimal and while substantial benzene utilization occurred, methanol biodegradation was hindered, presumably as a result of m-xylene inhibition. Regardless of soil depth, microcosms in which m-xylene degradation did not occur were characterized by a lack of methanol utilization which seemed to be indicative of m-xylene inhibition. The effect of m-xylene on the biodegradation of methanol will be addressed in the following section.

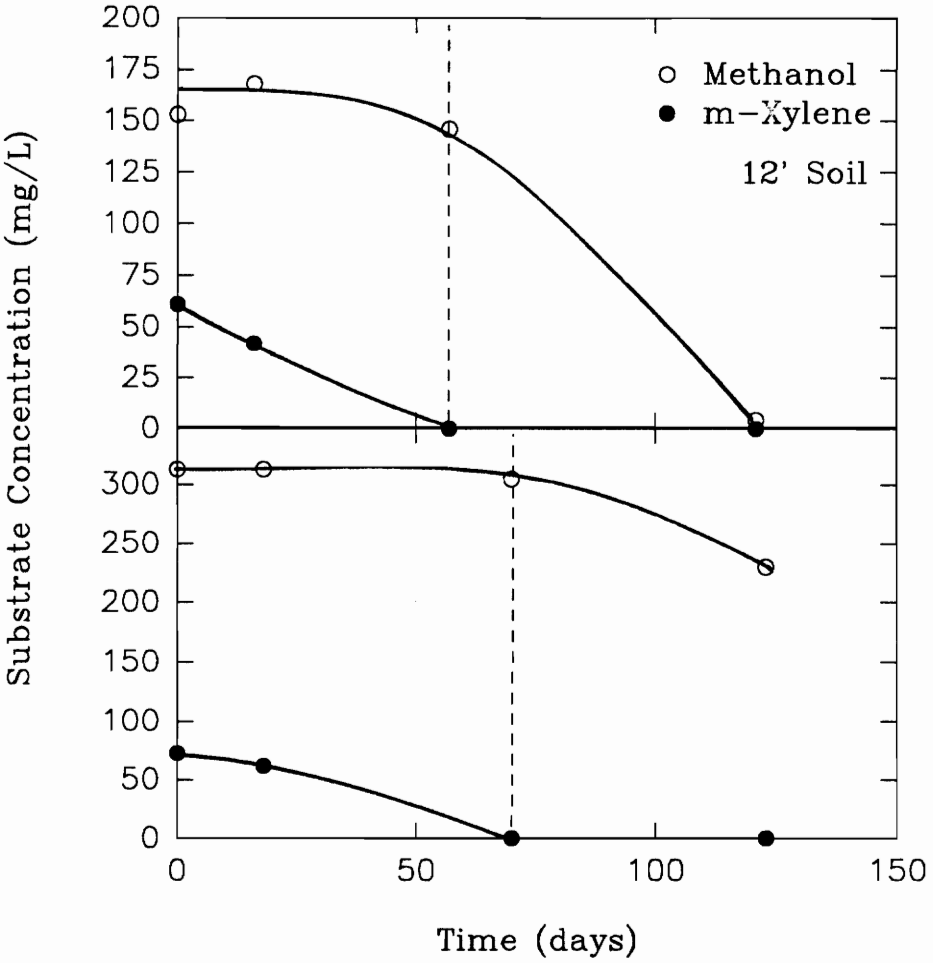


Figure 22. Sequential substrate utilization in microcosms containing 12 foot soil. Indigenous microorganisms + Benzene degraders --Pseudomonas putida and Pseudomonas fluorescens E.

4.5.3 M-xylene inhibition

In those soils which exhibited sequential substrate utilization, substantial methanol degradation occurred only after m-xylene utilization was completed. Even within depths that experienced sequential substrate utilization, there were microcosms in which m-xylene utilization did not occur. Differences in microbial responses within the same depth were experienced, in particular for microcosms exhibiting sequential substrate utilization. Figures 23 and 24 show differences in microbial responses in duplicate microcosms, for some 12 and 16.5 foot soil microcosms. While one replicate exhibited considerable biodegradation of all compounds, the other replicate did not. M-xylene degradation in replicate 2 did not occur, and while benzene utilization did not seem to be affected by m-xylene, methanol degradation did not occur.

Such variability may be attributable to the inoculation method employed in this study and/or to possible variations in the environmental conditions sustained within each microcosm. The inoculation method utilized in this study followed the guidelines utilized by Gullic (1990). Gullic utilized a pure culture of toluene degrading bacteria to dose autoclaved soil microcosms. Since the soil microcosms in his experiment were autoclaved before inoculation, the toluene degrading bacteria was not exposed to the indigenous microbial population. Variations observed in this study may have been the result of interference by the indigenous microbial population. Farmer (1989) also conducted microcosm studies by inoculating autoclaved soil with toluene acclimated bacteria. Although Farmer reported considerable biodegradation rates in most microcosms, some

