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PRELIMINARY INVESTIGATION ON ANAEROBIC BIODEGRADATION OF BENZENE IN CONTAMINATED AQUIFER SEDIMENT

by
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ABSTRACT

The objectives of this research were to document the anaerobic biodegradation of benzene in aquifer sediment recovered from a petroleum-contaminated aquifer near Rocky Point, N.C., and to identify the microbial conditions controlling its biodegradation. Laboratory-scale microcosms were constructed with contaminated sediment recovered from a section of the plume (mid-plume) from which previously excavated sediment had exhibited benzene biodegradation. Microcosms were also constructed with sediment from another section of the plume (end-plume) that had previously exhibited benzene biodegradation. Most probable number (MPN) assays were also initiated with these sediments to enumerate the total populations of iron and sulfate reducers and the sub-populations capable of benzene and toluene conversion to carbon dioxide.

No benzene mineralization was measured in the microcosms constructed with mid-plume sediment, and benzene biodegradation decreased to an undetectable rate with repeated benzene additions to the end-plume microcosms. The cessation of benzene biodegradation activity generally corresponded with a shift in the microcosms to methanogenic conditions, although the mechanism for this relationship could not be determined.

The MPN assays showed very low populations of both iron and sulfate reducers, leading to questions about whether the commonly used MPN media employed here were appropriate for this aquifer sediment. Radiolabeled benzene and toluene were used in the MPN assays to increase their sensitivity. However, the production of $^{14}CO_2$ in the benzene- and toluene-specific MPN assays did not exceed the amounts that could be attributed to impurities in these substrates. Thus, the MPN data did not provide evidence for the presence of sub-populations of iron or sulfate reducers that could degrade benzene or toluene.

Key Words: intrinsic bioremediation, biodegradation, benzene, toluene, iron reducers, sulfate reducers, MPN, groundwater

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SUMMARY AND CONCLUSIONS

Previous research has shown that benzene biodegradation occurred in microcosms constructed with mid-plume and end-plume sediment from a petroleum-contaminated aquifer near Rocky Point, N.C. However, additional hydrocarbons (TEX) were also added in most cases, and benzene loss occurred only after a lengthy lag period (> 100 days). In this study, additional microcosm experiments were performed to document mineralization of benzene and to understand the importance of iron and sulfate as electron acceptors for benzene biodegradation. A microcosm experiment was initiated containing added ^{14}C-benzene with sediments obtained from the mid-plume area of the aquifer. Additional work was carried out with existing microcosms containing end-plume sediment in which benzene loss had been measured. The purpose of this work was to determine if once initiated, repeated benzene loss would continue with no lag period between re-additions of benzene and to determine if dissolved sulfate was consumed during biodegradation.

Most Probable Number (MPN) assays were also conducted with contaminated sediment from each microcosm experiment as a source inoculum. The primary objective of this experiment was to study and enumerate the indigenous sulfate- and iron-reducing organisms from each area of the aquifer. The second objective was to determine if a difference in the numbers of benzene degraders could be measured from two different inocula: one from mid-plume microcosms in which benzene biodegradation had not yet occurred and the other from end-plume microcosms in which benzene loss had been repeatedly measured. Assays utilizing "readily degradable" substrates were prepared to enumerate the total iron-and sulfate-reducing populations. In additional MPN assays, ^{14}C-toluene and benzene were used to represent more and less degradable hydrocarbons in the aquifer sediment, respectively.

BENZENE BIODEGRADATION

Although $^{14}CO_2$ production was measured in the mid-plume microcosms, mineralization of benzene could not be verified due to impurities in the commercially supplied ^{14}C-benzene. Total organic carbon decreased in all microcosms, indicating that indigenous microorganisms were active and utilizing other carbon present on the contaminated sediments and groundwater.

In end-plume microcosms in which previous biodegradation was measured, relatively high amounts (>10 mg/L) of benzene loss were repeatedly measured with no lag period in five of six microcosms. Although a small increase in the decay rate was apparent after the second respiking in two microcosms, the decay rate did not increase significantly with successive benzene additions. Benzene loss ceased in all replicates after 560 days, concurrent with the detection of methane. The biodegradation of benzene, toluene, ethylbenzene, and xylene isomers (BTEX) ceased when methane was produced in previous experiments and suggests that a shift in the dominant electron-accepting process occurred during the monitoring period.

MPN ASSAYS

Overall, the MPN assays did not prove to be a useful tool to elucidate the number or type of indigenous organisms(s) responsible for benzene or toluene degradation. The MPNs of the

various populations were, in general, unrealistically low to undetectable. Even in assays where organisms were detected, a pattern of growth was not evident because positive tubes were spread throughout several dilutions. Complex microbial associations such as co-metabolic or syntrophic relationships involving more than one type of organisms may be present in this aquifer, preventing successful culturing on selective media. Two lines of evidence suggest limitations to the MPN assays: (1) the failure to detect sulfate reducers in the standard assays containing "readily degradable" substrates and (2) the number of positive tubes containing ^{14}C-benzene was comparable in both the mid-and end-plume inocula.

With respect to the negative results in the sulfate-reducing medium containing common substrates (Section 3.2, Table 4), it is apparent that organisms capable of sulfate reduction are present in the aquifer as evidenced by both black precipitate formation and $^{14}CO_2$ production in the tubes containing hydrocarbons as substrates. Thus, the particular type(s) of indigenous sulfate reducer(s) in the aquifer sediment were not successfully cultured in the medium used. This poses a significant drawback in using a standard medium to assess populations in some sedimentary environments.

A second line of evidence also suggests a limitation in the medium: the inoculum used for the end-plume MPN assays was obtained from a microcosm in which three successive additions of benzene had been readily degraded (Section 2.4.1, Figure 1). Thus, a substantial number of organisms capable of benzene biodegradation should have been present in the end-plume inocula. Yet, the number of bacteria detected in the end-plume inoculum was comparable to the mid-plume inoculum (Section 3.2, Table 4) in which no benzene biodegradation was evident in microcosms after 263 days (Section 3.1.1, Figure 2). Given this, a lower benzene-degrading population in the mid-plume inoculum relative to the end-plume inoculum would be expected if the medium provided an adequate environment to support growth of this population. The complete conversion of benzene or toluene to CO_2 by the cultured organism(s) is assumed for detection in the ^{14}C MPN assays, since the results were based on the production of $^{14}CO_2$ from ^{14}C substrates. Thus, if toluene or benzene degraded in the MPN but did not mineralize, the outcome would be negative. Impurities in the radiolabeled substrates also limited the usefulness of the substrate-specific MNP data.

CONCLUSIONS

The conclusions of this study are as follows:

- The extent of biodegradation varied in the aquifer sediment obtained from different areas of the plume. Large- and small-scale variations in contaminant degradation and geochemical conditions along the length of the plume make direct comparison of decay rates measured over different scales difficult.

- Microbial enumeration by MPN assays did not prove to be a useful tool to elucidate the number or type of indigenous organism(s) responsible for benzene or toluene degradation. Although clear evidence for toluene and benzene degradation is found in field data and in microcosm studies, the responsible organism(s) were not successfully cultured under the conditions provided in the MPN assays.

- Sulfate-reducing bacteria were not enumerated in a medium containing commonly used substrates considered to be "readily degradable."

- The factors governing benzene degradation in the subsurface are poorly understood. Physical, geochemical, and environmental characteristics in the subsurface can vary significantly not only from site to site but also on an intrasite level.

RECOMMENDATIONS

- Research is needed to better understand the factors causing spatial variations in contaminant degradation rates and to develop more accurate models for simulating these variations.

- Further knowledge is needed to understand the significance of ambient characteristics, their impact on ecology, the metabolic capabilities of indigenous microorganisms, and the factors that can influence microbial activity in the environment to reliably predict the fate of anthropogenic contaminants in the subsurface.

1.0 INTRODUCTION

Aromatic hydrocarbons such as benzene are ubiquitous in nature and occur as a result of natural processes as well as anthopogenic releases. It is generally accepted that most aromatic hydrocarbons in the environment are naturally formed from the pyrolysis of organic compounds (Gibson and Subramanian, 1984). The benzene ring is among the most widely distributed units of chemical structure in the environment (Dagley, 1981). It is not surprising that microorganisms have evolved that can utilize hydrocarbons as energy sources and, thus, naturally degrade these compounds in the environment.

