Analysis of microbiological and geochemical processes controlling biodegredation of aromatic hydrocarbons in anaerobic aquifers. [DNR-143] 2001

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Summary

Title: Analysis of Microbiological and Geochemical Processes Controlling Biodegradation of Aromatic Hydrocarbons in Anaerobic Aquifers

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Background: Benzene, toluene, ethylbenzene and xylenes (BTEX) are among the groundwater contaminants of greatest concern because of their toxicity, their solubility in water, and their resistance to degradation in anaerobic environments. Prior to entry of hydrocarbon pollutants, shallow aquifers are often aerobic with relatively low levels of dissolved organic carbon. Indigenous aerobic bacteria readily metabolize hydrocarbon pollutants (e.g. fuels) entering these systems, and quickly deplete the available oxygen. Most enhanced bioremediation systems involve the addition of oxygen, however, the feasibility of aerobic biodegradation is often limited by its low water solubility; in addition, oxygen injection may induce iron oxide precipitation. Thus, bioremediation using alternate electron acceptors, such as NO_3^-, Fe^{+3}, and SO_4^{-2}, has been the subject of much recent research. A better understanding of underlying mechanisms of these anaerobic processes is needed for them to be effectively employed for aquifer bioremediation. This requires identification of the organisms mediating the reactions, the metabolic pathways by which they degrade BTEX, and the effects of available electron acceptors (and other environmental factors) on their activities.

The study aquifer at Fort McCoy, Wisconsin, underlies a former Petroleum, Oils, and Lubricants Station, and is contaminated with BTEX from two 12,000-gallon leaking underground storage tanks (UST's) that were installed in 1943 and removed in 1989. Residual non-aqueous phase liquid concentrated in a 0.3 to 0.6 m thick zone near the water table continuously supplies BTEX to groundwater. The current plume is approximately 100 m long and 30 m wide at its maximum, with BTEX concentrations generally less than 20 mg/l.

Objectives: The overall goal of this project was to determine the kinetics of BTEX biodegradation in the presence and absence of NO_3^- and to establish links to changes in the microbial community structure that accompanied BTEX biodegradation. The project was organized into two concurrent tracks of investigation. The first was designed to yield process-level information on BTEX biodegradation. The second track focused on characterizing the physiological and phylogenetic properties of the BTEX-degrading microbial community.

Methods: These studies were conducted with sediment material collected from the Ft. McCoy site. The approach used for the Track 1 experiments was to establish a series of microcosms to monitor the degradation of BTEX and the use of NO_3^- and iron in a controlled laboratory setting. Rates of TEX degradation and NO_3^- use were calculated and compared to previously conducted field experiments. For

Track 2, at selected sampling points, the microcosm sediment was collected and used to inoculate enrichment cultures for BTEX degrading organisms. Aliquots of the sediment were also used for DNA extraction. The microbial community of the microcosms was analyzed by using molecular techniques including Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Denaturing Gradient Gel Electrophoresis (DGGE). We also designed and tested *in situ* microcosms (ISMs). The ISMs were investigated as a means to conduct controlled experiments *in situ*. We used the ISMs to study the BTEX biodegradation rates and TEA use under field conditions at the site.

Results and Discussion: In the laboratory microcosms, only those amended with BTEX and NO_3^- exhibited BTEX degradation. Toluene, *o*-xylene ethylbenzene and *m-,p*-xylenes degraded during the experiment. Benzene, however, was recalcitrant. Biodegradation rates were slightly higher in the field than in the lab. The sequence of TEX biodegradation in microcosms was similar to the field tracer experiments, but the lag times were different. The ISMs had a different sequence of TEX degradation, and had highly variable lag times. The differences in the lag times are most likely related to different competing electron donors in the field and in the lab, spatial heterogeneity in the field, and the re-establishment of acclimated microbial populations in the laboratory.

Molecular analysis indicated that the field samples and microcosm trials had distinctly different microbial communities. Nitrate amendment induced a significant alteration in the community structure. This structure appeared to be the same in microcosms treated with nitrate alone and in those receiving NO_3^- and BTEX. No organisms similar to known, cultured NO_3^-- or Fe^{+3} -reducing alkylbenzene degraders were identified in the microcosms. This suggests that the predominant TEX-degraders stimulated by NO_3^- amendment may comprise organisms not previously known to have alkylbenzene degrading abilities.

Conclusions/Recommendations: Results of this study suggest that NO_3^- can enhance overall BTEX biodegradation in the hydrocarbon-contaminated aquifer at Fort McCoy, WI, but it should be used in combination with other electron acceptors, either under intrinsic or enhanced conditions, to treat benzene. The molecular analysis indicated that distinct populations were enhanced by NO_3^- amendment, which probably use other electron donors as well as TEX. These banding patterns highlight populations in the microbial community that can be targeted for identification of the microorganisms involved with BTEX biodegradation and NO_3^- reduction in the field. Because the microbial communities of the microcosms and the field samples were different, future studies exploring impact of nitrate on specific microbial populations *in situ* would help describe impacts remediation efforts have on the microbial community.

Related Publications:

Schreiber, M.E. (1999) Experimental and modeling approaches to evaluating anaerobic biodegradation of petroleum-contaminated groundwater. Ph.D. dissertation. Department of Geology, University of Wisconsin-Madison, Madison, WI.

Taglia, P.J. (2001) Using *in situ* microcosms to evaluate the spatial heterogeneity of BTEX biodegradation under nitrate-reducing conditions. M.S. Thesis. Department of Geology, University of Wisconsin-Madison, Madison, WI.

Zwolinski, M.D., Harris, R.H., and Hickey, W.J. (2001) Microbial consortia involved in the anaerobic degradation of hydrocarbons. *Biodegradation* **in press.**

Key Words: BTEX, anaerobic biodegradation, denitrification, iron-reduction, molecular ecology, bioremediation, biostimulation

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Final Report: A final report containing more detailed information on this project is available for loan at the Water Resources Center, University of Wisconsin - Madison, 1975 Willow Dr., Madison, WI 53706. (608)262-3069.

Final Report

Wisconsin Department of Natural Resources, Groundwater Research Program

Analysis of Microbiological and Geochemical Processes Controlling Biodegradation of Aromatic Hydrocarbons in Anaerobic Aquifers

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method was used to construct the tree in Clustal X. 1000 bootstrap replicates were conducted also with Clustal X, bootstrap values greater than 50% are provided on the branches.

Fig. 14. Average percent toluene, *m-&p*-xylenes, and *o*-xylene remaining in enrichment cultures over time. Panel A. Individual compounds, Panel B. Mixed compounds.

Fig. 15. Concentrations of NO_2^- and benzene in enrichment cultures amended with NO_3^- and benzene. Cultures were inoculated with sediment from the microcosms amended with BTEX only (B) or BTEX + NO_3^- (NB). Abiotic enrichments were autoclaved and amended with sodium azide (0.1%). The enrichments that had transient NO_2^- accumulation also had oxidized resazurine. Error bars indicate one standard deviation for measurement of three replicated enrichments. For clarity, only the positive error bars are shown.

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I. ABSTRACT

In this study, we used a combination of chemical and molecular analysis to characterize BTEX biodegradation at a gasoline-contaminated site. The study aquifer at Fort McCoy, Wisconsin is contaminated with BTEX from two 12,000-gallon leaking underground storage tanks (UST's) that were installed in 1943 and removed in 1989. Residual non-aqueous phase liquid concentrated in a 0.3 to 0.6 m thick zone near the water table continuously supplies BTEX to groundwater. The project was organized into two concurrent tracks of investigation. The first track was designed to yield process-level information on BTEX biodegradation using laboratory and *in situ* microcosms. The second track focused on characterizing the physiological and phylogenetic properties of the BTEX-degrading microbial community.

The strategy used for the Track 1 experiments was to establish a series of microcosms to monitor the degradation of BTEX and the use of NO_3^- and iron in a controlled laboratory setting. The trials were established to test the ability of the sediment organisms to degrade BTEX with and without NO_3^- amendment, and to monitor NO_3^- loss with and with out BTEX. These experimental trials were compared to abiotic and non-amended controls. The microcosms were sampled for BTEX, NO_3^-, Fe^{+2} and DNA over a one-year period. The first part of the ISM experiments involved the design and testing of the ISMs. Each ISM consisted of a stainless-steel cylinder and sampling equipment, and enclosed a two-liter column of sediment. The ISM design went through several rounds of testing and design refinement before a final model was built. The ISMs were then used to examine BTEX degradation and NO_3^- use in three different areas of the aquifer.

For Track 2, microcosm sediment was collected at selected sampling points and used to inoculate enrichment cultures for BTEX degrading organisms. Aliquots of the sediment were also used for DNA extraction. The microbial community of the microcosms was analyzed by using molecular techniques including Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Denaturing Gradient Gel Electrophoresis (DGGE).

In the laboratory microcosms, only those amended with BTEX and NO_3^- exhibited BTEX degradation. Toluene was completely depleted by day 42, *o*-xylene by day 70, and ethylbenzene by day 266. By day 147 *m*-& *p*-xylene began to degrade but were still detectable at the end of the experiment. Benzene was recalcitrant throughout the experiment. Comparison of biodegradation rates, sequences, and lag times between field tracer tests, the ISMs, and the laboratory microcosm experiments demonstrate that the rates were slightly higher in the field than in the lab; differences were less than a factor of 2 for toluene and ethylbenzene, and 4 for *m,p*-xylenes. The overall sequence of biodegradation was similar, but the lag times were different. In the field, toluene, ethylbenzene, and *m,p*-xylenes degraded within 10 days while in the microcosm experiment and in the initial dosing of the enrichment cultures, there was a distinct order of degradation, with toluene degrading first, followed by *o*-xylene, *m,p*-xylenes, and ethylbenzene. Overall the ISMs also had a different sequence of TEX degradation than the lab microcosms, and had highly variable lag times. The differences in the lag times are most likely related to different competing electron donors in the field and in the lab, spatial heterogeneity in the field, and the re-establishment of acclimated microbial populations in the laboratory.

The field samples and microcosm trials had distinctly different microbial community banding patterns in the DGGE analysis. Nitrate amendment also produced banding patterns not seen in the BTEX only treatment. This indicates that NO_3^- may have allowed proliferation of a specific group of organisms that were not abundant in the absence of NO_3^-. Enhancement of this community in the laboratory experiments may explain the increased lag times in the degradation of xylenes and ethylbenzene compared to those seen in the tracer experiments. These banding patterns highlight populations in the microbial community that can be targeted for identification of the microorganisms involved with BTEX biodegradation and NO_3^- reduction in the microcosms and in the field.

II. INTRODUCTION

Groundwater is the primary drinking water source for about 50% of people living in the United States. Unfortunately, in some areas this valuable resource is threatened by chemical contamination from leaking underground storage tanks. EPA estimates that there are at least 386,000 confirmed releases of gasoline from leaking tanks (US Environmental Protection Agency, 1999). In Wisconsin, where 63% of the population relies on groundwater, 13,000 leaking underground storage tanks have been reported (US Environmental Protection Agency, 1999).

Prior to entry of hydrocarbon pollutants, shallow aquifers are often aerobic with relatively low levels of dissolved organic carbon (Anderson and Lovley, 1997). Indigenous aerobic bacteria readily metabolize hydrocarbon pollutants (e.g. fuels) entering these systems. These bacteria use molecular oxygen to initiate substrate transformation via the activity of oxygenases, and as the terminal electron acceptor (TEA) in respiratory energy generation. Collectively, the use of oxygenases and aerobic respiration allow aerobic bacteria to effectively degrade a wide spectrum of compounds present in fuels and other petroleum- and coal-products. However, levels of hydrocarbons introduced by spills are typically far in excess of the dissolved oxygen levels needed to support their degradation. Thus, aerobic organisms deplete oxygen and thereby transform the aquifer environment from aerobic to anaerobic (Vroblesky and Chapell, 1994; Anderson and Lovley, 1997; Lovley, 1997).

Although most enhanced bioremediation systems involve the addition of oxygen, the feasibility of aerobic biodegradation is often limited by the low water solubility of oxygen; in addition, oxygen injection may induce iron oxide precipitation. Thus, bioremediation using alternate electron acceptors has been the subject of much recent research. While experiments involving NO_3^--reducing conditions have demonstrated degradation of toluene, ethylbenzene, and xylenes (e.g. Hutchins, 1991), the fate of benzene remains uncertain. Laboratory experiments using sulfate-reducing conditions report degradation of toluene and xylenes(Beller et al., 1992; Edwards et al., 1992; Reinhard et al., 1997) and benzene (Lovley et al., 1995; Phelps et al., 1996; Weiner et al., 1998; Weiner and Lovley, 1998). Field experiments with sulfate injection have also been conducted (Reinhard et al., 1997) but there are no reports of any pilot-scale or full-scale remediation systems using sulfate. Degradation under iron-reducing conditions is not well-understood; enhancement of degradation by addition of chelating agents to increase iron bioavailability has been conducted only in lab experiments (Lovley et al., 1994; Lovley and Woodward, 1996; Lovley et al., 1996). Collectively, these field and microcosm experiments have demonstrated the potential for anaerobic degradation of all BTEX constituents. However, given the variability in these studies, it generally cannot be explained why a given BTEX compound was, or was not, biodegraded in the systems evaluated.

A better understanding of underlying mechanisms is needed for anaerobic processes to be effectively employed for aquifer bioremediation. This requires identification of the organisms mediating the reactions, the metabolic pathways by which they degrade BTEX, and the effects of available electron acceptors (and other environmental factors) on their activities. Among the few studies that have focused on identification and analysis of anaerobic BTEX degraders are those involving coupling of benzene or alkylbenzene oxidation to dissimilatory reduction of NO_3^- (Fries et al., 1994), ferric iron (Lovley and Lonergan, 1990), or sulfate (Beller et al., 1996; Phelps et al., 1998). The literature on the metabolic pathways by which BTEX compounds are anaerobically degraded is also limited, but studies conducted to date suggest that there may be a variety of pathways (Beller et al., 1992; Evans et al., 1992; Fuch et al., 1994; Wilson and Bouwer, 1997; Rabus and Heider, 1998). If so, it is important to determine whether there is a pattern to the distribution of these pathways, and how these pathways differ with respect to substrate specificity, products, prevailing electron acceptors, and other environmental controls.

III. OBJECTIVES

The overall goal of this project was to determine the kinetics of BTEX biodegradation in the presence and absence of NO_3^- and to establish links to changes in the microbial community structure that accompanied BTEX biodegradation. The project was organized into two concurrent tracks of investigation. The first was designed to yield process-level information on BTEX biodegradation. Specifically, we tried to determine 1) if benzene biodegrades under anaerobic conditions at the field site, 2) the extent of BTEX degradation under intrinsic, iron-reducing conditions, and 3) if added NO_3^- replaces iron as an electron acceptor or if NO_3^- and iron are used concurrently.

The second track focused on characterizing the physiological and phylogenetic properties of the BTEX-degrading microbial community. We attempted to determine 1) the spectrum of BTEX components degraded by single bacteria and/or bacterial consortia isolated from selected microcosms, 2) how metabolism of BTEX components is affected by available electron acceptors, 3) the phylogenetic affiliation of the organisms enriched in the microcosm and culture studies, and 4) how well the microcosms represent the microbial community found *in situ*.

IV. APPROACH

The approach used for the Track 1 experiments was to establish a series of microcosms to monitor the degradation of BTEX and the use of NO_3^- and iron in a controlled laboratory setting. The trials were established to test the ability of the sediment organisms to degrade BTEX with and without NO_3^- amendment, and to monitor NO_3^- loss with and with out BTEX. These experimental trials were compared to abiotic and non-amended controls. Track 2 experiments began with the microcosm sampling for chemical analysis. At selected sampling points, the microcosm sediment was collected and used to inoculate enrichment cultures for BTEX degrading organisms. Aliquots of the sediment were also used for DNA extraction. The microbial community of the microcosms was analyzed by using molecular techniques including Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Denaturing Gradient Gel Electrophoresis (DGGE).

During the second year of the project, we began the design and testing of *in situ* microcosms (ISMs). The purpose for developing the ISMs was to create a controlled situation for conducting experiments in the field. We used the ISMs to begin studying the BTEX biodegradation rates and TEA use under field conditions at our site. The objective of these experiments was to evaluate the spatial variability of *in situ* rates and extent of BTEX biodegradation resulting from addition of NO_3^- as an electron acceptor. The first part of the ISM experiments involved the design and testing of the ISMs. Each ISM consisted of a stainless-steel cylinder and sampling equipment, and enclosed a two-liter column of sediment. The ISM design went through several rounds of testing and design refinement before a final model was built. The ISMs were then used to examine BTEX degradation and NO_3^- use in three different areas of the aquifer.

