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Project Summary

Microbial Degradation of Alkylbenzenes under Sulfate-Reducing and Methanogenic Conditions

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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 near the Patuxent River (MD) and a creosote-contaminated aquifer near Pensacola (FL), were studied most extensively.

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. To our knowledge, this is one of the first reports of the degradation of alkylbenzenes under sulfate-reducing conditions. 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.

This Project Summary was developed by EPA's Robert S. Kerr Environmental Research Laboratory, Ada, OK, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

In 1986, the U.S. EPA reported that up to 35 percent of the nation's underground fuel storage tanks may be leaking. 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. In recognition of the fact that it is not always feasible to introduce oxygen into the subsurface to maintain aerobic microorganisms, and that consumption of oxygen by indigenous micro-

organisms often results in the development of anaerobic conditions, this study was undertaken to assess microbial degradation of fuel-derived aromatic hydrocarbons under 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 reported by various investigators for denitrifying conditions, methanogenic conditions, and ferric iron-reducing conditions. Very recently (since 1990), pure cultures of organisms that can degrade alkylbenzenes under nitrate-reducing conditions (Dolfing et al., 1990)[2] and ferric iron-reducing conditions (Lovley and Lonergan, 1990)[3] were isolated. 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.

This report summarizes research on the degradation of alkylbenzenes under sulfate-reducing and fermentative/methanogenic conditions, with an emphasis on the former conditions. Notably, definitive evidence of the coupling of alkylbenzene degradation to sulfate reduction has not yet been published. However, some co-authors of this summary and researchers at the R.S. Kerr Environmental Research Laboratory (U.S. EPA) have observed degradation of toluene under sulfate-reducing conditions in contaminated subsurface materials. In addition, other researchers (including Bak and Widdel,[1] 1986; Szewzyk and Pfennig, 1987)[4] have shown that 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, and hydroquinone).

Procedure

The initial goals of the project were to enrich ferric 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. Sulfate was present in Patuxent River materials and eventually became a suspected electron acceptor. 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.

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. The remaining volume of the bottles was headspace. Thirty grams (wet wt.) of solids were used in Patuxent River microcosms and 100 grams were used in Pensacola microcosms. Five aromatic hydrocarbons (benzene, toluene, ethylbenzene, and *o*- and *p*-xylenes) were initially spiked at concentrations in the range of 40 to 100 µM per compound. Sterile controls were removed from the glove box after sealing and were autoclaved at 121°C. Incubation was carried out at 35°C in an anaerobic glove box. Replicate microcosms and controls were used in all the experiments. After degradation had begun, regular re-spiking with aromatic hydrocarbons and sulfate was performed as necessary.

Growth Media and Ferric Iron Source

The composition of the defined mineral medium used for all microcosms and enrichments in the ferric iron-reduction and sulfate reduction studies (Medium 1) was based on medium used by Lovley and coworkers. This medium included the following compounds at the concentrations (mM) specified in parentheses: $NaHCO_3$ (30), NH_4Cl (28), $NaH_2PO_4 \cdot H_2O$ (4.4), NaCl (1.7), KCl (1.3), $CaCl_2 \cdot 2H_2O$ (0.68), $MgCl_2 \cdot 6H_2O$ (0.49), $MgSO_4 \cdot 7H_2O$ (0.41), $MnCl_2 \cdot 4H_2O$ (0.025), and $Na_2MoO_4 \cdot 2H_2O$ (0.004). The final pH of the medium was approximately 7.

A medium designed to support fermentative/methanogenic bacteria (Medium 2) included the following compounds at the concentrations (mM) specified in parentheses: $NaHCO_3$ (14.3), NH_4Cl (10), KH_2PO_4 (2.0), K_2HPO_4 (2.0), $MgSO_4 \cdot 7H_2O$ (0.51), $CaCl_2 \cdot 2H_2O$ (0.48), $FeCl_2 \cdot 4H_2O$ (0.1). In addition, Medium 2 contained trace minerals, vitamins, resazurin (a redox indicator), and amorphous ferrous sulfide as a reducing agent. 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.

