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Biotransformation of Gasoline-Contaminated Groundwater Under Mixed Electron-Acceptor Conditions

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Biotransformation of Gasoline-Contaminated Groundwater Under Mixed Electron-Acceptor Conditions

by

Jeffrey R. Barbaro, Barbara J. Butler, and James F. Barker
University of Waterloo
Waterloo, Ontario, CANADA

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Project Officer
Stephen R. Hutchins
Subsurface Protection and Remediation Division
National Risk Management Research Laboratory
Ada, OK 74820

National Risk Management Research Laboratory
Office of Research and Development
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Notice

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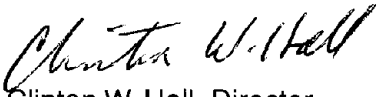
All research projects funded by the U.S. EPA are required to participate in the Agency Quality Assurance Program. This research was conducted under an approved Quality Assurance Project Plan (June, 1993) and Field Sampling Plan (April, 1995). For some of the laboratory and field activities, modified experimental objectives, unanticipated difficulties encountered during set up, and equipment availability necessitated deviations from procedures specified in these documents. Actual procedures used in the laboratory and field experiments performed in this study are described in detail in Chapter 3 and Appendix C, and are consistent with common research practices as reported in the peer-reviewed literature. Information on the Plans and documentation of the quality assurance activities and results are available from the Principal Investigator.

Foreword

The U.S. Environmental Protection Agency is charged by Congress with protecting the Nation's land, air, and water resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet these mandates, EPA's research program is providing data and technical support for solving environmental problems today and building a science knowledge base necessary to manage our ecological resources wisely, understand how pollutants affect our health, and prevent or reduce environmental risks in the future.

The National Risk Management Research Laboratory (NRMRL) is the Agency's center for investigation of technological and management approaches for reducing risks from threats to human health and the environment. The focus of the Laboratory's research program is on methods for the prevention and control of pollution to air, land, water, and subsurface resources; protection of water quality in public water systems; remediation of contaminated sites and ground water; and prevention and control of indoor air pollution. The goal of this research effort is to catalyze development and implementation of innovative, cost-effective environmental technologies; develop scientific and engineering information needed by EPA to support regulatory and policy decisions; and provide technical support and information transfer to ensure effective implementation of environmental regulations and strategies.

This project represents a cooperative effort between the University of Waterloo and the Environmental Protection Agency. This report summarizes research conducted using both laboratory batch microcosms and field-scale sheet-piling cells to evaluate whether bioremediation of monoaromatic fuel hydrocarbons can be enhanced using mixed rather than single electron acceptors. The studies focused on nitrate for anaerobic bioremediation and oxygen for aerobic bioremediation, and experiments were designed to test the hypothesis that low levels of oxygen may enhance biodegradation of more recalcitrant compounds (such as benzene) under denitrifying conditions. The findings from this project are directly applicable to the field-scale remediation of subsurface environments contaminated by petroleum hydrocarbons.



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National Risk Management Research Laboratory

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The gasoline used in this study was kindly provided by the American Petroleum Institute, with the assistance of Dr. Bruce Bauman. Dr. Mike Barcelona of the National Center for Integrated Bioremediation Research and Development, University of Michigan, extended the services of his laboratory for the analysis of organic acids in Borden groundwater. Additional analytical work on groundwater and core-extract samples from the Borden field site was performed at NRMRL, as well as the entire ^{14}C mineralization experiment. The contributions and technical support of these organizations are gratefully acknowledged.

EXECUTIVE SUMMARY

Controlled releases of API 91-01 gasoline within steel sheet-piling cells at CFB Borden and laboratory microcosm experiments were used to investigate the biotransformation of the aromatic hydrocarbons benzene, toluene, ethylbenzene, xylene isomers, trimethylbenzene isomers and naphthalene (referred to as BTEXTMB in this report) under mixed NO_3^- / O_2 electron-acceptor conditions. The main objective of the research was to evaluate nitrate-based bioremediation as a remedial technology in a gasoline source area. Dissolved oxygen was added to potentially enhance the mass loss of the soluble compounds that are recalcitrant under denitrifying conditions. The controlled field experiment also provided some insight into the interactions that occur between electron acceptors and soluble organics when a hydrocarbon source area is flushed with electron acceptors to stimulate *in situ* biotransformation.

In laboratory microcosm experiments, the effect of limited (microaerophilic) O_2 was found to depend on the concentrations of aromatic hydrocarbons and other carbon compounds present in the system. When aqueous concentrations of the aromatics were low (10x dilution of gasoline-saturated water), and there were no other sources of labile carbon, the mass of O_2 in a microcosm was fairly large relative to the mass of carbon, and aerobic mass losses were observed. Notably, however, benzene mass losses were typically minimal under these conditions. On the other hand, in gasoline-contaminated microcosms less extensive mass losses were observed, presumably as a result of O_2 consumption by other gasoline hydrocarbons. When aqueous concentrations of the aromatic hydrocarbons were increased to gasoline-saturated levels to reflect field conditions, negligible losses of the aromatics were observed despite rapid consumption of microaerophilic O_2. Under these conditions, the mass of O_2 was probably too low to observe any losses, even if the aromatics were utilized in preference to other gasoline hydrocarbons.

Nitrate utilization under anaerobic conditions was observed in most laboratory experiments, but rates were low relative to O_2 consumption, and losses were limited to toluene, ethylbenzene, and less consistently, *m*-xylene. After *in situ* exposure, NO_3^--reducing activity was not inhibited in gasoline-contaminated aquifer material, but the labile aromatic hydrocarbons were apparently not the preferred substrates in this carbon-rich environment. In contrast, other laboratory experiments with gasoline-contaminated material showed that benzene, toluene, ethylbenzene, *m*-xylene, *p*-xylene, 1,2,4-trimethylbenzene, and naphthalene would degrade at the expense of O_2. Under mixed electron-acceptor conditions, patterns of O_2, and NO_3^-, and aromatic-hydrocarbon concentrations suggested that O_2 and NO_3^- were used sequentially; most aromatic-hydrocarbon biotransformation occurred early, likely at the expense of microaerophilic O_2, with additional losses of toluene and ethylbenzene occurring under denitrifying conditions over longer time periods. When the initial concentrations of the aromatic hydrocarbons were low, there was a beneficial effect of dual electron acceptors: mass losses in microaerophilic / NO_3^- microcosms were more extensive than in comparable microaerophilic and anaerobic, denitrifying microcosms. This showed that under certain conditions the extent of mass loss could be maximized by the presence of these two electron acceptors. However, in experiments with gasoline-contaminated aquifer material, which were more representative of *in situ* conditions, mass losses were either very small or negligible under mixed microaerophilic / NO_3^- conditions.

API 91-01 gasoline was released into two treatment cells (70 L per cell) in the Borden aquifer to create gasoline-contaminated source areas below the water table, and then water amended with different combinations of electron acceptors was flushed vertically through the cells under highly-controlled flow

conditions. The Nitrate Cell received a mixture of microaerophilic O_2 and NO_3^-, and the Control Cell microaerophilic O_2 only. Aromatic-hydrocarbon and electron-acceptor utilization was monitored during both flushing and static periods over a 13 month period.

Data from multilevel piezometers showed that dissolved O_2 was consumed rapidly to a non-zero threshold concentration in both treatment cells. Because dissolved O_2 was not detected at sampling ports located 60 cm below ground surface, and water was injected at about 50 cm bgs, O_2 was apparently consumed within the first 10 cm of the vertical flowpath. In contrast to the rapid O_2 consumption, NO_3^- utilization was low, but the production of NO_2^- suggested that some biological NO_3^--reduction had been induced. A mass balance indicated that only 12% of added NO_3^- was consumed over the 174-day flushing experiment. Dissolved aromatic hydrocarbon concentrations remained fairly high (near gasoline-saturated levels) in both cells throughout the experiment. The depletion of these compounds was generally consistent with the dissolution of a multicomponent liquid (i.e., relatively rapid depletion of soluble compounds such as benzene) with no clear evidence of preferential removal of labile compounds from microbial activity. After the experiments were completed cores were collected from the cells to measure the mass of BTEXTMB remaining in the residual gasoline and mass balances were completed. In terms of total BTEXTMB, 81% and 83% of the initial mass was recovered in the Control and Nitrate Cells, respectively, which correspond to roughly 2,500 g of unrecovered mass per cell. These losses probably resulted from a combination of physical losses and error associated with the mass balance procedure, with only minor contributions from biotransformation.

Mass balance results were used to estimate the amount of aromatic-hydrocarbon mass loss that could reasonably be attributed to biotransformation. The results suggested that the mass of microaerophilic O_2 injected into the treatment cells was too low to observe any losses even if all of the O_2 was consumed in mineralization reactions with aromatic compounds. Similarly, given the limited NO_3^- utilization, the mass loss of compounds such as toluene that are labile under denitrifying conditions was likely very small relative to the size of the toluene pool in the Nitrate Cell. Consequently, although there was evidence from metabolite formation that some biotransformation of aromatic hydrocarbons had occurred, mass losses appeared to be quite low in both cells. Given these results, gasoline dissolution was the dominant mass removal mechanism from the treatment cells.

The laboratory microcosm experiments, microbial characterization results, and field data suggested that with *in situ* exposure, the aerobic microbial population in the Borden aquifer acclimatized to the gasoline phase and associated high aqueous concentrations of BTEXTMB. However, on the basis of the small quantity of O_2 available for mineralization reactions relative to the size of the carbon pool and the limited utilization of NO_3^-, the majority of the mass of recalcitrant compounds (i.e., benzene) would have been flushed into the aquifer without undergoing appreciable biotransformation. Therefore, in this aquifer there appeared to be no advantage associated with the microaerophilic / NO_3^- amended cell relative to the control during the short flushing period investigated in this study; NO_3^- utilization may have continued further downgradient, however, providing some benefit to an engineered or intrinsic remediation strategy. Despite these conclusions, it is possible that mixed electron acceptors would be more effective in other circumstances. For example, it is conceivable that this approach, particularly with respect to the effects of microaerophilic O_2, would be more effective during the latter stages of an enhanced bioremediation project when source-area concentrations were lower. Similarly, although microaerophilic O_2 was not found to be beneficial in our experimental system, the contribution to *in situ* mass loss could be significant in other applications. For example, mixed electron acceptors would potentially be effective for downgradient plume control using a reactive wall or other semi-passive remedial technology.

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CHAPTER 1. INTRODUCTION

1.1 Objectives

In North America leaking gasoline storage tanks are one of the major sources of groundwater contamination. Gasoline and other fuels contain several regulated compounds, most notably benzene, a suspected human carcinogen. Spilled gasoline is typically trapped in the vicinity of the water table as immobile, non-aqueous phase liquid (NAPL). Because soluble gasoline constituents partition to groundwater, the NAPL is a long-term source of groundwater contamination. Consequently, over the past two decades remediation of gasoline spills has become an important groundwater quality issue. The aromatic hydrocarbons benzene, toluene, ethylbenzene, xylene isomers, trimethylbenzene isomers, and naphthalene (referred to as BTEXTMB in this report) constitute the majority of the mass that partitions to groundwater. These constituents are more toxic and mobile than other fuel constituents.

Engineered systems incorporating free product recovery in conjunction with *in situ* bioremediation have often been used to remediate fuel spills. Providing sufficient oxygen (O_2) to the contaminated area is the major limitation of this approach. The problem of adding sufficient O_2 is more severe if residual fuel is present near the water table. If a plume is biodegrading intrinsically, O_2 can only be replenished by the relatively-weak dispersive mixing at the plume boundaries (MacQuarrie et al., 1989), and consequently anaerobic conditions can persist in the core of the plume. For both engineered and intrinsic remediation approaches, alternate electron acceptors may therefore play an important role in limiting contaminant migration. Nitrate (NO_3^-) has been investigated as a possible alternate electron acceptor for aromatic hydrocarbon biotransformation. It is very soluble, less reactive in anaerobic environments, and provides a high energy yield to denitrifying bacteria; as such it may be useful as a replacement or supplement to O_2 in oxygen-limited environments. As the sole electron acceptor, NO_3^- also has limitations, most significantly the frequent recalcitrance of benzene. In addition, NO_3^- is a regulated compound, and therefore its fate must also be considered.

To circumvent the limitations of single electron acceptors, recent research has focused on enhanced bioremediation approaches that rely upon mixed electron-acceptors. In this study we investigated the use of NO_3^- and O_2 mixtures to bioremediate dissolved-phase and residual gasoline. Dissolved oxygen was added to potentially enhance the mass loss of compounds that are recalcitrant under denitrifying conditions. It has been hypothesized (e.g., Wilson and Bouwer, 1997) that so-called "microaerophilic" dissolved O_2 concentrations (defined here as concentrations below 2 mg/L) could be utilized for initial oxidation of recalcitrant compounds such as benzene. This would yield partially-oxidized intermediates susceptible to oxidation under anaerobic, denitrifying conditions further downgradient, and result in enhanced mass removal. At the field scale, however, there are potential limitations, such as O_2 utilization by bacteria growing on non-target organic compounds and abiotic O_2 demand from reduced metal species. To date, relatively little research has been performed under mixed electron-acceptor conditions, and both the fundamental biological processes and field-scale controls are poorly understood.

The main objective of the research was to evaluate nitrate-based bioremediation as a remedial technology in a gasoline source area. It was hypothesized that a denitrifying population capable of rapid aromatic-hydrocarbon biotransformation would develop in the source area in response to extended NO_3^- exposure. The field research was conducted in the Borden aquifer located at Canadian Forces Base Borden, Ontario. Two controlled gasoline spills were used to generate source areas, and then water amended with different combinations of NO_3^- and O_2 was flushed through the gasoline-contaminated regions to evaluate the extent of mass loss of the soluble, plume-forming aromatic

hydrocarbons. Although both laboratory and field work were performed, the emphasis of the research was to perform an *in situ* evaluation of source-area bioremediation under controlled, dynamic conditions.

The laboratory data were used primarily in support of the field experiment, i.e., to provide data that could be used in the design of the field experiment, and in the interpretation of *in situ* results. In addition, by examining the extent of biotransformation under a wide range of electron-acceptor and organic substrate conditions, the laboratory studies provided supplementary data that could not be obtained at the field scale. A third purpose of the laboratory studies was to characterize the microbial population in the aquifer material and investigate the extent of acclimation to the gasoline spills over the course of the study. These disparate lines of field and laboratory evidence were then used to evaluate the effectiveness of this remedial approach in the Borden aquifer. A detailed description of the experimental activities, including the chronology, rationale, and specific objectives of each experiment, is provided below in Section 1.2.

In addition to the research performed at the University of Waterloo, this project involved substantial collaboration with the U.S. EPA National Risk Management Research Laboratory (NRMRL) at the Robert S. Kerr Environmental Research Center in Ada, Oklahoma. This included experimental work with Borden sand performed at NRMRL, analytical work (i.e., metabolites, determination of hydrocarbon classes) on water and core-extracts obtained from the treatment cells at Borden, and on-site collaboration, both at NRMRL (first author), and at a U.S.EPA research project at Eglin AFB, Florida. The results from the nitrate-based bioremediation project at Eglin AFB will be reported elsewhere.

All research projects funded by the U.S. EPA are required to participate in the Agency Quality Assurance Program. This research was conducted under an approved Quality Assurance Project Plan (June, 1993) and Field Sampling Plan (April, 1995). For some of the laboratory and field activities, modified experimental objectives, unanticipated difficulties encountered during set up, and equipment availability necessitated deviations from procedures specified in these documents. Actual procedures used in the laboratory and field experiments performed in this study are described in detail in Chapter 3 and Appendix C, and are consistent with common research practices as reported in the peer-reviewed literature. Information on the Plans and documentation of the quality assurance activities and results are available from the Principal Investigators.

The report is organized as follows: The Borden field site is described in Chapter 2. Descriptions of the field and laboratory methods used in the study are included in Chapter 3. Results and discussion of laboratory experiments, including both microcosm studies and microbial characterization work, are provided in Chapter 4. Results and discussion of the field experiment are provided in Chapter 5. The overall conclusions and implications of the research are discussed in Chapter 6.

1.2 Overview of Experimental Approach

The major laboratory and field activities are summarized in Table 1-1, and described briefly below. Discussions in Sections 1.2.1 and 1.2.2 describe the experiments in chronological order and also include the rationale behind each series of experiments.

1.2.1 Laboratory Experiments

Prior to the field experiments, a series of preliminary laboratory experiments were performed using pristine Borden aquifer material. The first experiment (Experiment 1) was performed to obtain preliminary data on the effects of dissolved O_2, and varying concentrations of NO_3^-, as well as the addition of inorganic nutrients (NH_4-N, PO_4-P), in this aquifer material. This experiment utilized neat BTEX (approximately 10 mg/L total BTEX). A second microcosm experiment (Experiment 2) was run to compare denitrifying activity in Borden aquifer material with two U.S. EPA research sites (Eglin AFB, Florida and Park City, Kansas) where nitrate-based bioremediation has also been evaluated. The next microcosm experiment (Experiment 3) was performed to determine whether BTEX biotransformation could be enhanced under mixed electron-acceptor conditions by comparing the extent of mass loss under denitrifying, microaerophilic, and mixed microaerophilic / NO_3^- conditions. This experiment utilized a tenfold dilution of gasoline-contacted water for a more realistic representation of anticipated field conditions. A microcosm experiment with ^{14}C ring-labelled aromatics was also completed to determine the extent of mineralization under anaerobic, denitrifying conditions. Enumerations of aerobic heterotrophs and denitrifiers in samples from several cores, and assays of

Table 1-1. *Summary of Major Experimental Activities. Activities are Listed in Chronological Order. Chronology is Approximate Because Some Preliminary Experiments were Run Concurrently. Report Sections in which Results are Discussed are also Shown, Along with Microcosm Experiment Number, where Appropriate.*

Report Section	Experiment	Objectives	Description
4.3.1	Preliminary microbial characterization of pristine Borden sand	Density of requisite microorganisms, effect of gasoline-saturated groundwater on aerobic microbial activity	Pristine Borden sand; aerobic heterotrophs, denitrifiers; aerobic dehydrogenase activity
4.1.1	Preliminary microcosm study (Experiment 1)	Effect of nutrients, O_2, and NO_3^- concentration on extent of biotransformation	Pristine Borden sand; neat BTEX mixture; e^- acceptors: NO_3^- only, high O_2 only, mixtures
4.1.2	Microcosm study, microbial enumerations (Experiment 2)	Comparison of BTEX-degrading activity under denitrifying conditions in Borden aquifer material with other petroleum-hydrocarbon contaminated sites	Aquifer material from Borden, Eglin AFB, FL, and Park City, KS; neat BTEX mixture; e^- acceptors: NO_3^- only
4.1.3	Microcosm study (Experiment 3)	Comparison of BTEX biotransformation under microaerophilic, denitrifying, and mixed e- acceptor conditions	Pristine Borden sand; low substrate conc. (1/10 gasoline-saturated); local gasoline source; e^- acceptors: NO_3^- only, low O_2 only, NO_3^- / low O_2 mixture
4.1.4	Microcosm study (Experiment 4)	Extent of biotransformation under microaerophilic O_2/NO_3^- conditions (high substrate concentrations)	Pristine Borden sand; variable substrate conc. (1/10 to gasoline-saturated); API 91-01 gasoline; e^- acceptors: NO_3^- / low O_2 mixture
4.1.5	Microcosm study, microbial enumerations (Experiment 5)	Extent of biotransformation under fully aerobic conditions (high substrate concentrations)	Pristine Borden sand; high substrate conc. (gasoline-saturated); API 91-01 gasoline; e^- acceptors: NO_3^- / high O_2 mixture, high O_2 only
5.0	Field demonstration	In situ demonstration of gasoline source area remediation under mixed e- acceptor conditions	Nitrate-amended and unremediated control treatment cells; API 91-01 gasoline source area; e^- acceptors: NO_3^- / low O_2 mixture, low O_2 only
4.2.1	Follow up microcosm study (Experiment 6)	Confirmation of field results; effects of long-term gasoline exposure on aromatic-hydrocarbon degrading activity	Gasoline-contaminated sand from Nitrate Cell; variable substrate conc. (1/10 to gasoline-saturated); e^- acceptors: NO_3^- / variable O_2 mixtures, unamended; acetylene block
4.3.2	Microbial enumerations and activity assays	Microbial characterization; demonstration of adaptation response	Gasoline-contaminated and pristine locations; aerobic heterotrophs, denitrifiers, benzene-toluene degraders; aerobic dehydrogenase activity

microbial activity, were also performed prior to the field experiment to obtain baseline information on the numbers and activity of the indigenous microbial populations.

After the gasoline was spilled in the field, it became apparent that aromatic-hydrocarbon concentrations would be higher than a tenfold dilution of gasoline-saturated water throughout the field treatment cells, and so an additional series of microcosm experiments was performed using gasoline-saturated groundwater (about 100 mg/L total aromatics) and pristine aquifer material. The first such experiment (Experiment 4) was performed to compare the response of the aquifer material to high (gasoline-saturated water) and low (10x dilution of gasoline-saturated water) concentrations under the mixed electron-acceptor conditions of the field trials (microaerophilic O_2 plus NO_3^-). Because the added O_2 was rapidly consumed in Experiment 4, a second experiment (Experiment 5) was then performed to determine whether high aqueous concentrations of aromatics were degradable under fully aerobic conditions.