Fe^{+2}/Fe^{+3}. Ferrous iron concentrations were determined by absorbance measurement at 562 nm after reaction with ferrozine (1 g/l in 50 mM HEPES buffer) (Stookey, 1970). Five ml of ferrozine was added to 100 µl of 0.2 um filtered sample. The ferrozine and sample were mixed for 15 s prior to measurement. The amount of total Fe was determined by adding 500 µl of 0.5 N hydroxylamine hydrochloric acid (HX-HCl) to the sample/ferrozine mixture and measuring absorbance.

Ferrous iron in sediment was extracted by adding 10 ml of 0.5 M HCl to 0.2 g of sediment and extracting for 1 h. Total Fe was estimated by an HX-HCl extraction method, in which the extractant was 0.25 M of HX-HCl in 0.25 M HCl. (Lovley and Phillips, 1987). Acid extracts were filtered through 0.25 um filters. Ferrous iron and total Fe of the acid extracts were then determined by reaction with ferrozine, as described above.

Headspace gases. Carbon dioxide, methane, and nitrogen were analyzed on day 210 in 1 ml headspace samples on a Hewlett Packard 5890A GC fitted with a thermal conductivity detector (TCD). Isothermal (45°C) separation was achieved with a Haysep R packed column (6 ft x 1/8 inch, Alltech) and a helium carrier gas flow rate of 25 ml/min. The injector and detector were held at 80°C and 100°C, respectively.

Dissolved oxygen (DO). DO was measured using a colorimetric method (Chemets, Chemetrics, Inc.). DO measurements were not taken until day 98; concentrations at this time point were all less than 0.1 mg/l, which was the detection limit. A lower-level measurement (10 to 100 µg/l) was used for the later times.

Total Organic Carbon (TOC). TOC in sediment was measured by the UWSPL using a chromic acid oxidation and back titration with a standard ferrous solution. The detection limit for this method is 0.1%.

Laboratory Microcosms

Sediment Collection. Sediment for the laboratory microcosm experiment was collected from a 0.6 m-thick zone near the fringe of the BTEX plume (**Fig. 1**). The total BTEX concentration in the groundwater at an adjacent multilevel sampling well was ≤ 1000 µg/l. The groundwater from this zone contained no detectable dissolved oxygen (DO) or NO_3^-, and had Fe^{+2} concentrations up to 15 mg/l, and SO_4^{-2} concentrations of less than 10 mg/l. A sample of the sediment was analyzed for total organic carbon (TOC) and grain size. The distribution of grain size was 97.7% sand, 0.4% silt, and 1.9% clay. TOC concentration was approximately 0.2%.

To obtain aquifer material, a Geoprobe® (Geoprobe® Systems, Salina, KS) was used to core to a depth of 1.2 m. The coring tube was retrieved, a sterile acrylic sleeve inserted, and then the device driven an additional 0.6 m into the aquifer. The acrylic sleeve was then removed, capped, wrapped in aluminum foil, and stored on ice for transport to the laboratory. After overnight storage at 4°C, the sediment cores were opened under an atmosphere of 80% N_2, 16% CO_2, and 4% H_2 in an anaerobic glovebox (Coy, Ann Arbor MI). Sediment at the end of the cores was removed, and the rest of the sediment was extruded and homogenized. The homogenized sediment remained overnight in the glovebox prior to microcosm construction. A sample of the sediment (~100 g) was taken immediately after homogenization for DNA extraction.

Construction. Microcosms were established in the anaerobic glovebox on the day following sediment collection. These contained 35 g of the homogenized, sediment material and 35 ml of base medium in 50 ml (nominal volume) serum bottles. Headspace in the bottles was less than 5 ml. The

medium contained (per liter): 0.43 g KH_2PO_4 and 0.38 g NH_4Cl, and 2.5 g $NaHCO_3$. For NO_3^- amendments, 0.65 g KNO_3 was added per liter of base medium. For the abiotic controls, the base + NO_3^- media was made in a 1% sodium azide solution. The media was autoclaved and flushed with a mixture of N_2/CO_2 that had been passed through a heated copper column to remove traces of O_2 before being introduced into the glovebox.

Five treatments were prepared and amended with BTEX, NO_3^-, or BTEX and NO_3^-, or as a sediment control (no NO_3^- or BTEX). The abiotic controls were made in a 1% azide base + NO_3^- media, and autoclaved at 120°C for one hour on three subsequent days prior to spiking with BTEX. Addition of BTEX compounds to the microcosms was accomplished by spiking the BTEX-amended microcosms with 0.9 ml of 40 mg/l BTEX stock solution. The stock solution was prepared by autoclaving deionized water, flushing with N_2/CO_2 mixture, and spiking with approximately 2.4 µl of neat compound. Once the media and amendments were added to the sediment, the serum bottles were sealed with Teflon-coated butyl rubber stoppers affixed with aluminum crimp caps. In order to allow for destructive sampling, 224 microcosms were constructed. The first four amendments were prepared in triplicate and the abiotic controls were prepared in duplicate. The microcosms were incubated in the dark in pressurized cans under an N_2/CO_2 atmosphere at room temperature (25°C).

Sampling. Destructive sampling of microcosms was conducted weekly or biweekly during the first two months of incubation, and monthly or bimonthly afterwards. Two days before sampling, the microcosms were removed from the pressurized cans, shaken, and put in the glovebox to let the fine particles settle. During sampling, the BTEX samples were collected first by gently pouring the microcosm liquid into 2 ml vial that contained 100 µl HCL as a preservative. Aqueous samples for NO_3^- /NO_2^- and Fe analyses were collected next, and were filtered using 0.2 µm filters. Once the liquid was emptied, 0.2 g of sediment was collected for acid extraction. The remaining sediment material was collected for DNA extraction and/or was used to construct enrichment cultures. Some sediment material for DNA analysis was stored in sterile 40 ml centrifuge tubes at -80°C. Chemical analysis was done as described above.

Molecular analysis of the microbial community

DNA extraction.

Sediment from the microcosm experiment was collected for DNA extraction when the microcosms were sampled for chemical analysis. Sediment material was collected in the anaerobic hood, transferred into sterile 35 ml centrifuge tubes, and stored at -80°C until extracted. Sediment samples extracted for DNA were taken from all of the BTEX and BTEX+NO_3^- treated microcosms from day 42 to day 364, and from the NO_3^- amended trial days 322 and 364. DNA was extracted by lysing the cells with lysozyme, SDS, and five freeze-thaw cycles. Following cell lysis, the DNA was separated from the sediment with slow centrifugation (4000 rpm, 10 min) and the DNA was precipitated from the supernatant with isopropanol, sodium acetate, and overnight incubation at -20°C. The DNA was resuspended in 10 mM Tris buffer pH 8.0. At this stage, the DNA extracts were a dark brown color due to organic matter contamination. A two-step clean-up method was used to purify DNA for use in the polymerase chain reaction (PCR). The first step was phenol-chloroform extraction of the reprecipitated sample. The sample was mixed with one volume of Tris-buffered phenol (pH 8.0) and centrifuged at 14,000 rpm for 10 minutes; the supernatant was recovered for the next steps. This was repeated two more times using a phenol/chloroform/isoamyl alcohol (24/24/1) mix followed by a chloroform/isoamyl alcohol (24/1) mix instead of the phenol. The supernatant, still a brown color, was purified by using the Wizard DNA clean-up kit (Promega, Madison, WI). All of the samples were checked on 1% agarose gels prior to and following the Wizard DNA clean-up step. DNA was also extracted from the field sediment (F1) used to construct the microcosms and from a second, more contaminated, field site (F2). Field sediment samples were extracted within 24 h of collection.

ARDRA: Amplified Ribosomal DNA Restriction Analysis.

Approximately 1100 base pairs of the 16S rRNA genes from microorganisms inhabiting the field and microcosm sediment were amplified with primers that targeted sequences common to all bacteria. The objective was to amplify the 16S rRNA gene from all of the organisms in the sample. The PCR was done in 50 µl final volume. Each reaction contained 2.5 U *Taq* polymerase, 1.5 mM $MgCl_2$, 0.3 µM primers EUB338F and EUB1492R, 200 µM dNTPs, and 40 µg BSA. DNA from pure cultures (positive controls) or sediment extracts (2 µl or 5 µl, respectively) was used as template for each reaction. The amplification reactions were done with a Delta Cycler I thermal cycler (Ericomp, San Diego, CA). The settings were 10 cycles of 94°C 1 min, 64°C to 54°C (decreasing 1°C every cycle) 1 min, and 72°C 3 min; 20 cycles of 94°C 1 min, 54°C 1min, and 72°C 3 min; and a final extension of 72°C 10 min. Amplification products were visualized on a 1% agarose gel, purified (Qiagen PCR purification kit), and digested with one or more restriction enzymes (*AluI*, *HinfI*, *RsaI*, or *TaqI*). Enzyme digests contained 16 µl template DNA, 0.5 U restriction enzyme, and were performed in the appropriate buffer supplied with the enzyme. All of the enzymes had incubation temperatures of 37°C except *TaqI*, which was 65°C. All enzyme digests were done for 3-4 hours.

Digested products were separated by electophoresis in a 4% Metaphore:Nusieve (3:1) gel (FMC Bioproduct, Rockland, ME) in TAE buffer. Gels were run for 4 h at 7V/cm. Bands were visualized after staining with ethidium bromide.

DGGE: Denaturing Gradient Gel Electophoresis

PCR amplification of Microcosm DNA. The extracted DNA was amplified with primers targeting a 569 bp sequence of the 16S–rRNA gene. A 40-bp GC clamp (Muyzer et al., 1993; Muyzer et al., 1996; Muyzer and Smalla, 1998; Rooney-Varga et al., 1999) was added to the forward primer, EUB338F, to stabilize the DNA in the gradient. The GC clamp allows the DNA fragment to remain double stranded in denaturing concentrations that, otherwise, would quickly separate the fragment. The result is greater resolution between different DNA sequences. The reverse primer was EUB907R (Rooney-Varga et al., 1999). Amplification reactions, consisted of 5µl Taq buffer B (Promega, Madison, WI), 0.3 µM each primer, 200 µM dNTPs, 0.4 µg/µl bovine serum albumin, and between 1 and 2.5 µl DNA template. The total reaction volume was 50 µl. The mixtures were heated to 94°C before 1.5U of *Taq* (Promega, Madison, WI) was added. The amount of template added was determined by comparing the amount of DNA in the extract to that of a sample known to amplify well using 2µl of DNA. After adding Taq, the amplification reaction consisted of 30 cycles of 94°C for 30 s, 40°C for 30 s, and 72°C for 30 seconds, and one final extension cycle of 72°C for five minutes after which the samples were held at 10°C.

Optimization of DGGE. DGGE was done with a Bio-Rad D-Code electrophoresis unit (Bio-Rad, Hercules, CA), which was generously loaned to us by Dr. Lunn of the UW-Madison School of Veterinary Medicine. Variables that needed to be optimized prior to using this technique included the concentration of acrlyamide, the denaturing gradient concentration range, and the time and voltage for the run. Several preliminary gels were run to establish the optimal parameters as a 10% acrlyamide concentration, a denaturing gradient of 40-80%, and an electrophoresis run of 90 V for 13 h or 150 V for nine hours. A 100% denaturant concentration would consist of 7 M urea and 40% formamide. The denaturing gradient can be modified as needed to increase the resolution of different portions of the banding patterns. All DGGE gels were run at 60°C in TAE buffer. Gels were stained with either ethidium bromide or SYBR Green I nucleic acid stain (BioWhittaker, Rockland, ME) and visualized by illuminating with UV light.

DGGE: Microcosm comparison experiments. For the microcosm comparison experiments, two 50 µl amplification reactions for each sample were combined and concentrated to a final volume of 50 µl using the Qiagen PCR purification kit (Qiagen, Valencia, CA). After being checked on a 1% agarose gel, the samples were mixed with an equal volume of sample loading buffer. Each lane was filled with 30 µl

of a sample. DNA samples from the microcosms and the field samples were run in a series of combinations to highlight differences or similarities between the samples.

Sequencing and sequence analysis. Selected bands were removed from the DGGE gels for sequence analysis. The bands were removed with a sterile scalpel, and placed in a microcentrifuge tube containing 50 µl buffer. The DNA was allowed to elute from the agarose overnight at room temperature. This DNA sample was then used for another round of PCR and analyzed by DGGE to verify that only one band had been sampled. The desired band was re-extracted from the gel, and used as template for a final PCR using the same primer set, but excluding the GC clamp. This template was purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA). Sequences wee obtained for both strands of the product using the Big Dye terminator system sequencing reactions. Sequencing reaction products were analyzed at the University of Wisconsin-Madison Biotechnology Center on an ABI PRISM 373 DNA Sequencer (PE Applied Biosystems, Foster City, CA).

Phylogenetic analysis was begun by matching the sequences to sequences housed in the Ribosomal Database Project (RDP) (Maidak et al., 2000) using the SeqMatch program (http://www.cme.msu.deu/RDP/html/index.html). The sequences were then aligned to each other and to selected sequences form Genebank and RDP using the PileUp program of the GCG package (Genetics computing Group, Madison, WI). A neighbor-joining tree with Kimura distance correction was built in Clustal X (Thompson et al., 1994). Gram-positive, *Clostridium* species were used as the outgroup. Statistical analysis of the trees was done using 1000 bootstrap repetitions, also in Clustal X.

Isolation of BTEX degrading organisms

Enrichment cultures. As a first step toward isolating of BTEX degrading organisms, a series of enrichment culture experiments were designed to enhance growth and to test the ability of the microbial communities for degradation under either NO_3^- or Fe^{+3}-reducing conditions. Sediment from the lab microcosms was used to inoculate the enrichments. Each enrichment culture consisted of ~2 g microcosm sediment and 20 ml minimal medium. The medium was prepared using anaerobic techniques and contained (per liter): 2.5g $NaHCO_3$; 0.26g KH_2PO_4; 0.4g K_2HPO_4; 0.02g $MgCl_2.6H_2O$; 0.8g NH_4Cl; 0.5g H_3BO_4; 1ml resazurine. Vitamin and mineral solutions were also added to aid growth as essential elements or cofactors. Dithiothritol was added to a final concentration of 0.05% as a reducing agent. Trials were established to enhance growth of NO_3^- and Fe^{+3}-reducing organisms with one or more BTEX compounds as an electron donor. For each TEA 5 mM NO_3^- (KNO_3) or 50 mM Fe^{+3} (ferric-sodium salt of EDTA) were amended to the base medium. The BTEX compounds were added to a final concentration of 5 to 10 mg/l from sterile, anaerobic, aqueous stocks. Abiotic control cultures were established by adding 0.1% sodium azide and autoclaving three times on three consecutive days prior to amending with hydrocarbons. The enrichments were sampled for benzene and NO_3^- or Fe^{+2} as described for the microcosms. The cultures were reamended with electron donor or acceptor as needed during the enrichment process. The enrichments were stored at 25°C in anaerobic jars pressurized with N_2:CO_2 to 10 psi.

Data from two of the enrichment culture trials are included in this report. The objective of the first was to isolate organism able to degrade toluene, *o*-xylene, and/or *m*-xylene alone or in combination. Sediment for these enrichments was taken from the BTEX+NO_3^- trial from the microcosms sampled on day 98. The objective of the second was to test if amending the enrichments for benzene degradation when it was added as the sole hydrocarbon and either NO_3^- or Fe^{+3} was amended to the medium. Microcosm sediment from day 354 from the BTEX and the BTEX+NO_3^- trials were used to inoculate the enrichments.

Isolation of toluene and *o*-xylene degrading microbes. To obtain isolates of toluene and *o*-xylene degrading organisms a subsample of the enrichment cultures (the toluene and xylene experiment

described above) was serially diluted in warm NO_3^--reducing medium containing 0.8% agar. One dilution series was prepared for each hydrocarbon (toluene and *o*-xylene). Approximately 2 µl of toluene or *o*-xylene was added to the soft agar mixture for a final concentration of about 45 mg/l on each plate. The dilution mixtures were poured over solidified agar plates (1.5% agar) of the same composition (without the hydrocarbon). Plates were stored sealed in thick plastic bags in an anaerobic jar at 25°C. After two weeks of incubation, several colonies were observed on all of the toluene and *o*-xylene dilution plates. Several of the colonies from each plate were transferred onto fresh solidified medium. Each isolate was also tested for growth in the absence of toluene or xylene and in the presence of oxygen.

Colonies grown on the agar medium were transferred to fresh liquid medium. Two isolates each from the toluene and the o-xylene plates were selected for growth in NO_3^--reducing medium amended with 0, 5, 10, or 25 mg/l of toluene or *o*-xylene, respectively. The isolates were designated Tol2, Tol3, OXyl1 and OXyl2. A non-inoculated control with approximately 10 mg/l toluene or *o*-xylene was also included for each hydrocarbon. Resazurine was included in the medium as an indicator of oxygen contamination. Growth was monitored in all of the cultures by measuring optical density at 600 nm.