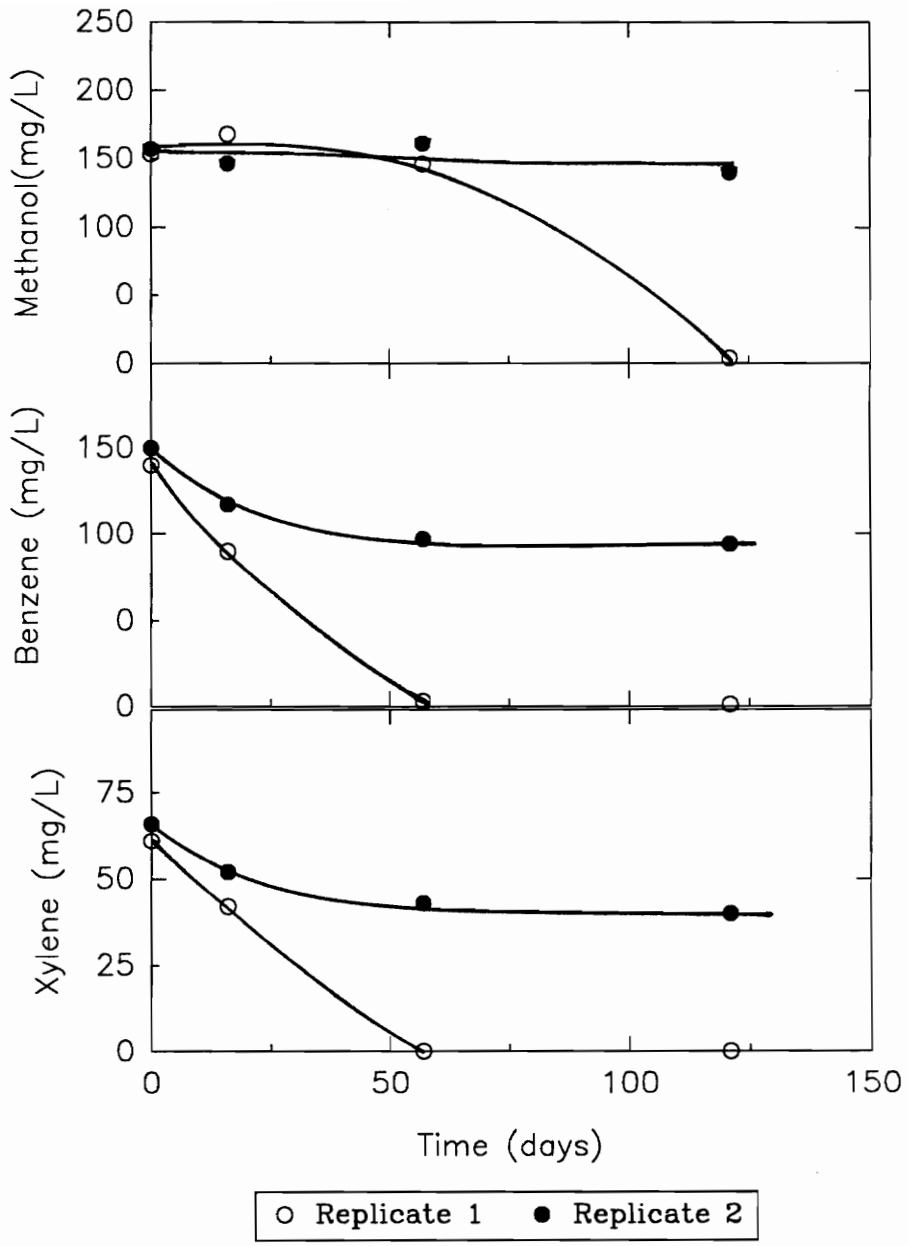


Figure 23. Differences in microbial responses in duplicate microcosms containing 12 foot soil. Indigenous microorganisms + Benzene degraders --Pseudomonas putida and Pseudomonas fluorescens E.

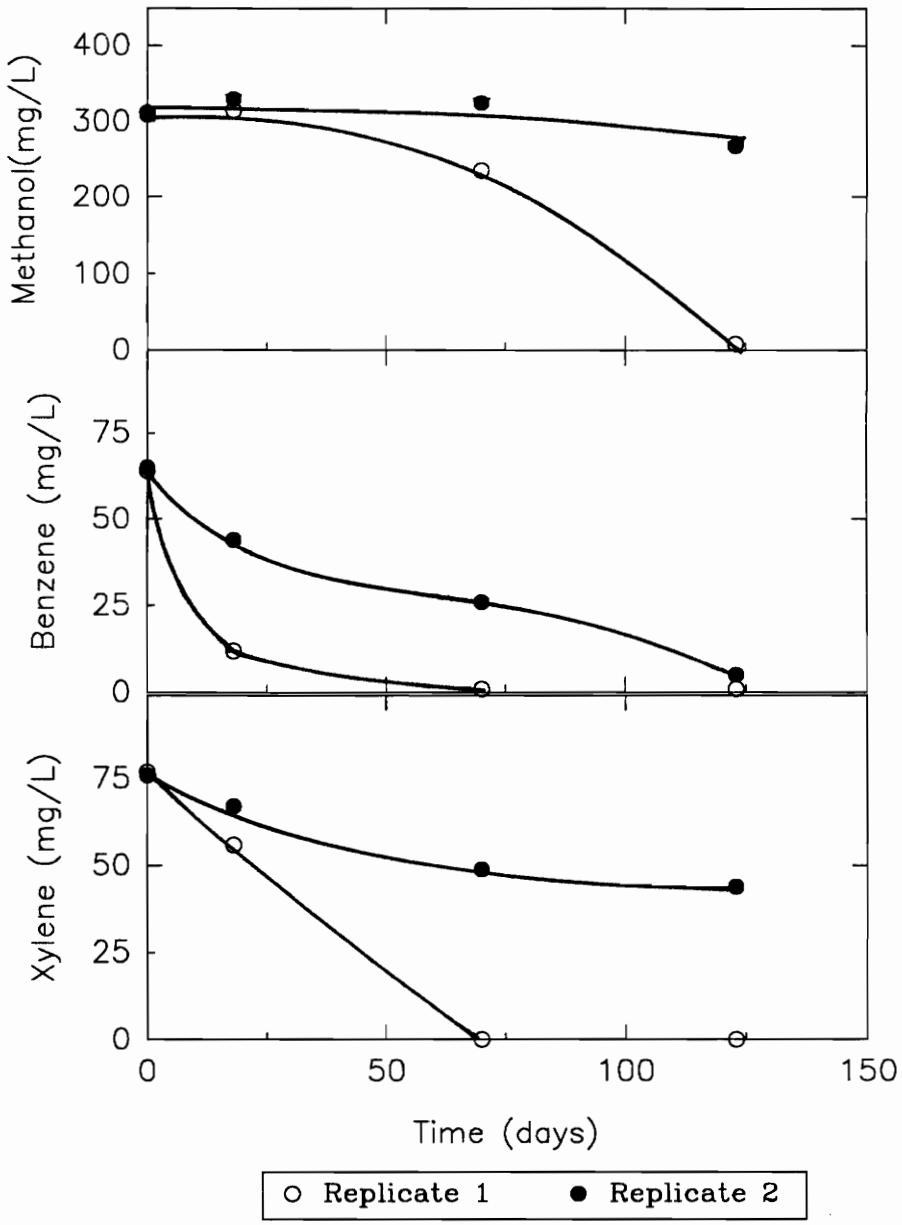


Figure 24. Differences in microbial responses in duplicate microcosms containing 16.5 foot soil. Indigenous microorganisms + Benzene degraders --Pseudomonas putida and Pseudomonas fluorescens E.

duplicate microcosms did not experience any degradation.