Iron was added to microcosms in the form of amorphous $Fe(OH)_3$. This iron phase was prepared by neutralizing a 0.1 M ferric chloride solution with sodium hydroxide. The amorphous $Fe(OH)_3$ was prepared with sterilized glassware and reagents that were prepared in sterilized Milli-Q water, but the iron phase itself could not be autoclaved because the elevated heat and pressure would have facilitated crystallization, which was not desired.

Soil and Sediment Inocula

Patuxent River soil was collected at the Naval Air Station, Patuxent River (MD) in September, 1987 and was provided by Ron Hoeppel of the Naval Civil Engineering Laboratory (Port Heuneme, CA). The Patuxent River 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 was fine-grained and appeared to be rich in organic matter (including some plant detritus).

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 groundwater at this depth contained tens of mg/L of nitrogen heterocycles, simple polynuclear aromatic hy-

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drocarbons, and phenols. 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 for this project were attempts to screen for activity of iron-reducing bacteria. Thus, experimental design typically consisted of setting up parallel series of microcosms, one with and one without added iron. Two microcosm experiments with this general design were performed with Patuxent River material (Experiments PR1 and PR2). Enrichments of certain iron-amended microcosms were prepared in an anaerobic glove box by shaking the microcosm, removing 20 percent (by volume) of the combined liquid and solids, adding this inoculum to a new bottle, and diluting to 200 mL with fresh medium. Series of enrichments were prepared after 88 and 235 days of incubation of selected microcosms from Experiment PR2.

In the experiments with fermentative/methanogenic bacteria, two types of mixtures of aromatic compounds were spiked to 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 ca. 40 μM. In the second case, *p*-cresol (0.46 mM), a putative intermediate of toluene transformation under anaerobic conditions, was added to the hydrocarbon mixture. No other organic amendments were made and no exogenous electron acceptors were added, except CO_2.

Analytical Methods

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 a Mininert valve with a 500 μL gas-tight syringe that included a PTFE plunger tip and a side-port needle.

HCl-extractable Fe(II) was measured as described by Lovley and Lonegran. 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) in 50 mM HEPES buffer (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).

Sulfate in filtered culture medium was determined by ion chromatography (Dionex Series 4000i with a Nelson Analytical Chromatography Software system) equipped with a 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.

Results and Discussions

Iron's Relationship to Toluene Degradation in Patuxent River Microcosms

Results of the first microcosm 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 autoclaved controls (one with added iron and one without), (2) three microcosms without added amorphous $Fe(OH)_3$, and (3) four microcosms with added amorphous $Fe(OH)_3$ [ca. 12 mM Fe(III) on Day 0]. Although rates of toluene degradation for microcosms with and 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. Note that the microcosms with added iron received additional toluene on Day 44, whereas those without added iron did not. The effect of the presence/absence of iron on toluene degradation rate was reproducible in this study. As an indication of the variability among replicates, the average amount of toluene that had been degraded by Day 60 in microcosms with added iron (0.35 ± 0.02 mM; mean $\pm$ s.d.) was significantly greater ($P < 0.001$) than the average among replicates without added iron (0.20 ± 0.02 mM).

In the second microcosm experiment (Experiment PR2), as in the first, the presence of amorphous $Fe(OH)_3$ had an effect on toluene degradation, but the effect was qualitatively different in the two experiments. Toluene concentration versus time is shown in Figure 2 for the first 47 days of incubation of five microcosms and two controls. 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.

Hypotheses were developed to explain the apparent stimulation of toluene degradation by iron, including the possible need for iron as a micronutrient required for the synthesis of enzymes involved in toluene degradation, the possible role of iron in reducing sulfide toxicity, and the possible presence of ferric iron-reducing bacteria that were syntrophically associated with sulfate-reducing bacteria. A preliminary experiment with Patuxent River enrichments was performed in an attempt to narrow down the range of plausible hypotheses. This experiment, which focused on the importance of iron oxidation state (ferric vs. ferrous) in stimulating toluene degradation, was not conclusive; however, the results suggested that the hypotheses proposing iron as a limiting micronutrient, or as an agent to reduce sulfide toxicity, were most plausible.