VI. RESULTS

In situ Microcosms

Groundwater conditions at ISM sites. Ground water at the site of Group 1 was contaminated with total BTEX concentrations from 2 mg/l to 15 mg/l. The concentration of dissolved oxygen was less than 0.05 mg/l, and the concentration of NO_3^- was generally less than 1 mg/l, indicating strongly reducing conditions. Sulfate concentrations were generally below 1.5 mg/l and dissolved Fe^{+2} concentrations varied from 0.1 to 17 mg/l. Based on these geochemical indicators, the prevailing TEAPs were probably SO_4^-- reduction and/or methanogenesis. All three ISMs at this site were placed between 6 and 8 feet below ground surface.

Groundwater at the site of Group 2 had much lower concentrations of total BTEX, ranging from 0.3 mg/l to 0.7 mg/l. The percentage of benzene, the most recalcitrant of the BTEX compounds under anaerobic conditions, was higher at this site than at others, indicating that natural attenuation of alkylbenzenes may be occurring upgradient. The dissolved oxygen concentration varied from 0.05 mg/l to 0.4 mg/l, and the NO_3^- concentrations were generally less than 1 mg/l. Sulfate concentrations ranged from 0.1 to 10 mg/l and dissolved Fe^{+2} concentrations ranged from 0.1 mg/l to 10.2 mg/l. The predominant TEAP at this site was inferred to be Fe^{+3}- or NO_3^--reduction. The ISMs at this site were placed between 5.5 and 7.5 feet below ground surface.

Groundwater at the site of Group 3 was contaminated with total BTEX concentrations from 0.01 to 0.55 mg/l. The dissolved oxygen varied from less than 0.05 mg/l to 0.2 mg/l and NO_3^- concentrations varied from 0.2 mg/l to 2.4 mg/l. Sulfate concentrations ranged from 0.1 mg/l to 4.4 mg/l and dissolved Fe^{+2} concentrations ranged from 0.5 mg/l to 8 mg/l. This site was also interpreted as Fe^{+3}- or NO_3^--reducing. The ISMs at this site were placed between 5.5 and 7.5 feet below ground surface.

In situ microcosm operation Monitoring the conservative tracer (Br^-) provided the best indicator of successful ISM operation. In the initial design and trials, dilution of the conservative tracer confirmed that the valves were leaking experimental water into the galvanized standpipe. During the ISM experiment, the concentration of the conservative tracer in the ISMs remained constant for 45 to 60 days (**Fig. 3-5**). Stable Br^- concentrations indicated uniform distribution of the loading water and confirmed that the injected pulse of water was isolated from the aquifer. The ISMs were considered operational during this period and the Br^- data was used to evaluate the degradation and consumption rates of the BTEX and TEAs.

Group 1. No clear evidence of degradation of BTEX was observed in any of the ISMs at Group 1. Incompletely dissolved laboratory spike was accidentally used to load the Group 1 ISMs, which caused inconsistent BTEX loading among these instruments. The first ISM loaded from the Tedlar™ bag (the BTEX ISM) showed an anomalously high BTEX concentration in one of two samples collected on day 14 (**Fig 3 and Table 1**). This was probably the result of injecting BTEX as a non-aqueous phase liquid (NAPL) into this ISM. The BTEX concentration of second ISM loaded (the BTEX + NO_3^- ISM) increased during the first three sampling days. This may also have been the result of loading this ISM with a NAPL. Incomplete displacement of ambient NAPL during loading may have also contributed the high BTEX concentrations in these ISMs.

Although BTEX degradation could not be quantified, the concentrations of NO_3^- and Br^- from the BTEX + NO_3^- ISM for the first 67 days indicated loss of NO_3^-. It was not possible to calculate a NO_3^- mass loss ratio since it was not possible to quantify TEX loss at this location.

The concentration of dissolved Fe^{+2} increased in the Group 1 BTEX ISM from approximately 20 mg/L on day 14 to approximately 26 mg/L on day 67. With the exception of day 14, the concentration of dissolved Fe^{+2} in the BTEX + NO_3^- ISM at Group 1 decreased from approximately 30 mg/L on day 6 to approximately 19 mg/L on day 67. The concentration of dissolved Fe^{+2} in the abiotic control at Group 1 remained relatively constant. It appeared that dissolved Fe^{+2} was being released in the BTEX ISM at

Group 1, suggesting that sediment-bound oxidized Fe^{+3} was being used as a TEA for the oxidation of organic matter. In the BTEX + NO_3^- ISM at Group 1, the loss of dissolved Fe^{+2} may have been the result of increasing the oxidizing potential of the sediment through the addition of NO_3^-. The relatively constant concentration of dissolved Fe^{+2} in the abiotic control at Group 1 suggests that the reduction of iron in the BTEX ISM and the oxidation of Fe^{+3} in the BTEX + NO_3^- are the result of microbe-mediated redox reactions.

Group 2. The BTEX ISM at Group 2 showed clear loss of toluene and *m,p*-xylenes, and possible loss of *o*-xylene (**Fig. 4 and Table 2**). The lag for *m,p*-xylenes was estimated to be approximately 10 to 15 d, and the lags for toluene and *o*-xylene were estimated to be approximately 32 to 40 d. The calculated rate constant for *m,p*-xylenes was 0.2 d^{-1} after a lag of 14 d. No clear evidence of ethylbenzene or benzene loss was observed in the BTEX ISM at this location.

The BTEX + NO_3^- ISM at Group 2 appeared to show degradation of *m,p*-xylenes, ethylbenzene and toluene (**Fig. 4 and Table 2**). As in the BTEX ISM, *m,p*-xylenes appeared to degrade first with no lag or with a lag time of approximately 10 d. The maximum rate constant calculated for *m,p*-xylenes with a 10 d lag time was 0.2 d^{-1}, identical to the value estimated for the Group 2 BTEX ISM. Thus, for *m,p*-xylenes, there was no obvious enhancement of degradation under NO_3^--reducing conditions. In the BTEX + NO_3^- ISM toluene had a shorter lag, approximately 15 to 20 days, but a similar degradation rate constant 0.07 d^{-1} when compared to the BTEX ISM. Thus, no obvious increase in the degradation rate occurred under NO_3^--reducing conditions, although the lag time was cut in half. *o*-Xylene appeared to show degradation, as in the BTEX ISM. However, the long lag time of between 20 and 45 days, and dilution of the conservative tracer by day 61 made confirmation difficult. Under NO_3^--reducing conditions, ethylbenzene showed degradation with a lag time of 10 to 15 days, intermediate between *m,p*-xylenes and toluene in this ISM. The degradation rate constant was estimated to be 0.05 d^{-1}.

Nitrate appeared to be consumed in the Group 2 BTEX + NO_3^- ISM starting within 10 days after amendment. The estimated first order rate constant for NO_3^- was 0.005 d^{-1}. There was an apparent decrease or even cessation of NO_3^- loss after approximately 30 days (**Fig. 4 and Table 2**). It is possible that NO_3^- use decreased when the *m,p*-xylenes, toluene and ethylbenzene reached low concentrations. By day 53, the concentration of NO_3^- declined from an initial concentration of approximately 200 mg/l to approximately 150 mg/l. The calculated NO_3^- mass loss ratio (the mass ratio of NO_3^- loss to TEX loss) was approximately 25.

Group 3. In contrast with the BTEX ISM at Group 2, only *m,p*-xylenes appeared to degrade in the BTEX ISM at Group 3 (**Fig. 5 and Table 3**) After a lag of 7 days, *m,p*-xylenes had a degradation rate constant of approximately 0.01 d^{-1}. No loss of toluene, *o*-xylene or ethylbenzene was observed. Sulfate concentrations, however, appeared to decline significantly during the first 15 days. The ambient ground water concentration of approximately 1.7 mg/l, present in the loading water, was reduced to a concentration of approximately 0.5 mg/l by day 45. Normalization of the SO_4^{-2} to both the Br^- concentration and the Cl^- concentration resulted in an estimated degradation rate constant of 0.03 d^{-1} during the first 15 days. The location of Group 3 at the plume fringe was expected to have Fe^{+3}-reducing conditions, but the depletion of bio-available Fe^{+3} at this site could have resulted in favorable conditions for reduction of the ambient SO_4^{-2}.

In the BTEX + NO_3^- ISM at Group 3 the *m,p*-xylenes, toluene and *o*-xylene degraded, but no loss of ethylbenzene or benzene was observed (**Fig. 5 and Table 3**). *m,p*-Xylenes degraded in the BTEX + NO_3^- ISM after a lag time of approximately 35 days, much longer than the lag time for the BTEX ISM at Group 3. Once degradation began, however, the rate constant, 0.2 d^{-1}, was least an order of magnitude greater than that of *m,p*-xylene in the adjacent BTEX ISM and was similar to the rate constant for *m,p*-xylenes in the Group 2 BTEX and BTEX + NO_3^- ISMs. Toluene concentrations were stable in the Group 3 BTEX ISM, but in the BTEX + NO_3^- ISM toluene degraded after a lag time of approximately 37 to 45 d, indicating enhancement under NO_3^--reducing conditions at this location. The estimated degradation rate constant, 0.2 d^{-1}, was identical to that estimated for *m,p*-xylene at this ISM and was much higher than the

degradation rate constants for toluene estimated in the BTEX and BTEX + NO_3^- ISMs at Group 2. No loss of ethylbenzene or benzene was apparent at the Group 3 BTEX + NO_3^- ISM.

Nitrate loss in the BTEX + NO_3^- ISM at Group 3 was constant throughout the experiment. The estimated degradation rate constant for NO_3^-, calculated from NO_3^- concentrations normalized to both bromide and chloride, was 0.003 d^{-1}. The total NO_3^- loss at the Group 3 BTEX + NO_3^- ISM was comparable to that at the Group 2 BTEX + NO_3^- ISM, approximately 50 mg/L. The corresponding NO_3^- use ratio, approximately 35, was slightly higher than that calculated for the Group 2 BTEX + NO_3^- ISM because less TEX was lost over the course of the experiment at Group 3.

In contrast with the sulfate loss observed in the Group 3 BTEX control, sulfate concentrations at the Group 3 BTEX + NO_3^- ISM increased over time. After a lag of approximately 8 to 13 days, the concentration of sulfate increased at a rate approximated by a first-order rate law. The estimated rate constant was in the range of -0.008 d^{-1} to -0.009 d^{-1}, using the sulfate concentrations normalized to chloride and bromide, respectively. It is possible that the addition of NO_3^- somehow induced microbial oxidation of sulfide that was present in the aquifer as a product of earlier sulfate reduction.

BTEX biodegradation in laboratory microcosms

Microcosms amended with BTEX and NO_3^-. Toluene, ethylbenzene, *m,p*-xylenes and *o*-xylene losses were measured in microcosms amended with NO_3^- (**Fig. 6**). After an estimated 7 d lag period, toluene degraded rapidly for 21 d and then degraded slowly for an additional 49 d, after which it was fully depleted. *o*-Xylene loss began after 28 d and continued to day 70. Ethylbenzene degradation was first measured after 98 d of incubation; full depletion did not occur until day 266. Loss of *m, p*-xylenes occurred after approximately 42 d of incubation; degradation was initially rapid until day 70 and was still present at low concentrations (<0.05 mg/l) after 266 d. Benzene loss was not measured during the 364 d incubation period. Nitrate loss in the BTEX amended microcosms was the most rapid during the first 42 d (**Fig. 7**). From days 48 to 266, NO_3^- loss slowed considerably. A low concentration of NO_2^--N (< 1 mg/l) was measured at early time points during the experiment, but was not detected past day 42. Concentrations of total dissolved Fe^{+2} increased in BTEX+NO_3^- microcosms during the first 28 d (**Fig. 8**), and then remained at a constant level of 0.15 mg/l. Total sediment Fe showed an overall decrease in concentration, but there was significant variability in the measurements of sediment iron from the triplicate microcosms. The variability reflects difficulty in collecting representative sediment samples from the microcosms, due to the heterogeneity of the sediment and to the small sample amount (0.2 g) used for acid extraction. Other electron acceptors measured periodically during the experiment were dissolved oxygen (DO) and methane (**Table 4**). At 144, 210, and 266 d, DO concentrations were between 30 and 50 µg/l. Headspace samples were analyzed for gases on day 210; no methane was detected.

Microcosms with BTEX only. Compared to the abiotic control, no significant loss of BTEX was measured without NO_3^- after incubating 364 d (**Fig. 6**). Total dissolved Fe^{+2} concentrations increased during the first 42 d and then remained at a constant level of 0.40 mg/l (**Fig. 8**), which was consistently higher than the ferrous iron measured in the BTEX+NO_3^- and the NO_3^- amended microcosms. DO, measured on days 144, 210, and 266, was below detection (less than 10 µg/l) in the BTEX microcosms (**Table 4**). Methane was measured on day 210 and none was detected.

Microcosms with NO_3^- only. In microcosms amended with NO_3^- but not BTEX, NO_3^- loss was similar to that measured in the BTEX+NO_3^- microcosms (**Fig. 7**), indicating that another electron donor was used for NO_3^- reduction. The original microcosm sediment contained total organic carbon of approximately 0.2 percent by weight. After 266 days, sediment samples from the BTEX+NO_3^-, NO_3^-, BTEX, and abiotic control were analyzed for TOC; concentrations ranged from 0.21 to 0.25 percent by weight. Thus, if organic carbon was lost, it was within experimental or measurement error. The dissolved Fe^{+2} increase in these microcosms was similar to the BTEX+NO_3^- microcosms (**Fig. 8**); concentrations increased to 0.15 mg/l and remained constant for the rest of the experiment. DO

concentrations remained constant at 20 µg/l between 144 and 266 days (**Table 4**). No methane was detected on day 210.

Abiotic microcosms with BTEX and NO_3^-. No BTEX loss was observed in the abiotic controls, although sorption appeared to have played a role during the early phases of incubation (**Fig. 6**). Sorption was more pronounced in the abiotic than in the live microcosms; autoclaving sediment has been observed to alter its sorption characteristics (Dao et al., 1982). In addition to changing sorption properties, autoclaving the sediment and media caused the color of the media to change to a dark reddish-brown. This color change caused interference with the colorimetric analysis of Fe. The ion chromatographs of the abiotic controls had a broad, unknown peak that interfered with the NO_3^- peak. Initially, azide was thought to be responsible, but subsequent analysis of azide did not produce the peak. Compounds that were produced during autoclaving of the sediment and media may have caused the unknown peak. Because the interfering peak could not be separated from the NO_3^- peak, NO_3^- in the abiotic controls was analyzed using a cadmium reduction method. This method uses colorimetric analysis to quantify concentrations, but the color of the sample did not interfere when the sample was sufficiently diluted. Results of the colorimetric analysis showed no loss of NO_3^- in the abiotic controls over time (**Fig. 7**). DO was not analyzed due to the color. Methane was not detected on day 210.

Molecular analysis

ARDRA

DNA recovered from the microcosms and field samples was successfully amplified with the conserved 16S rRNA gene primers, and digested with the restriction enzymes. The restriction digest patterns were the same for all of the samples tested (data not shown).

DGGE

DNA extraction and amplification. DNA was successfully recovered from all of the microcosm samples (**Fig. 9**). The amount of DNA used as template for the amplification reaction was quantified relative to the BTEX day 70 (**Fig. 9**, lane 14) sample. This sample was used to optimize the DGGE PCR, and 2 µl of this sample amplified well. The minimum amount of DNA used as template was 1 µl. All of the samples, except BTEX day 42, amplified well, and the amount of product was about the same for all samples.

Banding patterns, Field vs. Microcosms. When the field samples, F1 and F2 were compared to microcosm samples from days 322 and 364 of the BTEX, BTEX/NO_3^-, and NO_3^- microcosm treatments, some distinct banding patterns were seen (**Fig. 10**). Although some of the bands were faint, the overall patterns for different samples were clearly distinguishable. Samples F1 and F2 share one band (or band cluster) with all of the other samples (**Fig. 10**, solid black arrow). These samples also have other bands that are distinct from the microcosm samples (**Fig. 10**, open black arrows). This difference was also observed under different gradient conditions. The field samples repeatedly showed very few distinct bands.

Banding patterns and BTEX degradation. The BTEX/NO_3^- group was the only microcosm trial that showed TEX degradation. DNA from each of the time points from this trial were run in series to determine if any changes occurred during the experiment as TEX was degraded. A few bands appeared only at some time points (**Fig. 11**), however, most of the banding pattern remained constant throughout the degradation of BTEX in the BTEX+NO_3^- trial. It is difficult to discern if these bands correlate to the TEX degradation pattern of the microcosm experiment.

Banding patterns and sequence analysis of NO_3^- amended vs. non-amended microcosms. DNA amplified from the BTEX and BTEX+NO_3^- trials from several time points was run in parallel to determine if community differences occurred between the trials throughout the microcosm experiment. Samples from the final microcosms (day 364) from the BTEX, NO_3^- and BTEX+NO_3^- were also compared in a separate, but similar gel (**Fig. 12**). Selected bands from these gels were chosen for sequencing. Sequenced bands are indicated in the gel photo by white boxes. The phylogenetic tree (**Fig. 13**) demonstrates the relationships between the sequenced bands and representatives from the Genbank database. Sequences from bands at the same location are indicated by a letter and are very closely related (e.g., NB98 (B), NB 266 (B), and B98 (B)). These sequences have less than 1 nucleotide difference per 100 bases, and are probably from the same population. Small differences in the sequences may be due to sequencing errors. No sequences have been retrieved from the field sediment samples.

