The Effect of pH on the Biodegradation of Benzene, Toluene, Ethylbenzene, m-Xylene in Soils

by

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(Abstract)

Batch microcosms utilizing indigenous microorganisms were used to examine the effect of soil pH on the biodegradation of benzene, toluene, ethylbenzene, and m-xylene in subsurface soils. The biodegradation potential of both denitrifying and aerobic respiring microorganisms were assessed. Uncontaminated soil from five different soil depths was utilized. The pH of each soil was adjusted, such that a range of soil pH values existed at each soil depth. In this way, degradation rates of each of the aromatic compounds were determined at different soil pH values. Soil from each depth was characterized on the basis of: (1) soil particle size, (2) natural soil pH, (3) moisture content. It was suspected that the physical and chemical characteristics of a soil affected the microbial populations and the subsequent biodegradation potential of that soil.

At each soil depth, microcosms under aerobic conditions experienced a higher cumulative degradation rate of the BTEX compounds than did microcosms under denitrifying conditions. The results from this study suggest that the natural soil pH

did not provide an optimum environment for those aerobic respiring microorganisms which degrade BTEX. But rather, each depth in the soil profile had an optimum soil pH existing somewhere within the pH 5.4 to 6.6 range. Thus, the maximum combined degradation rate of BTEX was not found in soils at the natural soil pH. The 15 ft soil experienced the highest maximum combined degradation rate of BTEX. This soil had the highest moisture content, and relatively high sand and silt contents, which may have provided optimum conditions for microbial growth and subsequent biodegradation.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

INTRODUCTION

The ubiquity of petroleum hydrocarbon contamination of soils and groundwater and the subsequent threat these pose to potable groundwater supplies has necessitated the development of remediatory techniques for hydrocarbon removal. Enhanced in situ bioremediation is one of the techniques proposed for the removal of hydrocarbons from subsurface soils and groundwater (Wilson and Brown, 1989). In this method, indigenous microorganisms, some of which naturally decompose petroleum hydrocarbons, are stimulated upon receiving a supply of essential nutrients and a suitable electron acceptor. It has been suggested that microbial growth and subsequent biodegradation of organics will be at a maximum if the soil/microbe environment is maintained within a pH range of 6.0 to 8.5 (Atlas and Bartha, 1981; Bremmer and Shaw, 1958; Dupont et al., 1988). However, there appears to be few studies which measure the soil pH after it has been exposed to a neutral, nutrient-rich media. Recent studies suggest that differences in the degradation rates found in soils may be a function of the type of microbial population in that soil (Federle, 1988; Gullic, 1990). Similarly, the microbial population in a particular soil may be affected by the chemical and physical

characteristics of that soil, which are partially expressed by soil pH. Thus, site specific studies which monitor soil pH may help elucidate the source of variations in contaminant degradation rates.

Batch microcosms utilizing indigenous microorganisms were used to investigate the effect of soil pH on the biodegradation potential of monocyclic aromatic hydrocarbons. A pH range extending from acidic to alkaline was created for each of the five different soils utilized. This study was conducted to specifically assess:

- the effect of soil pH on the biodegradation of benzene, toluene, ethylbenzene, and m-xylene in subsurface soils.

- the suitability of hydrogen peroxide/supplied oxygen and nitrate as electron acceptors during enhanced in situ biodegradation of benzene, toluene, ethylbenzene, and m-xylene in subsurface soil by indigenous microorganisms.

Chapter 2

LITERATURE REVIEW

2.1 Gasoline Composition and Characteristics.

Gasoline is a mixture of over 100 chemical components, with the major chemical constituents being branched chain alkanes, cycloalkanes, and aromatics. In Table 1, the composition of the various hydrocarbons which comprise gasoline are listed (McDuffie, 1982). In table 2, variations in the chemical and physical properties of the major gasoline constituents are shown (McDuffie, 1982). These properties in turn govern contaminant/soil interactions (Nyer and Skladany, 1989).

Hydrocarbons are often found in four different phases when in a subsurface environment; a hydrocarbon plume existing above the water table, hydrocarbons adsorbed to soil particle surfaces and organic materials, hydrocarbon gases mixed with other soil gases, and dissolved hydrocarbons in groundwater (Falatko, 1991; Nyer and Skladany, 1989). The ultimate phase of a subsurface contaminant is affected by its potential for adsorption, abiotic decomposition, biodegradation, diffusion, and volatilization (Nyer and Skladany, 1989). The aromatic group, predominantly composed of benzene, toluene, ethylbenzene, and m-xylene (collectively termed BTEX), are

Table 1

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 2

Physical/Chemical Properties of Selected Gasoline Constituents

Compound	Molecular Weight	Vapor Pressure (25 0C, mm Hg)	Aqueous Solubility (25 0C, 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)

of particular interest due to their high percentage in gasoline, relatively high aqueous solubilities, toxicity, and carcinogenicity (Barker et al., 1987). The high water solubilities and corresponding low adsorption potentials (low K_{ow}) render the aromatics particularly susceptible to the mobile aqueous phase. Groundwater movement serves as the most prominent mechanism by which contamination is spread (Wilson and Brown, 1989). Consequently, BTEX compounds are commonly detected in groundwater supplies (Barker and Mayfield, 1988). The 1986 Safe Drinking Water Act mandated monitoring and/or removal of BTEX from water supplies due to their toxicity and carcinogenicity. As of January 1991, the maximum contaminant level (MCL) for benzene was 0.005 mg/L, toluene was 1.0 mg/L, ethylbenzene was 0.7 mg/L, and total xylene was 10 mg/L.

2.2 Aquifer Remediation.

The pump and treat remediation technique is one of two prominent methodologies often used to remove petroleum hydrocarbons from contaminated aquifers (Kerr, 1990; Nyer and Skladany, 1989). This technique employs at least one well which pumps free product floating on the aquifer and/or contaminated groundwater. The contaminated water is then brought to the surface and decontaminated by a specific treatment system. However, this pump and treat technology fails to remove the hydrocarbons which have adsorbed to soil

surfaces (Nyer and Skladany, 1989).

In situ bioremediation is the second major restoration technique utilized to remediate hydrocarbon contaminated aquifers (Nyer and Skladany, 1989). In situ treatment technology targets both adsorbed and dissolved contaminants for biodegradation (Dey et al., 1991; Wilson and Brown, 1989). In situ treatment practices implement the following design or a variation thereof to manipulate the soil/microbe system; nutrients and an appropriate terminal electron acceptor are supplied to the contaminated area through an injection well or infiltration gallery, producing wells are installed on the opposite end of the contaminated zone and pumped, such that the enriched media is drawn through the contaminated area (Technology Transfer Handbook, 1987). In this way, the microbial population in the contaminated region receives an increased supply of nutrients and is subsequently able to metabolize organic contaminants. Studies have shown that most of the constituents found in gasoline are biodegradable (Nyer and Skladany, 1989; Hinchee et al., 1991).

When abiotic hydrocarbon remediation techniques are used alone, they become less time and cost effective, especially when concentrations as low as the aforementioned MCL's must be attained (Kerr, 1990; Flathman, Jerger, and Bottomley, 1989). It has been suggested that in situ

bioremediation can be used in conjunction with abiotic processes to achieve maximum removal efficiency during restoration (Kerr, 1990). In this way, the contaminants in the adsorbed, aqueous, and free product phases are more completely removed.

2.3 Subsurface Microbiology.

In the past, it was commonly believed that microbial populations did not exist beneath the plant root zone (Technology Transfer Handbook, 1987). However, recent studies have documented the presence of indigenous microorganisms in uncontaminated groundwater at depths greater than 5906 ft (1800m) (Olsen et al. 1981). A study conducted by Goldsmith (1985) utilized soil from three different sampling sites, and at each site soil was collected at surface, 12, and 30 ft depths. His studies showed that the soils not only had nearly equal numbers of microbial cells but also a diverse microflora. As is commonly found, bacteria dominated a major portion of the microbial population in these soils (Hickman, 1985). Reduced morphological diversity and smaller body sizes are two of the characteristics which differentiate subsurface microflora from surface microflora (Hickman, 1988).

Indigenous bacteria in uncontaminated subsurface soil horizons are accustomed to low levels of nutrients and are thus considered oligotrophic (Kuhn et al., 1985; Poindexter,

1981). Subsurface bacteria have developed the ability to grow when very low nutrient concentrations exist; if the nutrient levels are increased, the bacteria must first acclimate to the increase and then commence growth. The low level of available nutrients may account for the slower microbial growth rates frequently found in subsurface soils (Thorn and Ventullo, 1988).

2.4 Basic Principles of In Situ Biodegradation of Aromatic Hydrocarbons.

In situ treatment utilizes indigenous microflora to biologically decompose organic contaminants in subsurface systems. When microorganisms metabolize these organic compounds, they acquire the energy and carbon needed to form and maintain additional microbial cells (McCarty and Rittmann, 1981). Optimal rates of indigenous microbial growth and subsequent maximum biodegradation rates may be achieved when hydrocarbon degrading microorganisms are in an environment which has a proper pH, temperature, moisture content, and sufficient supply of both mineral nutrients and suitable elctron acceptors (Flathman et al., 1989). In addition, the concentration of the substrate and subsequent metabolites should be low enough so as not to be toxic to the active microflora (Technology Transfer Handbook, 1987). During in situ treatment, the aforementioned factors are controlled within the soil/microflora subsurface

environment.

A balance of required substances, such as inorganic nitrogen, phosphorous, oxygen, and organic carbon, is naturally established in the soil/microflora environment prior to contamination (Dey et al., 1991). While in a balanced aerobic environment, microorganisms readily mineralize carbon, producing; energy, new cell material, CO_2, and water. However, the addition of organic carbon contaminants necessitates an additional increase in required nutrients if the nutritional balance is to be maintained in this system. As in activated sludge systems, micobial activity ceases if a sudden load of organic carbon is introduced to the system (Dey et al., 1991). Thus, a mineral-nutrient mixture is applied to the contaminated subsurface in an attempt to re-establish an appropriate balance amenable to metabolic activity.

2.4.1 Microbial Adsorption During In Situ Biodegradation.

Studies suggest that a greater number of microbes existing in subsurface hydrologic environments grow in microcolonies attached to solid surfaces as opposed to being suspended in the liquid phase (Ghiorse and Balkwill, 1983; McCarty and Rittmann, 1981). While subsurface microorganisms degrade hydrocarbons, most are either attached to soil particle surfaces or positioned within soil particle void spaces (McCarty and Rittmann, 1981). Some

studies show that transformations may occur while microorganisms are attached to droplets of contaminants (Atlas, 1981). However, a study conducted by Harvey et al. (1984) found that more than 95% of the total biomass in a contaminated aquifer were adsorbed to soil particles.

2.4.2 Aerobic Respiration.

Microbial degradation of aromatic hydrocarbons can be conducted in an oxygenated environment. Studies have been conducted which document in situ mineralization of aromatic compounds while under aerobic conditions (Atlas, 1981; Chiang et al., 1989; Dey et al., 1991). Oxidation of an organic substrate and concomitant reduction of oxygen characterize microbially mediated aerobic respiration (Hickman, 1988). Molecular oxygen serves two different functions during aerobic degradation of organic compounds. It serves as a final electron acceptor for those electrons liberated during the degradation of organics. Oxygen may also function as the reactant during initial decomposition of organic substrates. The supply of available molecular oxygen often limits the extent of biodegradation in contaminated aquifer systems (Barker et al., 1987).

2.4.3 Hydrogen Peroxide as Oxygen Source.

Field and laboratory studies have documented biodegradation of aromatics while utilizing hydrogen peroxide (H_2O_2) as the oxygen source (Trizinsky and Bouwer,

1990). Hydrogen peroxide dissociates into molecular oxygen and subsequently reaerates the subsurface environment (Pardieck et al., 1990). Equation (1) shows H_2O_2 decomposition and resultant end products (Hinchee et al., 1991):

$$H_2O_2 \longrightarrow H_2O + 1/2\ O_2 \qquad (1)$$

Equation (2) shows the oxygen requirements for aerobic biodegradation of benzene (Chiang et al., 1989):

$$C_6H_6 + 7\ 1/2\ O_2 \longrightarrow 6CO_2 + 3\ H_2O \qquad (2)$$

The oxygen to benzene molar ratio is 3.75:1; significantly more oxygen than aromatic contaminant must be available for complete mineralization to occur. Hydrogen peroxide is commonly used to aerate contaminated subsurface systems because: (1) it is relatively inexpensive and easily obtainable, (2) the molecular oxygen derived from it is readily utilized by microorganisms, (3) higher concentrations of oxygen can be generated by it then can be achieved by pure oxygen gas, and (4) its short half-life does not allow it to accumulate in the environment (Pardieck et al., 1990).

However, there are some disadvantages with the use of H_2O_2 in bioremediation. Hydrogen peroxide is toxic to microorganisms at high concentrations (Pardieck et al., 1990). In addition, H_2O_2 is unstable and therefore rapidly decomposes (Pardieck et al., 1990; Spain et al., 1989). The

resultant rapid production of dissociated molecular oxygen renders some of it insoluble. This fraction of the molecular oxygen is not only unavailable to microorganisms but may also become lodged in pore spaces, reducing soil permeability (Pardieck et al., 1990).

Hydrogen peroxide is both biotically and abiotically decomposed (Aggarwal et al., 1991 citing Schumb et al., 1955). Aqueous species of iron and copper are abiotic H_2O_2 decomposing catalysts commonly found in soils and groundwater. Bacterially produced peroxidase and catalase enzymes serve as the biotic H_2O_2 decomposing catalysts in hydrologic systems. Studies have shown that H_2O_2 decomposes at a faster rate than microorganisms can utilize oxygen for microbial growth (Hinchee et al., 1991). Thus, implementing processes which slow the rate of H_2O_2 decomposition may be beneficial, such as inhibiting catalase activity since its catalytic activity is much stronger than that of both peroxidase and non-enzymatic catalysts (Aggarwal et al., 1991). Studies have found that catechol, an intermediate of aerobic biodegradation of benzene and toluene, inhibits catalase activity (Aggarwal et al., 1991 citing Alyea and Pace, 1933). Thus, the rate of H_2O_2 decomposition may decrease once biodegradation of benzene and/or toluene is initiated and catechol is produced. As a result, there is a greater chance that active microorganisms will have the

opportuniy to utilize more oxygen before the H_2O_2 supply decomposes.

2.4.4 Degradation Pathways of Aromatics Under Aerobic Conditions.

Results from laboratory analyses indicate the general aerobic degradation pathways of homocyclic monoaromatic hydrocarbons (Dagley et al., 1964; Gibson et al., 1968). Figure 1 shows some of the intermediates formed during benzene biodegradation under aerobic conditions. The degradation pathways of toluene, ethylbenzene, and m-xylene are similar to that of benzene prior to ring cleavage (Gibson, et al., 1973; Claus and Walker, 1964; Davey and Gibson, 1974). During the first step, oxygenase enzymes insert oxygen onto the ring structure resulting in the formation of catechol or a catechol derivative. The number of oxygen atoms which are inserted onto the ring structure is dependent upon the type of oxygenase enzyme which is present. Once oxygen is directly attached to the ring structure, ring cleavage can commence. Ortho-cleavage is attained when fission occurs between the two adjacent carbon atoms bearing the hydroxyl groups. Alternatively, the fission may take place between one of the carbons which is bonded with a hydroxyl group and an adjacent carbon which does not have a hydroxyl group bonded to it. This molecular configuration is referred to as meta-cleavage. The type of

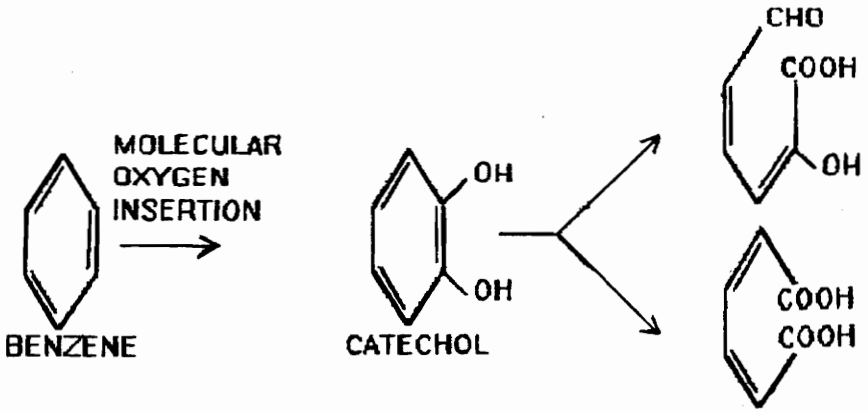


Figure 1. Aerobic Degradation pathway of benzene.

ring fission which occurs during biodegradation depends upon the type of substrates and microorganisms that are present (Hou, 1982). Further catabolism of the cleaved ring ultimately results in ATP production. The microorganisms incorporate a portion of the carbon into cell biomass.

2.4.5 Hydrocarbon Utilizing Aerobic Microorganisms.

Several petroleum hydrocarbon degrading microorganisms have been isolated and identified. Both heterotrophic bacteria and fungi have been found to degrade gasoline hydrocarbons. Pseudomonas, Archromobacter, Arthrobacter, Micrococcus, and Nocardia are some of the bacteria which degrade petroleum hydrocarbons in marine environments (Bartha and Atlas, 1977). Additional laboratory studies have isolated several species of Pseudomonas bacteria which are capable of aromatic hydrocarbon degradation (Gibson et al., 1968; Nozaka and Kusunose, 1968; Claus and Walker, 1964; and Davey and Gibson, 1974).

2.4.6 Anaerobic Soil Conditions and Denitrification.

The oxygen supply in subsurface systems often becomes depleted. Microorganisms may use the oxygen to metabolize organic compounds at a faster rate than oxygen could be replenished or it is possible that the heterogeneity of the soil provides for anoxic soil microsites in a generally aerobic soil matrix. Denitrification will occur in a subsurface system if substrate is available, nitrate is

present, and a viable population of denitrifying microorganisms are present (Hickman, 1988).

Denitrification is defined as the oxidation of an energy source, such as BTEX, coupled with the reduction of nitrate or nitrite to gaseous nitrogen oxides, such as nitric and nitrous oxide (Knowles, 1982). Further reduction of these oxides in semi-anoxic soil environments results in the formation of N_2 gas. In strict anoxic environments, reduction of the oxides results in ammonia formation. Denitrifying bacteria are facultative, and can thus function in both aerobic and anoxic environments. However, aerobic respiration yields more energy and is thus preferred over denitrification processes (Brock, 1974).

2.4.7 Degradation Pathways of Aromatics Under Anaerobic Conditions.

Although aerobic biodegradation processes have been thoroughly studied and are now widely accepted methods of hydrocarbon remediation in contaminated aquifers, the extent of aquifer remediation under denitrifying conditions remains to be verified (Hutchins et al., 1991). However studies have been conducted which document the degradation pathway of aromatic compounds under anerobic conditions. It was believed that the reduction of the benzene ring was the first step in this sequential degradation process of aromatic compounds; this step is followed by the insertion

of oxygen onto the ring structure (Young, 1984). The last step consists of ring cleavage. However relatively recent studies by Vogel and Grbic-Galic (1987), have challenged this conventional degradation pathway of benzenoid compounds. Their studies suggest that ring oxidation rather than ring reduction is the first step in the biodegradation of aromatic compounds. Subsurface in situ bioremediation systems have utilized mixed microbial cultures under denitrifying conditions to successfully degrade BTEX compounds (Major, et al., 1988, Kuhn et al., 1985, and Hutchins et al., 1991). However, Lovley and Lonergan (1990) have only recently isolated the first two microorganisms known to oxidize an aromatic hydrocarbon under anaerobic conditions. A microbially mediated degradation pathway employed by GS-15 utilized toluene as the electron donor and poorly crystalline Fe^{3+} oxide as the electron acceptor. A second microbe, Pseudomonas sp., oxidized toluene and may have concomitantly reduced either NO_3 or N_2O.

Unlike aerobic biodegradation of BTEX compounds, the resultant intermediates formed during BTEX metabolism under anaerobic conditions have not yet been confirmed (Lovley and Lonergan, 1990; Grbic-Galic and Vogel, 1987). However two pathways for toluene metabolism under denitrifying or methanogenic conditions have been proposed (Grbic-Galic and Vogel, 1987). The first pathway consists of aromatic ring

hydroxylation. This step results in the formation of the p-cresol intermediate, which is then oxidized to p-hydroxybenzoate. Additional studies by Grbic-Galic and Vogel (1987) suggested that benzene may also experience an initial ring oxidation step, producing phenol as the intermediate. Oxidation of the methyl group on the aromatic ring characterizes the second proposed pathway for toluene degradation. This step results in the sequential production of benzylalcohol, benzaldehyde, and lastly benzoate. Complete mineralization of benzoate under anaerobic conditions has been observed (Lovley et al., 1989). The proposed degradation pathways for toluene may serve as models of the degradation pathways for other aromatic hydrocarbon compounds which are under similar conditions.

2.5 Soil Buffering Capacity and pH.

Soil buffering capacity is defined as the ability of a soil to resist change in the soil solution pH. Clays and organically rich soils have net negative charges which attract cations. These adsorbed cations are in equilibrium with cations in the soil solution. The total quantity of exchangeable cations that a soil can adsorb is known as the cation exchange capacity (CEC).

Dissolution of soil minerals including feldspars and micas provide some of the sources of the exchangeable cations and hydroxyl ions which are found in soil solutions

(Dupont et al., 1988). When equal concentrations of cations are present in a soil solution, trivalent cations have greater adsorption potential than divalent cations and divalent cations have greater adsorption potentials than monovalent cations (Gerba and Bitton, 1984). Likewise, dissociated hydrogen ions contribute to exchangeable hydrogen in the soil solution. Soils with a large CEC generally have significant soil buffering capacities (Dupont et al., 1988). In this way, those soils with the greatest fractions of clay and humics have maximum buffering capacities (Gerba and Bitton, 1984).

However, the CEC and subsequent buffering capacity of organic matter and clays is also affected by the soil solution pH (Bitton and Gerba, 1984; Dupont et al., 1988). The acidic (highly reactive) functional groups present on humic materials become positively and negatively charged when in acidic and alkaline environments respectively (Soil Survey, 1980). In clay structures, broken bonds at crystal edges and dissociated amorphous minerals and hydrous oxides undergo protonation and deprotonation when in acidic and alkaline environments respectively (Soil Survey, 1980). Although both clay and organic matter have pH dependent surface charges, they also simultaneously possess a permanent net negative charge (Bitton and Gerba, 1984). As a result of pH dependent surface charges, the soil buffering

capacity of clays and organic matter increases at high pH and decreases at low pH (Bitton and Gerba, 1984).

2.5.1 Optimum Soil pH Conditions.

It has been shown that soil pH crucially effects microbial decomposition of organic contaminants (Atlas and Bartha, 1981; Dupont et al., 1988). For example, nutrient solubility, microbial adsorption, and metabolic pathways are all sensitive to soil pH. It has been suggested that the maximum rate of in situ bioremediation of organic compounds generally exists in soils which have a pH ranging from 6.0 to 8.0 (Atlas and Bartha, 1981; Bremmer and Shaw, 1958) and from 6.5 to 8.5 (Dupont et al., 1988). A review of the literature indicated that only some of the enhanced in situ bioremediation studies used a nutrient solution buffered to approximately pH 7.0 (Flathman, et al., 1989; Trizinsky and Bouwer, 1990; Parkin, et al., 1985; Payne and Floyd, 1990). Many of the studies failed to note the pH of the media and/or measure the resultant pH of the contaminated soil once inoculation with the nutrient media had occurred.

An enhanced in situ biodegradation study by Gullic (1989) found that soils with different natural pHs had different toluene biodegradation rates although each soil was dosed with the same buffered nutrient media (pH 7.3). A follow up study used a non-buffered nutrient media to dose those naturally acidic soils which demonstrated the lowest

toluene biodegradation rates; these soils were also inoculated with acclimated toluene degrading microorganisms. However, toluene biodegradation rates were still inhibited in these naturally acidic soils, suggesting that low soil pH was either directly or indirectly inhibiting biodegradation.

It is not unusual to find that chemical, physical, and biological characteristics are different for each subsurface environment and in turn may be reflected by soil pH. Since different soil systems may have anywhere from slightly to substantially different chemical, physical and microbial reactions when exposed to similar stimulus, it may be possible to optimize biodegradation rates if more studies were conducted which examined site specific optimum soil pHs.

Chapter 3

METHODS AND MATERIALS

This chapter describes the sampling and analytical protocol employed during a microcosm study which examined the effects of pH on the biodegradation of BTEX under both aerobic and denitrifying conditions. Both BTEX concentrations and pH were monitored in each sample throughout the course of this study.

3.1 Experimental Approach.

A microcosm study utilizing indigenous microorganisms was used to examine the effect of soil pH on biodegradation of BTEX. BTEX compounds were utilized in this study because of their relatively high water solubilities with respect to the other constituents found in gasoline. Hence, they are more readily transported through the subsurface and tend to be more prevalent in contaminated groundwater sources.

A pH range extending from acidic to alkaline was created for each soil depth/soil type such that their potential for BTEX biodegradation at different pH values could be examined. Both aerobic and denitrifying conditions were established at each depth, such that BTEX degradation rates for both metabolic groups in similar environments could be compared.

The sample matrix consisted of two sets of samples, a

set of microcosms under aerobic conditions and a set of microcosms under denitrifying conditions. Each set contained soil from 5 different depths. Triplicates at each depth and for each metabolic group were dosed with a spiked nutrient-buffer solution. Five different spiked nutrient-buffer solutions were created for each metabolic group, with each having a different pH.

3.2 Soil Collection.

The soil used in this study was collected from a dairy farm on Virginia Tech property. Two 17 ft auger holes were dug adjacent to those drilled by Gullic. An oak hickory forest was located up-gradient to the site and a pasture was located down-gradient to the site. This soil had no history of chemical contamination.

All of the materials utilized in this study required sterilization. Sterilization methods employed consisted of one or a combination of the following: autoclaving for 30 minutes at 121° C and 15 psi, ethanol rinse, alcohol-flame sterilization.

Unsaturated soil was collected at the surface, 3, 6, 15, and 17 ft. Seventeen ft was the greatest depth sampled since refusal occurred there upon interception with the underlying Rome formation bedrock. Soil from each depth was removed from the auger with a metal spatula and then placed into a metal tray. The soil was then poured into its

respective sterilized glass jar and covered with an airtight teflon lid. Upon collection, the soils were transported to the laboratory and stored in an incubator at 10° C.

3.3 Nutrient Solution Preparation.

A nutrient solution consisting of 1.9 mg/L KH_2PO_4, 6.25 mg/L K_2HPO_4, 0.5 mg/L $(NH_4)_2HPO_4$, 0.5 mL salts solution, and 1000 mL sterile distilled water was added to each microcosm. The nutrient solution used to dose the denitrifying microcosms also contained 30 mg/L KNO_3 as NO_3 and was sparged with nitrogen gas for 30 minutes to remove dissolved oxygen.

3.4 pH Adjustment and Addition of BTEX to Nutrient Solution.

Prior to microcosm dosing, the pH of the nutrient-buffer solution was altered to create five sets of samples, each at a different pH. The pH of the nutrient-buffer solutions were adjusted to 8.8, 7.5, 6.5, 5.6, and 4.8 for the denitrification media and 8.7, 7.6, 6.6, 5.6, and 4.6 for the aerobic media.

Before the pH of the nutrient-buffer solution was adjusted, it ranged from 7.0 to 7.3. Concentrated NH_4OH was added to the nutrient-buffer solution to increase the pH and concentrated HCL was added to decrease the pH. After pH adjustment was achieved, the solution was placed on a magnetic stir plate and drops of BTEX were added to the

center of the vortex. The bottle containing the solution was immediately sealed and stirred for 12 hours. A target concentration of 40 mg/L of each organic compound was added to the nutrient solution. However, the total concentration of BTEX was not uniform in each of the nutrient buffer solutions. Minimum headspace was maintained in the bottle to prevent excessive BTEX volatilization.

Upon completion of the mixing period, the spiked nutrient-buffer solution was pumped to a sterile, collapsible teflon bag. The solution was then pumped from the teflon bag to the microcosms.

3.5 Microcosm Preparation and Dosing.

Each microcosm from the two sets of samples was constructed in a similar manner. Five grams (+/- 0.1 g) of soil were aseptically transferred to a glass screw cap test tube (13 x 100 mm) which was sealed with a one-piece, teflon lined lid (12 mm). The aerobic microcosms were dosed in an ethanol rinsed glovebox, while the denitrifying microcosms were dosed in a nitrogen purged, ethanol rinsed glovebox. The glovebox helped shield both sets of microcosms from microbial contamination and minimized re-oxygenation of the denitrification solution in the case of the denitrifying microcosms. After partially filling the microcosm, it was mixed on a vortex mixer to displace trapped air and to evenly distribute the liquid media. The test tube was then

filled to its maximum capacity to eliminate the remaining headspace.

Variations in initial concentrations of BTEX compounds between each set of the nutrient-buffer media were incurred.

3.5.1 Addition of Electron Acceptors.

Each of the aerobic microcosms was dosed with 5 microliters of a 30% H_2O_2 solution immediately following dosing with the BTEX spiked nutrient solution. A sterilized 10 microliter syringe submerged just below the surface was used to transfer the H_2O_2 directly to the microcosm such that premature decomposition was minimized. This amount of H_2O_2 yielded 0.03% (by volume) concentration in the microcosm, which Gullic (1990) found was non-toxic to the microorganisms. In addition, Britton (1985) found H_2O_2 did not become toxic until a level of 0.05% (by volume) was attained. Additional H_2O_2 was periodically added to aerobic microcosms in 2 microliter doses.

After approximately 100 days, each denitrifying microcosm was dosed with 170 mg/L KNO_3 as NO_3 in addition to the 30 mg/L NO_3 they initially received. Stoichiometric calculations indicated this additional nitrate insured that nitrate was not a limiting factor in the complete mineralization of the BTEX compounds.

3.6 Sterile Control Microcosms.

Two sets of sterile control microcosms were

constructed, one without an electron acceptor and one with an electron acceptor. For each of the two sets, two microcosms at each of the five depths were constructed. Each of the sets contained 5.0 grams (+/- 0.1 g) of sterilized soil. This soil was autoclaved 4 times during a 2 day period; after which, the soil was transferred to sterile test tubes and was again autoclaved 4 times during a 2 day period. One set was dosed with a sterile BTEX spiked nutrient solution with no electron acceptor and the other set was dosed with a sterile BTEX spiked nutrient solution with H_2O_2 as the electron acceptor. It was considered that adsorption of the non-polar BTEX compounds would not be substantially affected by soil solution pH. As a result, the pH of the BTEX spiked nutrient solutions used to dose the sterile controls was not adjusted.

3.7 Soil Characterization.

The soil characteristics determined by Gullic (1990) were applicable to this study as well since the soils were collected from adjacent boreholes. Method 21-2.2.2 from "Methods of Soil Analysis" was employed to quantify soil moisture content. A premeasured soil mass was dried in an oven at 104° C for a period of 24 hours and then cooled in a dessicator. The soil was weighed again and any mass loss was attributed to a loss of moisture. The VPI&SU Soil Physics Lab performed a particle size analysis on samples

from each of the soil depths studied. This lab utilized the pipette method found in section 15-4 of "Methods of Soil Analysis" to quantify the percentages of silt, sand, and clay at each of the soil depths.

