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MICROBIAL DEGRADATION OF ALKYLBENZENES UNDER SULFATE-REDUCING AND METHANOGENIC CONDITIONS

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16. ABSTRACT

Aquifer solids and soils obtained from various hydrocarbon-contaminated sites were used to investigate the ability of indigenous microorganisms to degrade monoaromatic hydrocarbons under strictly anaerobic conditions. In anaerobic microcosms inoculated with fuel-contaminated soil from the Patuxent River site, toluene degradation occurred concomitantly with sulfate reduction and ferric iron reduction. Similar results were obtained with suspended enrichments derived from the microcosms. Stoichiometric data and other observations suggested that sulfate reduction was closely linked to toluene degradation, whereas iron reduction was a secondary, potentially abiotic, reaction between ferric iron and biogenic hydrogen sulfide. The presence of millimolar concentrations of amorphous $Fe(OH)_3$ in Patuxent River microcosms and enrichments either greatly facilitated the onset of toluene degradation or accelerated the rate once degradation had begun. Fermentative/methanogenic microcosms and enrichments that degraded toluene and o-xylene without added exogenous electron acceptors (except CO_2) were developed from creosote-contaminated Pensacola samples. The microcosms initially underwent an acclimation lag of several months; however, once the degradation of aromatic hydrocarbons was initiated, it proceeded at a relatively rapid rate, and it was complete (resulting in mineralization to CO_2 and CH_4). Benzene, ethylbenzene, and p-xylene were not degraded.

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FOREWORD

EPA is charged by Congress to protect the Nation's land, air, and water systems. Under a mandate of national environmental laws focused on air and water quality, solid waste management and the control of toxic substances, pesticides, noise and radiation, the Agency strives to formulate and implement actions which lead to a compatible balance between human activities and the ability of natural systems to support and nurture life.

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This report describes research conducted to evaluate the ability of indigenous aquifer- and soil-derived microorganisms to degrade simple aromatic hydrocarbons, such as toluene, under anaerobic conditions. Monoaromatic hydrocarbons are pervasive groundwater contaminants in areas near leaking underground fuel storage tanks and other uncontrolled releases of gasoline and aviation fuel. The research specifically assesses degradation under sulfate-reducing and methanogenic conditions, and evaluates the effects of certain geochemical factors, such as the presence of iron minerals, on degradation rates.

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ABSTRACT

Aquifer solids and soils obtained from various hydrocarbon-contaminated sites were used to investigate the ability of indigenous microorganisms to degrade monoaromatic hydrocarbons under strictly anaerobic conditions. Hydrocarbon-degrading microflora from two sites, an aviation fuel storage facility located near the Patuxent River (MD) and a creosote-contaminated aquifer near Pensacola (FL), were studied most extensively.

Anaerobic microcosms containing fuel-contaminated soil from the Patuxent River site displayed evidence of toluene degradation, sulfate reduction, and ferric iron reduction; all three processes appeared to be strongly linked and proceeded concomitantly. Stoichiometry and other considerations indicated that toluene degradation was closely linked to the activity of sulfate-reducing bacteria. To our knowledge, this is one of the first reports of the degradation of alkylbenzenes under sulfate-reducing conditions. Stoichiometric data were also used to evaluate whether ferric iron reduction was more closely linked with toluene oxidation (where ferric iron could serve as a terminal electron acceptor for toluene) or with sulfate reduction (where ferric iron could serve as the electron acceptor for the oxidation of biogenic hydrogen sulfide). Although the data do not yield a definitive conclusion, they indicate that toluene was oxidized to carbon dioxide by sulfate-reducing bacteria and that the resulting hydrogen sulfide was oxidized in a secondary, potentially abiotic, reaction with ferric iron.

The presence of millimolar concentrations of amorphous $Fe(OH)_3$ in Patuxent River microcosms either greatly facilitated the onset of toluene degradation (observed in one experiment) or accelerated the rate once degradation had begun (observed in another experiment). Several hypotheses are proposed in this report to explain the effect of added iron on toluene degradation.

Active enrichments were successfully generated from Patuxent River microcosms. These enrichments behaved similarly to the microcosms in that (1) strongly linked toluene degradation, sulfate reduction, and iron reduction were observed, and (2) the presence of amorphous $Fe(OH)_3$ markedly enhanced toluene degradation rates.

Fermentative/methanogenic microcosms that degraded toluene and *o*-xylene without added exogenous electron acceptors (except CO_2) were developed from creosote-contaminated aquifer solids derived from the Pensacola site. The microcosms initially underwent an acclimation lag of several months; however, once the degradation of aromatic hydrocarbons was initiated, it occurred at a relatively rapid rate, and it was complete (resulting in mineralization to CO_2 and CH_4). Benzene, ethylbenzene, and *p*-xylene were not degraded.

Active methanogenic enrichments were obtained from the Pensacola microcosms. These enrichments, upon stabilization, mineralized 50 μM of toluene and *o*-xylene to CO_2 and CH_4 in one to two weeks; and slowly degraded *m*-xylene. The estimated doubling time of the consortium was nine days. The presence of a small amount of aquifer solids supported the degradation of aromatic hydrocarbons and increased the degradation rate. Exogenous electron acceptors (nitrate and sulfate) slowed down the transformation.

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SECTION 1

INTRODUCTION

The U.S. EPA has reported that up to 35 percent of the nation's underground fuel storage tanks may be leaking (U.S. EPA, 1986). Together with surface spill accidents and landfill leachate intrusion, such leaks greatly contribute to groundwater contamination by gasoline and other petroleum derivatives. Although most gasoline constituents are readily degraded in aerobic surface water and soil systems, similar processes in the subsurface are significantly retarded because of insufficient concentrations of oxygen and/or nutrients, and consequently low numbers of active aerobic microorganisms. If oxygen and essential microbial nutrients are added, however, the aerobic biotransformation of major gasoline constituents in groundwater can proceed rapidly. This was demonstrated by Raymond (1974) and was applied for *in-situ* treatment of a contaminated aquifer in the early 1970's (Raymond et al., 1975). This bioreclamation process, with variations, has been used repeatedly since then (Lee et al., 1988). Unfortunately, it is not always feasible to introduce enough oxygen into the subsurface. Such limitations lead to the complete consumption of oxygen by indigenous microorganisms and result in the development of anaerobic conditions.

In the absence of oxygen, degradation of gasoline constituents can take place only with the use of alternative electron acceptors, such as nitrate, sulfate, or ferric iron, or fermentatively in combination with methanogenesis. To date, complete degradation of benzene, toluene, and/or xylene isomers by aquifer-, sediment-, or sewage-derived microorganisms has been found under denitrifying conditions (Hutchins et al., 1991; Kuhn et al., 1985, 1988; Major et al., 1988; Zeyer et al., 1986, 1990), methanogenic conditions (Grbić-Galić and Vogel, 1987; Vogel and Grbić-Galić, 1986; Wilson et al., 1986, 1987), and ferric iron-reducing conditions (Lovley and Lonergan, 1990; Lovley et al., 1989). Very recently, pure cultures of organisms that can degrade alkylbenzenes anaerobically have been isolated (Dolfing et al., 1990; Lovley and Lonergan, 1990). Despite this research activity, anaerobic degradation of monoaromatic hydrocarbons is not as well understood as aerobic degradation; further study of anaerobic degradation of these compounds for the range of potential electron-accepting conditions is warranted.

For this project, the initial objective was to investigate the degradation of benzene and selected alkylbenzenes under fermentative (including methanogenic) and ferric iron-reducing conditions. Sulfate-reducing and nitrate-reducing conditions were to be studied only as environmental factors that could influence fermentative and iron-reducing processes. As it turned out, active fermentative/methanogenic microcosms (and subsequently stable methanogenic consortia) that could degrade toluene and *o*-xylene were indeed successfully enriched. However, iron-reducing hydrocarbon-degrading communities could not be obtained. Instead, screening for iron reducers led to the enrichment of a sulfate-reducing consortium that could degrade toluene, which is the focus of this report. Notably, definitive evidence of the coupling of alkylbenzene degradation to sulfate reduction has not yet been published. However, some co-authors of this report have observed degradation of toluene and xylene isomers under sulfate-reducing conditions in studies of fuel-contaminated sediments from Seal Beach, California (Haag et al., in press; Edwards et al., 1991) and Sewell et al. (1991) recently reported toluene degradation under sulfate-reducing conditions. In addition, sulfate-reducers are capable of degrading a number of oxygen-containing aromatic compounds, some of which may be intermediates in anaerobic toluene degradation (e.g., *p*-cresol, benzoic acid, 2- and 4-hydroxybenzoic acid, phenol, catechol, resorcinol, hydroquinone; Bak and Widdel, 1986; Cord-Ruwisch and Garcia, 1985; Schnell et al., 1989; Szewzyk and Pfennig, 1987; Widdel et al., 1983).

SECTION 2

CONCLUSIONS

PATUXENT RIVER SOILS

Anaerobic microcosms containing contaminated soil from an aviation fuel storage facility located near the Patuxent River (MD) displayed evidence of toluene degradation, sulfate reduction, and ferric iron reduction; all three processes appeared to be strongly linked and proceeded concomitantly. Stoichiometry and other considerations strongly suggested that toluene degradation was closely linked to the activity of sulfate-reducing bacteria. To our knowledge, this is one of the first reports of the degradation of alkylbenzenes under sulfate-reducing conditions. Degradation of benzene, ethylbenzene, and *o*- and *p*-xylenes was not observed in these experiments.

Stoichiometric data were used to evaluate whether ferric iron reduction was more closely linked with toluene oxidation (where ferric iron could serve as a terminal electron acceptor for toluene) or with sulfate reduction (where ferric iron could serve as the electron acceptor for hydrogen sulfide oxidation). The data suggested that toluene was oxidized to carbon dioxide by sulfate-reducing bacteria and that the resulting hydrogen sulfide was oxidized in a secondary, potentially abiotic, reaction with ferric iron.

The presence of millimolar concentrations of amorphous $Fe(OH)_3$ in Patuxent River microcosms either greatly facilitated the onset of toluene degradation (observed in one experiment) or accelerated the rate once degradation had begun (observed in another experiment). Several hypotheses were proposed to explain the effect of added iron on toluene degradation, including the assimilation of iron as a limiting micronutrient that was required for the synthesis of enzymes involved in toluene degradation.

Active enrichments were successfully generated from Patuxent River microcosms. These enrichments behaved similarly to the microcosms in that (1) strongly linked toluene degradation, sulfate reduction, and iron reduction were observed, and (2) the presence of amorphous $Fe(OH)_3$ markedly enhanced toluene degradation rates.

PENSACOLA AQUIFER MATERIALS

Fermentative/methanogenic microcosms capable of degrading toluene and *o*-xylene were obtained from creosote-contaminated aquifer material (Pensacola, FL). The initial acclimation lag before the onset of degradation was two to three months. The addition of *p*-cresol, a putative intermediate of toluene transformation, may have shortened the acclimation period relative to microcosms that were not amended with *p*-cresol. Once the degradation was initiated, it was relatively rapid, and the rate increased upon each subsequent re-feeding. Benzene, ethylbenzene, and *p*-xylene were not degraded.

Stable suspended methanogenic consortia were enriched from the Pensacola microcosms. These cultures degraded 50 μM toluene and *o*-xylene to CO_2 and CH_4 in one to two weeks. The doubling time of the cultures was nine days. The presence of a trace amount of aquifer solids enhanced the biodegradation of aromatic hydrocarbons, indicating the importance of attachment surfaces and/or trace nutrients for the microorganisms. Exogenous electron acceptors (nitrate or sulfate) slowed down the degradation of toluene, suggesting that the degrading community was a well-acclimated fermentative/methanogenic consortium that operated optimally under the conditions of methanogenic fermentation.

GENERAL CONCLUSIONS

Of the various microbial inocula used in this study, the microflora from some of the examined sites displayed anaerobic catabolism of aromatic hydrocarbons that can be characterized as anaerobic respiration (e.g., sulfate reduction or denitrification in Patuxent River microcosms). These microbial communities required an exogenous electron acceptor to degrade aromatic hydrocarbons as electron donors/carbon sources/energy sources. The microflora from other sites, however, exhibited fermentative/methanogenic catabolism of aromatic hydrocarbons (Pensacola microcosms), which did not require any exogenous electron acceptors except CO_2. Furthermore, the addition of exogenous electron acceptors to some of these communities (Pensacola cultures) even slowed down the transformation. It should be noted that the aquifer solids from the Pensacola site were derived from actively methanogenic zones of this aquifer, which can explain the enrichment of fermentative/ methanogenic activity.

All of the inoculum sources tested showed at least some kind of anaerobic catabolism of alkylbenzenes (toluene, xylenes, ethylbenzene), which indicates that anaerobic degradation of these compounds might be widespread in the subsurface environment. In contrast, degradation of benzene was not observed using any of the inocula tested in this project, and benzene has proven to be highly recalcitrant in other studies as well. The type of anaerobic degradation of aromatic hydrocarbons that will occur at a specific site will depend on the contamination history of that site, the presence of specific microbial groups, the availability of exogenous electron acceptors, and a variety of environmental factors, including the geochemical characteristics of the site. Additional efforts are warranted in order to learn more about the physical and chemical factors that influence anaerobic catabolism of aromatic hydrocarbons.

SECTION 3

RECOMMENDATIONS

The results of this project leave open several avenues of research that were not explored because of time constraints. General areas of research that would be productive extensions of this project include:

- Isolation and characterization of pure, toluene-degrading cultures from Patuxent River and Pensacola enrichment cultures, or, if pure cultures cannot be obtained, examination of syntrophic relationships that appear to be required for toluene degradation.

- Determination of the metabolic pathways of toluene degradation under methanogenic and sulfate-reducing conditions, and examination of the effect of iron on the metabolic pathway in the sulfate-reducing (Patuxent River) cultures.

- Examination of the role of iron in facilitating toluene degradation in Patuxent River enrichments and closer examination of the relevance of this process to *in-situ* toluene degradation in aquifers under sulfate-reducing conditions.

The results of this study suggest that anaerobic transformation of alkylbenzenes may be ubiquitous in the subsurface. Anaerobic microorganisms could be taken advantage of at contaminated sites where it is not possible to introduce sufficient oxygen for aerobic degradation. However, samples from such sites would require preliminary laboratory investigation to determine whether (and which) exogenous electron acceptors were required, whether the addition of these acceptors and/or nutrients was necessary at the site, and which environmental factors were most important in controlling the anaerobic biodegradation process.

SECTION 4

MATERIALS AND METHODS

The primary goals of the project were to enrich iron-reducing or fermentative microbial communities that could degrade monoaromatic hydrocarbons. To enrich iron-reducing bacteria, the initial experimental approach consisted of screening sediments for hydrocarbon-degrading activity using a basal mineral medium that was either amended with ferric iron or was not amended with significant concentrations of any potential electron acceptor. Monitoring originally focused on aromatic hydrocarbons (the substrates) and the appearance of Fe(II) (as an indication of ferric iron reduction). Sulfate was present in some sediment inocula (e.g., Patuxent River) and eventually became a suspected electron acceptor; thus, subsequent monitoring included analysis for sulfate as well. To enrich fermentative/methanogenic microorganisms, aquifer solids (Pensacola, FL) were incubated with basal mineral medium and vitamins; carbon dioxide was added as the only exogenous electron acceptor. Monitoring focused on aromatic hydrocarbons and production of gaseous products (CO_2 and CH_4).

CONSTRUCTION AND MAINTENANCE OF MICROCOSMS AND ENRICHMENTS

Microcosms and enrichments were prepared under strictly anaerobic conditions in an anaerobic glove box (Coy Laboratory Products, Inc., Ann Arbor, MI). The microcosms and enrichments were contained in glass, 250-mL, screw-cap bottles that were sealed with Mininert PTFE valves (Alltech Associates, Inc, Deerfield, IL); the Mininert valves provided a tight seal for the bottles while allowing for sampling of headspace and culture medium via syringe. The combined volume of medium and wet solids (in microcosms) or medium and culture inoculum (in enrichments) was 200 mL in most experiments, although 170 mL combined volume was used in early experiments with Seal Beach and Traverse City materials. The remaining volume of the bottles was headspace. All weighing of medium and sediment was performed in the anaerobic glove box. Five aromatic hydrocarbons (benzene, toluene, ethylbenzene, and *o*- and *p* -xylenes) were initially spiked at concentrations of roughly 40 to 100 μM per compound. Sterile controls were removed from the glove box after sealing and were autoclaved at 121°C. Replicate

microcosms and controls were used in all the experiments. Incubation was carried out at 35°C in an anaerobic glove box. Although 35°C is higher than typical aquifer temperatures, the choice of this temperature was based in part on the practical consideration that experiments would probably proceed more rapidly. In addition, the average temperature of the Pensacola aquifer was relatively high (near 25°C).

After degradation had begun, regular re-spiking with aromatic hydrocarbons and sulfate was performed as necessary. Aromatic hydrocarbons (in neat form) were injected directly into active microcosms with a 10-µL syringe when depleted (typically weekly or biweekly in Patuxent River and Pensacola samples). For Patuxent River samples, an anaerobic solution of $MgSO_4$ was injected via 3-mL syringe as necessary (addition was performed inside the anaerobic glove box) and a slurry of amorphous $Fe(OH)_3$ was occasionally added to microcosms and enrichments via syringe.