Increasing the number of replicates utilized would be desirable to gain more confidence in the observed responses so that variations in the data can be statistically accounted for. However, such an increase would considerably increase the time required for the study. Moreover, the difficulty in mimicking natural environmental conditions in the laboratory may be greater with the use of small microcosms since considerably more handling of the soil is required. Larger microcosms may allow for better chances of obtaining an "undisturbed sample" and therefore, may be more representative of the actual natural environment. By using larger microcosms, microsites with substantially different characteristics within the same depth may not have as significant an impact on overall degradation rates as is the case with small microcosms. As a result, variations within replicates and consequently the number of replicates required may not be as great as with small microcosms. Even though variations occurred, it was apparent that for some soils, m-xylene hindered methanol biodegradation. In the 18 foot soil microcosms, for example, biodegradation of m-xylene was minimal, and while considerable benzene biodegradation occurred, methanol biodegradation rates were very low or non existent, in all duplicate microcosms.

In order to determine the effect of m-xylene on the degradation of methanol, microcosms containing only benzene and methanol were constructed. Figure 25 compares average biodegradation rates obtained in microcosms containing benzene, m-xylene, and

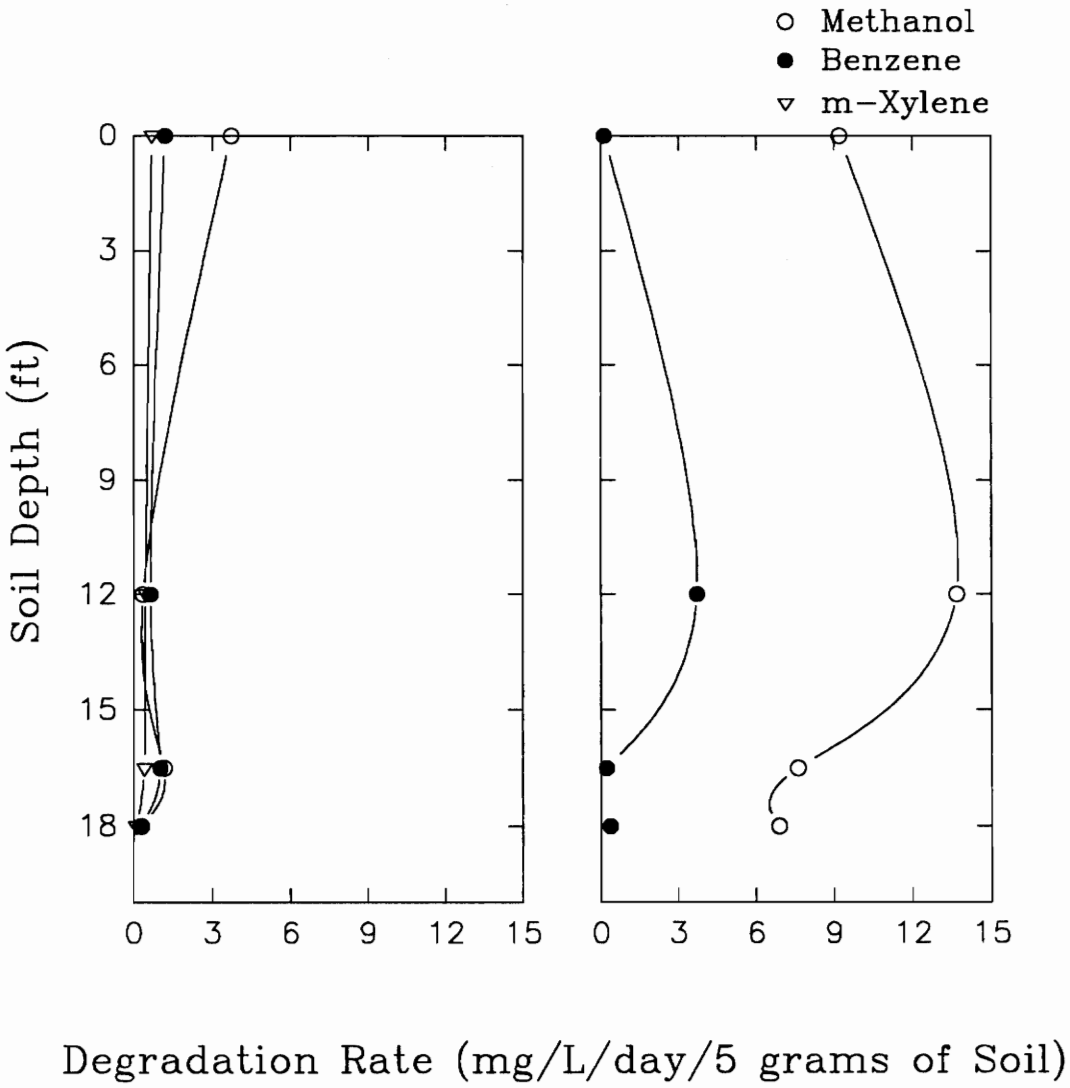


Figure 25. Effect of m-xylene on the degradation of methanol (average degradation rates). Indigenous microorganisms + Benzene degraders --Pseudomonas putida and Pseudomonas fluorescens E.

methanol to the biodegradation rates obtained from microcosms in which m-xylene was not present. Since biodegradation rates of methanol were considerably higher for all soil depths in the absence of m-xylene, it is clear that the presence of m-xylene hindered methanol utilization.

In Figure 26, the actual methanol biodegradation rates obtained in the presence of m-xylene are compared to those obtained without m-xylene (these biodegradation rates were obtained from microcosms to which m-xylene was not added, and from microcosms in which sequential substrate utilization occurred --only after complete m-xylene utilization was detected). As it can be seen, biodegradation rates of each soil depth were always lower in the presence of m-xylene.

As mentioned before, the benzene acclimated bacteria utilized in this study was not a pure culture. It is possible that a population of facultative methylotrophs able to utilize benzene as well as methanol existed in the culture, and that these organisms may be sensitive to m-xylene. No such effects were detected in microcosms containing indigenous microorganisms.

4.5.4 Effect of methanol on the biodegradation of benzene

In order to determine the effect of methanol on the degradation of benzene, microcosms containing benzene only were constructed, and their biodegradation rates were compared to the rates obtained from microcosms containing methanol and benzene.