3.8 pH Measurements.

The natural pH of the soil was measured according to "Methods of Soils Analysis", method 12-7.6. Five grams of soil was thoroughly mixed with 5 mL of distilled water for 5 sec. After 10 minutes, a Fisher Scientific combination electrode pH meter was used to stir the soil suspension and simultaneously measure its hydrogen ion concentration.

One of the triplicate microcosms was used solely for pH measurements rather than for GC analyses. Prior to the pH measurement, the microcosm was inverted and hand shaken for a period of 5 seconds, and then allowed to settle for a period of 10 minutes. After which, a ColorpHast pH strip was submerged in the supernatant. The microcosm was resealed immediately after insertion. After the pH stabilized, the pH paper was removed with a sterilized syringe needle and read immediately. This sequence of steps, in comparison to a direct pH measurement with no mixing, yielded a pH reading closer to that measured by the electrode. The precision of the pH paper was +/- 0.3 pH units.

3.9 Analytical Methods.

The following sampling protocol was employed to monitor BTEX concentrations throughout the course of this study: two microliters of supernatant was withdrawn from each sample and injected directly into a Hewlett Packard Model 5880A gas chromatograph (GC) with a flame ionization detector (FID). An oven temperature program was utilized such that proper separation of output peaks could be achieved. The oven temperature was held at 150° C for 3 minutes and then increased to 170° C at 30°/minute and held there for 20 minutes. The injector port and FID temperatures were maintained at 150 and 225° C respectively. Nitrogen carrier gas flowed at 30 ml/min through the 6 ft (1.829 m) x 1/8 in (3.175 mm) stainless steel column. The column was 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).

In addition, a Dionex 2010i ion chromatograph was used to calculate the final nitrate and nitrite concentrations in some of the denitrifying samples. Supernatant from the microcosm was injected onto an AS4A column which was used in conjunction with a conductivity detector and a Dionex 4270 integrator.

Chapter 4

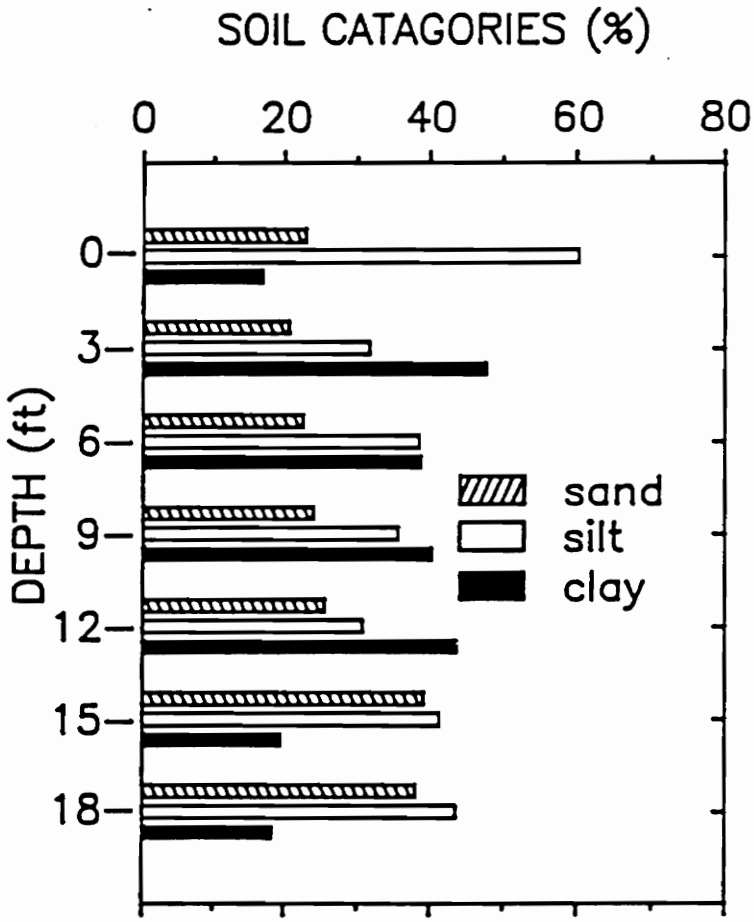
RESULTS AND DISCUSSION

A microcosm study utilizing indigenous microorganisms to degrade BTEX was conducted. Soil samples from 5 different depths were used and each of the soil pHs were adjusted such that the a range of soil pHs were created. In this way, the effects of pH on biodegradation were examined.

4.1 Soil Characteristics.

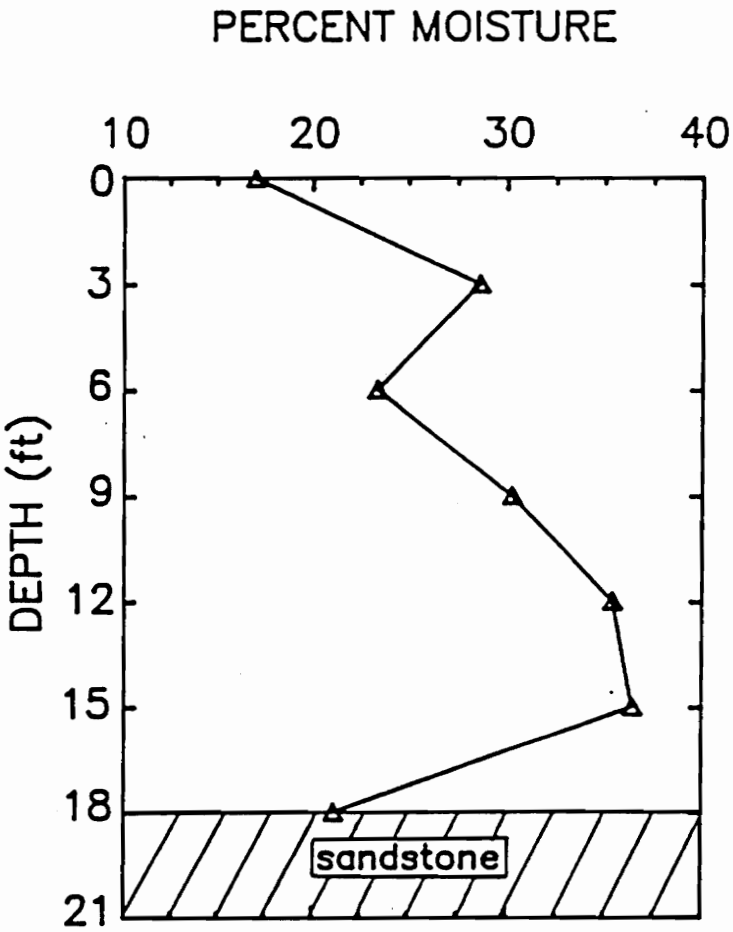
The soil used in this study was of the groseclose soil series, which is classified as a clayey, mixed, mesic Typic Hapludult. The soil characterizations which were determined by Gullic (1990) were applicable to this study since the soil used in this study was collected from a site immediately adjacent to Gullic's sampling site. Figure 2 from Gullic (1990) shows the soil particle size analysis which was performed on the soils from this site. This analysis indicates that the surface soil was greater than 60% silt and had a comparatively low sand and clay content. In contrast, both the 3 and 6 ft soils contained less than 60% sand and silt, and approximately 40 to 50% clay. The 15 and 17 ft soils had greater than 80% sand and silt and relatively low amounts of clay. Refusal occurred at 17 ft, where highly weathered limestone bedrock was encountered.

Figure 3 from Gullic (1990) quantifies soil moisture



(after Gullic, 1990)

Figure 2 . Percentages of sand, silt and clay at each depth of the sampling site.



(after Gullic, 1990)

Figure 3. Soil moisture content with increasing depth.

content. The surface soil moisture content is largely dependent on precipitation and can vary considerably on a short term basis (Gullic, 1990). The large clay content in 3 and 6 ft soils accounts for their high water content. The 15 ft soil had the highest moisture content. The 17 ft soil was well drained with a relatively low moisture content.

4.2 pH Stabilization.

In figures 4 and 5, examples of the variation in pH with time in samples under both aerobic and denitrifying conditions are shown. In Appendix A, similar figures for each soil studied are provided. The pH equilibria data for the 3 ft soil is plotted on Figure 4. One set of the 3 ft soil samples was dosed with a nutrient media buffered to approximately the same pH value (pH 4.6) as the natural soil (pH 4.7). The pH of the 3 ft soil solution immediately after initial dosing was 3.2. The pH gradually increased until it stabilized at approximately pH 4.0. The behavior of soil which is strongly acidic by nature may explain why this soil system did not re-equilibriate at the natural soil pH but rather at an even more acidic pH.

Under strongly acid soil conditions, the hydrogen ions adsorbed to permanently charged exchange sites of clay surfaces also serve as a source of exchangeable hydrogen ions (Brady, 1974). Those adsorbed hydrogen ions are also in equilibrium with the hydrogen ions in the soil solution

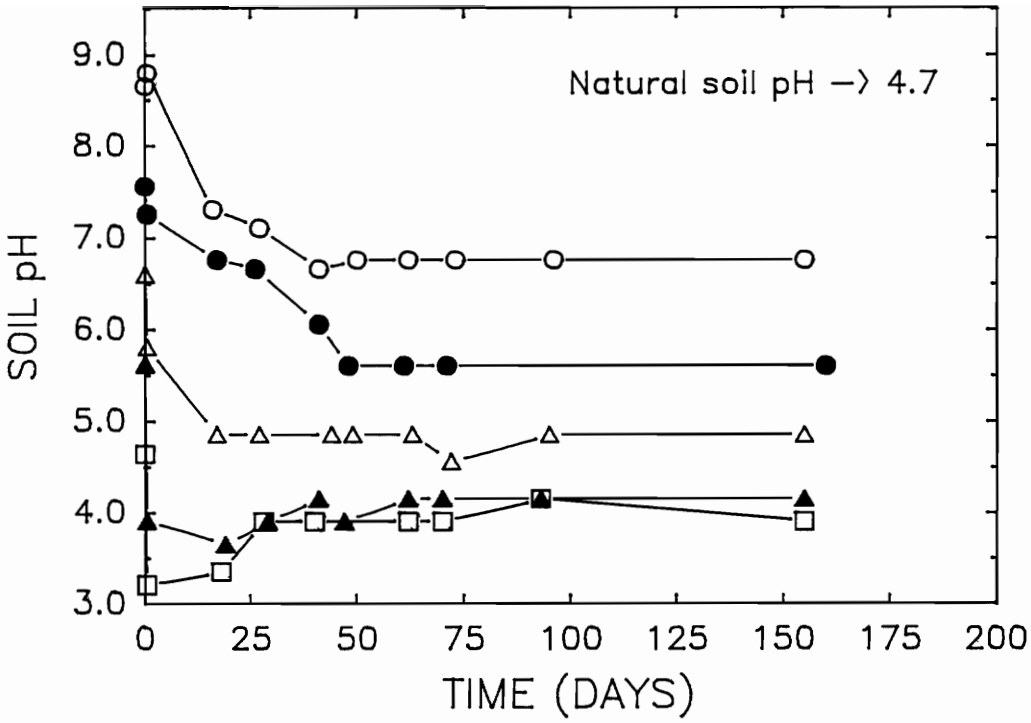


Figure 4. The pH equilibria attained in aerobic, 3 ft soil samples with adjusted pH.

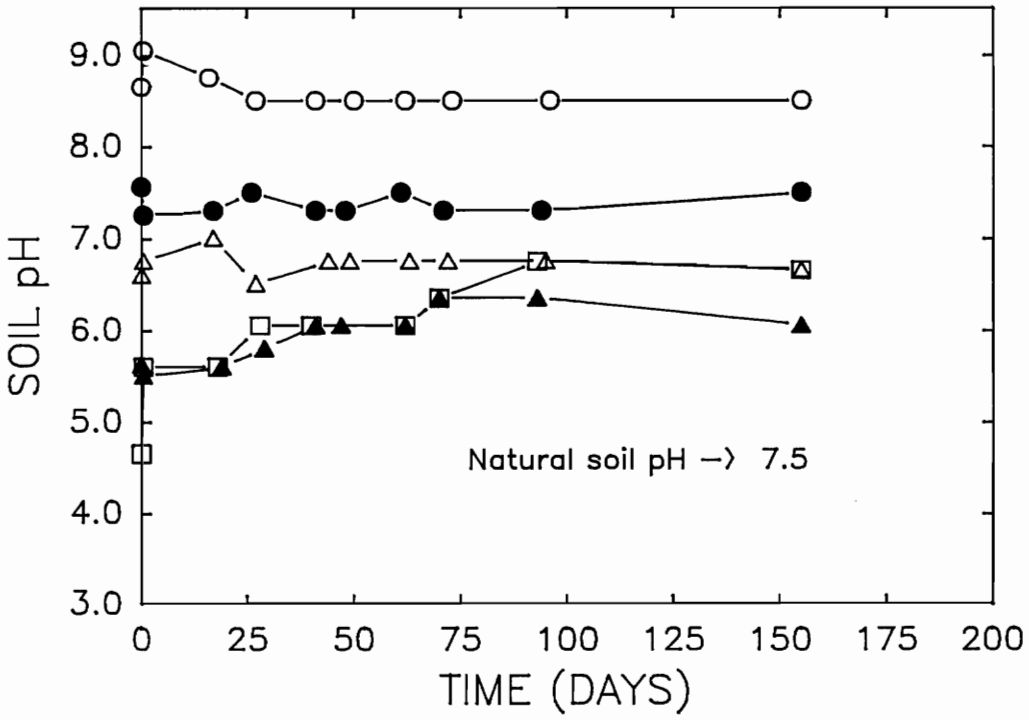


Figure 5. The pH equilibria attained in aerobic, 17 ft soil samples with adjusted soil pH.

(Brady, 1974). Thus, there is an indirect relationship between the amount of adsorbed hydrogen ions and soil solution pH. The amount of adsorbed aluminum ions also affects the hydrogen ion concentration in the soil solution.

When clay soils have a pH lower than 6, aluminum ions are released from the clay lattice and adsorbed to the clay surface (Dupont et al., 1988). At pH 4.7 and lower, the aluminum ion becomes the dominant aluminum species as opposed to a hydroxylated aluminum species (Brady, 1974). The adsorbed aluminum ions are in equilibrium with aluminum ions in the soil solution (Brady, 1974). The aluminum in solution tends to undergo the following reaction:

$$Al^{3+} + H_2O \rightarrow Al(OH)^{2+} + H^+ \qquad (3)$$

The generation of hydrogen ions lowers the soil solution pH.

When the 3 ft soil was dosed with a nutrient media slightly more acidic than the natural soil pH, the soil system equilibria was disturbed. More hydrogen ions may have been present in the both the soil solution and adsorbed to clay surfaces after dosing, thereby slightly lowering the pH of the soil solution. The lower pH of the soil solution could result in a greater amount of aluminum ions released from the octahedral layer of the clay structure. These ions may have further lowered the pH of the soil solution to 3.2.

However, the 3 ft soil sample did not stabilize at pH 3.2. Instead, the pH gradually increased, and then

stabilized at a pH below the natural soil pH. The low amount of alkalinity generating cations in strongly acidic soils may explain why the soil solution pH stabilized at 4.0. In strongly acid soils, a relatively small amount of metallic cations such as sodium, calcium, and magnesium can adsorb to clay surfaces (Brady, 1974). These cations replace the exchangeable hydrogen and aluminum ions adsorbed to the clay, causing an increase in the soil solution pH (Brady, 1974). However, the relative amounts of alkalinity generating metallic cations is much smaller than the amount of acidity generating aluminum and hydrogen ions in the soil solution, thus the net result is an acidic soil solution (Brady, 1974).

In this way, the adsorption of metallic cations in the 3 ft clay samples may have caused the observed increase in soil solution pH. The relatively small amount of metallic cations which strongly acid soils can adsorb limits the degree to which the pH of the soil solution can be increased. Thus, the pH of the soil solution increased from approximately 3.2 to 4.0, but may have stabilized there due to limited availability of alkalinity producing cations.

4.3 BTEX Adsorption.

In figure 6, the concentration of BTEX over time in a single biologically active microcosm is shown. The initial loss of BTEX which occurred during the first 32 days is

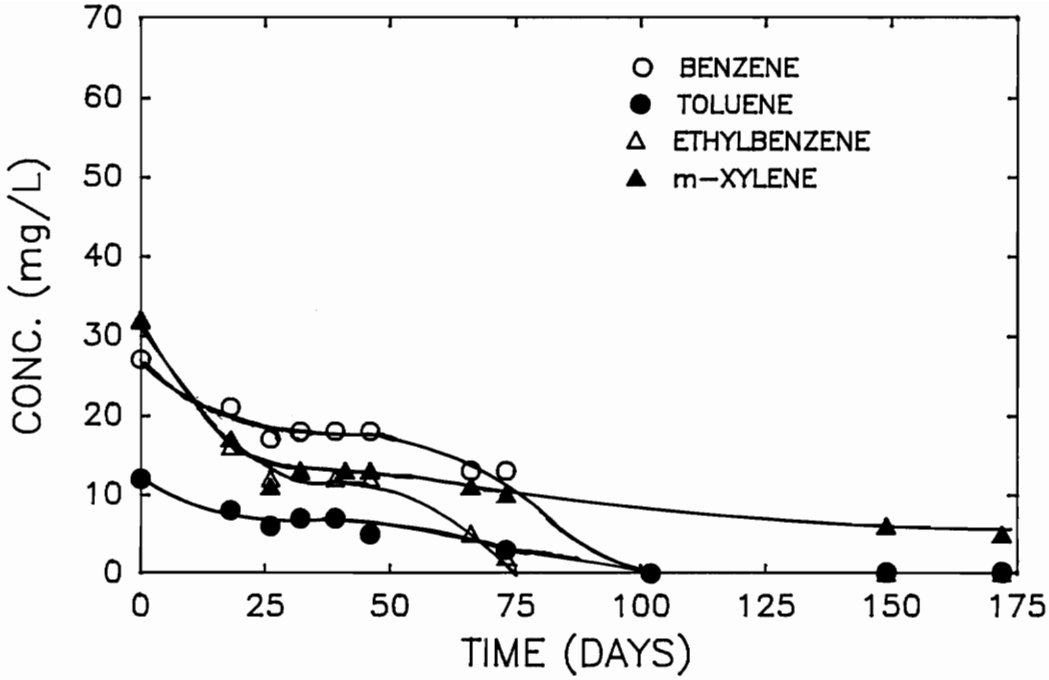


Figure 6. Initial adsorption phase and acclimation period of BTEX in a representative aerobic sample.

thought to be due to adsorption and is typical of the data from each microcosm. In both the aerobic and denitrifying microcosms, similar initial decreases in BTEX were observed during the first 25 to 40 days. However, not all microcosms lost equal amounts of BTEX, probably because the soils differed in their adsorptive characteristics even for soils taken from the same depth. Data from adsorption studies conducted on toluene by Farmer (1989) showed similar results.

Farmer (1989) found that sterile microcosms dosed with toluene experienced an initial period of rapid toluene adsorption followed by a period of slow adsorption. Adsorption studies using BTEX compounds in sterile microcosms were conducted during this study and similar fast and slow adsorption phases occurred. In figure 7, the variations in BTEX losses over time for both 3 and 17 ft H_2O_2 amended sterile controls are shown. In Appendix B, similar figures for each sterile control are provided. There appears to be greater losses in the high clay soil (3 feet) than in the sandy soil (17 feet). BTEX losses were attributed to abiotic processes such as adsorption and volatilization. Volatilization occurred while the test tubes were briefly uncovered during sampling. The 3 and 6 ft soils experienced the greatest net loss of BTEX in both amended and non-amended sterile controls. The high ionic

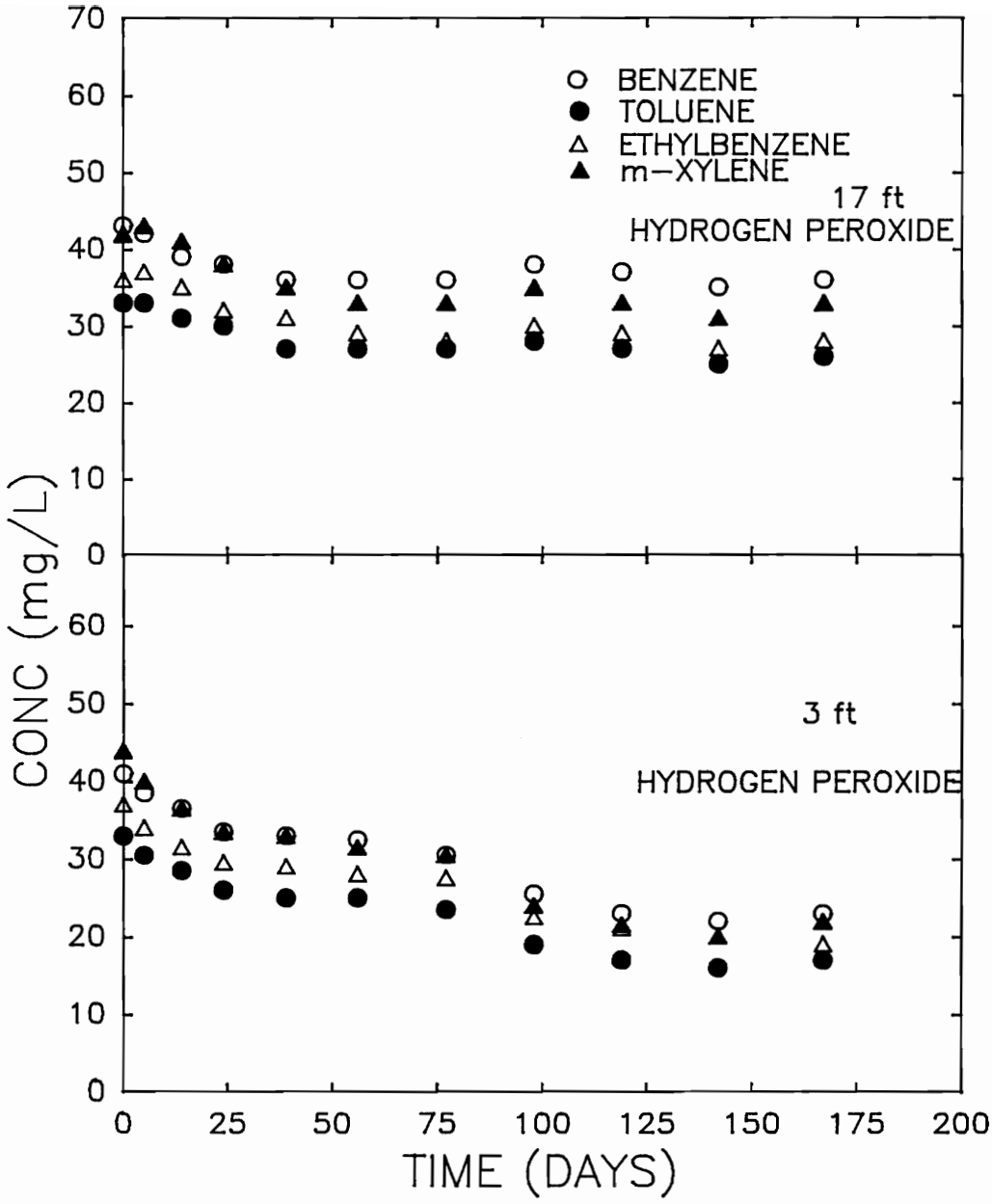


Figure 7. Abiotic losses in sterile hydrogen peroxide amended 17 ft soil (top) and 3 ft soil (bottom).

strength characteristic of clayey materials increases the adsorptive capabilities of the 3 and 6 ft soils (Krone, 1968). This explains why the 3 and 6 ft soils adsorbed a greater concentration of the BTEX compounds than the less clayey soils found at the surface and at 15 ft and 17 ft.

4.3.1 Use of Sterile Controls to Correct Biodegradation Rates.

The sterile controls were used to determine both when the rapid initial adsorption phase had ceased and the amount of BTEX lost due to slow adsorption and/or volatilization. Consequently, BTEX degradation rates in the samples were not calculated until after the initial rapid adsorption phase had ceased. The loss of each compound which was measured in the sterile controls was subtracted from the organic removal of each compound in the viable microcosms. The resulting value was considered to be the loss due to biodegradation. Data from duplicate microcosms were averaged, such that each data point reflects two samples.

4.4 Acclimation in Aerobic and Denitrifying Samples.

Many of the aerobic samples which experienced biodegradation, first experienced a period in which BTEX was degraded slowly or not at all; this phase was immediately followed by an accelerated loss of BTEX which was considered to be the result of biodegradation (Gullic, 1989; Hickman, 1989). The period of relatively slow BTEX

losses was characterized as the acclimation or lag period. During an acclimation period, microorganisms adapt to the newly exposed substrates. In figure 6, it is shown that the acclimation period lasted through day 50 for benzene, ethylbenzene, and m-xylene, and through day 40 for toluene. The acclimation period for each aerobic and denitrifying sample can be determined from the figures in Appendix C. However, the acclimation period in the denitrifying samples is not as easily identified as that in aerobic samples. This is perhaps due to the overall low degradation rates observed in the denitrification samples.

4.5 BTEX Degradation under Aerobic Conditions.

Figure 8 shows degradation rates of each of the BTEX compounds at each soil depth and at its corresponding pH range. Ranges are used because the pH changed slightly over time in most microcosms. The highest degradation rates were in soils with pH values ranging from 4.5 to 7.5. However, similar soil depths within this range often had widely varying BTEX degradation rates. For example, 17 ft soil samples with pH values ranging from 5.5 to 6.5 had combined degradation rates of BTEX which ranged from 0.275 to 0.864 mg/L/day/5 grams of soil. In addition, 15 ft soil samples with pH values ranging from 5.5 to 6.5 had combined degradation rates of BTEX which ranged from 0.188 to 0.732 mg/L/day/5 grams of soil. Some of the variation in

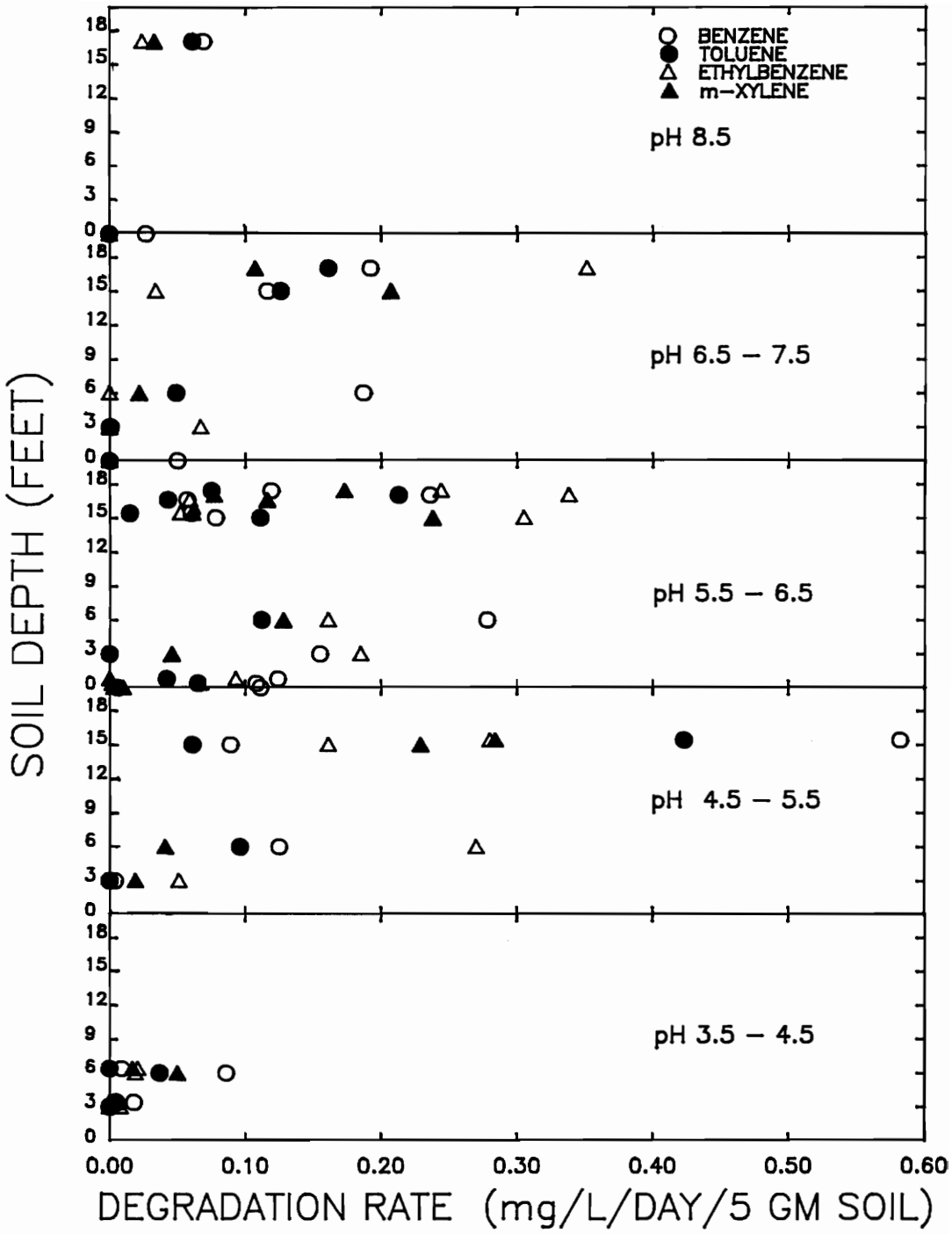


Figure 8. Highest BTEX degradation rates occurs in those soil depths where adjusted pH is in the range 4.5 to 7.5.

degradation rates in 15 and 17 ft soil samples with this pH range may be attributed to m-xylene effects. A more thorough discussion of the potential inhibitory effects of m-xylene is provided in section 4.5.2. However, m-xylene effects did not account for the variation in BTEX degradation rates found in 15 ft soil microcosms which had pH values ranging from 4.5 to 5.5.

In addition, 15, 17, and sometimes 6 ft soil samples with pH values ranging from 4.5 to 7.5, demonstrated higher degradation rates than both 3 ft and surface soil samples with the same pH range. Samples with the lowest pH values, 3.5 to 4.5, and samples with the highest pH value, 8.5, always showed relatively low degradation rates.