Growth medium

The composition of the growth medium used for all microcosms and enrichments in the ferric iron-reduction studies (Medium 1) was based on Lovley and Phillips (1986a) and is shown in Table 1. The final pH of the medium was approximately 7.

TABLE 1. MEDIUM 1 COMPOSITION

Compound	Conc. (g/L)	Conc. (mM)
$NaHCO_3$	2.5	30
NH_4Cl	1.5	28
$NaH_2PO_4 \cdot H_2O$	0.6	4.35
NaCl	0.1	1.7
KCl	0.1	1.3
$CaCl_2 \cdot 2H_2O$	0.1	0.68
$MgCl_2 \cdot 6H_2O$	0.1	0.49
$MgSO_4 \cdot 7H_2O$	0.1	0.41
$MnCl_2 \cdot 4H_2O$	0.005	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.001	0.004

The composition of the growth medium used for fermentative/methanogenic degradation studies (Medium 2) was a modification of several previous recipes (Owen et al., 1979; Shelton and Tiedje, 1984; Godsy, 1980; Ridgway et al., 1989; Tong et al., 1990). The composition is

shown in Table 2. The final pH of the medium was approximately 7, but was later adjusted to 6 because the particular methanogenic communities operated better at a lower pH.

TABLE 2. MEDIUM 2 COMPOSITION

Compound	Conc. (mg/L)	Conc. (mM)
KH_2PO_4	272	2.0
K_2HPO_4	348	2.0
NH_4Cl	535	10.0
$MgSO_4 \cdot 7H_2O$	125	0.51
$CaCl_2 \cdot 2H_2O$	70	0.48
$FeCl_2 \cdot 4H_2O$	20	0.1
Trace Minerals:		
H_3BO_3	0.6	9.7×10^{-3}
$ZnCl_2$	0.2	1.5×10^{-3}
$Na_2MoO_4 \cdot 2H_2O$	0.2	0.8×10^{-3}
$NiCl_2 \cdot 6H_2O$	1.5	6.3×10^{-3}
$MnCl_2 \cdot 4H_2O$	2.0	10.1×10^{-3}
$CuCl_2 \cdot 2H_2O$	0.2	1.2×10^{-3}
$CoCl_2 \cdot 6H_2O$	3.0	12.6×10^{-3}
Na_2SeO_3	0.04	0.2×10^{-3}
$Al_2(SO_4)_3 \cdot 18H_2O$	0.2	0.3×10^{-3}
Buffer:		
$NaHCO_3$	1,200	14.3
Resazurin	1.0	0.004
Reducing Agent:		
FeS (amorphous*)	54.8	0.62
Vitamins:		
Biotin	0.002	
Folic acid	0.002	
Pyridoxine hydrochloride	0.02	
Riboflavin	0.005	
Thiamine	0.005	
Nicotinic acid	0.005	
Pantothenic acid	0.005	
p-Aminobenzoic a. (PABA)	0.005	
Cyanocobalamin (B_{12})	0.005	
Thioctic (Lipoic) acid	0.005	
Coenzyme M (Mercaptoethanesulfonic a.)	0.1	

*Brock and O'Dea (1977)

Both media (excluding $NaHCO_3$, FeS, and vitamins, where applicable) were sterilized in an autoclave at 121°C for 20 minutes and were then purged with an oxygen-free mixture of N_2(79%)/CO_2 (21%) for 45 minutes. Medium 1 was amended with $NaHCO_3$ immediately after purging, and was flushed in the antechamber of an anaerobic glove box; the antechamber sequence consisted of evacuation under vacuum, flushing with N_2, evacuation, flushing with N_2, and flushing with the glove box atmosphere [N_2 (80%)/CO_2 (10%)/ H_2 (10%)]. Medium 2 was immediately sealed after autoclaving and purging with N_2 /CO_2 and was taken into an anaerobic glove box. Vitamins, FeS, and $NaHCO_3$ were added to the medium from sterile, anaerobic stock solutions.

Amorphous $Fe(OH)_3$

Iron was added to microcosms in the form of amorphous $Fe(OH)_3$. Amorphous $Fe(OH)_3$ was used rather than more crystalline iron phases because the rate of biological ferric iron reduction tends to increase significantly with decreasing degree of crystallinity (Lovley, 1987 and references therein). This iron phase was prepared by neutralizing a 0.1 M ferric chloride solution with sodium hydroxide. The precipitate was aged for four hours after neutralization; during this period, the pH was adjusted periodically with sodium hydroxide to neutralize acidification resulting from ferric iron hydrolysis. The precipitate was then rinsed with Milli-Q water in a 2-L Büchner funnel to reduce the residual chloride concentration to a level that would correspond to less than 1 mM in a 200 mL microcosm or enrichment. The residual chloride concentration of the filtrate was determined by measuring conductivity against NaCl standards. The amorphous $Fe(OH)_3$ was prepared with sterilized glassware and reagents prepared in sterilized Milli-Q water, but the iron phase itself could not be autoclaved because the elevated heat and pressure would facilitate crystallization, which was not desired.

Soil and sediment inocula

Patuxent River soil used for experiments PR1 and PR2 was collected at the Naval Air Station, Patuxent River (MD) in September, 1987. The site was extensively contaminated with aviation fuel (e.g., JP-5). The sample was collected near a hydrocarbon seep in a marshy area and was received in water-saturated form in a plastic, screw-capped container. It was stored at 4°C until its use in microcosms roughly two years after collection. The soil appeared to be fine-grained and rich in organic matter, and contained some plant detritus. More recently (in July 1990), additional material (soil and aquifer sediment) was collected from the site and was used in

Experiment PR3. The soil used in Experiment PR3 (Samples D7 - D10) was collected near the hydrocarbon seep that was sampled earlier.

Samples of gasoline-contaminated aquifer material from the Seal Beach (CA) site were collected in May, 1987 with a hollow stem auger at a depth of 2.54 m above the water table. After collection, the contents of the auger were transferred to a 20-L plastic bucket with an air-tight snap lid. The sediment was stored at 4°C until use.

Core samples of coarse-grained aquifer material contaminated by aviation gasoline from the U.S. Coast Guard Air Station site in Traverse City (MI) were provided by the U.S. Environmental Protection Agency (Ada, OK). The samples were collected aseptically with an auger under anaerobic conditions in August, 1989. The core sample used in this study was taken from below the water table, at 5.89-6.15 m below ground surface, from an actively methanogenic zone. It contained very low concentrations of nitrogen and phosphorus (μg/L quantities), and 9.5 mg/L sulfate (Stephen Hutchins, personal communication). The sample was stored at 4°C in a sealed Mason jar until use.

Aquifer solids from Pensacola (FL) were provided by E.M. Godsy (U.S. Geological Survey, Menlo Park, CA). The Pensacola aquifer (which is a U.S. Geological Survey national research demonstration area) consists of fine-to-coarse sand deposits interrupted by discontinuous silts and clays. The upper 30 m of the aquifer are contaminated by creosote and pentachlorophenol. The samples were obtained from an actively methanogenic, sandy zone of the aquifer, downgradient from the contamination source, at a depth of approximately 6 m. The ground water at this depth contained tens of mg/L of nitrogen heterocycles, simple polynuclear aromatic hydrocarbons, and phenols (Goerlitz et al., 1985). The sampling was performed with a hollow-stem auger and a split-spoon core sampling device. After the sampler was withdrawn from the borehole and split lengthwise, a portion of the core was removed with a sterile spatula. The center of the core was then subsampled by pushing a sterile brass tube into the core, extruded with a sterile syringe plunger, and stored (at 4°C) in sterile, sealed containers previously flushed with argon.

EXPERIMENTAL DESIGN

Many experimental trials were attempts to screen for activity of iron-reducing bacteria. Thus, experimental design often consisted of setting up parallel series of microcosms, one with and one without added iron. The following series of tables (Tables 3 - 11) describes the initial conditions of series of microcosms and enrichments that involved iron amendments. Tables 3 through 9 are ordered chronologically with respect to the date of preparation of microcosms or enrichments. The final table in the series (Table 11) shows amendments made to microcosms in Experiment PR2; this table was included because the detailed history of the amendments is useful in interpreting the data.

In the experiments with fermentative/methanogenic bacteria, two types of mixtures of aromatic compounds were spiked into the microcosms. In the first case, the mixture consisted only of aromatic hydrocarbons (toluene, ethylbenzene, *o*-xylene, and *p*-xylene), each at a concentration of 40 μM. In the second case, *p*-cresol (0.46 mM), a putative intermediate of toluene transformation under anaerobic conditions (Grbić-Galić and Vogel, 1987), was added to the hydrocarbon mixture. No other organic amendments were made and no exogenous electron acceptors were added, except CO_2.

TABLE 3. INITIAL CONDITIONS FOR SEAL BEACH MICROCOSMS

Microcosm	Autoclaved	am. $Fe(OH)_3$ [ca. 12-16 mM Fe(III)]	Sediment (ca. 24-26 g)	Acetate (280 μM)/ benzoate (77 μM)
1	X			
2	X		X	
3	X		X	
4			X	X
5			X	
6			X	
7			X	
8	X	X		
9	X	X	X	
10	X	X	X	
11		X	X	X
12		X	X	
13		X	X	
14		X	X	

TABLE 4. INITIAL CONDITIONS FOR TRAVERSE CITY MICROCOSMS

Microcosm	Autoclaved	am. $Fe(OH)_3$ [ca. 12-16 mM Fe(III)]	Sediment (ca. 24-26 g)	Acetate (280 μM)/ benzoate (77 μM)
15	X			
16	X		X	
17	X		X	
18			X	X
19			X	
20			X	
21			X	
22	X	X		
23	X	X	X	
24	X	X	X	
25		X	X	X
26		X	X	
27		X	X	
28		X	X	

TABLE 5. INITIAL CONDITIONS FOR EXPERIMENT PR1 MICROCOSMS (PATUXENT RIVER)

Microcosm	Autoclaved	amFe(OH)$_3$ [ca. 12 mM Fe(III)]	Sediment (ca. 30 g)	*p*-cresol (95 μM)	Fatty acid mixture*
A1	X				
A2	X				
A6	X		X		
A8	X	X	X		
A17			X	X	
A18			X		X
A19			X		
A21		X	X	X	
A22		X	X		X
A23		X	X		
A24		X	X		

Note: * Fatty acid mixture was 2:1:1 (molar) mixture of acetic acid, propionic acid, and butyric acid with a total acid concentration of ca. 0.47 mM.

TABLE 6. INITIAL CONDITIONS FOR ENRICHMENTS OF EXPERIMENT PR1

Enrichment	Autoclaved	amFe(OH)$_3$ [ca. 20 mM Fe(III)]	Fe(III)-citrate (2.5 g/L)	Inoculum (15 mL)*
B1	X	X	X	
B2	X	X	X	X
B3		X		X
B4		X		X
B5		X		X
B6			X	X
B7		X	X	X
B8		X	X	X

Note: * Inoculum was a mixture of microcosms A19, A21, A22, and A23 taken on Day 58 of incubation.

TABLE 7. INITIAL CONDITIONS FOR EXPERIMENT PR2 MICROCOSMS (PATUXENT RIVER)

Microcosm	Autoclaved	amFe(OH)$_3$ [ca. 20 mM Fe(III)]	Sediment (ca. 30 g)	Nitrate (5 mM)
C1	X		X	
C2	X	X	X	
C3			X	X
C4			X	
C5			X	
C6			X	
C8		X	X	
C9		X	X	
C10		X	X	

TABLE 8. INITIAL CONDITIONS FOR ENRICHMENTS (DAY 88) OF EXPERIMENT PR2

Enrichment	Autoclaved	amFe(OH)$_3$ [ca. 2 mM Fe(III)]	Inoculum* (40 g)	Hydrogen sulfide (ca. 1.5 mM)
E4	X	X	X	X
E6		X	X	X
E7		X	X	

Note: * Inoculum was Microcosm C9 taken on Day 88.

TABLE 9. INITIAL CONDITIONS FOR ENRICHMENTS (DAY 235) OF EXPERIMENT PR2

Enrichment	Autoclaved	amFe(OH)$_3$ [ca. 2 mM Fe(III)]	$FeSO_4$ (ca. 2 mM)	$MgSO_4$ (ca. 2 mM)	Inoculum* (35 g)
F1				X	X
F2				X	X
F3		X		X	X
F4		X		X	X
F5		X		X	X
F6		X		X	X
F7			X		X
F8			X		X
F9			X		X
F10			X		X

Note: * Inoculum was Microcosms C8 & C10 (homogenized) taken on Day 235.

TABLE 10. INITIAL CONDITIONS OF EXPERIMENT PR3 (PATUXENT RIVER)

Microcosm	Autoclaved	am. $Fe(OH)_3$ [ca. 10 mM Fe(III)]	Sediment (50 g)
D3		X	X
D4		X	X
D5		X	X
D6		X	X
D7	X	X	X
D8		X	X
D9		X	X
D10		X	X

Note: The site of collection for soil used in Microcosms D7 - D10 was near the site of collection for soil used in Experiments PR1 and PR2.

TABLE 11. AMENDMENTS FOR EXPERIMENT PR2 MICROCOSMS (DAY 0 - 70)

Time (days)	Bottle C3	Bottle C4	Bottle C5	Bottle C6	Bottle C8	Bottle C9	Bottle C10
0	N (5mM)				amFO (20mM)	amFO (20mM)	amFO (20mM)
14		N (2.5mM)	Ac (0.35mM)				
17	T,X						
24	N (5mM)						
31					amFO (10 mM)		
34	amFO (10 mM)						
35	T					amFO (10mM)	amFO (10mM)
40					T		
47	T	T			T	T	T
48	N (5mM)	amFO (10mM) Mo (20mM)	amFO (10mM)	S (0.40mM)	amFO (10mM) S (0.80mM)		
54					T		
55					S (1.2mM)	S (1.2mM)	S (1.2mM)
58		N (5mM)	S (0.40mM)		S (0.40 mM),T	T	T
67					S (0.80 mM), T	S (0.80 mM), T	S (0.80 mM), T
70	N (5mM), T	N (5mM), T	S (1.0mM)	S (1.0mM)	S (1.0 mM), T	S (1.0 mM), T	S (1.0 mM), T

where:

Ac = acetate
amFO = amorphous $Fe(OH)_3$
Mo = molybdate
N = nitrate
S = sulfate
T = toluene (typically 0.15 to 0.2 mM addition, but variable)
X = *o* -xylene (0.04 mM)

ANALYTICAL METHODS

Aromatic hydrocarbon analysis

Aromatic substrates were measured by a static headspace technique using an HP Model 5890A gas chromatograph (Hewlett-Packard Company, Palo Alto, CA) with a HNU Model PI 52-02A photoionization detector (10.2 eV lamp; HNU Systems, Inc., Newton, MA) and a 30-m DB-624 megabore fused silica capillary column (3.0 µm film thickness; J & W Scientific, Folsom, CA). Analyses were isothermal (65°C) and splitless. Sampling and analysis of headspace from microcosms, enrichments, and standards was performed identically: 300 µL of headspace was sampled through the Mininert valve of microcosms, enrichments, or standards with a 500 µL gas-tight syringe that included a PTFE plunger tip and a side-port needle.

Standards for headspace analyses were prepared by spiking a methanolic stock solution of the aromatic analytes into a Mininert-sealed bottle that contained 200 mL of water. The transfer of the methanolic stock solution was made with a gas-tight 500-µL syringe. The amount of stock solution (and correspondingly, the mass of each analyte) added to the standard bottle was determined gravimetrically by weighing the syringe immediately before and after spiking. The aqueous concentration of aromatic compounds in standards was estimated by using Henry's Law constants obtained from the literature (Mackay and Shiu, 1981), and Equation 2 below, which was derived from the definition of Henry's Law (Equation 1) and a mass balance expression.

Eq. 1 $$H_c = C_g/C_w$$

Eq. 2 $$C_w = m_T/(V_w + H_c * V_g)$$

where:

H_c = Henry's Law constant (dimensionless)

C_g = Gaseous solute concentration (moles/L)

C_w = Aqueous solute concentration (moles/L)

V_g = Volume of headspace in bottle (L)

V_w = Volume of water in bottle (L)

m_T = Total amount of solute added to standard (moles).

The aqueous concentration of aromatic compounds in microcosms and enrichments was determined by comparison of peak areas to standards. Note that the mass of analyte sorbed to solids was not represented in this measurement.

Based on detailed studies of Henry's constants of other volatile organic compounds that entailed spiking with methanolic stock solutions (Gossett, 1985), it was assumed in the present study that the presence of methanol at the applicable methanol concentrations would not significantly affect the gas/liquid partitioning of the analytes in the standards.

There was a noteworthy shortcoming of the headspace analyses in microcosms, which related to the presence of sediment. In microcosms, the calculated amount of aromatic substrate degraded was underestimated when the substrate was completely consumed (a regular occurrence in some Patuxent River microcosms). This underestimate resulted because the toluene that was sorbed to sediment immediately after spiking would progressively desorb and be degraded but would not be accounted for in the headspace analysis that immediately followed spiking. Controlled sorption experiments were not performed to quantify this effect. However, it is roughly estimated that the reported amount of toluene consumed in Patuxent River microcosms (Experiment PR2) could be lower by as much as 15 to 25 percent based upon (1) the known amount of pure toluene spiked into Patuxent River microcosms, (2) the corresponding aqueous concentrations as determined by headspace analysis, and (3) the assumption that the mass of toluene that sorbed just after spiking eventually desorbed and was degraded by the time of the next analysis, when toluene was undetectable. In contrast, the relatively low concentrations of solids in enrichments minimize this concern for enrichment cultures. Thus, toluene consumption reported in Patuxent River enrichments should be considered to be more accurate than in microcosms in terms of potential sorption effects.