Interrelationships Among Toluene Degradation, Sulfate Reduction, and Iron Reduction

In Patuxent River microcosms and enrichments, toluene degradation, sulfate reduction, and ferric iron reduction appeared to be strongly linked. An example of these relationships in a Patuxent River microcosm is shown in Figure 3, in which cumulative appearance of Fe(II) (an indication of ferric iron reduction) is shown on the far left *y* axis, and cumulative sulfate reduction and cumulative toluene degradation are shown on the right hand side of the figure. As shown in the figure, not only do these processes appear to proceed simultaneously, but their relative rates

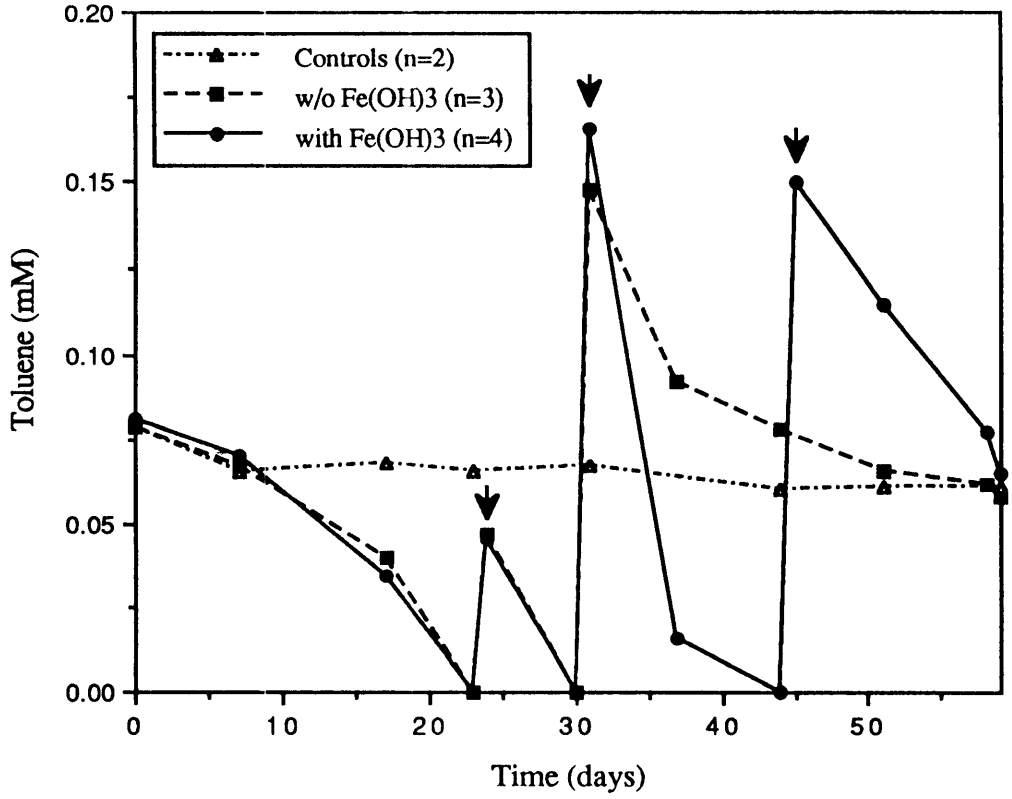

Figure 1. *Average toluene concentrations vs. time in Patuxent River (Experiment PR1) microcosms. Arrows indicate amendments of toluene.*