In figures 9 through 13, three dimensional plots of the total initial concentration of BTEX within each microcosm, individual BTEX degradation rates, and soil pH for each soil depth studied are shown. The total BTEX concentration and soil pH axes are not arithmetic. The pH values represent the pH range in which biodegradation activity occurred. In these figures, it is shown that at each depth, there was one pH value which exhibited a combined degradation rate of BTEX higher than that at other pH values. These figures can also be used to show the poor correlation between relatively low degradation rates and high total BTEX concentrations. This result is especially evident in the figure 12. Two 15 ft

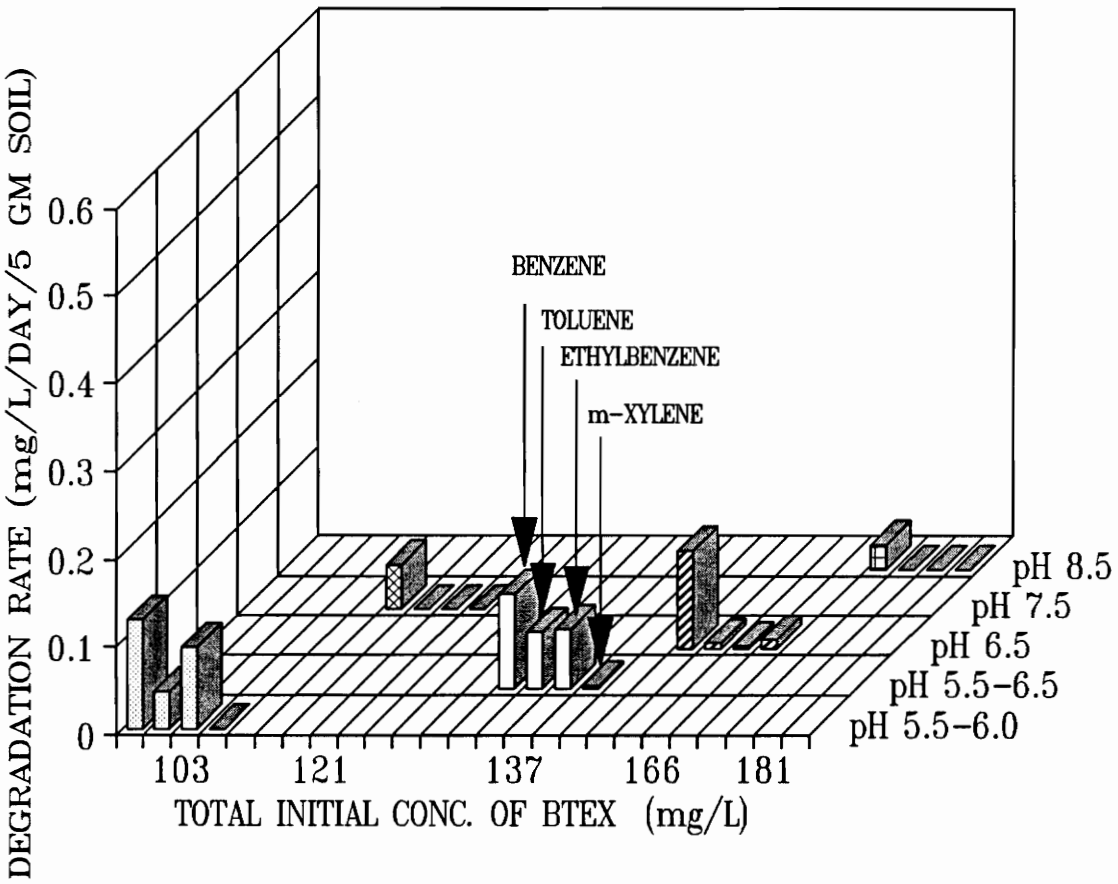


Figure 9. Comparison of BTEX degradation rates in surface soil samples which are under aerobic conditions and have adjusted soil pH. Natural soil pH is 6.7.

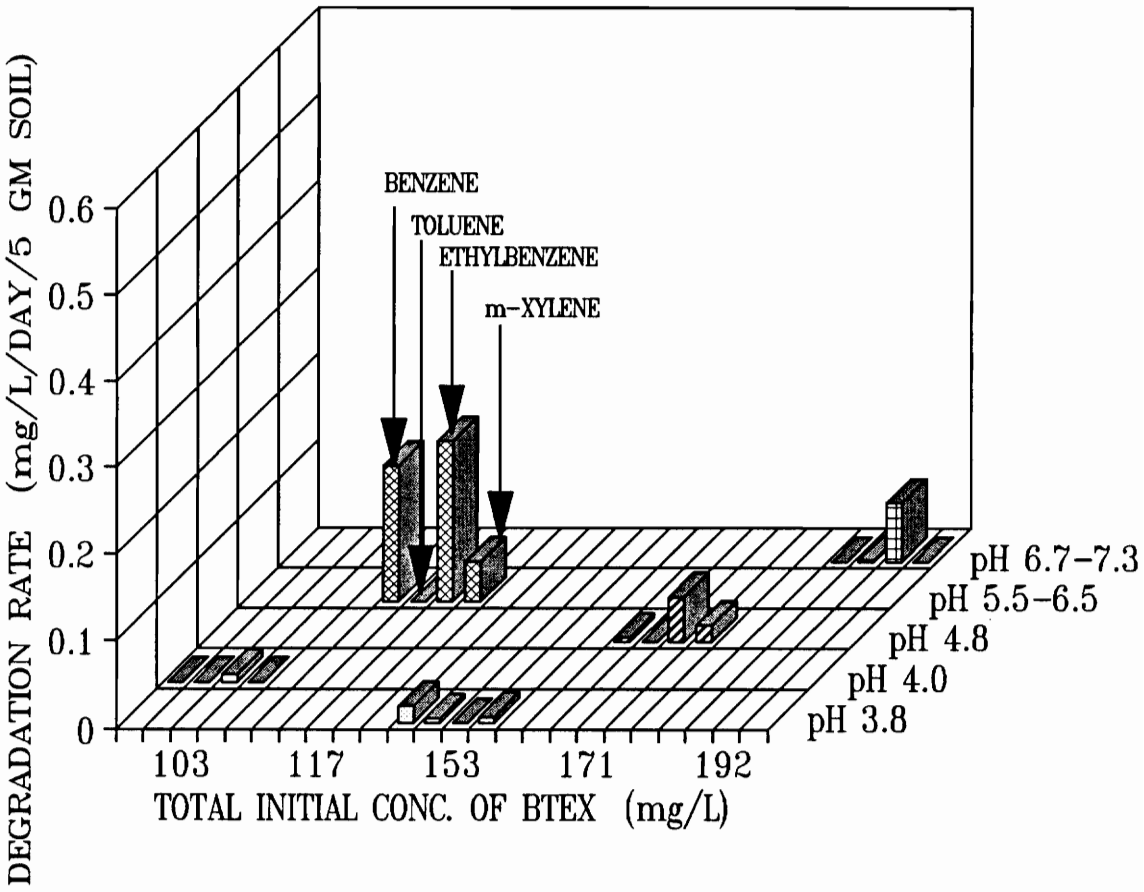


Figure 10. Comparison of BTEX degradation rates in 3 ft soil samples which are under aerobic conditions and have adjusted soil pH. Natural soil pH is 4.7.

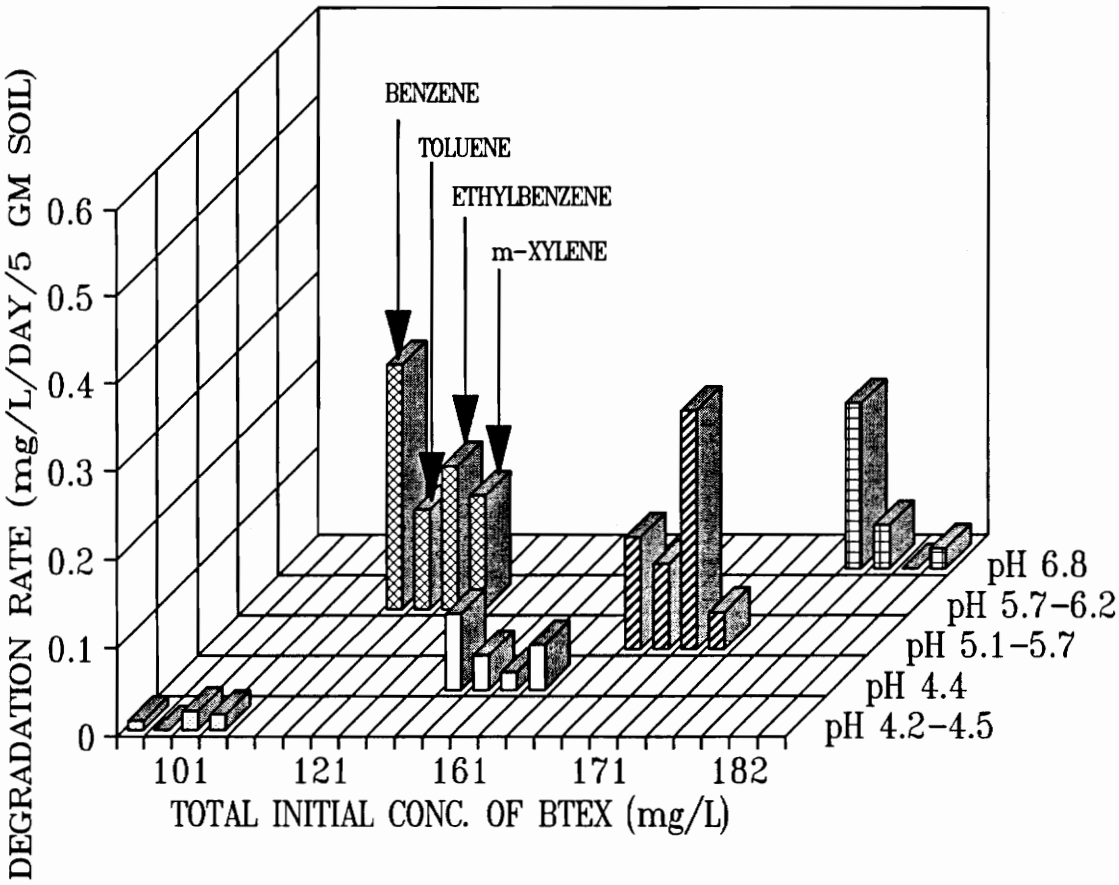


Figure 11. Comparison of BTEX degradation rates in 6 ft soil samples which are under aerobic conditions and have adjusted soil pH. Natural soil pH is 4.5.

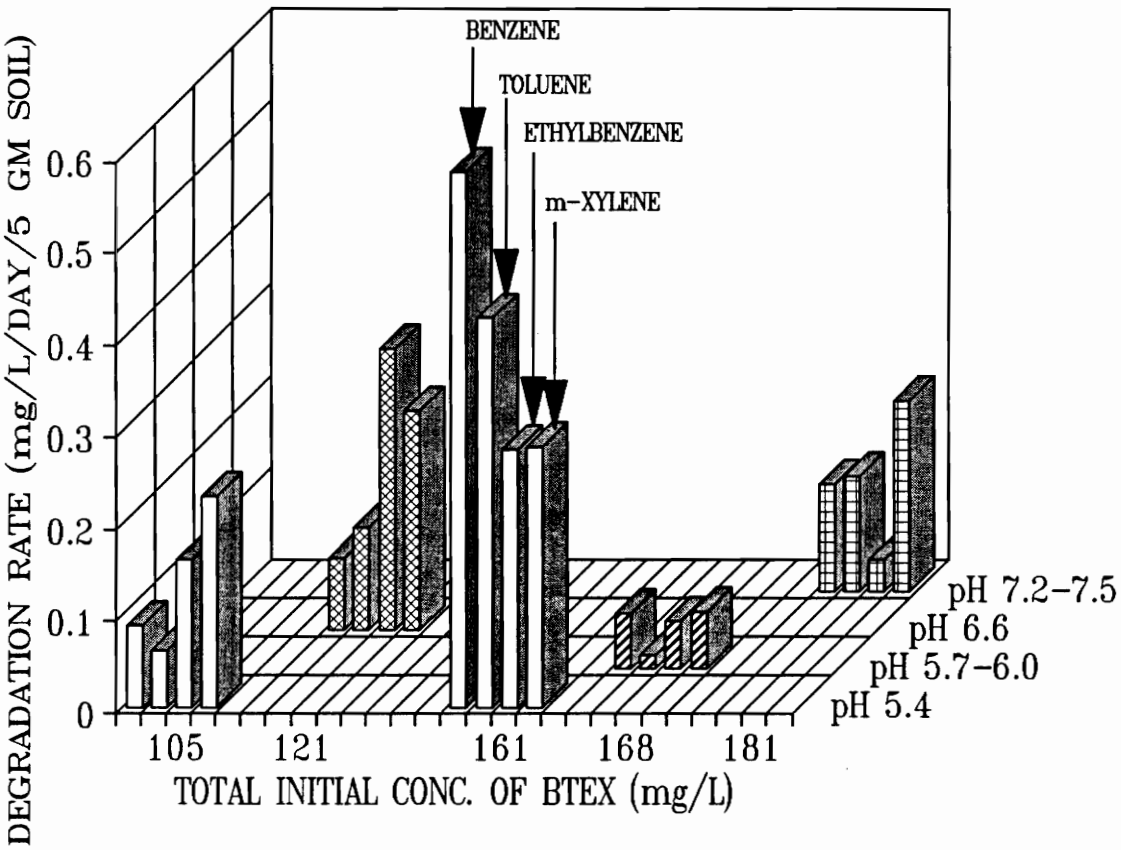


Figure 12. Comparison of BTEX degradation rates in 15 ft soil samples which are under aerobic conditions and have adjusted soil pH. Natural soil pH is 6.5.

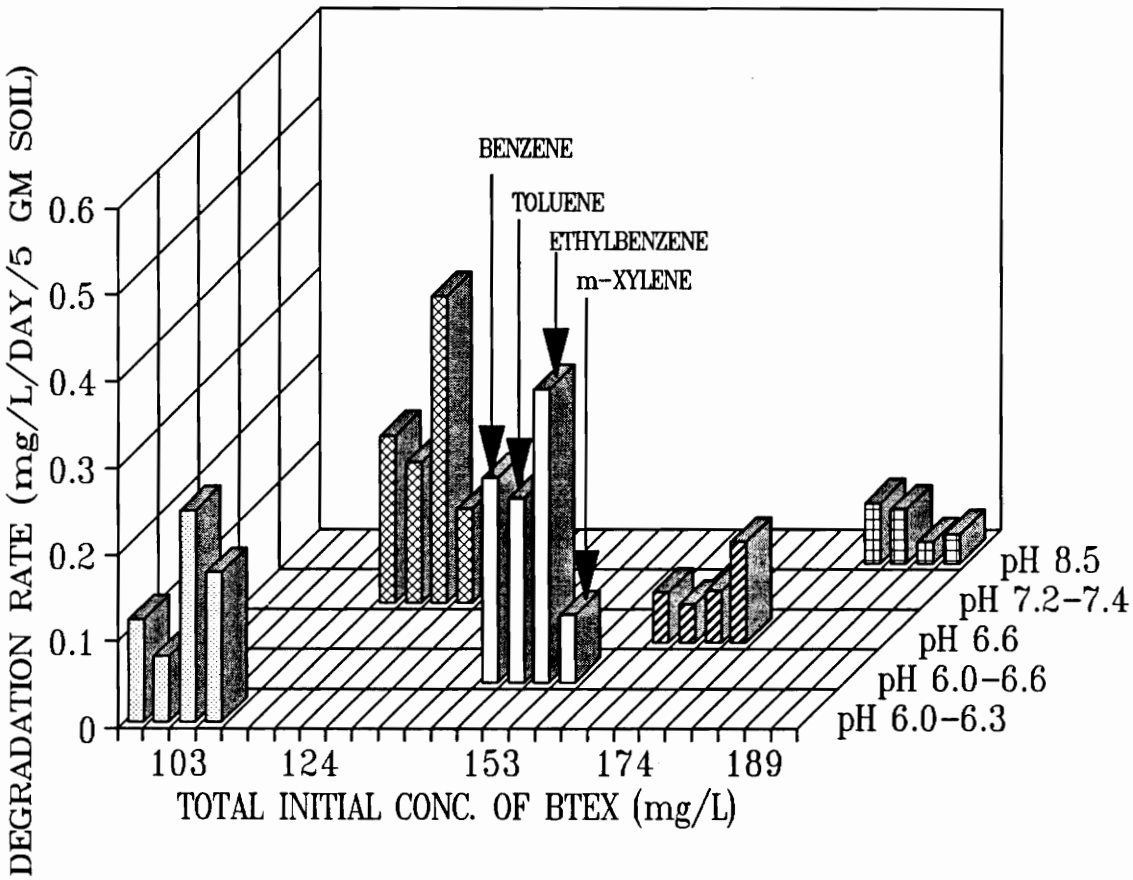


Figure 13. Comparison of BTEX degradation rates in 17 ft soil samples which are under aerobic conditions and have adjusted soil pH. Natural soil pH is 7.5.

soil microcosms have significantly different BTEX degradation rates yet have approximately equal total initial concentrations of BTEX, 161 and 168 mg/L. These microcosms also have virtually equal soil solutions pH values, 5.4 and 5.7 to 6.0. These results suggest that some other factor besides total BTEX concentration may be contributing to low degradation rates in the microcosms.

In figure 14, the maximum combined degradation rate of BTEX for both surface and 3 ft soils is shown to occur in pH range 5.5 to 6.5, the maximum combined degradation rate of BTEX for 6 ft soil is shown to occur in pH range 5.7 to 6.2, the maximum combined degradation rate for 15 ft soil is shown to occur at pH 5.4, and the maximum combined degradation rate of BTEX for 17 ft soil is shown to occur in pH range 6.0 to 6.6. It appears that the pH range in which microbial degradation rates were highest for the entire soil profile occurred between pH 5.4 and 6.6. This optimum pH range falls in the lower end of the 6.0 to 8.5 optimum pH range commonly recommended for maximum biodegradation rates (Atlas and Bartha, 1986; Bremmer and Shaw, 1958; Dupont et al., 1988).

In figure 15a, the degradation rate of the BTEX compounds in the sample which had the maximum combined BTEX degradation rate/optimum pH is shown for each soil depth. In figure 15b, the degradation rate of each of the BTEX

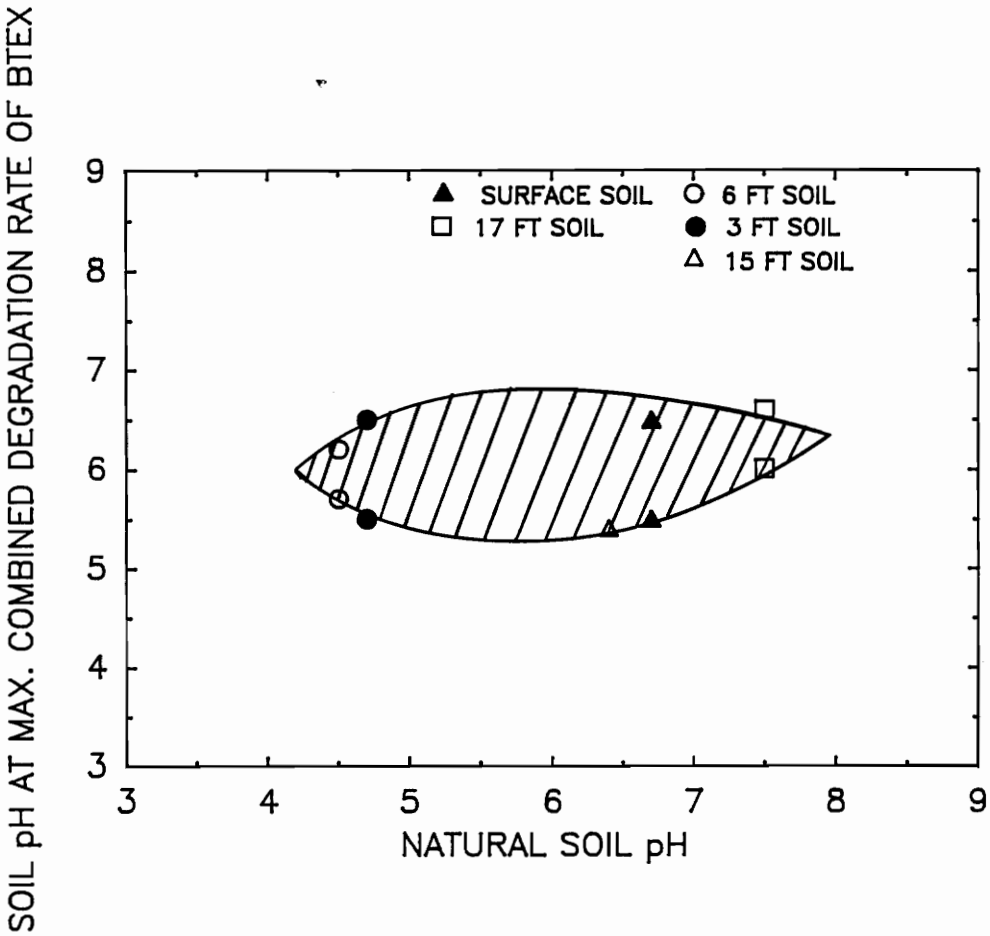


Figure 14. The pH range at which maximum degradation occurred for each soil depth while under aerobic conditions.

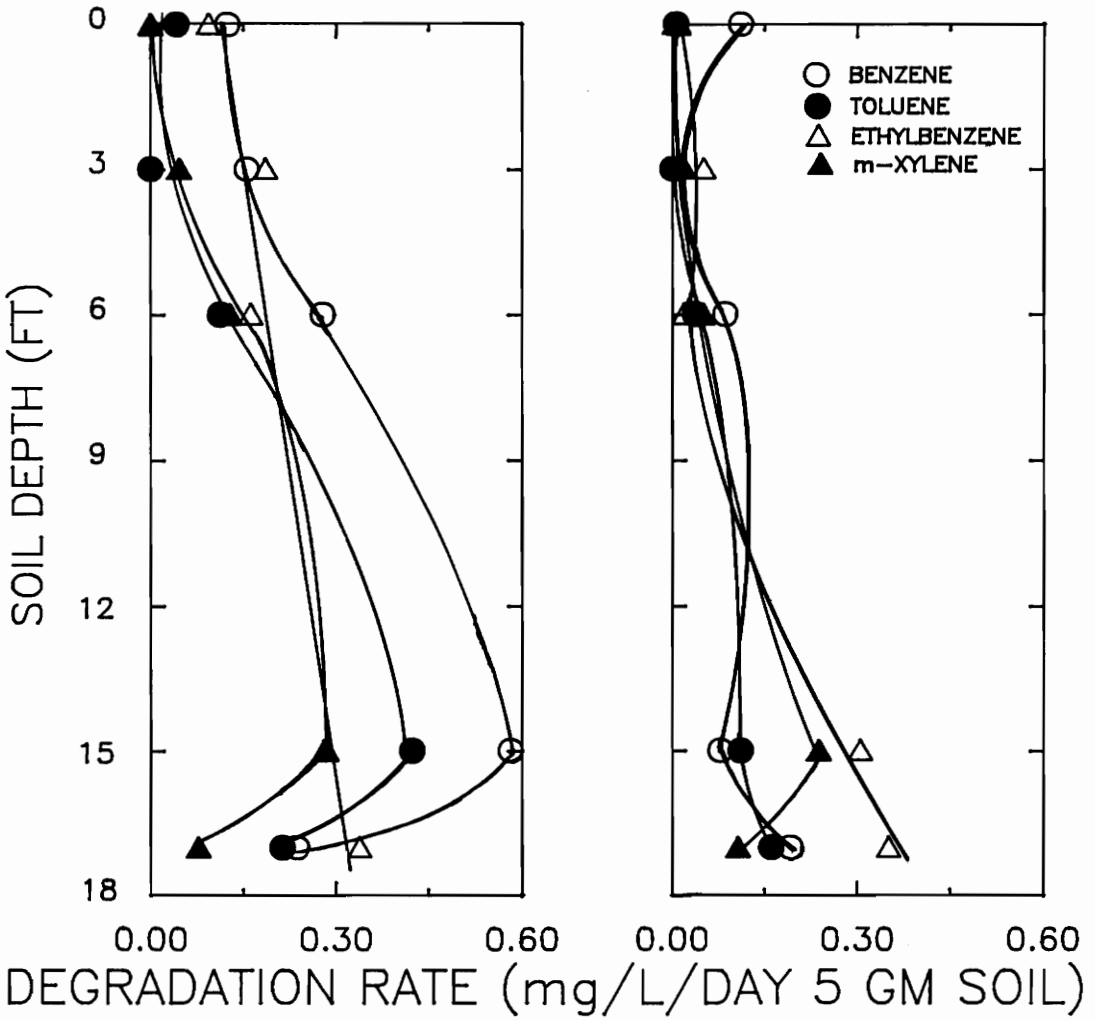


Figure 15a. Maximum degradation rate of BTEX while at optimum pH and under aerobic conditions.

Figure 15b. Degradation rate of BTEX while at natural soil pH and under aerobic conditions

compounds in the soil sample with soil pH equal to the natural soil pH is shown. For each of the soil depths, the degradation rate of benzene was lower at the natural soil pH than at the optimum soil pH. The degradation rate of toluene was lower at the natural soil pH than at the optimum soil pH for all but the 3 ft soil. Toluene degradation was not detected in the 3 ft soil. The surface, 3, and 6 ft soil samples at the natural soil pH had lower ethylbenzene degradation rates than corresponding rates at the optimum soil pH. Whereas, the 15 and 17 ft soil samples at the natural soil pH had higher ethylbenzene degradation rates than those at the optimum soil pH. The 3, 6, and 15 ft soils had lower m-xylene degradation rates at the natural soil pH than at the optimum soil pH. The m-xylene degradation rate in 17 ft soil was higher at the natural soil pH than at the optimum soil pH. Degradation of m-xylene did not occur in the surface soil.

In figure 16a, the maximum combined degradation rate of BTEX for each depth is compared to its respective combined degradation rate of BTEX at the natural soil pH. At each of the soil depths, the degradation rate at the natural soil pH is less than the maximum combined BTEX degradation rate. In figure 16b, the natural soil pH is compared to the pH at which the maximum combined BTEX degradation rate occurred for each soil depth. It appears that the combined

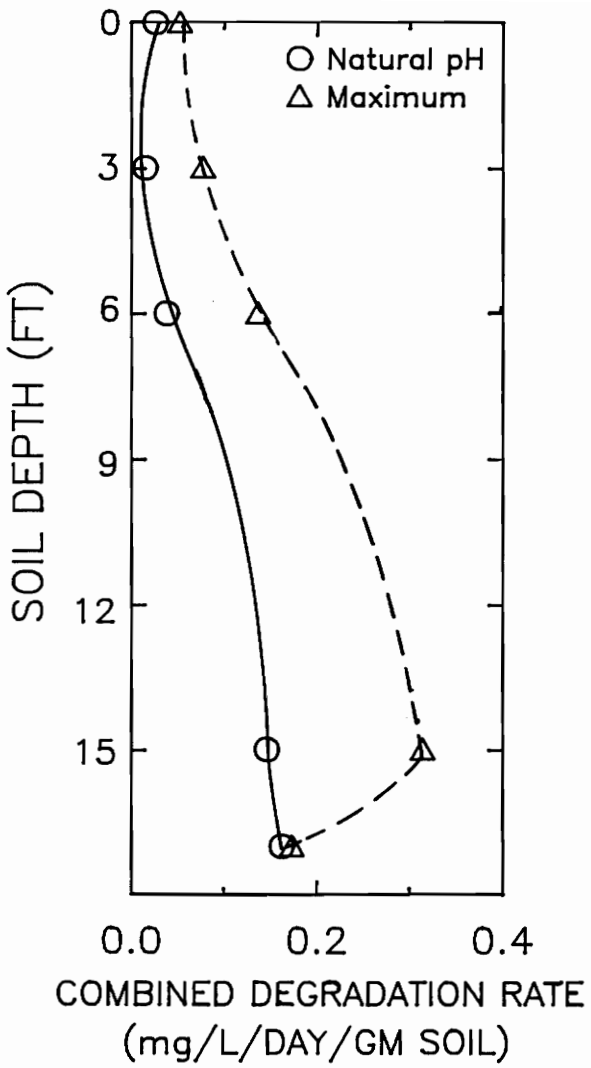


Figure 16a. Soil depth vs. maximum combined degradation rate and combined degradation rate at natural pH.

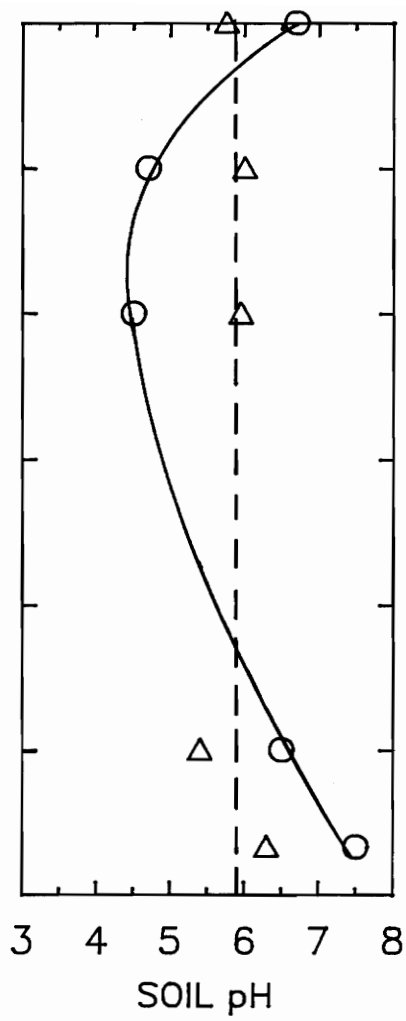


Figure 16b. Soil depth vs. natural pH and adjusted soil pH at maximum degradation.

degradation rate of BTEX in soils at the natural pH is consistently lower than the combined degradation rate of BTEX in soils at soil pH ranging from 5.4 to 6.6.

Hydrocarbon degrading microorganisms may have been inhibited at pH values less than the lower limit of the pH 5.4 to 6.6 range in which maximum degradation rates were observed. Cuthbert et al. (1955) found that pH values of approximately 5.6 and below adversely affected the survival of Bacterium coli. type I and Streptococcus faecalis in the soil. They proposed that pH indirectly inhibited biodegradation by limiting nutrient availability and/or promoting growth of predator organisms. Although the above mentioned bacteria are not recognized as BTEX degraders, it is reasonable to suggest that hydrocarbon degrading bacteria and actinomycetes would react similarly. For example, these microorganisms have been shown to terminate growth when their environment is at approximately pH 5 and below (Gray, 1971). Thus, it is not unlikely to find that both natural soil and adjusted soil pHs below 5.4 have relatively low biodegradation rates.

The relatively low biodegradation rates found in soils outside the optimum pH range 5.4 to 6.6 may be partially attributed to the lack of available inorganic phosphorus. Phosphorus is an essential nutrient used in the generation of ATP (Atlas and Bartha, 1986). It has been suggested that

optimum degradation will occur in environments where the carbon to nitrogen to phosphorus ratio is approximately 120:10:1 (Dupont et al., 1988). Thus, the concentration of phosphorus is crucial for optimum degradation. The phosphorus based nutrient media served as a significant supply of inorganic phosphorus. However, the pH of the soil solution dictates the solubility and subsequent availability of this phosphorus (Brady, 1974). Both acidic soils and alkaline soils frequently have low amounts of soluble phosphorus due to precipitation reactions and/or chemical fixation by hydrous oxides such as hydrous iron and aluminum (Brady, 1974). It has been suggested that soils in the pH 6.0 to 7.0 range have the highest amount of available phosphorus (Brady, 1974; Dupont et al., 1988). However, the pH range of optimum phosphorus solubility varies somewhat depending upon what type of phosphorous containing mineral is present in the soil (Brady, 1974). Thus, it is possible that soil pH ranging from 5.4 to 6.6 could yield the appropriate amount of soluble phosphorus such that the 120:10:1 nutrient ratio was satisfied. Therefore, it may not be unreasonable to find that both natural soil pHs and adjusted soil pHs lower than 5.4 and higher than 6.6 experienced relatively low biodegradation rates.