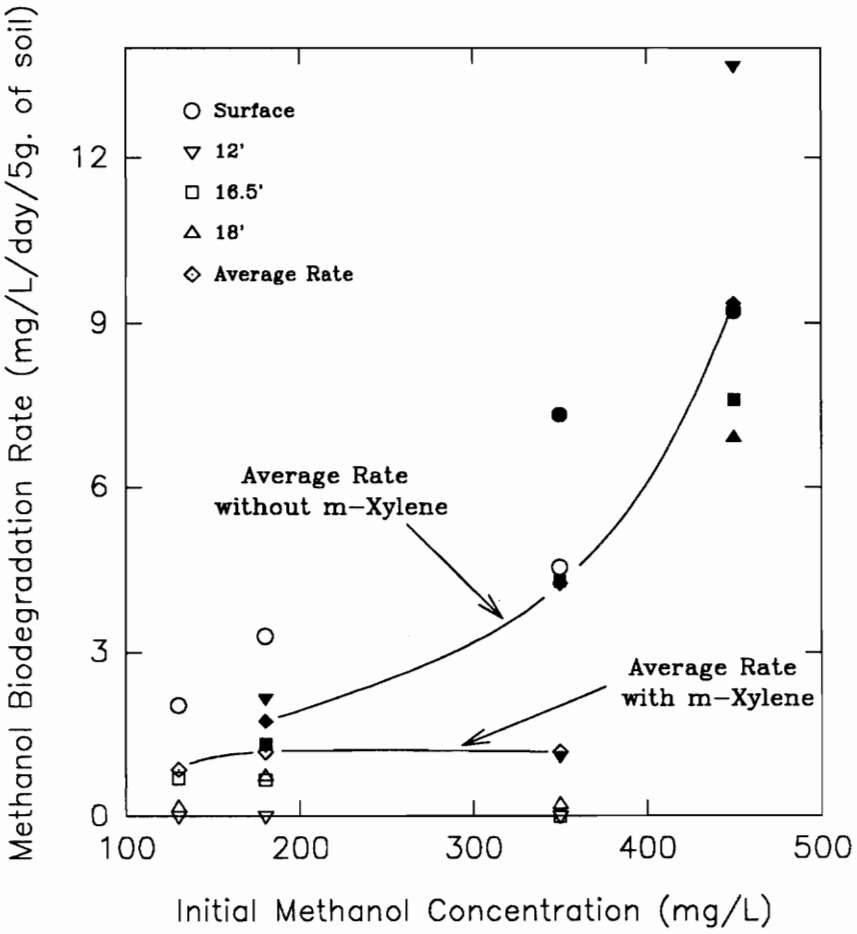


Figure 26. Effect of m-xylene on the degradation of methanol. Hollow symbols represent biodegradation rates obtained in the presence of m-xylene, for each soil depth. Filled symbols represent biodegradation rates obtained in the absence of m-xylene. Indigenous microorganisms + Benzene degraders --Pseudomonas putida and Pseudomonas fluorescens E.

Figure 27 compares these biodegradation rates. For all soil depths, with the exception of 12 foot soil, biodegradation rates of benzene were higher in microcosms to which methanol was not added. It is possible that as rapid utilization of methanol occurred, depletion of nutrients and oxygen occurred at a rate faster than supplied, causing a decrease in the biodegradation rates of benzene. These results seem to contradict those obtained in phase I of this study, in which the addition of increasing concentrations of methanol to microcosms containing indigenous microorganisms seemed to increase benzene and m-xylene biodegradation rates, in particular for surface soils. Such difference in microbial response may be caused by the addition of benzene acclimated bacteria to microcosms utilized in phase II. Benzene degraders may be responsible for most of the increase in the biodegradation rates of phase II of this study. In surface soils, for example, substantial biodegradation of all compounds occurred simultaneously in microcosms containing benzene concentrations of about 160 mg/L, which is well above that determined to inhibit biodegradation by the indigenous microorganisms. Since the microbial populations of microcosms utilized in phase I differed from those utilized in phase II, it is not unusual to find variations in their responses to similar substrate concentrations. Different type of bacteria do not always react similarly to the same combination of substrates (Harder and Dijkhuinzen, 1982). Figure 28 shows biodegradation curves for microcosms containing benzene and methanol in representative microcosms. This pattern was typical of surface, 16.5 and 18 foot soils. In the 12 foot soil, on the other hand, biodegradation rates of benzene seemed to have been stimulated by methanol. Figure 29 shows the data obtained from these microcosms.

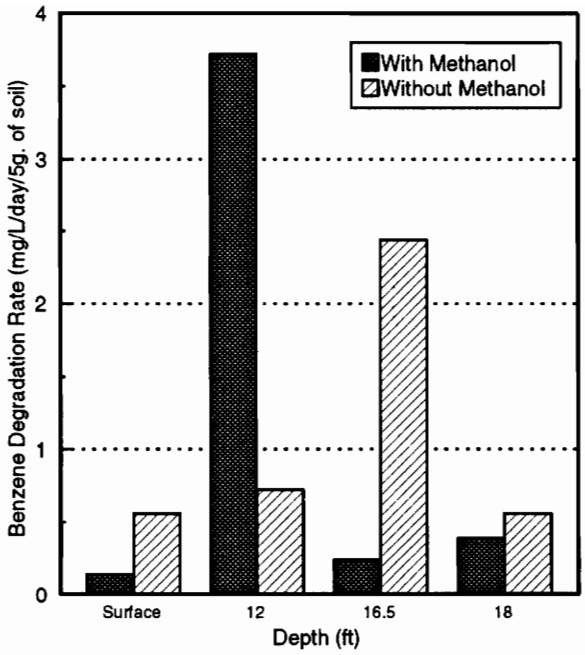


Figure 27. Effect of methanol on the degradation of benzene. Indigenous microorganisms + Benzene degraders --Pseudomonas putida and Pseudomonas fluorescens E.