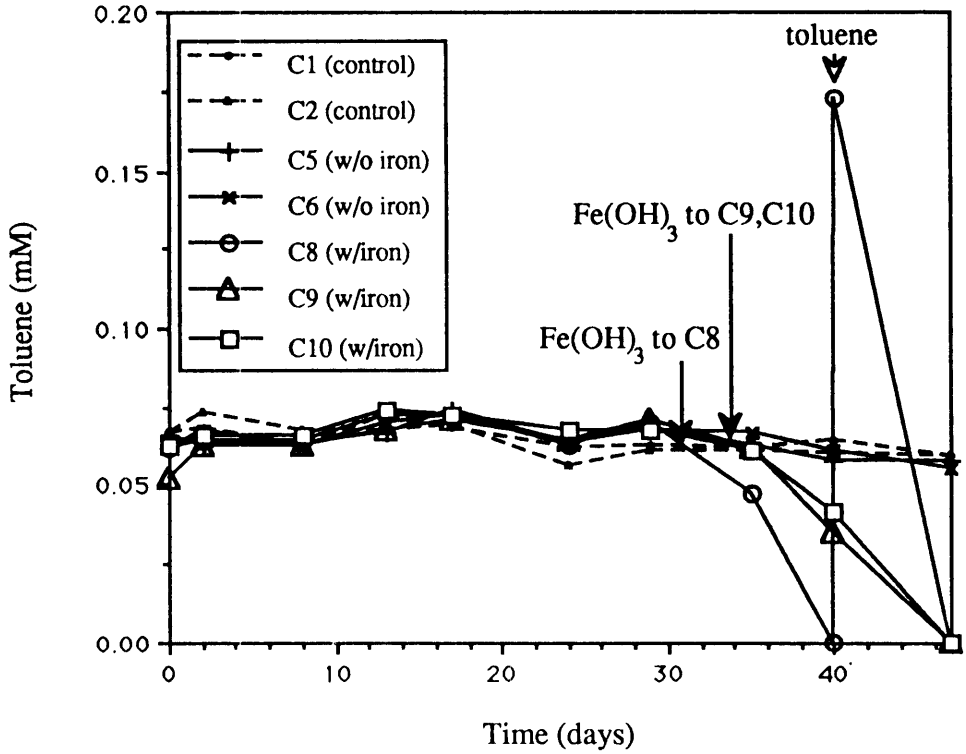

Figure 2. *Toluene vs. time in Patuxent River (Experiment PR2) microcosms. Filled arrows represent amendment of ca. 10 mM Fe(III). The original amount of Fe(III) added to Microcosms C8, C9, and C10 was ca. 20 mM.*

appear to be constant. Similar plots were obtained for other microcosms and enrichments.

Several lines of evidence suggest, but do not prove, that toluene degradation was directly linked to sulfate reduction in microcosms and enrichments: (1) the two processes were synchronous, (2) the observed stoichiometric ratios of sulfate consumed/toluene consumed were consistent with the theoretical ratio for the complete oxidation of toluene to bicarbonate coupled with the reduction of sulfate to hydrogen sulfide, and (3) toluene degradation ceased when sulfate was depleted, and conversely, sulfate reduction ceased when toluene was depleted.

The synchronism of toluene degradation and sulfate reduction is apparent in Figure 3 and is supported by the very strong correlation coefficients for regressions of cumulative toluene degradation vs. cumulative sulfate reduction over time (typically $r^2 > 0.95$ for microcosms and enrichments). The ratio of sulfate consumed/toluene consumed for the microcosm shown in Figure 3 was 4.2. In Patuxent River enrichments, which were less susceptible than the microcosms to complicating effects related to the presence of soils, the ratio typically ranged from 3.5 to 4.2 in various experiments. These values approximate the theoretical ratios ranging from 4.5 (toluene oxidation to bicarbonate with no bacterial cell growth; Equation 1) to 4.0 (toluene oxidation to bicarbonate with estimated cell growth; Equation 2).

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. Further evidence of the link between toluene degradation and sulfate reduction was the apparent dependence of toluene degradation on the presence of sulfate, and conversely, the apparent dependence of sulfate reduction on the presence of toluene (shown for an enrichment in Figure 4).