4.5.1 Inhibited Degradation in Surface Soils.

As is shown in Figure 16a, surface soil microcosms

under aerobic conditions had the lowest maximum net BTEX degradation rate when compared to the maximum rates of the other soil depths. Low microbial activity in surface soils is somewhat unusual since aerobic biodegradation studies often find greater microbial activity in surface horizons than in underlying soil horizons (Gullic, 1990; Hickman, 1988). However, this rather unexpected result may be explained in the following manner. Surface soils naturally receive an influx of water with higher concentrations of both oxygen and organic materials than do underlying soil horizons (Hickman, 1988). Thus, the aerobic microbial population existing in the surface soil acclimate to the most readily degradable organic compounds and leave the more refractory compounds to be further transported through underlying soil horizons (Hickman, 1988). Consequently, the microbial community in the subsurface soils have acclimated to those organics which are not biodegraded as easily (Hickman, 1988).

This study also found that benzene degradation rates in surface soil, although low, were nevertheless greater than degradation rates for toluene, ethylbenzene, and m-xylene. Gibson et al. (1968) found that aerobic biodegradation of benzenoid compounds by Pseudomonas putida was hindered by those compounds with relatively large alkyl substituents. A study by Mueller et al. (1991) found that aerobic, surface

soil microorganisms degraded phenol more readily than more complicated chemical structures such as heterocyclics, PAHs, and PCP. These researchers attributed the rapid biodegradation of phenol to its higher aqueous solubility and relatively simple chemical structure. Thus, it is possible that the relatively high aqueous solubility and lack of functional groups on the benzene ring allows for a simpler degradation pathway for benzene than for toluene, ethylbenzene, and m-xylene. Thus, the microbial community in the surface soil may be more acclimated to the relatively simple chemical structure of benzene, rendering the benzene degradation rate higher than the degradation rate of the other compounds in the surface soil.

In addition, as shown in Figure 16a, there is a direct relationship between the maximum combined degradation rate of BTEX and increasing soil depth, with the exception of 17 ft soil. This trend suggests that the microorganisms at progressively greater soil depths may become more acclimated to recalcitrant organic compounds with higher molecular weights and lower water solubilities. The 3 and 6 ft soils have the highest clay content and relatively high moisture contents. The combination of these soil characteristics may indicate that there is a low flux of water and soluble organics in these soils. In addition, most bacteria in low flow clay soils are attached, thus they are less likely to

come in contact with soluble organic material. Even though populations of attached bacteria are generally larger than populations of unattached bacteria, the former micoorganisms have comparatively low activities (Arvin et al., 1988). Consequently, microbes in clayey 3 and 6 ft soils may require relatively long acclimation periods and may also have slower degradation rates (Gullic, 1990). It is possible that both hydraulic conditions and numbers of bacteria are factors which affect biodegradation (Arvin et al., 1988)

The highest combined degradation rate of BTEX occurs in the soil which also has the highest natural moisture content. However, since this 15 ft soil also has a low clay content and relatively high sand and silt contents, it may be inferred that this soil does not retain water over an extended period as higher clay soils might. The 15 ft soil may retain water and soluble organics in periods of heavy precipitation, thereby allowing microorganisms to acclimate to the organics. Thus, the relationship between high moisture content and low clay content may indicate that microorganisms in this 15 ft soil have a history of acclimating to and then substantially degrading the majority of the organic compounds which infiltrate to this depth. The relatively low soil moisture content found in the 17 ft soil may indicate that this soil received a lower influx of

water and soluble compounds than did the 15 ft soil. The low moisture content in addition to the high sand and silt contents in the 17 ft soil may indicate that water and soluble organics flowed rapidly through this strata. As a result, the microorganisms in the 17 ft soil may not have had a sufficient opportunity to acclimate to the remaining organic compounds which infiltrated to this depth. As a consequence of not having a history of acclimation and subsequent degradation, the 17 ft soil has a lower net BTEX degradation rate than the overlying 15 ft soil.

4.5.2 Potential m-Xylene Inhibition.

As was shown in figure 8, the 15 and 17 ft soil microcosms with virtually equal pH values exhibited significantly different combined degradation rates of BTEX. In addition, it is shown in figures 12 and 13 that relatively high total BTEX concentrations do not always directly correlate with relatively low degradation rates. As a result, the effect of individual compounds on degradation rates was considered. These results suggest the possibility of inhibition and upon further inspection, m-xylene inhibition seemed to be a possibility. Unlike m-xylene, the concentration of benzene, toluene, and ethylbenzene did not show a correlation with low BTEX degradation rates.

From figures 17 and 18, it may be inferred that

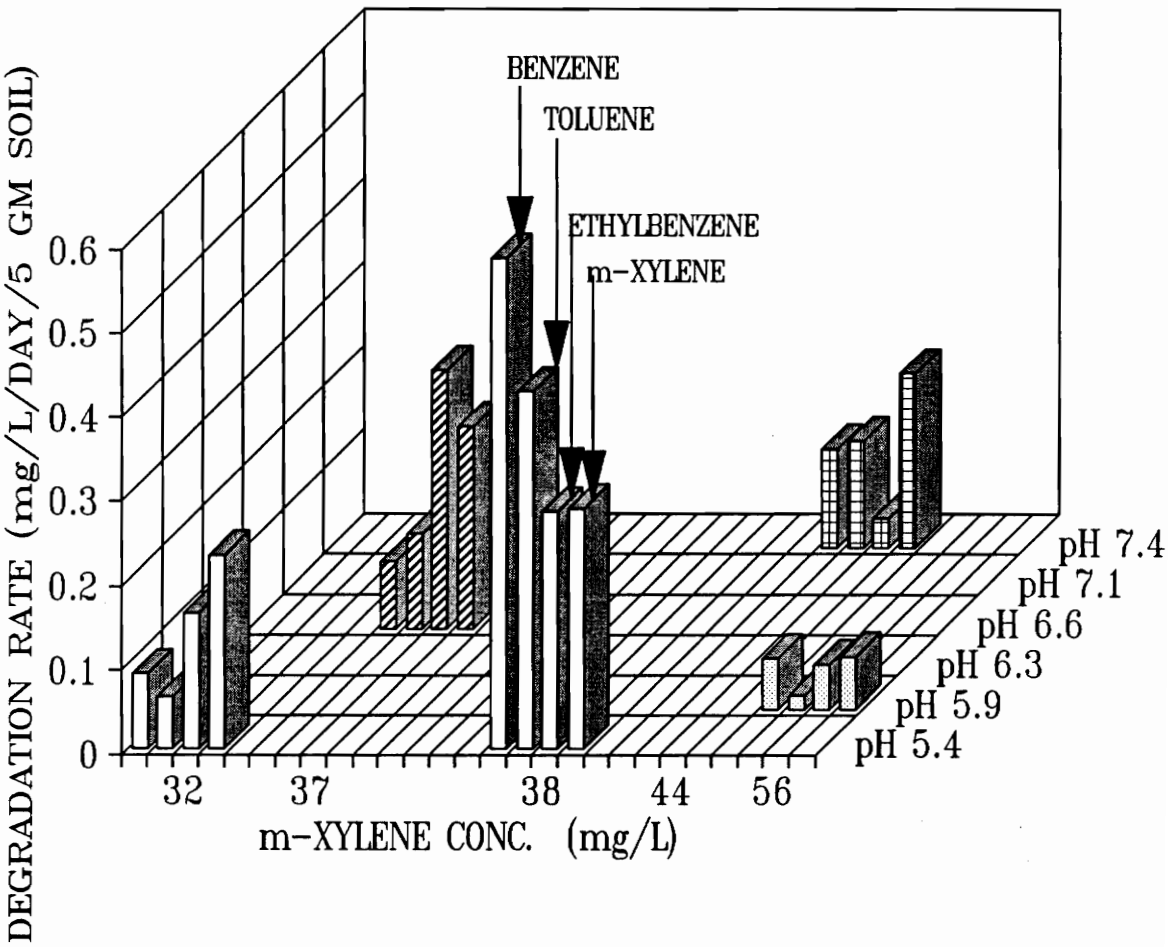


Figure 17. Effect of m-xylene on degradation of BTEX in 15 ft soil samples.

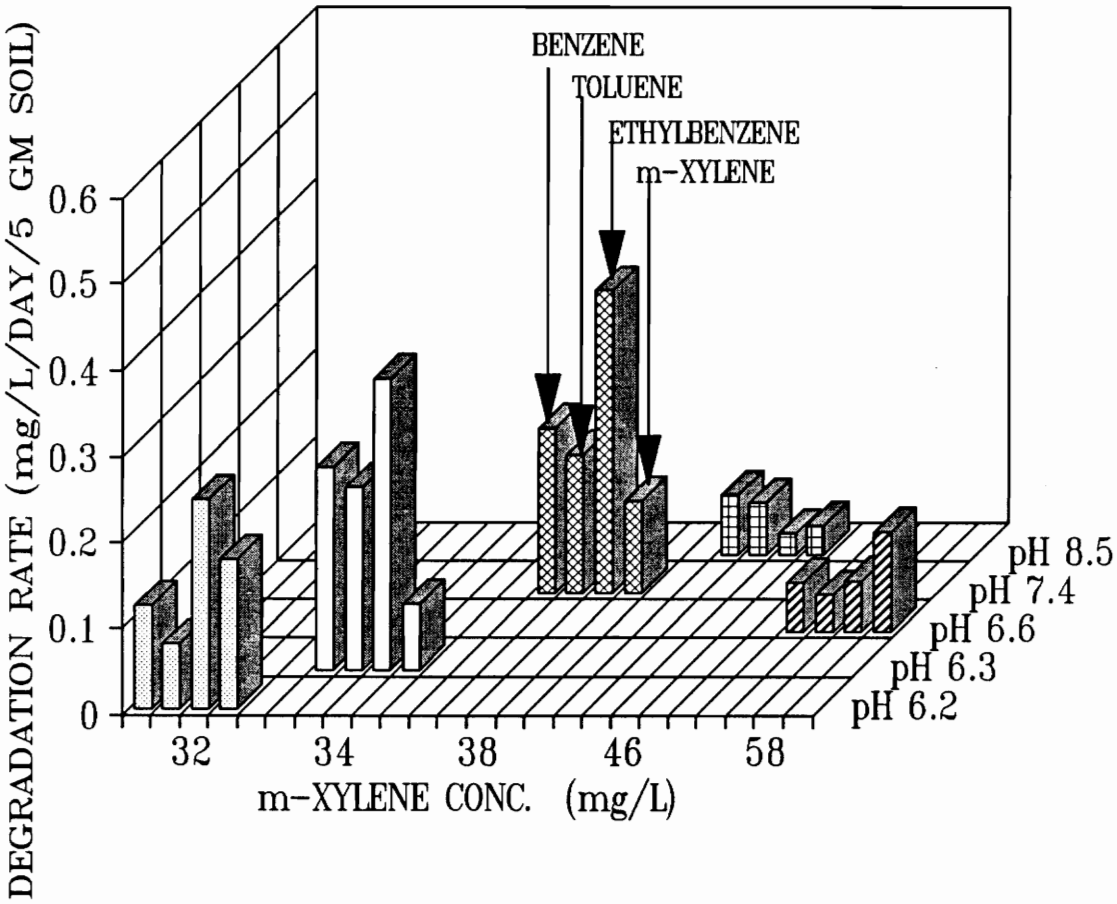


Figure 18. Effect of m-xylene on degradation of BTEX in 17 ft soil samples.

relatively high m-xylene concentrations lowered degradation activity in both 15 and 17 ft soils. A concentration of 56 mg/L of m-xylene was present in the 15 ft soil sample which showed lowered degradation rates and 58 mg/L of m-xylene was present in the 17 ft soil sample which also experienced lowered degradation rates. The m-xylene concentrations in the 15 and 17 ft soil samples which experienced the highest combined degradation rate of BTEX were 38 and 34 mg/L respectively. The pH range of the 15 and 17 ft samples with high m-xylene concentrations and low degradation rates were 5.7 to 6.9 and 6.6 respectively, and both samples were in the pH range at which maximum degradation occurred in this soil profile. The pH values were virtually equal in both the sample with the relatively low m-xylene concentration and corresponding highest combined degradation rate and the sample with the highest m-xylene concentration and corresponding low combined degradation rate. Thus, it appears that it may not be the pH of the soil, but perhaps the high m-xylene concentration which caused the reduced BTEX degradation rates in the 15 and 17 ft soils.

Researchers have yet to find the precise concentration at which the BTEX compounds become toxic to microorganisms. However, the disinfecting properties of toluene have been known for many years (Bartha and Atlas, 1977). Studies show that high concentrations of petroleum products, such as

xylene, can cause inhibitory biological effects. In one such study, the algal population died as a result of high concentrations of xylene (Kauss et al, 1973). The extent of microbial inhibition is dependent upon the concentration and solubility of individual petroleum components (Calder and Lader, 1976).

4.5.3 Sequential Substrate Utilization.

In figures 19 and 20, sequential substrate utilization characterized by complete benzene utilization followed by m-xylene utilization is shown. This type of response was observed only in those 3 and 6 ft soil samples which experienced the maximum combined degradation rate of BTEX. The benzene concentration in the other 3 and 6 ft soil samples never became equal to or less than the GC detection limit.

Only after the concentration of benzene became less than the GC detection limit did the concentration of m-xylene start to decrease in those samples showing sequential substrate utilization. This response suggests diauxic growth may be occurring between bacteria which metabolize benzene and m-xylene in both the 3 and 6 ft soils. In diauxic growth, catabolite repression results when organisms which metabolize the first substrate inhibit those enzymes and their related enzyme systems which metabolize the second substrate (Harder and Dijkhuizen, 1989). Following complete

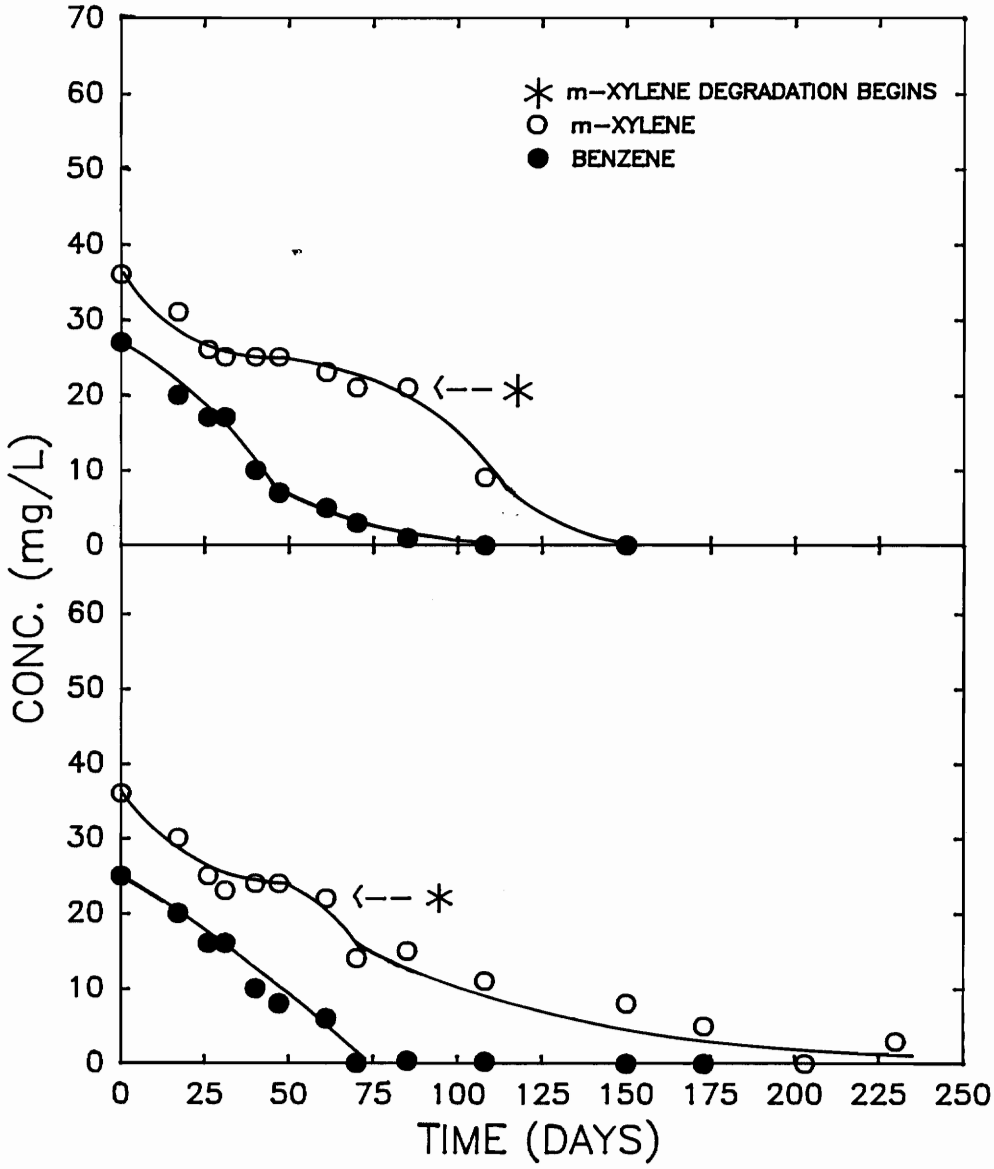


Figure 19. Sequential utilization of first benzene and then m-xylene in duplicate aerobic 3 ft soil samples.

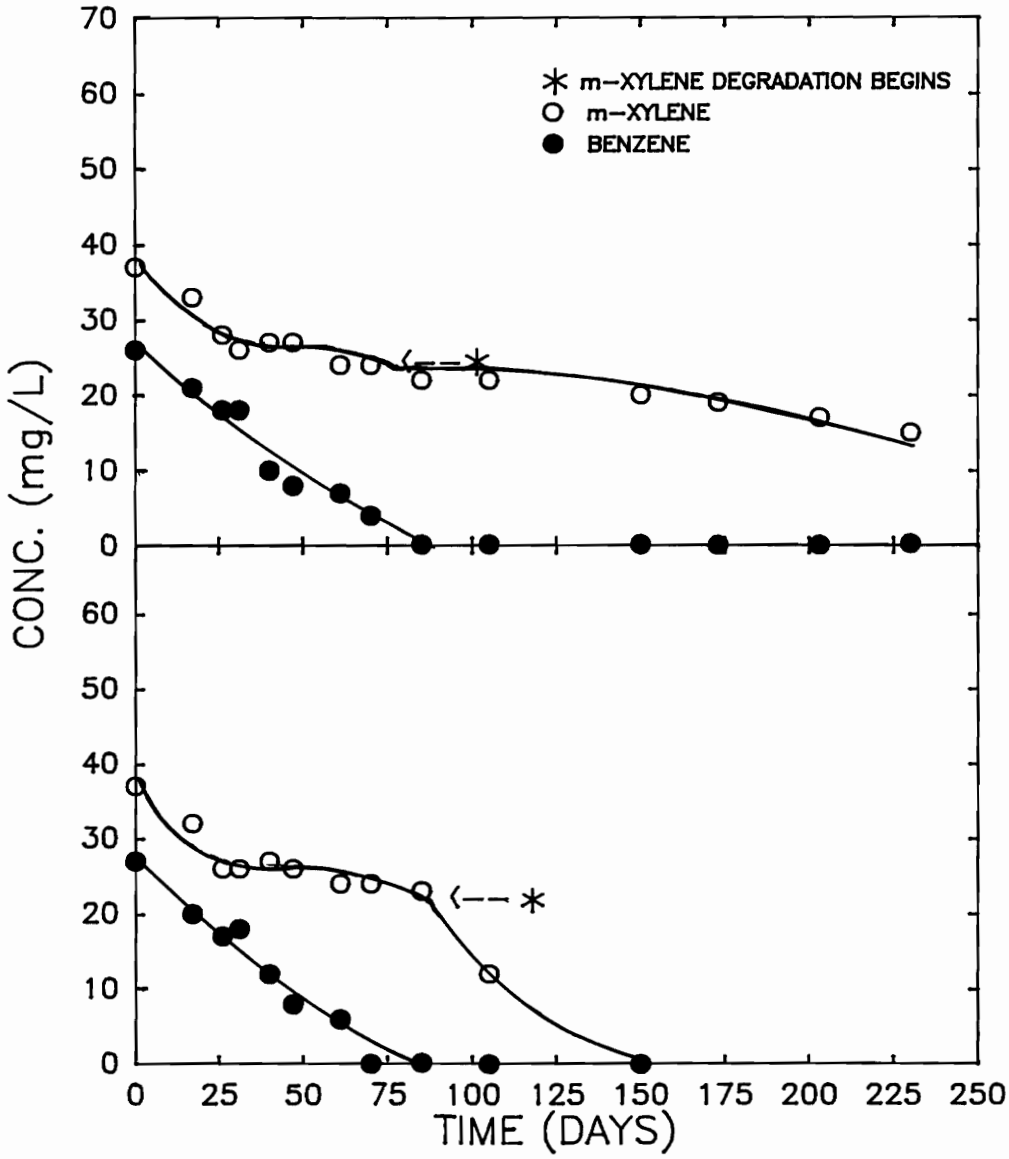


Figure 20. Sequential utilization of benzene first and then of m-xylene in duplicate aerobic 6 ft soil samples.

metabolism of the first substrate, enzyme inhibition is terminated, and after a lag phase of variable length all of the cells begin degrading the second substrate (Harder and Dijkhuizen, 1989).

Sequential utilization in mixed substrate environments does not always result in diauxic growth (Harder and Dijkhuizen, 1982). In fact, microorganisms and their corresponding enzymes which are needed to metabolize the second substrate may be present and uninhibited, however sequential substrate utilization could still occur (Harder and Dijkhuizen, 1982).

4.5.4 Differences in Microbial Responses.

The physical and chemical properties of soils can affect a variety of microbial properties. Walker and Colwell (1975) found that microbial populations from two different soil environments, responded differently when exposed to the same petroleum hydrocarbons; one of the microflora populations experienced inhibited growth, while the other experienced enhanced growth rates. In addition, Gullic (1990) found that when under similar environmental conditions, soils with the highest clay contents experienced lower toluene degradation rates than did sandy soils. Thus, different microbial populations may exist in different types of soils, thereby contributing to the variety of responses found in these soils.

Gullic's results were similar to those in this study in that soils with similar clay contents responded differently than did soils with similar sand and silt contents. For instance, with respect to potential m-xylene inhibition, only the 15 and 17 ft soils showed signs of inhibition. Microbial systems did not appear to be inhibited in surface, 3, and 6 ft soil microcosms which also had relatively high m-xylene concentrations. The quantity of clay, sand, and silt is different for all five soil depths studied and hence may affect the quantity and type of microorganisms present in these soils. However, the 15 and 17 ft soils had the most similar sand, silt, and clay contents. Thus, it is possible that similar types of microorganisms existed in these two soils, but they may not have been prevalent in the surface, 3, and 6 ft soils.

The occurrence of diauxic growth in 3 and 6 ft soils and not in surface, 15, and 17 ft soils may also be due to incongruous microbial populations. Different types of bacteria do not always have similar reactions to the same combination of substrates (Harder and Dijkhuizen, 1989). Thus, it is possible that surface, 15, and 17 ft soils have a microbial population which differs from those in 3 and 6 ft soils.

Sequential utilization in 3 and 6 ft soils may be attributed to the possibility that microorganisms present in

these soils may have a lower overall energy level than microorganisms in surface, 15, and 17 ft soils. As was mentioned earlier, the strong ionic strength of clay tends to cause nutrients to adsorb to its surface. Hence microorganisns must carry out biodegradation with only a limited supply of available nutrients or use energy to desorb the nutrients and then carry out biodegradation. However, the nutrient limitations of the former option would limit the amount of biodegradation that could occur. In addition, that energy used in the desorption process is no longer available for the metabolism of BTEX, thus less biodegradation can be conducted. The lower net energy levels of the microorganisms in 3 and 6 ft soils may render them less capable of degrading the BTEX compounds all at once; thus sequential utilization was observed in these clayey soils. The surface, 15, and 17 ft soils had lower clay contents and may have been better able to sustain concomitant degradation of BTEX compounds.

4.6 Nitrite Accumulation In Samples Under Denitrifying Conditions.

The degradation rates of BTEX compounds under denitrifying conditions were not statistically different from corresponding losses in the sterile controls. Thus, it appears that degradation of BTEX compounds did not occur in the soils under denitrifying conditions. The lack of BTEX

degradation under denitrifying conditions may be the result of nitrite accumulation in the microcosm. A study by Rake and Eagon (1980) revealed that the inhibitory effect of nitrite resulted in certain bacteria being less efficient at using substrate to synthesize ATP and generate biomass. This may ultimately slow substrate biodegradation rates. Mulheren (1985) found that 5 mg/L nitrite slowed and eventually inhibited methanol biodegradation in closed microcosm systems. However, Rake and Eagon (1980) found relatively high nitrite levels caused complete inhibition, which in turn terminated microbial growth and subsequent biodegradation. Figure 21 shows the nitrite level in each of the samples which experienced maximum combined degradation rates. It may be possible that the nitrite levels measured in the samples were high enough to cause complete inhibition in the microcosms.

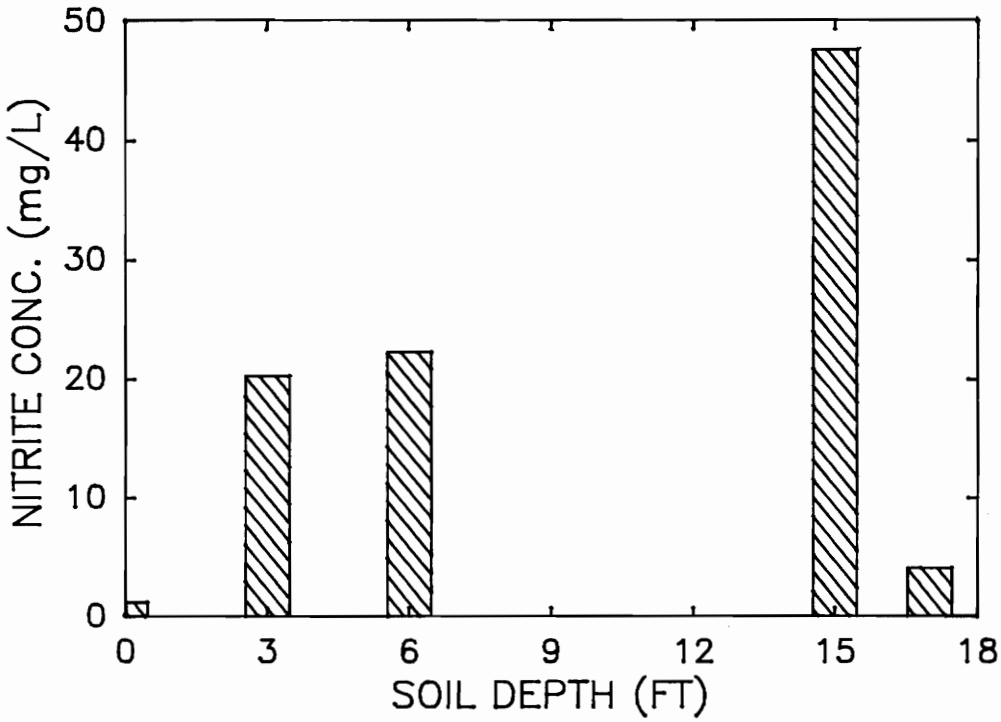


Figure 21. Concentration of nitrite in each soil sample which experienced the maximum combined degradation rate of BTEX.

Chapter 5

CONCLUSIONS

Soil pH partially reflects chemical and physical properties of soil. These soil characteristics are thought to affect the biodegradation potential of soils. Batch soil microcosms containing indigenous microorganisms were used to examine the relationship between soil solution pH and BTEX biodegradation potential. In addition, two separate systems were constructed, one used H_2O_2 as an electron acceptor and the other used NO_3 as an electron acceptor. In this way, the suitability of both electron acceptors during BTEX biodegradation was investigated. At each soil depth, the maximum combined degradation rate of BTEX occurring under aerobic conditions was identified. Of each soil within the entire soil profile, the soil with the highest moisture content and relatively low clay content appeared to support an optimum combined degradation rate of BTEX. The degradation rates of BTEX in each soil under denitrifying conditions were not statistically different from the abiotic BTEX losses which occurred in the sterile controls.

A range of soil pHs was created for each soil. This enabled the determination of BTEX biodegradation rates at different soil pHs. In this way, the soil pH which exhibited the maximum combined degradation rate of BTEX was

identified. The natural soil pH was not the optimum soil pH for the maximum combined degradation rate of BTEX under aerobic conditions. It appeared that BTEX degradation under aerobic conditions was at a maximum in slightly acidic soils.

The following specific conclusions were determined based on the results from this thesis:

1) Aerobic conditions utilizing H_2O_2 as a terminal electron acceptor and oxygen source provide a more suitable environment for BTEX degradation than do denitrifying conditions utilizing NO_3 as an electron acceptor.

2) The natural soil pH did not always provide optimum conditions for BTEX degradation when the soils employed in this study were in an aerobic environment.

3) The microbial population in the 15 ft soil exhibited the highest maximum combined degradation rate of BTEX when compared to degradation rates in the other aerobic soils.

4) Of the soil solution pH ranges established at each soil depth, microcosms in pH range 5.4 to 6.6 experienced the highest combined degradation rate of BTEX. Thereby

suggesting that BTEX degrading microorganisms under aerobic conditions function better in a soil environment at this optimum range rather than in a soil environment at a pH outside of this range.

5) Sequential substrate utilization occurred only in those aerobic soils with high clay contents (3 and 6 ft soils). Benzene was the primary substrate and m-xylene served as the secondary substrate.

6) Potential m-xylene inhibition occurred in 15 and 17 ft soils.

7) Soils with similar sand, silt, and clay contents exhibited similar trends. This may indicate that similar microbial communities exist in soils with similar characteristics.

Chapter 6

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APPENDIX A

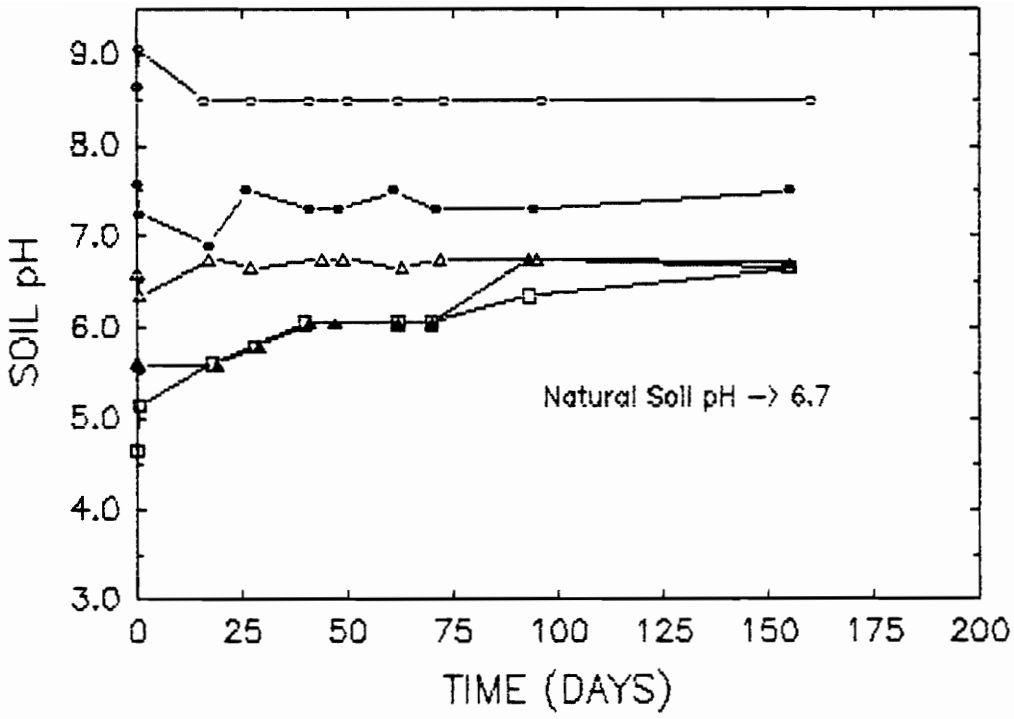

Figure A-1. The pH equilibria attained in aerobic, surface soils with adjusted pH.