After the flushing experiments were completed, cores were collected from the treatment cells amended with NO_3^- to investigate the extent of biotransformation in aquifer material that had been exposed to gasoline for nearly two years. A final microcosm experiment (Experiment 6) was performed to investigate the response in gasoline-contaminated aquifer material under various electron-acceptor and substrate conditions, and to confirm the results from the treatment cell that was amended with NO_3^- and O_2. Additional microbial enumerations and activity assays were then performed to evaluate the extent of acclimation to the gasoline contamination.

1.2.2 Field Experiments

Flushing experiments began in May, 1996, approximately six months after the gasoline-contaminated zones were created, and continued for 174 days. During this period groundwater amended with electron acceptors was injected continuously into the treatment cells. Target vertical groundwater velocities and cell residence times were about 25 cm/day and 10 days, respectively. The Nitrate Cell received mixed electron acceptors (NO_3^- and dissolved O_2), and the Control Cell dissolved O_2 only to investigate NO_3^- as an alternate electron acceptor in a low O_2 environment. To perform mass balance calculations, flow rates, electron acceptor and dissolved BTEXTMB concentrations were measured periodically. In November, 1996, pumps were shut off and the cells were sampled periodically under static conditions for organics and electron acceptors. In May, 1997, flushing experiments were initiated again for 24 days to re-establish conditions similar to the previous year. During this period, a nutrient solution was pumped into both cells to investigate whether microbial activity was nutrient limited, and the injection concentration of dissolved O_2 was increased. For clarity, these phases are referred to in this report as "the 174-day flushing experiment", "the static period", and "the 24-day flushing experiment". Groundwater samples were also collected near the end of the experiment for analysis of partially-oxidized intermediates (metabolites). In July and August, 1997, cores were collected for microbial characterization work, laboratory microcosm studies with exposed aquifer material, and a mass balance on the aromatic compounds to determine the extent of remediation.

1.3 Background Literature

1.3.1 Biotransformation Under Single Electron-Acceptor Conditions

Aerobic Conditions. Under aerobic conditions, petroleum hydrocarbons, including aromatic hydrocarbons, are readily degraded by indigenous groundwater microorganisms (e.g., Barker et al., 1987). Aerobic degradation rates are controlled at the plume scale by the aquifer properties that control O_2 transport to the contaminated area (MacQuarrie et al., 1989), rather than by limitations of microbial metabolism. As a remediation technology, the major limitation of aerobic biotransformation is the inability to deliver sufficient O_2 to the contaminated area. Oxygen replenishment is limited by its low solubility (ca. 10.9 mg/L at 10°C) and high reactivity with reduced species such as iron (Fe) (Morgan and Watkinson, 1992).

At low concentrations, the kinetics of dissolved O_2 utilization may dramatically limit the rate of oxygen uptake and aromatic-hydrocarbon degradation. It has been commonly observed that the rate of O_2 uptake is independent of concentration at high O_2 concentrations, but below some critical value, uptake rates become dependent on concentration (e.g., Johnson, 1967). The concentration of dissolved O_2 has also been observed to limit substrate degradation rates (Larson et al., 1981; Shaler and Klecka, 1986; Chiang et al., 1987; Leahy and Olsen 1997). The threshold or critical O_2

concentration is often characterized by the half-saturation constant (K_{do}), which is defined as the concentration at which the respiration rate is one half the maximum rate. Shaler and Klecka (1986) compiled K_{do} values for various oxygenases responsible for either ring fission or initial hydroxylation of the aromatic ring. They found that these values were high (up to 2.2 mg/L) relative to those for metabolism of small, easily-degraded compounds such as glucose or acetate, for which O_2 is required mainly as the terminal electron acceptor for the cytochrome oxidase. They postulated that high concentrations were consistent with the dual role of O_2 as both cosubstrate (i.e., addition of oxygen atoms to the aromatic ring during initial oxidation steps) and electron acceptor in this type of transformation reaction. On the other hand, microorganisms adapted to a low O_2 environment may synthesize enzyme systems designed to more efficiently utilize O_2 (e.g., a monooxygenase system with lower half-saturation constants) (Leahy and Olsen, 1997). Wilson and Bouwer (1997) found that critical O_2 concentrations, compiled from sixteen studies, ranged from 0.013 to 1.5 mg/L, with the lower values generally corresponding to utilization of simple substrates such as glucose.

If broadly applicable, these findings have important implications for a remedial approach that relies upon low levels of dissolved O_2 to initiate oxidation of recalcitrant organics. If the half-saturation concentrations for O_2 uptake are high, rates of substrate utilization will begin dropping at relatively high O_2 concentrations, and substantial threshold O_2 concentrations may persist, or the remaining O_2 may be utilized by other strains growing on simpler non-target substrates. More work is needed to clarify this issue, however, because even a small mass-loss enhancement may contribute significantly to the overall success of a remediation program.

Denitrifying Conditions. Several studies suggest that NO_3^- addition is a potentially viable bioremediation technology (e.g., Kuhn et al., 1988; Hutchins et al., 1991a, 1991b; Barbaro et al., 1992). With NO_3^- as sole electron acceptor, however, results among various studies have not been consistent, and several potential limitations have been identified.

Perhaps the major limitation associated with NO_3^- addition is the frequent recalcitrance of benzene under anaerobic conditions (Berry-Spark et al., 1986; Hutchins, 1991a, 1991b; Barbaro et al., 1992). On the other hand, benzene has been shown to biodegrade under denitrifying conditions in the Major et al. (1988) study which utilized Borden aquifer sediment. While the possibility of experimental artifact (e.g., microcosm leakage) cannot be ruled out, benzene loss was not observed in active, anaerobic controls incubated under identical conditions. In other studies with Borden aquifer material (Berry-Spark et al. 1986; Barbaro et al., 1992), as well as at other sites (Hutchins, 1992), it was noted that the addition of acetylene gas substantially inhibited aromatic hydrocarbon biotransformation. In the Major et al. (1988) study, however, BTX-degrading activity was less affected by the addition of acetylene. Their data showed that the accumulation of nitrous oxide corresponded to the period when BTX was declining, and that NO_3^- was required for BTX disappearance. These results raise the possibility that the experimental design of Major et al. (1988) selected for a distinct denitrifying population with the metabolic capability to biodegrade benzene.

Previous studies have also shown varying levels of removal of the toluene, ethylbenzene, and the xylene isomers. For instance, using aquifer sediment from Park City, Kansas, Hutchins et al. (1995) found that toluene, ethylbenzene, *m*-xylene, and *p*-xylene were biodegraded under denitrifying conditions to below 5 µg/L in batch microcosms. In contrast, in the field experiment performed by Barbaro et al. (1992), a toluene threshold concentration of 50 to 100 µg/L persisted throughout the experiment, and ethylbenzene and xylenes removal was on the order of only 50% of injection concentrations, despite the continued presence of NO_3^-. Because the aromatic hydrocarbons are regulated compounds, threshold concentrations are potentially problematic.

A second limitation involves the incomplete mineralization of monoaromatic hydrocarbons under limited O_2 or anaerobic conditions. Partially-oxidized compounds may form under both aerobic (Barker et al., 1987) and anaerobic conditions (Cozzarelli et al., 1995; Barbaro et al., 1992; Cozzarelli et al., 1990), but they appear to be most persistent, and therefore accumulate, in O_2-depleted environments. Metabolite production under denitrifying conditions has been demonstrated in laboratory studies with pure cultures (Evans et al., 1992; Kuhn et al., 1988). These compounds are mobile and geochemically reactive. If persistent, their presence may adversely affect bioremediation systems based on anaerobic, NO_3^- utilization.

Another potential problem is the degradation of non-target organic compounds in preference to aromatic hydrocarbons. Hutchins et al. (1991a) noted that the extent of BTEX mass loss was lower in aquifer material contaminated with JP-4 jet fuel relative to uncontaminated material spiked with BTEX. The NO_3^- demand was much larger in the JP-4 contaminated material, suggesting that non-target organics were being utilized in preference to BTEX. Similar observations were made by Reinhard et al. (1995) in the hydrocarbon-contaminated Seal Beach aquifer. Barbaro et al. (1992) also arrived at similar conclusions, although in that study it appeared that NO_3^- was being used either as an assimilatory source of N and/or as an electron acceptor to oxidize naturally-occurring organic matter in preference to low concentrations of BTEX. Although the biotransformation of other fuel constituents can be considered a positive result, insufficient microbial growth on the mobile, regulated compounds may limit this technology.

1.3.2 Biotransformation Under Mixed Electron-Acceptor Conditions

Background. The major anticipated advantage of biotransformation under mixed electron-acceptor conditions is enhanced mass loss, particularly of compounds such as benzene that are recalcitrant under denitrifying conditions. Because much less work has been done under these conditions, however, the advantages and limitations discussed in this section are still quite speculative, particularly for *in situ* applications where an indigenous microbial population mediates reactions.

From research on their population ecology and growth strategies, most denitrifiers are facultatively-anaerobic, heterotrophic bacteria that grow readily under aerobic conditions and prefer to utilize O_2 as electron acceptor. Historically there has been considerable debate regarding the effect of O_2 on denitrifying activity. The conventional view was that denitrifying activity did not begin until O_2 was nearly depleted (Tiedje, 1982; Tiedje, 1988), and many researchers considered denitrification a strictly anaerobic process. There is also considerable recent evidence that denitrification proceeds in the presence of substantial amounts of O_2 (Krul, 1976; Robertson and Kuenen, 1984; Lloyd et al., 1987; Bonin and Gilewicz, 1991; Lloyd, 1993; Patureau et al., 1994; Carter et al., 1995). Although aerobic denitrification is now a well-established phenomenon, the regulating mechanisms and physiological significance of the process are still not well understood, and the biochemical diversity of denitrifiers makes generalization difficult. Wilson and Bouwer (1997) provide a comprehensive review of the aerobic denitrification literature.

Under mixed O_2/ NO_3^- conditions, O_2 appears to be the most important variable. If the dissolved O_2 concentration is initially high, then there will be considerable aerobic biotransformation, but denitrifying activity will be reduced or completely inhibited. Current research indicates that the critical dissolved O_2 concentration above which denitrification is completely inhibited varies over a broad range (0.02 to 7.7 mg/L) (Wilson and Bouwer, 1997). The critical concentration is thought to be species, enzyme, and substrate specific, and probably dependent on growth conditions. Alternatively, if the dissolved O_2 concentration is low, rates of aerobic respiration will be low or negligible, and denitrifying activity will probably dominate. As discussed in Section 1.1.2, the critical O_2 concentration for supporting aerobic degradation of aromatic substrates appears to be in the range of 1-2 mg/L, with considerable variation among species and substrates. It should be noted that additional complexity may be present *in situ*; dissolved O_2 concentrations measured in bulk pore water may not be indicative of the O_2 levels in microsites or biofilms.

If the O_2 concentration is initially high (e.g., 10 mg/L), NO_3^- could be used to enhance aerobic microbial activity and growth by serving as an assimilatory source of nitrogen (Van 'T Riet et al., 1968), but, as discussed above, dissimilatory NO_3^- reduction would probably be inhibited. Once dissolved O_2 concentrations fell below the critical value for inhibiting denitrifying activity, NO_3^- could then be utilized as the electron acceptor. The major advantage of high initial O_2 concentrations would appear to be relatively extensive aerobic biotransformation. A second possible advantage would be a larger population of denitrifiers resulting from aerobic growth of facultative anaerobes (Su and Kafkewitz, 1994). It should be noted, however, that the regions of aerobic and denitrifying activity would probably be separated spatially at the plume scale, diminishing the benefit of this effect, with most O_2 depletion occurring near the point of injection, and NO_3^- depletion over a much longer, downgradient flow path.

Of greater interest for nitrate-based bioremediation is the behavior of a mixture consisting of NO_3^- and microaerophilic dissolved O_2 concentrations. Under microaerophilic conditions, it is less likely that

the concentration of dissolved O_2 would be inhibitory to denitrifiers. As a consequence, denitrifying activity could occur in the presence of low levels of O_2; the rate, however, may be significantly reduced. There are two potential advantages to a mixture consisting of NO_3^- and microaerophilic O_2. If the target compound is degradable under denitrifying conditions, NO_3^- can be used by facultative anaerobes to relieve the electron-acceptor deficit imposed by O_2 consumption (Mikesell et al., 1993; Leahy and Olsen, 1997); this circumvents the problem of re-oxygenating the subsurface. More importantly for bioremediation of aromatic hydrocarbons, it is hypothesized (Britton, 1989; Wilson and Bouwer, 1997) that the O_2 could participate in reactions and contribute to the complete oxidation of otherwise recalcitrant compounds such as benzene.

Based on the current literature, the benefits associated with microaerophilic O_2 are unclear. If the concentration of dissolved O_2 is below the critical value to support O_2-linked respiration, then there would be negligible aerobic degradation of recalcitrant compounds like benzene (Section 1.3.1). Even if all of the O_2 was consumed (i.e., the K_{do} was low), the stoichiometry of the reaction would appear to constrain the extent of aerobic oxidation. For example, if the aerobic reaction proceeds to CO_2, complete utilization of 2 mg/L O_2 mineralizes only 0.65 mg/L benzene to CO_2. Benzene mass loss would be reduced further if the O_2 consumption was spread among other aromatic hydrocarbons. In fact, in laboratory experiments with Borden sand, *m*-xylene is commonly biodegraded first under aerobic conditions (Section 4). Alternatively, some researchers have hypothesized that, rather than supporting aerobic respiration, low levels of dissolved O_2 could be utilized only as a substrate for oxygenase enzymes (Britton, 1989). This mechanism has been proposed as a means of initiating aromatic ring oxidation, yielding greater quantities of partially oxidized intermediates susceptible to further oxidation by a denitrifying pathway. There is currently no evidence that the so-called "sparing effect" has enhanced the biotransformation of aromatic compounds *in situ*.

From an engineering perspective, a mixture of microaerophilic O_2 / NO_3^- is the optimal combination. This circumvents the problems associated with adding high O_2 concentrations and relies mostly on soluble NO_3^- as the oxidant. At the field scale, however, there are potential limitations to adding microaerophilic O_2 concentrations. First, in most contaminated aquifers multiple substrates are present, including aromatics, other hydrocarbons, and natural organic material. It is conceivable, therefore, that the O_2 added to an aquifer will be utilized completely by bacteria growing on non-target compounds. Second, the abiotic O_2 demand must be determined. As noted by Kennedy and Hutchins (1992), if the aquifer is initially anaerobic, reduced metal species may exert a large O_2 demand, leading to scavenging of O_2 intended for bioremediation, and formation plugging. Finally, some strains of denitrifiers have been shown to be very sensitive to dissolved O_2 (Hernandez and Rowe, 1987), so it is possible that even a low concentration of O_2 *in situ* may inhibit denitrifying activity.

Application to Fuel-Contaminated Sites. The published laboratory studies on biotransformation of aromatic hydrocarbons under mixed electron acceptor conditions have yielded ambiguous results. Major et al. (1988) found that BTX mass loss in microcosms with Borden aquifer sediment was slightly enhanced in the presence of both NO_3^- and O_2 relative to losses in aerobic microcosms. They speculated that the NO_3^- alleviated a nitrogen limitation during aerobic metabolism, or that denitrification was occurring within anaerobic microsites. In a pure culture study, Su and Kafkewitz (1994) found that *Pseudomonas maltophilia* was capable of degrading toluene and xylene isomers in the presence of NO_3^- and 2 percent O_2. Miller and Hutchins (1995) used laboratory columns to study BTEX removal from three different aquifer sands under NO_3^- only and then NO_3^- / O_2 conditions. In two of the columns, adding low levels of O_2 did not enhance BTEX removal, and in the third, O_2 had an inhibitory effect. Under all conditions, benzene was completely recalcitrant. Hutchins et al. (1992) also used laboratory columns to study the effects of various combinations of NO_3^-/O_2 on the removal of BTEX from aquifer material. They found that adding NO_3^- to the column with low O_2 decreased TEX breakthrough by an order of magnitude, demonstrating that NO_3^- was needed to increase substrate utilization. There were no adverse effects associated with low levels of O_2. Benzene removal was low and independent of electron acceptor conditions. In contrast, Wilson et al. (1995) observed benzene degradation in the presence of 2 mg/L dissolved O_2 and NO_3^-, but only toluene degradation under anaerobic, denitrifying conditions.

Anid et al. (1993) investigated BTEX removal in aerobic columns amended with either hydrogen peroxide or NO_3^-. In columns amended with NO_3^-, effluent dissolved O_2 concentrations dropped from

9 mg/L to 2 mg/L and 164 mg/L NO_3^- was consumed. Benzene was recalcitrant in these columns, but in additional experiments with NO_3^- and <1 mg/L O_2, approximately 25 percent of added benzene appeared to biodegrade. In this aquifer material, strict anaerobic conditions did not appear to be required for NO_3^- reduction, although the formation of anaerobic microsites cannot be ruled out. In addition, benzene removal was enhanced under anaerobic rather than aerobic conditions. Leahy and Olsen (1997) showed that NO_3^- enhanced the rate of toluene utilization by denitrifying strains after the dissolved O_2 fell below a critical concentration. Denitrifying strains were able to maintain a higher rate of toluene utilization by switching to denitrifying activity when the availability of dissolved O_2 was low. Similarly, Mikesell et al. (1993) and Hutchins (1991a) both demonstrated in the laboratory that, under limited O_2 conditions, biotransformation of certain aromatics could be enhanced by the presence of NO_3^-. In the Hutchins (1991a) study, benzene losses were observed in microcosms amended with both O_2 / NO_3^- after the apparent removal of O_2.

Since the late 1980s, only a handful of nitrate-based bioremediation field studies have been completed (Sheehan et al., 1988; Hutchins et al., 1991b; Battermann and Meier-Lohr, 1995; Hutchins et al., 1995; Reinhard et al., 1995). Hutchins et al. (1991b) added NO_3^- and O_2 via an infiltration gallery to an aquifer in Traverse City, Michigan contaminated with JP-4 jet fuel. Both electron acceptors were consumed, and more NO_3^- was consumed than required for BTX degradation, indicating that other compounds were being utilized under denitrifying conditions. Toluene and xylenes degradation appeared to be stimulated in the aquifer, but benzene removal was apparently due only to flushing. Hutchins et al. (1995) describe the performance of a nitrate-based bioremediation system applied to a petroleum spill in Park City, Kansas. In this study, NO_3^- utilization was confirmed but site heterogeneities obscured the performance evaluation. Although laboratory data indicated that the aromatic hydrocarbons would biodegrade under denitrifying conditions, this could not be confirmed in the field. This study showed the problems that can arise in evaluating the extent of bioremediation in heterogeneous aquifers.

Battermann and Meier-Lohr (1995) describe the performance of a large-scale NO_3^- plus O_2 bioremediation system at an abandoned refinery site contaminated with residual hydrocarbons (dissolved BTEX concentrations 10 to 100 mg/L). After three years of operation, about 300 metric tons of hydrocarbons were removed, of which 80 percent were attributed to biotransformation and 20 percent to flushing. During pilot-scale tests at the same site, Battermann et al. (1994) found that hydrogen peroxide was attenuated near the infiltration point. Nitrate, on the other hand, was distributed over much larger contaminated areas; in the first year of operation, approximately 100 mg/L NO_3^- was consumed over a 50-day residence time.

These field studies have suggested that ambient dissolved O_2 in the injected water does not inhibit denitrifying activity, but there were no observable benefits associated with O_2 either. The extent of aerobic biotransformation near source areas or the effects of lower concentrations of O_2 further downgradient are not easily assessed in uncontrolled field situations. Moreover, it is difficult to determine the contribution of flushing as a mass removal mechanism. The highly-controlled field experiments used in this study provided additional information on the *in situ* utilization of O_2 in this type of bioremediation system.

CHAPTER 2. STUDY AREA

The field research was conducted at CFB Borden, Ontario (Figure 2-1). The research area is located in an abandoned sand pit. The depth to groundwater varies from ground surface to about 1.5 meters below ground surface depending on location within the pit and the season. The site has been studied extensively over the past 17 years. The geology and local hydrogeology were investigated by MacFarlane et al. (1983). The hydrogeology and groundwater chemistry of the sand pit were discussed in detail by Mackay et al. (1986).