If toluene oxidation and sulfate reduction were directly linked, what is the explanation for ferric iron reduction, which occurred concurrently with these two processes? Two possible explanations for ferric iron reduction are (1) direct coupling with toluene oxidation (where ferric iron could have served as a terminal electron acceptor for toluene) and (2) coupling with sulfate reduction (where ferric iron could have served as the electron acceptor for the oxidation of biogenic hydrogen sulfide). Stoichiometric ratios were used to examine the likelihood of these two explanations, although data for other variables would be required to reach more definitive conclusions. The ratio of Fe(III) reduced/toluene consumed was used to investigate the possibility that ferric iron-reducing bacteria were oxidizing toluene. The observed ratios were considerably lower than the theoretical ratio of 36 expected for toluene oxidation to bicarbonate coupled to ferric iron reduction (e.g., Equation 3).

For example, the observed ratio for the microcosm shown in Figure 3 was 12, which suggested that not enough ferric iron had been reduced to account for complete toluene oxidation. Thus, stoichiometry suggested either that ferric iron reducers were not involved in toluene oxidation or that they carried out incomplete oxidation. Indeed, the ratio of sulfate consumed/toluene consumed (discussed earlier) further suggests that ferric iron reducers played little, if any, role in toluene degradation.

A different stoichiometric ratio supported the contention that ferric iron reduction could have been the result of an oxidation-reduction reaction between ferric iron and biogenic hydrogen sulfide. The observed ratio of Fe(III) reduced/sulfate reduced was used to investigate the possible reactions between hydrogen sulfide and amorphous $Fe(OH)_3$. For this analysis, it was assumed, based on extensive literature about sulfate-reducing bacteria, that all reduced sulfate was converted to hydrogen sulfide. Potential reactions between amorphous $Fe(OH)_3$ and hydrogen sulfide that were considered for this analysis included the oxidation of hydrogen sulfide to either elemental sulfur or thiosulfate. The reactions were chosen based on reports in the geochemical literature of the abiotic, anoxic or oxygen-limited reactions of goethite or amorphous $Fe(OH)_3$ with hydrogen sulfide; in these studies, the rapid formation of ferrous sulfide, elemental sulfur, and/or thiosulfate was observed. For the microcosm shown in Figure 3, the ratio of Fe(III) reduced/sulfate reduced was consistent with the formation of thiosulfate but not elemental sulfur. Overall, the observed stoichiometric ratios relating Fe(II) appearance, toluene disappearance, and sulfate disappearance for that microcosm were consistent with Equation 4, which represents toluene oxidation coupled with sulfate reduction, and the reduction of ferric iron by hydrogen sulfide to form thiosulfate.

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). Two microcosms developed activity toward toluene and subsequently toward *o*-xylene. Toluene degradation started after approximately 100 - 120 days of incubation, whereas *o*-xylene degradation started after 200 - 255 days of incubation. Benzene, ethylbenzene, and *p*-xylene were not degraded in any microcosms. Autoclaved controls exhibited no degradation of any aromatic substrates. Upon re-feeding active microcosms with toluene (100 µM) and *o*-xylene (100 µM), the degradation of both compounds resumed immediately.

After the third re-feeding, 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 re-feeding 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.

Eq.1 $C_7H_8 + 4.5\ SO_4^{2-} + 3\ H_2O = 2.25\ H_2S + 2.25\ HS^- + 7\ HCO_3^- + 0.25\ H^+ \Delta G^{o\prime} = -49$ kcal/reaction

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

Eq. 3 $C_7H_8 + 36\ Fe(OH)_3$ (s) $+ 29\ HCO_3^- = 36\ FeCO_3$ (s) $+ 58\ H_2O + 29\ OH^-$

Eq. 4 $C_7H_8 + 11.8\ Fe(OH)_3$ (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) $+ 18.5\ H_2O + 9.34\ OH^-$