Refined petroleum-based products can contain up to 30% of the water-soluble aromatics benzene, toluene, ethylbenzene, and xylene isomers (BTEX). BTEX compounds and are often identified as the major groundwater contaminants from underground storage tank (UST) releases and other fuel spills. Their presence in groundwater used for drinking water poses a threat to human health. The estimated number of USTs containing gasoline is 1.4 million, with 85% of the tanks constructed of steel without corrosion protection (Bowlen and Kosson, 1995). Cleanup costs for these sites using conventional technologies typically exceed $250,000.

Intrinsic bioremediation is a remedial alternative in which indigenous microorganisms are used to biodegrade subsurface contaminants without direct human intervention. Its use can greatly reduce the cost and risk of exposure relative to conventional groundwater treatment alternatives. However, to gain regulatory acceptance, an understanding of the underlying processes controlling the rate and extent of intrinsic biodegradation must be demonstrated. Assessing the potential for intrinsic bioremediation in the subsurface is complicated by site-specific conditions such as environmental factors and the indigenous microbial population.

Previous microcosm experiments using sediments obtained from a petroleum-contaminated aquifer near Rocky Point, N.C. (Arvida) verified that indigenous microorganisms in the contaminated sediments and groundwater can biodegrade BTEX under anaerobic conditions (Hunt et al., 1997b). A sequential pattern of BTEX degradation occurred and was consistent with groundwater monitoring data along the plume length. Toluene and *o*-xylene degraded first, followed by *m*- and *p*-xylene, benzene, and finally ethylbenzene. In microcosms using mid-plume sediments, ferric iron was the dominant electron acceptor when BTEX loss was measured (Hunt et al., 1997b). However, given the sequential pattern of degradation and detectable amounts of dissolved sulfate, sulfate reduction could not be completely excluded as an electron sink. Additionally, groundwater monitoring indicates sulfate decreases in the upper portion (source area) of the plume, suggesting that sulfate reduction occurs *in situ*. Surprisingly, highly variable sulfate levels were discovered in both mid- and end-plume sediments as a result of the microcosm experiments, indicating that sulfate may be available and thus utilized as an electron acceptor throughout the aquifer. Therefore, based on previous laboratory and field research, several questions remained:

- Is benzene completely mineralized by sediment from the Rocky Point site?
- Why is there a lag prior to the onset of benzene degradation in microcosm studies?

- What electron acceptor controls benzene biodegradation?

1.1 BENZENE LOSS

Of particular interest was the consistent loss of benzene in the microcosm experiments. This hydrocarbon is especially problematic since it is a carcinogen, is regulated at very low levels in drinking water [49 FR 114 (1984)], and is often recalcitrant under anaerobic conditions (Acton and Barker 1992; Beller et al., 1991; Evans et al., 1991; Hutchins et al., 1991; Edwards et al., 1992; Edwards and Grbic-Galic, 1994). Although other researchers have reported anaerobic benzene biodegradation (Kazumi et al., 1997), most often the growth environment provided did not reflect ambient conditions, and thus the potential for *in situ* biodegradation cannot be directly assessed. In all Rocky Point microcosm experiments, there was a significant lag period (100 to 200 days) prior to the onset of benzene loss (Hunt, 1997). Moreover, the first-order decay rate during the time of measurable benzene loss was two orders of magnitude higher in laboratory and *in situ* column experiments (Hunt et al., 1997a) than as estimated from field data (Borden et al., 1995; Hunt et al., 1997a). This discrepancy poses limitations in relating experimental data to *in situ* decay and raises questions regarding the occurrence of *in situ* benzene biodegradation. In microcosms prepared with source area sediment from the same plume, an extended lag period (>300 days) was evident prior to measurable toluene and *o*-xylene loss. During this lag period, there was a very slow degradation rate for these hydrocarbons. This raises the possibility that a very slow rate occurs during the lag period and may reflect *in situ* decay rates in some portions of the plume.

1.2 LAG PERIOD

Researchers have reported the presence of a lag period prior to biodegradation of newly introduced organic compounds (Corseuil and Weber, 1994). It is hypothesized that this lag period is attributable to insufficient numbers of contaminant-adapted indigenous microbes, that is, low initial populations when experiments are initiated. Aelion et al. (1987) further discusses several potential mechanisms for an extended lag period prior to compound degradation.

> In some cases, it appears that time is required for a population to increase to the extent required to produce a measurable biodegradation rate (Spain et al., 1980; Ventullo and Larson, 1986; Wiggins et al. 1987). Other possible explanations include the time necessary for enzyme induction (Schmidt et al., 1987) or genetic alterations (Wyndham, 1986). Environmental factors have also been suggested as significant influences, such as insufficient nutrients, and the preferential utilization of organic compounds other than the pollutant (Lewis et al., 1986; Swindoll et al., 1988). Finally it is possible that time is required for the degraders to acclimate to toxins or inhibitors in their environment (Wiggins et al., 1987).

Groundwater contamination was first detected in 1987 at the Rocky Point site. At the time of microcosm construction, the microcosms had been exposed to benzene for a minimum of 5 years and would have had ample time for "contaminant adaptation." Laboratory microcosm experiments showed that the addition of nutrients (nitrogen, phosphors, and trace vitamins or

minerals supplements), sulfate, sodium molydbate, and BES did not decrease this lag period. In fact, most nutrients increased the lag period or inhibited benzene biodegradation (Hunt, 1997). In addition, the lag period did not decrease in additional microcosm experiments with benzene as the only added hydrocarbon, indicating that the presence of TEX did not directly influence the lag period (Hunt, 1997). Consequently, none of the mechanisms listed by Aelion et al. (1987) fully explains the observed lag period.

1.3 ELECTRON ACCEPTOR

Sediment characterization documented the presence of significant amounts of solid phase Fe (III) in the mid-plume aquifer sediment. However, given the presence of small, but highly variable concentrations of sulfate, it has not been possible to delineate the electron acceptor that supports benzene biodegradation on the basis of previous microcosm experiments (Hunt et al., 1997b).

2.0 EXPERIMENTAL METHODS

2.1 EXPERIMENTAL DESIGN

To further examine the anaerobic biodegradation of benzene, microcosm experiments using sediments from two areas of the plume were established. One set of microcosm experiments was prepared from mid-plume sediment and contained ^{14}C-benzene. This set was used to determine whether (1) benzene was mineralized, (2) the initial concentration of benzene influenced biodegradation, and (3) a slow mineralization rate could be measured by means of radiolabeled benzene during the period prior to detecting benzene loss by gas chromatography (i.e., during the lag period). A second set of microcosms, containing end-plume sediment from previous microcosm experiments in which benzene loss had been measured, was monitored for continued benzene loss. These microcosms were spiked with unlabeled benzene several times to evaluate whether (1) repeated benzene loss could be measured, (2) the lag period prior to benzene loss could be reduced, and (3) the decay rate was altered during this process. Indigenous microorganisms present on the aquifer sediment and in the groundwater were used in all microcosm experiments. The design of the ambient microcosm experiments was based on the U.S. Environmental Protection Agency protocol for "anaerobic microbiological transformation rate data for chemicals in the subsurface" [53 FR 115 (1988)]. Microcosms simulated ambient conditions to the maximum extent possible.

Most probable number (MPN) assays were also conducted with the contaminated sediments used to initiate each microcosm experiment to better enumerate the indigenous microorganisms present that were capable of hydrocarbon degradation. Since iron reduction and sulfate reduction were the dominant electron sinks in the anaerobic portion of the contaminated aquifer (Borden et al., 1995), two media were used: the first specific to the growth of sulfate reducers, and the second specific to the growth of iron reducers. Assays containing standard or "readily degradable" substrates were prepared. Hydrocarbon-specific assays also were prepared. In these assays, ^{14}C-toluene and benzene were chosen to represent more and less degradable hydrocarbons in the aquifer sediment, respectively. The objectives of this experiment were (1) to enumerate indigenous sulfate- and iron-reducing organisms from the two different areas of the aquifer and (2) to determine if a difference in the numbers of benzene degraders could be measured from two different inocula: one from mid-plume microcosms in which benzene biodegradation had not yet occurred (lag phase) and the other from end-plume microcosms in which benzene loss had been repeatedly measured. Computation of MPN values was made with a FORTRAN-based program (Russek and Colwell, 1983).