The sand pit is underlain by a relatively homogeneous, unconfined sand aquifer composed of interbedded fine- to medium-grained glaciolacustrine sand. Analysis of a bulk sample indicates that the aquifer material is composed of 58% quartz, 19% feldspars, 14% carbonates, 7% amphiboles, and

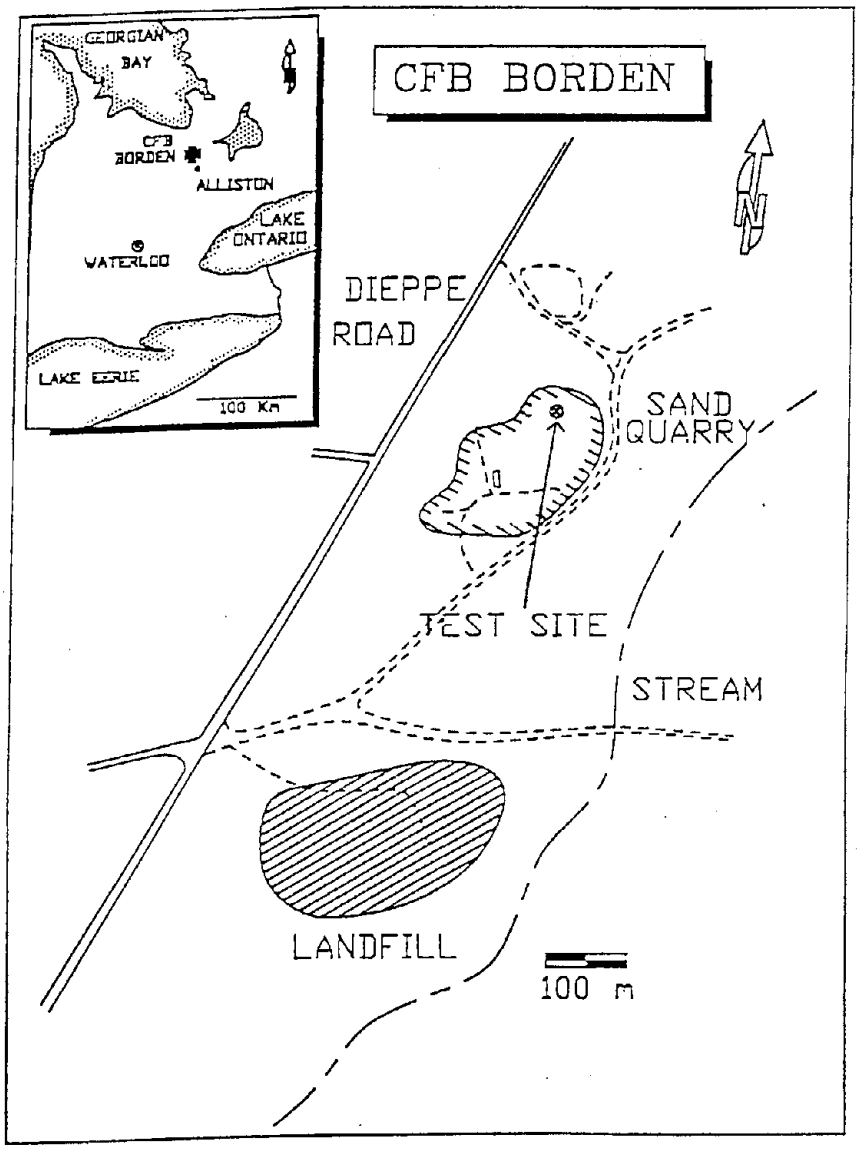


Figure 2-1 Location map of the study area at CFB Borden (from Oliveira (1997)).

2% chlorite (Mackay et al., 1986). The aquifer is about nine meters thick, extending from ground surface to a clay layer. Detailed coring has shown that the deposit contains discontinuous lenses of sand that vary from 0.02 to 0.1 m in thickness and 2-5 m in length (Sudicky, 1986). The mean hydraulic conductivity is 7 x 10^{-3} cm/sec, with variations between layers occasionally exceeding two orders of magnitude (Sudicky, 1986). The mean weight-fraction of organic carbon (f_{oc}) was estimated at 0.00018, and the porosity at 0.33 (Mackay et al. 1986). The spatial variability of porosity based on 36 samples was found to be small (coefficient of variation = 0.05).

A dense, woody peat layer was encountered at the experimental site at a depth of about 2.7 m below ground surface. Based on cores and other drilling information, this layer was about 10 cm thick, fairly continuous, and overlain by about 25 cm of dark gray, dense silty sand. Elevated dissolved methane (CH_4) and sulfide (HS^-) were frequently detected in groundwater samples collected near the peat layer, and cores of the peat sequence had a sulfide odor and were dark gray with orange oxidation zones on the upper and lower boundaries. To avoid pumping water across this sequence, all experiments were conducted within the top 2.5 m of the aquifer. As a low-hydraulic-conductivity layer, this sequence appeared to limit the flux of water from the underlying aquifer when the flushing experiments were in progress.

Groundwater flows in a northeasterly direction at about 9 cm/day, and remains near 10°C throughout the year. A leachate plume originating from an abandoned municipal landfill is present at the base of the unconfined aquifer, at depths ranging from five to seven meters below ground surface. Groundwater above the leachate plume is unaffected by the landfill.

Based on locations of previous experimental activities, the site has never been exposed to aromatic hydrocarbons. Although the presence and depth of the landfill leachate plume beneath the site was not determined, groundwater chemistry data collected from a shallow well (2.49 m depth) indicated that the landfill leachate, if present, was below the experimental zone. Dissolved O_2 levels in shallow groundwater were unexpectedly low at about 0.2 mg/L; in other regions of the sand pit, shallow groundwater has substantially higher concentrations of dissolved oxygen (e.g., Barbaro et al., 1994). There was no detectable Fe or NO_3^- in background groundwater, and dissolved organic carbon was low (<2 mg/L). Dissolved Mn was detected, however, and may have exerted an abiotic O_2 demand. The chemistry of the groundwater is summarized in Table 2-1.

Table 2-1. *Chemistry of Unamended Groundwater at the Experimental Site. Samples Collected May, 1996 from Shallow, Upgradient Supply Well. Concentrations in mg/L.*

Parameter	***Concentration***
Si	2.88
Cl	3.58
SO_4	6.31
NO_3	<0.1
PO_4	<0.2
Br	<0.1
HCO_3	258
Ca	80
Mg	3.55
Na	4.21
K	1.16
NH_4	0.82
Fe	<0.1
Mn	1.24
pH	7.48
DO	0.2
DOC	<2
BTEXTMB	n.d.[1]
Temp (°C)	12.5

[1] n.d. - not detected at individual compound method detection limit (Appendix C).

CHAPTER 3. EXPERIMENTAL METHODS

The procedures used in the laboratory and field experiments are described in this chapter. Descriptions of the procedures used for laboratory microcosm experiments, microbial characterization of aquifer materials, the controlled gasoline spills in the field, and the design and operation of the field experiments are included here. A description of the physical and chemical characteristics of the API 91-01 gasoline is included in Appendix A. A description of procedures and results of the field tracer test designed to evaluate the flow of injected water in the treatment cells is provided in Appendix B. Appendix C includes descriptions of laboratory sampling and analytical procedures, as well as analytical procedures used for field samples. Appendix D includes results of the groundwater sampling performed downgradient of the wastewater treatment mound and treatment cells to monitor potential releases to the aquifer.

3.1 Aquifer Core Collection

Aquifer sediment was collected at various times during the study for different purposes. Core material was used for logging the stratigraphy, microcosm and microbial characterization studies, and mass balances on the gasoline injected into the field treatment cells. Cores were obtained in 5.08 cm (2 in) diameter aluminum tubes using either a piston or core catcher. The coring system is described in detail in Zapico et al. (1987).

Aluminum core tubes designated for microbiology experiments were rinsed with methanol and flamed in the field prior to use. After the cores were collected they were sealed tightly, transported within 4 hr to the University of Waterloo, and stored at 4°C. In preparation for laboratory experiments, aquifer material was removed from core tubes either in an anaerobic chamber or a sterile, laminar flow cabinet (Nuaire, Inc., Model NU-408FM-300), depending on *in situ* O_2 concentrations, and refrigerated in sterile mason jars. Material adjacent to core-tube walls was discarded or used in sterilized control microcosms.

3.2 Laboratory Microcosm Experiments

3.2.1 General Set Up Procedures

All microcosm experiments in this study had one of two basic designs. The most common approach, designated Design 1, consisted of a series of crimp-sealed microcosms (60 ml glass hypovials). For each sampling event, a set of microcosms was sacrificed for analysis. A set contained replicate microcosms for each treatment group in the experiment. Generally there were three to five different treatment groups, including a sterile control, per experiment. The second approach, designated Design 2, utilized a smaller number (typically 1-3 bottles per treatment) of 100 ml screw-top bottles designed for repetitive sampling through a threaded mininert™ valve (Dynatech Precision Sampling Corp., Baton Rouge, LA). For each sampling event, a sample of headspace gas was obtained from these microcosms. This design was used for the first series of preliminary experiments only (Section 4.1.1). A summary of the microcosm designs is provided in Table 3-1. Sterile equipment and aseptic technique were used in all aspects of microcosm preparation and sampling.

Each microcosm received 20-25 g of homogenized aquifer material, groundwater spiked with organics, nutrients, and electron acceptors, if required, and was sealed with a Teflon™-lined septum and an aluminum crimp seal, or mininert™ valve. All Design 2 microcosms contained a headspace, from which gas-phase samples were collected. Depending on the intended O_2 status of Design 1 microcosms, a 2-4 ml headspace was present. Preliminary work with this type of set-up indicated that a pure O_2 or air filled headspace was the most reliable means of supplying dissolved O_2. If O_2 was not required, Design 1 microcosms were prepared with no headspace.

Table 3-1. *Summary of Microcosm Designs Used in the Study*

Type	Description
Design 1	60-ml glass hypovials; crimp-sealed; 20-25 g aquifer material; 2-4 ml pure oxygen or air headspace present in some microcosms; analytes measured in fluid phase (organics, e^- acceptors); set of replicates sacrificed for each sampling event
Design 2	100-ml screw-top bottles; threaded mininert™ valves; 20-25 g aquifer material; anaerobic or air headspace present; analytes measured in headspace gas (organics, O_2), and fluid phase (e^- acceptors); single or replicate bottles sampled repetitively over time

The source of the organic substrates was either gasoline or pure solutions of benzene, toluene, ethylbenzene and xylene isomers (6:4:2:2 mg/L concentration ratio). To prepare gasoline-saturated water, an appropriate volume of aerobic Borden groundwater was first gassed for at least 3 hr with either sterile nitrogen gas or sterile air, depending on the treatment. An aliquot of this water was transferred to a sterile separatory funnel to prepare gasoline-saturated groundwater. Gasoline was added to yield a 10:1 water to gasoline volume ratio. The separatory funnel was then shaken manually three times (5 min each). After 24 hr, gasoline-saturated water was removed from the funnel to glass bottles for dispensing to microcosms. The dissolved O_2 concentration of the prepared water was determined prior to dispensing to microcosms. If lower concentrations were required for an experiment, a 10x dilution of the gasoline-saturated water was performed using an aliquot of the gassed, hydrocarbon-free groundwater. Measured concentrations in gasoline-saturated water ranged from 70-130 mg/L, depending on the experiment. Concentrations differed because different brands of gasoline were used.

The set-up procedures were similar for all experiments. Microcosms were prepared either in the anaerobic chamber or in the sterile flow cabinet. For anaerobic or low O_2 conditions, aquifer material was typically dispensed within the anaerobic chamber to avoid any contact with air. For aerobic conditions, aquifer material was dispensed into microcosms in the flow cabinet. Aquifer material was dispensed into sterile control microcosms several days in advance to allow for sterilization (1/2 or 1 hr autoclave run on three consecutive days). After all the aquifer material was dispensed, contaminated groundwater and amendments were added. Microcosms were then filled completely with appropriate solutions and crimp sealed. If required, microcosms received inorganic nutrients (NH_4Cl and KH_2PO_4) from concentrated, sterile, anaerobic stock solutions to yield 5 mg/L as N, and 2 mg/L as P. Nitrate, where required, was added as a concentrated stock solution of KNO_3 to yield concentrations of 5-50 mg/L as N. Sterile controls received an additional 0.5-0.6 ml of 10% (w/v) sodium azide solution or 0.25 ml of a 4% $HgCl_2$ solution. In some cases, the nutrients and NO_3^- were spiked into the groundwater before dispensing to microcosms.

Aerobic Design 2 microcosms were prepared with aerobic groundwater in equilibrium with atmospheric O_2. Design 1 microcosms requiring dissolved O_2 were moved to the sterile flow cabinet after construction, decrimped, and the appropriate volume of water removed with a sterile syringe. "Low" dissolved O_2 microcosms, also referred to as microaerophilic microcosms, typically received a 2-4 ml ambient air headspace, and "high" dissolved oxygen microcosms, also referred to as aerobic microcosms, contained a 4-ml pure O_2 headspace. Pure O_2 was added by purging the headspace at a rate of approximately 300 ml/min for 30 seconds and quickly replacing the crimp seal. It should be noted that partitioning of the aromatic hydrocarbons to a headspace of this size is minimal. For example, partitioning calculations based on Henry's Law indicate that only 2% of the total mass of benzene would partition to a 4-ml headspace.

We attempted to establish "microaerophilic" conditions in two ways: First, by mixing aerated and O_2-stripped (by N_2-purging) waters in a 50:50 ratio, and second, by introducing a small air-filled headspace into otherwise anaerobically prepared microcosms, similar to the method of Hutchins (1991a). The first method should have provided an initial D.O. of 3.5-4 mg/L, based on numerous D.O. measurements of aerated water. However, the method essentially failed, as the available O_2 may have been consumed by the initial microcosm sampling on day 1. Alternatively, because day 1 BTEX levels in these and the

strictly-denitrifying microcosms were similar, the D.O. may have actually been consumed or lost during microcosm preparation in the anaerobic chamber. The microcosms prepared in this manner behaved identically to those set up under anaerobic conditions, and are not discussed further. The second method was used for all experiments requiring microaerophilic conditions. It provided a total mass of about 1.0 mg O_2 (assuming 20% by volume in air) in a microcosm. Measured initial aqueous concentrations in sterile controls were ca. 4.5 mg/L (Section 4.2.1). It is acknowledged that the initial D.O. concentration in microcosms prepared in this manner exceeded microaerophilic levels as defined previously in this study (2 mg/L or less). However, to obtain observable results it was necessary to provide a mass of O_2 sufficient to drive aerobic reactions in this static system. A strict adherence to the operational definition of microaerophilic conditions (2 mg/L initial concentration with no headspace), while ideal, was not tractable with our experimental methods. Considering the limitations associated with establishing microaerophilic conditions, we concluded that the air headspace provided the best analogue to the dynamic system in the field, where a continuous injection concentration of ca. 2 mg/L O_2 could be maintained.

Sterile aqueous controls (no aquifer material) were also prepared (Experiment 6) to investigate the rate of diffusive loss of dissolved O_2 and BTEXTMB. Design 1 microcosms were prepared aseptically with sterile groundwater and amended with 90 mg/L neat BTEXTMB, 0.6 ml sodium azide solution, and dissolved O_2. Two sets of microcosms were prepared: microaerophilic and fully-aerobic. Microaerophilic microcosms contained a 2-ml air headspace, and were incubated in the anaerobic chamber. Aerobic microcosms contained a 4-ml pure-O_2 headspace and were incubated in the laboratory cupboard. These microcosms were stored with other microcosms prepared for Experiment 6 and periodically sampled over a 159-day period to observe the loss of dissolved O_2 and organics from sterile microcosms.

After set-up microcosms were stored in the dark at room temperature (23±2°C) either in an anaerobic chamber (Lab-Line Instruments, Inc., Model 6550) supplied with a 1% CO_2, 2.5% H_2O, 96.5% N_2 mixed gas, or a laboratory cupboard (aerobic microcosms). Room temperature incubation was unavoidable because the temperature within the anaerobic chamber could not be controlled. Experimental temperatures were therefore roughly twice *in situ* temperatures. Our previous experience with Borden aquifer material suggests that temperature does not affect experimental results with respect to compound degradability; compounds that degrade at room temperatures also degrade at groundwater temperatures. Reaction rates are, however, faster at room temperature.

3.2.2 Acetylene Block

The cause of the rapid NO_3^- utilization observed in the final microcosm experiment (Experiment 6; Section 4.2) was investigated by assaying for denitrifying activity. Because acetylene inhibits the reduction of N_2O to N_2, the accumulation of nitrous oxide in the presence of acetylene is considered strong evidence of denitrification (Tiedje, 1982). Acetylene gas was added to duplicate microcosms from each treatment group that contained NO_3^- by injecting 1% (v/v) through the septa, shaking the vial for 1 min, storing inverted for 1/2 hr to equilibrate the aqueous phase with the headspace, and then quickly replacing the pierced septa. Prior to injection, the acetylene was passed through a series of flasks containing distilled water to remove any acetone that may have been present. These microcosms were incubated for 15 days and then analyzed for N_2O and acetylene, as well as dissolved O_2, NO_3^-, nitrite (NO_2^-), and BTEXTMB.

3.3 ^{14}C Mineralization Experiment

The conversion of benzene, toluene, *m*-xylene, and *o*-xylene to CO_2 (mineralization) under anaerobic, denitrifying conditions was investigated with standard $^{14}CO_2$ trapping techniques. The radiolabelled compounds (Sigma Chemical Co., St. Louis, MO) were obtained from pure solutions: [U-^{14}C]benzene, [ring-U-^{14}C]toluene, *m*-[ring-U-^{14}C]xylene, and *o*-[ring-U-^{14}C]xylene. Details of microcosm preparation, sampling, and analyses used in this experiment have been described previously (Hutchins, 1993). In brief, 10 g of pristine Borden sand with no known prior exposure to hydrocarbons was dispensed to 60-ml serum bottles designed for repetitive sampling. Each microcosm received a single radiolabelled aromatic compound (3-9 mg/L aqueous concentrations), 20 mg/L NO_3^--nitrogen if required, N and P as nutrients, and anaerobic water (distilled water mixed with groundwater from a spring near Ada, OK). Sterile controls received both mercuric chloride (250 mg/L) and sodium azide (500 mg/L). After spiking with the appropriate amendments, each microcosm was sealed without headspace using a Teflon™-lined butyl

rubber septum, mixed, and incubated at room temperature in an anaerobic glove box. Three replicate microcosms with corresponding sterile and unamended (no NO_3^-) controls were prepared for each of the four radiolabelled compounds.

Microcosms were sampled five times over a 63 day incubation period. All sampling was done in the anaerobic glove box. The headspace created by removing water for analysis was eliminated by adding sterile 6-mm glass beads. Microcosms were then resealed, mixed, and again incubated in the glove box. Samples were analyzed for aromatic hydrocarbons, NO_3^-, and NO_2^-, as described in Hutchins (1993). The distribution of the radiolabel was determined with a modification of the method used by Grbić-Galić and Vogel (1987). This method involves measuring the sum of $^{14}CO_2$ and nonvolatile intermediates, nonvolatile intermediates only, and then calculating $^{14}CO_2$ by difference. Total ^{14}C activity in the aqueous phase was also estimated. This approach accounts for radiolabel distribution, and therefore extent of biotransformation, in the aqueous phase only; activity of the solids was not determined.

3.4 Microbial Characterization

3.4.1 Enumerations

Aerobic Heterotrophs. Enumerations of viable, aerobic heterotrophic and denitrifying microorganisms were conducted by the standard spread plate and most-probable-number (MPN) methods, respectively, using 0.1% Na-pyrophosphate (pH=7.0) to suspend the aquifer material, and phosphate-buffered saline (1.18 g Na_2HPO_4, 0.22 g $NaH_2PO_4@H_2O$, 8.5 g/L NaCl, pH 7) to dilute the suspension as required. R2A medium was used for the aerobic heterotrophic plate counts (HPCs) (Reasoner and Geldreich, 1985). R2A medium is a relatively low-nutrient medium that was developed for the enumeration of microorganisms in potable water. All plates were prepared in triplicate for each dilution, and incubated at room temperature for up to 30 days.

Denitrifiers. For the denitrifier MPN procedure, 18-ml vials were filled with 12 ml 1/10-strength nutrient broth (Difco Laboratories, Detroit, MI), and amended with 2 mM KNO_3 and 0.17% Noble agar (Difco). Immediately prior to inoculation, the medium was deaerated by placing the vials in flowing steam for 5-10 min, then quickly cooled to room temperature and inoculated. Inoculated vials were sealed with sterile, slotted butyl rubber stoppers (Wheaton), and the headspace of each vial was flushed with sterile nitrogen for 30 sec by loosening the stopper slightly and inserting a sterile syringe needle into the stopper slot. Vials were then sealed permanently with an aluminum crimp lid. Finally, 0.6 ml acetylene gas was injected into the headspace using a sterile 1-ml syringe fitted with a membrane filter (0.2 µm pore size). All vials were prepared in triplicate and incubated at room temperature for up to 61 days. After incubation, 0.1-0.2 ml of culture fluid was removed from each vial and tested with diphenylamine reagent for NO_3^- and/or NO_2^- (Tiedje, 1982). Denitrification was confirmed in approximately 10% of vials with depleted NO_3^- by analyzing for the presence of accumulated N_2O. A 2-ml sample of microcosm headspace gas was analyzed for N_2O as described in Appendix C.

Aerobic Benzene-Toluene Degraders. A suspension of aquifer material was prepared by aseptically adding 10 g (wet wt) of aquifer material to 90 ml 0.1% Na pyrophosphate solution (pH 7). The suspension was shaken for 10 min at 400 rpm on a rotary shaker, then diluted further in phosphate-buffered saline. One ml aliquots of selected dilutions were added to triplicate tubes of a mineral medium (Furukawa et al., 1983) in screw-capped test tubes (10 ml medium/tube). Each tube was then amended with 1µL of a neat, filter-sterilized benzene/toluene mixture (1:1 concentration ratio) using a micropipettor. The tube was closed and shaken to dissolve the hydrocarbons. Tube caps were covered with a layer of Parafilm and plastic wrap to minimize losses of volatiles during incubation. Resulting maximum aqueous concentrations were about 50 mg/L for both benzene and toluene, although actual concentrations were probably lower due to partitioning into the tube headspace, and to losses to the atmosphere during the amendment procedure. Tubes were incubated at room temperature for 77 days, and during the incubation, they were shaken periodically to keep the medium oxygenated. At the end of the incubation, tubes were scored for growth (culture turbidity) by visual inspection, and an MPN of benzene-toluene degraders was determined from the appropriate 3-tube MPN table (Mayou, 1976).