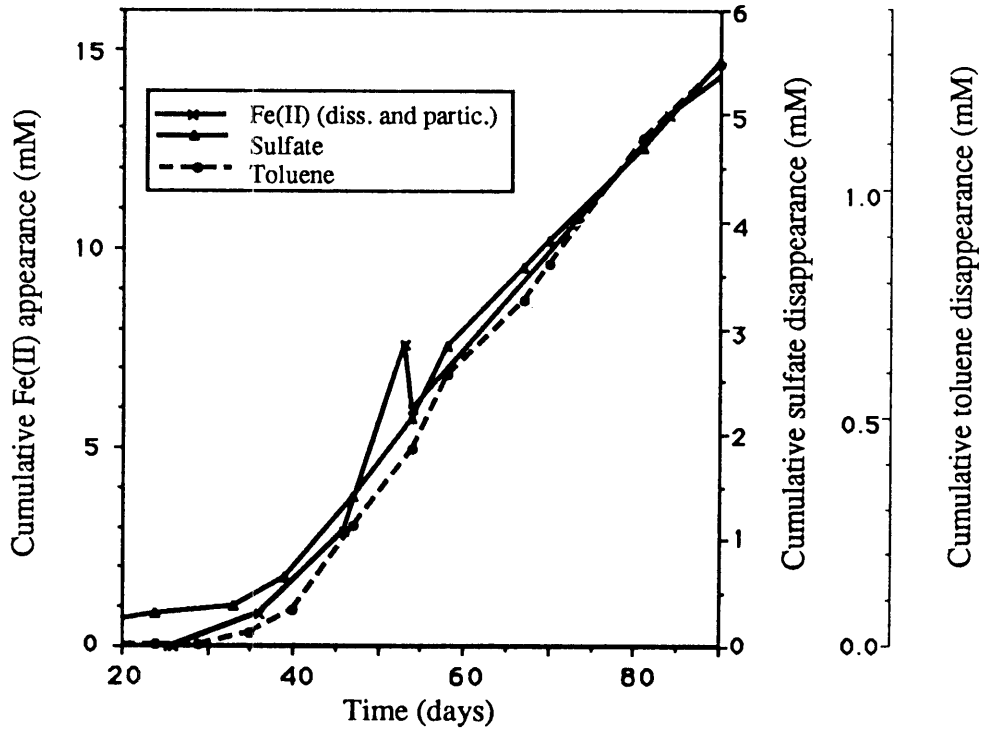

Figure 3. *Cumulative ferric iron reduction, sulfate reduction, and toluene degradation vs. time for a Patuxent River microcosm.*