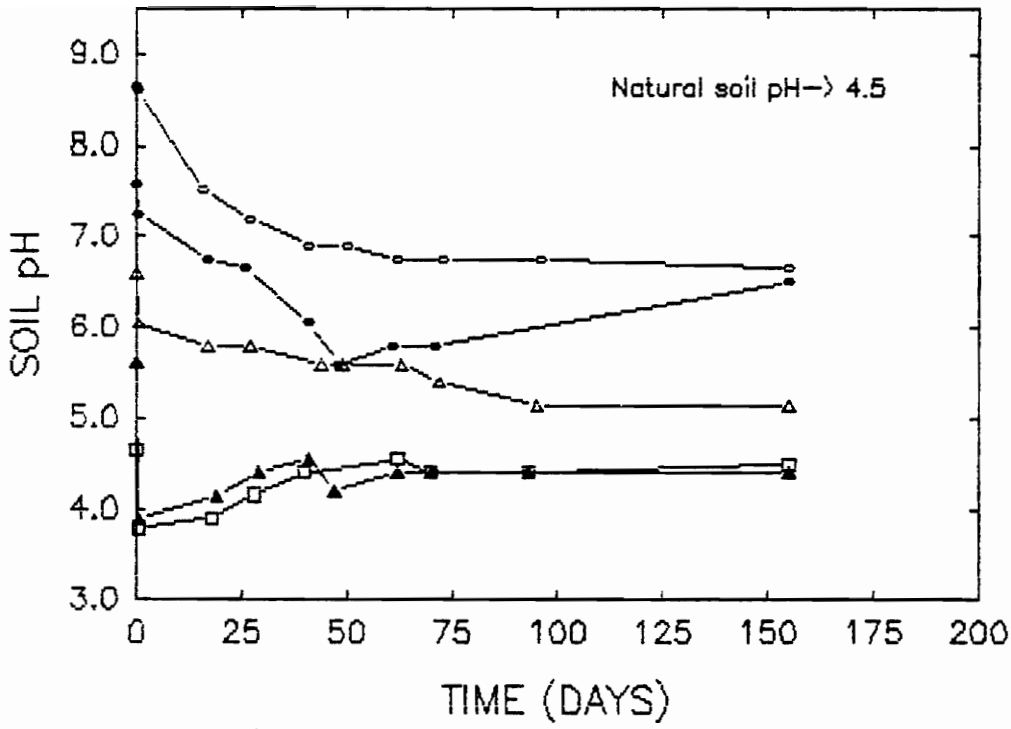

Figure A–2. The pH equilibria attained in aerobic, 6 ft soil with adjusted pH.

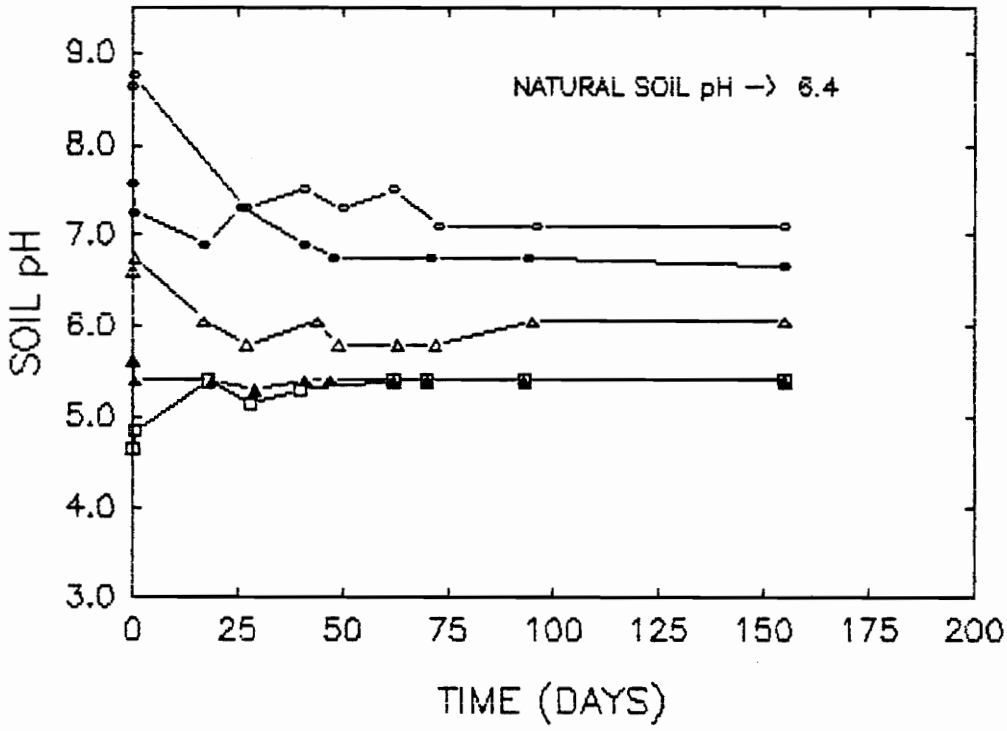

Figure A–3. The pH equilibria attained in aerobic, 15 ft soils with adjusted soil pH.

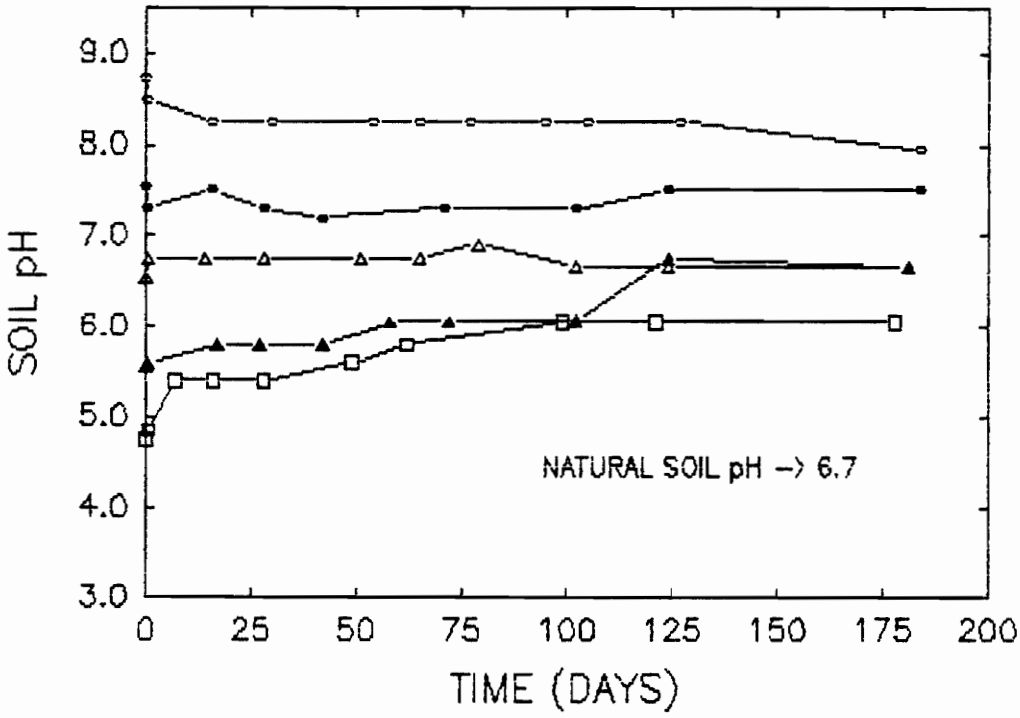

Figure A-4. The pH equilibria attained in surface soil under denitrifying conditions and with adjusted soil pH.

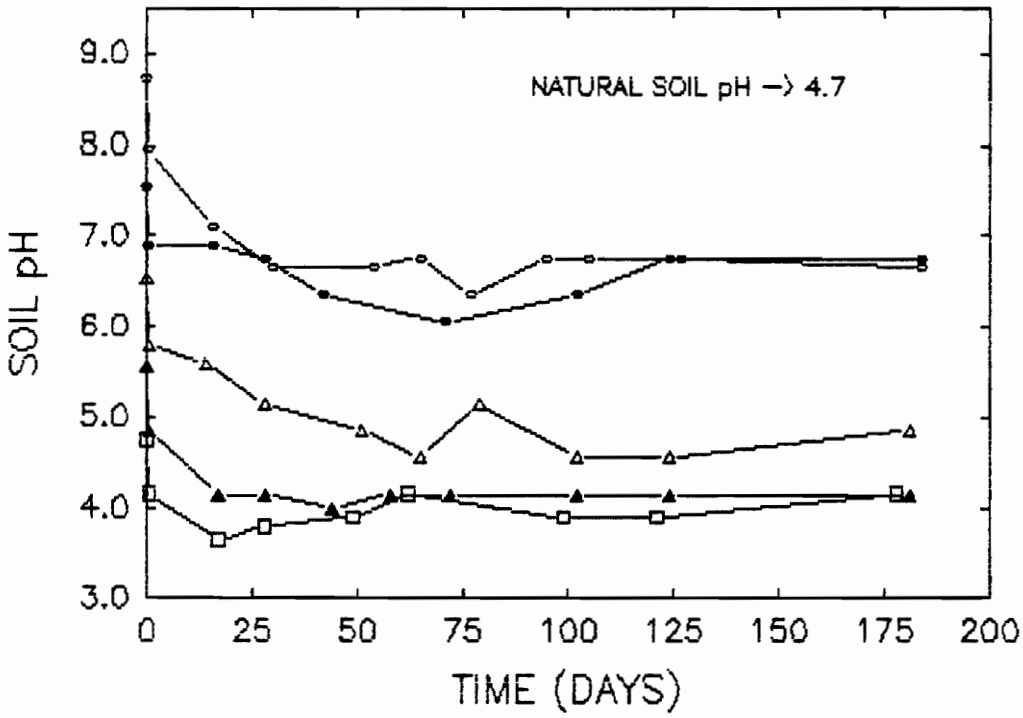

Figure A-5. The pH equilibria attained in 3 ft soil under denitrifying conditions and with adjusted soil pH.

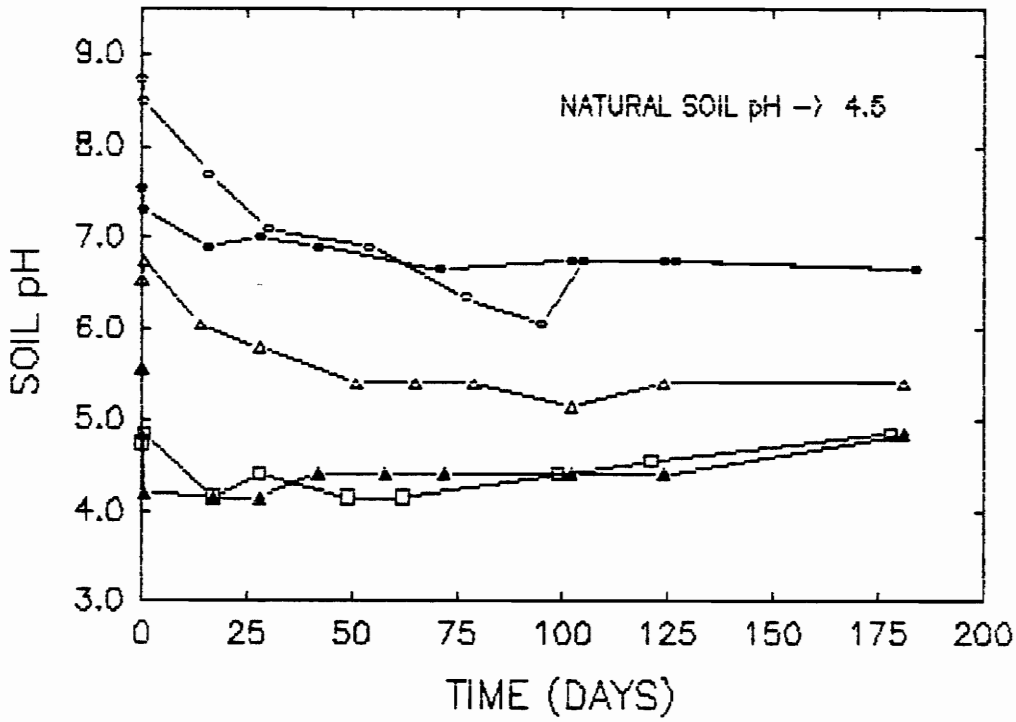

Figure A-6. The pH equilibria attained in 6 ft soil under denitrifying conditions and with adjusted soil pH.

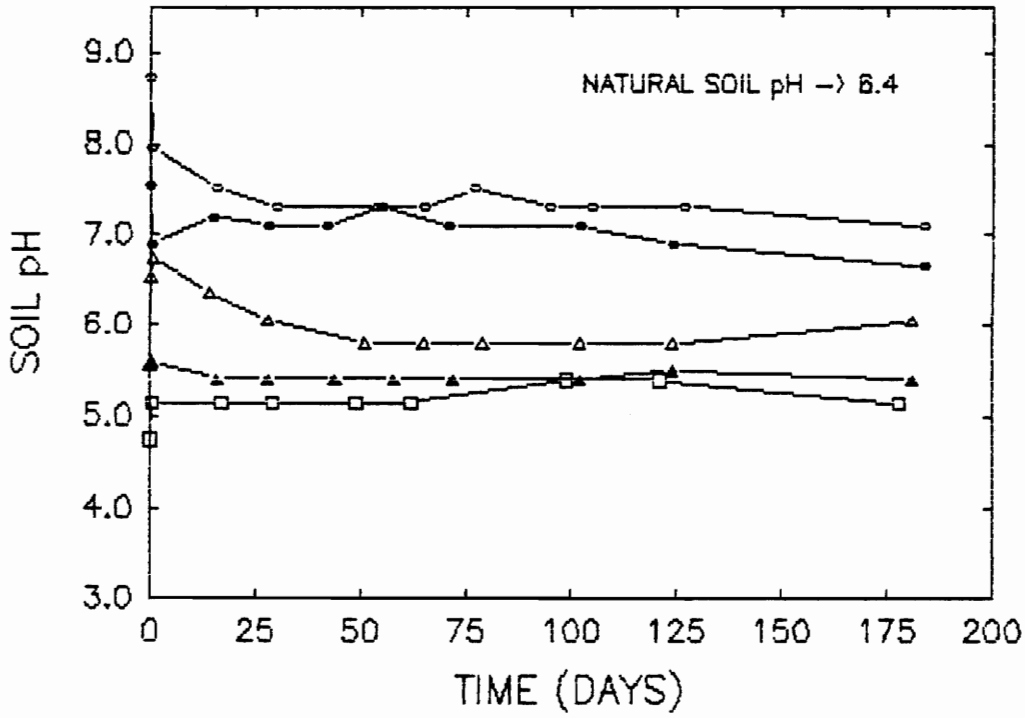

Figure A–7. The pH equilibria attained in 15 ft soil under denitrifying conditions and with adjusted soil pH.

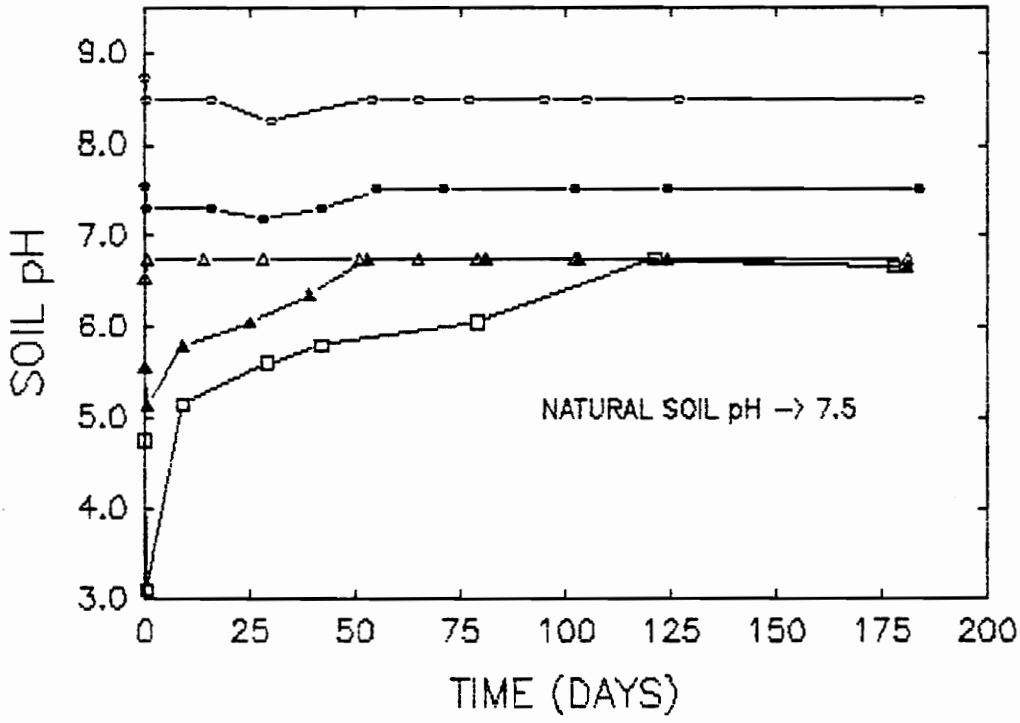

Figure A–8. The pH equilibria attained in 17 ft soils under denitrifying conditions and with adjusted soil pH

APPENDIX B

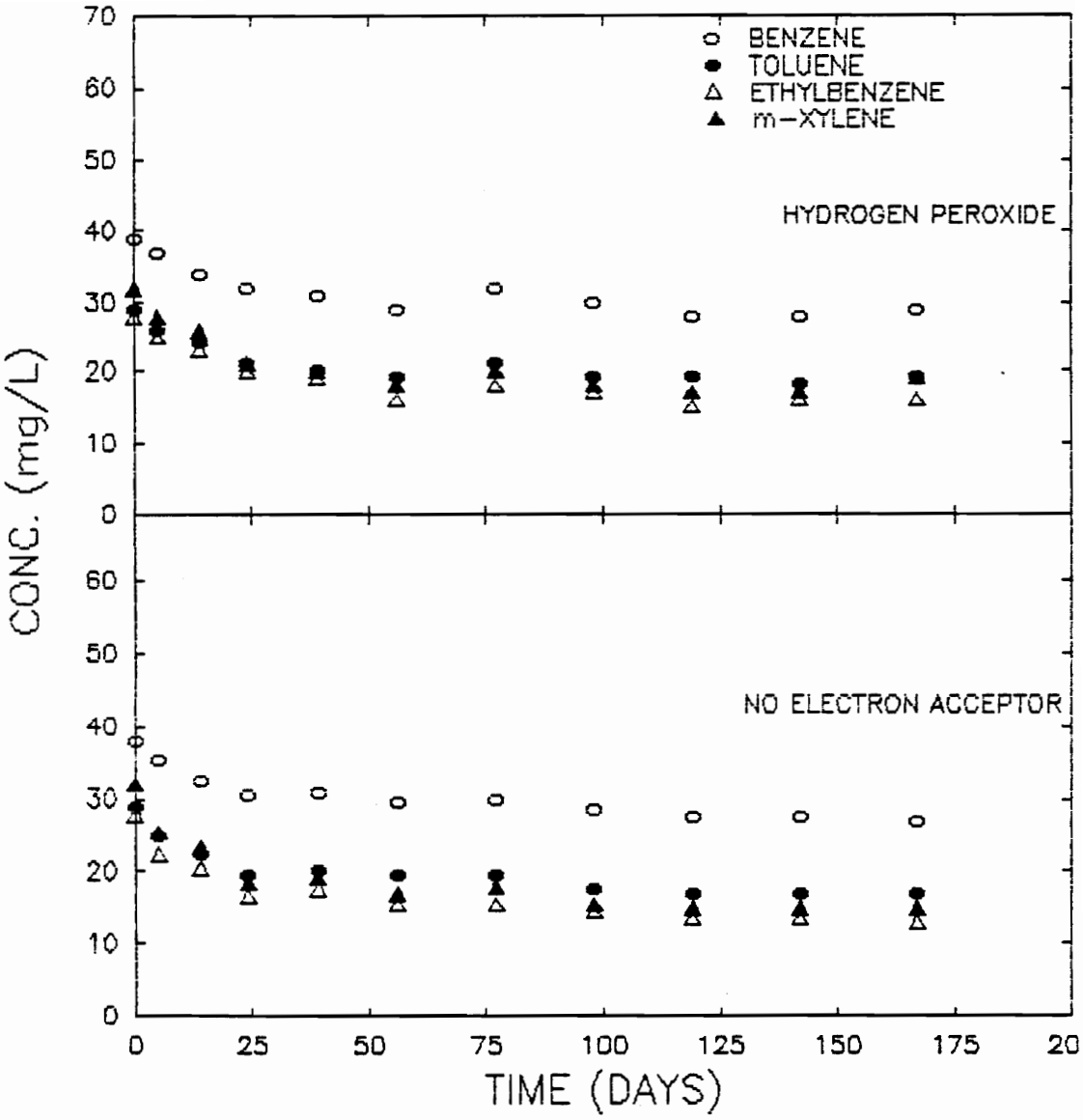

Figure B-1. Abiotic losses in sterile surface soil with hydrogen peroxide as electron acceptor (top) and with no electron acceptor (bottom).

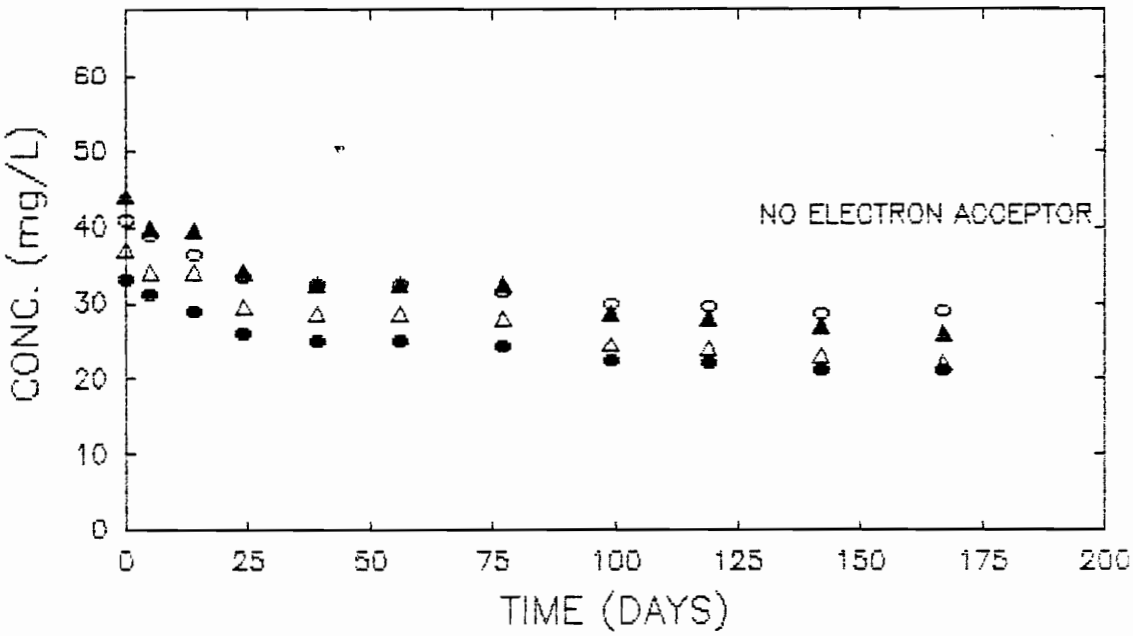

Figure B–2. Abiotic losses in sterile 3 ft soil with no electron acceptor.

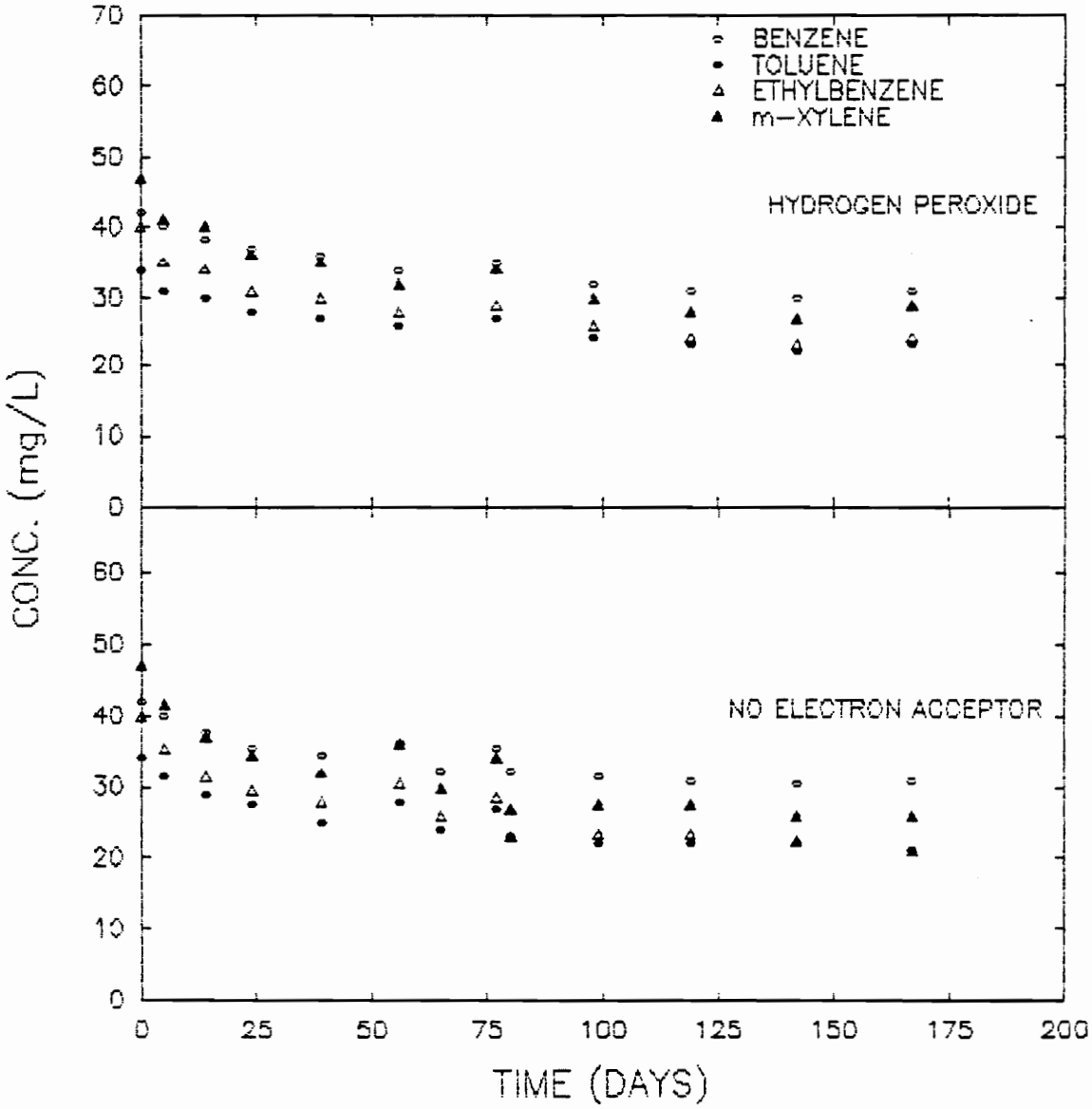

Figure B-3. Abiotic losses in sterile 6 ft soil with hydrogen peroxide as electron acceptor (top) and with no electron acceptor (bottom).

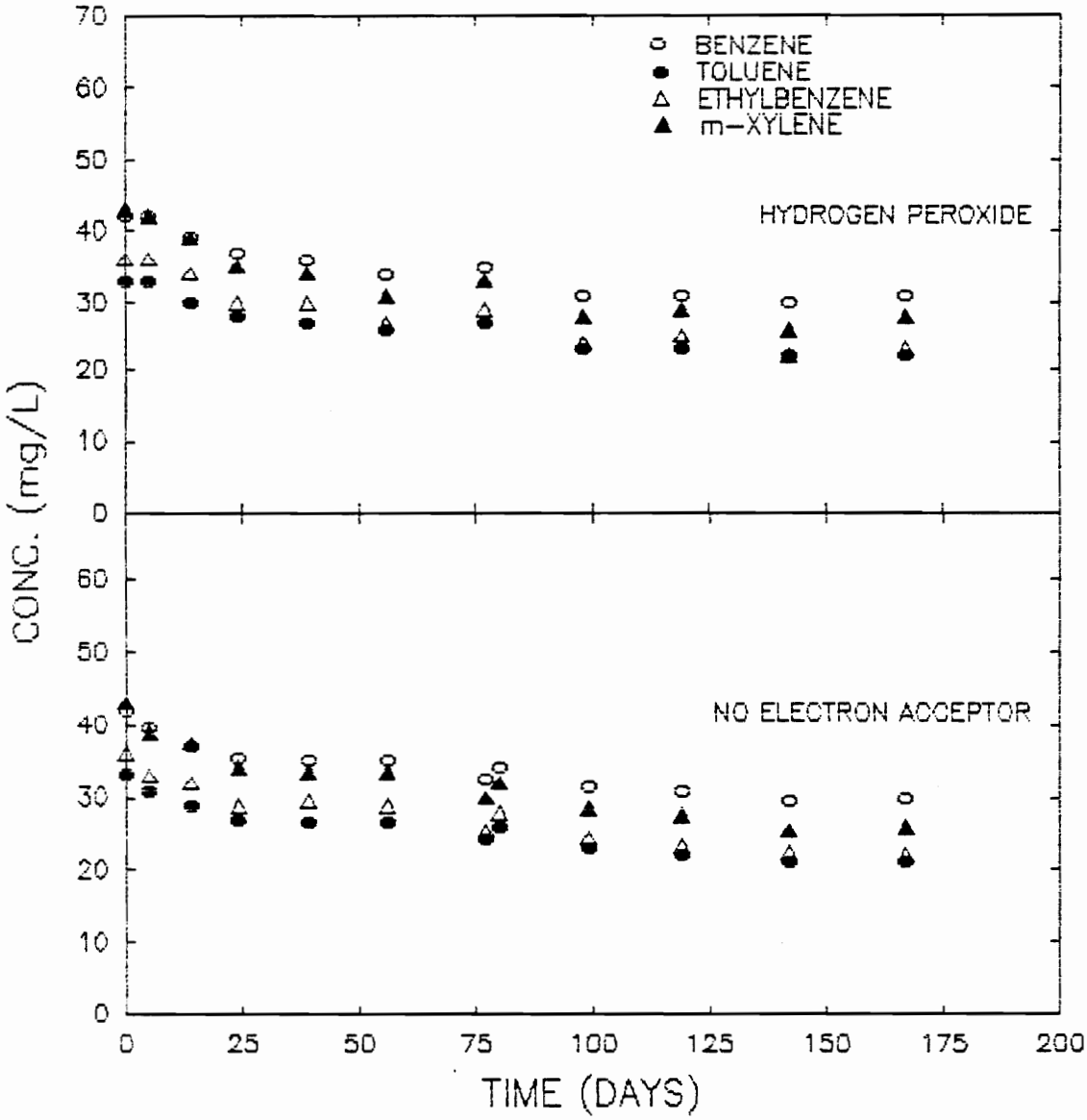

Figure B-4. Abiotic BTEX losses in sterile 15 ft soil with hydrogen peroxide as electron acceptor (top) and with no electron acceptor (bottom).