3.4.2 Microbial Dehydrogenase Activity.

The electron transport system (ETS) test developed by Trevors et al. (1982) allows for a comparison of microbial activity in soil or sediments under varied incubation conditions. This test measures aerobic,

microbial dehydrogenase activity in the aquifer material. Experiments were run to compare activities between different sample locations under similar substrate conditions (e.g., gasoline-saturated water), and between different substrate conditions for the same sample location. The test is based on the reduction of water-soluble 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl chloride (INT) to methanol-extractable red iodonitrotetrazolium formazan (INT-formazan) by the microbial dehydrogenase activity of the aquifer sediment.

Ten grams (wet weight) of aquifer material were incubated in crimp-sealed 60 ml hypovials. Active, blank and sterile control treatments were included in each experiment. Active treatments received 1 ml of 0.4% (w/v) sterile INT solution, one or more carbon sources (gasoline-saturated water, 1/10 gasoline-saturated water, sterile 0.2% (w/v) glucose solution, or sterile R2A broth), nutrients (5 mg/L as N and 2 mg/L as P), and sterile distilled water, as required, so that each flask or vial received a total of 2.5 ml or 3.0 ml liquid. Sterile controls contained aquifer material that had been autoclaved for 1/2-1 hr on three consecutive days, and received 0.5 ml of 10% (w/v) sodium azide solution in place of sterile water. Blank treatments received water in place of INT solution. Each treatment was prepared in duplicate. Experiments were performed under aerobic conditions.

Flasks were sampled several times over ca. a 30 day incubation period by removing approximately 1 g of sand slurry, extracting the slurry with 5 ml methanol, and measuring the INT-formazan content spectrophotometrically (Trevors et al., 1982). An INT-formazan standard curve was generated using standards consisting of reagent INT-formazan in methanol. Blank-corrected INT-formazan content was reported on a dry weight basis.

3.5 Borden Field Experiment

3.5.1 Instrumentation

The field trials were performed within 2m by 2m by 3.5m deep Waterloo Barrier™ sealable, sheet-piling cells. Cells were installed by vibrating the individual sections of sheet piling to the target depth. The joints were then sealed with bentonite grout to isolate the interior of the cell from the surrounding aquifer. The base of the cell was open to the underlying aquifer.

Two treatment cells were used in these experiments: a Nitrate Cell (NO_3^-/O_2 amended) and a Control Cell (O_2 amended). Each cell contained instrumentation for groundwater sampling, water level measurements, gasoline injection, dewatering, geophysical measurements, and the addition and extraction of water. Cell instrumentation is shown on Figure 3-1. Most instrumentation was installed by advancing a steel casing to depth, washing the cuttings from the inside of the casing with flowing water, lowering the instrument, and then removing the steel casing. The formation was then allowed to collapse into the annular space. Drive points were vibrated into the ground using a pneumatic hammer.

The five multilevel piezometers in each cell were constructed of a series of 3.2 mm (1/8 in) stainless-steel tubes soldered to a 2.54 cm (1 in) stainless-steel center tube. Each piezometer had nine sampling ports, spaced 30 cm apart, between 30 and 270 cm bgs (below ground surface of cell). To avoid creating a preferential vertical flow path along the piezometer wall, the 3.2 mm tubes were placed inside the 2.54 cm center tube so that sample ports were flush with the center tube outside wall. Each cell contained five 2.86 cm (1-1/8 in) diameter stainless-steel drive point piezometers (depth 2.4 m; screen length 18 cm), used primarily for water level measurements. Each cell also contained three access tubes for geophysical measurements. The access tubes were constructed of 5.08 cm (2 in) diameter Schedule 40 PVC pipes (2.4 m depth) with solid 3.2 mm (1/8 in) stainless-steel rods embedded into the outside of the pipe. The rods were used to measure water content prior to the gasoline spill with time domain reflectometry (TDR).

To flush water vertically through the cells, both injection and extraction wells were also installed. Extraction wells were constructed of 2.86 cm (1-1/8 in) diameter stainless steel tubing flush-soldered to a 50-cm long drive-point screen; tops of screens were located 200 cm bgs. One centrally-located well was used in each cell for extraction of water (Figure 3-1). Water was injected into a high hydraulic conductivity layer located near the top of each cell. This layer was used as a means of distributing injected water horizontally without the use of multiple injection wells. It was installed by excavating the cells to ca. 60 cm, levelling the surface, and then backfilling with 8 cm of coarse sand, followed by 8 cm of 0.95 cm

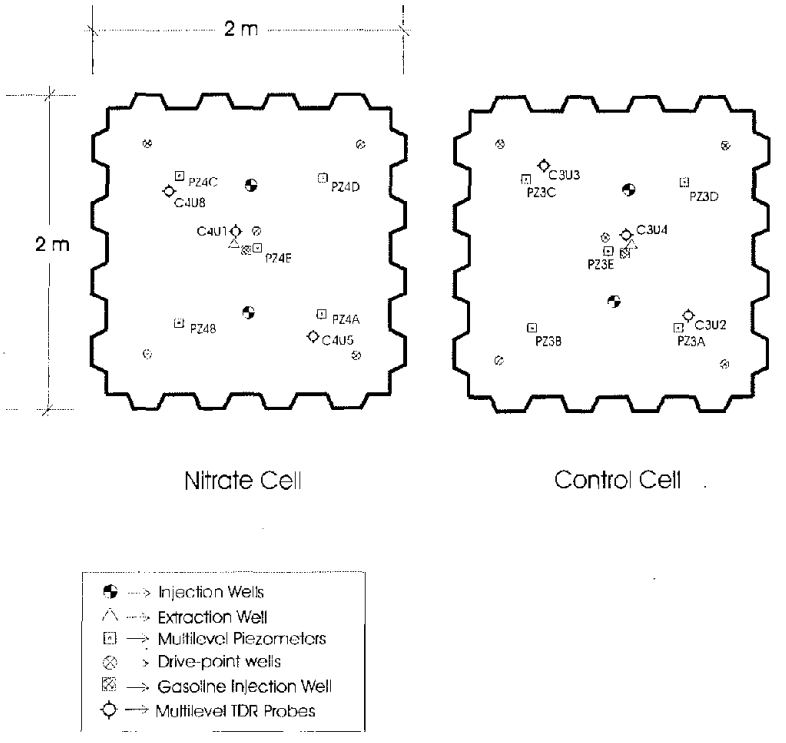


FIGURE 3-1 Plan view of treatment cells with surveyed locations of instrumentation.

(3/8 in) diameter pea gravel. A commercial filter fabric was then placed over the pea gravel and the cells backfilled with 5 cm of coarse sand followed by native Borden sand to surface. Prior to backfilling, a 5.08 cm (2 in) PVC injection well with a 30.5 cm (12 in) well screen was installed in each cell to a 65-cm depth so that the screened interval straddled the pea gravel layer. Both of these wells gradually lost transmitting capacity over the first 3.5 months of the experiment, and were replaced by stainless steel drive point wells with No. 10 slot well screens which operated for the remainder of the experiment (Figure 3-1).

A centrally-located drive-point gasoline injection well (depth 150 cm bgs; screen length 18 cm) and a 1.9 cm (3/4 in) diameter PVC dewatering well (depth 250 cm bgs; screen length 86 cm) were also installed in each cell (for clarity not shown on figure).

After the equipment was installed, plastic tarps were placed on cell surfaces as vapor barriers. Gaps around well casings were sealed with roofing tar or silicone. These sealants were not in contact with injected water or gasoline. The outer edges of the cells were sealed by packing a wedge of thick bentonite grout along the crenulated wall of the sheet piling. The grout was then covered with sand to slow desiccation. A monitoring and pumping-well network was also installed downgradient of the cells to detect releases and control the plume, if necessary (Figure 3-2). Periodic sampling of downgradient multilevel piezometers indicated that gasoline releases did not occur (Appendix D). To prevent infiltration of rainwater, a greenhouse was constructed over the cells.

3.5.2 Gasoline Injection

The objective of the spills was to emplace a source of gasoline below the water table as a spatially-uniform, residual phase so abiotic losses (volatilization, physical removal during high water table events) and preferential flow of water around gasoline-contaminated zones would be minimized. To emplace the

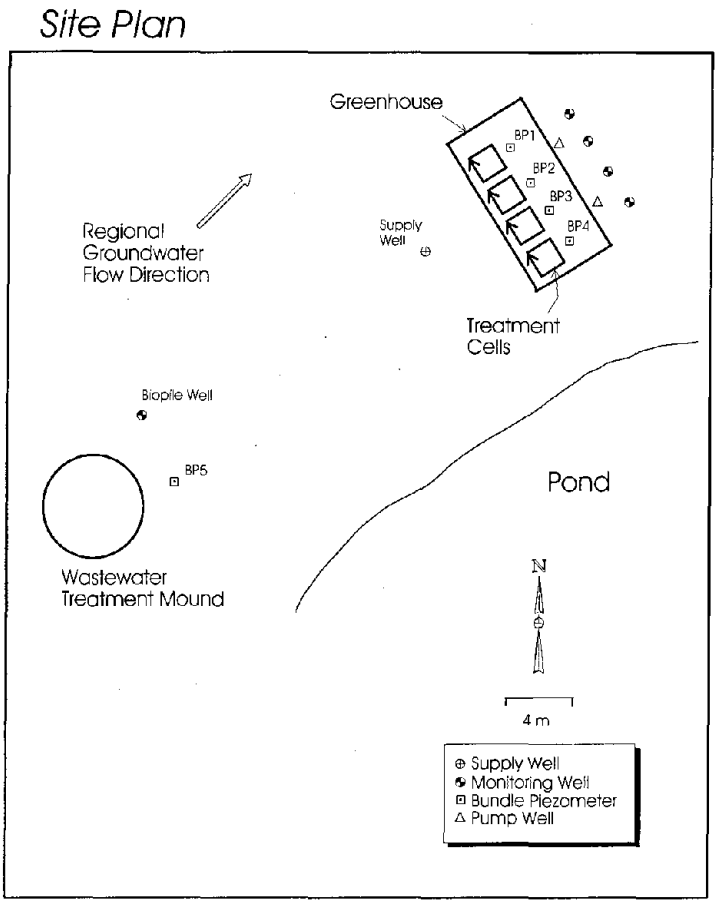


FIGURE 3-2 Plan view of study area.

sources, the water table was lowered, the gasoline gravity-fed into the gasoline injection wells, and the water table allowed to recover to the pre-spill elevation.

Seventy liters of API 91-01 gasoline (Appendix A) were injected into each cell during October, 1995. Prior to injection, the ambient water table was approximately 70 cm bgs. The water table was lowered using pumps connected to the four corner drive point wells, and the PVC dewatering well. The Control Cell was dewatered for 29 hrs and the Nitrate Cell for 47 hours prior to injection (injections occurred on successive days). In both instances target water table depths (Nitrate Cell: 175 cm bgs; Control Cell: 180 cm bgs) were reached and maintained for several hours prior to gasoline injection to give the moisture content profile time to respond to the falling water table and approach a condition of static equilibrium. The target water table depth was chosen so that the top of the capillary fringe would be roughly coincident with the base of the gasoline injection well. The thickness of the capillary fringe is about 30 cm in the Borden aquifer (Nwankwor et al., 1992). Water content profiles collected with the multilevel TDR probes 1-3 hrs before injection show that the top of the capillary fringe was slightly higher than anticipated in both cells (Figure 3-3). A description of the methods used to collect and process the TDR data is provided by Oliveira (1997).

Gasoline was gravity-fed into the gasoline injection wells from a sealed, polyethylene tank. The durations of the injections were 5 hr 45 min for the Nitrate Cell, and 7 hr 45 min for the Control Cell. A partially-clogged flow meter was responsible for the slower rate of injection into the Control Cell. Groundwater extraction from the center drive-point well (screen depth 222-240 cm bgs) continued throughout the injection. However, these wells were incapable of sustaining the required extraction rate, and the water table rose ca. 10 cm in both cells during the injections. Extraction wells were turned off immediately after the injection of gasoline was completed. After about two weeks, the water table had

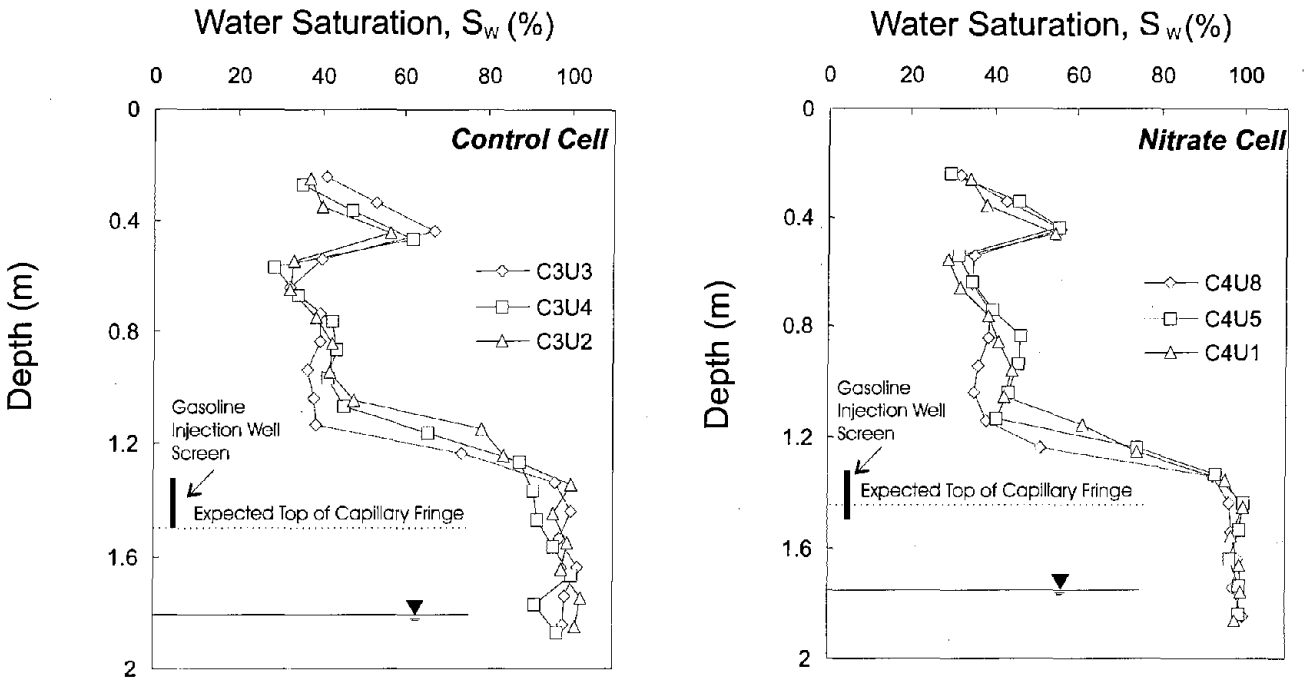


FIGURE 3-3 Vertical profiles of water saturation prior to the gasoline injections. Profiles were obtained 1-3 hours before injection with multilevel TDR probes.

recovered to pre-injection levels. Because the injection procedure involved dewatering, a trapped air phase was probably also present below the water table. The mass of O_2 in the trapped air phase was estimated and incorporated into mass balance calculations (Section 5.2). The extent of volatilization that occurred during gasoline injection could not be quantified, but based on relatively short contact times with the atmosphere during injection and analysis of groundwater from gasoline-contaminated zones one month after injection (see below), volatilization losses of target compounds appeared minor.

Detailed measurement of the *in situ* magnitude and structure of the trapped gasoline and air phases was considered beyond the scope of the research. A detailed characterization of their distribution was therefore not attempted, but a general understanding of the distribution of the injected gasoline was developed from field observations, groundwater samples, and core extract data. The distribution of aqueous-phase BTEXTMB concentrations during November, 1995, one month after injection, is shown in Figures 3-4 and 3-5. These figures suggest that the gasoline phase did spread radially outward from injection wells as intended, and was present mainly in the 120 cm to 60 cm bgs depth interval. The gasoline contaminated zones were thickest in the vicinity of the injection wells, extending to a depth of approximately 150 cm bgs. These initial observations were confirmed by the results of the post-experiment coring (Chapter 5). However, complete trapping below the water table apparently did not occur; after recovery to the pre-injection head, a thin layer of gasoline was observed on the water table in the Control Cell. This indicated that the 110 cm recovery following injection did not trap all of the gasoline below the water table. During the November sampling round, it was also observed that a mobile gasoline phase (i.e., above residual saturation) was present near some of the sampling ports; some samples contained a gasoline/water mixture, as indicated by field observations and concentrations well above those in gasoline-saturated water (Figures 3-4 and 3-5). Overall, however, the injections were successful in emplacing a source below the water table, and the gasoline distributions were suitable for meeting the goals of the experiment.

During the following Spring snowmelt, groundwater with a sheen of gasoline was observed above the surfaces of the cells. The amount of gasoline removed from the cells during this event could not be determined, but visual observations and samples of the standing water suggested that losses were minor. In addition, the high water table may have trapped some gasoline above the gasoline-contaminated zone

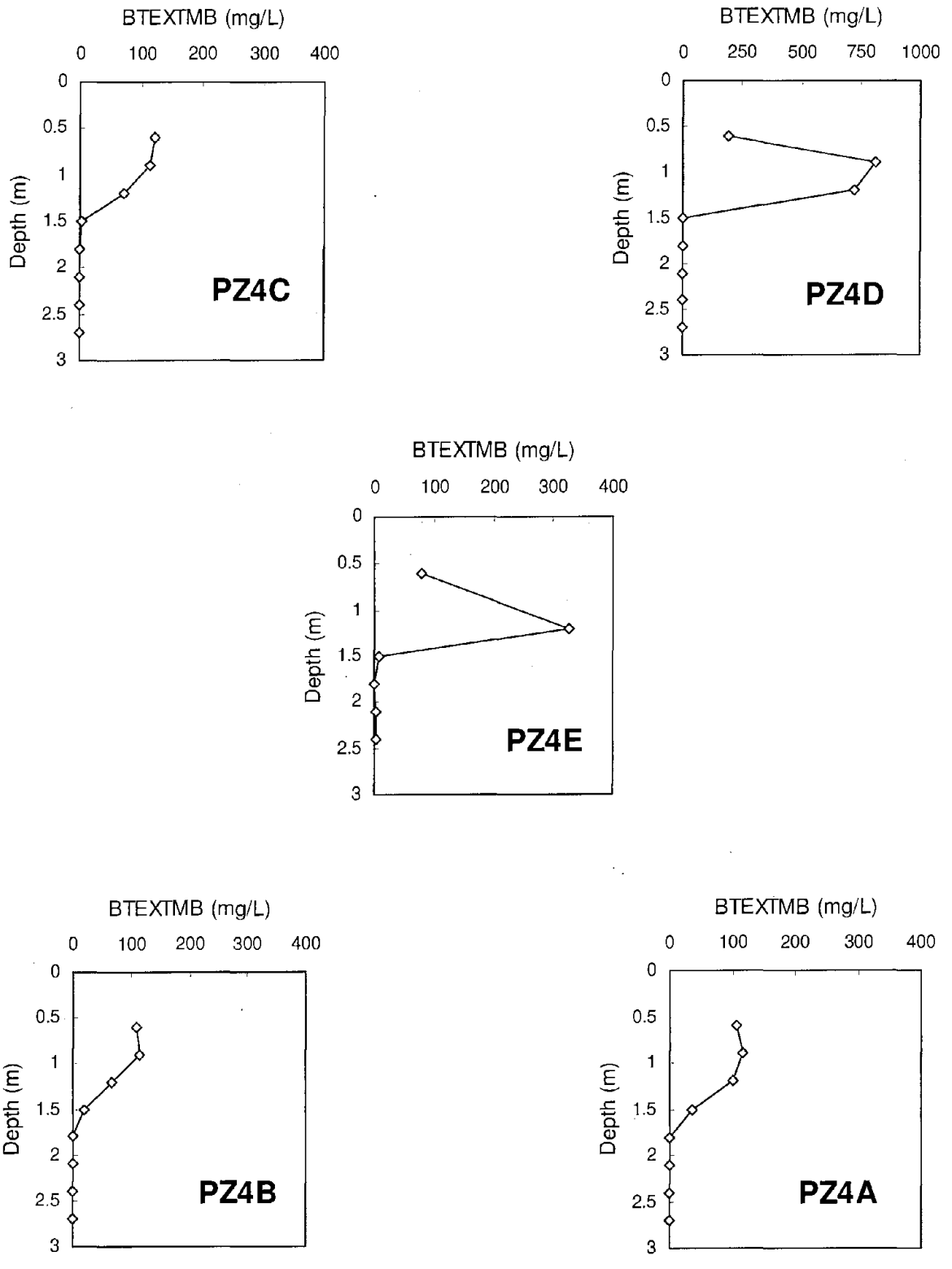


FIGURE 3-4 Vertical profiles of total dissolved BTEXTMB in the Nitrate Cell on November 22-26, 1995, approximately one month after gasoline injection. The water table depth was approximately 50 cm bgs. Note scale change for piezometer PZ4D.