These gels demonstrated that the organisms enriched in the microcosms were different from the field sediment (F1, day 0) (**Figs. 10-12**). The BTEX+NO_3^- and NO_3^- trials shared several bands that were not seen in the BTEX only trial (**Fig. 12**, bands A and F). Bands B and E were detected in most of the NO_3^- amended samples, but were not detectable in the BTEX only trials after days 147 and 70 respectively. Sequences of the bands A and B were tightly clustered within the β-Proteobacteria, while the bands E and F did not closely align with any identified organism (**Fig. 13**).

Some bands, such as G and H, were only detectable in the BTEX only trial. These sequences clustered loosely within the *Flexibacter-Cytophaga-Bacteroides* group. However, when aligned with sequences in the RDP, the similarity values are very low (S-ab<0.5). Others, such as C, may have been present in all of the trials. These sequences were most closely related to the *Acidobacterium.*

Isolation of BTEX degrading organisms

Enrichment cultures

Toluene and xylene degrading enrichment cultures. Degradation of toluene, *o*-xylene, and *m*-xylene occurred in all of these enrichment cultures. The compounds degraded in an order similar to that seen in the microcosms, toluene followed by the xylenes (**Fig. 14A**). In addition, the xylenes degraded faster in the cultures amended with a mixture of the hydrocarbons than in those with only one compound (**Fig. 14B**). After 128 of incubation, additional compound was added to the enrichments. After spiking, all of the compounds degraded at the same rate regardless of the presence of multiple compounds.

Benzene enrichments. Benzene was quickly depleted from the NO_3^-- and Fe^{+3}-reducing enrichments. However, no benzene was degraded in the microcosms from which the inocula were taken, suggesting that the enrichments may have been contaminated with oxygen. The removal of benzene, however, was biological because the abiotic controls showed no loss of benzene after the initial amendment (**Fig. 15**). The enrichments were left for about one month to allow for the complete consumption of oxygen. The cultures were then amended with fresh anaerobic NO_3^-- or Fe^{+3}-reducing medium and benzene solution. Resazurine was included in the NO_3^- medium to indicate oxygen contamination. Oxidation of resazurine, however, can occur with reaction to oxygen or NO_2^-. The resazurine did become oxidized, but this was concurrent with a slight accumulation of NO_2^- (**Fig. 15**). This apparent reduction of NO_3^- however was not detectable by loss of NO_3^- and did not correlate with any loss of benzene. After the second and third amendments, benzene loss was detected in the abiotic controls. There was also no detectable loss of benzene or reduction of Fe^{+3} in the Fe^{+3} amended enrichments beyond that seen in the abiotic controls.

Isolation of toluene and xylene degrading anaerobes

Isolates from the enrichment cultures were maintained on solid anaerobic medium, and grew sparsely with or without toluene or *o*-xylene. Thus, it was difficult to determine if growth was improved with addition of the hydrocarbons as a carbon and electron donor source because the colonies were very

small regardless of the growth conditions. In the liquid cultures, growth was sporadic although the hydrocarbon concentrations in the isolate trials were similar. Only a few cultures demonstrated growth detectable by increased cell density. None of the cultures without hydrocarbon had a detectable increase in cell density, however none of the cultures had a detectable decrease in toluene or *o*-xylene, regardless of any increases in density. In several of the cultures, the resazurine became oxidized. However, NO_2^- was detected only in the cultures that changed color. Thus, the cultures may have been accumulating NO_2^- (from NO_3^- reduction) and using an electron donor other than the hydrocarbons. DNA from one of the cultures, Tol2, was isolated and the 16S-rRNA gene sequence (~600bp) was determined. A Genbank BLAST search revealed that this sequence was very closely related (100% sequence match) to *Bradyrhizobium* sp.

VII. DISCUSSION

Biodegradation under NO_3^--reducing conditions: field vs. laboratory

The overall patterns of BTEX biodegradation were generally similar between the field and the lab experiments, but the sequence of alkylbenzene degradation differed. In NO_3^- tracer experiments conducted for a previous project, toluene, ethylbenzene and *m, p*-xylenes losses were observed 10 days after injection; *o*-xylene loss was not observed until approximately 20 days and could not be quantified (Schreiber and Bahr, 1999). In the BTEX+NO_3 microcosm, toluene began to degrade after an apparent lag of 7 days, *o*-xylene after 28 days, *m,p*-xylenes after 42 days, and ethylbenzene after 98 days. Benzene degradation was not observed in the field, laboratory, or ISM experiments. In the ISM experiments, enhancement of toluene, xylene and ethylbenzene degradation with NO_3^- was unpredictable and appeared to be influenced more by location than amendments. In Group 3 (low groundwater hydrocarbon concentration) toluene, *m- & p*-xylenes and *o*-xylene degradation was enhanced by NO_3^-. However, in Group 2 (high groundwater hydrocarbon concentration) only ethylbenzene degradation was stimulated by NO_3^- even though NO_3^- consumption was approximately the same in both groups (**Table 5**). Overall, first-order rate constants estimated from the field experiment and the ISMs were higher than those measured during the laboratory experiment (**Table 5**).

Differences between the rate constants, lag times, and the sequence of degradation could reflect differences in BTEX and NO_3^- concentrations, or other geochemical or microbiological conditions. In the tracer experiment, the groundwater had a pH of 6 while the laboratory microcosm medium had a pH of 7. Because denitrification is more favorable at higher pH values (Delwiche and Bryan, 1976), the pH differences would not explain the higher rates measured in the field. The overall higher rate constants measured in the field also cannot be explained by temperature differences; the higher incubation temperature in the lab (25°C vs. 15°C in the field) should have resulted in higher lab rates. Differences in BTEX concentrations that were added (6 mg/l in field, 2 mg/l in lab) were probably not great enough to affect reaction rates.

Although phosphate and ammonium were added to the microcosm media and not to the tracer solution or ISM medium, several studies have demonstrated that addition of nutrients does not significantly enhance biodegradation rates (Hutchins, 1991; Ball and Reinhard, 1996; Nales et al., 1998). However, in the Ball and Reinhard (1996) (Ball and Reinhard, 1996) experiments, the order of ethylbenzene and toluene biodegradation appeared to be controlled by whether groundwater or basal salts medium was used. In microcosms where groundwater was used as the media and was only amended with NO_3^-, toluene degraded before ethylbenzene. In media containing a variety of salts and NO_3^-, ethylbenzene degraded before toluene. In our experiments, the opposite pattern was observed. Toluene and ethylbenzene degraded at the same time in the field tracer experiment, in which no nutrients were added, while in the lab toluene degraded much sooner (less than 7 d lag) than ethylbenzene (98 d lag).

Cometabolic reactions and/or competing non-BTEX electron donors may have also influenced the relative order of biodegradation. In laboratory experiments conducted by Evans et al. (1991), *o*-xylene did not degrade when present alone under NO_3^--reducing conditions, but did degrade in the presence of toluene. Other studies have suggested that *o*-xylene degradation is inhibited by the presence of other petroleum compounds (Trinzinsky and Bouwer, 1990). In our enrichment cultures, however, there did not appear to be significant differences in degradation sequence between the individual and mixed compound cultures of toluene, *m,p*-xylenes, and *o*-xylene (**Fig. 14A**), suggesting that the presence or absence of individual compounds did not affect degradation of other compounds. Benzene degradation may have been inhibited by the presence of competing electron donors, such as the TEX compounds or sediment organic carbon (Nales et al., 1998). In the field experiment, sediment organic carbon content was low (< 0.05%), but many other hydrocarbon compounds were present. The concurrent degradation of toluene, ethylbenzene, and m,p-xylene suggest that the presence of other non-BTEX hydrocarbons did not inhibit degradation of TEX. In the lab, only the BTEX compounds were added, but the sediment contained

approximately 0.25% organic carbon. The longer lag periods for ethylbenzene and *m,p*-xylenes in the lab experiment may be related to an inhibitory effect of organic carbon on TEX degradation.

The sequence of toluene and xylene degradation in the enrichment cultures can be used to gain insight into some of the microbial processes that may have led to the differences between the tracer and microcosm results. The initial sequence of alkylbenzene degradation in the cultures was similar to sequence in the microcosms; toluene degradation occurred most rapidly, followed by the xylenes. However, when added in combination the xylenes degraded faster than when they were amended individually. When the cultures were re-dosed with hydrocarbon, all three compounds degraded rapidly and the loss rates were comparable, similar to the patterns observed in the field (**Fig. 14A**) indicating acclimation to the chemicals. These results indicate that the presence of toluene may help establish the microbial community responsible for degrading the aromatic compounds or initiate degradation of the xylenes through cometabolic reactions. During the tracer experiment, degradation occurred relatively rapidly, within approximately 10 days, likely because the microorganisms living in the contaminated aquifer had been exposed to BTEX for approximately 30 to 50 years and were acclimated to degradation of these chemicals. The sediment collected for the microcosm study was also from the contaminant plume; thus, the microbes should have been acclimated to BTEX. The longer lag period observed in the lab may be related to sediment collection and microcosm construction. Spatial relationships between different microbial populations may have been disturbed as the sediments were cored and subsequently homogenized. Re-establishment of these communities may have been required before degradation could occur at rates similar to what was observed in the field.

BTEX degradation coupled to other electron acceptors.

Degradation in the microcosm experiments did not appear to be coupled to other available electron acceptors. Trace concentrations (less than 50 µg/l) of DO were measured in BTEX+NO_3^- and NO_3^- microcosms, indicating that some DO was present in the initial media. DO was not detected in the BTEX microcosms or the sediment control, suggesting that the original DO from the media was used in these treatments. However, as shown in **Fig. 6**, the consumption of less than 50 µg/l did not contribute to BTEX loss in the BTEX microcosms. Low but consistent concentrations of DO in the microcosms amended with NO_3^- suggest that DO concentrations were too low to be used efficiently. Thus, NO_3^- became the dominant electron acceptor. Sulfate was not added to the media nor was it present in the sediment; thus sulfate reduction was not possible. Methane was not detected in any of the microcosms at Day 210, indicating that degradation coupled to methanogenesis was not a source of BTEX loss.

Because iron reduction is likely the dominant electron-accepting process in the plume, as evidenced by dissolved ferrous iron concentrations of up to 50 mg/l in groundwater, it was expected that that Fe^{+3} from the microcosm sediment would be reduced in the BTEX microcosms that were not amended with NO_3^-. However, although Fe^{+3} reduction did occur, it was not significant (dissolved iron concentrations were less than 1 mg/l) and did not appear to be coupled to BTEX degradation. The likely reason for lack of Fe^{+3} reduction in the microcosms is that bioavailable Fe^{+3} had been depleted in sediment over the approximately 30 to 50 years of the lifetime of the plume. Analyses of acid extractions of sediment from pristine and contaminated portions of the aquifer at the site suggest that Fe^{+3} in the plume is depleted with respect to uncontaminated sediment. Depletion of Fe^{+3} has been reported in other studies of contaminant plumes (Lovley and Phillips, 1987; Heron and Christensen, 1995).

Microbial mediation of Fe^{+3} reduction was detected in the BTEX ISM at Group 1. However, because BTEX loss could not be monitored, pairing Fe^{+3} reduction with degradation was not possible. Depletion of the bio-available Fe^{+3} in the Group 3 ISMs may have allowed SO_4^{-2} to become an important TEAP in the BTEX ISMs at this site.

Electron acceptor use in the absence of BTEX.

Nitrate, Fe^{+3}, and trace concentrations of DO were used in microcosms not amended with BTEX. In the NO_3^- microcosms, NO_3^- loss was almost as extensive as it was in the BTEX+NO_3 microcosms. In the

sediment control, ferrous iron concentrations increased to the same concentration as in the BTEX microcosm. The lack of trace concentrations of DO in the BTEX microcosms and sediment control compared to the NO_3^- amended microcosms indicate that DO was also used. The electron donor responsible for the use of these electron acceptors is most likely sediment organic carbon, but other reduced compounds may be present in the sediment and could contribute to electron acceptor use.

Nitrate mass loss.

The NO_3^- mass loss ratio, the mass of NO_3^- required to degrade a certain mass of toluene, is approximately 4.9. This value was calculated according to the equation $C_7H_8 + 7.8NO_3^- + 7.8H^+ \rightarrow 7CO_2 + 3.6N_2 + 7.6\ H_2O$. If the other BTEX compounds are included, the value becomes approximately 5. If bacterial cell production is included, as in the equation $C_7H_8 + 3NO_3^- + H^+ \rightarrow C_5H_7O_2N(cells) + 2CO_2 + N_2 + 3H_2O$ the nitrate mass loss ratio becomes approximately 2. In the field tracer experiment, the NO_3^- mass ratio, ranged from 90 to 157, twenty to fifty times higher than what was needed for BTEX oxidation alone (Schreiber and Bahr, 1999). Because sediment organic carbon in the tracer zone was less than 0.05%, we hypothesized that the additional loss was primarily due to oxidation of non-BTEX hydrocarbon compounds in the plume. In the microcosm experiment, only BTEX compounds were added, so NO_3^- loss in addition to what was required for BTEX oxidation was assumed to be related to oxidation of organic carbon or other reduced compounds in the sediment. The NO_3^- mass ratio for TEX loss in the microcosm experiment ranges from 20 if NO_3^- loss was corrected for non-BTEX losses, to 150 if non-BTEX losses are not considered (**Table 5**). The NO_3^- mass ratios for the ISM amended with nitrate were from 25 to 33, indicating that non-BTEX losses are also involved with NO_3^- reduction in the ISMs.

Implications for biodegradation under enhanced NO_3^--reducing conditions.

Addition of NO_3^- to the BTEX-contaminated aquifer at the Fort McCoy enhances biodegradation of toluene, ethylbenzene, and xylenes, but not of benzene (**Table 5**). Comparison of field and laboratory results indicated that the patterns of biodegradation were similar, but the estimated lag times were longer and first-order rate constants lower in the lab experiments than in the field experiments. In the ISMs, the lag times for some compounds (e.g. toluene) were much longer than in the tracer experiments. However, in the ISMs the degradation rates were generally higher than in either the microcosm or the tracer experiments. The differences between the experiments may reflect 1) competing electron donors in the experiments and 2) disruption of the microbial communities in sediment collection and homogenization for the microcosm experiment. In the enrichment cultures, several hydrocarbon doses were required before degradation rates and patterns were similar to what was observed in the field.

Nitrate losses in both field and lab experiments were significantly higher than would be predicted by stoichiometric relationships. In the field, the mass ratio of NO_3^- loss to TEX loss ranged between 90 to157, 25 to 33 in the ISMs, and 20 to 150 in the lab. The excess NO_3^- losses were caused by oxidation of other electron donors, such as sediment organic carbon, other hydrocarbons, organic acids, ferrous iron, and trace sulfides. If NO_3^- is under consideration for use in an enhanced bioremediation system, the effect of other electron donors on excess NO_3^- loss must be evaluated. Because NO_3^- is itself a groundwater contaminant, adding higher concentrations to groundwater may not be acceptable in some areas.

Although iron reduction occurred to a limited extent, it did not contribute measurably to BTEX loss in the microcosm experiments. The similar patterns of Fe^{+2} production in the BTEX microcosm and the sediment control suggest that the iron reduction was coupled to oxidation of electron donors in the sediment. Low levels of ferrous iron were measured in NO_3^--amended microcosms, suggesting that some iron reduction occurred during NO_3^- reduction.

Although two recent studies have demonstrated that benzene degradation coupled to NO_3^- reduction is possible in some sediments (Nales et al., 1998; Burland and Edwards, 1999), most studies suggest that benzene is recalcitrant under enhanced NO_3^--reducing conditions. Based on results of the field and laboratory experiments at the Fort McCoy site, benzene appeared to be recalcitrant to degradation under enhanced NO_3^--reducing conditions. However, NO_3^- does offer several advantages to other electron

acceptors in enhancing biodegradation of TEX compounds, including high solubility and non-toxic byproducts (N_2). One potential bioremediation method is to use multiple electron acceptors to enhance BTEX degradation, for example, NO_3^- and oxygen mixtures (Hutchins, 1991; Anid et al., 1993) or NO_3^- and sulfate mixtures (Ball and Reinhard, 1996). It is important to realize that application of enhanced anaerobic biodegradation can potentially remove significant masses of total BTEX, but treating the individual compounds may require enhancement of several electron-accepting processes, or use of coupled enhanced and intrinsic biodegradation.