2.2 SAMPLE COLLECTION

Aquifer sediment was obtained from the mid-plume area of a petroleum-contaminated plume near Rocky Point, N.C. All sediment was obtained under anaerobic conditions by drilling below the water table with a hollow-stem auger and then advancing a sterile coring tube (Dunlap et al., 1984). The tube was brought to the surface, immediately capped with sterile butyl rubber stoppers, and transported to the laboratory on ice where it was extruded into an anaerobic glovebox (Ray Products, El Monte, Calif.) under N_2 within 24 h. The first and last 10 cm of the core were removed, and the outer portions of sediment were pared away. The remaining

sediment was anaerobically transferred into sterile mason jars and stored at 4°C. Prior to use, the wet sediment from each core was mixed and passed through a No. 8 sieve in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.).

Groundwater for laboratory experiments was collected anaerobically from an adjacent well at the time of sediment collection. The well screen interval was selected to correspond to the sediment sampling depth. The well headspace was sparged continually with argon for 10 min. before and during sample collection. Groundwater was pumped from the well by means of an inertial pump (Waterra, Buffalo, N.Y.) connected to a closed system of polyethylene tubing and collected in a nitrogen-sparged 2.3-L (80-oz.) glass bottle. The collection bottle was sealed with a two-holed rubber stopper that was connected to a second nitrogen-sparged bottle. The second bottle released pressure as water entered the collection bottle, thus preventing air from entering the system on the backstroke of the pump. The water was transported to the laboratory on ice where it was stored at 4°C prior to use. All equipment and containers in contact with the water and sediment were presterilized.

2.3 MICROCOSM CONSTRUCTION AND ANALYSIS

2.3.1 Mid-Plume Sediment. Multiple replicate microcosms with no headspace were prepared within two weeks of sample collection. Anaerobic and aseptic conditions were rigorously maintained during all manipulations and storage. All containers, stoppers, and instruments coming in contact with the sediment or water were sterilized by autoclaving prior to use. Construction and sampling was performed in a Coy anaerobic chamber containing N_2:H_2 (95:5). The microcosms were prepared in 60-mL (75-mL working volume) serum bottles and contained 95 g saturated sediment and 45 mL of total liquid. The final dry sediment to water ratio was 1.8 g/mL. This design resulted in 18 mL of free liquid available for sampling. Several sets of multiple replicate microcosms were constructed.

Microcosms were filled with groundwater to eliminate all headspace; spiked first with the stock solution containing sterile anaerobic deionized water, resazurin (0.0002% final concentration), and benzene (final concentration ~7 mg/L); and then spiked with a second solution containing uniformly labeled ^{14}C-benzene (Moravek Biochemicals, Inc., Brea, Calif.) and sterile anaerobic deionized water (~300,000 dpm/mL final concentration). The microcosms were then sealed with Teflon-lined gray butyl stoppers (West Co., Phoenixville, Pa.) and aluminum crimps. A second set of microcosms was prepared to represent ambient benzene concentrations in the sediment and contained uniformly labeled ^{14}C-benzene (Sigma Chemical Company, Saint Louis, Mo.) (100,000 dpm/mL) but no additional unlabeled benzene (final concentration ~ 275 µg/L from the groundwater and saturated sediments). Sterile anaerobic water was prepared by boiling deionized water under nitrogen followed by autoclaving in sealed serum bottles containing a nitrogen headspace for 20 min. at 121°C. The ^{14}C-benzene spike solution was prepared by aseptically dispensing the contents of the sealed ampoule from the vendor into a 160-mL serum bottle containing sterile anaerobic deionized water in the anaerobic chamber. The serum bottle was capped with a black butyl stopper and aluminum crimp seal and stored for 1 week at 4°C prior to use. To allow for supplemental analyses, a separate set of microcosms that did not contain the ^{14}C-benzene spike was monitored in parallel for ions, methane, and total organic carbon (TOC).

The abiotic controls were prepared in the anaerobic chamber, closed with a black butyl stopper, and autoclaved at 121°C for 30 min. on two consecutive days prior to adding the spike solutions. The microcosms were incubated in the dark at 16°C in anaerobic incubation jars with oxygen-scavenging catalyst envelopes containing sodium borohydride (BBL Gas Pak Jar System, Fisher Scientific, Raleigh, N.C.) and dry redox indicator strips (BBL Microbiology Systems, Becton Dickinson and Co., Md.). The incubation jars were evacuated and refilled with nitrogen three times whenever the microcosms were removed for sampling. The resazurin redox indicator remained colorless throughout all experiments, indicating that anaerobic conditions were maintained. Triplicate live and abiotic microcosms were destructively sampled at monthly intervals.

The microcosms were sampled in the anaerobic chamber by simultaneously removing approximately 5 mL of free liquid in a gas-tight syringe (Hamilton Co., Reno, Nev.) while puncturing the stopper with a needle, so a vacuum did not develop. The microcosms were shaken 24 h prior to sampling to ensure a well-mixed sample and to allow time for the sediment fraction to settle. Care was taken to remove a sample that did not contain visible particulate material.

In microcosms without labeled benzene, the first mL, used for methane analysis, was injected into a 10-mL vial sealed with a black butyl rubber stopper (Geomicrobial Technologies, Inc., Ochelata, Okla.) and stored at 4°C. Within 48 h, a 1-mL headspace sample was removed from this vial while simultaneously pressurizing the vial slightly by injecting into it 3 mL of high-purity deionized water. This sample was immediately injected into a Shimadzu 9A gas chromatograph (GC) equipped with a 1.5-m by 3.2-mm stainless steel column packed with Hayesep T 100/120 mesh (Altech, Deerfield, Ill.) and a flame ionization detector (FID). The dissolved methane concentration was calculated by assuming that all the dissolved methane in the 1-mL aliquot volatilized into the vial headspace. For BTEX analyses, between 0.25 and 1 mL of the remaining 1-mL sample from the microcosm was placed in a closed test tube and diluted to 5 mL. BTEX analyses were performed by using a Tekmar Purge-and-Trap Model LSC 2000 and a Perkin Elmer Model 900 autosystem GC or a Tekmar Purge-and-Trap Model LSC 3000 with Model 2016 Purge and Trap and a Shimadzu GC-14A. Both GCs were equipped with a 75-m DB-624 Megabore capillary column (J & W Scientific, Folsom, Calif.) and an FID. Peak areas were compared with external standards.

Dissolved TOC analysis was performed by injecting the remaining liquid into a small vial so that no headspace resulted. The vials were sealed and stored at 4°C. The total carbon and inorganic carbon in the liquid samples were measured directly with a DC-190 High Temperature TOC Analyzer (Rosemont Analytical Inc., Santa Clara, Calif.). Total organic carbon was calculated as the difference between these two measurements.

An additional 3 mL of liquid was removed from the microcosms for analysis of dissolved iron, total sulfur, sulfate, thiosulfate, and sulfite. This liquid was filtered (0.2-μm nylon acrodisk™, Gelman Sciences, Ann Arbor, Mich.) into vials containing 0.5N HCl. Samples were stored at 4°C and analyzed within 2 weeks. Iron and total sulfur were analyzed with a Perkin Elmer II inductively coupled plasma argon emission spectrometer (ICP-AES). Sulfate, thiosulfate, and sulfite were analyzed with a Dionex 2010i ion chromatograph (IC) with a Dionex AS4A column. The mobile-phase flow rate was 2 mL/min. and contained 1.8 mM Na_2CO_3 plus 1.7 mM

$NaHCO_3$. The pH of the microcosms was measured in the anaerobic chamber after the liquid was removed for all other analyses.

Microcosms containing uniformly labeled ^{14}C-benzene, BTEX, and pH were analyzed by the procedures described above. The first 3 mL (or 4 mL) of sample were subjected to three (or four) different treatments to measure the ^{14}C activity in the microcosms. Sample processing methods to determine ^{14}C activity were similar to those described by Grbic-Galic and Vogel (1987) with some modification to facilitate use of a scintillation fluid with a high flashpoint and to estimate the contribution of sorbed ^{14}C to particulates (if present) in the sample. Four samples were processed:

- Sample One: A 1-mL sample was injected directly into a vial containing 18 mL of Ultima Gold scintillation fluid (Packard Instrument Co., Inc., Meriden, Conn.) and shaken vigorously for 30 s. This sample included benzene, volatile intermediates, nonvolatile intermediates, CO_2, and particulates (if present).

- Sample Two: A second 1-mL sample was injected into a vial containing 1 mL 2N NaOH (pH > 11) and then purged with N_2 for 30 min. (flow rate 150 mL/min.). After purging, 17 mL of Ultima Gold scintillation fluid was added, and the vial was shaken vigorously for 30 s. This sample represented the sum of dissolved $^{14}CO_2$, nonvolatile intermediates, and particulates (if present).