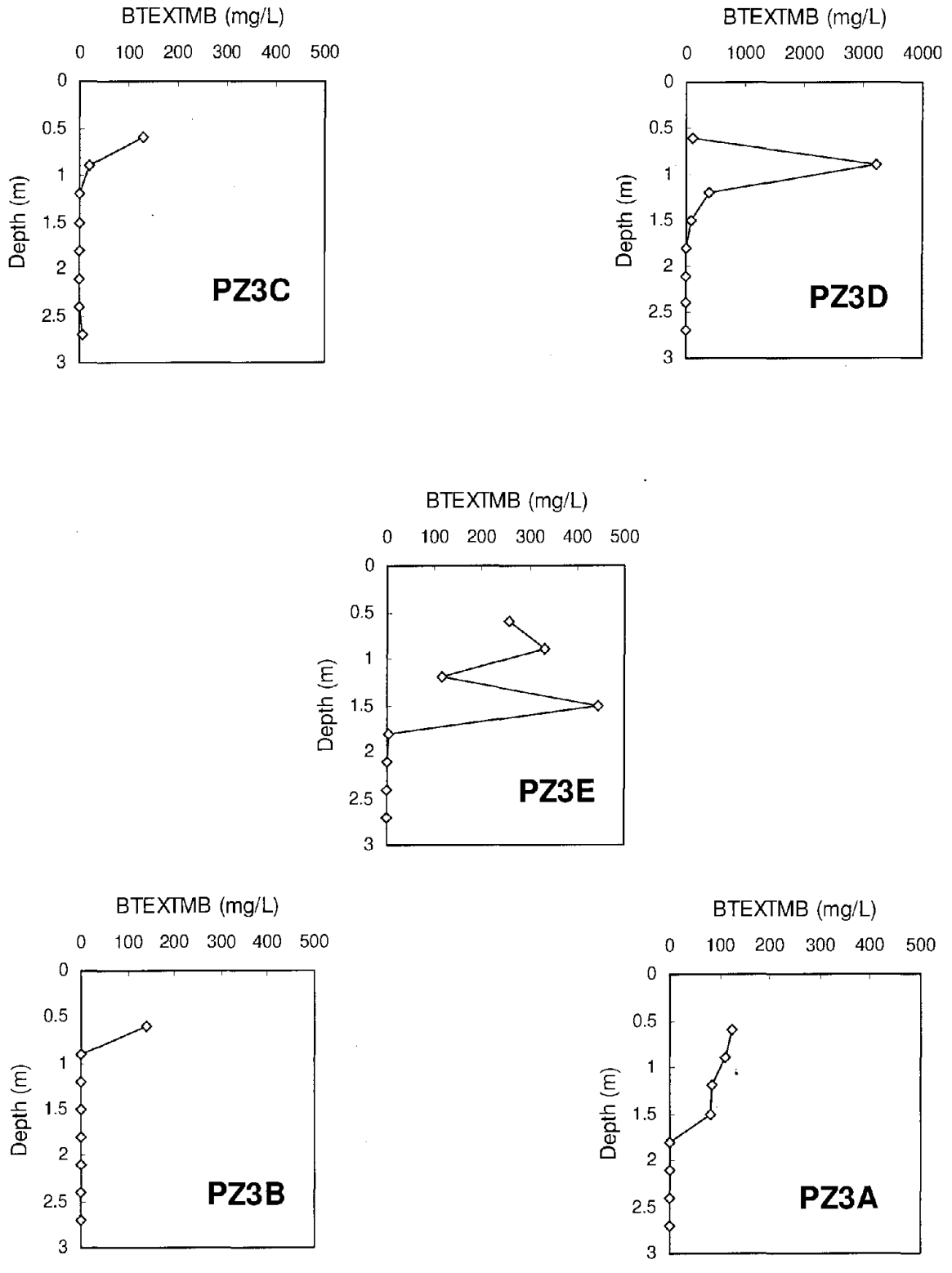


FIGURE 3-5 Vertical profiles of total dissolved BTEXTMB in the Control Cell on November 22-26, 1995, approximately one month after gasoline injection. The water table depth was approximately 50 cm bgs. Note scale change for piezometer PZ3D.

contacted by amended water during the flushing experiments. These regions were cored at the end of the experiment and any mass that was present was included in mass balance calculations.

3.5.3 Experimental Design

Injection Procedures. To obtain data that could be used for a mass balance, injection systems were designed to flush water continuously through the cells at a constant flux. A schematic of the injection system is shown in Figure 3-6. A vertically-downward flow field was created by injecting into the pea gravel layer (50 cm bgs), and extracting from the extraction well (screen: 200-250 cm bgs) (Figure 3-6). The length of the vertical flowpath was about 1.75 m. Constant lower head conditions were maintained by water level controllers (SSAC Inc., LLC5 Series) which automatically cycled the extraction pumps on and off. The injection pumps operated continuously. By adjusting the elevation of the lower heads, upper heads in both cells were maintained at about 50 cm bgs under constant injection conditions, despite fluctuating water table elevations in the aquifer.

During the first week of the experiment, water was obtained from a shallow upgradient well located in a flooded area. This well appeared to be pumping surface water which was fully oxygenated. To obtain groundwater with lower dissolved O_2, a shallow PVC well (2.49 m total depth) located approximately 6 m upgradient of the cells was used as the water supply for the remainder of the experiment (Figure 3-2). To inject the same dissolved oxygen concentration into both cells, a single 0.635 cm (1/4 in) diameter polyethylene tube was installed in the well as the intake line. This line was then split with a Y-connector, and lines were run to individual injection peristaltic pumps (Masterflex L/S Series). The Control Cell received no additional amendments prior to injection; the Nitrate Cell received NO_3^- via a Swagelok® fitting located downstream of the injection pump.

Dissolved oxygen was controlled by placing an aerator tube in the supply well. The aerator was located just above the water intake tube to minimize incorporation of bubbles. The target dissolved O_2 concentration range was 2-4 mg/L. This arrangement provided reasonable control over dissolved O_2 concentrations, but also appeared to stimulate growth of unidentified bacteria (possibly Fe oxidizers) in the supply well, injection tubing, and injection wells. During the final 24-day flushing experiment (May 1997), the injected dissolved O_2 concentration was increased to ca. 5 mg/L by switching the water supply from groundwater to the pond adjacent to the site.

The injection NO_3^- concentration was controlled by pumping a continuously-stirred, concentrated NO_3^- stock solution into the injection flow line. A dedicated peristaltic pump (Masterflex L/S Series) was used to feed stock solution into the line. The pumping rate was set to yield a 100x dilution. The stock tank held 40 L of solution and was replenished every two weeks. The initial target injection concentration was 150 mg/L NO_3^-, but because utilization was low, the target concentration was lowered to 100 mg/L on day 67. During the 24-day flushing experiment, the possibility that biotransformation was nutrient-limited was investigated by pumping a diluted, modified Bushnell-Haas (MBH) medium (HK_2PO_4, H_2KPO_4, NH_4NO_3, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$) (Mueller et al., 1991) into both cells using a concentrated stock solution and dedicated feed-pump as above. Because of pump malfunctions, the solution was pumped in intermittently over the 24 day period. Actual injected concentrations are not known because complete dissolution of the concentrated stock solution could not be maintained.

Injection flow rates were set by calibrating the peristaltic pump controllers, and measured periodically (n=22) using a stopwatch and graduated cylinder. During the first two months of the experiment, target flow rates were adjusted periodically to achieve steady flow conditions and the desired residence time. Target flow rates and actual measurements are shown on Figure 3-7. To calculate a mean flow rate representative of the entire 174-day flushing experiment, a mean and standard deviation were calculated for each of the three target injection-rate periods (Figure 3-7). An overall mean was then calculated from the individual mean injecti1on rates, and a pooled standard deviation from the individual standard deviations. These calculations yielded rates of 250 ± 14 ml/min and 237 ± 23 ml/min for the Nitrate and Control cells, respectively. The uncertainty is the standard deviation of the temporal distribution of injection rates. These injection rates correspond to residence times of about 9 days, or 20 treatment-cell pore volumes over 174 days. On the basis of a measured rate of 200 ml/min (n=1), an additional three pore volumes were removed from each cell during the 24-day flushing period the following spring.

Injection System

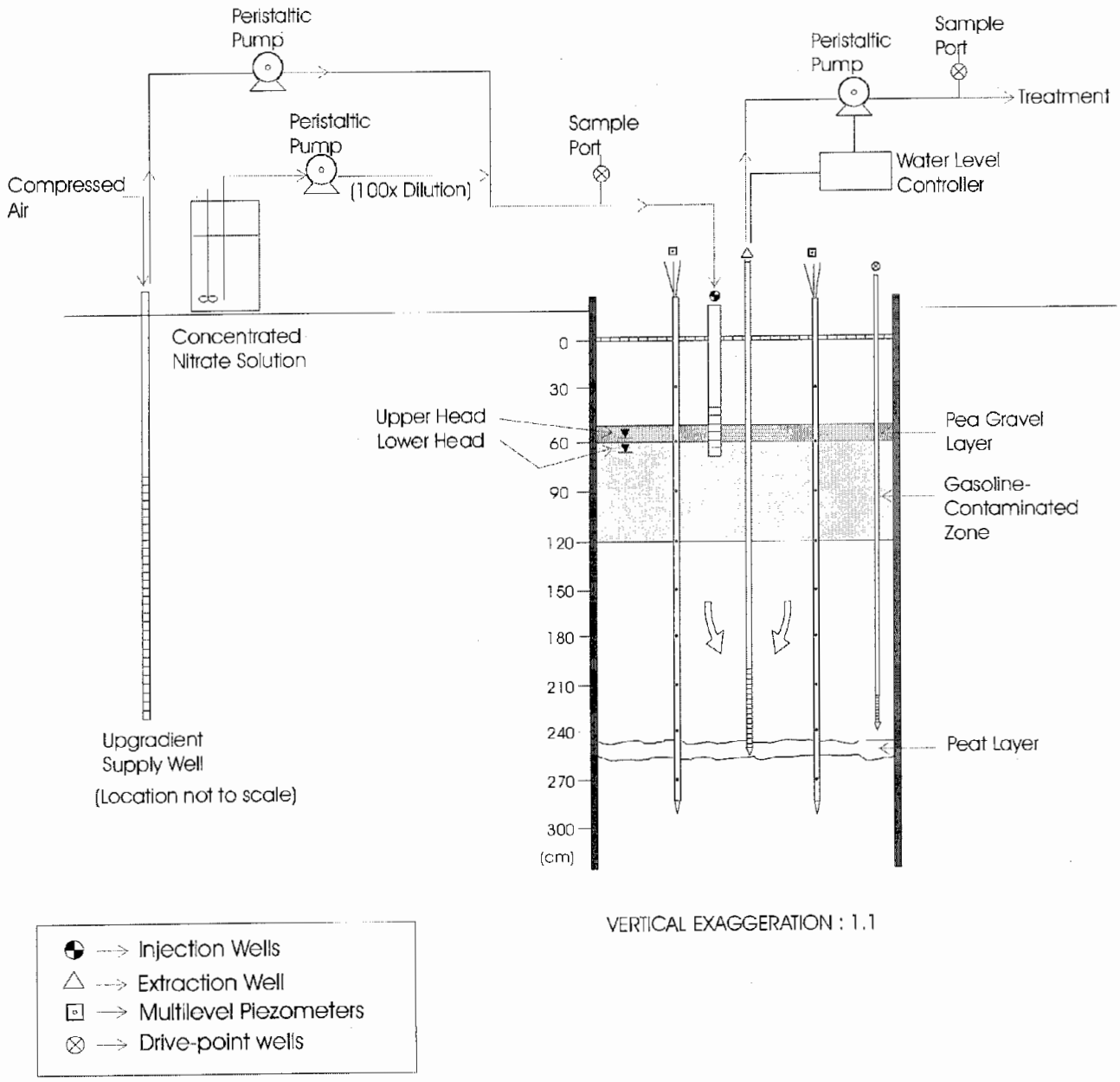


FIGURE 3-6 Injection system schematic for the Nitrate Cell. Cross section of cell shows selected instrumentation to illustrate positions of injection/extraction wells and multilevel piezometer ports. The system for the Control Cell was identical except for NO_3^- addition equipment.

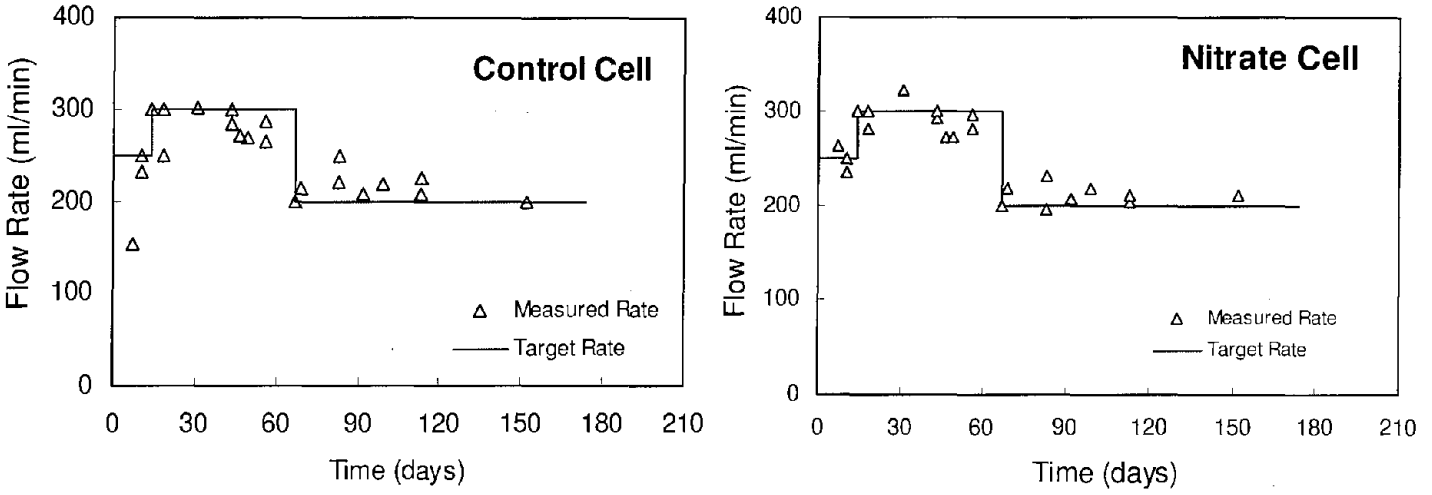


FIGURE 3-7 Record of injection rates over the 174-day flushing experiment.

Peristaltic pumps (Masterflex L/S Series) were also used to extract groundwater. Extracted groundwater was pumped into a polyethylene holding tank, and then to an unsaturated soil mound located 30 m upgradient of the treatment cells (Figure 3-2). In previous studies at Borden, infiltration through the unsaturated zone was shown to be an effective means of stimulating aerobic biotransformation of aromatic hydrocarbons (O'Leary et al., 1995). The absence of detectable BTEXTMB in a downgradient multilevel piezometer and well showed that infiltration through the mound effectively attenuated these compounds (Appendix D).

To operate continuously the injection system required regular maintenance, mainly pump tubing replacement and injection/extraction well cleaning. During the first few months of the experiment, unanticipated problems such as power outages led to several shutdowns on the order of 24 hours duration. Routine maintenance, however, required the injection systems to be shut off a few times a week for only 1 hour.

Tracer Test. During July, 1996, tracer tests were performed in both treatment cells (Appendix B). The main objectives of the test were to confirm that the injected water was distributed uniformly in the cells (i.e., minimal short-circuiting), and to calculate dispersivities and linear groundwater flow velocities.

3.5.4 Field Sample Collection

Groundwater Samples. Transport of dissolved organics and added electron acceptors was monitored by routine sampling for BTEXTMB, dissolved O_2, NO_3^-, and NO_2^-. Samples were collected biweekly from the five piezometer ports at the 60 cm bgs and 180 bgs depths. Monthly vertical profiles from the center piezometers were also determined. Changes in geochemical conditions were determined by less frequent analysis of one or more of the following: pH, temperature, alkalinity, Fe, sulfate (SO_4^{2-}), HS^-, and CH_4.

Groundwater samples were collected from the small piezometer tubes with a peristaltic pump. BTEXTMB samples were collected in 18-ml crimp-top glass vials clamped onto an in-line stainless-steel sampling head (Figure 3-8). Previous tests indicated that there was no significant bias introduced by this sampling procedure (Barker et al., 1987). In this experiment cross-contamination between sampling ports was minimized by proceeding from low-concentration to high concentration locations, and cleaning the sampling head with acetone and distilled water after sampling highly-contaminated ports. In addition, because the 18-ml vial remained in-line while the D.O. and inorganic samples were collected, a minimum of 100 ml of groundwater were flushed through the system prior to obtaining an organics sample. Blanks collected periodically by flushing organic-free distilled water through the sampling apparatus confirmed that cross-contamination was negligible. Each BTEXTMB sample was preserved with 0.1 ml of 10% (w/v) sodium azide solution, sealed without headspace with a Teflon-faced septum and aluminum crimp seal. Samples were refrigerated until analysis, which typically occurred within 3 days of collection.

After pumping ca. 25 ml of water to flush the piezometer tubes of stagnant water, dissolved O_2 concentrations were measured colorimetrically in the field (indigo-carmine method (Gilbert et al., 1982)). Samples were collected under low-flow conditions from a flow-through sample cup attached to the end of the peristaltic-pump outlet tube (Figure 3-8). Samples were typically colorless, and free of turbidity. To obtain a sample, a CHEMetrics Vacu-Vial® O_2 ampoule was inserted in the flowing stream of water and filled. The concentration was read in a CHEMetrics VVR spectrophotometer, using an O_2-specific filter that automated sample quantification. Two filters were used in this study: a 0-15 mg/L range filter, and a 0-2 mg/L range filter. Each filter contained a pre-programmed calibration curve, and did not require daily calibration. The accuracy of the calibration was tested in the laboratory using air-sparged water (aerobic) and a 2% (w/v) sodium sulfite solution (O_2-free). The ambient temperature of the laboratory was 24°C. This test yielded concentrations of 7.54±0.18 mg/L (n=5) for the aerobic water, and 0.0±0.0 (n=5) for the O_2-free water. At this temperature the concentration of dissolved O_2 in equilibrium with air (one atmosphere total pressure) is 8.25 mg/L (Drever, 1992). The majority of the field samples were analyzed with the 0-2 mg/L range filter. The specified method detection limit (MDL) for this filter was 0.05 mg/L dissolved O_2.

If required, samples for inorganic parameters were then collected from the outlet tube of the peristaltic pump (Figure 3-8). Nitrate samples were collected in 18-ml scintillation vials and preserved with two drops of formaldehyde. Iron and SO_4^{2-} samples were collected in 30-ml glass syringes and then filtered (0.45 µm) into scintillation vials. Samples for Fe analysis were acidified with 2-3 drops of concentrated hydrochloric acid. Groundwater samples for dissolved CH_4 analysis were collected without headspace in 40-ml screw-top glass vials. All of these samples were shipped back to the University of Waterloo for analysis (Appendix C).

Total dissolved sulfide concentrations were also measured in the field using a CHEMetrics colorimetric analysis (MDL=0.06 mg/L) in a manner similar to D.O. Total alkalinity as $CaCO_3$ was measured in the field with a Hach Digital Titrator (Model 16900).

Injection/Extraction Samples. Concentrations of electron acceptors injected into the cells were determined by frequent (daily to bi-weekly) sampling of injection water for NO_3^-, NO_2^-, and dissolved O_2.

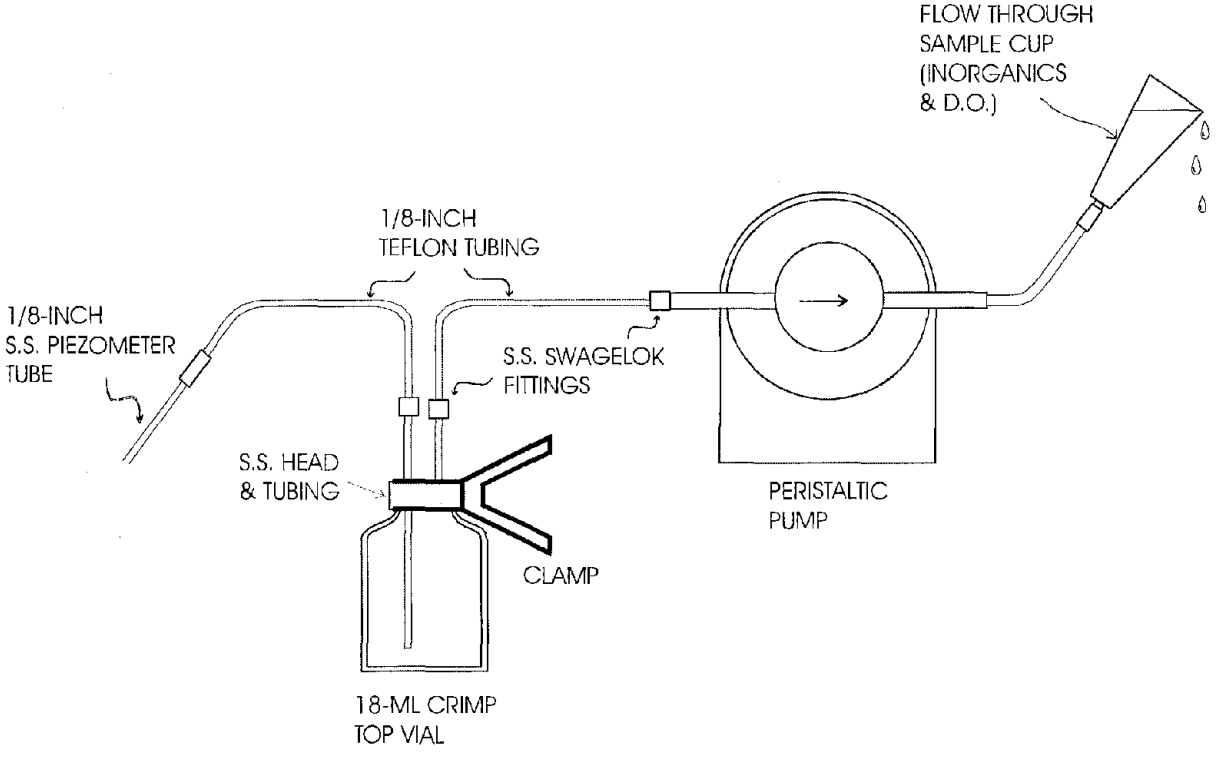


FIGURE 3-8 Schematic of groundwater sample collection apparatus. Dissolved O_2 samples collected from sample cup under flowing stream of water.