Fe(II) Analysis

HCl-extractable Fe(II) was measured as described by Lovley and Phillips (1986b). An aliquot of 0.1 - 0.2 g of culture medium was removed via syringe and was weighed into a glass vial containing 5.0 mL of 0.5 M HCl. After approximately 15 minutes of acid extraction, 0.2 mL of the mixture was added to a glass vial containing 5.0 mL of ferrozine [1 g/L; 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid, monosodium salt] in 50 mM HEPES buffer [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] (adjusted to pH 7). After being mixed for 15 seconds, the mixture was filtered through a 0.2 μm, Nylon 66 syringe filter and the absorbance at

562 nm was measured with an HP Model 8451A diode array spectrophotometer (Hewlett-Packard Company, Palo Alto, CA). The advantages of first dissolving culture medium in HCl are that: (1) Fe(II) in poorly crystalline precipitates (such as amorphous ferrous sulfide) could be determined in addition to soluble Fe(II), which is important because precipitates were undoubtedly the predominant Fe(II) phases in the systems studied, and (2) under the moderately oxidizing conditions of analysis, Fe(II) is thermodynamically stable at acidic pH but not at neutral pH. Standards were prepared by dissolving a known amount of ammonium iron (II) sulfate hexahydrate (stored in the dark in an anaerobic glove box) in 0.5 M HCl, and were analyzed as described above.

Sulfate and nitrate analysis

Sulfate and nitrate in filtered culture medium were determined by ion chromatography (Dionex Series 4000i with a Nelson Analytical Chromatography Software system) equipped with an HPIC-AS4A column (Dionex, Sunnyvale, CA), an anion micro membrane suppressor, and a conductivity detector. Analyses were isocratic, with a sodium bicarbonate (0.75 mM) / sodium carbonate (2.2 mM) eluant. Ions were identified and quantified by comparing retention times and peak areas to those of external standards.

Gas chromatography/mass spectrometry (GC/MS) analysis

GC/MSD (mass selective detector) analyses for potential metabolites were performed on samples from some microcosms. An aliquot of 2.75 mL of culture liquid was acidified and extracted with 1 mL of diethyl ether. Derivatization was not attempted. Analyses were performed on an HP Model 5890 GC with a 60-m DB-5 column (J & W Scientific, Folsom, CA) coupled with a 5970 Series MSD (Hewlett-Packard Company, Palo Alto, CA). The temperature program was as follows: 40°C (hold 5 min) to 120°C (at 2°C/min) and then to 200°C (at 4°C/min). The mass scanning range was from 50 to 250 amu.

^{14}C Analysis

^{14}C-labeled toluene was used in the experiments with methanogenic Pensacola microcosms and suspended cultures. [^{14}C-ring]toluene and [^{14}C-methyl]toluene were purchased from Sigma Co. (St. Louis, MO). ^{14}C-Activity in liquid and gaseous samples from the cultures was determined on a Tricarb Model 4530 scintillation spectrometer (Packard Instrument Co., Downers

Grove, IL). Counting efficiency corrections were made using the external standard channels ratio method (Bell and Hayes, 1958). Three separate 1-mL liquid samples were counted for each analysis (Grbić-Galić and Vogel, 1987). All of them were mixed with 10 mL of liquid scintillation cocktail (Universol TM Biodegradable-Nontoxic-Nonflammable, ICN Biomedicals, Inc., Irvine, CA), but one was pretreated with 1 mL of 1N HCl, the other with 1 mL of 1N NaOH, whereas the third received no pretreatment. The pretreated samples were nitrogen-stripped before adding the scintillation cocktail. These measurements were used to determine the ^{14}C-activity in the volatile, nonvolatile, and CO_2 fractions, as described by Grbić-Galić and Vogel (1987).

SECTION 5

RESULTS AND DISCUSSION

This section will focus primarily on studies performed with Patuxent River material, including microcosm experiments (Experiments PR1 and PR2) as well as enrichment cultures. Microcosms containing Patuxent River material and enrichment cultures of these microcosms showed evidence of toluene degradation, sulfate reduction, and ferric iron reduction. In addition, the presence of amorphous $Fe(OH)_3$ had a marked effect on toluene degradation rates.

Although some degradation of aromatic substrates (e.g., toluene and ethylbenzene) was observed in Seal Beach and Traverse City microcosms, the activity could not be sustained and the presence of iron appeared to have little or no effect on degradation rates. Data for these materials will be summarized briefly in this section.

The studies of Pensacola microcosms showed that toluene and *o*-xylene were completely degraded under the conditions of methanogenic fermentation. The effects of *p*-cresol addition and other factors on the length of the acclimation period to toluene were investigated. Stable methanogenic consortia that were enriched from the microcosms degraded toluene, *o*-xylene, and *m*-xylene.

SULFATE REDUCTION AND FERRIC IRON REDUCTION IN PATUXENT RIVER SOILS

Experiment PR1 - Iron's relationship to toluene degradation rates

Results of the first experiment with Patuxent River material (Experiment PR1) are shown in Figure 1 in terms of toluene concentration versus time over the first two months of incubation. The data in Figure 1 represent averages of three groups of microcosms: (1) two controls (A6 and A8), (2) three microcosms without added amorphous $Fe(OH)_3$, and (3) four microcosms with added amorphous $Fe(OH)_3$. Although rates of toluene degradation for microcosms with and

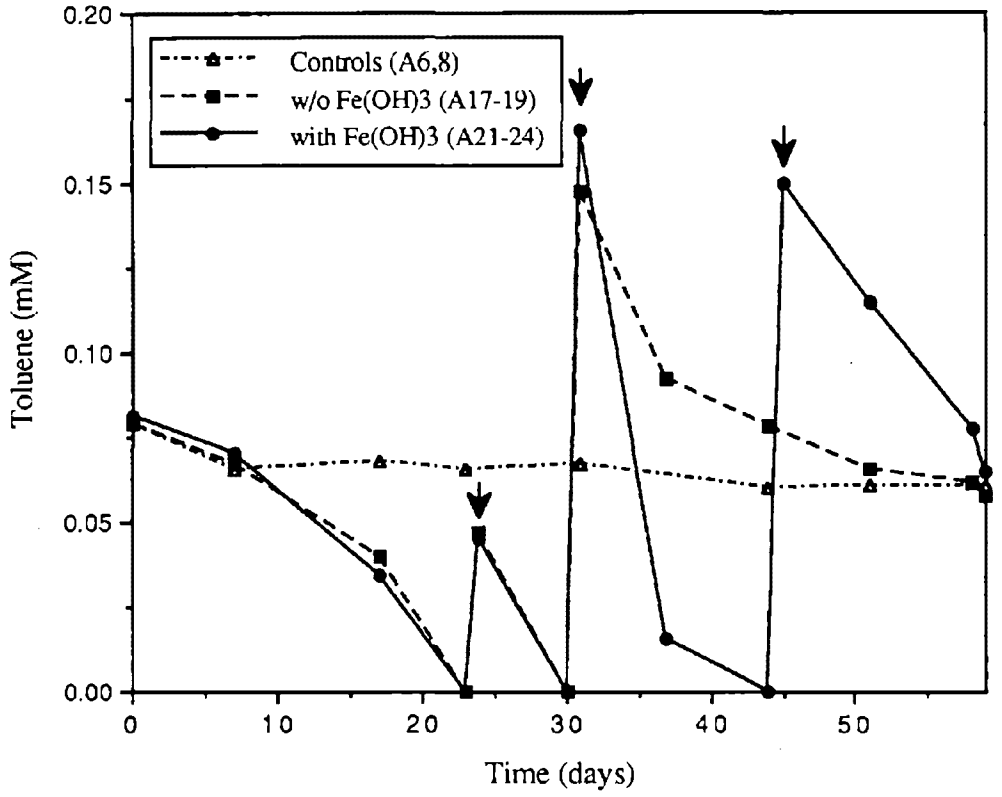

Figure 1. Average toluene concentrations vs. time in Experiment PR1 microcosms. Arrows indicate amendments of toluene.

without added iron were very similar for the first 3 to 4 weeks of incubation, the microcosms with added iron clearly had faster toluene degradation rates starting at Day 30. These differences in rates continued throughout the next 30 days of incubation, after which toluene degradation effectively ceased (based on later evidence, it is likely that this cessation of activity was due to depletion of sulfate, the electron acceptor).

The replicability of the effect of amorphous $Fe(OH)_3$ on toluene degradation rate is apparent in Figure 2, in which individual microcosms from Experiment PR1 are represented in terms of cumulative toluene degradation over time. The presence/absence of added iron appears to be the primary variable that distinguishes the toluene degradation rates of these seven microcosms, despite the fact that other variables were applicable (i.e., the presence of additional substrates, such as *p*-cresol, in certain microcosms; see Table 5). Because the range of variables measured was limited in this preliminary experiment, a more detailed assessment is not possible (e.g., determination of the electron acceptor). Dissolved/colloidal Fe(II) (i.e., that which could pass though a 0.2 μm filter) was measured, but equilibrium modeling suggested that solid forms of Fe(II) would predominate over dissolved forms in aqueous systems with the composition of the growth medium. Also, sulfate was not measured regularly in this experiment. In the follow-up experiment using the same soil inoculum (Experiment PR2), particulate Fe(II) and sulfate (the suspected electron acceptor) were also measured.

Experiment PR2 - Interrelationships among toluene, sulfate, and iron

In Experiment PR2, as in Experiment PR1, the presence of amorphous $Fe(OH)_3$ had an effect on toluene degradation, but the effect was qualitatively different in the two experiments. Toluene and sulfate concentrations versus time are shown in Figure 3a and b for the first 47 days of incubation of five microcosms and two controls. As shown in Figure 3a, toluene was not degraded in any microcosms during the first month of incubation. However, the addition of ca. 10 mM amorphous $Fe(OH)_3$ to the three microcosms that initially contained added iron (Microcosms C8, C9, and C10) initiated toluene degradation within a few days in each of those microcosms. Two microcosms that did not receive amorphous $Fe(OH)_3$ over the period shown (Microcosms C5 and C6) did not degrade toluene until roughly 40 days later than the iron-amended microcosms (as discussed later in this section).

Sulfate reduction was observed in all active microcosms (Figure 3b) but, over the period shown, was most rapid in the microcosms without added iron (Microcosms C5 and C6). Sulfate

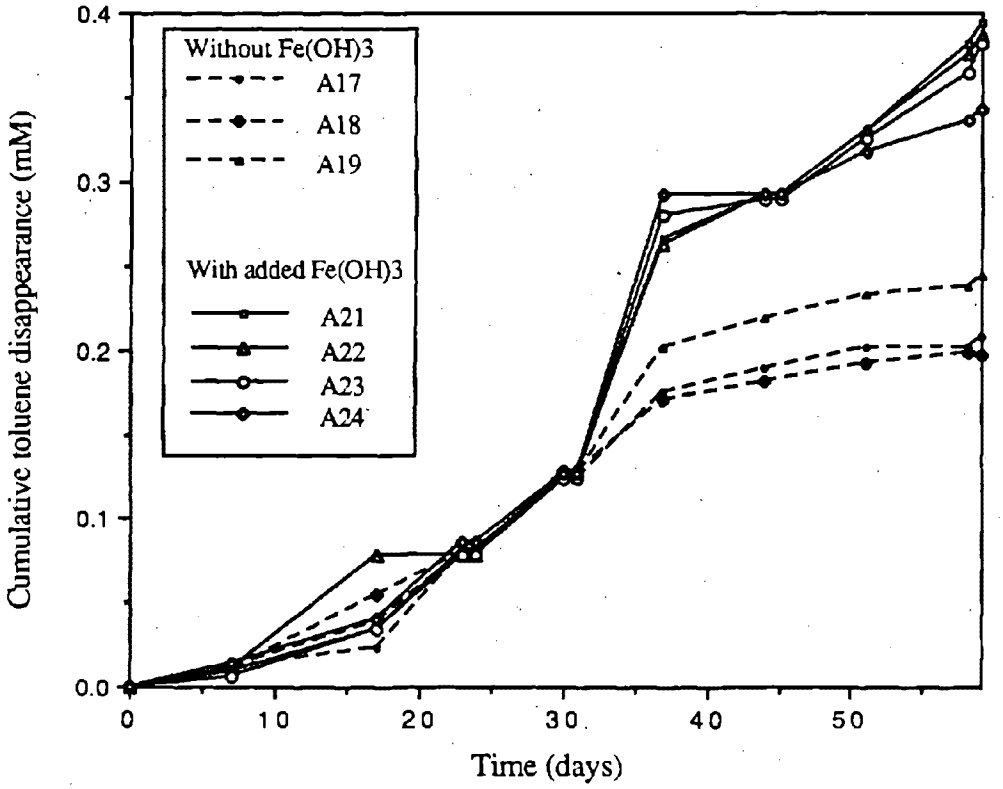

Figure 2. Cumulative toluene disappearance vs. time in Experiment PR1 microcosms

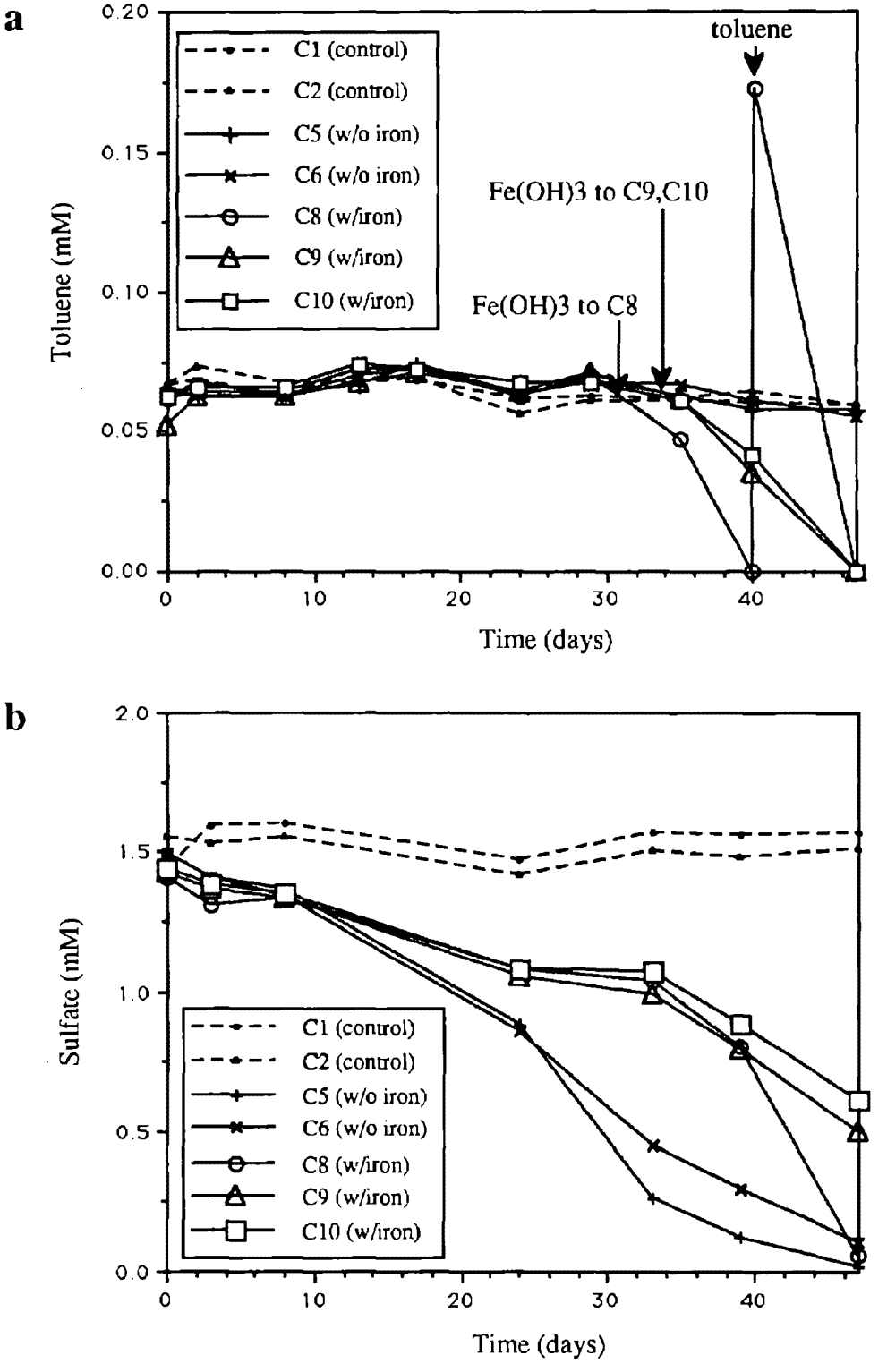

Figure 3. Toluene (a) and sulfate (b) vs. time in Experiment PR 2 microcosms.

reduction in the microcosms with added iron had virtually ceased between Days 24 and 33, before rapid toluene degradation began. Once toluene degradation began in the Patuxent River microcosms, the rate of sulfate reduction increased and there was a strong correlation between sulfate disappearance and toluene disappearance. Figure 4 depicts this correlation for Microcosm C8 over the first three months of incubation. Similarly strong regressions were observed for the other active microcosms. The ratio of sulfate consumed/toluene consumed in Microcosm C8 was 4.2 (Day 81); similar ratios (4.1 to 4.2) were observed for the other iron-amended bottles, C9 and C10. These values, which were corrected for the amount of sulfate reduced before toluene degradation began, approximate the theoretical ratios ranging from 4.0 (toluene oxidation to bicarbonate with bacterial cell growth; Equation 4) to 4.5 (toluene oxidation to bicarbonate with no cell growth; Equation 3); the estimation of cell growth in Equation 4 was derived using methods described by McCarty (1971, 1975).