- Sample Three: A third 1-mL sample was injected into a vial containing 3 mL 0.1 N HCL (pH < 2) and then purged with N_2 for 30 min. (flow rate 150 mL/min.). After purging, 15 mL of scintillation fluid was added, and the vial was shaken vigorously for 30 s. This sample represented the activity from nonvolatile intermediates and particulates (if present).

- Sample Four: A fourth 1-mL sample was passed through a 0.2-µm filter (Gelman Sciences, Ann Arbor, Mich.) and then analyzed by following the procedure described for Sample Three. This fraction represented the ^{14}C activity of nonvolatile intermediates. The particulate contribution was calculated as the difference between the purged acid-filtered sample (Sample Four) and the purged acidified sample (Sample Three). This measurement was not found to be significant unless visible particulates were present; thus, this analysis was not performed on every sample.

$^{14}CO_2$ was calculated as the difference between the ^{14}C dpm of the purged NaOH-treated sample (Sample Two) and the purged HCl-treated sample (Sample Three). Blanks containing scintillation fluid, acid, and base also were prepared; sparged (base or acid samples); and analyzed concurrently. The background levels (~30 dpm) were subtracted from the results of each sample. The ^{14}C activity was measured with a Tri-Carb Liquid Scintillation Analyzer, Model 2100TR (Packard Instrument Co. Inc., Meridan, Conn.).

The biochemical purity of the ^{14}C-benzene was specified by the vendor as > 98%. The total amount of counts (dpm/mL) and purity of the spike solution were verified prior to initiating the

experiments by analyzing the total counts, the volatile fraction, and the nonvolatile fraction by using the protocol described above. The results are shown in Table 1.

Table 1. Purity of Radiolabeled Benzene and Toluene.[a]

Fraction	Benzene-1[b]	Benzene-2[b]	Toluene
Nonvolatile ^{14}C	1.8	0.09	8
$^{14}CO_2$	0.7	0.03	0.1

[a]Data are expressed as a % of the total dpm of benzene or toluene.

[b]Benzene-1 was used in the microcosms containing ambient benzene concentrations and in the benzene MPN tubes. Benzene-2 was used in the microcosms containing an elevated benzene concentration.

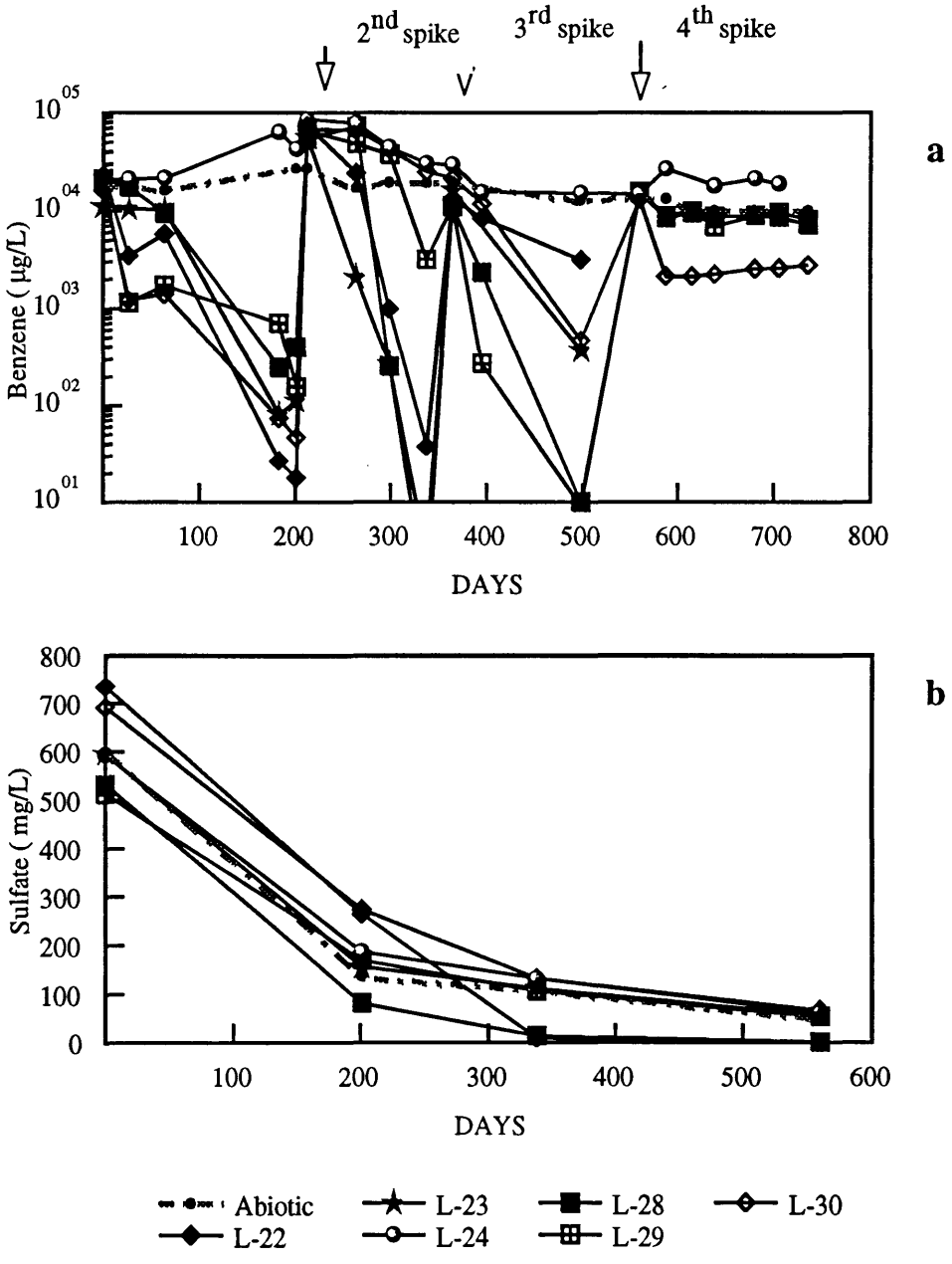
2.3.2 End-Plume Sediment. Seven separate microcosms were prepared from previously destructively sampled microcosms containing end-plume sediment in which benzene loss had been measured. The microcosms consisted of the three live (L22–L24) and one abiotic (A24) end-plume microcosms destructively sampled after 253 days of incubation and three live microcosms (L28–L30) destructively sampled after 327 days (Hunt, 1997). The microcosms were prepared by adding 3 mL of a stock solution containing sterile anaerobic deionized water, resazurin (0.0002% final concentration), and benzene (final concentration ~ 15,000-40,000 ug/L); topped with autoclaved groundwater from the adjacent monitoring well; and capped with a mininert valve (Supelco Inc., Pheonixville, Pa.). The valves were sterilized by soaking them in methanol stock solution for 24 h prior to use. The concentration of benzene was < 500 µg/L in all microcosms before respiking. Sulfate concentrations were >500 mg/L in all replicates, and trace levels of methane had been measured in all replicates (<0.1 mg/L). The microcosms were covered to prevent exposure to light and incubated in the anaerobic chamber (28°C) where they were sampled for benzene 19 times over the course of 765 days. The analytical procedures were identical to those used for the mid-plume microcosms (Section 2.3.1). Sampling was performed in the Coy anaerobic chamber by removing approximately 0.2 mL of liquid with a gas-tight syringe. The liquid that was removed was replaced with sterile anaerobic deionized water by means of a separate sterile syringe. Duplicate analyses were performed on every fifth sample.

Benzene was periodically re-added to the microcosms. This was accomplished by removing 3 mLs of liquid from each microcosm and replacing the liquid with the benzene stock solution. The withdrawn liquid was filtered (0.2 µm) and acidified for dissolved iron and sulfate analysis by IC and ICP-AES as described in Section 2.3.1.

2.4 MICROBIAL ENUMERATION

2.4.1 Inocula Preparation. There were two sources of inocula used in the assays. The first source was mid-plume aquifer sediment used to construct the microcosms containing ^{14}C-benzene (Section 2.3.1). The contents of one live microcosm (containing unlabeled benzene) were emptied onto a sterile glass dish in the anaerobic chamber. The sample was blended and 10 g was used to prepare a single inoculum in the assays. The second inoculum consisted of 10 g of end-plume sediment from a respiked microcosm in which benzene degradation had been measured (Figure 1).