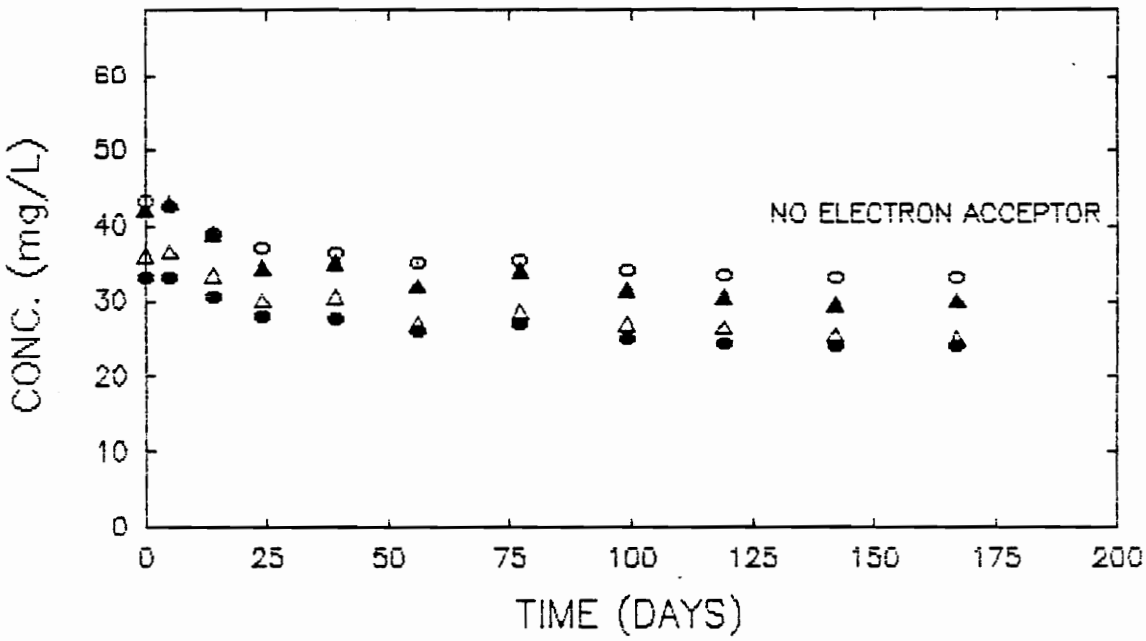

Figure B-5. Abiotic BTEX losses in sterile 17 ft soil with no electron acceptor.

APPENDIX C

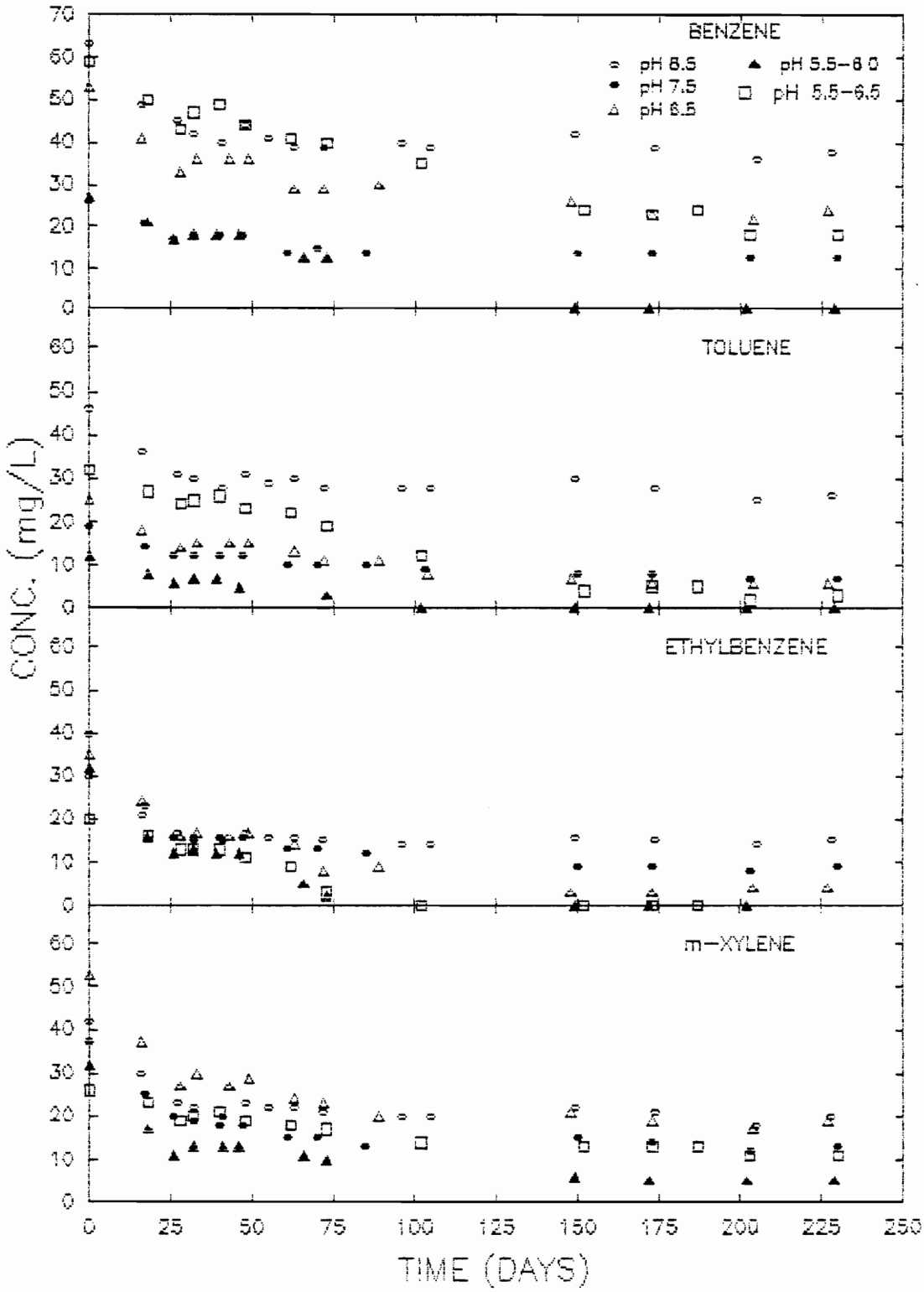

Figure C–1. Loss of BTEX in aerobic–surface soil microcosm (duplicate).

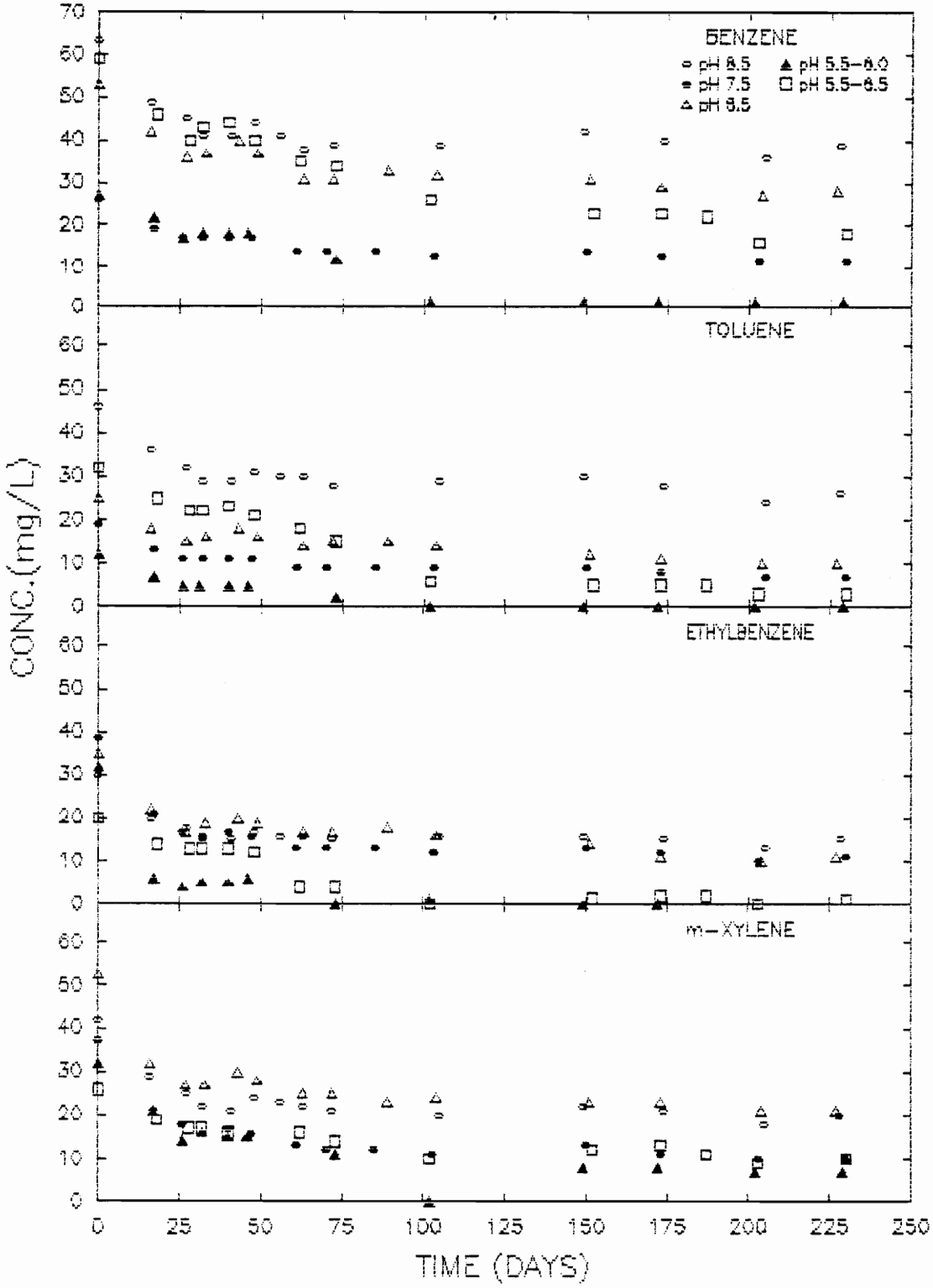

Figure C–2. Loss of BTEX in aerobic–surface soil microcosms (duplicate).

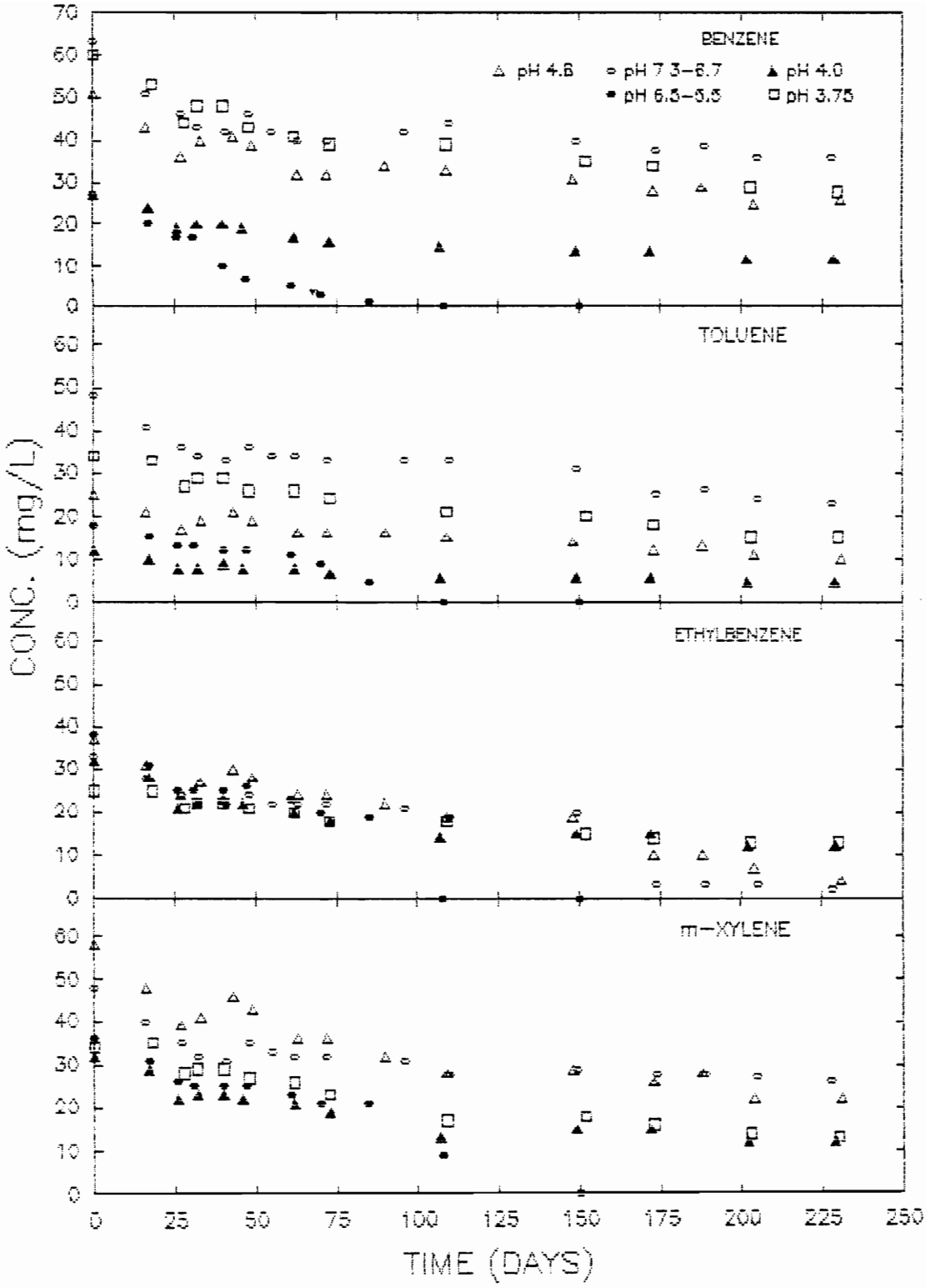

Figure C–3. Loss of BTEX in aerobic–3 ft soil microcosm (duplicate).

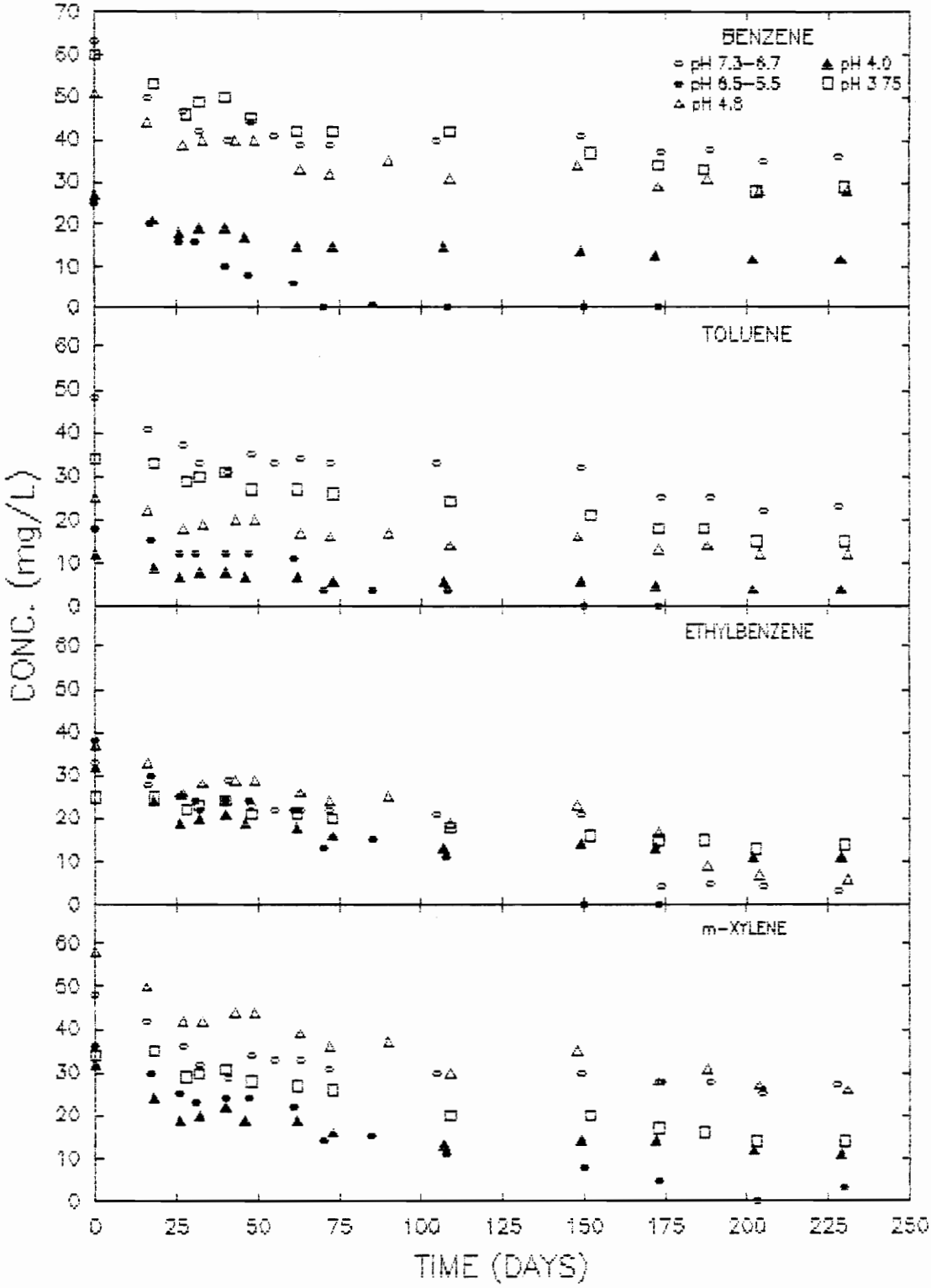

Figure C–4. Loss of BTEX in aerobic–3 ft soil microcosms (duplicate).

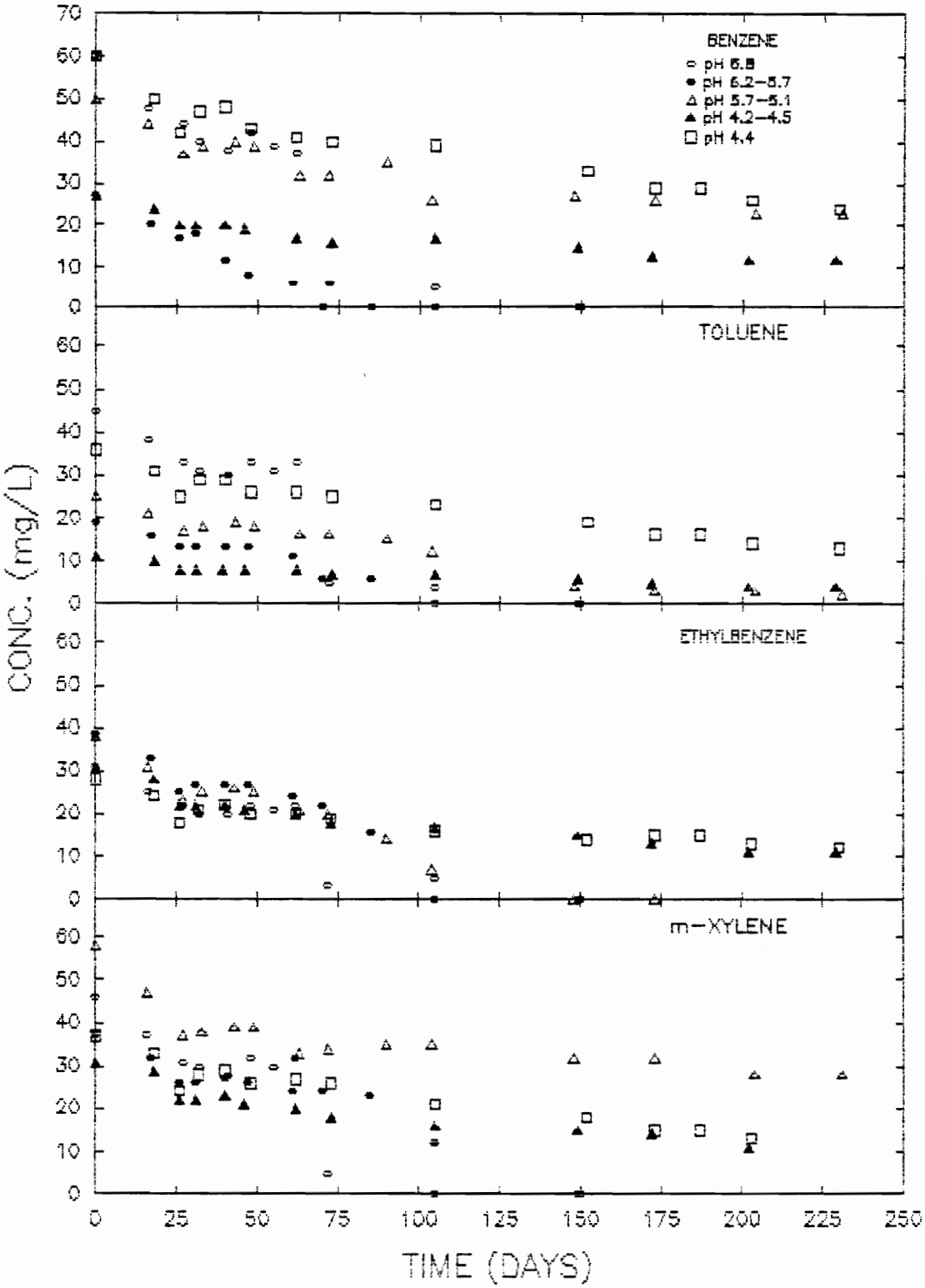

Figure C–5. Loss of BTEX in aerobic–6 ft sicrocosm (duplicate).

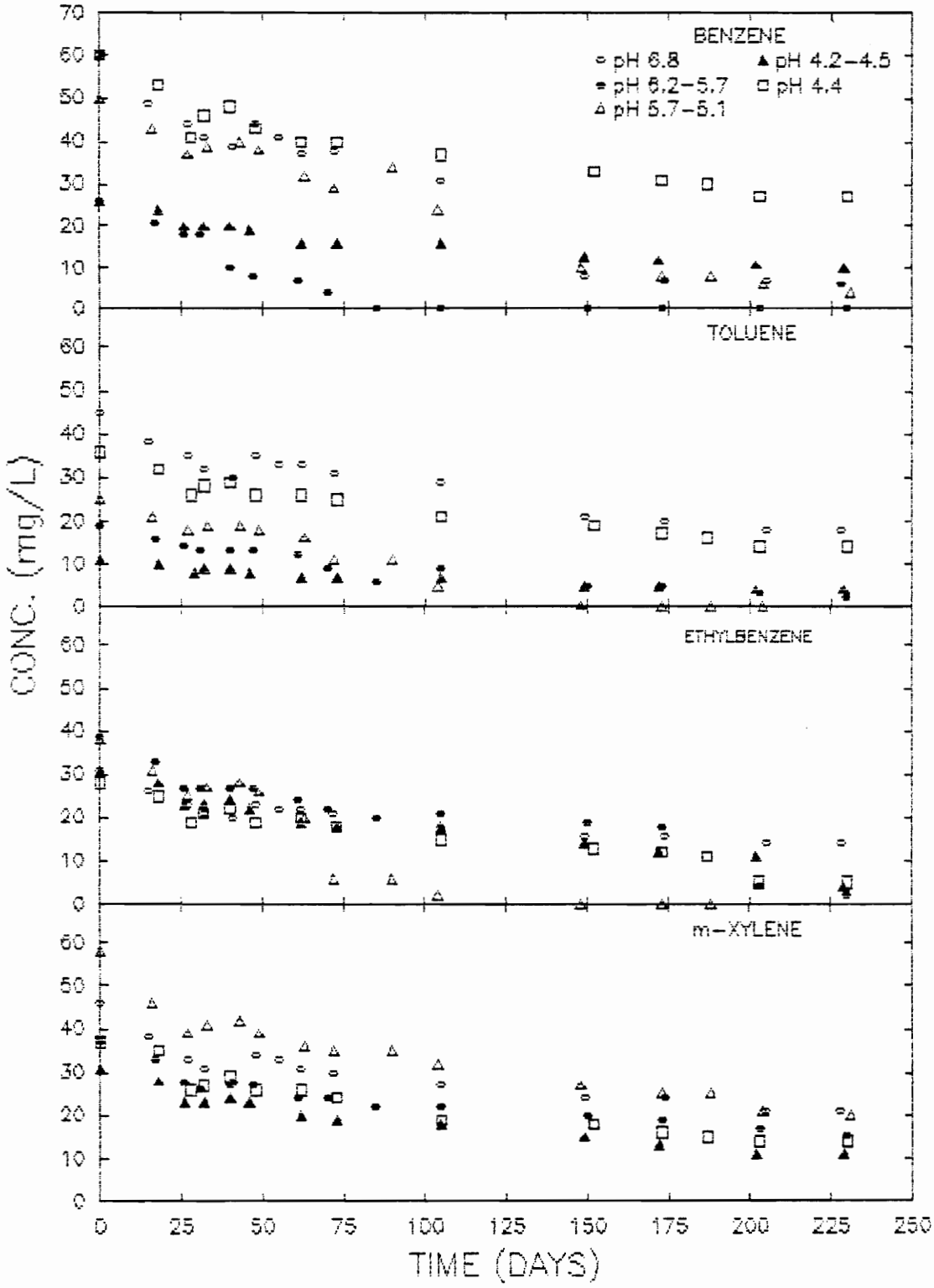

Figure C–6. Loss of BTEX in aerobic–6 ft soil microcosms (duplicate).

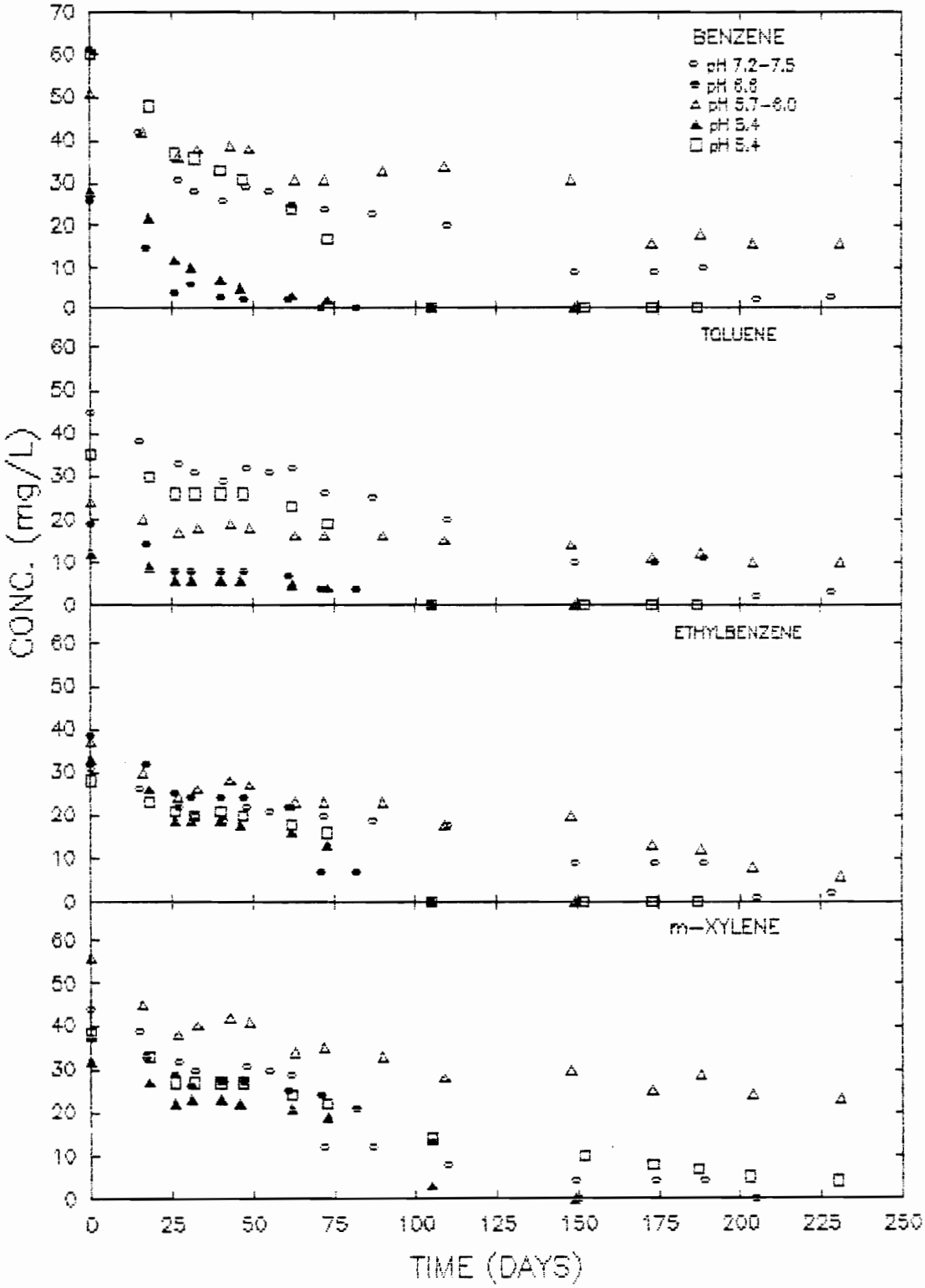

Figure C-7. Loss of BTEX in aerobic-15 ft soil microcosm (duplicate).