Microbial Community analysis

The objective of the second track of this project was to link the chemical processes to changes in the microbial community. Organisms involved with the degradation of hydrocarbons can be treated as a "black-box" for determining the rate, sequence and extent of biodegradation. However, in order to improve bioremediation technologies information about the organisms mediating the reactions are needed so the metabolic potential of these organisms can be optimized. The most direct way to accomplish this is to isolate organisms from the environment that can mediate the activities seen in the field. Obtaining isolates of BTEX-degrading organisms, however, has proven to be a difficult task. Isolation of organisms from complex environments such as soil or groundwater has been a continuing challenge for microbial ecologists. In these isolation experiments, most of the isolates grew on plates in the absence of hydrocarbons. This indicates that the organisms were able to use some components of the medium for carbon and energy sources. These components could have been the reducing agent or the vitamin mix. Trace amounts of H_2 in the flask headspace may also have served as an energy source. Interdependence of organisms, with diverse physiologic requirements and consortia mediated degradation processes compound the difficulty of the isolation process (Zwolinski et al., 2001). Relatively few BTEX degrading organisms have been successfully isolated from these environments. In addition to these difficulties, isolation of organisms also provides limited information about the environment. Culturing may select for organisms that are not the predominant mediators of degradation in the field.

In addition to attempting to culture the organisms from the microcosms, we also explored several molecular techniques for monitoring the BTEX degrading microbial community. The amplified ribosomal DNA restriction analysis (ARDRA) technique has been used by other researchers to demonstrate changes occurring in microbial communities correlating with some environmental change (Smit et al., 1997). Our objective for using this technique was to differentiate when changes in the microbial community occurred during the microcosm study. However, no changes in the microbial community of the microcosms were detected. Microcosms, field sediment, and even aerobic non-contaminated surface soils had the same restriction digest patterns. The ARDRA technique did not produce high enough resolution to meet our objective. Because we did not know what organisms were present in the sample and represented in the amplified DNA, we could not choose a restriction enzyme that would cut the DNA into fragments and produce unique patterns between the samples. Another cause of pattern similarity may have been due to the specificity of the PCR primers. Although these primers were designed to amplify the 16S rRNA gene from all bacteria, in reality they may have selectively amplified only a few sequences. These results led us to explore higher resolution techniques such as DGGE.

NO_3^- mediated microbial community changes. The DGGE and sequence analysis successfully identified differences between the microcosm trials. Nitrate appeared to cause more of a community change than BTEX amendment, even in the microcosms where TEX was actively degraded. This result is supported by the identification of the β-Proteobacteria sequences that were consistently found in the BTEX+NO_3^- and NO_3^--amended microcosms, but not in the BTEX only trials. These sequences were closely related to organisms such as *Zooglea*, which are facultative anaerobes that can grow as denitrifiers. One related sequence (B 98(B)) was confirmed in the BTEX only sample, however this band was not detected in the BTEX only trials after day 147 (**Fig. 12 & 13**). Bands E and A may also been

detected in the BTEX only until days 70 and 98, respectively, but these were not confirmed with sequencing. The similarities between the communities in the BTEX + NO_3^- and NO_3^- amended microcosms is consistent with the pattern of nitrate consumption seen in the microcosm chemical data. Nitrate-reducing organisms that can degrade TEX may also be able to catabolize a range of electron donor sources. These data suggest that NO_3^- (or TEA availability) was a major force in microbial population selection.

Most of the cultured and identified NO_3^--reducing, alkylbenzene degrading organisms described in the literature have belonged to either the *Azoarcus* or *Thauera* groups within the β-Proteobacteria (Anders et al., 1995; Zhou et al., 1995; Song et al., 1999). Neither of these groups was represented in our analysis of the microcosm community so far. The distribution of alkylbenzene degrading activity in NO_e^--reducing organisms is largely unknown. Our results, however, suggest that alkylbenzene degradation in the NO_3^- amended microcosms could have been mediated by several other groups of organisms. Because these groups were also detected in the microcosms not amended with BTEX, these organisms can also use other carbon and electron donor sources. More specific primer sets can be used to investigate the presence of known alkylbenzene degraders in the microcosms and in the field can.

The samples used for the DGGE analysis, were taken after toluene and *o*-xylene had completely degraded and ethylbenzene and *m,p*-xylenes were slowly degrading. It is interesting that no major differences were identified over time in the microcosms as ethylbenzene and *m,p*-xylenes degraded. This may have been expected, however, because the degradation mechanisms of these compounds are similar (Evans et al., 1991; Beller and Spormann, 1997; Wilson and Bouwer, 1997; Rabus and Heider, 1998; Krieger et al., 1999). It is possible that a specific population of TEX degraders was enhanced in these microcosms, and they consumed TEX in the most beneficial (or easiest) order. However, because similar organisms were identified in the microcosms that were not amended with BTEX, the TEX degrading population must have been enhanced by alternative carbon sources.

The two sequenced organisms that may have been found in all of the microcosm trials were closely related to *Acidobacterium. A. capsulatum* is the only cultured representative of this bacterial phyla. However, related sequences have been identified in many different environments including soil, freshwater and marine sediments, and hydrothermal vents (Barns et al., 1999). Similar to many other microbial community studies, this project identified several sequences that were not closely related to any cultured representative. NB 364 (E & F) and N 364 (E) were related to *Chlorobium vibrioforme,* a Green sulfur bacteria, and a sequence identified from a methanogenic tricholorbenzene (TCE) degrading community in a fluidized bead reactor (**Fig. 13**) (von Wintzingerode et al., 1999). Sequence B 364 (G) was loosely related to the *Cytophagales* division and a related clone (WCHB1-53) that was isolated from a hydrocarbon and TCE contaminated aquifer (Dojka et al., 1998). Thus, the organisms identified from the microcosms represent a diverse set of phylogenetic linkages and potential physiologic properties even after they have been incubated with NO_3^- and/or BTEX for a year.

Microcosm alteration of the field microbial community. Another important finding of the DGGE analysis was the distinct differences in the microbial community band patterns between the field and the microcosm trials. Only a few bands from the field sample DGGE were visible. Since the PCR generated approximately the same amount of product from field and microcosms, this may indicate that the field samples had greater diversity than the microcosm samples, and not enough of any single sequence was represented in the product to produce a clear band. We were not able to determine if the organisms enhanced by NO_3^- in the microcosms were also those responsible for the TEX degradation. In the field, where TEX degradation does occur, other organisms may be responsible for hydrocarbon degradation and it is unknown how NO_3^- amendment would affect these processes. Further studies combining the *in situ* microcosms and the DGGE/sequencing approach, could be used to explore how NO_3^- changes the microbial community of the sediments under field conditions.

VIII. SUMMARY AND CONCLUSIONS

Results of this study suggest that NO_3^- can enhance overall BTEX biodegradation in the hydrocarbon-contaminated aquifer at Fort McCoy, WI, but it should be used in combination with other electron acceptors, either under intrinsic or enhanced conditions, to treat benzene. Excess NO_3^- losses due to oxidation of other electron donors was measured in both the field and laboratory experiments, indicating that reliance on standard stoichiometric relationships should be reevaluated. Although intrinsic biodegradation under Fe^{+3}-reducing conditions is currently occurring in the field, results of the microcosm experiment and from measurements of Fe^{+3} in sediment extracts indicate that the amount of bioavailable Fe^{+3} left in the sediment is depleted with respect to uncontaminated sediments. This has important implications for assessments of intrinsic biodegradation of BTEX because although the other electron acceptors are either brought in with groundwater or through recharge (DO, NO_3, SO_4) or can be considered unlimited (CO_2), Fe^{+3} reserves are limited; after many years of contamination, the extent of iron reduction contributing to BTEX oxidation will decrease. These results suggest that more research in intrinsic biodegradation should focus on the amount as well as the bioavailability of Fe^{+3} in BTEX-contaminated aquifers.

Comparison of biodegradation rates, patterns, and lag times between the field, ISMs, and laboratory experiments demonstrate that the rates were slightly higher in the field than in the lab; differences were less than a factor of 2 for toluene and ethylbenzene, and 4 for *m,p*-xylenes. The overall pattern of biodegradation was similar, but the lag times were different. In the field, toluene, ethylbenzene, and *m,p*-xylenes degraded within 10 days while in the microcosm experiment and in the initial dosing of the enrichment cultures, there was a distinct order of degradation, with toluene degrading first, followed by *o*-xylene, *m,p*-xylenes, and ethylbenzene. Overall the ISMs also had a distinct pattern of TEX degradation, different from the lab microcosms, and had highly variable lag times. The differences in the lag times are most likely related to different competing electron donors in the field and in the lab, spatial heterogeneity in the field, and the re-establishment of acclimated microbial populations in the laboratory.

The molecular analysis of the microcosm sediments indicated that distinct populations were enhanced by NO_3^- amendment. Because many of the same populations were identified in the microcosms treated with NO_3^- but not BTEX, these populations may or may not be directly involved with the TEX degradation seen in the microcosms, but are most likely involved with the oxidation of other electron donor sources. No organisms related to known Fe^{+3}-reducers have been identified from the microcosms, however further analysis with more specific probes (e.g. for the *Geobacter*) may reveal their presence.

As was expected, the microcosm community was distinct from that found in the field. The NO_3^- and incubation conditions probably enhanced a sub-community from the field. The importance of these organisms in the degradation of TEX under enhanced and intrinsic field conditions is unknown. However, the changes in the microbial community probably caused the different degradation rates seen in the field and microcosm experiments. Future experiments combining the ISMs with DGGE and sequencing may reveal which microbial groups are most impacted by treatment methods and how this affects TEX degradation.

X. Figure

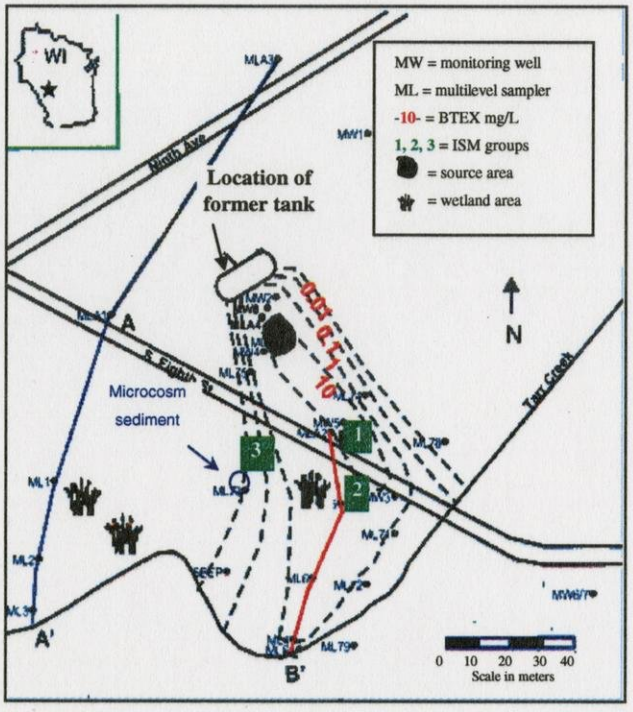


Fig. 1. Map of Fort McCoy, WI. BTEX concentrations for the plume are indicated by the dashed contours. The location of the microcosm sampling site and the locations of the ISMs are indicated. Section A-A' is the non-contaminated transect and section B-B' is the contaminated transect used for the tracer experiment.

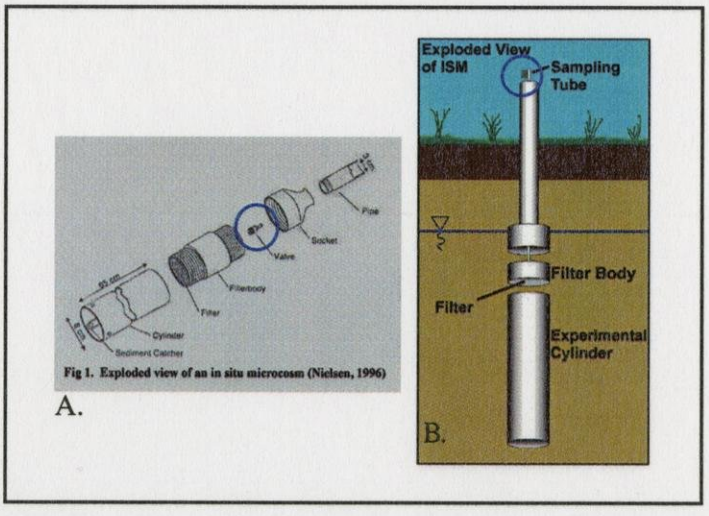


Fig. 2. Design of *in situ* microcosms. Panel A. Main ISM components in the original design. The valve is within the ISM itself. Panel B. Diagram of the ISM components in the improved design. The sampling valve is now at the top of the sampling tube, above the ground surface.

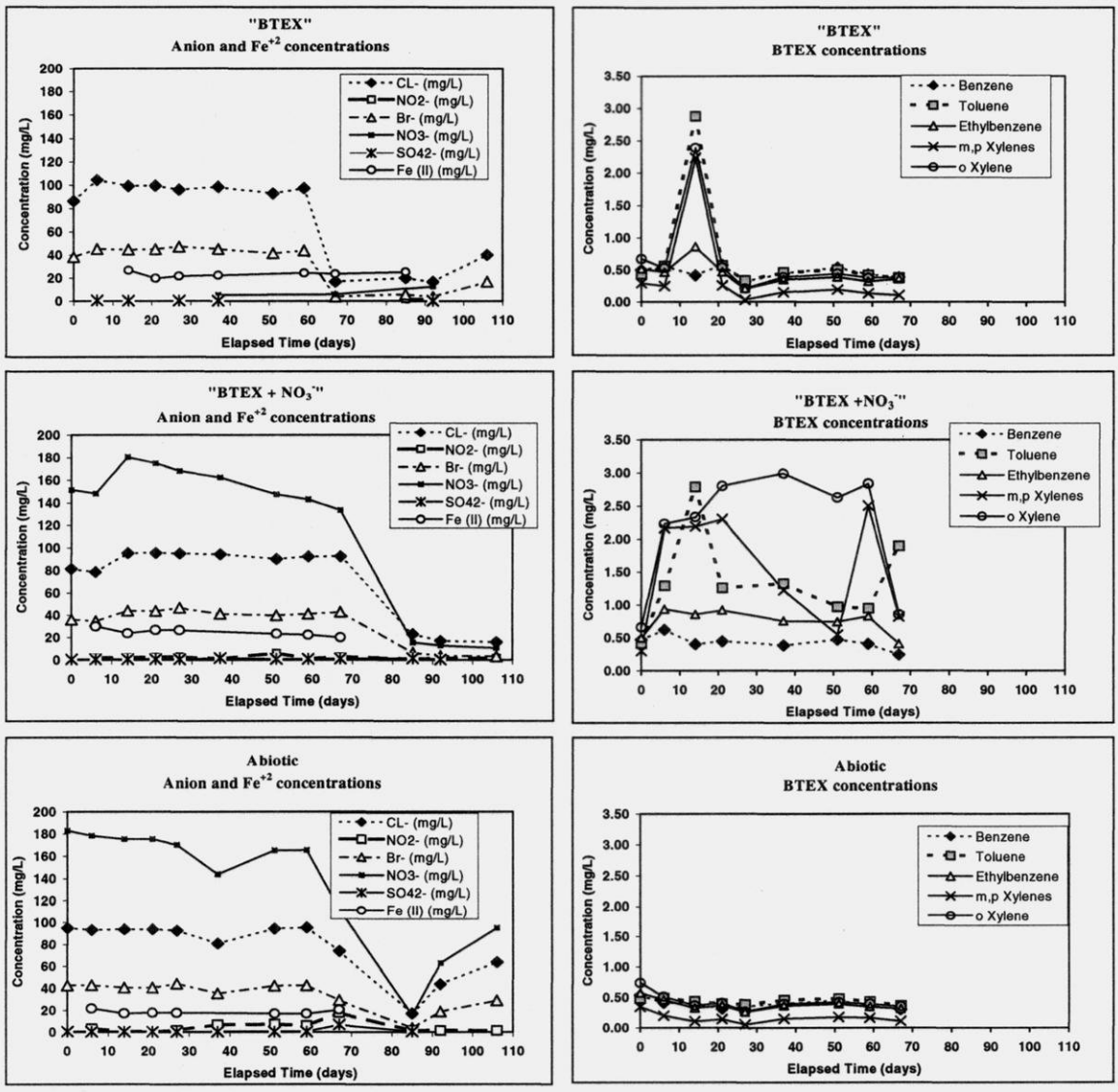


Fig. 3. Group 1 ISMs. Concentrations of BTEX, anions, and Fe^{+2} in ISMs located at Group 1. ISM was located between 6 and 8 feet below ground surface. The data used to construct these graphs are also available in Table 1.