To provide a baseline, the injection stream was also sampled at least once for dissolved organic carbon (DOC), pH, temperature, alkalinity, Fe, SO_4^{2-}, HS^-, and BTEXTMB. Similarly, extraction water was analyzed regularly for NO_3^-, NO_2^-, dissolved O_2, and BTEXTMB, and less frequently for the inorganic parameters listed above. Samples of injection water were collected directly from the end of the injection tubing, and extraction water from a sampling port (Figure 3-6). Collection procedures and preservatives were as described in the preceding paragraphs.

Metabolites. Evidence for biotransformation was obtained by analyzing samples for hydrocarbon metabolites. These compounds (aromatic and aliphatic acids and phenolic compounds) result from incomplete mineralization of various hydrocarbon constituents. Three sets of samples were collected, all under static conditions: May 1997, early June 1997, and late June 1997. The first two sets of samples were shipped to the National Center for Integrated Bioremediation (NCIBRD) at the University of Michigan, Ann Arbor for analysis, and the third to the NRMRL, Ada, Oklahoma.

Samples were collected from two locations within each cell, and the groundwater supply well. A fresh sample of API 91-01 gasoline was also analyzed by NRMRL for these compounds. Samples were collected from the peristaltic pump outlet tube in 500 ml amber bottles, preserved with either KOH to pH=11 or 1% (w/v) trisodium phosphate solution, and refrigerated until shipment.

Aquifer Cores From Treatment Cells. When the field experiments were completed, cores were collected to estimate the mass of BTEXTMB remaining in the treatment cells. Core subsamples were immersed in solvent immediately after collection to obtain an estimate of total contaminant mass in the aquifer sample, including mass in the sorbed, aqueous, and residual gasoline phases. Because a non-wetting phase liquid at residual saturation in a homogeneous sand occurs as disconnected singlet and multi-pore blobs (Chatzis et al., 1983), which are readily transferred to a contacting solvent phase, a one-step extraction was used in this study: Prior to analysis, gasoline was extracted into the methanol phase by shaking samples for 15 min at 300 rpm on an orbital shaker, and then sonicating for 3 min to remove any emulsification that may have been present in the methanol. This method was similar to other published field solvent-extraction approaches (Ball et al., 1997; Vandegrift and Kampbell, 1988). For aquifer samples with low concentrations of organics, the efficiency of the extraction, particularly of the sorbed phase, is critical for obtaining accurate results (Ball et al., 1997). In this experiment, on the other hand, there was a large absolute amount of mass (ca. 7 kg BTEXTMB recovered in each cell), and a high percentage of this mass was necessarily present within the gasoline phase; the sorbed and aqueous phases contributed negligible mass to the total estimate. Based on the above reasoning, if a negative bias was associated with the extraction technique (i.e., less than 100 % recovery), it was probably small relative to the total mass in the sample, as well as other uncertainties in the mass balance.

Grids consisting of nine equal areas were used to determine core locations. One core was collected from the center of each area. An additional core was collected from a random location near the center of each cell. Core runs were typically from ground surface to 152 cm (5 ft) with recoveries averaging 117 cm and 124 cm from the Control and Nitrate cells, respectively. A deeper interval, 152 cm to 305 cm (5 ft to 10 ft), was also obtained from the cores at cell centers (cores 3J and 4K). For most cores eight uniformly-spaced subsamples were collected, corresponding to one subsample per 15-cm interval. Mass balance calculations were therefore based on approximately 80 sample locations per cell.

Subsamples of aquifer material were collected within 15 minutes of core extraction. To obtain these samples, a 2-cm wide strip of the core barrel was removed with a circular saw to expose the aquifer material. At each sampling location the outer layer of sand was scraped away, and a ca. 10-g plug of sand was obtained by inserting the barrel of a dedicated, plastic 10-cc syringe into the core. The plug of sand was then quickly extruded into a pre-weighed 40-ml screw-top glass vial containing 15 ml methanol, re-capped, and refrigerated until analysis. Sample bottles were weighed before and after methanol addition to accurately determine the mass of methanol. After sample collection, bottles were weighed again to determine the wet weight of the aquifer sample. Methanol was dispensed into sample bottles no more than 36 hours prior to use, and stored in the refrigerator to minimize volatilization. There were no samples with visible loss of methanol. Samples were analyzed one week after collection.

To estimate depletion of other gasoline constituents, a total of six additional subsamples of core material were collected from randomly-selected locations in both cells for a semiquantitative analysis of gasoline components (i.e., grouped as alkanes, aromatics, bicycloalkanes, naphthenes, olefins, and PNAs). The core subsamples were extruded into 40-ml screw-top glass vials containing 25-ml deionized water and refrigerated until shipment to the NRMRL for analysis. For comparison, fresh API 91-01 gasoline and an aliquot of Borden sand spiked with API 91-01 were also analyzed for these component groups. Details of the analytical method are provided elsewhere (Hutchins et al., 1998).

CHAPTER 4. LABORATORY EXPERIMENTS

The results of the laboratory experiments are summarized in this chapter. Each of the microcosm experiments listed in Table 1-1 is described in detail here. Section 4.1 includes all of the microcosm experiments performed with pristine Borden sand. Experiments 1, 2, and 3 were performed before the field experiment design was finalized. Experiments 4 and 5 were set up after the field experiment was designed and the gasoline spilled at Borden, but before the field data collection was initiated. Section 4.2 includes the follow-up microcosm experiment performed with aquifer material collected from the Nitrate Cell. Because of the large amount of detailed information, a results and discussion section is provided for each individual microcosm experiment described in these sections. Section 4.3 contains results of all of the microbiological characterization work performed over the course of this study. The major conclusions from all of the microcosm experiments and microbiological characterization studies are summarized in Table 4-1. The final section (Section 4.4) includes a general discussion of the experimental work performed in the laboratory.

In discussing these experiments, the term "denitrifying activity" is used in a general sense to define any dissimilatory NO_3^--reducing activity occurring under anaerobic conditions. The term "denitrification" is only used when an assay was performed to confirm that NO_3^- was reduced to gaseous end products. Except where noted in Section 4.2, NO_3^- depletion under anaerobic conditions is assumed to be a dissimilatory process where NO_3^- is utilized as an electron acceptor. This assumption is consistent with previous experiments in which Borden aquifer material was amended anaerobically with aromatic hydrocarbons and NO_3^- and denitrification was confirmed with an assay (e.g., Barbaro et al., 1992).

4.1 Microcosm Experiments: Pristine Borden Sand

Because this study included a controlled release of gasoline in the field, Borden aquifer material from an existing spill was not available for preliminary laboratory experiments. Thus the experiments that provided baseline information on electron-acceptor and aromatic hydrocarbon utilization were performed with aquifer material that had no known prior exposure to hydrocarbon contamination. The microbial community in a low-carbon environment such as the Borden aquifer may require time to adapt to the unfavorable conditions created by a hydrocarbon spill (Chapelle, 1993). It was anticipated therefore that the hydrocarbon-degrading capabilities of contaminated aquifer material would increase with exposure. This provided an opportunity to compare aromatic-hydrocarbon and electron-acceptor utilization in pristine and contaminated Borden sand for evidence of acclimation of the indigenous microbial population.

4.1.1 Experiment 1: Effect of Oxygen, Nitrate Concentration, and Nutrients

Biotransformation of aromatic hydrocarbons under NO_3^--reducing conditions was previously demonstrated in the Borden aquifer , in the deeper, anaerobic zone affected by leachate emanating from an abandoned landfill (Barbaro et al., 1992). The current field experiment was designed to take place in the upper, pristine aquifer, which is generally aerobic in character, although low dissolved O_2 zones are present. This laboratory experiment was designed to evaluate the potential for aromatic hydrocarbon utilization at the expense of NO_3^- and O_2 in this upper aquifer material. Treatments were set up to screen for O_2-supported and NO_3^--supported BTEX degradation, to investigate the extent of mass loss under a range of NO_3^- concentrations, and to determine whether added inorganic nutrients (N and P) influenced BTEX biotransformation.

Design 2 microcosms (Section 3.2.1) were prepared using pristine Borden sand obtained in the saturated zone approximately 1 m to 1.5 m below ground surface, and were amended with an aliquot of a neat BTEX mixture (B:T:E:*p*-X:*m*-X:*o*-X in ratios of 3:2:1:1:1:1) to about 12.4 mg/L BTEX. Microcosms designed for repeated sampling were advantageous for this application because a broad range of experimental conditions could be evaluated simultaneously in an experiment of practical size. The

Table 4-1. *Summary of Major Conclusions from Laboratory Experiments*

Report Section	Experiment	Major Conclusions
4.1.1	Effect of O_2, NO_3^- concentration, and inorganic nutrients (Experiment 1)	Aerobic BTEX biotransformation initiated readily in Borden aquifer material; biotransformation under denitrifying conditions more limited, with only toluene and ethylbenzene degrading consistently; addition of inorganic N and P was needed for optimal aerobic degradation of 12.4 mg/L BTEX, but had no discernable effect on anaerobic activity
4.1.2	Comparison of BTEX biotransformation under denitrifying conditions in CFB Borden, Eglin AFB and Park City aquifer microcosms (Experiment 2)	Aromatic hydrocarbon biotransformation under denitrifying conditions was very limited in pristine Borden material compared to two other petroleum-hydrocarbon contaminated sites
4.1.3	Biotransformation of BTEX in gasoline-contaminated groundwater under denitrifying, microaerophilic, and mixed electron-acceptor conditions (Experiment 3)	Patterns of BTEX, O_2, and NO_3^-/NO_2^- concentrations suggested that O_2 and NO_3^- were used sequentially under mixed electron-acceptor conditions; most BTEX biotransformation occurred early, likely at the expense of microaerophilic O_2; additional TEX losses occurred later under mixed electron-acceptor, and denitrifying conditions; benzene losses under microaerophilic conditions were minimal
4.1.4	Effect of BTEXTMB concentration under microaerophilic / NO_3^- conditions in pristine Borden aquifer material (Experiment 4)	Negligible BTEXTMB losses at gasoline-saturated aqueous concentrations, likely at the expense of microaerophilic O_2; patterns of NO_3^- uptake suggested denitrifying population in pristine material inhibited by high substrate concentrations
4.1.5	Effect of O_2 concentration under high BTEXTMB concentration conditions in pristine Borden aquifer material (Experiment 5)	Aerobic biotransformation at gasoline-saturated aqueous concentrations highly variable in pristine aquifer material; may indicate patchy distribution of aerobic populations tolerant of high substrate concentrations
4.2.1	Extent of biotransformation under various substrate and mixed electron-acceptor conditions in gasoline-contaminated Borden aquifer material extracted from Nitrate Cell (Experiment 6)	Large O_2 demand and BTEXTMB mass loss relative to pristine aquifer material; nitrate possibly used as N source during aerobic degradation; relatively rapid continued NO_3^- utilization following O_2 depletion, but no discernable utilization of aromatics; differences between pristine and contaminated aquifer period suggested acclimation had occurred with large potential O_2 demand; minor mass loss under microaerophilic / NO_3^- conditions consistent with *in situ* observations
4.3.1 and 4.3.2	Microbial characterization results	Numbers of culturable aerobic heterotrophs and denitrifiers in Borden aquifer variable but consistent with other pristine shallow aquifers; microbial numbers in treatment cells moderately elevated relative to pristine background locations; aerobic dehydrogenase activity assays suggested that microbial activity was partially inhibited by gasoline phase

Table 4-2. *Design Summary, Experiment 1*

Treatment	NO_3-N (mg/L)	Inorganic Nutrients[1]	Replicates
Aerobic			
Active	0	+ N, P	1
Active	0	- N, P	1
Active	25	+ N, P	4
Anaerobic			
Sterile[2]	25	+ N, P	2
Sterile	25	- N, P	1
Active	0	+ N, P	2
Active	0	- N, P	1
Active	5	+ N, P	2
Active	5	- N, P	1
Active	25	+ N, P	7
Active	25	- N, P	1
Active	25	+ mineral salts	2
Active	25	- mineral salts	1
Active	50	+ N, P	1
Active	50	- N, P	1

[1] N, P: 5 mg N + 2 mg P per L, as NH_4Cl and KH_2PO_4 mineral salts: 2 mg K_2HPO_4, 2 mg KH_2PO_4, 3.3 mg NH_4Cl, 0.4 mg $MgSO_4.7H_2O$, 0.04 mg $CaCl_2.2H_2O$, 0.01 mg $FeCl_3.6H_2O$ per L (0.8 mg P + 0.86 mg N per L).

[2] autoclaved aquifer solids + poisoned (0.01% $HgCl_2$) groundwater.

experimental design is summarized in Table 4-2. The design called for multiple replicates for each treatment, but during microcosm preparation, stock solution was incorrectly added to some of the aerobic microcosms. As a result, an aerobic treatment that contained NO_3^--N but was lacking N and P was not prepared. Consequently, utilization of NO_3^--N as a nutrient during aerobic biotransformation could not be evaluated in this experiment. Strong evidence for assimilatory NO_3^- reduction was obtained in a subsequent experiment (Experiment 6; Section 4.2.1). Microcosms were sampled periodically over a 250 day period.

Microcosms incubated within the anaerobic chamber were removed for sampling, which was conducted by syringe under flowing argon gas, then returned to the chamber. It was hoped that use of threaded rather than older push-type mininert™ valves might minimize microcosm leakage, but problems were encountered with some vials, presumably as they were passed through the evacuation chamber of the anaerobic glovebox after sampling. All subsequent laboratory experiments were therefore conducted using sacrificial (Design 1) microcosms. The $HgCl_2$ solution used in sterile control microcosms was found to interfere with the NO_3^- analysis, and therefore, concentration data were not obtained for controls. Consequently, a sodium azide solution was used in sterile control microcosms in all subsequent laboratory experiments (Experiments 2 through 6). On the basis of these later experiments, NO_3^- utilization was negligible in sterile controls.

Results and Discussion. As expected, based on earlier CFB Borden studies (e.g., Barker et al., 1987), BTEX concentrations decreased rapidly in active, aerobic microcosms to below detection by day 8 (Figure 4-1). A second BTEX amendment on day 32 (Figure 4-1, Arrow) was also rapidly metabolized in NH_4-N and PO_4-P-containing microcosms, in both presence and absence of NO_3^-. Inorganic nutrient addition was necessary to obtain maximum degradation under the experimental conditions evaluated here. In the absence of N and P, only slow biotransformation occurred (Figures 4-1 and 4-2), with *m*- and

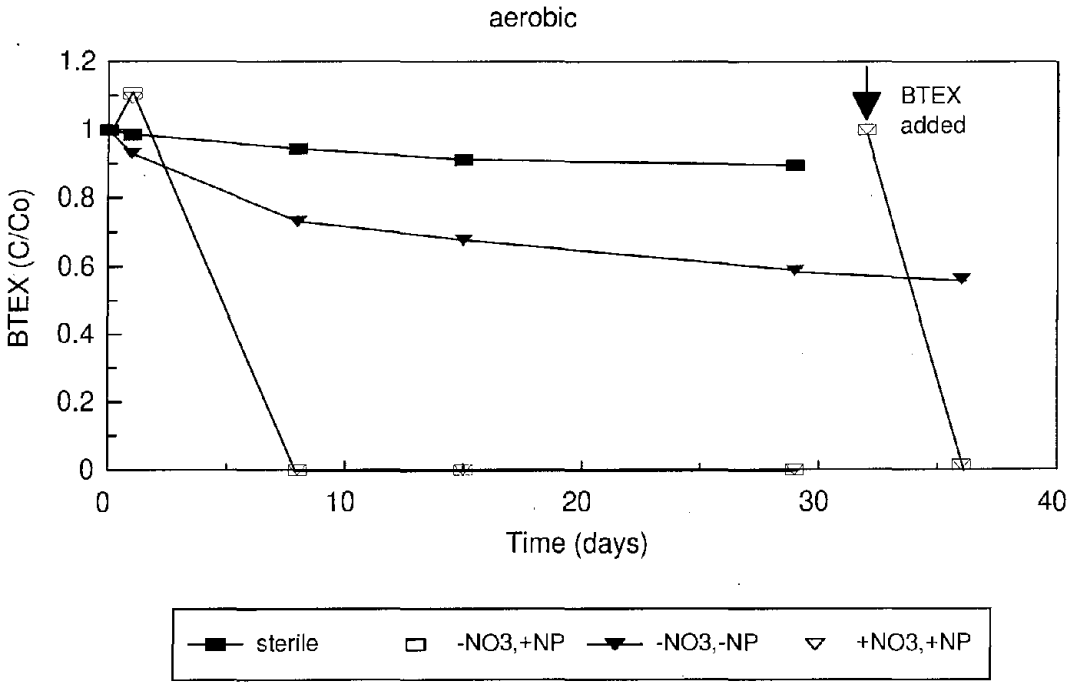


FIGURE 4-1 Aerobic BTEX biotransformation in the presence or absence of NO_3^- and/or NH_4-N and PO_4-P. Active, +N, P microcosms were reamended with 1 μL of BTEX on day 32 (arrow).

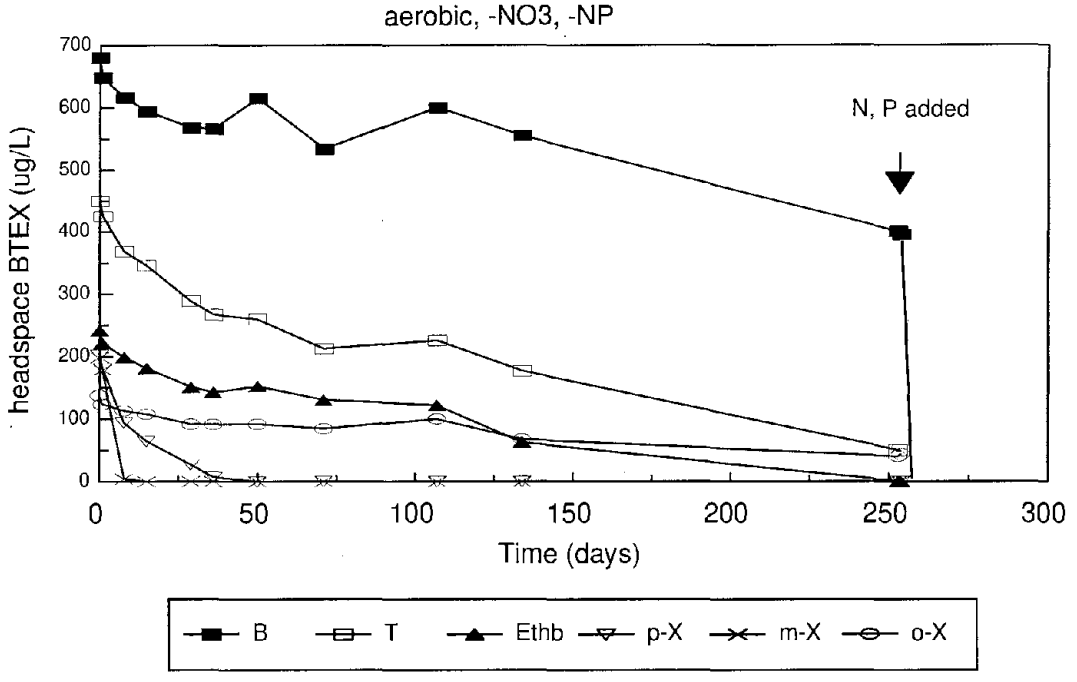


FIGURE 4-2 Aerobic BTEX biotransformation under NO_3^--free, N,P-free conditions. NH_4Cl and KH_2PO_4 were added on day 253 (arrow).

p-xylene declining first (Figure 4-2). Addition of N and P to this microcosm on day 253 (Figure 4-2, Arrow) resulted in total BTEX depletion within 6 days, providing clear evidence that microbial activity was restricted in the absence of inorganic nutrients. Note that in these microcosms O_2 was available in excess.

In contrast, far less BTEX biotransformation occurred under anaerobic conditions, although the addition of NO_3^- did stimulate some aromatic hydrocarbon depletion. No BTEX loss was observed in anaerobic, NO_3^--free microcosms (Figure 4-3), but ethylbenzene degraded to below detection in all NO_3^--amended microcosms, and toluene degradation was also observed in some, but not all NO_3^--amended microcosms (Figure 4-4). The other aromatic hydrocarbons appeared recalcitrant. As noted, microcosm leakage was a problem; therefore Figure 4-4 shows data only from selected microcosms that appeared to maintain integrity. Ethylbenzene degradation began in the NO_3^--amended microcosms after a lag period of 50-70 days. In contrast, when toluene loss occurred a lag period was not apparent, but a plateau effect was always observed, where ~0.5-2% of the initial toluene persisted for an extended period before declining to below detection (e.g., Figure 4-4b).