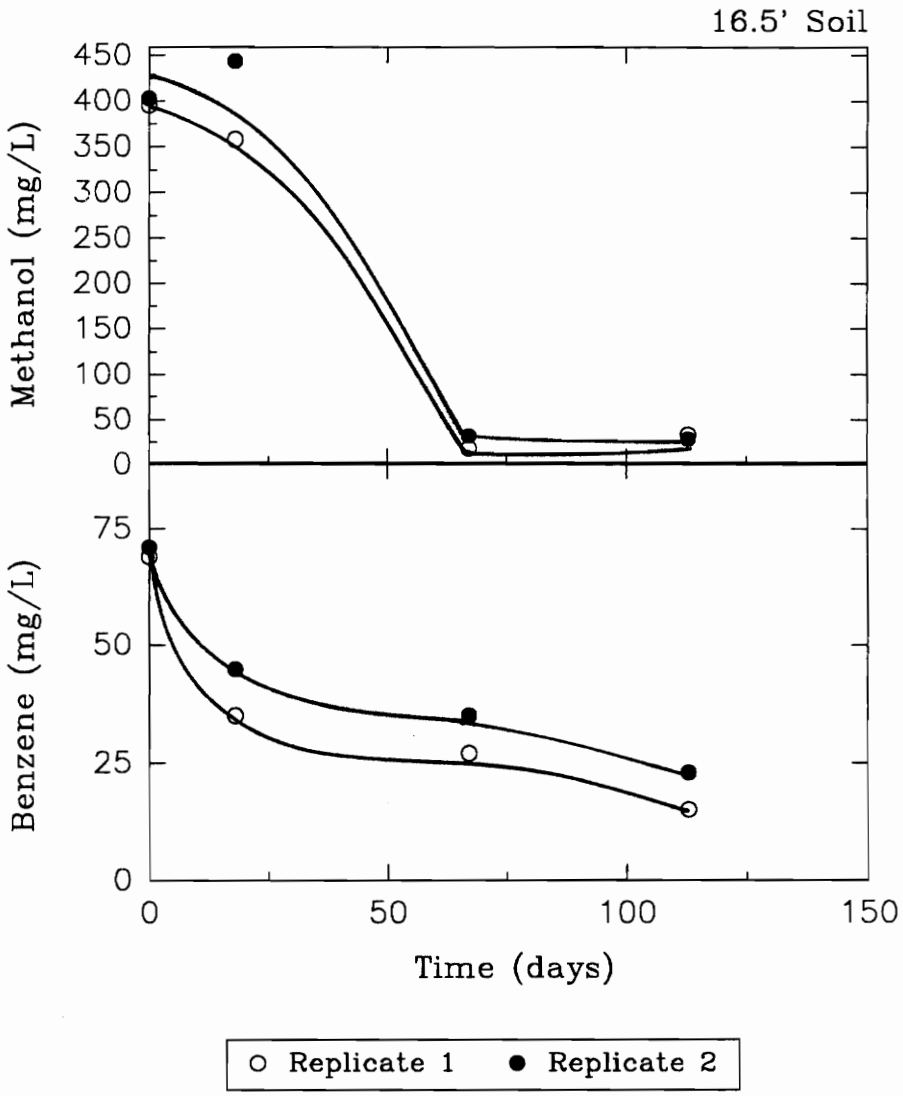


Figure 28. Biodegradation in microcosms containing 16.5 foot soil and dosed with benzene and methanol only. Indigenous microorganisms + Benzene degraders --Pseudomonas putida and Pseudomonas fluorescens E.

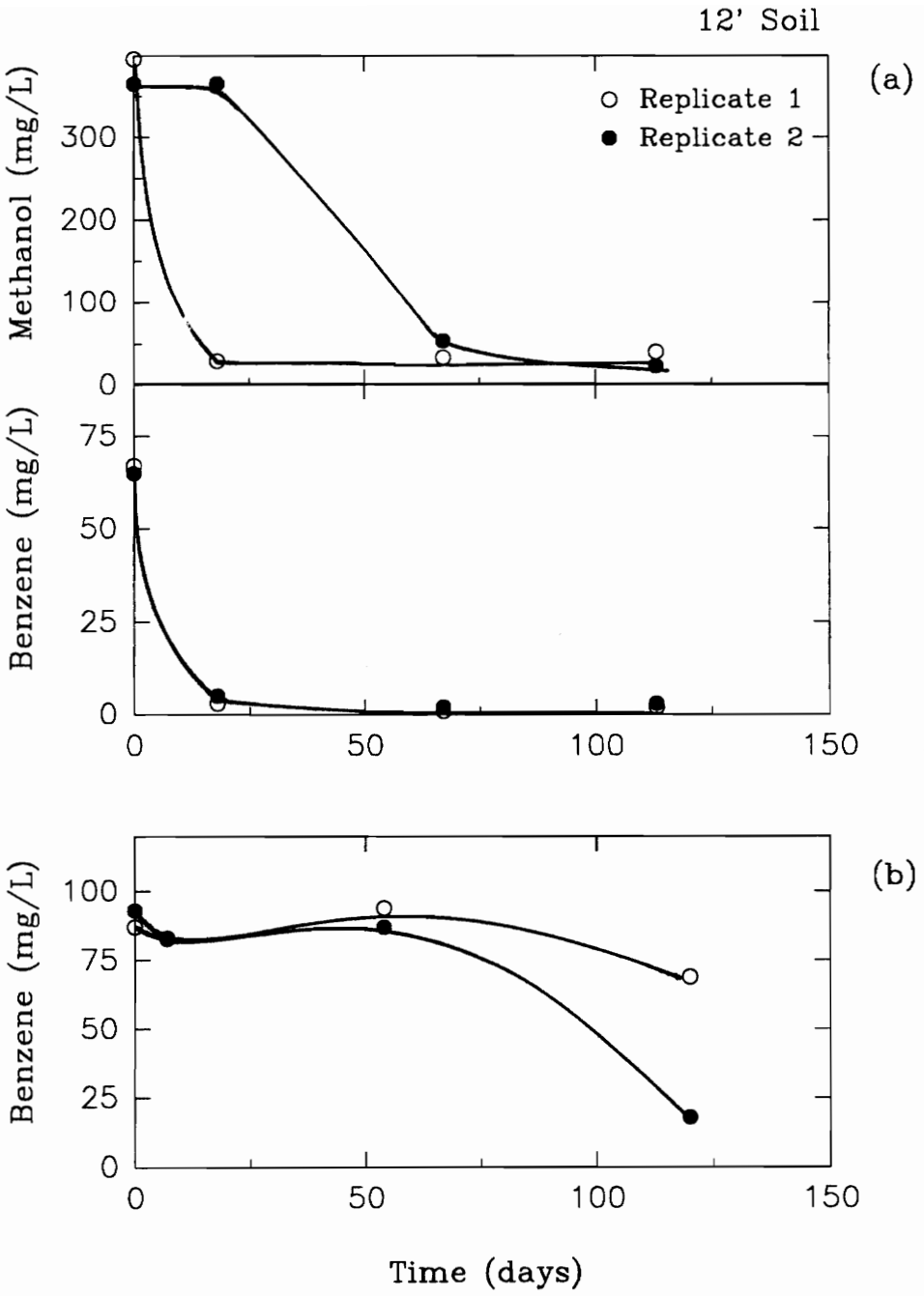


Figure 29. Biodegradation in microcosms containing 12 foot soil. (a)--Microcosms dosed with methanol and benzene. (b)--Microcosms dosed with benzene only. Indigenous microorganisms + Benzene degraders --Pseudomonas putida and Pseudomonas fluorescens E.