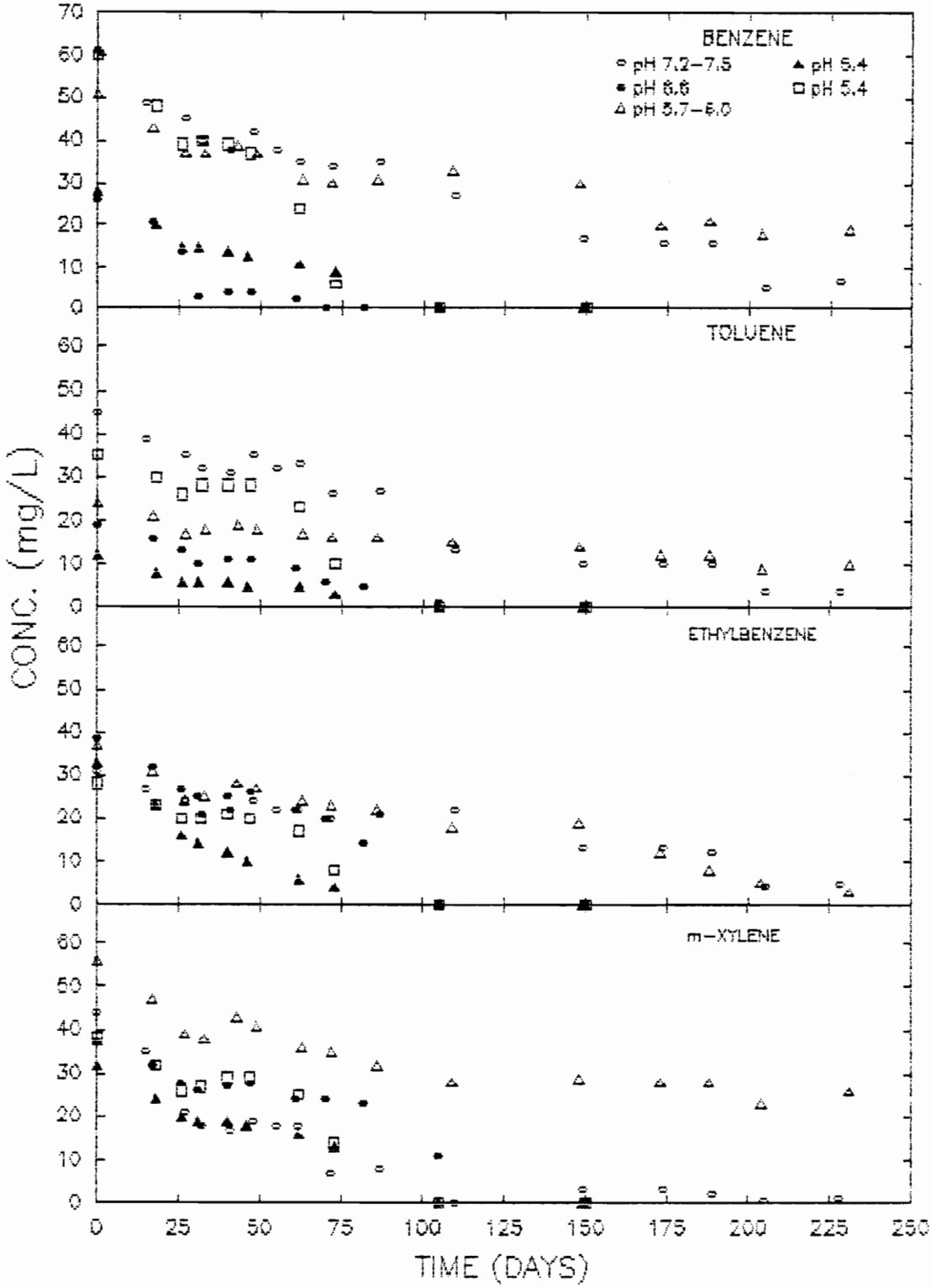

Figure C–8. Loss of BTEX in aerobic–15 ft soil microcosms (duplicate).

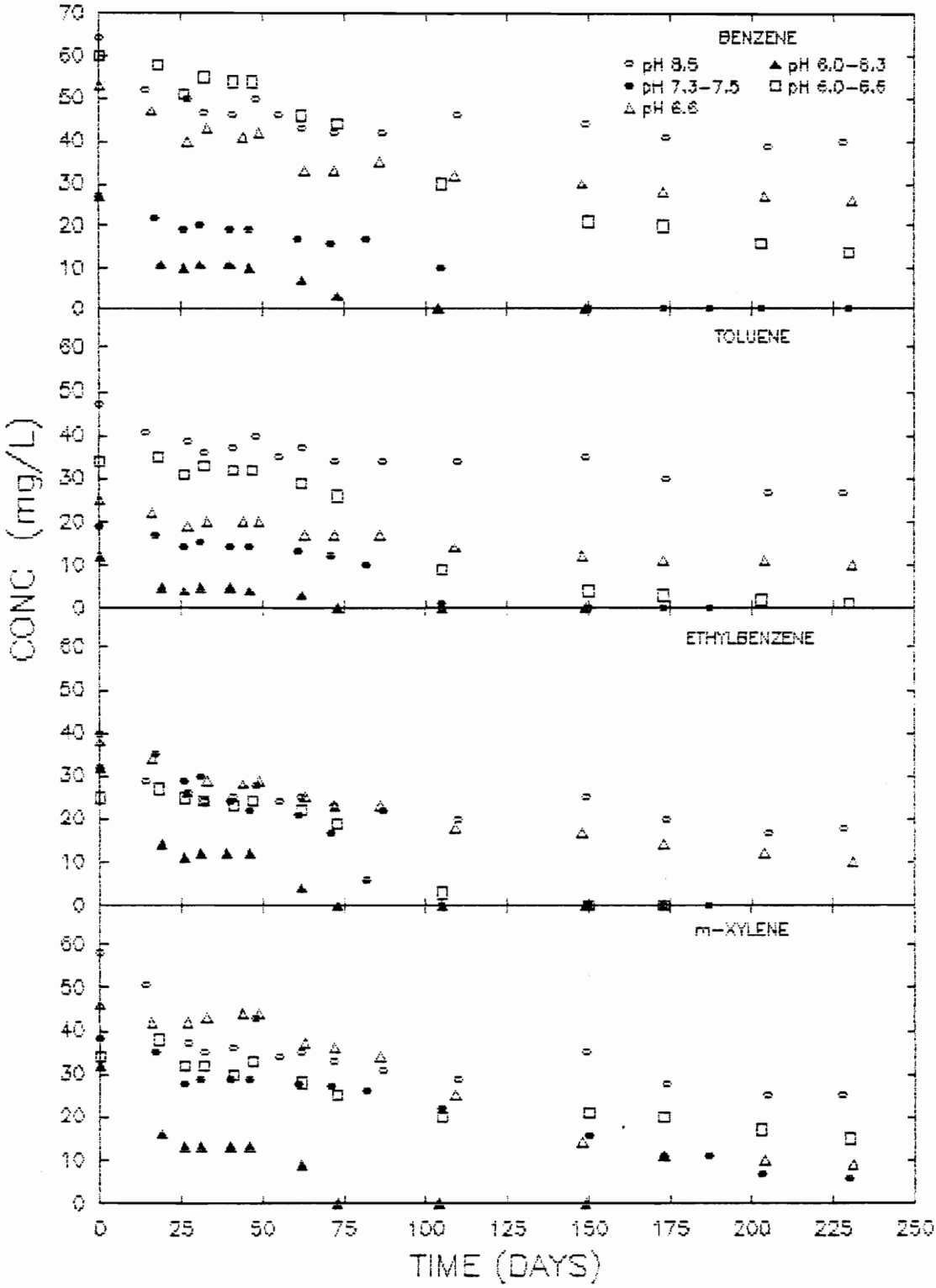

Figure C–9. Loss of BTEX in aerobic–17 ft soil microcosms (duplicate).

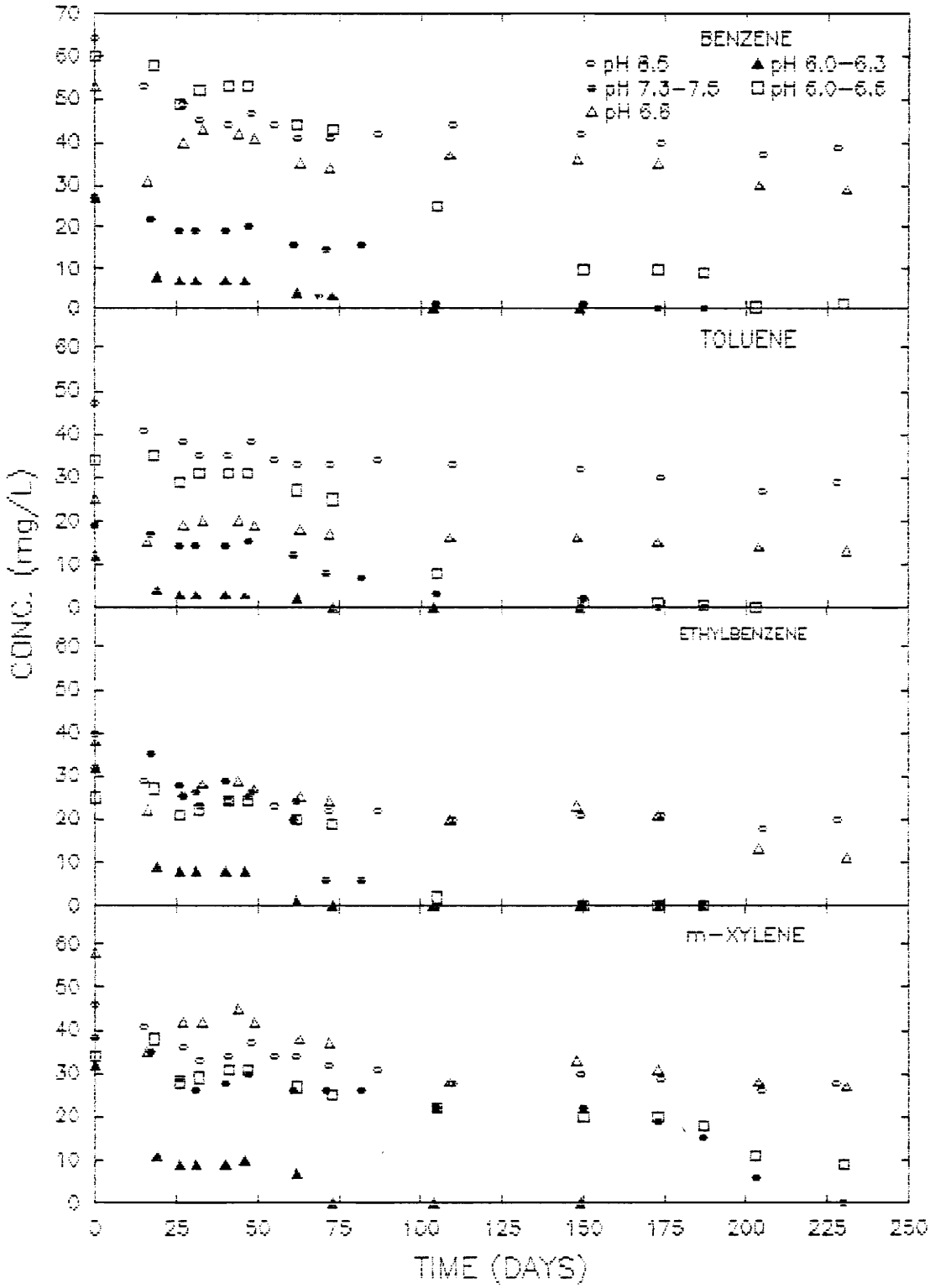

Figure C–10. Loss of BTEX in aerobic–17 ft soil microcosms (duplicate).

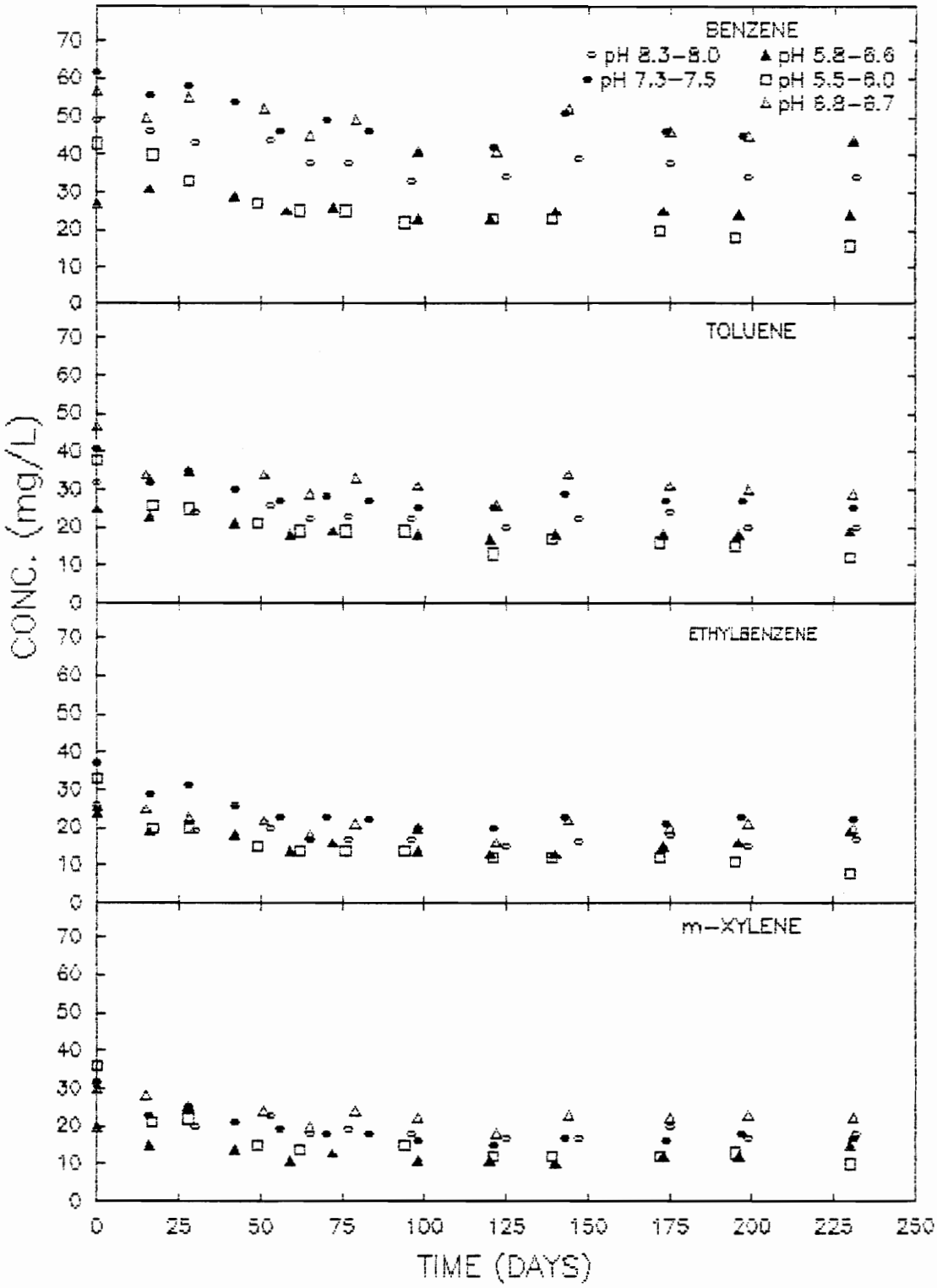

Figure C–11. Loss of BTEX in surface soil microcosms under denitrifying conditions (duplicate).

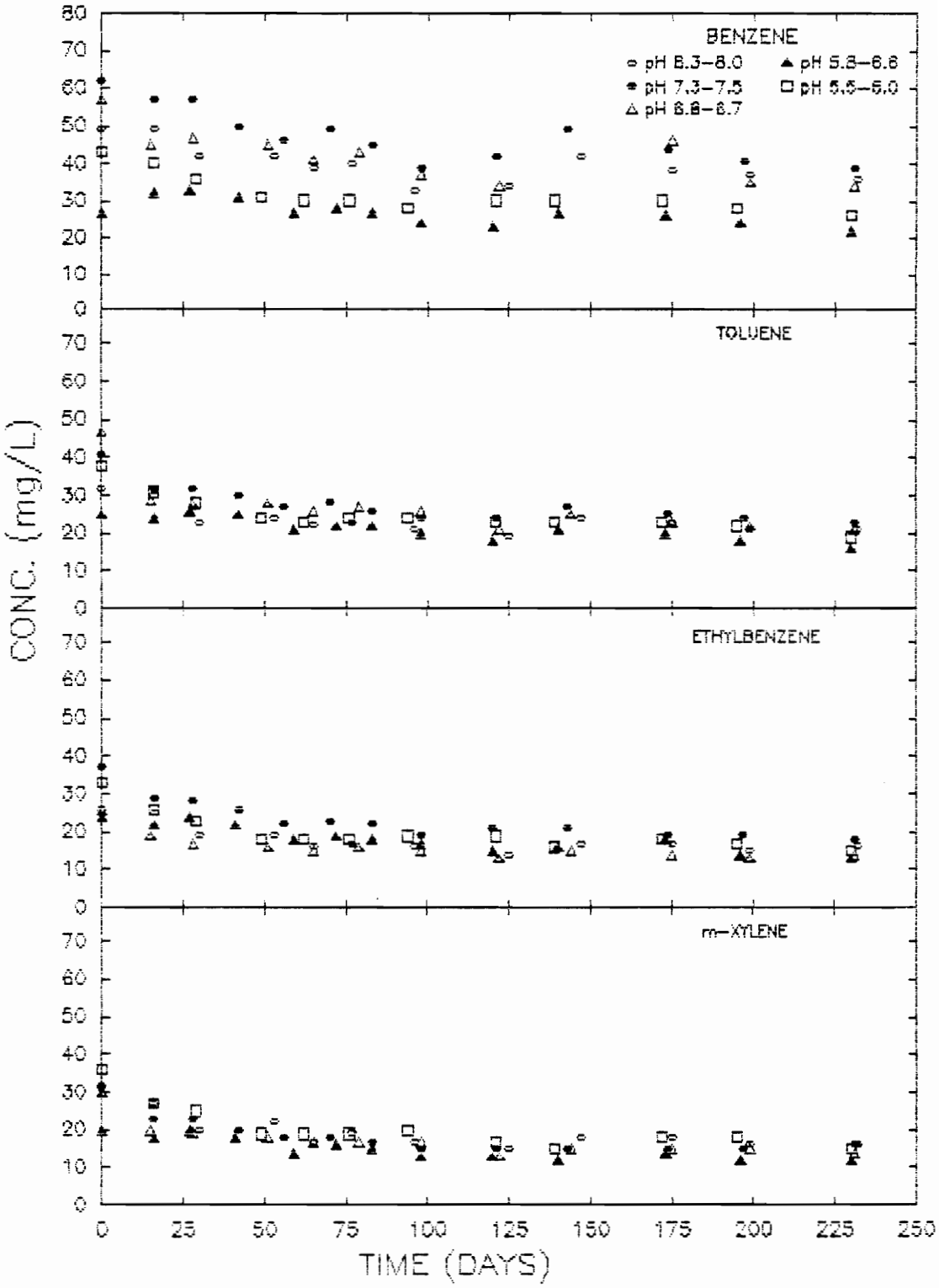

Figure C–12. Loss of BTEX in surface soil microcosms under denitrifying conditions (duplicate).

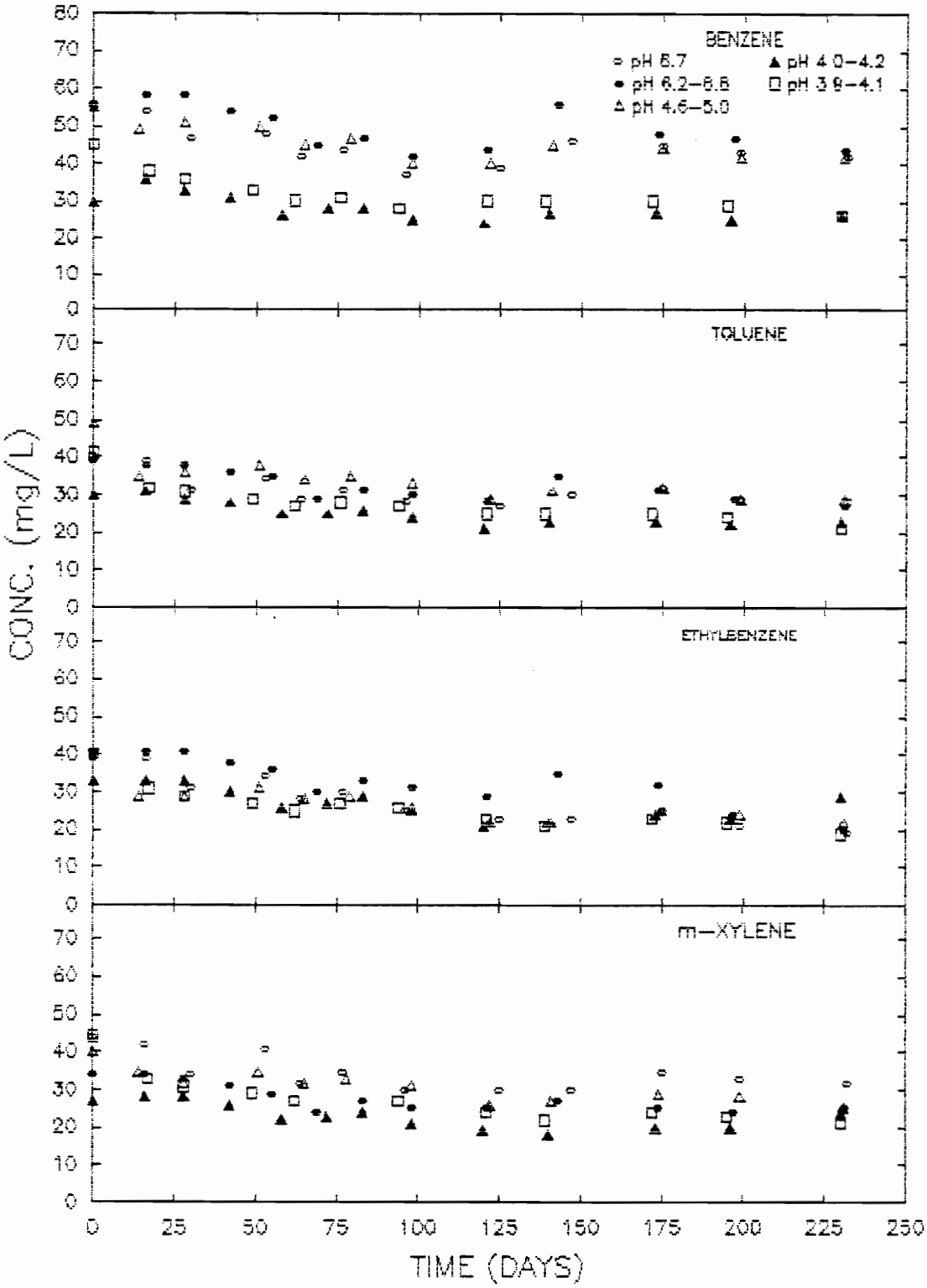

Figure C–13. Loss of BTEX in 3 ft soil microcosms under denitrifying conditions (duplicate).

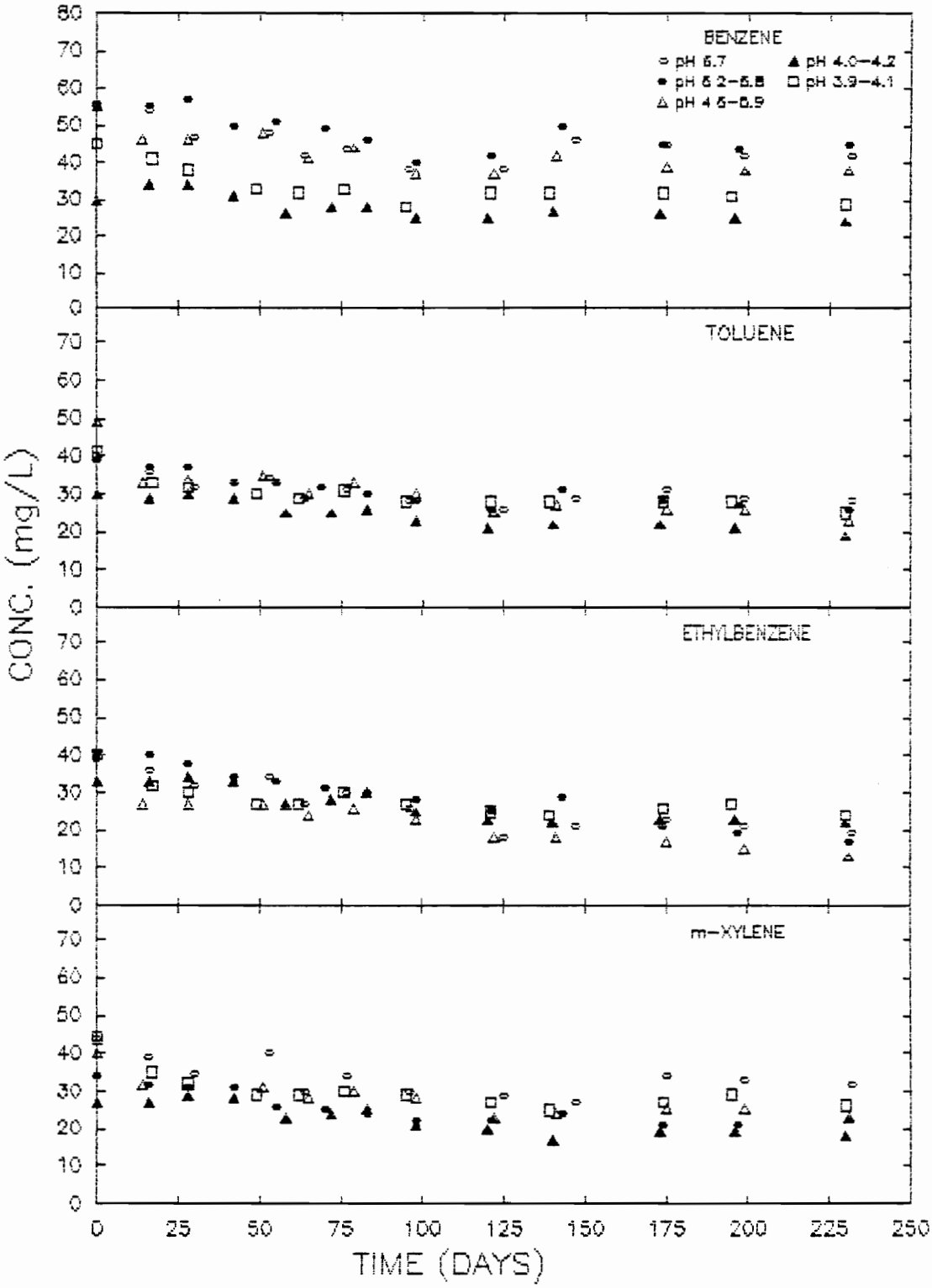

Figure C–14. Loss of BTEX in 3 ft soil microcosms under denitrifying conditions (duplicate).

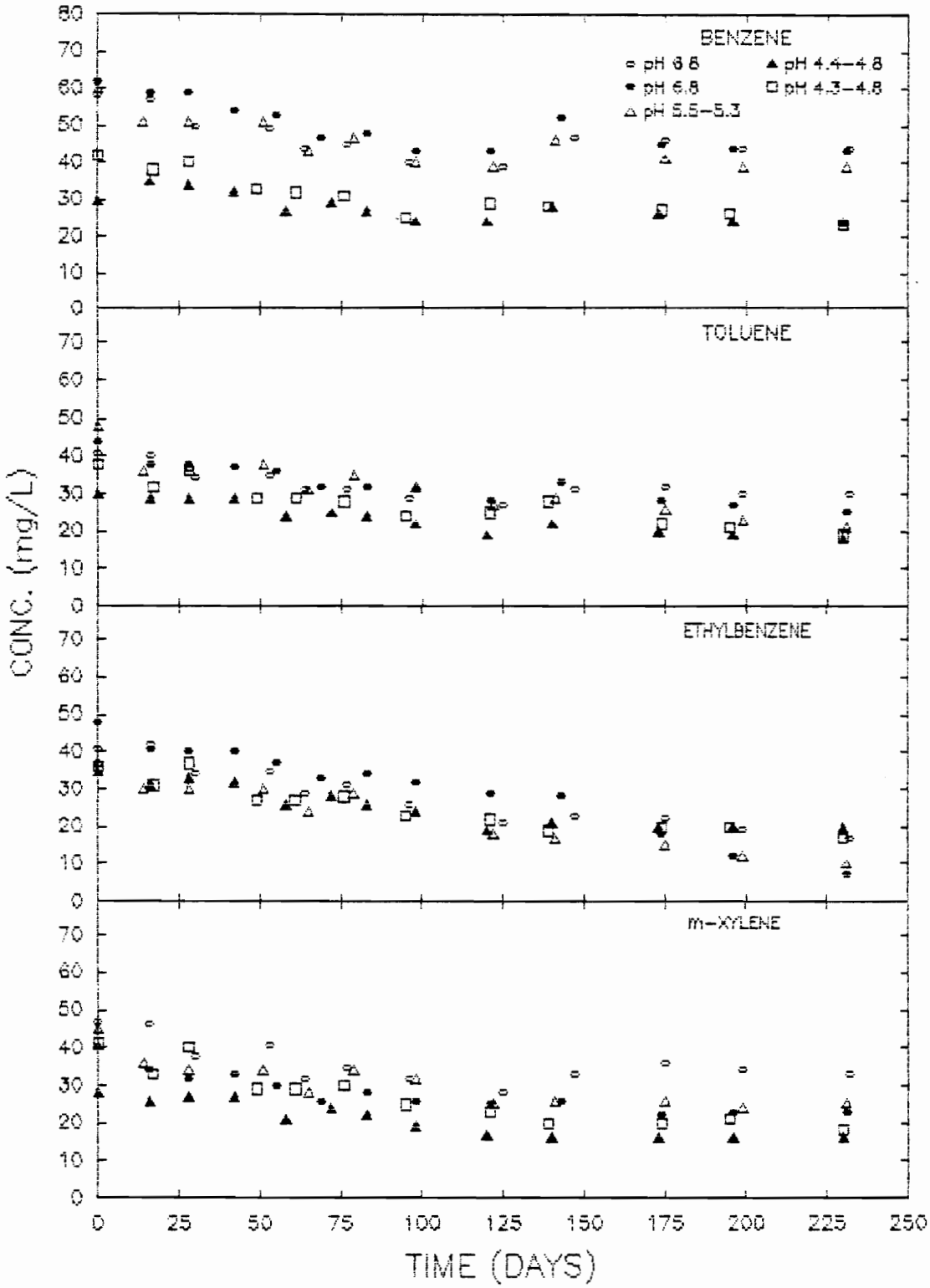

Figure C–15. Loss of BTEX in 6 ft soil microcosms under denitrifying conditions (duplicate).