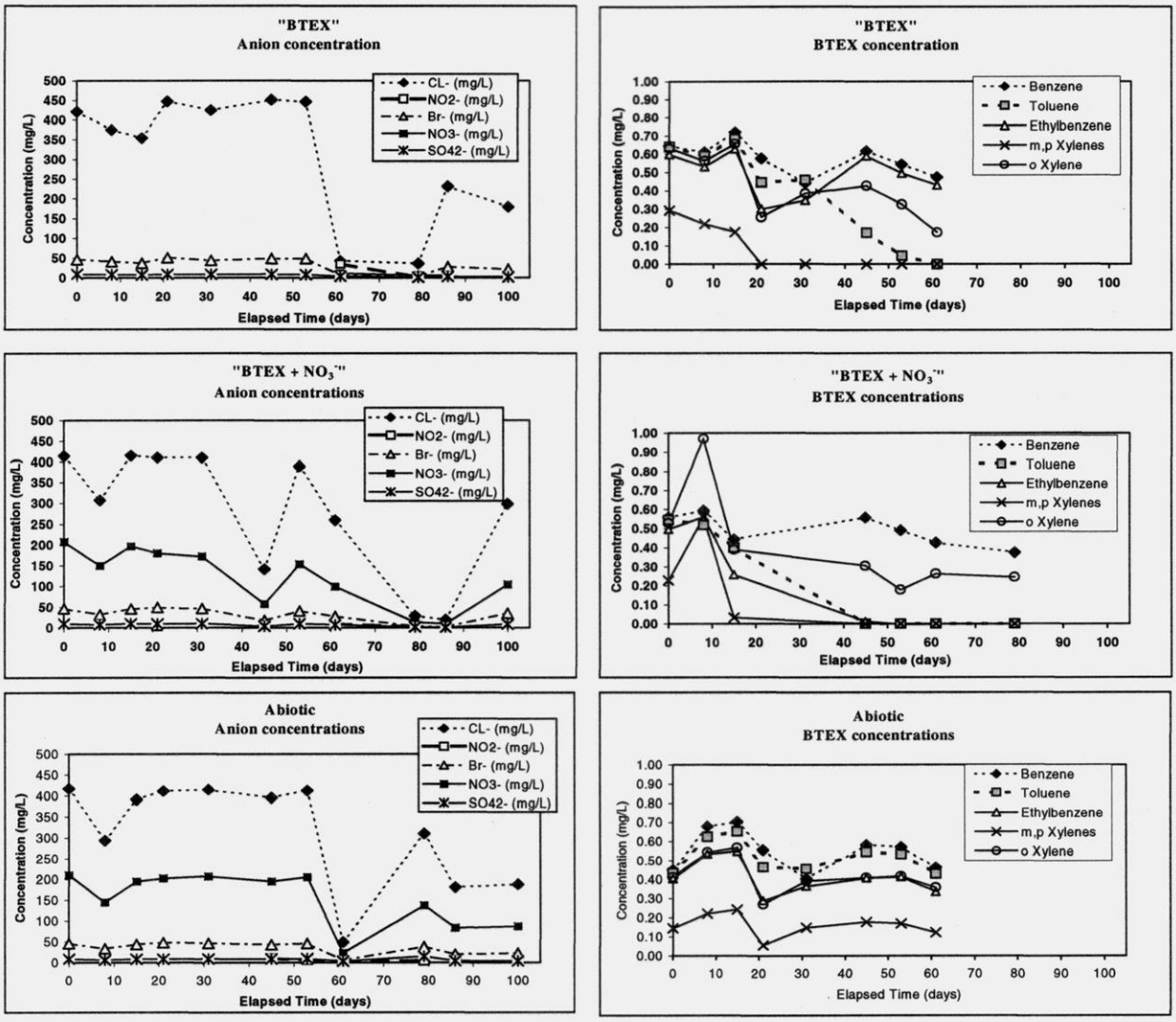


Fig. 4. Group 2 ISMs. Concentrations of BTEX and anions in ISMs located at Group 2. ISM was located between 5.5 to 7.5 feet below ground surface. The data used to construct these graphs are also available in Table 2.

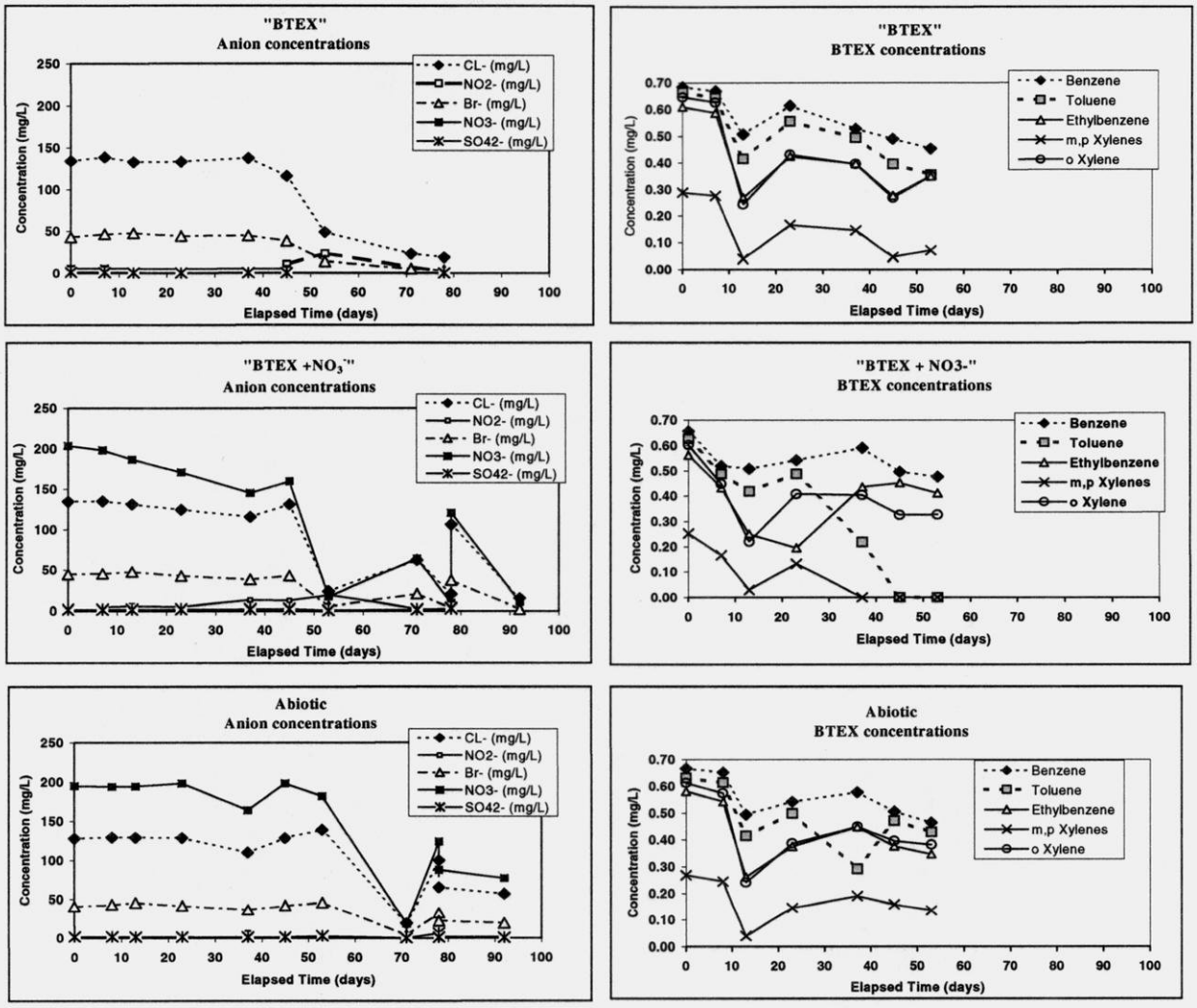


Fig. 5. Group 3 ISMs. Concentrations of BTEX and anions in ISMs located at Group 3. ISM was located between 5.5 to 7.5 feet below ground surface. The data used to construct these graphs are also available in Table 3.

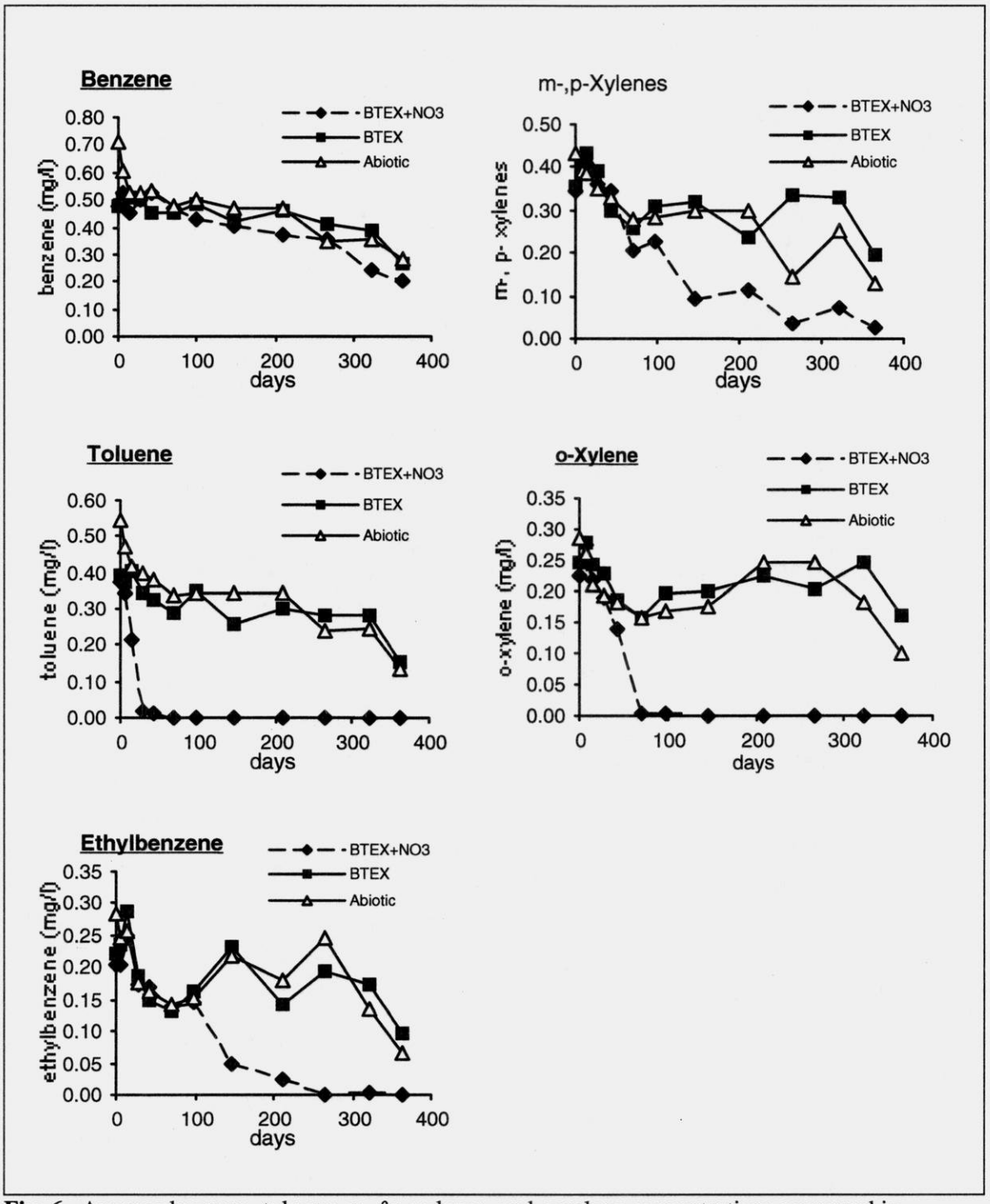


Fig. 6. Average benzene, toluene, *m-&p*-xylenes, and *o*-xylene concentrations measured in microcosms (with and without NO_3^-) and abiotic controls. Error bars represent standard deviations of triplicate measurements.

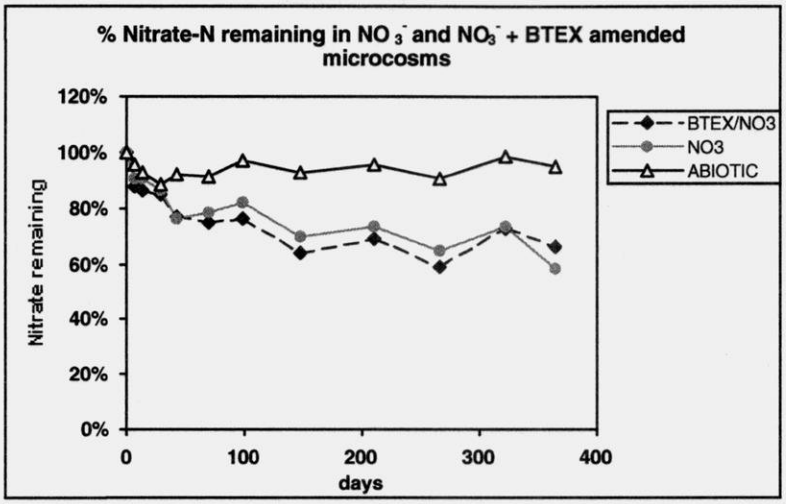


Fig. 7 Average percent of NO_3^- remaining in microcosms amended with NO_3^-, with and without BTEX.

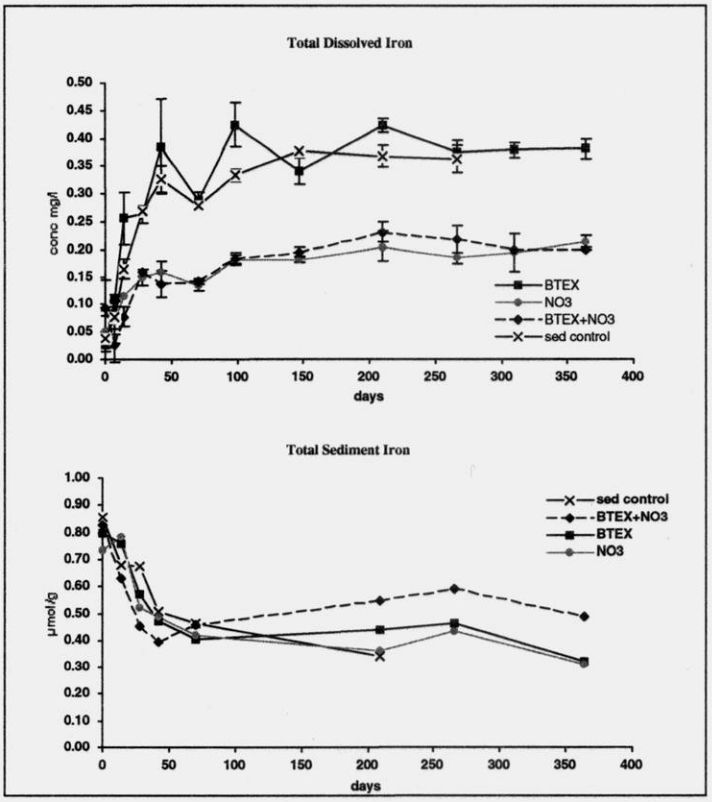
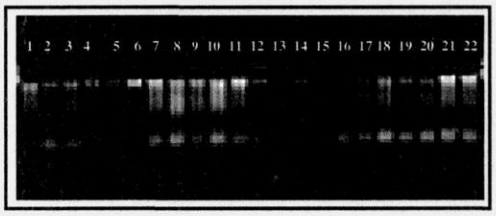
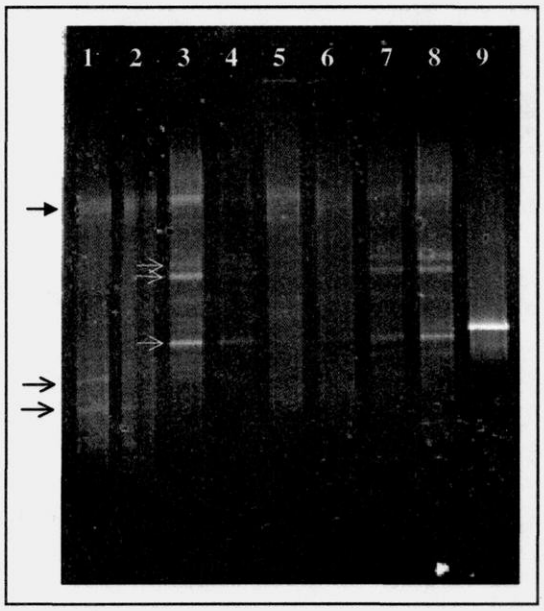


Fig. 8 Average total dissolved and sediment Fe concentrations in microcosms. Abiotic controls were not analyzed for iron because of colorimetric interference. Error bars represent standard deviations of triplicate measurements.