As mentioned before, the 12 foot soil consisted mostly of clay. The high ionic strength of clay tends to increase adsorption of nutrients and organics to its surface. As a result, microorganisms must carry out biodegradation with limited nutrient availability, or must use energy to desorb the nutrients first, and then proceed to biodegrade the substrates. Under limited nutrient availability, methanol may have served as a more readily available energy source. In addition, since m-xylene and benzene are moderately hydrophobic, they tend to adsorb to soil surfaces, in particular to clay soils. Since methanol is completely miscible in water, its presence may have increased the aqueous solubility of benzene, possibly increasing contact between the benzene degraders and benzene. Farmer (1989) found that toluene acclimated bacteria could readily utilize aqueous toluene, and that biodegradation of the remaining sorbed toluene was limited by its rate of desorption into the aqueous phase. It is possible for biodegradation by the benzene acclimated bacteria used in this study to be governed by the same mechanism, and that most degradation takes place in solution. This would explain why the biodegradation rates of benzene in microcosms containing only benzene were much lower than in microcosms containing methanol and benzene. Since the other soils have a relatively smaller surface area than clay soils, this effect may have not been as significant.

Furthermore, if in fact the overall biodegradation rates in the 12 foot soil were hindered by a lower pH (5.8 to 6.0), it is possible that the high methanol concentrations in microcosms containing benzene and methanol partially compensated for the pH stress on the microorganisms. A study conducted by Haines (1970) on the biodegradation of

cellobiose by a Pseudomonas species utilizing glass beads of different diameters to simulate a soil matrix, found that at low substrate concentrations (0.1% w/v), bacterial growth was limited by high solution pHs (8.0 to 10.0). An increase in the substrate concentration to 1.0% w/v partially compensated for the pH stress on the organisms, reflected by a considerable increase in CO_2 production rates. Therefore, it is possible that the biodegradation rates observed in this study in microcosms containing benzene only were lower as a result of the acidic pH of the 12 foot soil (Figure 29 (b)), and that when high concentrations of methanol were added (Figure 29 (a)) the pH stress diminished and higher biodegradation rates were observed for both compounds.

CHAPTER V

CONCLUSION

Microbial degradation of petroleum hydrocarbons is a complex process subject to localized environmental factors. The physical, chemical, and biological characteristics of an aquifer have a marked influence on the indigenous microbiota, and consequently, on their potential for the biodegradation of organic pollutants. In order for enhanced in situ bioremediation to be successfully implemented, a good understanding of such parameters is needed.

In order to incorporate the effect that some of these parameters may have on degradation rates, soil microcosms containing benzene, m-xylene, and methanol were utilized in this study. The results obtained indicate that under aerobic conditions, benzene, m-xylene, and methanol can be degraded by indigenous microorganisms. A three fold increase in the biodegradation rates of all compounds in the mixture was detected as a result of the addition of benzene acclimated bacteria. The following specific conclusions were drawn based on the results obtained by this study:

1. Biodegradation of benzene, m-xylene, and methanol occurred at all subsurface soil depths.

2. In all soil depths, biodegradation rates of methanol were higher than for benzene and m-xylene.

3. Methanol did not adversely affect the biodegradation of benzene and m-xylene by indigenous microorganisms, and if anything, may be responsible for an increase in biodegradation rates, in the surface and 16.5 foot soils.

4. Benzene inhibited methanol biodegradation by the indigenous microorganisms at concentrations of about 125 mg/L.

5. The addition of benzene acclimated bacteria increased the biodegradation rates of benzene, methanol, and m-xylene.

6. Sequential substrate utilization characterized by complete m-xylene utilization followed by methanol utilization occurred in some microcosms dosed with benzene acclimated bacteria.

7. In all soil depths, m-xylene inhibited the biodegradation of methanol in microcosms dosed with benzene degraders, and did not adversely affect the degradation of benzene.

CHAPTER VI

REFERENCES

Aamand, J., Jorgensen, C., Arvin, E., and Jensen, B. (1989). Microbial Adaptation to Degradation of Hydrocarbons in Polluted and Unpolluted Groundwater. Journal of Contaminant Hydrology, v. 4, pp. 299-312.

Aggarwal, P. K., Means, J. L., Downey, D. C., and Hinchee, R. E. (1991). Use of Hydrogen Peroxide as an Oxygen Source for In-Situ Biodegradation. Part II. Laboratory Studies. Journal of Hazardous Materials, v. 27, pp. 301-314.

Arvin, E., Jensen, B., Aamand, J., and Jorgensen, C. (1988). The Potential of Free-Living Ground Water Bacteria to Degrade Aromatic Hydrocarbons and Heterocyclic Compounds. Water Science and Technology, v. 20, n. 3, pp. 103-118.

Atlas, R. M. (1981). Microbial Degradation of Petroleum Hydrocarbons: An Environmental Perspective. Microbiological Reviews, v. 45, pp. 180-209.

Atlas, R. M. (1978). An Assessment of the biodegradation of Petroleum in the Arctic. In: Microbial Ecology, Loutit, M.W., and Miles, J.A.R., eds., Springer-Verlag, Berlin, pp. 86-90.

Atlas, R. M. and Bartha, R. (1986). Microbial Ecology: Fundamentals and Applications. The Benjamin/Cummings Publishing Company, Inc., Menlo Park, California.

Barker, J.F., Patrick, G.C., and Major, D. (1987). Natural Attenuation of Aromatic Hydrocarbons in a Shallow Sand Aquifer. Ground Water Monitoring Review, v. 8, pp. 64-71.