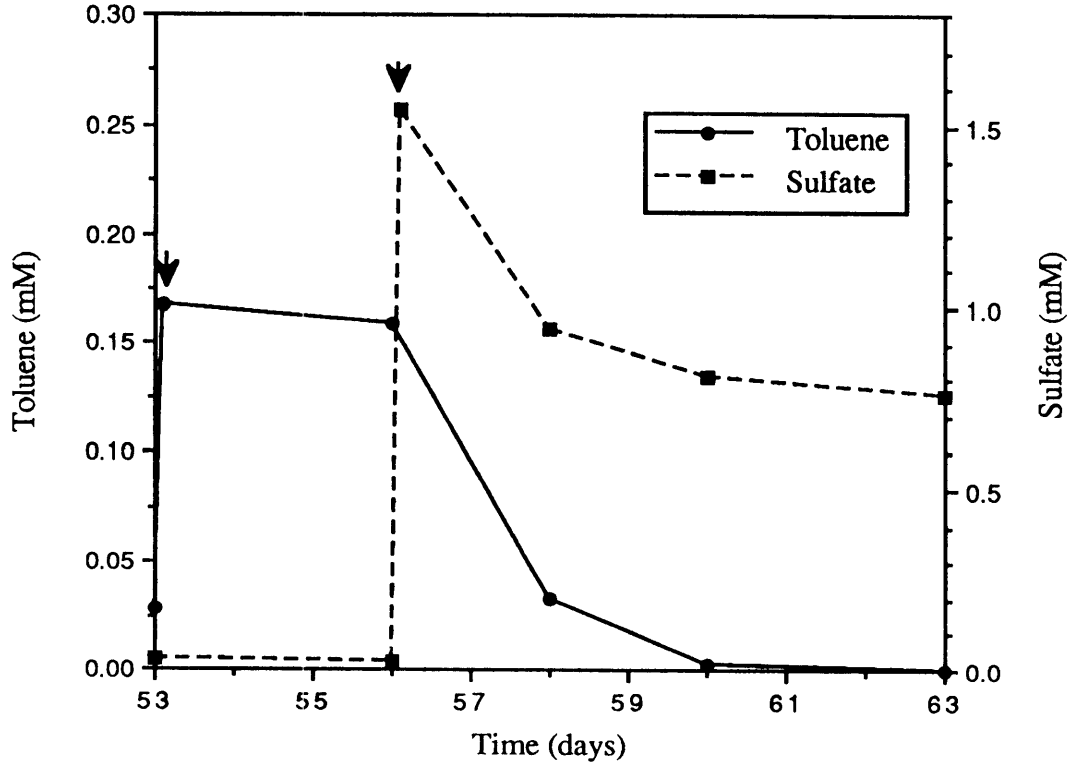

Figure 4. *Mutual dependence of toluene degradation and sulfate reduction for a Patuxent River enrichment. Arrows indicate amendment of toluene or sulfate. Data points represent the averages of duplicates.*

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 at an approximate rate of 4 μM/day (Figure 5). The substrates were continuously converted to CO_2 and CH_4. The original microcosms and the first transfers from the microcosms, which still contained a small amount of aquifer solids, 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). It is suspected that the solids were necessary for attachment of the microorganisms, or that the solids contained a trace nutrient which might have been essential for the transformations.

The degradation of toluene and *o*-xylene in suspended mixed cultures was associated with cell growth. Cell counts were determined by acridine orange staining in conjunction with epifluorescence microscopy. 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 highly dependent on initial cell count.

The effects of certain environmental conditions on the rate of toluene degradation were investigated. The cultures degraded toluene faster at 35° C than at 20° C, which is to be expected for enzyme-mediated reactions. pH 6 was more favorable than pH 7, and pH 8 appeared to be unfavorable. This result was consistent with the conditions in the contaminated aquifer, where a groundwater pH of 6 or below was predominant. 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 observations of other investigators that sulfide is inhibitory to methanogens.

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 (80 μM) alone, with 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 lag time before the onset of toluene degradation was approximately 50 days for both static and agitated microcosms. The acclimation lag for toluene was shorter in these microcosms than in the first study (in which the acclimation lag for toluene was 100 - 120 days) because the aquifer material used for these adaptation studies had already undergone an enrichment step during creosote-degradation studies. 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. Therefore, the addition of *p*-cresol, a putative intermediate in toluene degradation, did not appear to shorten the acclimation lag. 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.

Conclusions and Recommendations

Patuxent River Soils

The work with microflora enriched from hydrocarbon-contaminated Patuxent River soil resulted in two general findings: (1) degradation of toluene under sulfate-reducing conditions appears to occur despite the relatively small amount of energy that this process can yield to bacteria, and (2) the presence of iron [in the form of amorphous $Fe(OH)_3$] can either greatly facilitate the onset of toluene degradation under sulfate-reducing conditions or can accelerate the rate once degradation has begun. Both findings relate geochemical site conditions (e.g., oxidation-reduction potential, the presence of sulfate, the presence of iron-containing minerals) to the potential for *in-situ* biological restoration of aquifers contaminated with refined petroleum products, such as gasoline and aviation fuel.

Pensacola Aulfer Materials

In fermentative/methanogenic microcosms containing creosote-contaminated aquifer material (Pensacola, FL), the initial acclimation lag before the onset of toluene and *o*-xylene degradation was two to three months. Although the initial adaptation period was long, the degradation, once initiated, was relatively rapid, and the rate increased upon each subsequent re-feeding. Benzene, ethylbenzene, and *p*-xylene were not degraded. Stable suspended methanogenic consortia enriched from Pensacola aquifer microcosms degraded 50 μM of toluene and o-xylene to CO_2 and CH_4 in one to two weeks. The length of the adaptation lag and the rates of degradation were strongly influenced by environmental conditions, most notably the presence of other more readily degradable substrates.