The presence or absence of inorganic nutrients (either N and P, or the mineral salts suite) did not affect anaerobic BTEX behavior (Figure 4-4b vs 4-4d). Presumably this was because the total hydrocarbon loss in even the most active NO_3^--amended microcosms was small compared to that in the aerobic BTEX-degrading microcosms. In addition, NO_3^- may have satisfied an assimilatory N requirement in these microcosms. Evidence of a nutrient limitation, therefore, was not observed. Nevertheless, addition of NH_4-N and PO_4-P was continued in subsequent microcosm experiments, to ensure that inorganic nutrient limitation would not impede anaerobic hydrocarbon biotransformation in the event of greater degradative activity. Provision of NH_4-N was intended to satisfy biomass production requirements so that NO_3^- would be available as a potential electron acceptor (Hutchins, 1991b).

Nitrate concentration had no discernable effect on ethylbenzene biotransformation, except when no NO_3^- was provided. Eventual toluene biotransformation to undetectable levels was observed in seven of 11 microcosms amended with 25 mg/L NO_3-N, but in none of three 0 mg/L NO_3-N microcosms, three 5 mg/L NO_3-N microcosms or two 50 mg/L NO_3-N microcosms. On this basis, of the concentrations tested in this experiment, the 25 mg/L level of NO_3-N was judged optimal. After the day 253 BTEX analyses were completed, all of the monitored microcosms were sacrificed and analyzed for pH and NO_3^- / NO_2^- concentrations. The final microcosm pH ranged from pH 7.0 to pH 7.6. The NO_3^- / NO_2^- data are summarized in Table 4-3.

A few 25 mg/L NO_3-N amended microcosms were analyzed on day 0, but then incubated continuously within the anaerobic chamber until sacrificed for BTEX, D.O., NO_3^- and NO_2^- analysis on days 71 or 134 (Table 4-4). Aromatic hydrocarbons lost due to passage through the glovebox evacuation chamber should therefore have been minimal; hence these microcosms confirmed the occurrence of toluene and ethylbenzene biotransformation under denitrifying conditions. The removal of these compounds is consistent with previous results obtained under anaerobic, denitrifying conditions (Barbaro et al., 1992).

Overall this initial screening experiment provided results that were consistent with previous experiments. In the Borden sand, BTEX biotransforms readily in the presence of O_2, and, in the presence of excess O_2, the extent of biotransformation can be limited by inorganic- nutrient availability. The experimental setup did not allow for a determination of whether a particular nutrient, phosphorous or nitrogen, limits the reaction. Under anaerobic, denitrifying conditions, only toluene and ethylbenzene biotransform at an appreciable rate relative to the length of the incubation period, and there is no discernable increase in mass loss in the presence of inorganic nutrients.

4.1.2 Experiment 2: Comparison of BTEX Biotransformation Under Denitrifying Conditions in CFB Borden, Eglin AFB and Park City Aquifer Microcosms

In this experiment, the BTEX-degrading capacity under denitrifying conditions of pristine Borden aquifer material was compared to that of aquifer material obtained from two petroleum hydrocarbon-exposed sites: Park City, Kansas (petroleum-hydrocarbon mixture), and Eglin AFB, Florida (jet fuel). The subsurface microbiota from both US sites are known to degrade aromatic hydrocarbons under denitrifying conditions (e.g., Kennedy and Hutchins, 1992; Thomas et al., 1995).

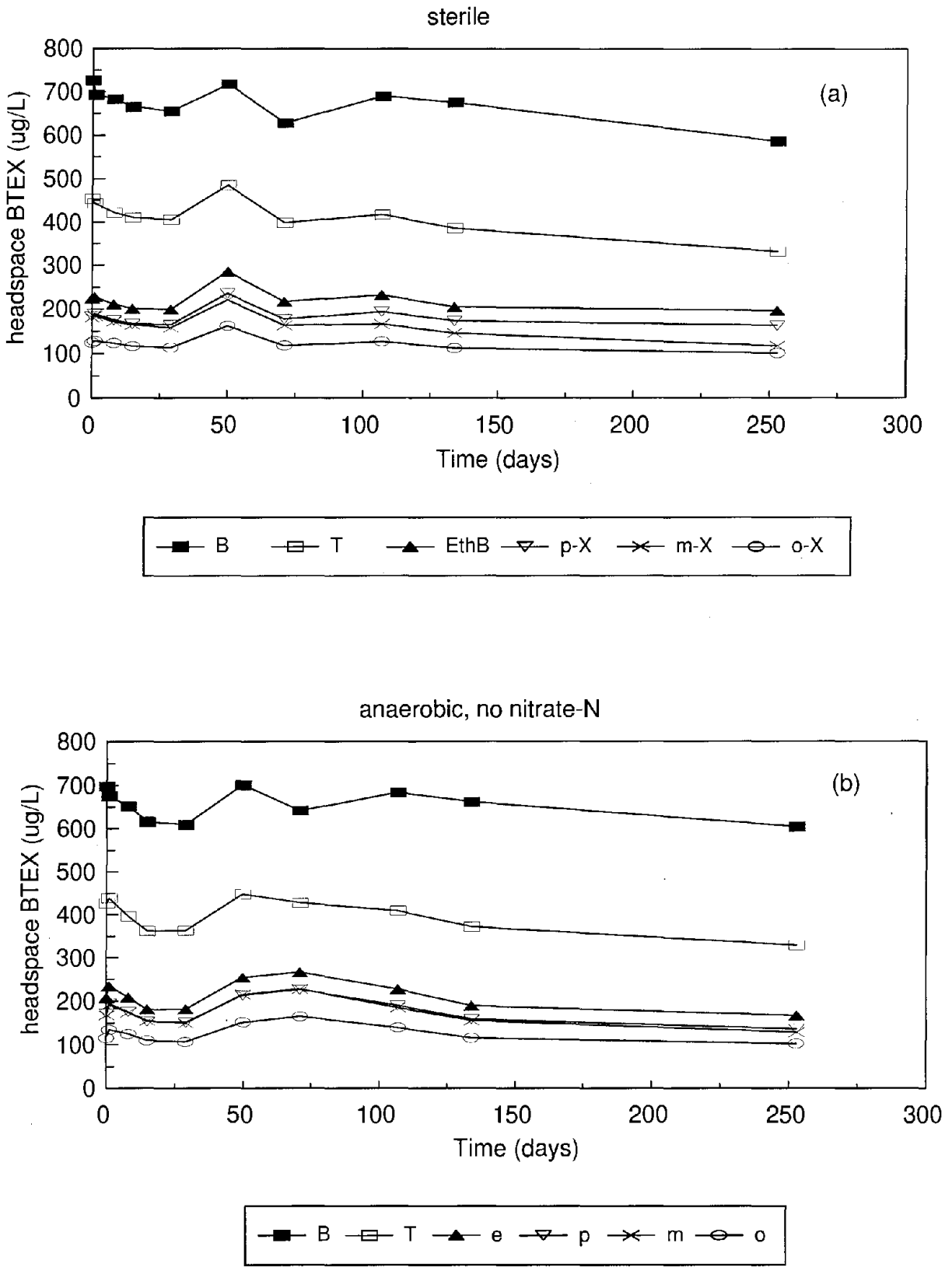


FIGURE 4-3 BTEX biotransformation in (a) anaerobic sterile control and (b) anaerobic NO_3^--free microcosms. Plotted values are the mean of the three microcosms (two +N, P, one -N, P).

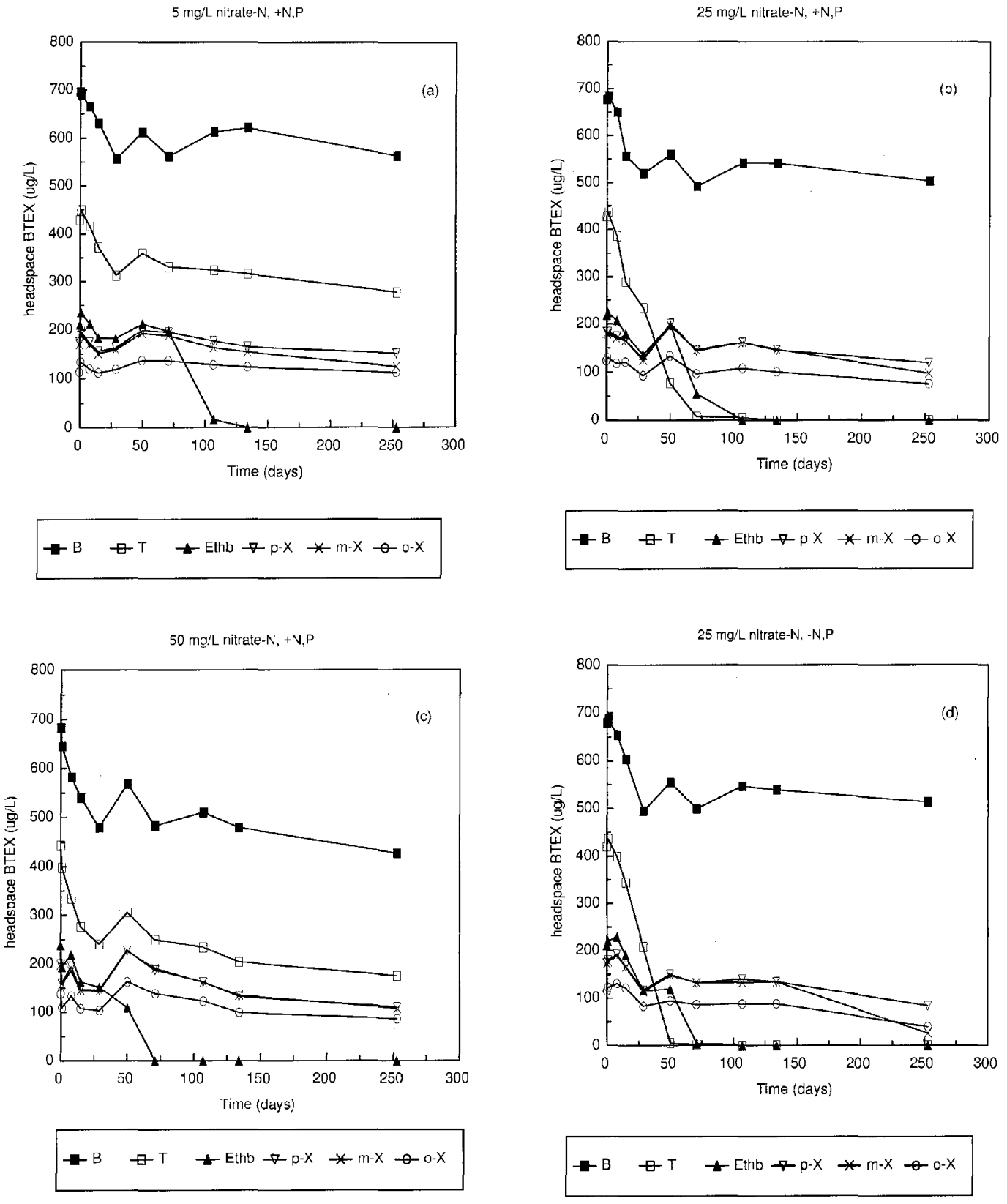


FIGURE 4-4 BTEX biotransformation in selected anaerobic microcosms: (a) 5 mg/L NO_3-N+N,P, (b) 25 mg/L NO_3-N,+N,P, (c) 50 mg/L NO_3-N, +N, P, (d) 25 mg/L NO_3-N, -N,P.

Table 4-3. *Nitrate-N and Nitrite-N in Anaerobic Microcosms on Day 253*

Microcosm Condition	NO_3-N (mg/L)	NO_2-N (mg/L)
Sterile, 25 mg/L NO_3-N amended (n = 3)	n.t.[1]	n.t.
Active, 0 mg/L NO_3-N amended (n = 3)	n.d.[2]	n.d.
Active, 5 mg/L NO_3-N amended (n = 3)	<0.06 [3]	0.06 (s.d. = 0.10)
Active, 25 mg/L NO_3-N amended (n = 9)	15.45 (s.d. = 1.9)	0.38 (s.d. = 0.19)
Active, 50 mg/L NO_3-N amended (n = 2)	38.77 (s.d. = 1.01)	0.45 (s.d. = 0.33)

[1] n.t., not tested.
[2] n.d., none detected.
[3] small peak detected, but below quantifiable limit.

Table 4-4. *Initial and Final BTEX, D.O., and Nitrate/Nitrite Concentrations in Denitrifying Microcosms*

Time (days)	Headspace Gas BTEX (µg/L)						D.O. (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)
	Ben	Tol	Eben	*p*-X	*m*-X	*o*-X			
Microcosm 1									
0	716	471	253	211	207	143	n.t.	n.t.	n.t.
2	n.t.[1]	n.t.	n.t.	n.t.	n.t.	n.t.	0.54	n.t.	n.t.
71	427	8	172	157	159	112	0.24	15.4	n.d.
Microcosm 2									
0	697	460	251	210	208	147	n.t.	n.t.	n.t.
71	624	2	180	188	191	116	0.32	8.1	n.d.[2]
Microcosm 3									
0	698	462	249	207	205	142	n.t.	n.t.	n.t.
134	665	n.d.	n.d.	147	150	93	n.d.	6.5	0.13

[1] n.t., not tested.
[2] n.d. none detected.

Table 4-5. *Park City, Kansas and Eglin AFB, Florida Subsurface Materials*

Park City, Kansas	Sample labelled 60G38/39; collected 25 ft below grade, several feet below significant contamination; coarse sand
Eglin AFB, Florida	Sample labelled 80KC6; collected from 5.5-6.3 ft below grade, downgradient of residual contamination source; fine sand

Design 1 microcosms were constructed with pristine Borden aquifer material, and aquifer material provided by the U.S. EPA NRMRL from the Eglin AFB, Florida and Park City, Kansas sites. Information available for the latter two materials is summarized in Table 4-5. Groundwater was not available from the US sites, so Borden groundwater amended to about 12.4 mg/L BTEX with a neat BTEX mixture (as in section 4.1.1) was used in all microcosms. All microcosms were provided with 25 mg/L NO_3-N, 5 mg/L NH_4-N and 2 mg/L PO_4-P. One sterile control and three active microcosms were prepared for each material.

Results and Discussion. Benzene was recalcitrant in all microcosms, and *o*-xylene was also persistent, although there was evidence that *o*-xylene was subject to cometabolism in the Eglin and Park City materials (Figure 4-5). The most striking feature of Figure 4-5 is the lack of activity in the Borden microcosms compared with Eglin and Park City microcosms. Substantial toluene depletion (to a plateau

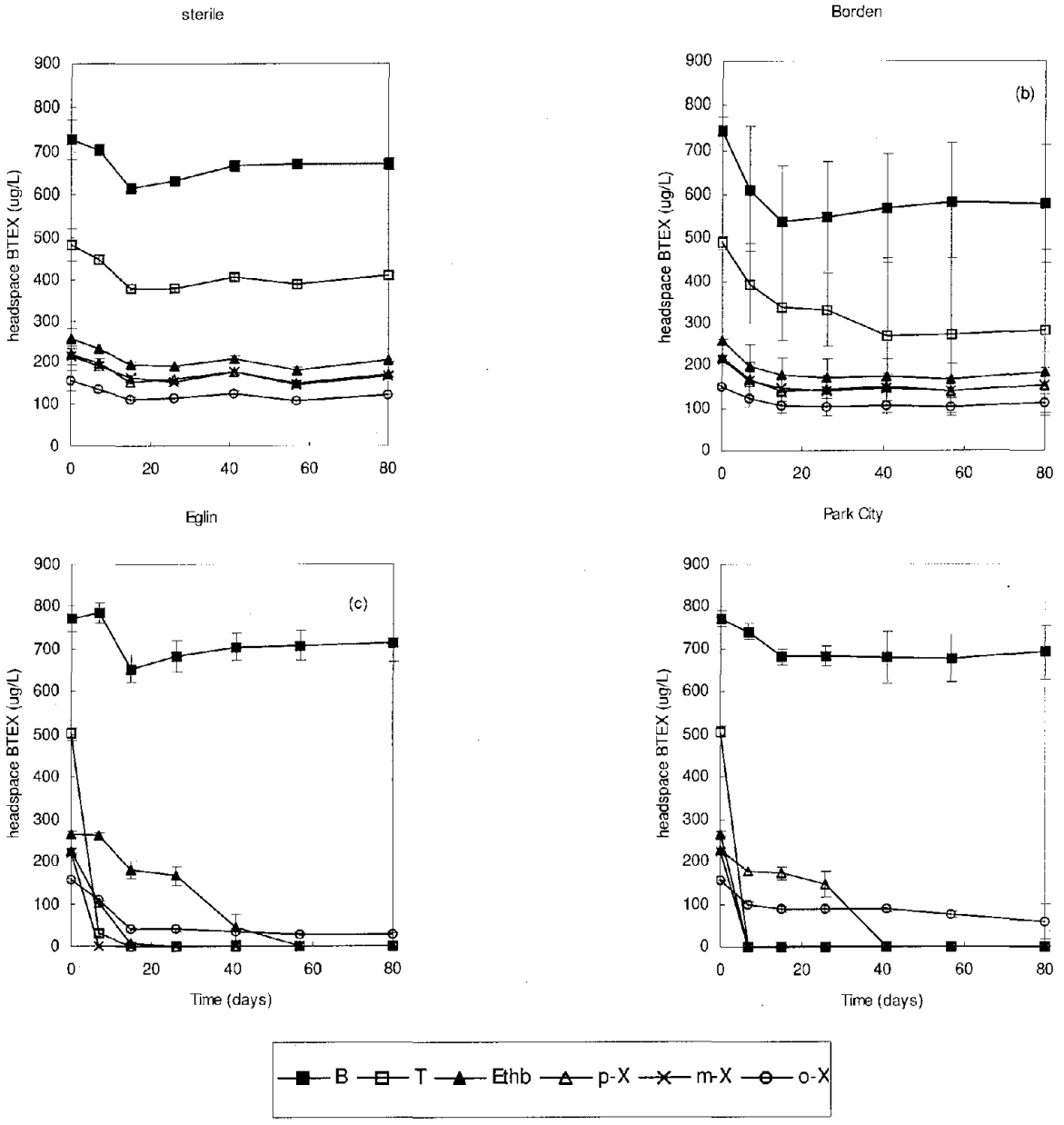


FIGURE 4-5 Comparison of BTEX biotransformation in Borden, Eglin and Park City microcosms under denitrifying conditions. Plotted values are the mean ± s.d. of three replicate microcoms (active) or three single microcosms from each site (sterile control).

of ca. 10 µg/L headspace toluene) did occur, but in only one of the three active Borden microcosms. This experiment was continued for 168 days, but all compounds persisted in the three active Borden microcosms, with the exception of the one toluene-degrading microcosm, which continued to degrade toluene upon reamendment on day 106 (data not shown)

Reamendment of single Eglin and Park City microcosms with toluene on day 106 led to further declines in *o*-xylene levels, although interestingly, reamendment of other microcosms with ethylbenzene (Eglin) or *p*-xylene (Park City) did not stimulate further *o*-xylene losses (data not shown). The TEX loss pattern differed in Park City and Eglin microcosms; the former were least efficient in *p*-xylene removal, whereas ethylbenzene was transformed most slowly by the Eglin microbiota (Figure 4-5). The degree of activity in the Eglin and Park City microcosms confirmed that the provided N, P levels and 25 mg/L NO_3-N would support anaerobic TEX biotransformation at the concentrations tested here.

Aerobic heterotrophic plate counts for the three aquifer materials were fairly similar, but the denitrifier counts for these particular samples were 10-100x larger in the exposed materials than in the pristine Borden material (Table 4-6). As discussed in Section 4.3.1, denitrifiers recovered from pristine Borden aquifer material sometimes exceeded 10^4/g. Nevertheless, robust hydrocarbon-degrading activity, comparable to that in the Eglin or Park City aquifer material, was not observed under denitrifying conditions in any of our experiments with pristine Borden sand. These data demonstrated, therefore, that the size of the denitrifier population, as represented by denitrifier counts, was not a reliable indicator of aromatic-hydrocarbon degrading activity in pristine Borden aquifer material.

In this experiment, denitrifying activity in aquifer material that had been exposed to hydrocarbons was compared with material with no known prior exposure. It was therefore not unexpected that TEX losses were more extensive in the exposed materials. Given the weak denitrifying activity observed in the laboratory, and assuming that the results in static laboratory microcosms are reasonably representative of a dynamic field setting (Barbaro et al., 1992), the Borden aquifer would appear to be a poor candidate for nitrate-based bioremediation. However, we hypothesized that more extensive hydrocarbon-degrading activity would develop *in situ* after a period of exposure to gasoline hydrocarbons, O_2, and NO_3^-. The laboratory data obtained with exposed Borden aquifer material are presented in Sections 4.2 and 4.3, and discussed in Section 4.4.