Figure 1. Benzene and Sulfate in Respiked End-Plume Microcosms



The 10-g sediment sample was diluted in 90 mL of a sterile basal salts medium (BSM) containing approximately 100 3-mm-diameter glass beads. The container was vigorously shaken for 1 min. to separate the organisms from the sediment. This procedure was shown to increase enumeration of lactose-degrading bacteria from a chemostat (Chartrain and Zeikus, 1986). A 10-mL volume of this suspension was serially diluted in 90 mL BSM to a 10^{-5} dilution and treated in the same manner as the primary dilution. A 1-mL portion from each dilution was inoculated into the MPN tubes for a total of 10 or 5 replicates. The BSM was prepared by dissolving the following in deionized water (g/L): NH_4Cl (1.5), NaH_2PO_4 H_20 (0.6), KCl (0.1), $NaHCO_3$ (2.5), $CaCl_2*2H_20$ (0.1), NaCl (0.1), Mg Cl_2 $*6H_2O$ (0.1), and $MnCl_2*4H_2O$ (0.005).

The solution was then bubbled under a stream of N_2 and stored in the anaerobic chamber overnight. The next day, 90 mL of solution was transferred to serum bottles containing the glass beads, sealed with black butyl stoppers (Geomicrobial Technologies, Inc., Ochelata, Okla.), and then autoclaved for 20 min. at 121°C. The pH was adjusted to 7.

2.4.2 Total Sulfate Reducers. Sulfate reducers were enumerated by a five-tube MPN assay based on a phosphate buffered basal medium used by Chartrain and Zeikus (1986) to enumerate sulfate reducers in a chemostat. The medium contained the following (g/L): KH_2PO_4 (1.5), $K_2HPO_4*3H_2O$ (2.9), $MgCl_2*6H_2O$ (0.2), $CaCl_2*2H_2O$ (0.1), NaCl (0.9), and NH_4Cl (1.0). The medium also contained these vitamins (µg/L): biotin (10), pantothenic acid (25), lipoic acid (25), folic acid (10), thiamine (25), riboflavin (25), pyridoxine-HCL (50), cyanocobalamin (0.05), nicotinic acid (25), and *p*-aminobenzoic acid (25).

In addition, resazurin (0.0002% final concentration) and 10 mL/L of trace mineral solution were added. The trace mineral solution consisted of (g/L): nitrilotriacetic acid (15), $FeSO_4*7H_2O$ (0.1), $MnCl_2*4H_2O$ (0.1), $CoCl_2*6H_2O$ (0.17), $CaCl_2*2H_2O$ (0.1), $ZnCl_2$ (0.1), $CuCl_2*2H_2O$ (0.02), H_3BO_3 (0.01), $Na_2MoO_4*2H_2O$ (0.01), NaCl (1.0), Na_2SeO_3 (0.016), $NiSO_4*6H_2O$ (0.026), and $NaWO_4*2H_2O$ (0.033).

To prepare the MPN assay, the medium containing the trace mineral solution, Na lactate (2.24 g/L), $FeSO_4$ (3.02 g/L), and yeast extract (0.050%) was combined with deionized water in a beaker. The following additional carbon sources were added separately (g/L): Sodium acetate (2.0), propionic acid (0.7), *n*-butyric acid (0.8), and benzoic acid (0.5). The pH was adjusted to 7.0, and the medium boiled under a N_2:CO_2 (80:20) gas stream that had been passed through a heated copper column and autoclaved for 20 min. at 121°C. Next 0.020% sodium ascorbate, a filtered sterilized vitamin solution, and $NaHCO_3$ (3.5 g/L) were added. The medium (9 mL) was then dispensed into tubes and sealed with black butyl stoppers and crimp seals. The tubes were incubated at 25°C for 131 days in the dark. Tubes containing a black precipitate (Fe_2S) were interpreted as positive for sulfate reducers.

2.4.3 Total Iron Reducers. Iron reducers were enumerated by way of a ten-tube MPN assay based on medium described by Lovley and Phillips (1986). The medium contained the following (g/L): NH_4Cl (1.5), NaH_2PO_4 H_2O (0.6), KCl (0.1), $NaHCO_3$ (2.5), $CaCl_2*2H_20$ (0.1), NaCl (0.1), Na_2MoO_4 $*2H_2O$ (0.001), Mg Cl_2 $*6H_2O$ (0.1), $MnCl_2$ $*4H_2O$ (0.005), and yeast extract (0.050%).

Resazurin (0.0002%) also was included as a redox indicator. Trace vitamin and mineral solutions were added to the media to yield final concentrations equal to those in the sulfate-reducing media described in Section 2.4.2. Amorphous $Fe(OH)_3$ (250 mM) was added as the electron acceptor and sodium acetate (2.7 g/L) as the carbon source. $MgSO_4$ was excluded from the media to preclude the potential use of sulfate as an electron acceptor in the assay. Amorphous $Fe(OH)_3$ was prepared by neutralizing a 0.4-M solution of $FeCl_3$ to pH 7 with NaOH (Lovley and Phillips, 1986). The precipitate was washed with deionized water until the chloride concentration was less than 1 mM. Sterile conditions were maintained to the maximum extent possible. However, the iron phase itself was not autoclaved, since the elevated heat and pressure would facilitate crystallization, which was not desired.

The medium was prepared by dissolving all constituents (excluding the $NaHCO_3$ and amorphous Fe $[OH]_3$) in deionized water followed by autoclaving at 121°C for 20 min. The medium was then bubbled for 45 min. under a stream of N_2:CO_2 (80:20) that had been first passed through a heated column to remove all oxygen. $NaHCO_3$ and amorphous $Fe(OH)_3$ were added, and 9 mL of the medium was anaerobically dispensed into presterilized test tubes and sealed with black butyl stoppers and crimp seals (final pH = 7.02). The tubes were incubated at 25°C in the dark.

The concentration of ferrous iron in each MPN tube was determined after 131 days of incubation by a 3-h extraction with 0.5N HCl. In the anaerobic chamber, the MPN tubes were shaken to ensure all particulate matter was uniformly distributed, and 0.5 mL from each MPN tube was added to a glass vial containing 5 mL of 0.5N HCl in the anaerobic chamber. The vials were closed, mixed for 30 s, and allowed to stand for 3 h in the anaerobic chamber. After this time, approximately 3 mL was removed, passed through a 0.2-μm nylon acrodisc™ filter (Gelman Sciences, Ann Arbor, Mich.), and placed in a separate vial. The ferrous iron concentration in each extract was determined by absorbance measurements at 562 nm after reaction with ferrozine™ (Stookey, 1970) (1 g/L in 50 mM n-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], pH 7). A standard curve was prepared by using ferrous ethylenediammonium sulfate. Disposable glassware was used to prevent prior exposure to iron.

The two potential sources of background ferrous iron in the tubes came from the sediment extract used to inoculate the tubes and from impurities contained in the prepared $Fe(OH)_3$. To quantify the ferrous iron that could be attributed to either of these sources, triplicate control tubes were inoculated at each dilution and analyzed for ferrous iron within 3 h of initiating the MPN assay. Additional control tubes were prepared that did not contain inoculum and analyzed for ferrous iron after 131 days to assess the potential for abiotic reduction during the incubation period. The mean and variance were computed for the control tubes. Students *t* values were calculated for each tube and compared to the control values. Tubes were counted as positive if $t_{\text{calculated (for each tube)}} > t_{\text{table (of control)}}$ at α=0.05 (for a 1-tailed curve).

2.4.4 Benzene and Toluene Degraders. Five-tube MPN assays were used to enumerate microorganisms capable of mineralizing benzene or toluene in the defined sulfate- and iron-reducing media. The same general protocol used for total sulfate reducers and iron reducers, respectively, was followed. However, ^{14}C-toluene or benzene was used, and the production of $^{14}CO_2$ was monitored. To enumerate benzene degraders, unlabeled benzene (final concentration ~5 mg/L) and uniformly labeled benzene (Moravek Biochemicals, Inc., Brea, Calif.) (final count

50,000 to 100,000 dpm/mL) were substituted for the "readily" degradable carbon sources in each of the two base media. Similarly, to enumerate toluene degraders, unlabeled (final concentration ~5 mg/L) and uniformly labeled toluene (Sigma Chemical Company, St. Louis, Mo.) (20,000 to 25,000 dpm/mL) were added in separate tubes. In the iron-reducing assays, 10 mM of the prepared $Fe(OH)_3$ were added to the media. In the sulfate-reducing assays, 2 mM of $FeSO_4$ (0.3 g/L) were added. In each case, the added electron acceptor was five times the amount required for complete oxidation of the added substrates.