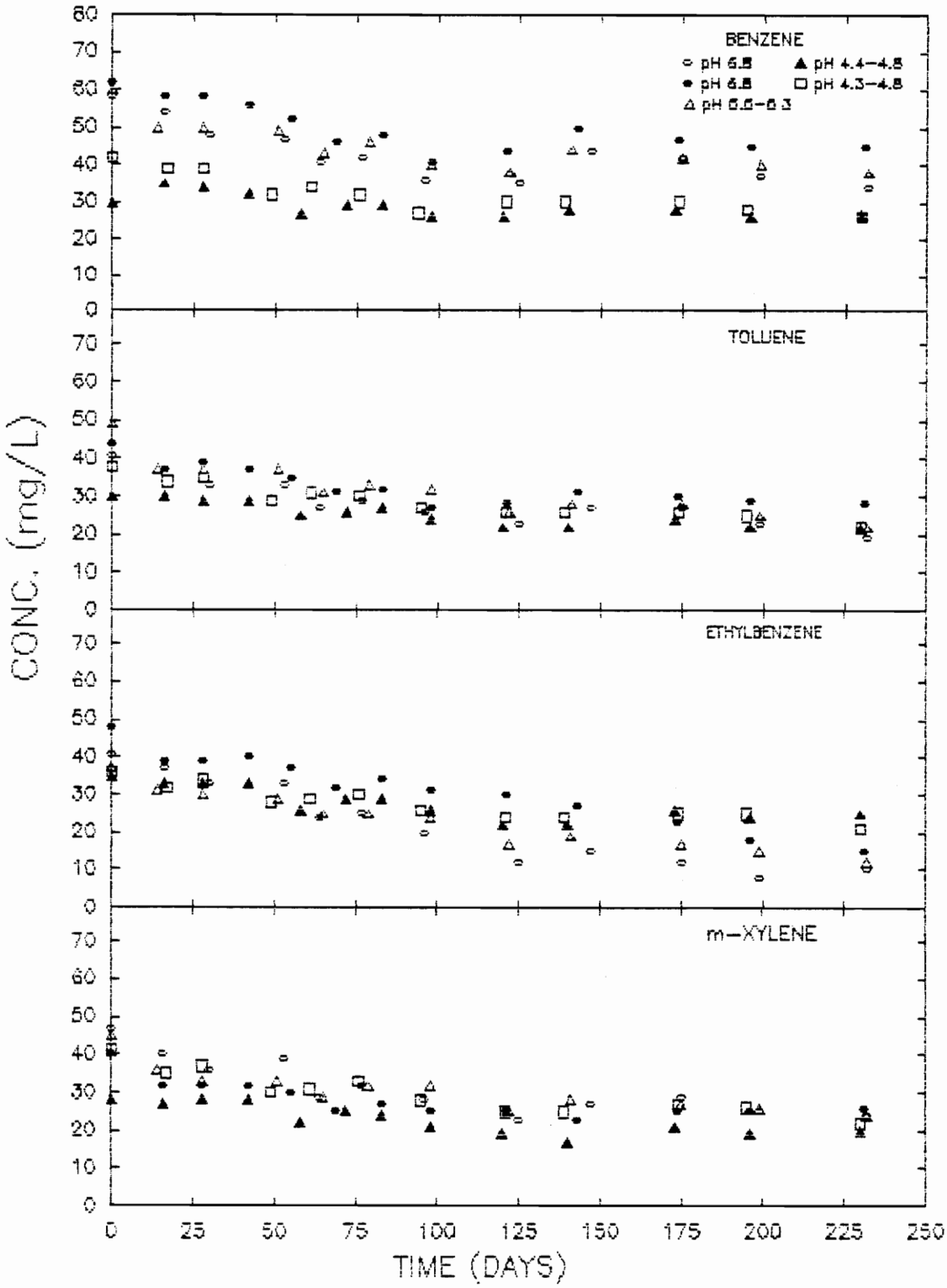

Figure C–16. Loss of BTEX in 6 ft soil microcosms under denitrifying conditions (duplicate).

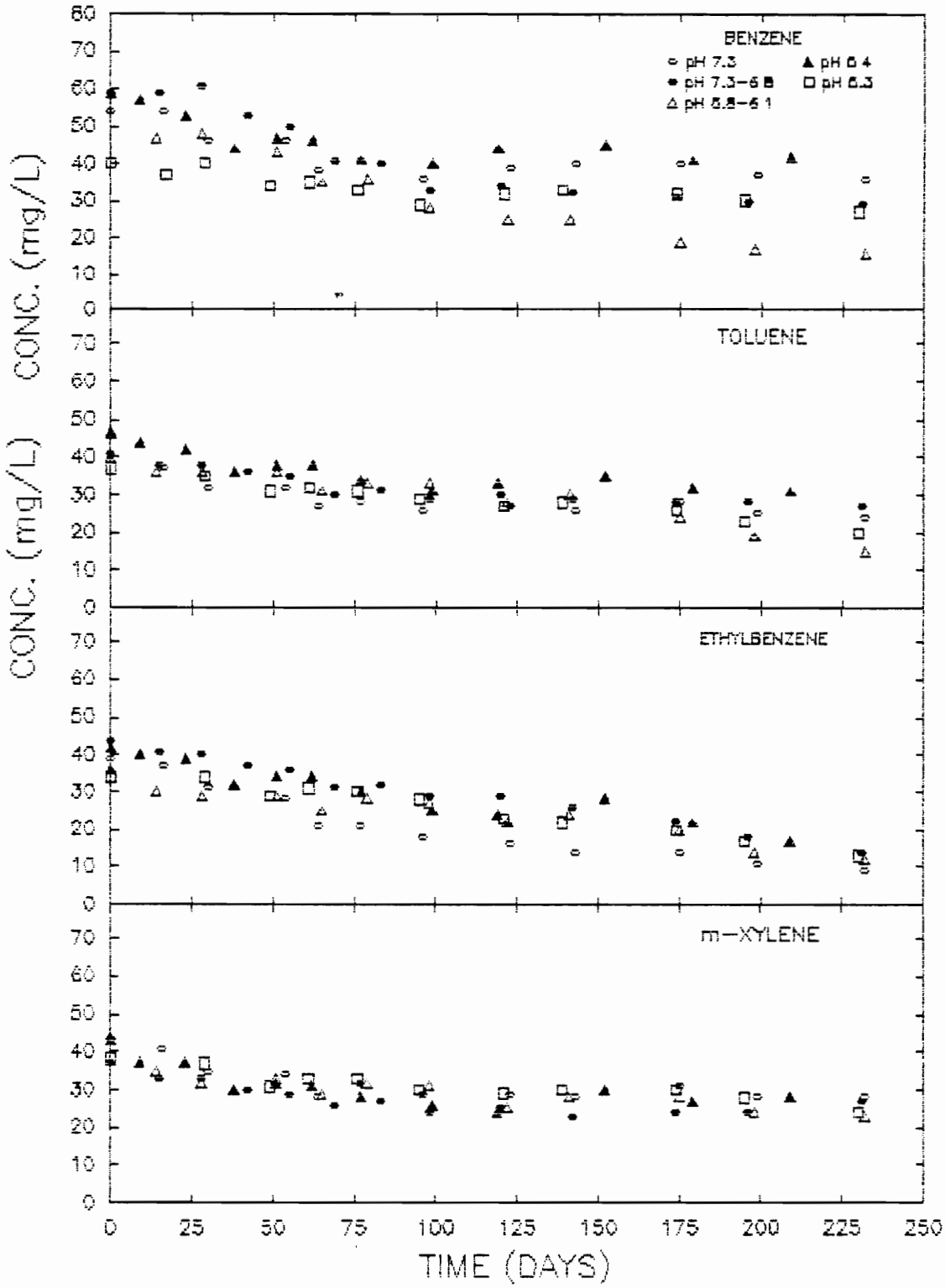

Figure C–17. Loss of BTEX in 15 ft soil microcosms under denitrifying conditions (duplicate).

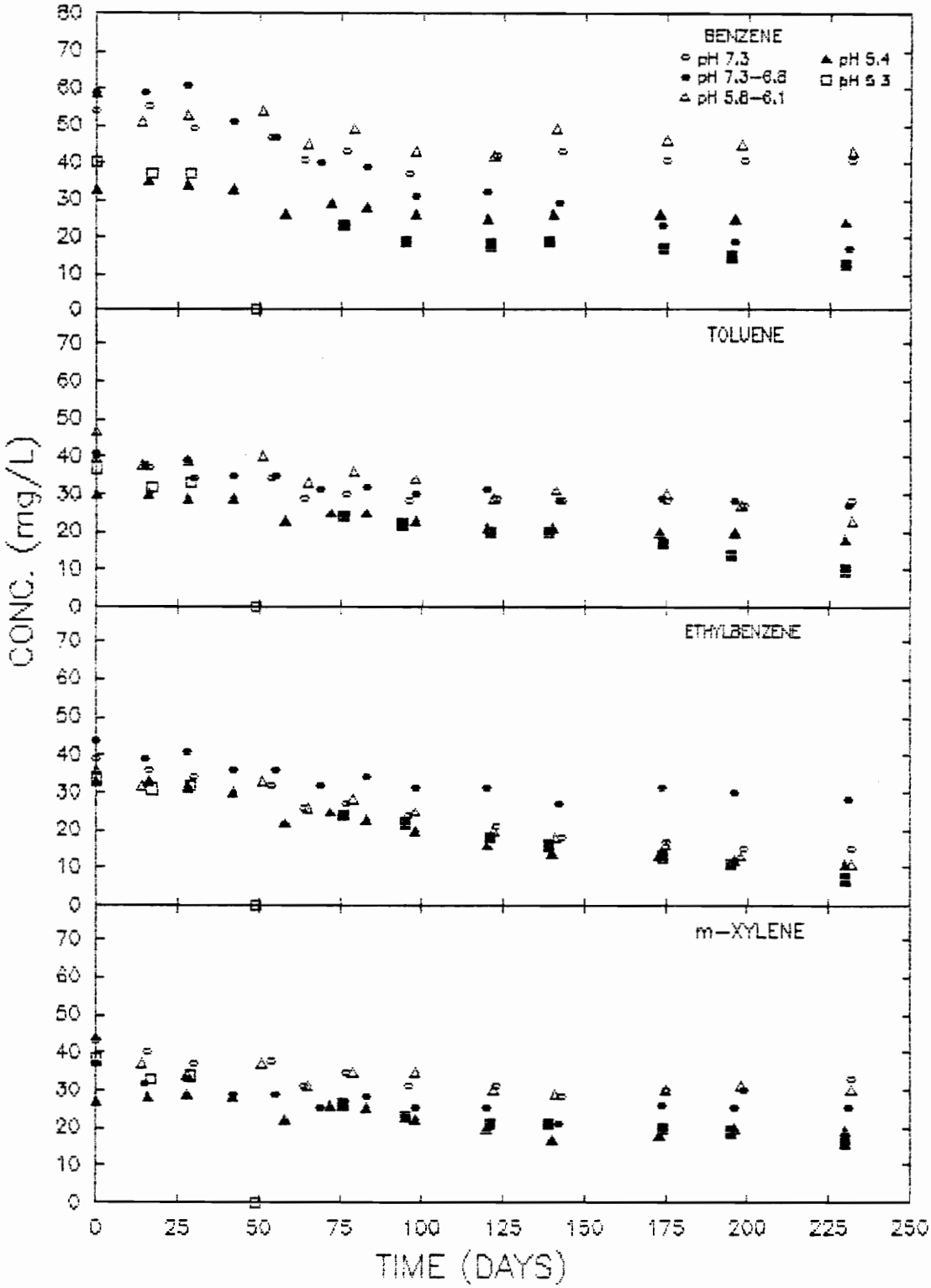

Figure C–18. Loss of BTEX in 15 ft soil microcosms under denitrifying conditions (duplicate).

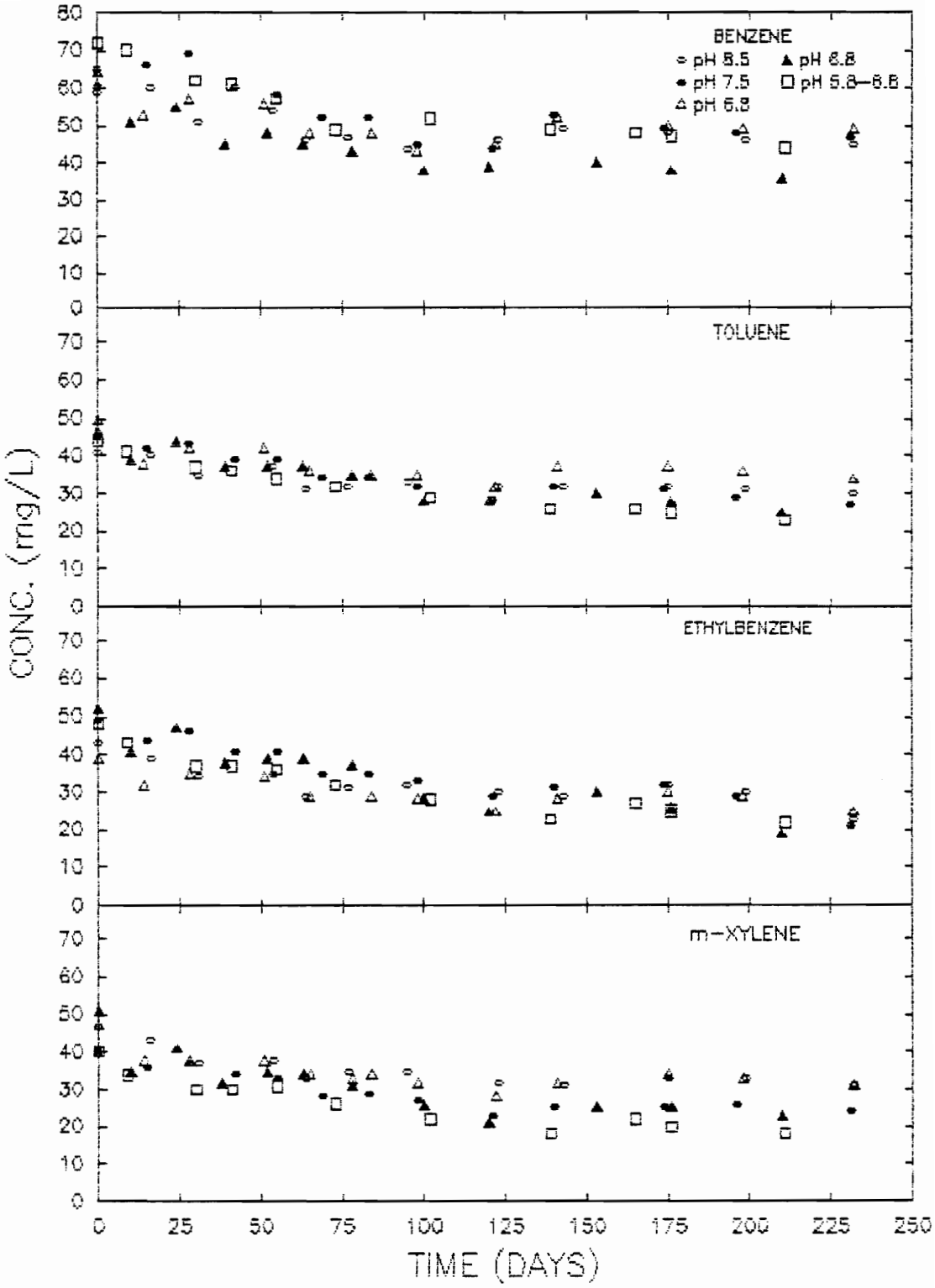

Figure C-19. Loss of BTEX in 17 ft soil microcosms under denitrifying conditions (duplicate).

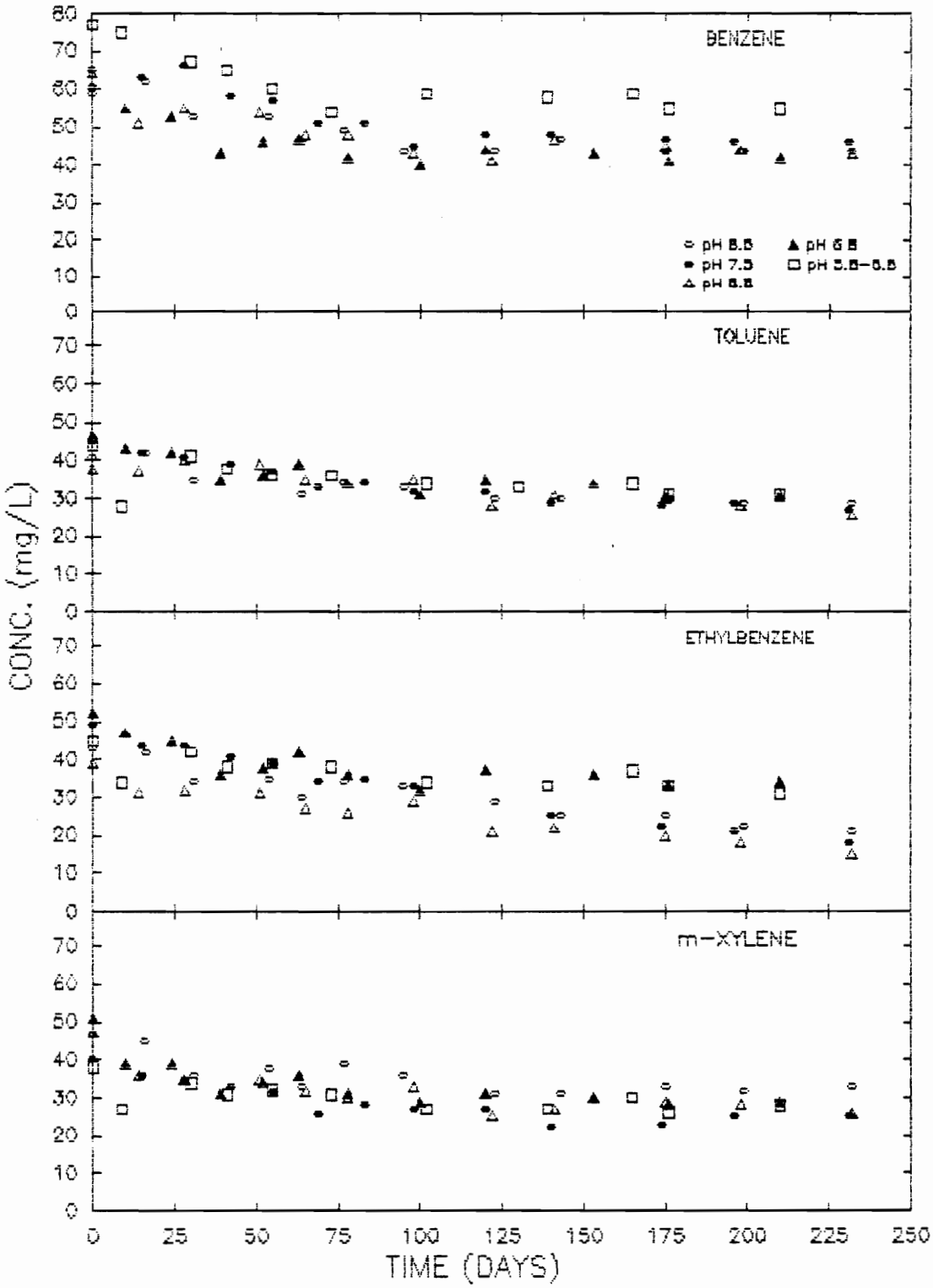

Figure C-20. Loss of BTEX in 17 ft soil microcosms under denitrifying conditions (duplicate).

APPENDIX D

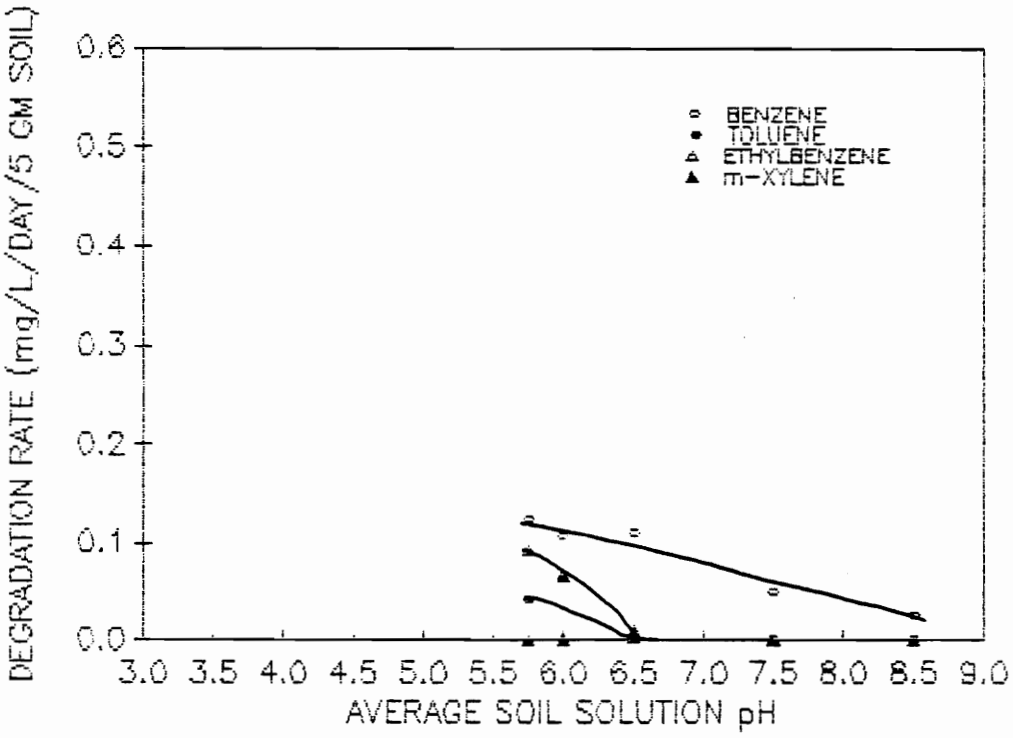

Figure D-1. Comparison of individual BTEX degradation rates in surface soil under aerobic conditions.

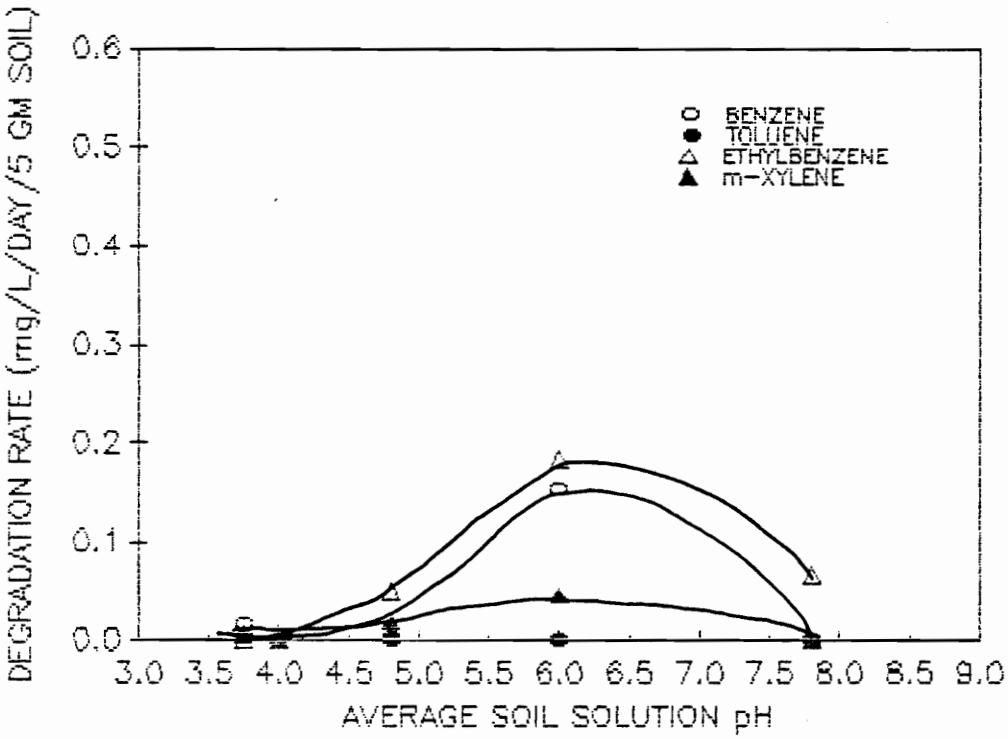

Figure D-2. Comparison of individual BTEX degradation rates in 3 ft soil and under aerobic conditions.

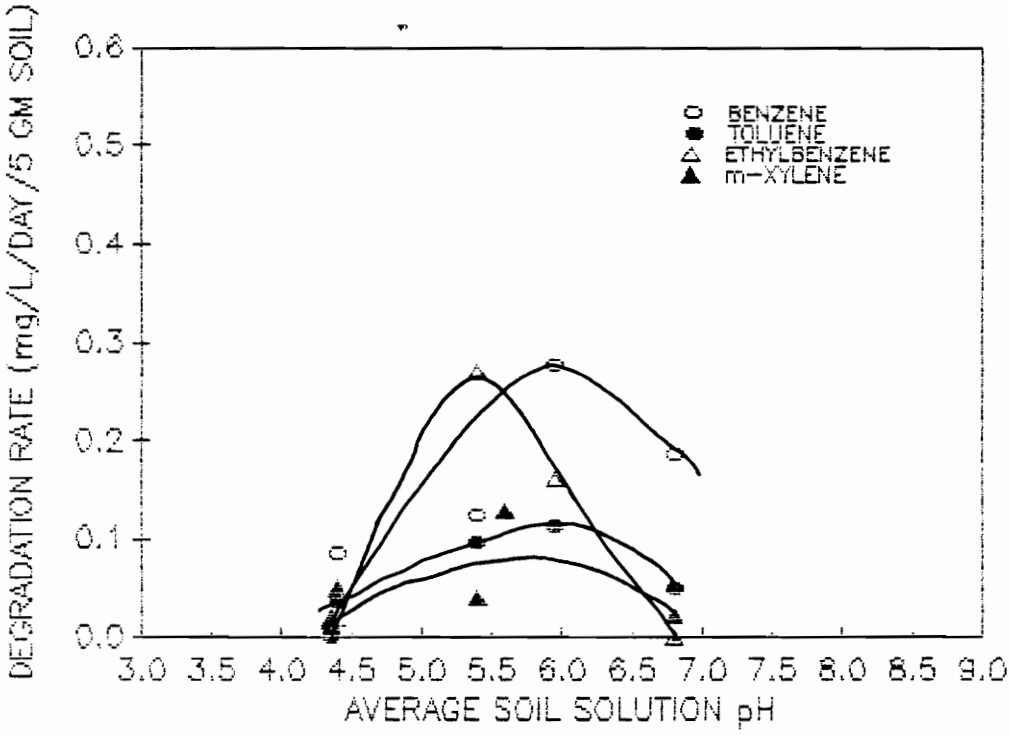

Figure D-3. Comparison of individual BTEX degradation rates in 6 ft soil and under aerobic conditions.

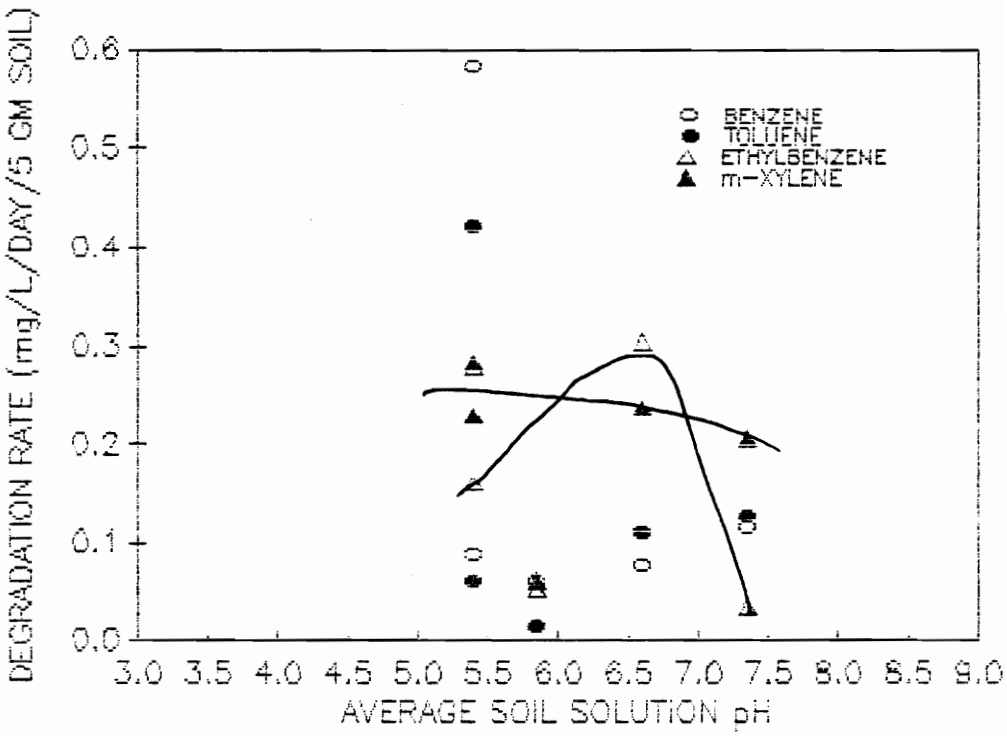

Figure D-4. Comparison of individual BTEX degradation rates in 15 ft soil and under aerobic conditions.

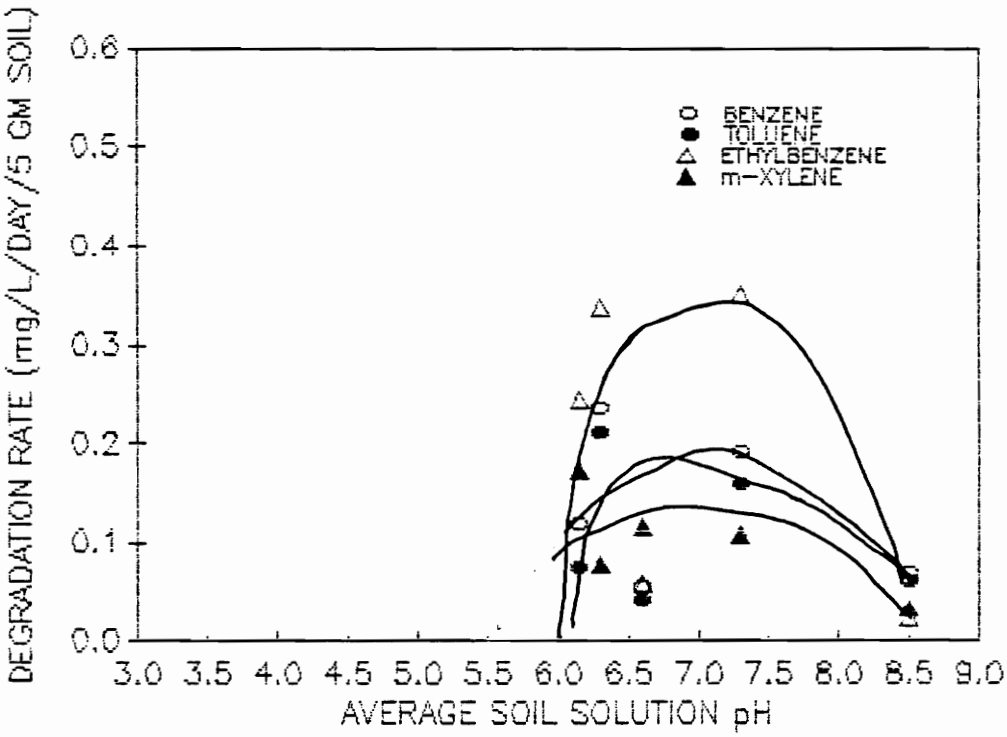

Figure D-5. Comparison of individual BTEX degradation rates in 17 ft soil and under aerobic conditions.

VITA

Amy Jeanne McCormick was born on January 28, 1967 in Saranac Lake, N.Y. She lived in Saranac Lake until she graduated highschool in June, 1985. She then moved to Greensboro, N.C. to attend Guilford College. In May, 1989 she graduated with a B.S. degree in physics. After completing her second summer of employment as an environmental engineering technician, she moved to Blacksburg, Virginia to attend graduate school at Virginia Polytechnic Institute and State University. She received her M.S. degree in environmental engineering in September, 1991.

Amy J. McCormick

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