Eq. 3 $$C_7H_8 + 4.5\ SO_4^{2-} + 3\ H_2O = 2.25\ H_2S + 2.25\ HS^- + 7\ HCO_3^- + 0.25\ H^+$$
$$\Delta G^{o\prime} = -49 \text{ kcal/reaction}$$

Eq. 4 $$C_7H_8 + 4.03\ SO_4^{2-} + 0.19\ NH_4^+ + 0.75\ CO_2 + 3.18\ H_2O$$
$$= 2.02\ H_2S + 2.02\ HS^- + 0.19\ C_5H_7O_2N \text{ (cells)} + 6.8\ HCO_3^- + 0.94\ H^+$$

The observed values of this ratio and the consistency of the ratio over months of monitoring provide preliminary evidence that toluene was oxidized to bicarbonate by sulfate-reducers. As further evidence of the link between toluene degradation and sulfate reduction, it was apparent that toluene degradation ceased when sulfate was depleted, and conversely, sulfate reduction ceased when toluene was depleted (such data are shown for an enrichment in the following section).

The reduction of Fe(III) [i.e., the appearance of Fe(II)] followed a similar trend as the disappearance of toluene and sulfate (shown for Microcosm C8 in Figure 5). Note that the Fe(II) concentration in Figure 5 represents the amount of iron reduced since the initiation of toluene degradation, not the absolute concentration. The available data cannot be used to demonstrate conclusively whether iron reduction was directly coupled with toluene oxidation (where ferric iron would have served as a terminal electron acceptor for toluene) or coupled with sulfate reduction (where ferric iron would have served as the electron acceptor for the oxidation of biogenic hydrogen sulfide). However, the observed stoichiometry gives some insight into this issue. The ratio of Fe(III) reduced/toluene consumed can be used to investigate the possibility that ferric iron-reducing bacteria are oxidizing toluene. As shown in Table 12, the stoichiometry of iron reduction

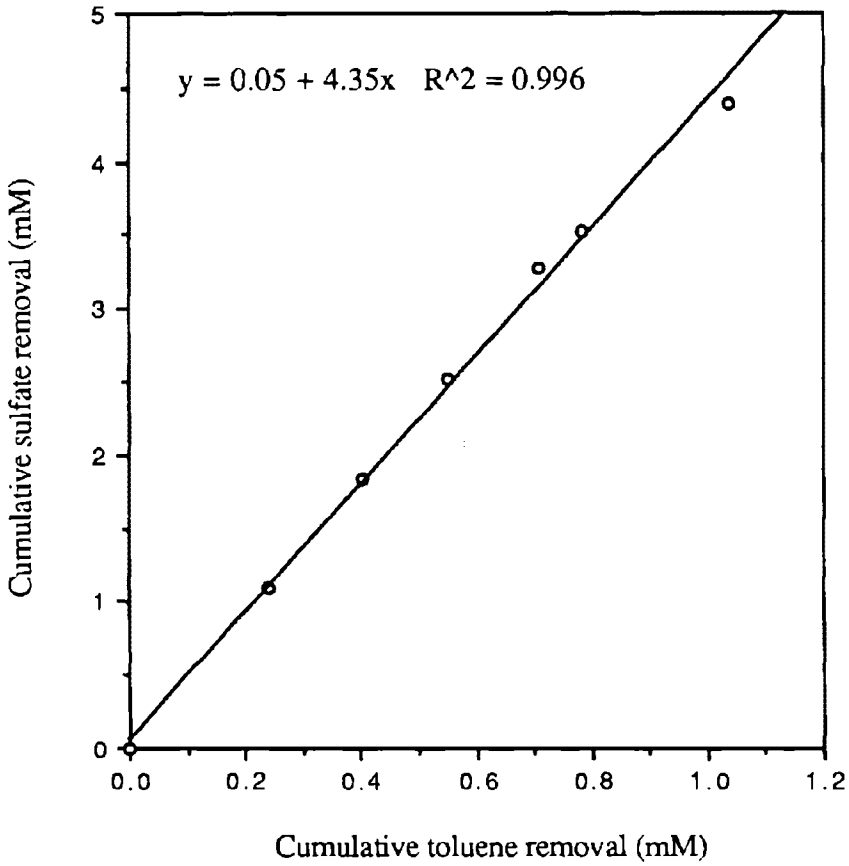

Figure 4. Sulfate vs. toluene consumed in Microcosm C8 (Experiment PR2; Day 0 to 85).

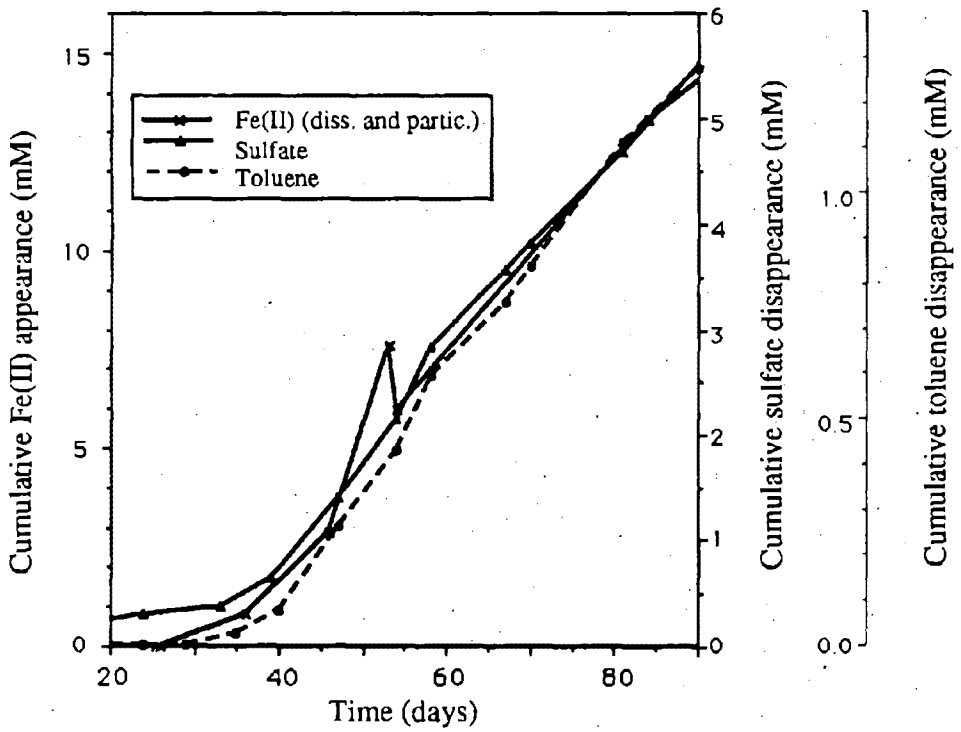

Figure 5. Cumulative Fe(II) appearance and sulfate and toluene disappearance in Microcosm C8 (Experiment PR2).

is not consistent with plausible biological processes [e.g., toluene oxidation to bicarbonate with amorphous $Fe(OH)_3$ being reduced to siderite; Equation 5] because the ratio is too low. Notably, the ratio of 36 shown for the iron reduction reaction is universal for any iron phases in the absence of cell growth, and would be the same for any Fe(III) phase being reduced to any Fe(II) phase in conjunction with toluene oxidation to carbon dioxide. Thus, two lines of stoichiometric evidence argue against biotic ferric iron reduction: (1) the ratio of Fe(III) reduced/toluene consumed and (2) the ratio of sulfate consumed/toluene consumed (which suggests the predominant role of sulfate reducers). Nonetheless, it is possible that ferric iron reducers were syntrophically associated with sulfate reducers and were performing only small steps in toluene oxidation rather than complete oxidation to carbon dioxide.

TABLE 12. THEORETICAL AND OBSERVED STOICHIOMETRY OF IRON REDUCTION AND TOLUENE OXIDATION

Reaction	Fe(III) reduced/ toluene consumed
Observed (Microcosm C8 - Day 90)	12
$Fe(OH)_3$ to $FeCO_3$ (without cells; Eq. 5)	36
$Fe(OH)_3$ to $FeCO_3$ (with cells)	21

Eq. 5 $$C_7H_8 + 36\ Fe(OH)_3\ (s) + 29\ HCO_3^- = 36\ FeCO_3\ (s) + 58\ H_2O + 29\ OH^-$$

The ratio of Fe(III)reduced/sulfate consumed provides an indication of the likelihood that ferric iron reduction resulted from an oxidation-reduction reaction between ferric iron and biogenic hydrogen sulfide. Table 13 presents the observed ratio (Microcosm C8) along with the theoretical ratios for various possible reactions between amorphous $Fe(OH)_3$ and hydrogen sulfide. The reactions were chosen based on studies of the abiotic, anoxic or oxygen-limited reactions of goethite or amorphous $Fe(OH)_3$ with hydrogen sulfide (Pyzik and Sommer 1981, Rickard 1974, Berner 1964) in which the observed products included ferrous sulfide, elemental sulfur, and, in Pyzik and Sommer (1981), thiosulfate. It is assumed in Table 13 and the related discussion that "sulfate consumed" is equivalent to "sulfide present" in the microcosms, that is, complete reduction of sulfate to sulfide is assumed; this assumption is well-founded based on the literature. Certain pairs of reactions in Table 13 (Equations 6 and 7; Equations 8 and 9) have the same sulfide oxidation product (i.e., elemental sulfur or thiosulfate) but different proportions of available sulfide relative to iron. For example, in Equations 6 and 8, there is sufficient sulfide present to precipitate

TABLE 13. THEORETICAL AND OBSERVED STOICHIOMETRY OF IRON REDUCTION VIA HYDROGEN SULFIDE

Reaction	Fe(III) reduced/ sulfate consumed
Observed (Microcosm C8 - Day 90)	2.6
Sulfide to S^o (Eq. 6)	0.67
Sulfide (limited) to S^o (Eq. 7)	2
Sulfide to thiosulfate (Eq. 8)	0.8
Sulfide (limited) to thiosulfate (Eq. 9)	4
Sulfide (limited) to thiosulfate (Eq. 10)	2.6

Eq. 6 $4\ Fe(OH)_3\ (s) + 3\ H_2S + 3\ HS^- = 2S^o + 4\ FeS\ (s) + 9\ H_2O + 3\ OH^-$

Eq. 7 $4\ Fe(OH)_3\ (s) + H_2S + HS^- = 2\ S^o + 4\ Fe^{2+} + 3\ H_2O + 9\ OH^-$

Eq. 8 $8\ Fe(OH)_3\ (s) + 5\ H_2S + 5\ HS^- = S_2O_3^{2-} + 8\ FeS\ (s) + 18\ H_2O + 3\ OH^-$

Eq. 9 $8\ Fe(OH)_3\ (s) + H_2S + HS^- = S_2O_3^{2-} + 8\ Fe^{2+} + 6\ H_2O + 15\ OH^-$

Eq. 10 $Fe(OH)_3\ (s) + 0.19\ H_2S + 0.19\ HS^- + 0.87\ HCO_3^-$
$= 0.125\ S_2O_3^{2-} + 0.13\ FeS\ (s) + 0.87\ FeCO_3\ (s) + 1.79\ H_2O + 0.81\ OH^-$

Eq. 11 $C_7H_8 + 11.8\ Fe(OH)_3\ (s) + 4.5\ SO_4^{2-} + 3.3\ HCO_3^-$
$= 1.48\ S_2O_3^{2-} + 1.54\ FeS\ (s) + 10.3\ FeCO_3\ (s) + 18.5\ H_2O + 9.34\ OH^-$

all reduced iron as ferrous sulfide, whereas in Equations 7 and 9 there is sufficient sulfide present only to reduce all available ferric iron, but not to precipitate any of the reduced iron. Intermediate amounts of sulfide would result in intermediate ratios. Equation 10 represents a reaction that agrees with the observed stoichiometry. In this reaction, there is insufficient sulfide present to precipitate all reduced iron as FeS, as was apparently the case in Experiment PR2 (compare *y* axes in Figure 5). The remainder of the reduced iron is precipitated as $FeCO_3$ in Equation 10, as would be expected based on equilibrium considerations. A combination of Equation 10 and Equation 3 results in an overall reaction (Equation 11) that is consistent with the observed stoichiometry in Microcosm C8 in terms of iron, sulfate, and toluene; this suggests, but does not prove, that toluene is being oxidized to carbon dioxide by sulfate-reducing bacteria and that the resulting sulfide is responsible for the observed iron reduction. The abiotic reaction proposed for hydrogen sulfide

and amorphous $Fe(OH)_3$ is only one of a number of possible reactions that could explain the stoichiometry.

Interpretation of the role of iron is complicated by results for Microcosm C6, which received no iron additions. Toluene degradation began in Microcosm C6 around Day 70 and has continued at a rate comparable to bottles that received additional iron (e.g., Microcosm C8; Figure 6). It should be noted that, although iron was not added to Microcosm C6, significant indigenous reduced iron was present in this microcosm: a relatively constant concentration of ca. 20 mM Fe(II) was measured in Microcosm C6 over several months. Since toluene degradation commenced in Microcosm C6, the correlation between toluene and sulfate disappearance has been strong ($r^2 >$ 0.98), although the ratio of sulfate consumed/toluene consumed (ca. 5.1) is somewhat higher than that observed for the iron-amended microcosms (i.e., C8, C9, and C10). The higher ratio may reflect the consumption of an additional electron donor that was present in the sediment, as indicated by the fact that sulfate was reduced in Microcosm C6 for roughly 70 days before toluene degradation began (Figure 3b). The microflora of Microcosm C6 have not been further studied, although comparisons of this assemblage with assemblages of enrichments that responded to iron addition could enhance our understanding of the role of iron in toluene-degrading, sulfate-reducing microorganisms.

Patuxent River enrichments - Reproduction of trends seen in microcosms

Attempts to make enrichment cultures from microcosms in Experiment PR1 were unsuccessful, however, active enrichments were generated from microcosms of Experiment PR2. Like the Patuxent River microcosms, enrichments generated from Microcosm C9 (see Table 8) demonstrated a strong relationship between the presence of amorphous $Fe(OH)_3$ and toluene degradation rate (Figure 7). Several interesting features are apparent in Figure 7: (1) Enrichment E6, which initially received 1.5 mM sulfide in addition to 2mM amorphous $Fe(OH)_3$, appeared to degrade toluene considerably more slowly than Enrichment E7, which initially received the same amount of iron but no sulfide, and (2) the addition of amorphous $Fe(OH)_3$ to both enrichments on Day 32 markedly increased toluene degradation rates, especially in Enrichment E7, in which toluene degradation had slowed to the rate of Enrichment E6 after Day 20. Considerably lower concentrations of Fe(III) were spiked into the Patuxent River enrichments than in the microcosms, and there is some evidence in Figure 7 that additional iron amendments were required to sustain toluene degradation rates (see Enrichment E7 between Days 20 and 30).

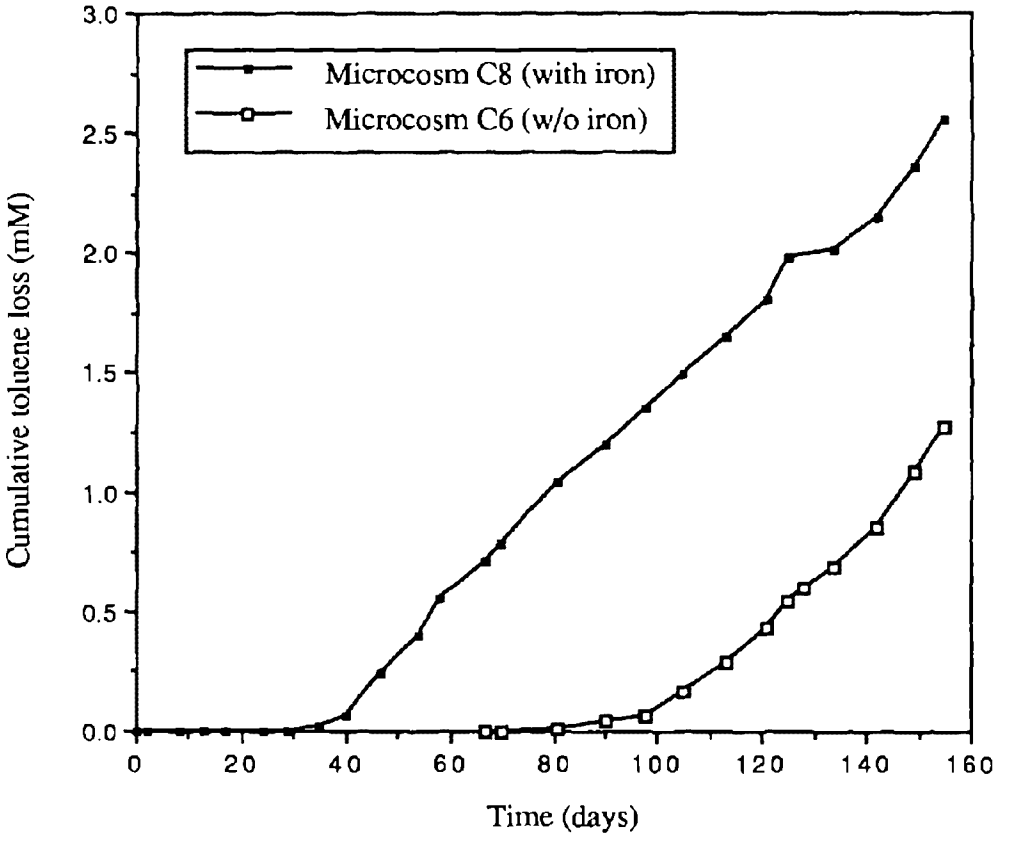

Figure 6. Cumulative toluene disappearance vs. time in Microcosms C6 and C8 (Experiment PR2).