The tubes were incubated at 25°C in the dark and analyzed for $^{14}CO_2$ production after approximately 4 months. To assess the potential for abiotic production of $^{14}CO_2$ and/or contaminants in the labeled substrate, separate uninoculated control tubes were prepared and analyzed for $^{14}CO_2$ production concurrent with the inoculated tubes. The tubes were sampled in the anaerobic chamber by withdrawing 3 mL of liquid in a gas-tight syringe while puncturing the stopper with a needle so that a vacuum did not develop. The activity of ^{14}C was measured in the samples according to the protocol described in Section 2.3.1. The mean and variance for $^{14}CO_2$ were computed for the control tubes. Students *t* values were calculated for each tube and compared to the control values. Tubes were counted as positive if $t_{calculated\ (for\ each\ tube)} > t_{table\ (of\ control)}$ at α=0.05 (for a 1-tailed curve).

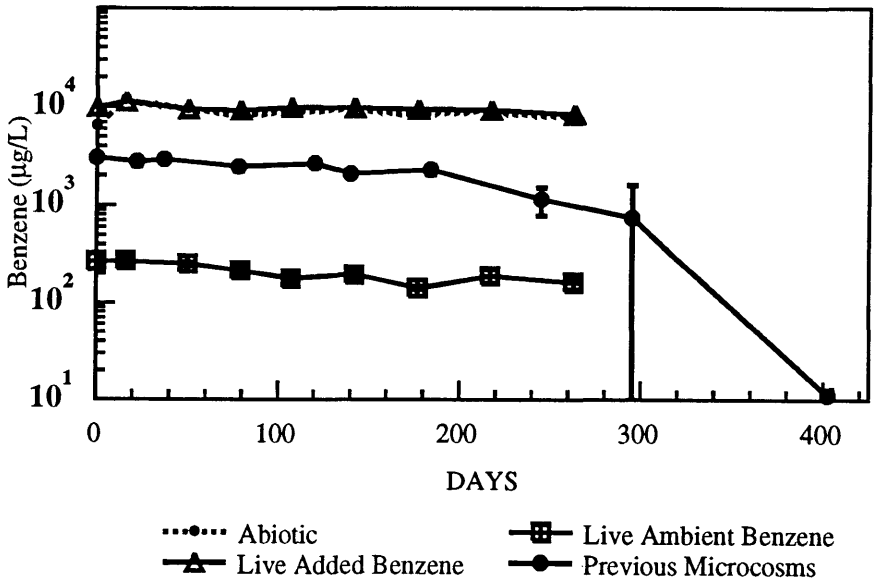
3.0 RESULTS AND DISCUSSION

3.1 BENZENE BIODEGRADATION

Results of previous work showed that benzene loss occurred in microcosms constructed with mid-plume and end-plume sediment from a petroleum-contaminated site near Rocky Point, N.C. (Hunt et al., 1997b). However, additional hydrocarbons (TEX) were also added in most cases, and benzene loss occurred only after a lengthy lag period (> 100 days). Thus, further experiments were performed to document mineralization of benzene and to explore favorable conditions for benzene biodegradation in the aquifer sediment. A microcosm experiment was initiated containing added ^{14}C-benzene with sediments obtained from the mid-plume area of the aquifer. Additional tests were conducted with existing microcosms containing end-plume sediment in which benzene loss had been observed. The purpose of this work was to determine if once initiated, repeated benzene loss would continue with no lag period between re-additions of benzene and to determine if dissolved sulfate was consumed during biodegradation.

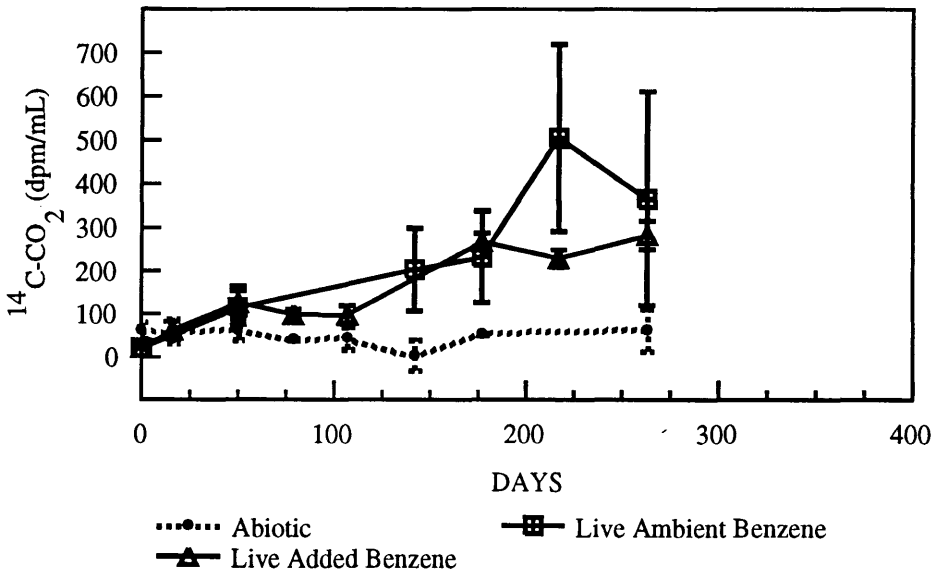
3.1.1 ^{14}C Mid-Plume Microcosms. After 263 days of monitoring, no measurable benzene loss was evident in the mid-plume microcosms containing ^{14}C-benzene at ambient levels (~ 0.275 mg/L) or in those spiked with elevated (~ 7 mg/L) levels (Figure 2). This time period is comparable to the lag period prior to the onset of benzene biodegradation measured in previous experiments containing mid-plume sediment with added BTEX (Hunt et al., 1997b). A small amount of $^{14}CO_2$ was produced in both sets of microcosms (Figure 3). The amount of $^{14}CO_2$ production corresponds to 0.1% (elevated benzene microcosms) and to 0.5% (ambient-level

Figure 2. Benzene Concentration in Mid-Plume Microcosms



Data are the average of two or three destructively sampled replicates. Standard deviation less than symbol size is not shown.

Figure 3. Total Counts ^{14}C-CO_2 During Lag Period in Mid-Plume Microcosms



Data are average of two or three destructively sampled replicates at each time point. Data were corrected for background $^{14}CO_2$.

benzene microcosms) of the total dpm present in the microcosms. However, this $^{14}CO_2$ may be attributed to impurities in the benzene as documented in Table 1. This $^{14}CO_2$ production does demonstrate that biological activity occurred during this time period, since $^{14}CO_2$ production was not detected in the abiotic control microcosms.

The results of microcosm analyses performed initially and at day 263 for CH_4, TOC, Fe, SO_4, and pH are presented in Table 2. Total organic carbon decreased by more than 50% in all live incubations and remained constant in the abiotic microcosms, providing further evidence for biological activity during the monitoring period. Methane was generated in all but one live microcosm at day 263 (in two of the three elevated benzene replicates and in all three ambient-level benzene replicates). Although methanogenic activity was not found to be significant from the site groundwater monitoring investigation (Gomez, 1993; Borden et al., 1995) and early microcosm studies using sediment from the mid-plume area of the aquifer (Hunt et al., 1997b), methane was detected in follow-up laboratory studies using mid-plume sediment (Hunt, 1997). In these follow-up studies, BTEX biodegradation was slowed or inhibited in microcosms when methane was detected. Groundwater samples obtained from a multilevel monitoring well in September 1995 showed the presence of methane in the mid-plume area at discrete intervals. The presence of methane in the microcosms confirms that a methanogenic consortium exists in the aquifer sediment and may be active in some sections of the aquifer.

Table 2. Select Microcosm Parameters at the Initial Sampling and after 263-Day Incubation[a]

Parameter	Day	Live, Added Benzene (mg/L)	Live, Ambient Benzene (mg/L)	Abiotic (mg/L)
Iron[b]	0	152 (12)	142 (15)	50 (16)
	263	135(32)	120 (11)	80 (9)
TOC[b]	0	126 (22)	54 (6)	63 (19)
	263	52 (41)	18 (2)	55 (7)
Sulfate[b]	0	376 (9)	370 (24)	148 (90)
	263	43 (74)	35 (51)	12 (14)
Methane[b]	0	<0.05	<0.05	<0.05
	263	4.36 (6.21)	2.01 (0.51)	<0.05
pH	0	5.0 (0.3)	4.8 (0.3)	4.5 (0.6)
	263	5.8 (0.1)	5.8 (0.1)	5.1 (0.4)

[a]All values reflect the average of three destructively sampled microcosms with the standard deviation shown in parentheses.

[b]Data represent dissolved concentrations.