Bossert, I. and Bartha, R. (1984). The Fate of Petroleum in Soil Ecosystems. In: Petroleum Microbiology, Atlas, R., ed., Macmillan Publishing Company, New York, pp. 436-473.

Brady, N. C. (1984). Nature and Properties of Soils. Macmillan Publishing Co., Inc. New York, N.Y.

Britton, L.N. (1985). Feasibility Studies on Use of H_2O_2 to Enhance Microbial Degradation of Gasoline. API Publication No. 4389.

Brock, T.D. and Madigan, M.T. (1991). Biology of Microorganisms. Prentice Hall Inc., Sixth Edition, Englewood Cliffs, New Jersey.

Brock, T.D., Smith, D.W., and Madigan, M.T. (1984). Biology of Microorganisms. Prentice Hall Inc., Englewood Cliffs, New Jersey.

Brookman, G.T., Flanagan, M., and Kebe, T.O. (1985). Literature Survey: Hydrocarbon Solubilities and Attenuation Mechanisms. API Publication Number 4414.

Canter, L.W., Knox, R.C., and Fairchild, D.M. (1990). Ground Water Quality Protection. Lewis Publishers, Inc., Chelsea, Michigan. Ch. 5 and 10.

Cerniglia, C.E. (1984). Microbial Transformation of Aromatic Hydrocarbons. In: Petroleum Microbiology, Atlas, R., ed., Macmillan Publishing Company, New York.

Chiang, C.Y., Salanitro, E.Y., Chai, E.X., Colthart, J.D., and Klein, C.L. (1989). Aerobic Biodegradation of Benzene, Toluene, Xylene in a Sandy Aquifer - Data Analysis and Computer Model. Ground Water, v.27, pp. 823-834.

Cuthbert, W.A., Panes, J.J., and Hill, E.C. (1950). Survival of Bacterium Coli Type I and Streptococcus Faecalis in Soil. Journal of Applied Bacteriology, v. 18, pp. 408-414.

Dagley, S., Chapman, P.J., Gibson, D.T., and Wood. J.M. (1964). Degradation of the Benzene Nucleus by Bacteria. Nature, v. 202, n. 4934, pp. 775-778.

Davey, J.F. and Gibson, D.T. (1974). Bacterial Metabolism of Para- and Meta- Xylene: Oxidation of a Methyl Substituent. Journal of Bacteriology, v. 119, n. 3, pp. 923-929.

Dupont, R.R., Sims, R.C., Sims, J.L., and Sorensen, D.L. (1988). In Situ Biological Treatment of Hazardous Waste-Contaminated Soils. In: Biotreatment Systems, v.2, Wise, D., ed., CRC Press, Inc., Boca Raton, Florida, pp. 23-94.

Eden, R.Q.E. (1985). Additives, Blending Agents and Alternative Fuels: their Manufacture, Properties and Uses. In: Technology of Gasoline, Hancock, E.G., ed., Blackwell Scientific Publications, Oxford. pp. 86-105.

Farmer, W.S. (1989). A Microcosm Study of the Biodegradability of Adsorbed Toluene by Acclimated Bacteria in Soils. M.S. thesis, VPI&SU, Blacksburg, VA.

Federle, T.W. (1988). Mineralization of Monosubstituted Aromatic Compounds in Unsaturated and Saturated Subsurface Soils. Canadian Journal of Microbiology,v. 34, pp. 1037-1042.

Focht, D.D. (1988). Performance of Biodegradative Microorganisms in Soil: Xenobiotic Chemicals as Unexploited Metabolic Niches. In: Environmental Biotechnology: Reducing Risks from Environmental Chemicals through Biotechnology, Omenn, G., ed., Plenum Press, New York, pp. 15-29.

Gandhidasan, P., Ertas, A., and Anderson, E.E. (1991). Review of Methanol and Compressed Natural Gas (GNG) as Alternative for Transportation Fuels. Journal of Energy Resources Technology, v. 113, pp. 101-107.

Gibson, D.T. (1977). Biodegradation of Aromatic Petroleum Hydrocarbons. In: Fate and Effect of Petroleum Hydrocarbons in Marine Ecosystems and Organisms, Wolfe, D.A., ed., Pergamon Press, New York.

Gibson, D.T., Gschwendt, B., Yeh, W.K., and Kobal, V.M. (1973). Initial Reactions in the Oxidation of Ethylbenzene by Pseudomas putida. Biochemistry, v. 12, n. 8, pp. 1520-1528.

Gibson, D.T., Koch, J.R., and Kallio, R.E. (1968). Oxidative Degradation of Aromatic Hydrocarbons by Microorganisms. I.Enzymatic Formation of Catechol from Benzene. Biochemistry, v. 7, n. 7, pp. 2653-2662.

Gibson, D.T., Mahedevan, V., and Davey, J.F. (1974). Bacterial Metabolism of para- and meta- Xylene: Oxidation of the Aromatic Ring. Journal of Bacteriology, v. 119, n. 3, pp. 930-936.

Goldsmith, C.D. (1985). Biodegradation of Methanol and Tertiary Butyl Alcohol in Previously Uncontaminated Subsurface Systems. Ph.D. dissertation, VPI&SU, Blacksburg, VA.

Goldstein, B.D. (1983). Clinical Hematotoxicity of Benzene. In: Carcinogenicity and Toxicity of Benzene, Mehlman, M.A., ed., Princeton Scientific Publishers, Inc., Princeton, New Jersey.

Grady, L.C. (1985). Biodegradation: Its Measurement and Microbiological Basis. Biotechnology and Bioengineering, v. 27, pp. 660-674.

Gray, T. R. G. and Williams, S. T. (1971). Soil Microorganisms. Hafner Publishing Company Inc., New York, N.Y.

Gullic, D.B. (1990). Variations in the Biodegradation Potential of Toluene with Increasing Depth in an Unsaturated Subsurface Environment. M.S. thesis, VPI&SU, Blacksburg, VA.

Haines, J.R. (1979). Effects of the Physical Microenvironment on Soil Microorganisms. Ph.D. dissertation. Cornell University, Ithaca, NY.

Hancock, E.G. (1985). The manufacture of Gasoline and the Chemistry of Its Components. In: Technology of Gasoline, Hancock, E.G., ed., Blackwell Scientific Publications, Oxford, pp. 20-56.

Harder, W. and Dijkhuizen, L. (1982). Strategies of Mixed Substrate Utilization in Microorganisms. Philosophical Transactions of Royal Society of London, b287, pp. 459-480.