General Conclusions and Recommendations

All of the inoculum sources tested in this project (including some materials not discussed in this summary) showed at least some evidence of anaerobic catabolism of alkylbenzenes, 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, chemical, and biochemical factors that influence anaerobic catabolism of aromatic hydrocarbons. With respect to the Patuxent River and Pensacola microflora studied for this project, important areas for future work include (1) isolation and characterization of pure, toluene-degrading cultures, or, if pure cultures cannot be obtained, examination of syntrophic relationships that appear to be required for toluene degradation; (2) detailed examination of the role of iron in facilitating toluene degradation in Patuxent River enrichments (e.g., the importance of iron oxidation state) and closer examination of the relevance of this process to *in-situ* toluene degradation in aquifers under sulfate-reducing conditions; and (3) determination of the metabolic pathways of toluene and *o*-xylene degradation under methanogenic and sulfate-reducing conditions.

The results of this study and the work of other researchers suggest that anaero-

bic 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.

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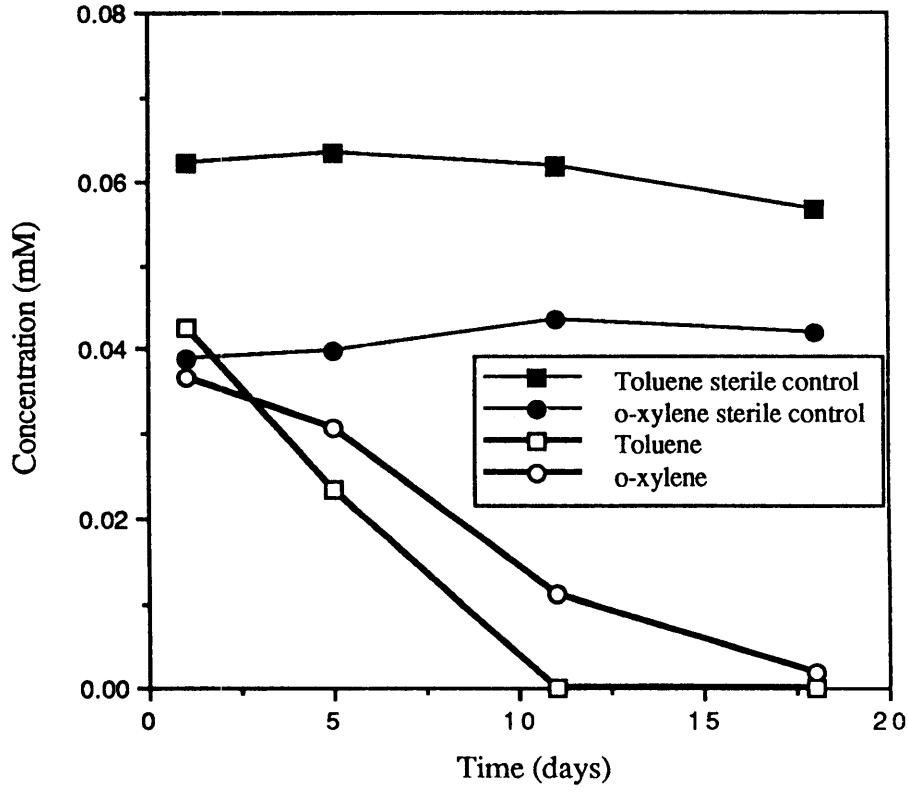

Figure 5. *Simultaneous degradation of toluene and o-xylene by a mixed culture enriched from Pensacola aquifer sediment under methanogenic conditions.*

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The complete report, entitled "Microbial Degradation of Alkylbenzenes Under Sulfate-Reducing and Methanogenic Conditions," (Order No. PB91-212 324/AS; Cost: $17.00, subject to change) will be available only from:

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