The concentration of dissolved sulfate in the microcosms was initially high (>100 mg/L) relative to previous microcosm experiments containing mid-plume sediment. The sediment was retrieved from a location approximately 1 m from sediments used in previous work that contained <5 mg/L sulfate, indicating small-scale heterogeneity in sulfate concentrations within the mid-plume area. Sulfate concentrations decreased to below detection (<1 mg/L) in both the microcosms containing ambient and elevated benzene concentrations in two of the three replicates at day 263, while the remaining live replicate in each set contained >95 mg/L sulfate. Sulfate consumption due to biological processes cannot be assumed, since the concentration in the abiotic microcosms also decreased. There was considerably more clay material visible in all microcosms relative to previous experiments. This observation was verified by grain size analysis that showed the sediment used to construct the microcosms contained a larger amount of fine material relative to previous microcosm experiments (Hunt, 1997). In general, adsorbed sulfate can occur in mineral soils that contain appreciable amounts of iron and aluminum clay colloids (Metson, 1979; Fuller et al., 1985). Given the presence of the alumino-iron-containing clay mineral glauconite in the aquifer, it is possible that the sulfate was released from the clay material during microcosm

construction and sorbed back to the sediment during the incubation. Thus, this produced a change in dissolved sulfate concentration over the course of time.

3.1.2 Respiked End-Plume Microcosms. Biodegradation of more than 10 mg/L of benzene occurred with no measurable lag period in five out of six live microcosms in approximately 200 days (Figure 1a). Biodegradation proceeded in these microcosms without adding exogenous substances such as electron acceptors, chelators, or additional carbon. However, unlike the previous experiments, the microcosms were incubated at room temperature (28°C) rather than at the ambient groundwater temperature (16°C). Room temperature may have altered the level of microbial activity relative to incubation at the ambient temperature. After 201 days, dissolved sulfate remained high in the microcosms (80 to 270 mg/L), while dissolved iron was low (0.5-11 mg/L) (Table 3). The pH was between 8.0 and 8.5 in all live microcosms in which benzene degraded but was 6.0 in the microcosm in which no degradation was measured (L-24). Although still high, the sulfate concentrations (>80 mg/L) were approximately 10 times lower than the sulfate concentrations in the original microcosms at the initial respiking. Since this decrease also occurred in the abiotic microcosm, it is attributed to removal of liquid in the microcosm prior to respiking with benzene, rather than depletion attributed to biological activity.

A second benzene addition (day 212) resulted in continued biodegradation of benzene after approximately 100 days in three of the six microcosms (L-22, L-23, and L-28) and after approximately 130 days in a fourth (L-29) (Figure 1a). This represents a factor of 2 decrease in the rate of degradation (Table 3) relative to the first benzene addition. Sulfate concentrations decreased in all microcosms but were comparable to concentrations in the abiotic microcosms at day 338. Interestingly, detectable decreases in dissolved iron were not evident in the microcosms with benzene biodegradation (Table 3), possibly due to precipitation of FeS as a result of the sulfate production. Benzene remained recalcitrant in microcosm L-24 (Figure 1a).

Continued benzene biodegradation occurred after a third benzene addition (day 365) in most microcosms. However, the rate of biodegradation did not continue to increase and was comparable to the initial rate of decay in most microcosms. Analysis just prior to a fourth addition (day 560) revealed that all live replicates except one (L-22) contained methane. This microcosm was sacrificed and used as inoculum for MPN assays (Section 2.4.1).

After the fourth benzene addition on day 560, biodegradation ceased in the remaining replicates (Figure 1a). Methane was not detected in any of the microcosms initially; however, it was not sampled a second time until day 560. Thus, the time period over which it was first produced cannot be verified. The highest level of methane was detected in the microcosm (L-24) in which benzene loss did not occur over the duration of the monitoring period. This observation is consistent with previous microcosm experiments that showed that the lack of BTEX biodegradation was correlated with the occurrence of methane production (Hunt, 1997). However, whether this observation represents a cause and effect or is the result of some other factor cannot be determined from the data available. It is also possible that the inability to sustain and increase the rate of benzene biodegradation over time resulted from the consumption of some vitamin or mineral required for this microbial activity.

In previous experiments, benzene biodegradation was inhibited in molybdate-amended

Table 3. Decay Rates and Dissolved Parameters in Respiked End-Plume Microcosms

Concentrations Immediately Prior to Second Benzene Addition, Day 201:

Microcosm ID	Benzene (µg/L)	Decay Rate (day^{-1}) Day 0-201	Iron (mg/L)	Sulfate (mg/L)	pH
L-22	<40[a]	0.035	11.2	265	8
L-23	112	0.027	1.2	156	8
L-24	42875	ND[b]	13	187	6
L-28	399	0.023	0.5	81.5	8.5
L-29	157	0.015	6.6	170	8
L-30	47	0.025	9	274	8
ABIOTIC	21098	ND	6.2	137	7

Concentrations Immediately Prior to Third Benzene Addition, Day 338:

Microcosm ID	Benzene (µg/L)	Decay Rate (day^{-1}) Day 212-338	Iron (mg/L)	Sulfate (mg/L)
L-22	<40[a]	0.060	1	9.9
L-23	250	0.069	3	113
L-24	30354	ND	43	132
L-28	<40[a]	0.011	0.1	14
L-29	3194	ND	6.4	107
L-30	25020	ND	34	132
ABIOTIC	19054	ND	0.5	110

Concentrations Immediately Prior to Fourth Benzene Addition, Day 560:

Microcosm ID	Benzene (µg/L)	Decay Rate (day^{-1}) Day 365-499	Iron (mg/L)	Sulfate (mg/L)	Methane (mg/L)
L-22[c]	3168	0.010	<0.1[a]	<0.5[a]	<0.02[a]
L-23	372	0.024	1.3	56.5	0.10
L-24	15224	ND	4.5	61.8	1.06
L-28	<40[a]	0.052	0.5	1.6	0.23
L-29	<40[a]	0.048	1.3	54.3	0.83
L-30	471	0.029	3	67	1.73
ABIOTIC	19497	ND	2.5	50	<0.02[a]

[a]Below quantitative detection limit.

[b]No decay.

[c]Used as inoculum for MPN enumeration.

microcosms (Hunt, 1997). However, high concentrations of sulfate (>1000 mg/L) did not stimulate benzene biodegradation, nor did biodegradation occur consistently when such concentrations were present (Hunt, 1997). Curiously, there was adequate sulfate present (>50 mg/L) at day 560, when methane was detected, to degrade approximately 10 mg/L of benzene in three of the active microcosms (L-23, L-29, L-30). This amount is based on simple stoichiometry that assumes the complete mineralization of benzene via sulfate reduction.

The specific electron-accepting conditions directly linked to benzene biodegradation remain unverified in these microcosms. The presence of sulfate appears to be a necessary but insufficient condition for benzene biodegradation to proceed. However, the potential importance of iron reduction and/or fermentative reactions cannot be determined.

3.2 MICROBIAL ENUMERATION

Two types of MPN assays were performed: standard assays in which "readily degradable" susbtrates were used and contaminant-specific assays in which toluene or benzene was the sole substrate. Toluene and benzene were chosen as examples of more and less degradable hydrocarbons, respectively. To determine if differences in populations could be measured, two separate inoculum sources were used: end-plume sediment in which repeated benzene loss had been measured (Section 3.1.2) and mid-plume sediment that was used to construct the microcosms containing ^{14}C (Section 3.1.1). The enumeration of indigenous microorganisms from mid- and end-plume sediments revealed that both iron and sulfate reducers were present in the sediment. However, only very low numbers were detected (Table 4).

Table 4. Population of Specific Groups of Microorganisms in MPN Assays (cells/dry g sediment)

Substrate	Sulfate Reducers	Iron Reducers
Mid-Plume Inoculum		
Standard[a]	None Detected	110 (50 to 239)[c]
^{14}C Toluene	27 (10 to 72)[b,c]	None Detected
^{14}C Benzene	13 (4 to 40)[b,c]	None Detected
End-Plume Inoculum		
Standard[a]	None Detected	19 (9 to 39)[c]
^{14}C Toluene	10 (3 to 29)[b,c]	None Detected
^{14}C Benzene	37 (15 to 91)[b,c]	None Detected

[a]The substrates used in standard sulfate-reducing media were sodium lactate, sodium acetate, propionic acid, *n*-butyric acid, and benzoic acid. The substrate used in the standard iron-reducing medium was sodium acetate.