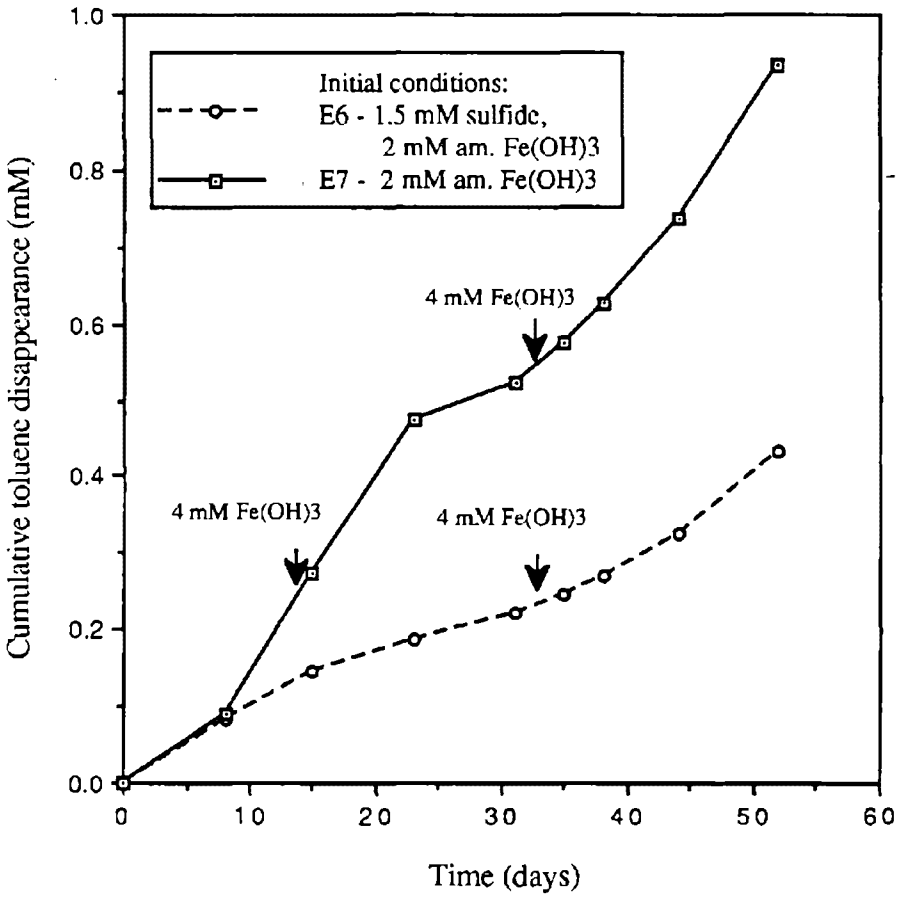

Figure 7. Cumulative toluene disappearance vs. time in two enrichments from Experiment PR2.

The strong relationship between Fe(II) appearance, toluene disappearance, and sulfate disappearance that was demonstrated for microcosms (Figure 5) was also evident in enrichments (data for Enrichment E7 are shown in Figure 8). Notably, the sulfate consumed/toluene consumed ratios in Enrichments E6 and E7 (ranging from 3.5 to 3.7; Day 84) were approximately 20 percent lower than the ratios observed for the microcosms. The lower ratio in enrichments may have been due to the lower concentrations of solids in the enrichments, which resulted in headspace analyses that were more representative of true toluene consumption because they were largely unaffected by sorption (recall discussion in Analytical Methods section). However, another set of enrichments (see Table 9) had typical ratios in the range of 3.9 to 4.2 (by Day 53), which is consistent with the ratios observed in Patuxent River microcosms. It is possible that the variability observed in these ratios was a result of analytical variability, or was related to variability among microbial assemblages in different systems (e.g., sulfate reducers may have played a different metabolic role in toluene degradation in different microcosms and enrichments).

As further evidence of the close linkage between toluene degradation and sulfate reduction, a controlled test of the effect of sulfate depletion on toluene degradation and the effect of toluene depletion on sulfate reduction was performed with Enrichment F9. The results are shown in Figure 9. When sulfate was depleted (Days 53 to 56), toluene was not appreciably degraded. After sulfate was spiked into the enrichment (Day 56), concurrent toluene degradation and sulfate reduction occurred until Day 60, when toluene was depleted. In the absence of toluene (Days 60 to 63), sulfate reduction was not apparent. The ratio of sulfate consumed/toluene consumed during this test was approximately 4.4 (between Days 53 and 60).

Experiment PR3 - Additional Patuxent River microcosms

As shown in Table 10, eight microcosms were generated with recently collected soil and aquifer sediment from various portions of the Patuxent River site. Some of the Patuxent River microcosms have shown toluene degrading activity (particularly D6 and D9), but the activity ceased when sulfate was depleted. In order to see if ferric iron reducers were present that could carry out toluene degradation, sulfate was not replenished but amorphous $Fe(OH)_3$ was added. Based on the cessation of toluene-degrading activity over weeks of monitoring, either ferric iron reducers were not present or their growth was extremely slow.

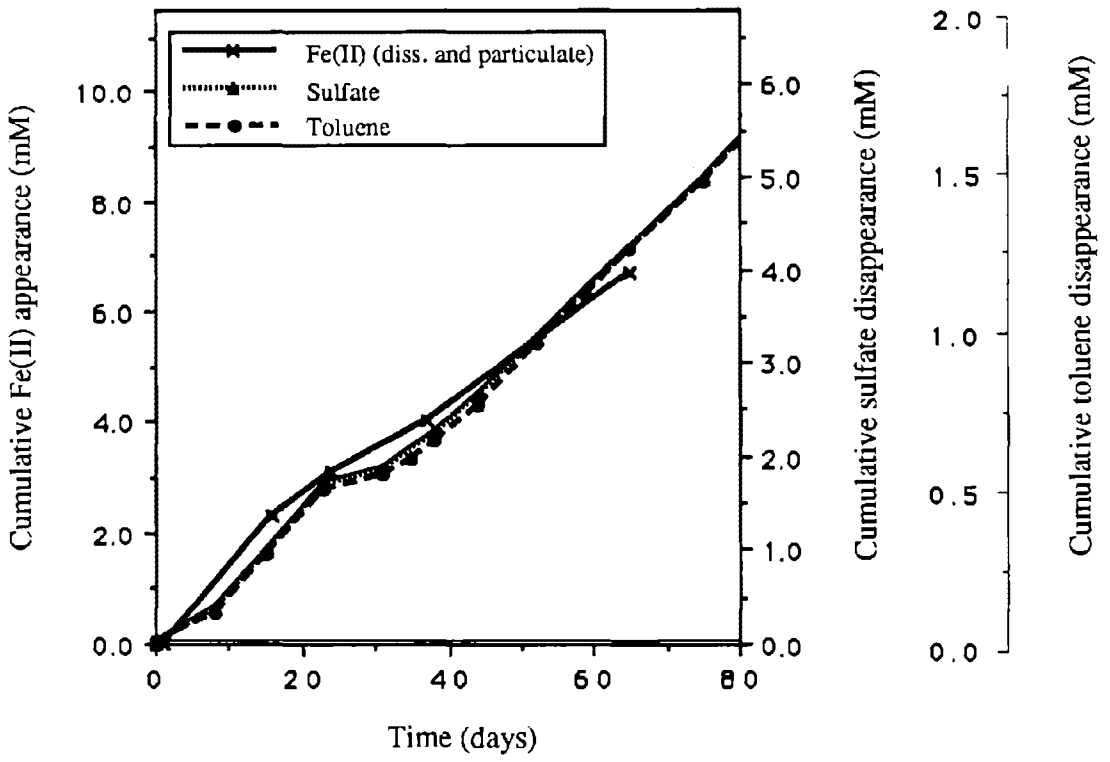

Figure 8. Cumulative Fe(II) appearance and sulfate and toluene disappearance in Enrichment E7 (Experiment PR2).

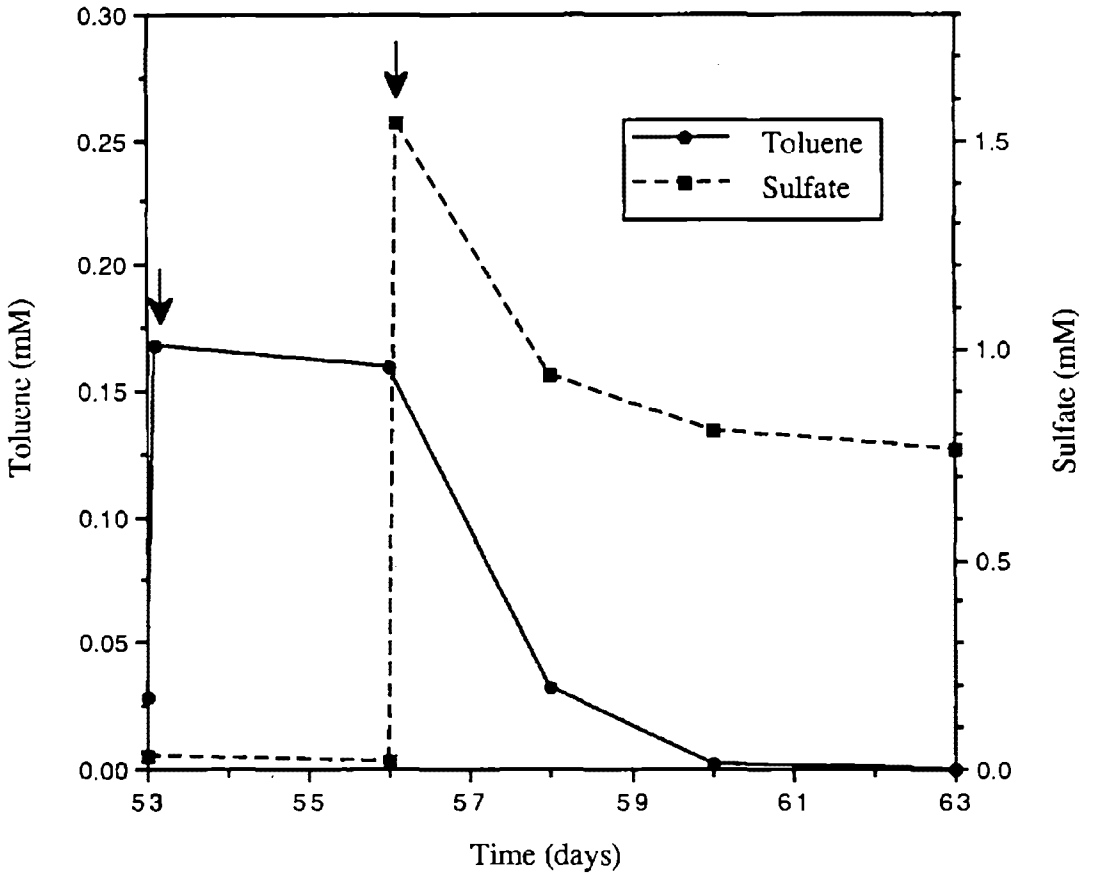

Figure 9. Mutual dependence of toluene degradation and sulfate reduction for a Patuxent River enrichment. Arrows indicate amendment of toluene or sulfate. Data points represent the averages of duplicates.

DISCUSSION OF THE ROLE OF IRON IN TOLUENE DEGRADATION (PATUXENT RIVER EXPERIMENTS)

The data presented in this report indicate the importance of iron in either initiating or accelerating toluene degradation, but do not explicitly indicate how iron is responsible for these effects. Five hypotheses have been formulated that could explain the nature of iron's effect; these hypotheses are described below and are discussed in relation to the available data.

Hypothesis 1- Reduction of sulfide toxicity

Fe(III) could be reducing sulfide toxicity in two ways: by serving as an electron acceptor in the oxidation of hydrogen sulfide (e.g., to elemental sulfur) and by precipitating sulfide as ferrous sulfide once the iron has been reduced to ferrous form (both processes are illustrated by Equation 6). Sulfide toxicity is often referred to in the literature but is not extensively documented. Postgate (1984) invoked sulfide toxicity to explain the linear rather than exponential growth that has been observed in batch cultures of sulfate-reducing bacteria; it was postulated that sulfide inhibits growth, and thus reduces the specific growth rate (typically represented as μ) over time as sulfide is produced.

Hypothesis 2 - Iron as a limiting nutrient

Iron might be a limiting nutrient that is required for toluene degradation. The concentration of soluble (biologically available) ferrous iron was decreased considerably by precipitation as ferrous sulfide. Note that the millimolar concentrations of Fe(II) presented in the Results and Discussion section are largely particulate, not dissolved.

Postgate (1984) noted that "*Desulfovibrio* shows an exceptionally high requirement for inorganic iron". As a quantitative indication of this requirement, Postgate (1956) reported that *D. desulfuricans* required on the order of 10 µM iron for optimal growth with lactate. It is likely that the iron requirement of sulfate-reducing bacteria is related to the synthesis of iron-rich proteins (e.g., cytochromes c and ferredoxin). For example, Tsuji and Yagi (1980), using *D. vulgaris*, found that the amount of cytochrome c3 in iron deficient (11 nM iron) medium was as high as in iron-rich (50 nM iron) medium, whereas other iron-rich proteins were present at much lower concentrations in the iron-deficient bacteria. Thus, cytochrome c3 was presumably critical for bacterial metabolism, as it was produced preferentially in iron-deficient cultures. Postgate (1965), citing unpublished data, noted that *Desulfovibrio sp.* changed from linear to exponential growth

when chelators were added (e.g., citrate or EDTA). This finding is at odds with the assumption discussed previously (Hypothesis 1) that sulfide toxicity is responsible for such growth patterns. In general, it is difficult to experimentally distinguish between the effects of sulfide toxicity and iron limitation in sulfate-reducing cultures, as both are related to the interaction between iron and sulfide.

Hypothesis 3 - Oxidation of sulfide to preferable electron acceptors

Ferric iron may oxidize sulfide to thiosulfate (Pyzik and Sommer, 1981) or sulfite; these sulfur oxyanions are intermediates (or potential intermediates, in the case of thiosulfate; e.g., Akagi, 1981) in sulfate reduction and both may be used as electron acceptors for the oxidation of aromatic compounds (Widdel, 1988; Cypionika et al., 1985). The use of these sulfur species would be energetically preferable to sulfate because the energy-requiring activation of sulfate to form APS (adenosine-5'-phosphosulfate) would not be necessary (e.g., Widdel, 1988; Badziong and Thauer, 1978).

Hypothesis 4 - Presence of ferric iron-reducing bacteria

Iron-reducing bacteria may be performing the initial (but incomplete) oxidation of toluene or in some other way may be engaged in a syntrophic relationship with sulfate-reducers. Recently, ferric iron-reducing cultures have been isolated that can mineralize toluene and can also degrade potential toluene intermediates, such as *p*-cresol (Lovley and Lonergan, 1990). Thus, the existence of such organisms is plausible in the Patuxent River samples.

Hypothesis 5 - Presence of fermentative bacteria

Fermentative bacteria that are highly dependent on iron could be performing the initial oxidation of toluene. This iron dependence differs from Hypothesis 2 in the sense that the bacteria being considered are not the predominant species involved in overall toluene degradation (i.e., sulfate reducers).

Since the experiments performed for this project were not designed to test the hypotheses stated above, the available data are of limited use in evaluating their validity. At this time, none of the hypotheses can be definitively ruled out. However, some speculation based on the data is warranted. Perhaps the most striking data regarding the effect of iron on toluene degradation was

the initiation of toluene degradation after the second addition of amorphous $Fe(OH)_3$ in Experiment PR2 (Figure 3). From the sulfate data, it is clear that sulfate reducers were active in these microcosms well before toluene degradation began. Thus, the addition of iron must have either allowed these sulfate reducers to switch substrates (to toluene) or promoted the very rapid development of iron-dependent sulfate reducers or fermenters, or iron-reducing bacteria. The observations are consistent with Hypothesis 2; it is plausible that iron was a limiting nutrient that was required for producing respiratory or other iron-containing enzymes specific to toluene degradation in sulfate reducers. However, it is difficult to explain why the iron addition at Day 0 was not sufficient to supply the needed iron.