Lane	Sample/treatment (days)	µl for PCR
1	F1	1
2	F2	1.5
3	F2[b]	1.5
4	BTEX/NO_3^- (42)	1.5
5	BTEX/NO_3^- (70)	2
6	BTEX/NO_3^- (98)	1
7	BTEX/NO_3^- (147)	1
8	BTEX/NO_3^- (210)	1
9	BTEX/NO_3^- (266)	1
10	BTEX/NO_3^- (322)	1
11	BTEX/NO_3^- (364)	1
12	BTEX (42)	1.5
13	BTEX (70)	4
14	BTEX (70)[b]	2
15	BTEX (95)	2.5
16	BTEX (147)	2.5
17	BTEX (210)	1.5
18	BTEX (266)	1
19	BTEX (322)	1.5
20	BTEX (364)	1.5
21	NO_3^- (322)	1
22	NO_3^- (364)	1

Fig. 9. DNA extracted from microcosms and electrophoresed in a 1% agarose gel. Sample F1 was extracted from the field sediment used to construct the microcosms. Sample F2 was from a second, more contaminated, field site.



Lane	Sample/treatment (days)
1	F1
2	F2
3	BTEX/NO_3^- (322)
4	BTEX/NO_3^- (364)
5	BTEX (322)
6	BTEX (364)
7	NO_3^- (322)
8	NO_3^- (364)
9	*Desulfovibrio desulfuricans*

Fig. 10. Microcosm DNA samples from the last two sampling points of each trial were amplified and separated on a 40-80% denaturing gradient gel. White arrows indicate potential band differences between the BTEX/NO_3^- and the BTEX trials. Black arrows indicate bands of interest in the field samples.

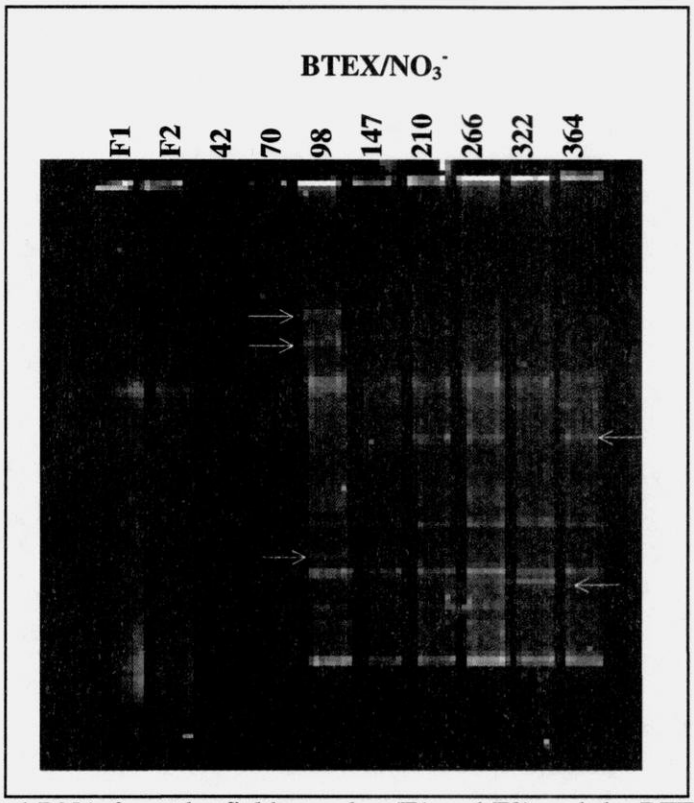


Fig. 11. Amplified DNA from the field samples (F1 and F2) and the BTEX/NO_3^- microcosms were separated on a 40-80% denatuing gradient. The white arrows indicate bands that are only appear at some of the microcosm time points.

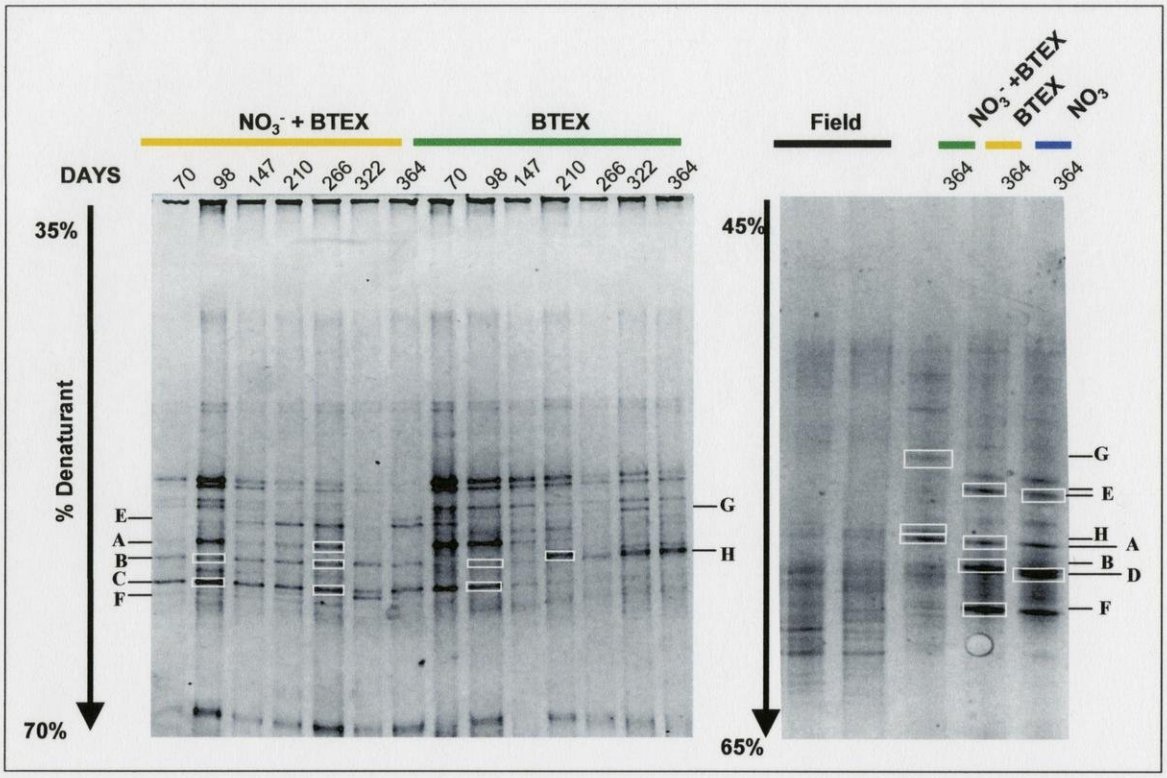


Fig. 12. DGGE gels (negative image) of DNA extracted and amplified with bacterial primers from microcosms amended with NO_3^-, BTEX, or NO_3^- + BTEX. Bands marked with a white box were extracted from the gel, and sequenced.

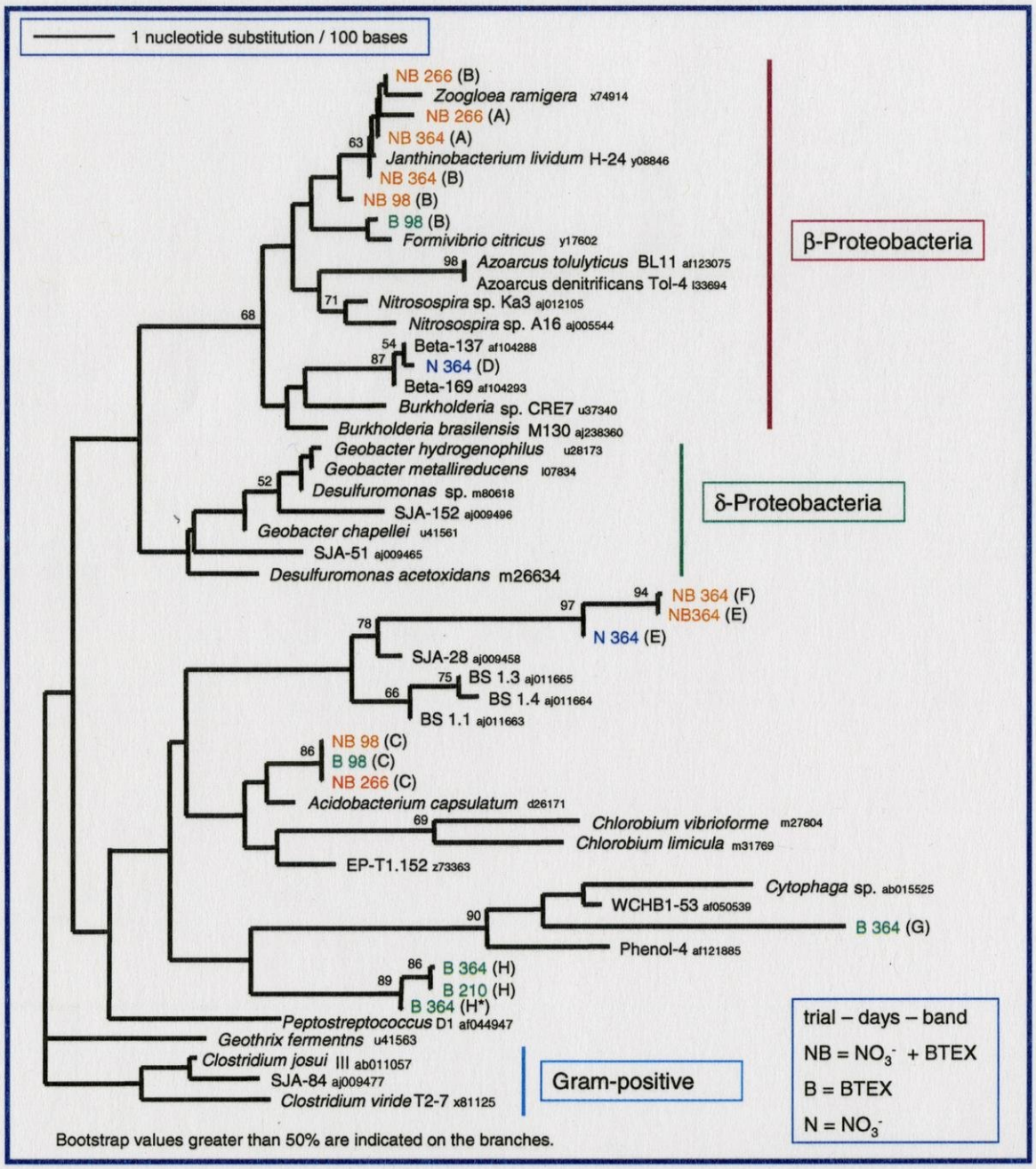


Fig. 13 Phylogenetic tree of selected DGGE bands from Fig . 12 and selected sequences from the Genebank database. Ascension numbers for database sequences are provided. The neighbor-joining method was used to construct the tree in Clustal X. 1000 bootstrap replicates were conducted also with Clustal X, bootstrap values greater than 50% are provided on the branches.

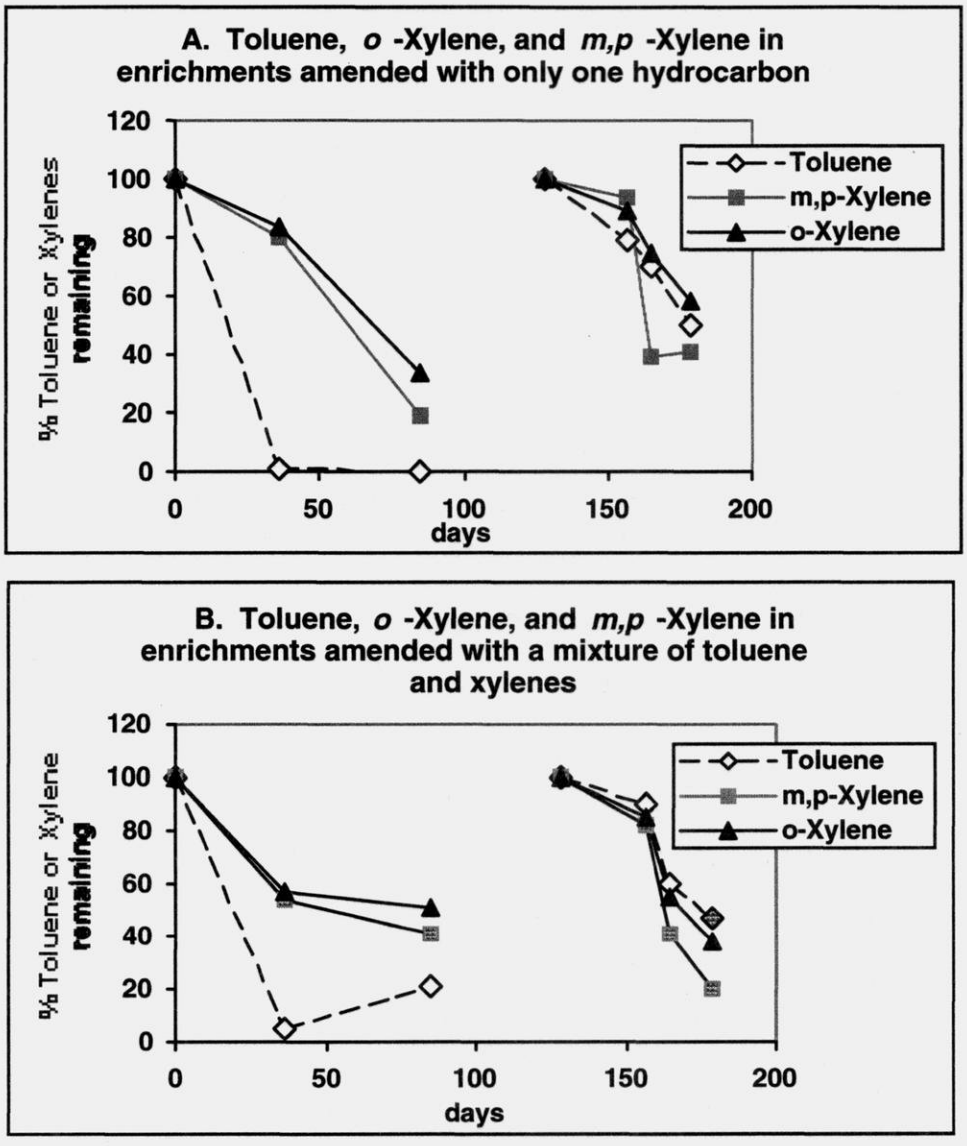


Fig. 14. Average percent toluene, *m*-&*p*-xylenes, and *o*-xylene remaining in enrichment cultures over time. Panel A. Individual compounds, Panel B. Mixed compounds.

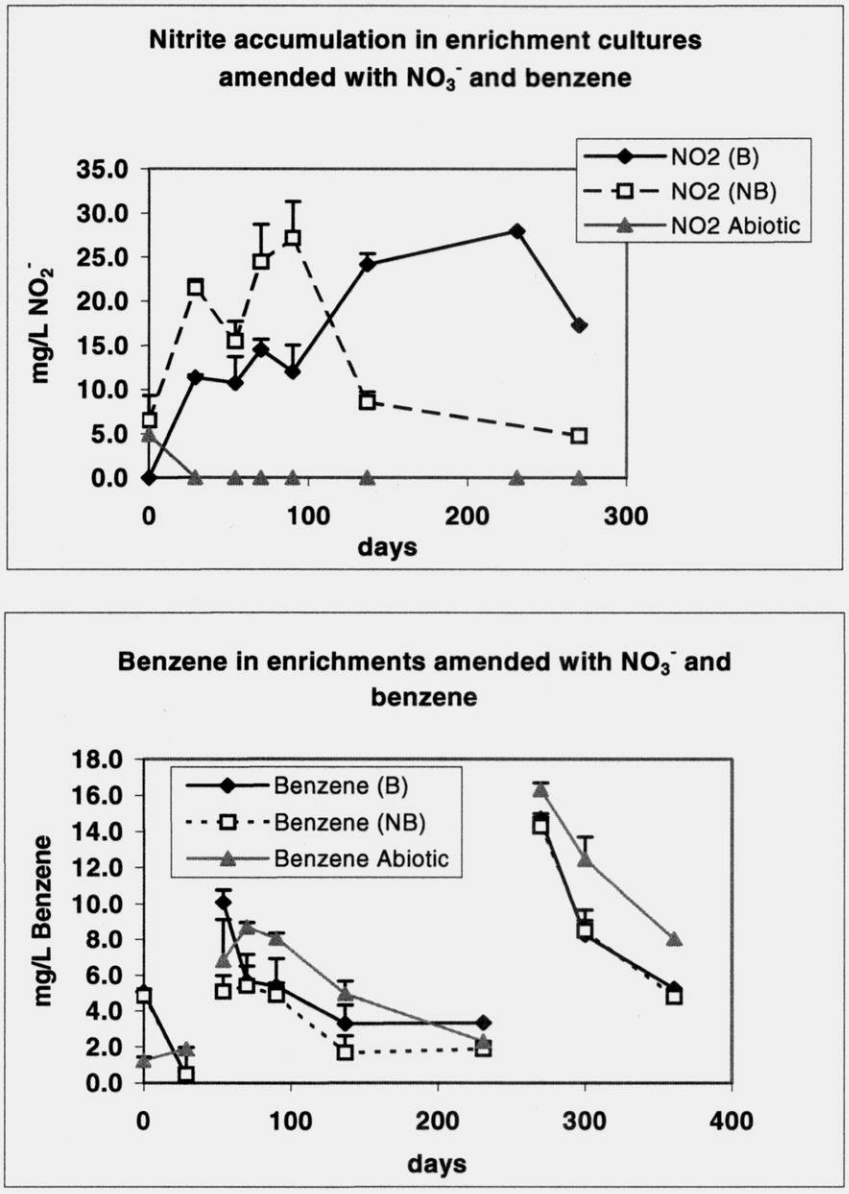


Fig. 15. Concentrations of NO_2^- and benzene in enrichment cultures amended with NO_3^- and benzene. Cultures were inoculated with sediment from the microcosms amended with BTEX only (B) or BTEX + NO_3^- (NB). Abiotic enrichments were autoclaved and amended with sodium azide (0.1%). The enrichments that had transient NO_2^- accumulation also had oxidized resazurine. Error bars indicate one standard deviation for measurement of three replicated enrichments. For clarity, only the positive error bars are shown.