[b]The presence of a nonvolatile component was detected in the substrates added, and growth may have resulted from the utilization of this component.

[c]Ninety-five percent confidence interval shown in parentheses.

3.2.1 Sulfate-Reducing MPNs. There were no positive tubes in the MPN enumerations after more than 4 months of incubation for sulfate reducers in the medium containing the "readily degradable" substrates from either mid-plume or end-plume inocula. Although a standard medium that contained a mixture of substrates commonly used to culture sulfate reducers was used, dissimilatory sulfate reduction is carried out by a diverse group of organisms and the choice of electron donor and carbon source can be decisive for the growth of different species (Pfennig et al., 1981). While acetate and pyruvate (two substrates used in the medium) are known to be used by sulfate reducers (Postgate, 1979), they were not found to stimulate growth of sulfate reducers in salt marsh sediments (Dicker and Smith, 1985). Thus, it is possible that the population present in sediments used here could not utilize the carbon sources provided. It is also possible that the absence of some other trace component of the MPN medium limited growth of bacteria from this aquifer sediment. In separate assays, microorganisms were detected in the same sulfate-reducing media when landfill leachate was used as the inoculum, indicating that the medium used was suitable for growth of sulfate-reducing bacteria present in the leachate (data not shown).

Contrary to the results in the standard assays, the presence of sulfate-reducing bacteria was evident in both mid-and end-plume sediment by the detection of $^{14}CO_2$ and the formation of a black precipitate in the tubes containing ^{14}C-toluene or ^{14}C-benzene when incubated over the same time period. However, the presence of small quantities of nonvolatile ^{14}C in both benzene (~2 %) and toluene spike solutions (~8%) prior to the initiation of the experiment (Table 1) raises the possibility that the $^{14}CO_2$ production may have resulted from the biotransformation of these impurities. The percentage of $^{14}CO_2$ recovery in many MPN tubes was not significantly above this level. Hence, the assays confirm the presence of a sulfate-reducing population in the inoculum but do not definitively confirm that the organisms cultured have the capability to mineralize the substrates provided (toluene or benzene).

The absence of sulfate reducers in the standard medium containing the "readily degradable" substrates is curious and suggests that the medium provided only a marginally suitable growth environment for the indigenous organisms. Problems were also noted in the MPN tubes containing ^{14}C toluene or benzene. These tubes did not follow a clear pattern of growth because, in most cases, positive tubes were scattered throughout several dilutions. This indicates a weak MPN test and suggests potential limitations in the growth medium used. Although all MPN results reported in Table 4 are based on *t* tests performed at $\alpha = 0.05$, MPNs calculated by using $\alpha = 0.01$ does not significantly change the results. For example, at a $\alpha = 0.01$, the MPN for the mid-plume inoculum grown on the ^{14}C-benzene is 8 sulfate reducers/gram of dry sediment. Given the accuracy of the MPN analysis, this is comparable to the value at $\alpha = 0.05$ of 13 sulfate reducers/gram of dry sediment.

In summary, it is questionable whether the MPN results for sulfate reducers reflect their population in the sediment. Frequently, less than 1% of the total population as enumerated by a direct count is recoverable by standard plating procedures (Bottomley et al., 1991). Similar relationships have been reported for both surface and subsurface soils (Colwell, 1989). Moreover, based on polymerase chain reaction patterns, which allows for a very sensitive detection of a given DNA fragment in a complex mixture of molecules, the soil contains bacteria that are virtually unknown as compared to what is found by isolation and culture procedures

(Torsvik et al., 1990). Both sources of homogenized sediment (mid-and end-plume) showed moderate to high sulfate concentrations (> 100 mg/L), indicating that the availability of sulfate as an electron acceptor would not have limited the development of sulfate-reducing organisms. Although environmental factors such as pH, the presence of toxins, or ecological pressures cannot be ruled out as potential factors limiting the growth of sulfate-reducing organisms in the aquifer, it is equally possible that the MPN assays greatly underestimated the actual numbers of sulfate-reducing organisms because of difficulties associated with culturing the existing population.

3.2.2 Iron-Reducing MPNs. Small populations of iron reducers were detected in the MPN assays containing acetate as a substrate for both mid- and end-plume inocula (Table 4). Consistent with the hydrocarbon degraders in sulfate-reducing media, a clear pattern of growth was not evident in the tubes because positive tubes were scattered throughout several dilutions.

The iron-reducing MPN assays containing toluene or benzene were negative for both mid-plume and end-plume inocula (Table 4). This was surprising considering results of previous microcosms constructed with mid-plume sediment that indicated that BTEX was degraded under predominately iron-reducing conditions (Hunt et al., 1997b). As was the case in the sulfate-reducing medium, questions regarding limitations of the medium must be considered. It is possible that hydrocarbon degraders capable of iron reduction were not cultured in the media. Although iron reducers were detected in the standard MPNs with acetate as the carbon source, the results of these MPNs were based on ferrous iron production and substrate degradation was not measured. In addition, more than one type of microorganism capable of iron reduction but using different carbon sources for growth may be present in the aquifer. It is possible that toluene and/or benzene biodegraded in the ^{14}C assays but that: (1) the compounds were not completely mineralized (thus $^{14}CO_2$ was not produced) or (2) the presence of more than one group of microorganism is required to mineralize these hydrocarbons and all were not successfully cultured in the selective media. The latter possibility further suggests that hydrocarbon (toluene and benzene) degradation in the aquifer studied may require syntrophy or a consortium of organisms to proceed. In a study by Jones et al. (1983), pure cultures of iron reducers produced less ferrous iron than the mixed cultures from which they were derived. This finding indicates that bacteria capable of iron reduction may function optimally in mixed populations (Ghiorse, 1988) and poses a significant drawback in using information gained from techniques utilizing selective cultures such as the MPN assays.

An unexpected outcome of the MPN assays was the substantial amount of $^{14}CO_2$ measured in both uninoculated and inoculated tubes containing ^{14}C-benzene in the iron-reducing MPNs using the two different inocula. The percentage $^{14}CO_2$ recovered represents 7 to 10 % of the total activity in the tubes. Although there was a small percentage of $^{14}CO_2$ present in the ^{14}C-benzene spike solution (Table 1), the amount produced after the three-to four-month incubation period was over an order of magnitude higher than that present initially in the spike solution and was consistent in all tubes (control and live), irrespective of dilution. While the same ^{14}C-benzene spike solution was used both in the sulfate-reducing MPN assays and in abiotic microcosms (Section 3.1.1), $^{14}CO_2$ production was not evident in these samples. Furthermore, the nonvolatile fraction increased in all tubes sampled in the iron-reducing media containing ^{14}C-benzene. This indicates that the ^{14}C benzene reacted with some component of the iron-reducing media such that it behaved as $^{14}CO_2$.

One potential explanation for this is that the ^{14}C-benzene sorbed to the solid phase iron in the MPN tubes. When samples were processed, $^{14}CO_2$ was removed by acidification and sparged as described in Section 2.3.1. This acidification step would have dissolved any iron present, resulting in the release and stripping of sorbed benzene. However, additional tests were conducted with filtered and unfiltered samples and this explanation could not be validated.

The results of all MPNs containing ^{14}C also demonstrate drawbacks in using radiochemicals for these assays. Limitations in detection of low levels of $^{14}CO_2$ production were apparent, given the purity of the radiochemicals. Thus, it is important to validate and report ^{14}C compound purity and monitor uninoculated controls. MPNs utilizing ^{14}C compounds have been used by other researchers (Lehmicke et al., 1979; Armstrong et al., 1991) on a limited basis, primarily with standard substrates, but control tubes, if made, are often not reported. A study by Gu et al. (1995) demonstrated that using ^{14}C radiochemicals can cause potential experimental error in studies using volatile organic compounds because of impurities in the chemical. Although present in relatively small quantities, these impurities may be utilized to produce $^{14}CO_2$ at the levels measured in the MPN assay.

In summary, data from the MPN assays do not allow identification of the dominant electron-accepting process involved in benzene or toluene biodegradation. The low populations enumerated based on the use of readily degradable substrates leads to questions about the appropriateness of the medium used for the acquifer sediment tested here. The presence of impurities in the radiolabeled benzene and toluene solutions precludes any conclusion that these substrates were mineralized in the MPN assays.

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LIST OF PATENTS AND PUBLICATIONS RESULTING FROM THE PROJECT

Hunt, M. J. 1997. Assessing intrinsic hydrocarbon bioremediation in anaerobic coastal plain aquifers. Ph.D. diss., Department of Civil Engineering, North Carolina State University.

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