The stimulation of iron-reducing bacteria (Hypothesis 4) is also a plausible explanation for the rapid initiation of toluene degradation in Experiment PR2; however, other data argue against the presence of iron-reducers. For example, the addition of amorphous $Fe(OH)_3$ to Microcosm C5 (Experiment PR2) on Day 48, when sulfate had been depleted, did not result in the initiation of toluene degradation over a ten-day period, after which sulfate was again added (data not shown). In addition, Experiment PR3 results suggested that, if iron reducers were present, they must have had very slow growth rates, which is not consistent with the observation of rapid initiation of toluene degradation in Experiment PR2. This discussion applies equally well to iron-reducing bacteria that could perform only a minor, initial toluene degradation and those that could completely oxidize toluene (the latter have been ruled out by stoichiometry).

The observation of the rapid initiation of toluene degradation by iron addition (Experiment PR2) also does not strongly support Hypotheses 1 or 3 (i.e., iron reduced sulfide toxicity or generated preferable electron acceptors) because these mechanisms would tend to accelerate toluene degradation rates (via enhanced growth rates) once degradation had begun, but would not necessarily be expected to initiate toluene degradation. With regard to sulfide toxicity, it seems unlikely that the ca. 0.4 mM of sulfide generated before toluene degradation began in Microcosms C8, C9, and C10 would have resulted in significant toxicity, especially in the presence of the 20 mM Fe(III) that was added initially .

Preliminary experiment to examine the role of iron

A preliminary test with Patuxent River enrichments was performed in an attempt to narrow down the list of plausible hypotheses for the role of iron in toluene degradation. The test was predicated on the fact that certain of the hypotheses stated above (namely Hypotheses 3 and 4) apply only to ferric iron, whereas the remaining hypotheses could apply to ferric or ferrous iron. Thus, if ferrous iron were added to the enrichments and had the same apparent effect as ferric iron, Hypotheses 3 and 4 (i.e., oxidation to preferable electron acceptors and the presence of ferric iron-reducing bacteria) could be ruled out. The experimental design (summarized in Table 9) consisted of initially adding either ferrous iron (as $FeSO_4$) or ferric iron [as amorphous $Fe(OH)_3$] and observing the relative toluene degradation rates. Live controls consisted of enrichments that were not amended with iron (unfortunately, the parent microcosms had a considerable amount of iron, particularly ferrous iron, that carried into the enrichments and may have compromised their role as controls).

The results for the first 40 days of incubation are shown in Figure 10, which shows average cumulative toluene degradation vs. time for the three different initial conditions. For the first three weeks of incubation, toluene degradation rates were extremely consistent among all enrichments (error bars represent one standard deviation). After Day 20, the Fe(II)-amended enrichments continued to degrade toluene as rapidly as it was supplied and variability remained very low; in contrast, the Fe(III)-amended enrichments began to degrade toluene less rapidly and with far greater variability. The trend for Fe(III)-amended enrichments was not unlike the trend for enrichments shown in Figure 7. The results are difficult to explain if one assumes that the same microbial assemblages were present in all enrichments, especially when the controls are considered. If all enrichments were assumed to contain the same assemblages, it would appear that ferric iron was actually inhibitory. It is possible that the different initial conditions provided selective pressure for different microbial assemblages, and the Fe(II)-amended and control enrichments may have had similar assemblages that were unlike the Fe(III)-amended assemblages. Notably, the Fe(II)-amended enrichments and controls began to slow down after Day 45 and were clearly stimulated by the addition of more Fe(II) (data not shown). Thus, these enrichments had a response to ferrous iron, possibly negating Hypotheses 3 and 4; however, these results cannot necessarily be applied to the earlier experiments with Patuxent River microcosms because different microbial assemblages may have been present.

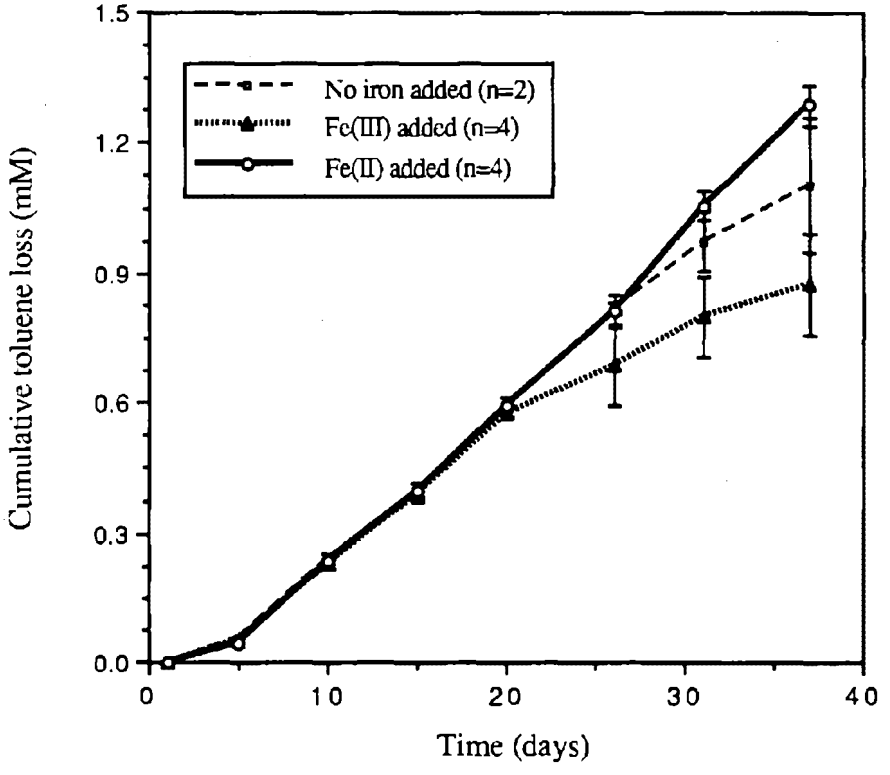

Figure 10. Test of the effect of iron oxidation state on toluene degradation using Patuxent River enrichments. Error bars represent one standard deviation.

NITRATE REDUCTION IN PATUXENT RIVER MICROCOSMS

One of the Patuxent River microcosms (C3; Experiment PR2) was amended with nitrate on Day 0 (Table 7). As shown in Figure 11a, almost immediate disappearance of toluene and nitrate was observed in this microcosm. Nitrate was likely the electron acceptor for toluene because Fe(II) and sulfate data (discussed later) indicate that iron and sulfate were not electron acceptors in this microcosm. The cumulative ratio of nitrate consumed/toluene consumed was about 35 at Day 70; the theoretical ratio when toluene degradation is coupled with the reduction of nitrate to nitrogen gas is 7.2. Thus, nitrate must have been participating in reactions (either biotic or abiotic) in addition to toluene oxidation. A very interesting trend was observed with sulfate in this microcosm (see Figure 11b): sulfate concentration <u>increased</u> from control levels, plateaued from Day 47 to 58, and then continued to rise, whereas control levels of sulfate were constant. The primary source of the sulfate was probably the oxidation of sulfide (which was present in the controls as well as in the active, sulfate-reducing microcosms, and thus must have been present at Day 0). The shape of the sulfate curve in Figure 11b suggests that a second source of reduced sulfur may have been oxidized starting after Day 58.

Nitrate may have been the electron acceptor in a reaction with sulfide; this reaction could explain the additional sink for nitrate mentioned above. The oxidation of reduced sulfur species may have been carried out by chemolithotrophic bacteria (e.g., *Thiobacillus denitrificans*). The observed stoichiometry is consistent with the reduction of nitrate to nitrite by hydrogen sulfide:

$$8\ NO_3^- + H_2S + HS^- = 8\ NO_2^- + 2\ SO_4^{2-} + 3\ H^+ \qquad \Delta G^{o'}\ (pH\ 7) = -\ 232\ kcal.$$

The theoretical ratio of [sulfate produced/nitrate consumed] in the above equation is 0.25, whereas the observed ratio was 0.256. Note that the amount of nitrate used in the observed ratio was corrected for the biological oxidation of toluene; that is, the amount of nitrate that would theoretically be required to completely oxidize the toluene was subtracted from the total amount of nitrate consumed. This correction of the amount of nitrate consumed (and the associated assumptions) are not of great significance in relation to the overall amount of nitrate consumed. Notably, the thermodynamically favorable reduction of nitrate to nitrogen gas in conjunction with sulfide oxidation

$$16NO_3^- + 5H_2S + 5HS^- + H^+ = 8N_2 + 10SO_4^{2-} + 8H_2O \qquad \Delta G^{o'}\ (pH\ 7) = -\ 1777\ kcal$$

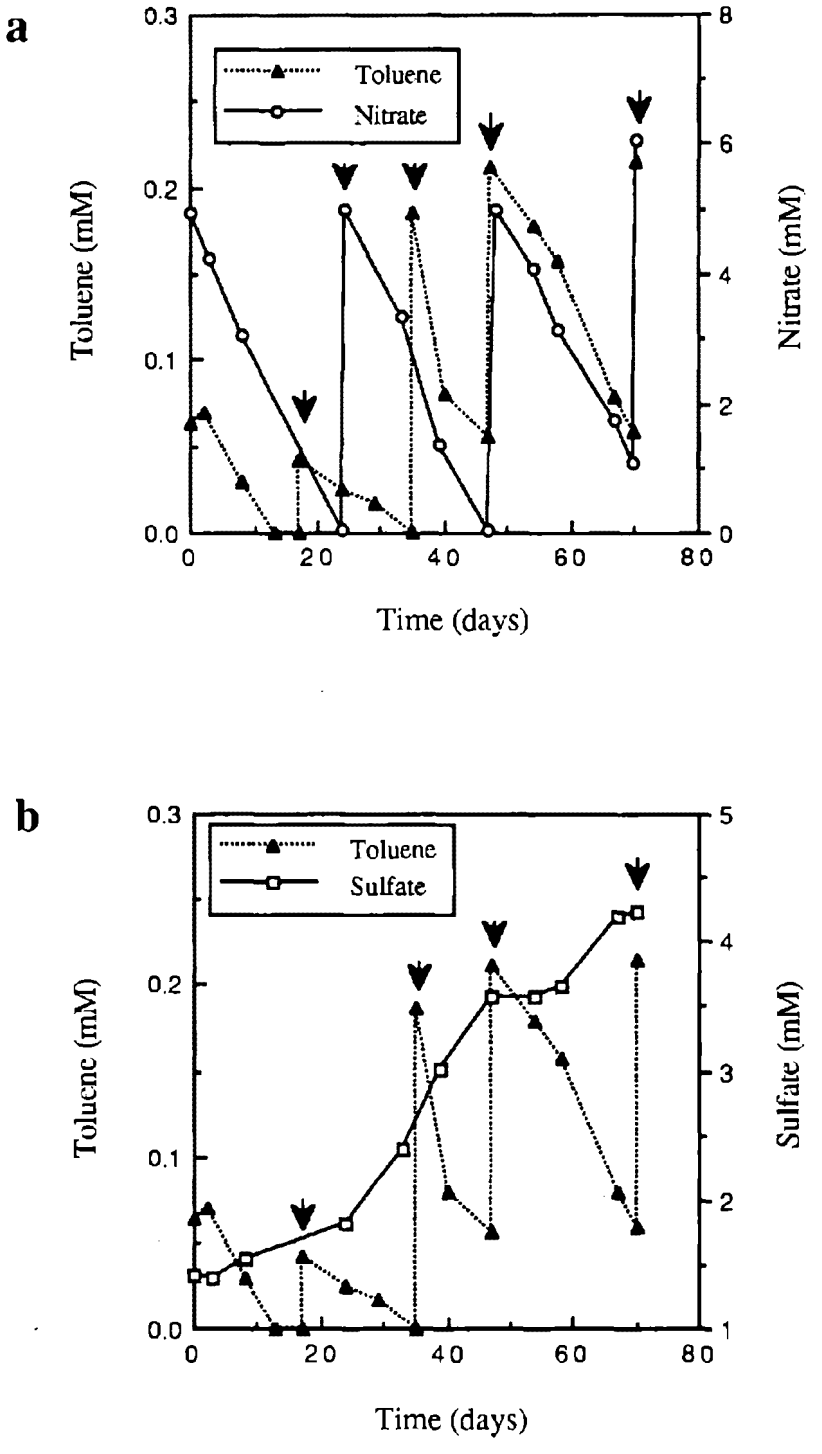

Figure 11. Toluene and nitrate (a) or toluene and sulfate (b) vs. time in Microcosm C3. Arrows indicate amendments of toluene and/or nitrate.

has a [sulfate produced/nitrate consumed] ratio of 0.63, which is not consistent with the observed ratio.

The total Fe(II) concentration in this microcosm was below control levels, suggesting some oxidation of Fe(II). However, the concentration of Fe(II) remained relatively constant (8.49±0.91), suggesting that Fe(II) oxidation was not an ongoing process (or was balanced with Fe(III) reduction).

ATTEMPTS TO ENRICH IRON-REDUCERS IN SEAL BEACH AND TRAVERSE CITY MICROCOSMS

Seal Beach Microcosms

After a lag period of one to three weeks, toluene degradation was observed in five of the eight Seal Beach microcosms. Of these five microcosms, two contained added amorphous $Fe(OH)_3$ and three did not. The toluene degradation rates were relatively slow (Figure 12) and activity could not be maintained over time. The electron acceptor in these samples is unknown, but the available data suggest that sulfate, not ferric iron, was the electron acceptor. Sulfate was not measured in this study, however, active microcosms turned noticeably darker (blacker) by Day 25, suggesting the possibility of ferrous sulfide formation. Dissolved Fe(II) (not dissolved plus particulate, as the culture medium was filtered through a 0.2 μm filter during the Seal Beach and Traverse City experiments) did not increase significantly in Seal Beach microcosms, and never exceeded one mole percent of the iron initially added. Although a microcosm with added iron showed a slightly greater extent of toluene degradation than the average of three microcosms without iron (Figure 12), the significance of this trend relative to experimental error is debatable.

Traverse City Microcosms

Degradation of ethylbenzene was clearly apparent in five of the eight active microcosms, and toluene degradation was also observed in two of these microcosms. Of the five microcosms with apparent degradation, two contained added amorphous $Fe(OH)_3$ and three did not. However, as observed for the Seal Beach microcosms, there was no significant appearance of dissolved Fe(II) or clear effect on substrate degradation that could be attributed to the presence of iron. In Figure 13, toluene and ethylbenzene degradation are shown for one microcosm without added iron and one with added iron.

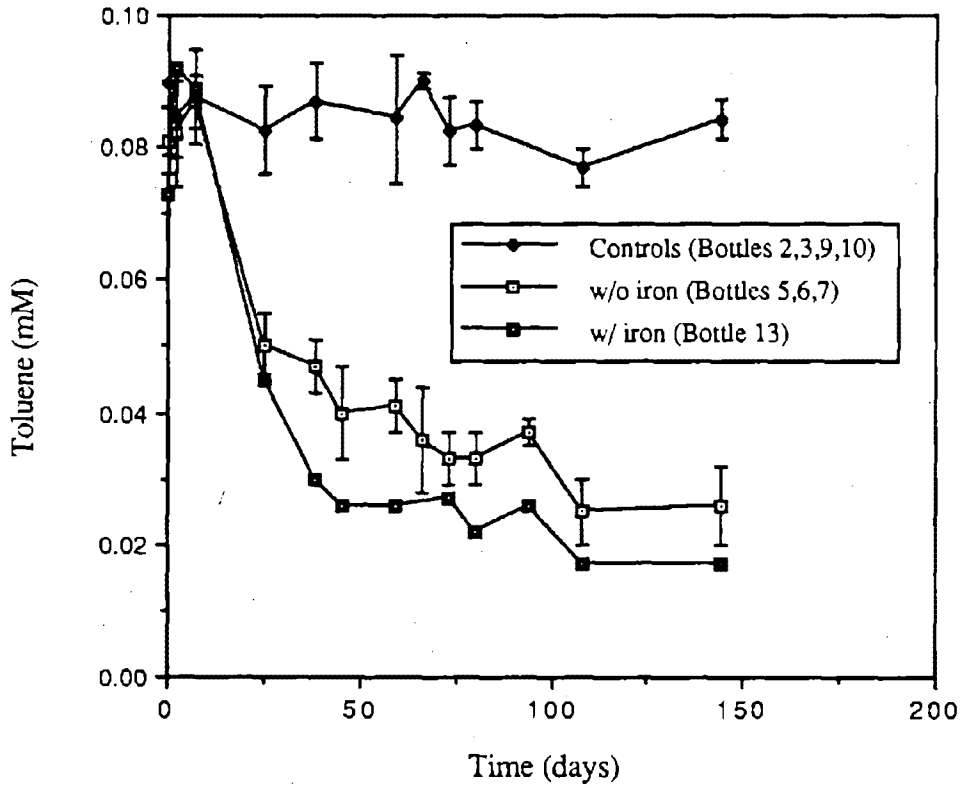

Figure 12. Toluene concentration vs. time in Seal Beach microcosms. Bars represent one standard deviation.