XI. TABLES

Table 1. Chemical data for Group 1 ISMs.

			Ion chromatography (mg/L)						Gas Chromatography (mg/L)					
	Sample day	cumulative volume sampled (mL)	CL^-	NO_2^-	Br^-	NO_3^-	SO_4^{-2}	Fe^{+2} colorimetric(mg/L)	Benzene	Toluene	Ethylbenzene	*m,p*-Xylene	*o*-Xxylene	Total BTEX
Multilevel	0	N.A.	91.1	60.2			0.7	34.2	0.57	0.43	0.52	0.24	0.56	2.32
BTEX amended	0	20	86.5		38.2				0.50	0.43	0.52	0.29	0.67	2.41
	6	55	104.4		45.2		0.7	26.9	0.56	0.56	0.47	0.25	0.53	2.36
	14	73	99.2		44.6		0.5	19.9	0.40	0.42	0.33	0.13	0.38	1.67
	14	88							0.42	2.88	0.86	2.23	2.39	8.78
	21	105	99.5		45.0			21.7	0.58	0.57	0.47	0.26	0.54	2.42
	27	122	96.0		47.1		0.4	22.5	0.32	0.33	0.21	0.03	0.21	1.10
	37	139	98.4		45.1	5.5	0.6		0.44	0.46	0.35	0.15	0.38	1.78
	51	156	93.0		41.6			24.6	0.54	0.51	0.39	0.20	0.44	2.08
	69	173	97.3		43.9			23.7	0.43	0.42	0.32	0.14	0.38	1.69
	67	190	16.9		4.0	5.8		25.1	0.39	0.38	0.36	0.11	0.38	1.62
	67	207	36.4	1.0	8.6	10.9	2.7	26.0	0.33	0.33	0.30	0.09	0.34	1.39
	85	224	19.9	1.7	5.7									
	92	241	16.8	1.6	3.7	12.9	0.4							
	106	258	40.2		16.9									
BTEX + NO_3^- amended	0	20	81.5		36.1	151.3	0.6		0.47	0.41	0.50	0.30	0.66	2.33
	6	55	78.7	2.0	34.7	148.0	0.5	30.1	0.62	1.29	0.94	2.17	2.23	7.25
	14	73	95.4	2.4	44.1	180.6	0.6	23.8	0.40	2.79	0.86	2.19	2.33	8.57
	21	88	95.5	2.7	43.7	175.0	0.6	26.7	0.45	1.26	0.92	2.30	2.80	7.73
	27	105	94.9	2.9	46.6	168.1	0.6	26.5						
	37	122	94.5	1.3	41.3	162.5	1.8		0.39	1.33	0.76	1.23	2.99	6.69
	51	139	90.2	6.0	39.9	147.3	0.9	23.4	0.47	0.97	0.75	0.55	2.63	5.37
	69	156	92.4	1.4	41.2	142.9	1.0	22.4	0.41	0.95	0.83	2.50	2.84	7.53
	67	173	21.7	43.4	4.2	15.2	3.0	20.1	0.09	0.96	0.17	0.80	0.86	2.89
	67	190	92.4	2.9	42.9	133.5	1.1	19.0	0.25	1.90	0.41	0.82	1.64	5.01
	85	207	23.1	0.9	6.3	15.5	1.6							
	92	224	16.8	1.6	3.7	12.9	0.4							
	106	241	15.8	1.7	3.1	10.7								
Abiotic	0	20	94.9		42.7	183.1	0.7		0.53	0.48	0.57	0.34	0.74	2.66
	6	55	93.2	3.4	42.8	178.6	0.5	21.8	0.41	0.49	0.46	0.20	0.51	2.07
	14	73	93.6	0.7	40.6	175.4	0.5	17.1	0.39	0.43	0.33	0.10	0.37	1.62
	21	88	93.6	0.7	40.6	175.4	0.5	17.9	0.31	0.39	0.37	0.14	0.40	1.62
	27	105	92.7	2.0	44.4	170.4	0.4	17.8	0.35	0.38	0.26	0.06	0.27	1.32
	37	122	80.8	7.0	35.4	143.8	0.6		0.40	0.46	0.36	0.15	0.39	1.76
	51	139	94.4	7.4	42.2	165.4	0.6	17.1	0.45	0.48	0.39	0.18	0.42	1.92
	69	156	95.3	6.0	42.7	165.7	0.6	16.8	0.36	0.43	0.35	0.16	0.39	1.70
	67	173	73.9	17.7	29.1	112.1	6.8	20.5	0.30	0.36	0.31	0.11	0.35	1.44
	67	190	44.3		17.4	67.7		20.4	0.29	0.34	0.27	0.10	0.33	1.33
	85	207	17.2	1.6	3.8	15.8	0.6							
	92	224	43.9	1.7	18.9	63.1								
	106	241	63.9	1.5	29.0	95.0								

Table 2. Chemical data for Group 2 ISMs.

			Ion Chromatography (mg/L)					Gas Chromatography (mg/L)					
	Sample day	cumulative volume sampled (mL)	CL^-	NO_2^-	Br^-	NO_3^-	SO_4^{-2}	Benzene	Toluene	Ethylbenzene	*m,p*-Xylene	*o*-Xylene	Total BTEX
Multilevel	0	N.A.	418.1	47.2			59.8	0.54	0.42	0.50	0.23	0.54	2.22
BTEX amended	0	10	421.1		46.8	3.4	9.6	0.64	0.64	0.60	0.29	0.63	2.81
	8	38	375.0		41.6		8.5	0.61	0.59	0.53	0.22	0.56	2.51
	15	53	354.2		37.8		7.6	0.72	0.68	0.63	0.17	0.66	2.86
	21	70	447.3		51.5		9.3	0.58	0.45	0.30	0.00	0.26	1.53
	31	87	424.6		44.4		9.0	0.44	0.46	0.35	0.00	0.38	1.78
	45	104	450.9		49.0		9.2	0.62	0.17	0.59	0.00	0.43	1.65
	53	121	446.7		48.9		8.6	0.54	0.05	0.50	0.00	0.33	1.26
	61	138	43.2	35.4	6.1	11.4	4.4	0.47	0.00	0.43	0.00	0.17	0.92
	79	155	36.7	1.7	4.4	6.4	1.2						
	86	172	231.2		28.4	6.3	3.4						
	100	189	179.6		21.8		2.1						
BTEX + NO_3^- amended	0	10	414.0		46.3	207.2	9.4	0.56	0.54	0.50	0.23	0.53	2.36
	8	38	307.3		32.0	149.7	7.3	0.59	0.52	0.56	0.56	0.97	3.2
	15	53	414.9		46.0	196.7	9.7	0.44	0.40	0.26	0.03	0.39	1.53
	21	70	410.1	4.9	49.0	180.1	9.5	0.36	0.11	0.02	0.00	0.15	0.60
	31	87	410.0		46.0	172.1	9.7	0.00	0.00	0.00	0.00	0.00	0.00
	45	104	141.6		17.5	57.8	3.7	0.56	0.00	0.01	0.00	0.31	0.70
	53	121	389.7		40.4	15309	9.6	0.49	0.00	0.00	0.00	0.18	0.49
	61	138	259.6	8.7	27.5	99.2	6.9	0.43	0.00	0.00	0.00	0.26	0.51
	61	155	31.3	43.7	2.3	12.5	2.6	0.38	0.00	0.00	0.00	0.25	0.45
	79	172	28.4	1.9	3.0	12.8	1.4						
	86	189	19.3		1.3	9.2	0.4						
	100	206	298.4		35.0	103.9	7.9						
Abiotic	0	10	417.7		46.0	209.6	9.1	0.45	0.43	0.40	0.14	0.41	1.84
	8	38	293.8		34.1	144.9	6.5	0.68	0.63	0.53	0.22	0.54	2.60
	15	53	391.7		44.3	194.6	8.7	0.70	0.65	0.55	0.24	0.57	2.72
	21	70	411.8		48.7	202.0	8.9	0.56	0.46	0.29	0.06	0.27	1.64
	31	87	414.9		47.3	206.8	9.6	0.40	0.46	0.36	0.15	0.39	1.76
	45	104	395.8	9.6	43.5	194.4	9.1	0.58	0.55	0.41	0.18	0.41	2.12
	53	121	412.8	6.8	46.1	204.5	9.7	0.57	0.53	0.41	0.17	0.42	2.10
	61	138	49.7	3.2	6.1	23.1	3.6	0.46	0.43	0.34	0.12	0.36	1.71
	79	155	310.1	5.0	38.4	137.3	15.2						
	86	172	180.7		20.4	83.8	4.3						
	100	189	187.6		22.8	87.6	4.3						

Table 3. Chemical data for Group 3 ISMs.

			Ion Chromatography (mg/L)					Gas Chromatography (mg/L)					
	Sample day	cumulative volume sampled (mL)	CL^-	NO_2^-	Br^-	NO_3^-	SO_4^{-2}	Benzene	Toluene	Ethylbenzene	*m,p*-Xylene	*o*-Xylene	Total BTEX
Multilevel	0	N.A.	147.9	27.4			2.61	0.03	0.00	0.04	0.00	0.01	0.07
BTEX amended	0	30	134.3		42.9	5.9	1.5	0.68	0.66	0.61	0.29	0.65	2.89
	7	50	138.7		46.4	6.0	1.1	0.67	0.64	0.59	0.28	0.63	2.80
	13	67	132.9		47.7		0.7	0.51	0.42	0.27	0.04	0.24	1.47
	23	84	133.4		44.3		0.6	0.61	0.56	0.42	0.17	0.43	2.19
	37	101	137.7		44.8		0.5	0.53	0.50	0.40	0.15	0.40	1.96
	45	118	116.4	10.4	38.3	5.5	0.6	0.49	0.40	0.28	0.05	0.27	1.48
	53	135	48.8	23.5	14.4			0.45	0.36	0.35	0.07	0.35	1.58
	71	152	23.1		5.3								
	78	169	18.7	1.6	3.8		0.3						
	78	186	70.5		23.7	6.4	0.6						
	92	203	15.1		1.7								
BTEX + NO_3^- amended	0	30	134.7		44.7	203.6	1.5	0.33	0.28	0.22	0.02	0.23	1.07
	7	50	135.0	4.3	45.3	198.3	1.5	0.52	0.49	0.43	0.17	0.45	2.06
	13	67	131.2	5.7	47.5	186.8	1.6	0.51	0.42	0.25	0.03	0.22	1.43
	23	84	124.6	4.9	42.8	170.9	1.8	0.54	0.49	0.20	0.13	0.41	1.77
	37	101	115.8	13.1	38.3	145.5	2.1	0.59	0.22	0.44	0.00	0.40	1.60
	45	118	131.4	12.4	43.0	159.8	2.5	0.50	0.00	0.45	0.00	0.33	1.10
	53	135	23.7	19.5	4.7	17.2	0.6	0.48	0.00	0.41	0.00	0.33	1.04
	71	152	62.4	2.2	20.4	64.0	1.5						
	78	169	20.5	1.5	2.8	9.3	2.5						
	78	186	106.1		37.2	120.7	3.0						
	92	203	15.2		1.9	10.7							
Abiotic	0	30	127.9		40.8	217.6	1.8	0.67	0.63	0.58	0.27	0.61	2.76
	7	50	129.2		43.3	195.0	1.7	0.65	0.61	0.54	0.25	0.57	2.63
	13	67	129.1		45.8	194.1	1.6	0.49	0.42	0.26	0.04	0.24	1.45
	23	84	128.7		41.8	194.5	1.6	0.54	0.50	0.38	0.15	0.39	1.95
	37	101	110.1	7.0	36.6	198.3	1.4	0.58	0.29	0.45	0.19	0.45	1.96
	45	118	128.6		42.1	163.8	1.7	0.51	0.47	0.38	0.16	0.40	1.91
	53	135	139.2	3.3	46.2	198.6	2.9	0.46	0.43	0.35	0.14	0.38	1.76
	71	152	19.1		3.9	182.0	0.3						
	78	169	99.8	3.0	32.4	18.8	6.2						
	78	186	65.2	1.6	21.9	123.9	1.2						
	92	203	56.8	1.6	18.9	87.7	0.8						

Table 4. Dissolved oxygen concentrations in microcosms

DO µg/l	day 98	day 144	day 210	day 266
BTEX+NO_3	<100	40	40	30 to 40
NO_3	<100	20	20	20
BTEX	<50	<10	<10	<10
sediment control	<50	<10	<10	<10

Table 5. Summary of calculated degradation rate constants at Fort McCoy.

Compound		Group 1		Group 2		Group 3		NO_3^- amended Field Tracer Tests	Lab Microcosms	
		BTEX	BTEX + NO_3^-	BTEX	BTEX + NO_3^-	BTEX	BTEX + NO_3^-	Intermediate	BTEX	BTEX + NO_3^-
toluene	degradation	no	?[1]	yes	yes	no	yes	yes	no	yes
	lag time (days)			32 to 40	15 to 20		37 to 45	10		7
	rate constant (day^{-1})			0.08	0.07		0.2	0.019		0.028
ethylbenzene	degradation	no	?[1]	no	yes	no	?[5]	yes	no	yes
	lag time (days)				10			10		98
	rate constant (day^{-1})				0.05			0.12		0.008
m,p-xylene	degradation	no	?[1]	yes	yes	yes	yes	yes	no	yes
	lag time (days)			10 to 15	10	7	35	10		42
	rate constant (day^{-1})			0.2	0.2	0.01	0.2	0.011		0.004
o-xylene	degradation	no	?[1]	yes	?[4]	no	yes	?	no	yes
	lag time (days)			32 to 40			37			28
	rate constant (day^{-1})			0.02			0.004			0.007
nitrate	consumption	N.A.	yes	N.A.	yes	N.A.	yes	yes	N.A.	yes
	lag time (days)		0 to 35		10		0	12-17		<7
	rate constant (day^{-1})		0.004		0.004		0.003	0.03 to 0.06		0.001 to 0.003
	Use ratio				25		33	90 to 117		140
sulfate	consumption	N.A.	N.A.	N.A.		yes	increase[3]		N.A.	N.A.
	lag time (days)					0	12			
	rate constant (day^{-1})					0.03	0.008 to 0.009			

notes:
N.A. not applicable due to the absence of this compound at the start of the experiment.
[1] Degradation could not be confirmed due to large fluctuations ifn BTEX concentration between sampling events.
[2] From Scheriber (1999)
[3] Increases in SO_4^{-2} concentrations were attributed to oxidation of SO_4^{-2} under NO_3^--reducing conditions.
[4] Concentrations for sampling events on days 45 and 53 show loss but later samples may have been influenced by dilution in this ISM.
[5] Concentrations for sampling events on days 23 and 37 show loss by concentrations are higher on days 45 and 53.

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XII. APPENDIX

Publications resulting from this project

Schreiber, M.E. (1999) Experimental and modeling approaches to evaluating anaerobic biodegradation of petroleum-contaminated groundwater. Ph.D. dissertation. Department of Geology, University of Wisconsin-Madison, Madison, WI.

Taglia, P.J. (2001) Using *in situ* microcosms to evaluate the spatial heterogeneity of BTEX biodegradation under nitrate-reducing conditions. M.S. Thesis. Department of Geology, University of Wisconsin-Madison, Madison, WI.

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Presentations

Schreiber, M.E., J.M. Bahr, M.D. Zwolinski, Y. Shi, W.J. Hickey, and K.A. Brownell. 1997. Field and laboratory studies of BTEX bioremediation under denitrifying conditions. Proceedings of the Fourth International Symposium on *In Situ* and On-Site Bioremediation, April 28-May 1, 1997, Vol. 5:13 17.

Zwolinski, M.D., M.E. Schreiber, W.J. Hickey and J.M. Bahr. 2000. Analysis of microbiological and geochemical processes degradation of aromatic hydrocarbons in anaerobic aquifers. *In* Abstr. 21St North American Annual Mtg. of Society of Environmental Toxicology and Chemistry. Society of Environ. Toxicol. Chem., Pensacola, FL.