Hathaway, D.L. and Andrews, C.B. (1990). Fate and Transport Modelling of Organic Compounds from a Gasoline Spill. Ground Water Managment, v. 4, pp. 563-576.

Hickman, G.T. and Novak, J.T. (1989). Relationship between Subsurface Biodegradation Rates and Microbial Density. Environmental Science and Technology, v. 23, n. 5, pp. 525-532.

Hinchee, R.E., Downey, D.C., and Aggarwal, P.K. (1991). Use of Hydrogen Peroxide as an Oxygen Source for In-situ Biodegradation. Part I. Field Studies. Journal of Hazardous Materials, v. 27, pp. 287-299.

Hou, C.T. (1982). Microbial Transformation of Important Industrial Hydrocarbons. In: Microbial Transformations of Bioactive Compounds, v. 1. Rosazza, J.P., ed., CRC Press, Inc., Boca Raton, Florida. pp. 81-107.

Karlson, U. and Frankenberger, W.T. Jr. (1989). Microbial Degradation of Benzene and Toluene in Groundwater. Journal of Environmental Contamination and Toxicity, v. 43, pp. 505-510.

Kim, K. (1987). Biodegradation of Benzene. M.S. thesis, VPI&SU, Blacksburg, VA.

Krone, R.B. (1968). Movement of Disease Producing Organisms Through Soils. In: Municipal Sewage Effluent for Irrigation, Wilson, C.W., ed., The Louisiana Tech Alumni Foundation, Louisiana. pp. 75-106.

Kuhn, E.P., Colberg, P.J., Schnoor, J.L., Wanner, O., Zehnder, A.J.B., and Schwarzenbach, R.P. (1985). Microbial Transformations of Substituted Benzenes During Infiltration of River Water to Groundwater: Laboratory Column Studies. Environmental Science and Technology, v. 19, pp. 961-968.

Lodaya, M., Lakhwala, F., Rus, E., Singh, M., Lewandowski, G., and Sofer, S. (1991). Biodegradation of Benzene and a BTX Mixture using Immobilized Activated Sludge.

Journal of Environmental Science and Health. Part A: Environmental Science and Engineering, v. 26, n. 1, pp. 121-137.

McCarty, P.L. (1988). Bioengineering Issues Related to In-Situ Remediation of Contaminated Soils and Groundwater. In: Environmental Biotechnology: Reducing Risks from Environmental Chemicals through Biotechnology, Omenn, G.S., ed., Plenum Publishing Corporation, New York, N.Y. pp. 143-162.

McCarty, P.L., Reinhard, M., and Rittmann, B.E. (1981). Trace Organics in Groundwater. Environmental Science and Technology, v. 15, n. 1, pp. 40-51.

McCormick, A.J. (1991). The Effect of Ph on the Biodegradation of Benzene, Toluene, Ethylbenzene, m-Xylene in Soils. M.S. thesis, VPI&SU, Blacksburg, VA.

McDuffie, B. (1982). Chemical Aspects of Oil/Gasoline Spills - Information and References.

McGill, W.B., Rowell, M.J., and Westlake, D.W.S. (1981). Biochemistry, Ecology, and Microbiology of Petroleum Components in Soil. In: Soil Biochemistry, v. 5. Paul, E.A. and Ladd, J.N., eds., Marcel Dekker, Inc., New York, N.Y., pp. 229-296.

Mckee, J.E., Laverty, F.B., and Hertel, R.M. (1972). Gasoline in Groundwater. Journal of the Water Pollution Control Federation, v. 44, n. 2, pp. 293-302.

Nozaka, J. and Kusunose, M. (1968). Metabolism of Hydrocarbons in Microorganisms. Agricultural and Biological Chemistry, v. 32, n. 8, pp. 1033-1039.

Nyer, E.K. and Skladany, G.J. (1989). Relating the Physical and Chemical Properties of Petroleum Hydrocarbons to Soil and Aquifer Remediation. Ground Water Monitoring Review, v. 9, pp. 54-59.

Pardieck, D.L., Bouwer, E.J., and Stone, A.T. (1990). Hydrogen Peroxide as a Source of Oxidant Capacity for the Biotransformation of Benzene, Toluene and Xylene in Biofilms. *Proceedings of the 1990 Specialty Conference*, Arlington, VA, July 8-11, 1990, pp. 374-381.

Richards, B. N. (1987). The Microbiology of Terrestial Ecosystems. John Wiley & Sons Inc., England, pp. 128-129.

Ridgway, H.F., Safarik, J., Phipps, D., Carl, P., and Clark, D. (1990). Identification and Catabolic Activity of Well-Derived Gasoline-Degrading Bacteria from a Contaminated Aquifer. Applied and Environmental Microbiology, v. 56, n. 11, pp. 3565-3575.

Robinson, K.G., Farmer, W.S., and Novak, J.T. (1989). Availability of Sorbed Toluene in Soils for Biodegradation by Acclimated Bacteria. Water Research, v. 24, n. 3, pp. 345-350.

Spain, J.C., Milligan, J.D., Downey, D.C., and Slaughter, J.K. (1989). Excessive Bacterial Decomposition of H_2O_2 During Enhanced Biodegradation. Ground Water, v. 27, pp. 163-167.

Thorn, P.M. and Ventullo, R.M. (1988). Measurement of Bacterial Growth in Subsurface Sediments Using the Incorporation of Tritiated Thymidine into DNA. Microbial Ecology, v. 16, pp. 3-16.

Vestal, R.J. (1984). The Metabolism of Gaseous Hydrocarbons by Microorganisms. In: Petroleum Microbiology, Atlas, R., ed., MacMillan Publishing Company, New York. pp. 129-152.

Wade, L.E., Gengelbactt, R.B., Trumbley, J.L., and Hallbawer, W.L. (1960). Methanol. Encyclopedia of Chemical Technology, v. 5, pp. 398-415.

White, K.D. (1986). A Comparison of Subsurface Biodegradation Rates of Methanol and Tertiary Butanol in Contaminated and Uncontaminated Sites. Ph.D. dissertation, VPI&SU, Blacksburg, VA.

Wilson, S.B. and Brown, R.A. (1989). In Situ Bioremediation: A Cost-Effective Technology to Remediate Subsurface Organic Contamination. Ground Water Monitoring Review, v. 9, pp. 173-179.

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