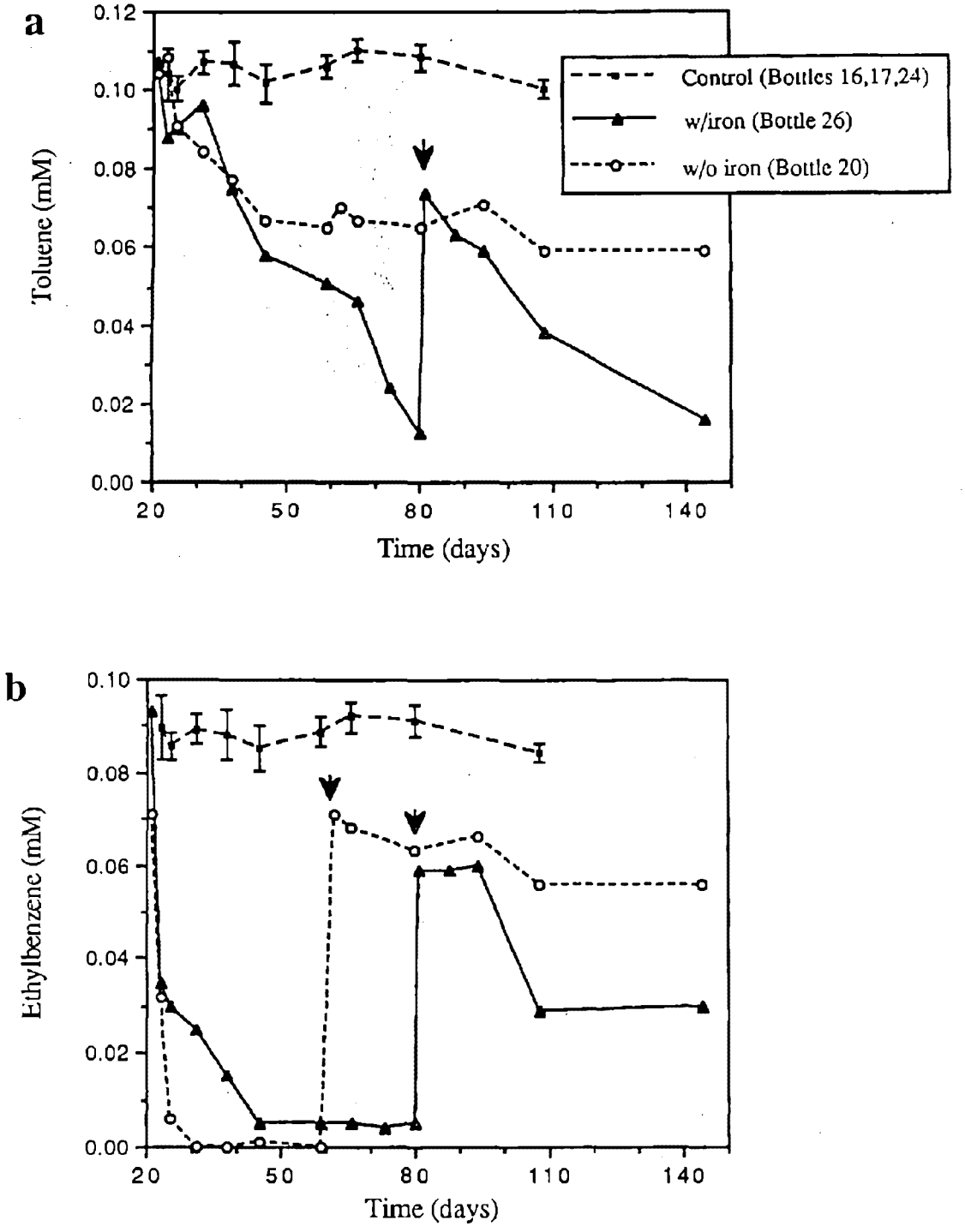

Figure 13. Toluene (a) and ethylbenzene (b) vs. time in two Traverse City microcosms. Arrows indicate amendments of toluene or ethylbenzene.

METHANOGENIC DEGRADATION OF TOLUENE AND *o*-XYLENE BY MICROORGANISMS FROM THE PENSACOLA AQUIFER

Pensacola microcosms were initially fed mixtures of benzene, toluene, ethylbenzene, *o*-xylene, and *p*-xylene at low concentrations (120 μM total hydrocarbons). One half of the microcosms fed substituted aromatic compounds (toluene, ethylbenzene, *o*-xylene and *p*-xylene) also received *p*-cresol (0.46 mM), a compound previously proven to be the crucial intermediate in methanogenic degradation of toluene (Vogel and Grbić-Galić, 1986). Two groups of microcosms (one with added *p*-cresol; one without) were set up to determine whether *p*-cresol would facilitate the adaptation to alkylbenzene degradation. Although duplicate microcosms were initially prepared, only one microcosm from each group developed activity toward toluene and subsequently toward *o*-xylene. In one of the microcosms without *p*-cresol, toluene degradation started after approximately 120 days of incubation, whereas one of the *p*-cresol-amended microcosms displayed toluene degradation after 100 days. The same microcosms had degraded *p*-cresol completely in less than 30 days of incubation. Upon refeeding of the microcosms with toluene (100 μM) and *p*-cresol (where appropriate; 0.46 mM), the degradation of both compounds resumed immediately. Furthermore, the *p*-cresol-amended microcosms also began to degrade *o*-xylene after 200 days of incubation, whereas the microcosms without *p*-cresol developed the *o*-xylene degrading capability only after 255 days. It appears that *p*-cresol may have been supporting growth of the complex microbial community, which allowed for development of more biomass and a faster acclimation to the aromatic hydrocarbons (regardless of the acclimation mechanism). Because of the limited number of active microcosms in this study, the experiment was repeated and is discussed later in this section. Benzene, ethylbenzene and *p*-xylene were not degraded in any microcosms. Autoclaved controls exhibited no degradation of any aromatic compounds.

After the third refeeding, the microcosms were used as the source for enrichment of suspended consortia degrading toluene and *o*-xylene. This was accomplished in two steps. In the first step, 10 g of aquifer solids and 20 mL of the culture fluid from the microcosms were transferred into 180 mL of Medium 2, and amended with 50 μM toluene and/or *o*-xylene. Transformation activity was detected after one to two weeks of incubation. Upon refeeding with the aromatic hydrocarbons, the degradation resumed and was completed in two to three weeks. In the second step of enrichment, only the liquid portion of these primary enrichments (30 mL) was transferred into Medium 2 (170 mL). The degradation activity was retained. Figure 14 shows ^{14}C

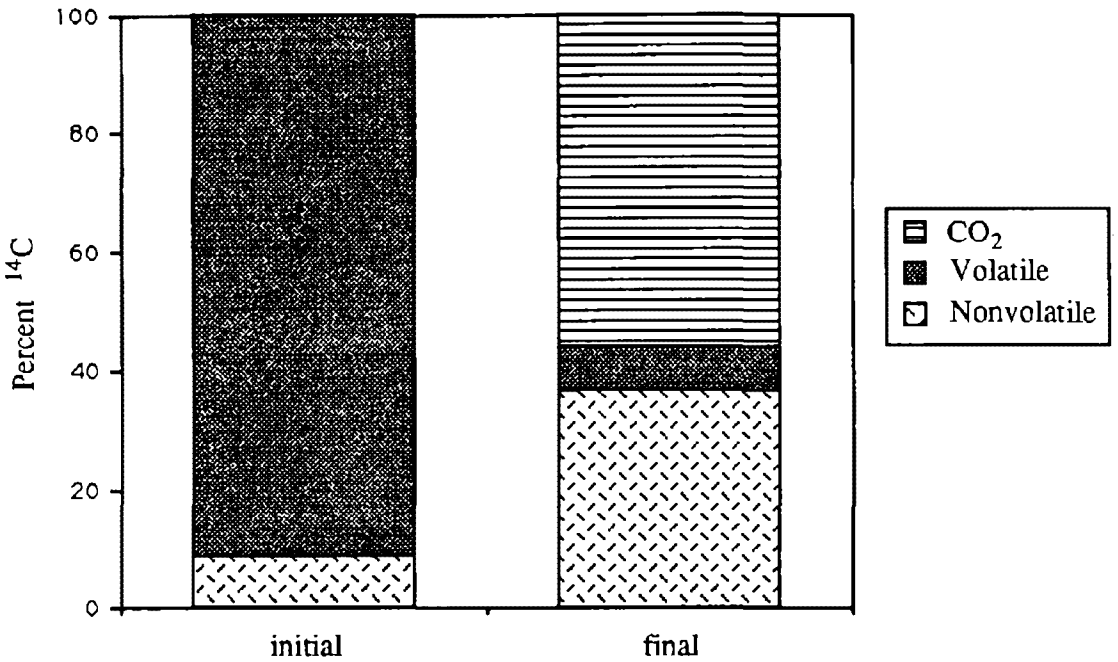

Figure 14. ^{14}C mass balance in suspended cultures fed ^{14}C-labeled toluene. The first column represents the initial distribution of the label; the volatile portion is toluene. The second column shows the distribution of ^{14}C after all the toluene was degraded. The large CO_2 fraction confirms complete degradation of toluene. The nonvolatile fraction contains degradation intermediates and cell-bound ^{14}C; this fraction was found to decrease with time. Both ring-labeled and methyl-labeled toluene yielded the same results.

mass balance in suspended cultures that degraded [^{14}C]toluene. After several refeedings, the cultures stabilized and increased their degradation rate (complete degradation of toluene and *o*-xylene in one to two weeks of incubation; see Figure 15). The substrates were continuously converted to stoichiometric amounts of CO_2 and CH_4 (Edwards and Grbić-Galić, 1990). Note that the data presented in Figure 15 (and in the remaining figures in this report, except Figure 21) represent the averages of two or three replicates. In these figures, the maximum difference between replicates was 25%, and most replicates agreed within 10%.

The stable mixed cultures (both primary and secondary enrichments) were maintained on toluene and *o*-xylene as sole carbon sources for over a year. They degraded toluene and *o*-xylene relatively rapidly. The cultures have been acclimated to an additional compound, *m*-xylene, which was degraded only slowly, after the other two compounds were completely depleted. The original microcosms and the first transfers from the microcosms, which still contained a minor amount of aquifer solids, were relatively active and degraded toluene and *o*-xylene (fed at a concentration of 50 μM each) completely in less than two weeks. Secondary transfers, which no longer contained a visible amount of aquifer solids, degraded the substrates at a considerably slower rate (three to four times slower). Subsequent transfers demonstrated no activity. It is suspected that the solids were necessary for attachment of the microorganisms, or that they contained a trace nutrient which might have been essential for the transformations.

Since the original headspace in the microcosms contained 10% molecular hydrogen, which could have influenced the activity of fermentative or obligate proton-reducing acetogens participating in the hydrocarbon transformation, the effect of different concentrations of hydrogen on toluene degradation and methane production was tested. In this experiment, 40-mL vials sealed with Mininert valves and containing 20 mL of Medium 2 were inoculated with 10 mL of stable mixed culture. The headspace (10 mL) was exchanged with gases containing different concentrations of hydrogen and each vial was spiked with toluene. The results are summarized in Table 14. As is evident from the table, 70% of H_2 in the headspace did not strongly inhibit toluene degradation, although degradation in the absence of H_2 in the headspace was the fastest. Some of the effects might have been due to differences in the initial CO_2 concentration in the headspace, which might have influenced the pH. However, the data strongly suggest that the toluene

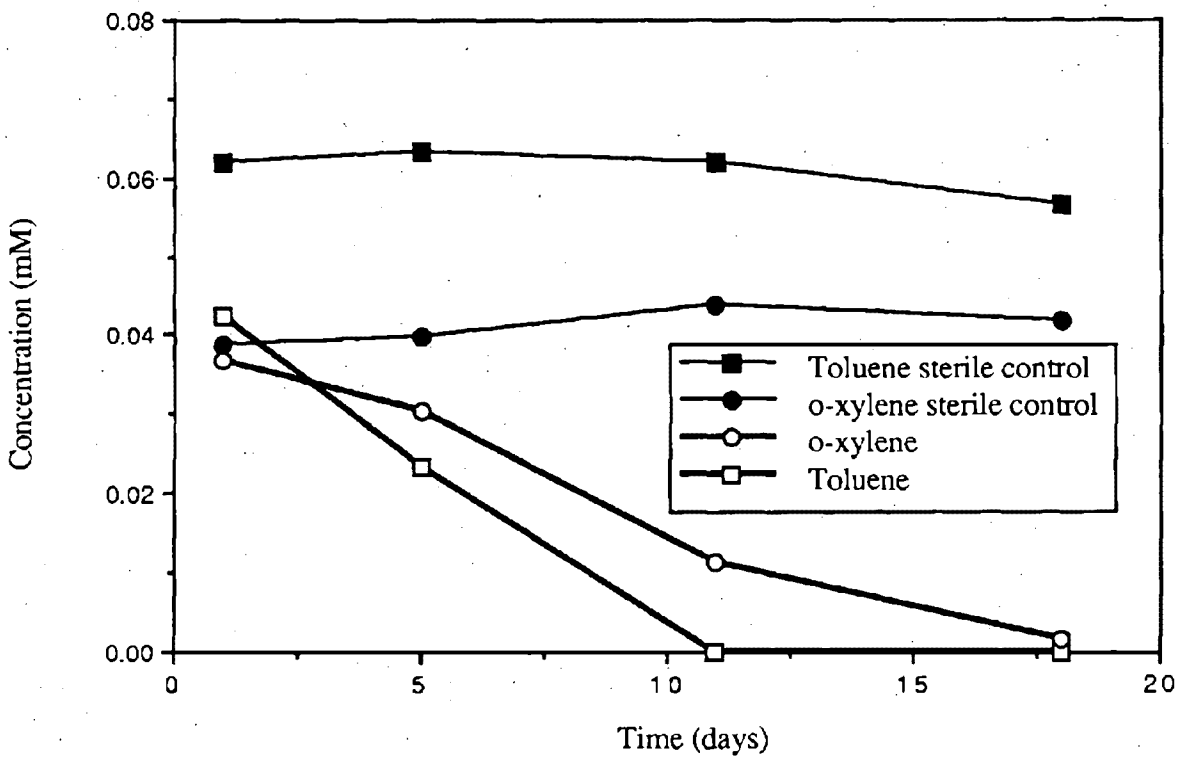

Figure 15. Degradation of toluene and o-xylene in suspended mixed culture. The degradation of both compounds proceeded simultaneously in mixed cultures amended with both compounds.

TABLE 14. INFLUENCE OF H_2 CONCENTRATION IN THE HEADSPACE ON TOLUENE DEGRADATION AND METHANE PRODUCTION IN SUSPENDED METHANOGENIC CULTURES DEGRADING TOLUENE AS THE SOLE SUBSTRATE

Time (Days)	10% H_2, 10% CO_2, 80% N_2	**NO H_2**, 21.3% CO_2, 78.7% N_2	70% H_2, 30% CO_2	10% H_2, 10% CO_2, 80% N_2, **NO Toluene** (Positive Control)	10% H_2, 10% CO_2, 80% N_2, **Sterile Control**
1	**TOL**:4.00[a] **CH_4**: 0.058	**TOL**: 4.05 **CH_4**: 0.014	**TOL**: 4.26 **CH_4**: 0.045	**CH_4**: 0.061	**CH_4**: 0.005
5	**TOL**: 1.79 **CH_4**: 0.299	**TOL**: 0.38 **CH_4**: 0.082	**TOL**: 0.84 **CH_4**: 1.18	**CH_4**: 0.529	**CH_4**: 0.005
7	**TOL**: 0.85 **CH_4**: 0.349	**TOL**: <0.01 **CH_4**: 0.106	**TOL**: 0.35 **CH_4**: 2.64	**CH_4**: 0.784	**CH_4**: 0.006
11	**TOL**: 0.01 **CH_4**: 0.362	**TOL**: -[b] **CH_4**:0.104	**TOL**: 0.54 **CH_4**: 4.06	**CH_4**: 0.814	**CH_4**: -[b]

[a] Toluene concentration is expressed in mg/L, and methane concentration as percentage in the headspace. Values are averages of three replicates.

[b] Not measured.

transformers were not affected by high concentrations of hydrogen, and also that the methanogens were performing well even in the complete absence (initially) of hydrogen in the atmosphere.

The degradation of toluene and *o*-xylene in suspended mixed cultures was associated with cell growth. Cell counts were determined by staining with acridine orange and observation under the epifluorescence microscope. Stable mixed cultures reached an average of 10^8 cells/mL at the peak of the logarithmic growth phase. The doubling time for the stable mixed culture utilizing toluene as the sole carbon and energy source was about 9 days. The initial rate of growth and the rate of toluene degradation were both dependent on the initial cell count, as shown in Figures 16 and 17.

We have studied the influence of some environmental factors (temperature, pH, presence of exogenous electron acceptors, presence of increased concentrations of sulfide) on the performance

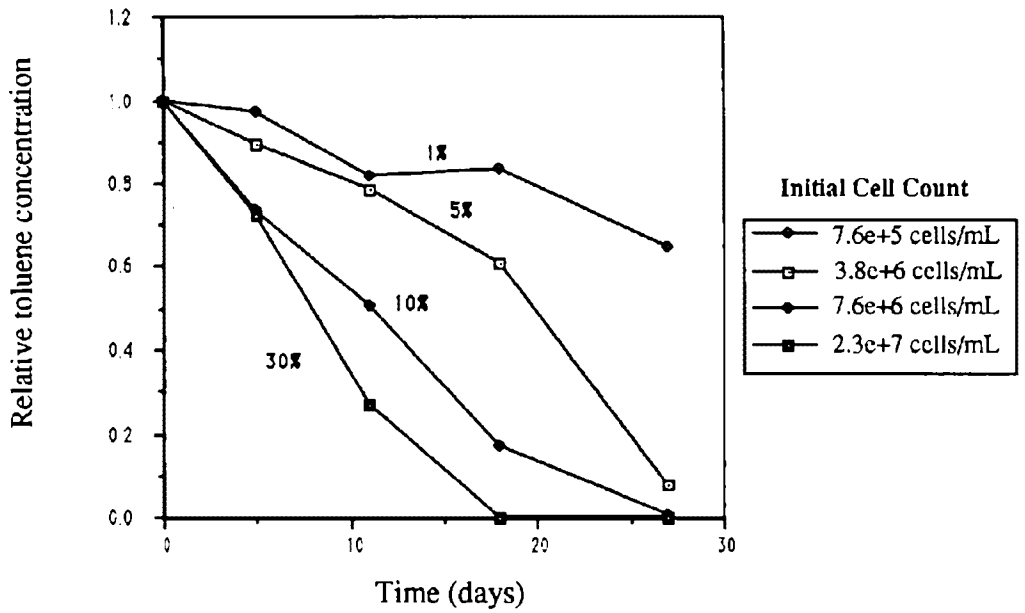

Figure 16. Effect of initial cell count on the rate of toluene degradation in a mixed methanogenic culture. Inoculum strength (as percent) is indicated.

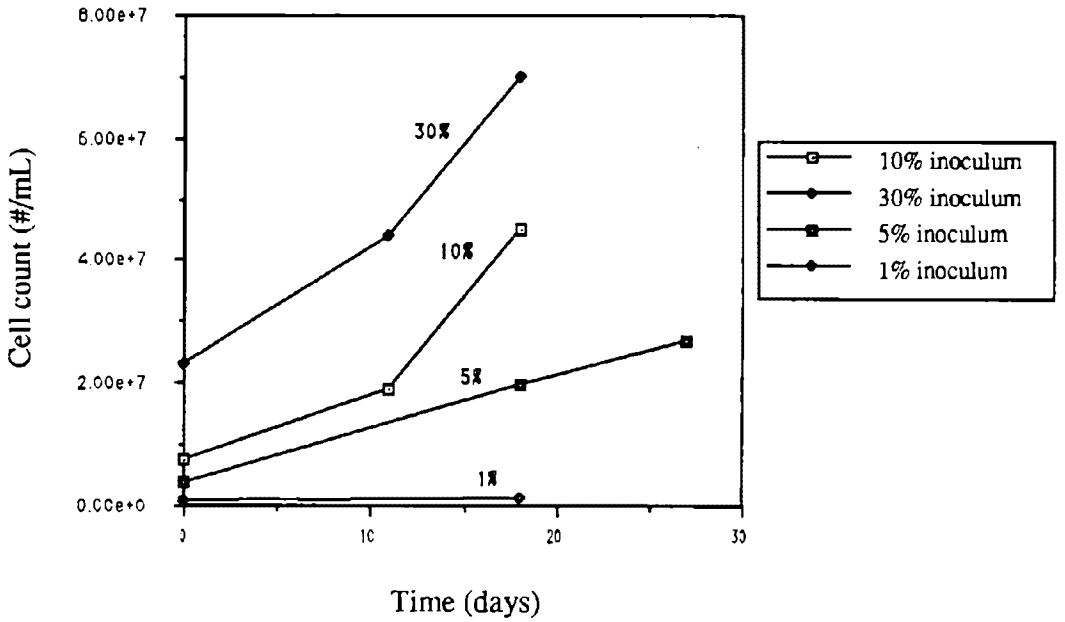

Figure 17. Cell count vs. time during toluene degradation by a mixed methanogenic culture.

of these cultures, and the results are summarized in Figures 18, 19, and 20. The cultures degraded toluene faster at 35°C than at 20°C. pH 6 was more favorable than pH 7, and pH 8 appeared to be unfavorable. This is consistent with the conditions in the contaminated aquifer, where a groundwater pH of 6 or below was predominant (E.M. Godsy, personal communication). The addition of exogenous electron acceptors (nitrate or sulfate) slowed down toluene degradation, indicating that the active community consisted of fermentative and methanogenic bacteria that were acclimated to the conditions of methanogenic fermentation. Sulfide also slowed the transformation down, in accordance with other reports that sulfide may be inhibitory to methanogens (Oremland, 1988).

In an attempt to isolate pure cultures from the mixed cultures, roll tubes were prepared using the same mineral medium with toluene and *o*-xylene, with the addition of 2% agar. The headspace in the roll tubes consisted of N_2 and CO_2 or H_2/CO_2. Colonies were observed after three to four weeks of incubation. Some were transferred to liquid medium with toluene and *o*-xylene. No activity toward aromatic substrates has been observed; however colonies transferred into liquid medium with H_2 in the headspace produced methane.

Some of the Pensacola aquifer material, previously used in experiments of degradation of creosote constituents (courtesy of E.M. Godsy, U.S. Geological Survey) was used to set up new microcosms for studying adaptation to toluene degradation under various environmental conditions. The following conditions were tested: 1) toluene (0.08 mM) alone, microcosms incubated statically as before; 2) toluene alone, with microcosms shaken vigorously once per day; 3) toluene with *p*-cresol (0.75 mM); 4) toluene with acetate (25 mM). The results are presented in Figure 21. Each data point in Figure 21 is the average of four replicate microcosms. The sediment used in this experiment was homogenized to the extent possible by mixing in a large beaker inside a glove box before distribution into bottles. As a result of homogenization, replicates in this experiment were reproducible, with a maximum coefficient of variation of 20%. The lag time before the onset of toluene degradation was about 50 days for both static and agitated microcosms. This lag time was increased to about 100 days if *p*-cresol was present, indicating that *p*-cresol was utilized as a preferential substrate. Toluene degradation started 50 days after all the *p*-cresol had been depleted; it is possible that the onset of toluene degradation required an enrichment of a different microbial population, containing the active enzymes. The *p*-cresol results conflict with the initial results obtained with the microcosms (see above). The difference is probably due to the fact that the aquifer material used for these adaptation studies had already undergone an enrichment step during creosote-degradation studies (E.M. Godsy, personal communication), so that specific

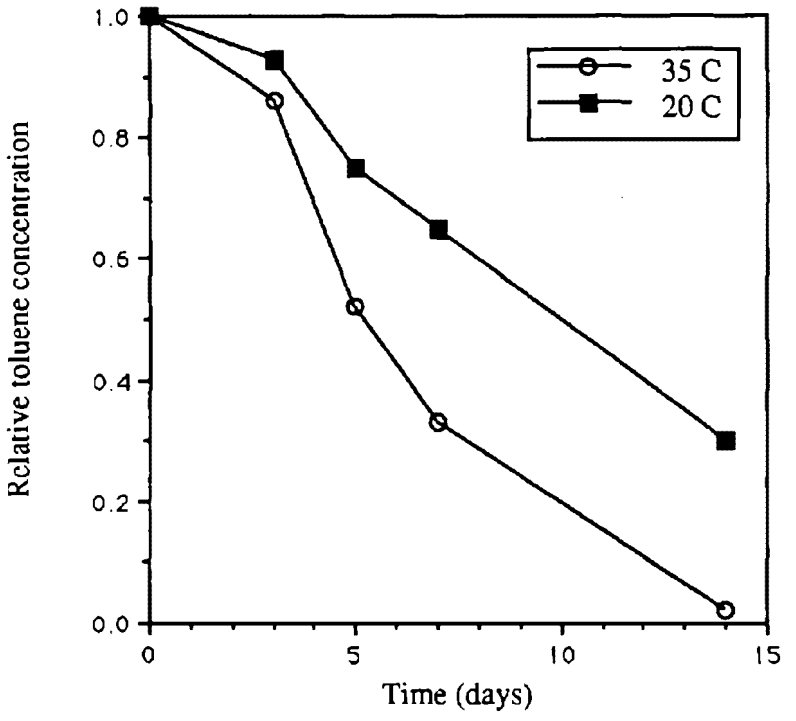

Figure 18. Effect of temperature on toluene degradation in suspended culture.

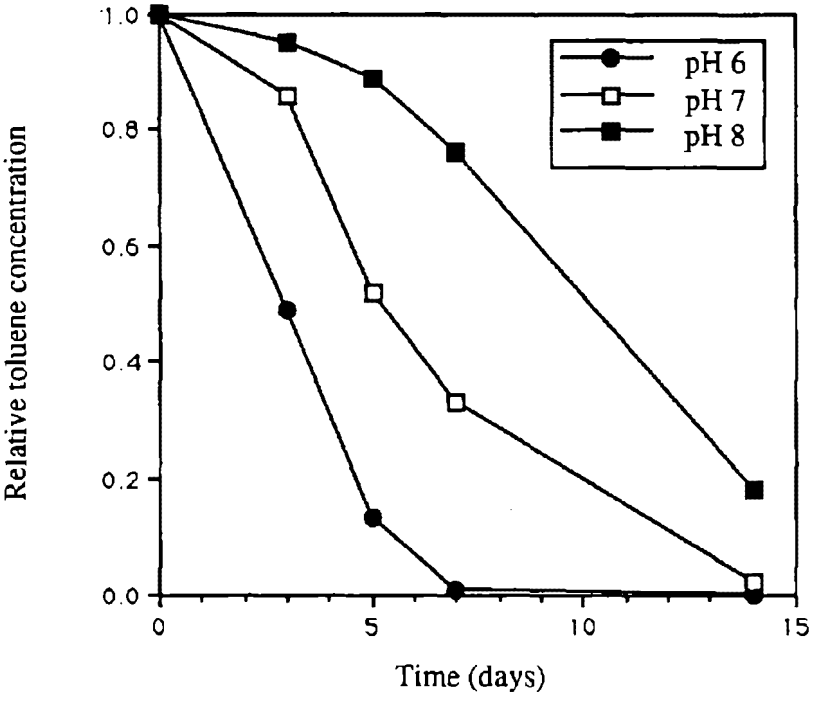

Figure 19. Effect of pH on toluene degradation in suspended culture.

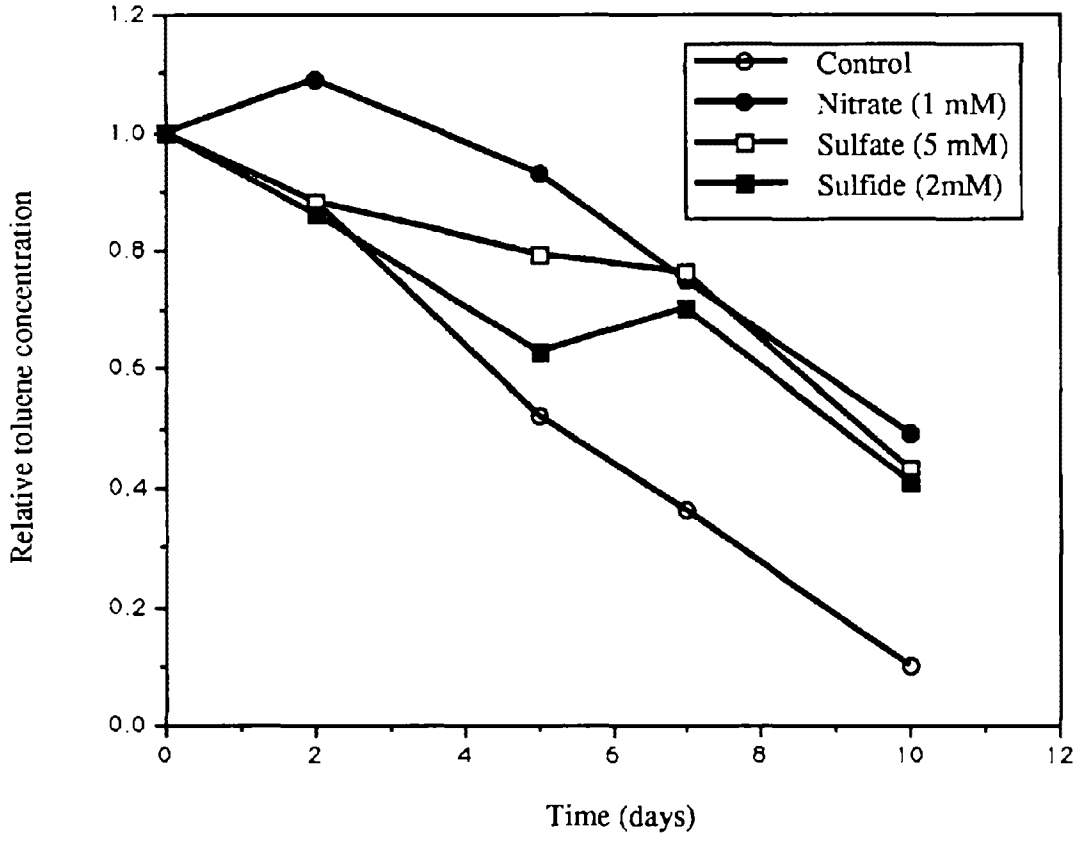

Figure 20. Effects of nitrate (1 mM), sulfate (5 mM), and sulfide (2 mM), all as sodium salts, on toluene degradation in suspended culture. The control is methanogenic degradation in the absence of any of these compounds, at pH 7.

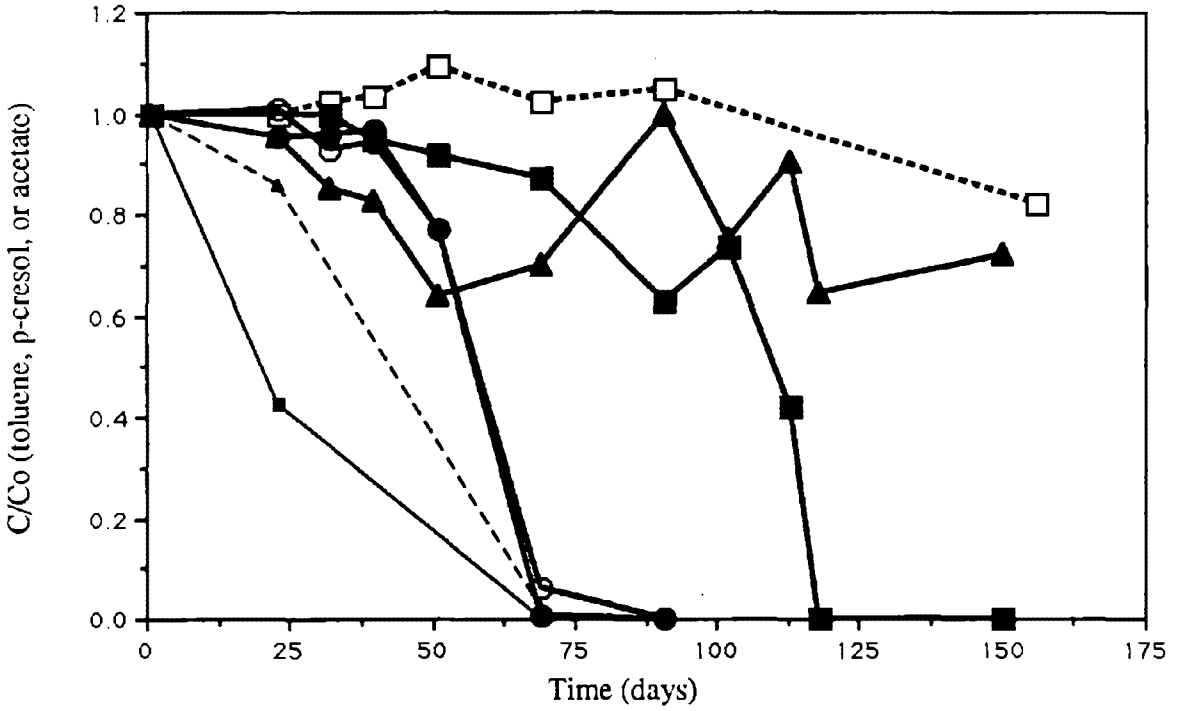

Figure 21. The length of the acclimation lag in toluene-degrading methanogenic microcosms under different environmental conditions.
Symbols:

- toluene concentration in killed biological control microcosms;
- toluene in microcosms incubated statically;
- toluene in microcosms incubated shaken;
- toluene and *p*-cresol in microcosms amended with both toluene and *p*-cresol;
- toluene and acetate in microcosms amended with both toluene and acetate.

microbial populations were enriched, whereas others were reduced or eliminated. The addition of acetate prolonged the acclimation lag: acetate-amended microcosms did not adapt to toluene degradation for more than 100 days after the depletion of acetate. This finding supports the hypothesis that the acetoclastic methanogens are not the rate-limiting population in the toluene degradation process, and suggests that acetate prevented the enrichment of the capable aromatic-degrading community on the aquifer material.

Attempts to detect some intermediates of toluene and xylene transformation using HPLC and GC/MS were unsuccessful because of the low concentrations involved. The simultaneous adaptation method (Stanier, 1947) is being used to determine which potential intermediates can be degraded by the enrichment culture.

In summary, active fermentative/methanogenic communities capable of degrading alkylbenzenes (i.e., toluene, xylenes) were successfully enriched from a methanogenic zone of a creosote-contaminated aquifer. The aromatic hydrocarbons were completely mineralized in a medium containing inorganic nutrients and vitamins, in the absence of accessory organic substrates and any electron acceptors other than CO_2. The conditions favorable for this degradation (the absence of oxygen, nitrate, and sulfate; the presence of solids; acidic pH) reflect the conditions in the zone of the aquifer from which the inoculum was obtained. The results demonstrate the significance of natural biotransformation processes in anoxic aquifers contaminated by aromatic hydrocarbons.

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