4.1.3 Experiment 3: Biotransformation of BTEX in Gasoline-Contaminated Groundwater Under Denitrifying, Microaerophilic and Mixed Electron-Acceptor Conditions

This experiment was performed to compare the extent of BTEX biotransformation under denitrifying, microaerophilic, and mixed electron-acceptor conditions. Design 1 microcosms were prepared with pristine Borden aquifer material, diluted (approximately 10x) gasoline-saturated groundwater, NO_3^-, and inorganic nutrients. Sets of replicate microcosms were prepared under either anaerobic denitrifying, microaerophilic, or mixed electron-acceptor (i.e., microaerophilic O_2 plus NO_3^-) conditions. This experiment was performed before the API 91-01 gasoline was acquired. Consequently, the gasoline-saturated groundwater was prepared with a locally-available unleaded gasoline, which was amended with pure benzene to provide a BTEX ratio similar to that investigated by Barbaro et al. (1992). The initial

Table 4-6. *Microbial Enumeration of Borden, Park City and Eglin AFB Aquifer Materials*

Sample	Heterotrophic Plate Count[1] CFU/g dry wt (s.d.)	Denitrifier Count[2] MPN/g dry wt (95% confidence)
Borden	1.1×10^5 (3.8×10^4)	2.8×10^2 ($24 - 1.5 \times 10^3$)
Park City	9.5×10^5 (7.2×10^4)	2.6×10^4 ($4.5 \times 10^3 - 1.5 \times 10^3$)
Eglin AFB	9.9×10^4 (1.6×10^4)	4.6×10^3 ($8.2 \times 10^2 - 1.5 \times 10^3$)

[1] plated on R2A medium.
[2] 3-tube MPN using KNO_3-1/10NB medium.

Table 4-7. *Design Summary, Experiment 3*

Treatment[1]	Replicates	Sampling Events
Sterile, denitrifying, 50 mg/L NO_3-N	3	7
Sterile, microaerophilic, 50 mg/L NO_3-N	3	7
Active, denitrifying, 25 mg/L NO_3-N	3	7
Active, denitrifying, 50 mg/L NO_3-N	3	7
Active, microaerophilic, 25 mg/L NO_3-N + air headspace	3	7
Active, microaerophilic, 50 mg/L NO_3-N + air headspace	3	7
Active, anaerobic, 0 mg/L NO_3-N	3	7
Active, microaerophilic, 0 mg/L NO_3-N + air headspace	3	7

[1] All microcosms contained 5 mg/L NH_4-N, 2 mg/L PO_4-P. A 4-ml air headspace was present as required.

BTEX concentration of the groundwater in the microcosms was approximately 13 mg/L. Microaerophilic conditions were established with a 4-ml air headspace (Section 3.2.1). For each treatment group multiple replicate microcosms were sampled periodically over a 348 day period for BTEX, dissolved O_2 (modified Winkler method (Appendix C)), NO_3^-, and NO_2^-. The experimental design is summarized in Table 4-7.

Results and Discussion (i). BTEX results from the microcosms are shown in Figures 4-6 and 4-7. Benzene persisted in all microcosms for the entire 348 days of monitoring, except for small initial losses under microaerophilic conditions. Substantial, rapid TEX losses occurred between days 1 and 8 in all active microaerophilic microcosms (Figure 4-6). The patterns of compound losses were typical of aerobic Borden microcosms (Section 4.1.1). *m*-Xylene was degraded to near or below detection levels, and substantial *p*-xylene and ethylbenzene losses also occurred. The considerable variability in the data is a common result when analyzing replicate samples of Borden aquifer material over time, but this factor makes the interpretation of early toluene and *o*-xylene behavior more uncertain. These early compound losses appeared to be more restricted in the NO_3^--free microaerophilic microcosms (Figure 4-6d) than in those amended with NO_3^- (Figures 4-6b and 4-6c). For example, ~45% of the *p*-xylene remained in the former, versus 5-10% in the latter. After day 8, degradative activity largely ceased in the microaerophilic and mixed electron acceptor microcosms (Figure 4-6). No BTEX biotransformation was observed in denitrifying microcosms over 140 days of incubation (Figure 4-7).

Sampling was suspended on day 140 because of the lack of BTEX-degrading activity. Approximately 7 months later (day 348 in Figure 4-6, Figure 4-7), another set of microcosms was sacrificed. The use of mean values obscures the results of this sampling round somewhat, because the results differed for individual microcosms within each set of three replicates. As apparent in some of the figures (e.g., toluene in Figures 4-6b, 4-6c, and 4-7c), further compound losses were initiated between day 140 and day 348 in individual microcosms. The six microaerophilic / NO_3^- microcosms analyzed on day 348 showed partial toluene degradation. Residual toluene ranged from 15 to 60% and from 25 to 64% of that present in the day 348 sterile controls, for the microaerophilic / 25 mg/L NO_3-N and microaerophilic / 50 mg/L NO_3-N conditions, respectively. Ethylbenzene was totally depleted in one of these microcosms, but the actual ethylbenzene fate was difficult to discern because concentrations varied substantially among replicates at all sampling events after day 1 (Figure 4-6). It is suspected that this compound sometimes degraded when microaerophilic O_2 was present to tens of µg/L which then persisted, and sometimes failed to degrade, so that hundreds of µg/L persisted. Three of the six denitrifying microcosms analyzed on day 348 had also degraded some toluene, but not ethylbenzene. All other compounds remained recalcitrant in both the microaerophilic / NO_3^- and denitrifying microcosms.

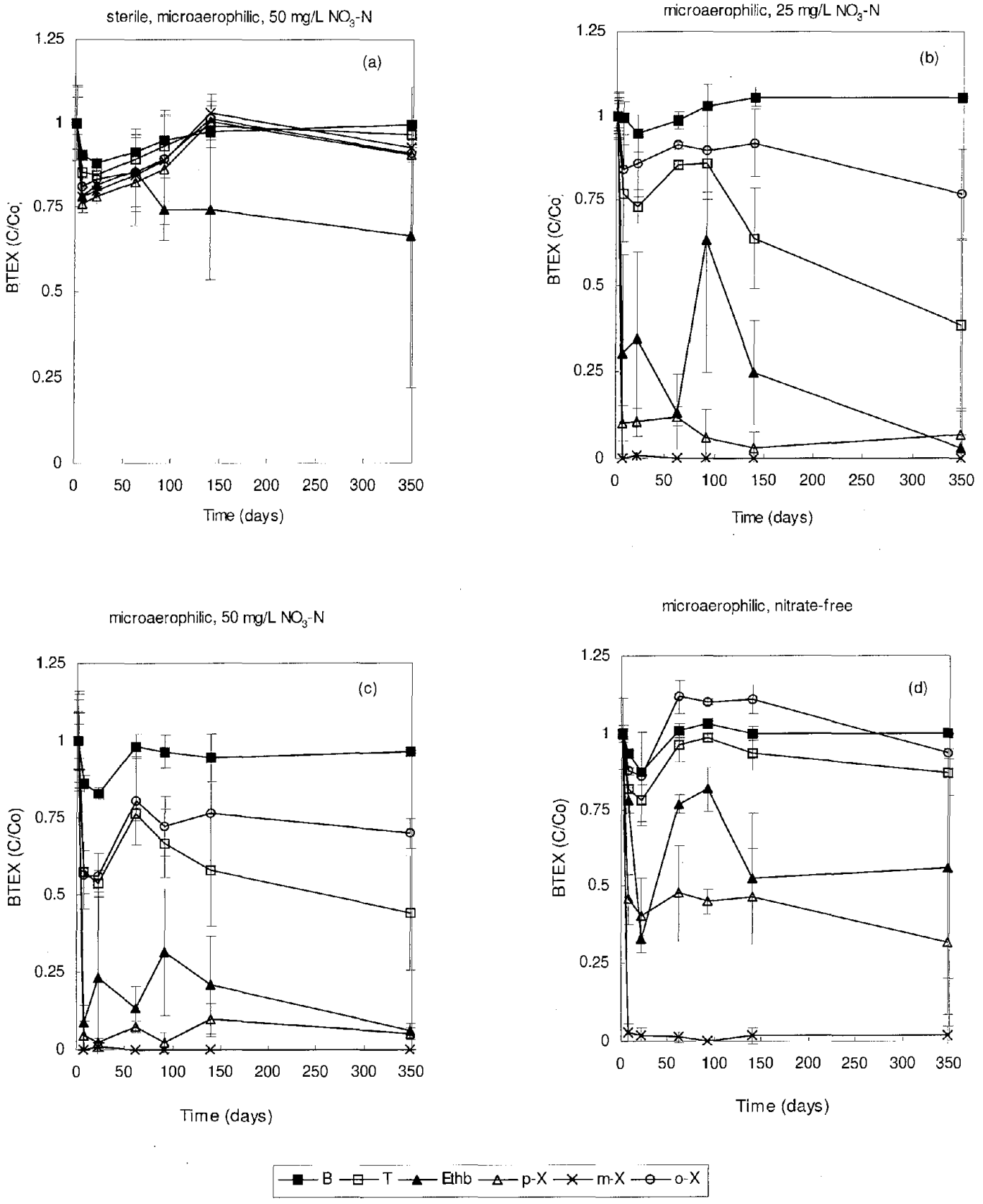


FIGURE 4-6 Biotransformation of BTEX in gasoline-contaminated groundwater under microaerophilic and mixed electron-acceptor conditions. Plotted values are the mean ± s.d. of three replicate microcosms.

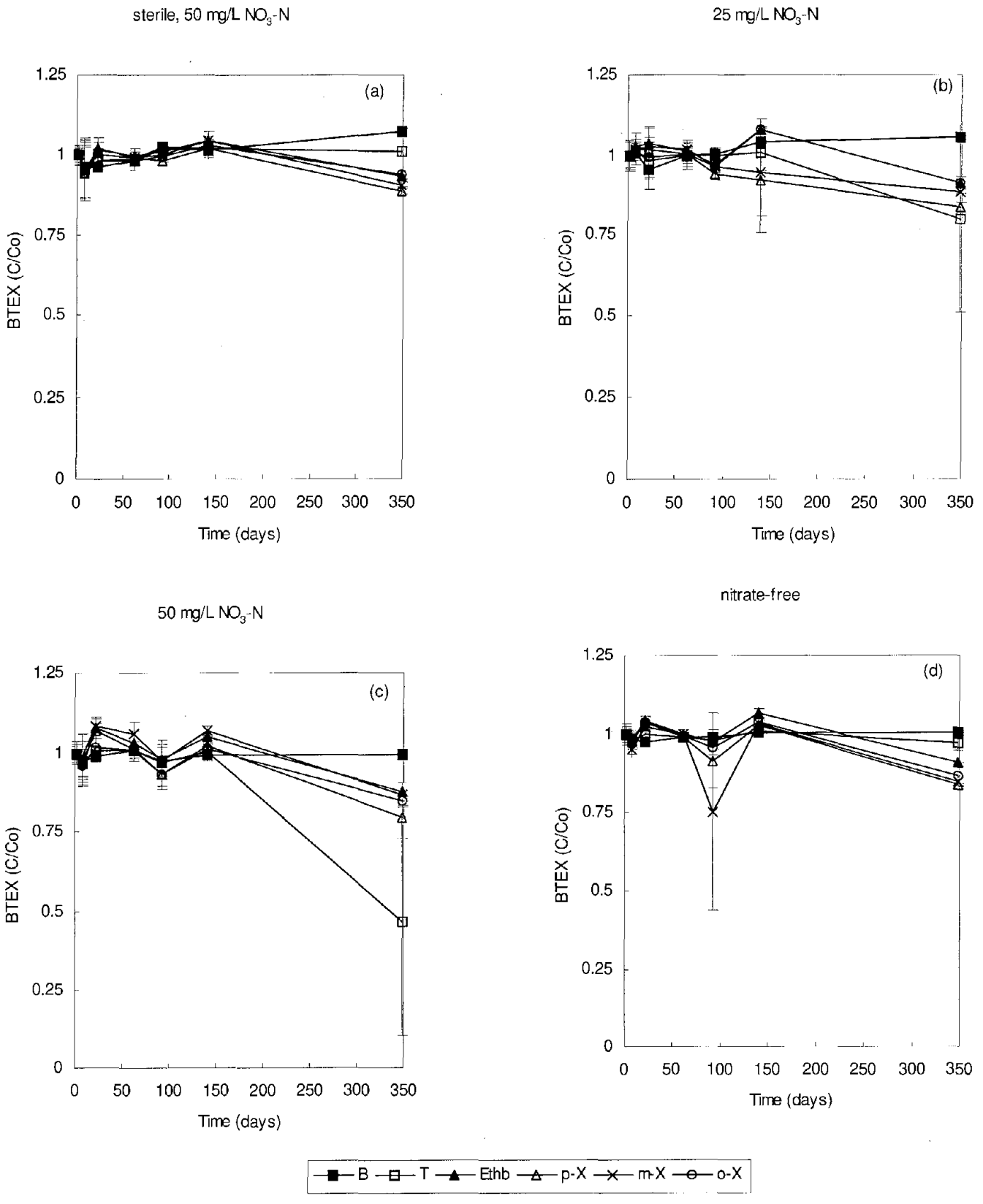


FIGURE 4-7 Biotransformation of BTEX in gasoline-contaminated groundwater under denitrifying and anaerobic, NO_3^--free conditions. Plotted values are the mean ± s.d. of three replicate microcosms.

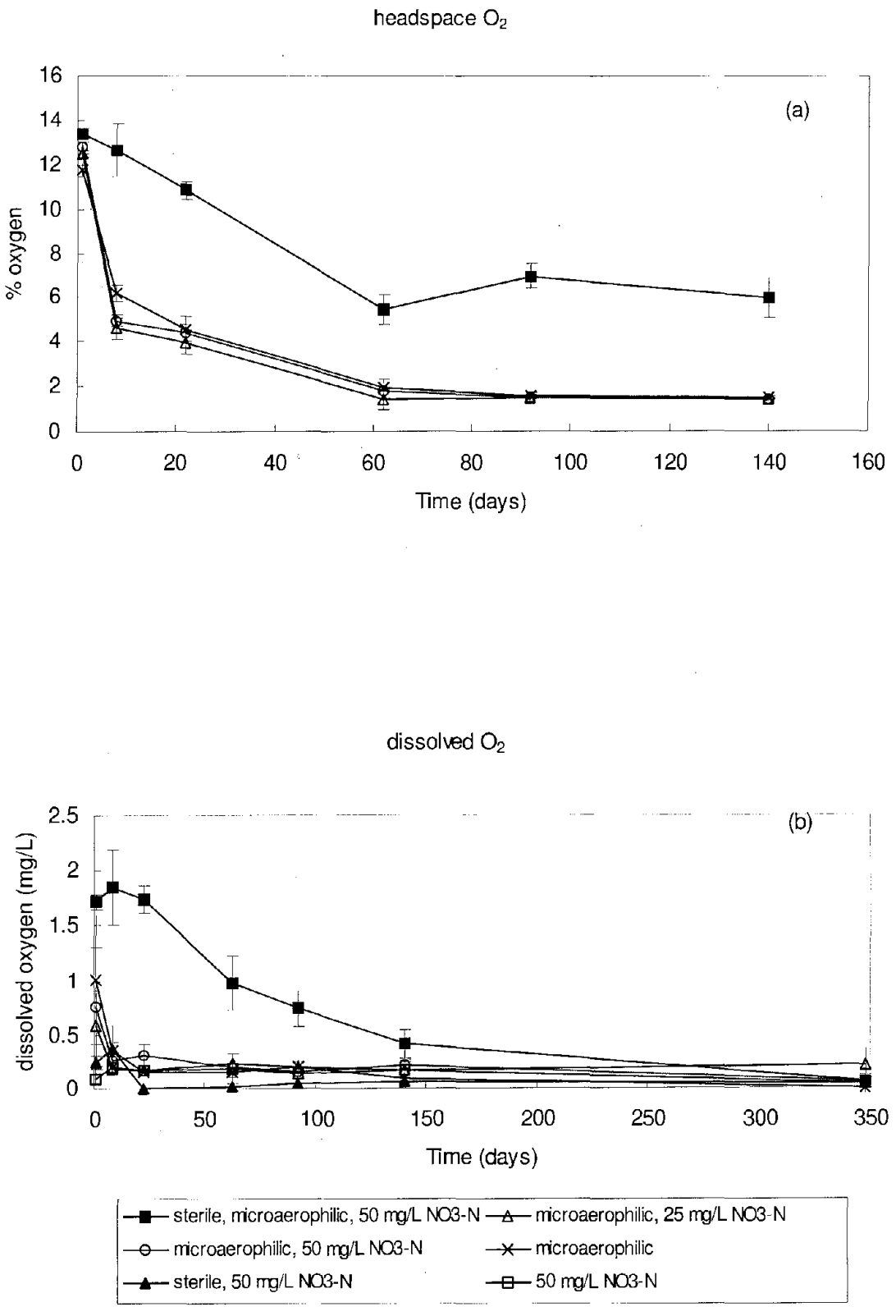


FIGURE 4-8 Headspace O_2 (a) and dissolved O_2 (b) in sterile and active microaerophilic microcosms. D.O. in sterile and active 50 mg/L NO3-N microcosms is also shown in (b) for comparison. Plotted values are the mean ± s.d. of three replicate microcosms.

The results of O_2 monitoring (Figure 4-8) support the conclusion that early BTEX losses in the microaerophilic and the mixed electron-acceptor microcosms were oxygen-linked. One difficulty with the use of an air-filled headspace to provide O_2 is that the D.O. level in the water of active microcosms cannot be measured continuously, and therefore is not known except at discrete measurement times. Oxygen partitioning between the gaseous, liquid and solids phases will be affected continually by microbial O_2 consumption, which will not likely be constant. In this experiment, both the headspace O_2 and dissolved O_2 contents were measured up to day 140 (Figure 4-8). Measurements on day 1 indicated a mean O_2 concentration of 12.6% (s.d.=0.67, n=12) in the headspace of sterile and active microaerophilic microcosms. The mean D.O. recorded for the same microcosms was 1.02 mg/L (s.d.=0.67, n=12). However, it is clear in comparing D.O. data for the sterile and the active microaerophilic conditions (Figure 4-8b) that a microbial O_2 demand was already exerted by the day 1 sampling event. In active microaerophilic microcosms the D.O. decreased to a mean level of 0.27 mg/L (s.d.=0.10) by day 8, and persisted at a similar level thereafter (Figure 4-8b). D.O. was maintained at ca. 1.7-1.8 mg/L up to day 22 in the microaerophilic, sterile controls, but decreased to a mean of 0.98 mg/L by day 62 and continued to slowly decrease thereafter. This may have reflected diffusion across or leakage around the septa and gradual equilibration with the atmosphere of the anaerobic chamber (See Section 4.2.1).

The NO_3^--N and NO_2^--N concentrations measured during the experiment are shown in Figure 4-9. Losses of NO_3^- were apparent. The mean NO_3-N concentrations of the microaerophilic / NO_3^--N microcosms on day 348 were 60% and 72% of initial concentrations for the 25 mg/L NO_3-N and 50 mg/L NO_3-N conditions, respectively (Figure 4-9a); the mean NO_3-N concentrations of the denitrifying microcosms were 38% and 76% of initial concentrations for the 25 mg/L NO_3-N and 50 mg/L NO_3-N conditions, respectively (Figure 4-9b). Nitrite accumulation was minimal, but small amounts of NO_2^- were occasionally recorded in these microcosm sets, with the exception of the microaerophilic / 50 mg/L NO_3-N microcosms (Figure 4-9). These results suggest that some denitrification occurred in the NO_3^--amended microcosms, which could have supported the late (i.e., after day 8) TEX losses that were observed under mixed electron-acceptor (Figures 4-6a and 4-6b) and denitrifying (Figures 4-7a and 4-7b) conditions, but were not apparent under microaerophilic, NO_3^--free (Figure 4-6d), or anaerobic, NO_3^--free conditions (Figure 4-7d).

The overall patterns of BTEX and electron-acceptor utilization under the conditions investigated in this experiment were most consistent with sequential utilization of O_2 and NO_3^- as electron-acceptors. Relatively rapid aerobic BTEX losses were followed by slower depletion of toluene and ethylbenzene under anaerobic, denitrifying conditions. However, a comparison of Figures 4-6b, 4-6c, and 4-6d (i.e., mixed electron-acceptor conditions and microaerophilic conditions) is revealing, because the early (between days 1 and 8) BTEX losses were more restricted under microaerophilic conditions. It's unlikely that nutrient limitation in the microaerophilic microcosms explains the observed difference; previous experiments (Section 4.1.1,Figure 4-1) demonstrated that the NH_4-N and PO_4-P present in both treatments exceeded assimilatory requirements for aerobic degradation of about twice the BTEX mass present in these microcosms. One possible explanation for the difference between these treatments is the occurrence of the so-called sparing effect in the mixed electron-acceptor microcosms (see Section 1.3.2; Hutchins, 1991a). After the addition of O_2 to aromatic hydrocarbon rings, the partially-oxidized TEX metabolites could have been rapidly metabolized by denitrifying microorganisms. Because considerably less O_2 is required to partially-oxidize the ring, this would 'spare' more of the limited O_2 for further initial oxidation of the parent compounds; hence more parent compound losses would potentially occur under these conditions. The sparing effect could not occur in the NO_3^--free microcosms, because the only electron-acceptor available to support continued degradation of any partially-oxidized TEX metabolites would be O_2. The possibility that this phenomenon might occur in the field was one of the reasons for undertaking this study (Section 1.3.2).

The occurrence of the sparing effect cannot be unequivocally proven or disproven with the available data because aromatic hydrocarbon metabolites were not monitored. But in this aquifer material the explanation seems very unlikely. The sluggish hydrocarbon-degrading activity of the Borden denitrifying community observed throughout this study suggests the occurrence of a concerted, rapid process of aerobic oxygenase plus denitrifier metabolism resulting in rapid TEX losses is implausible. Moreover, one can estimate the NO_3^- demand potentially exerted by these hypothetical, partially-oxidized TEX

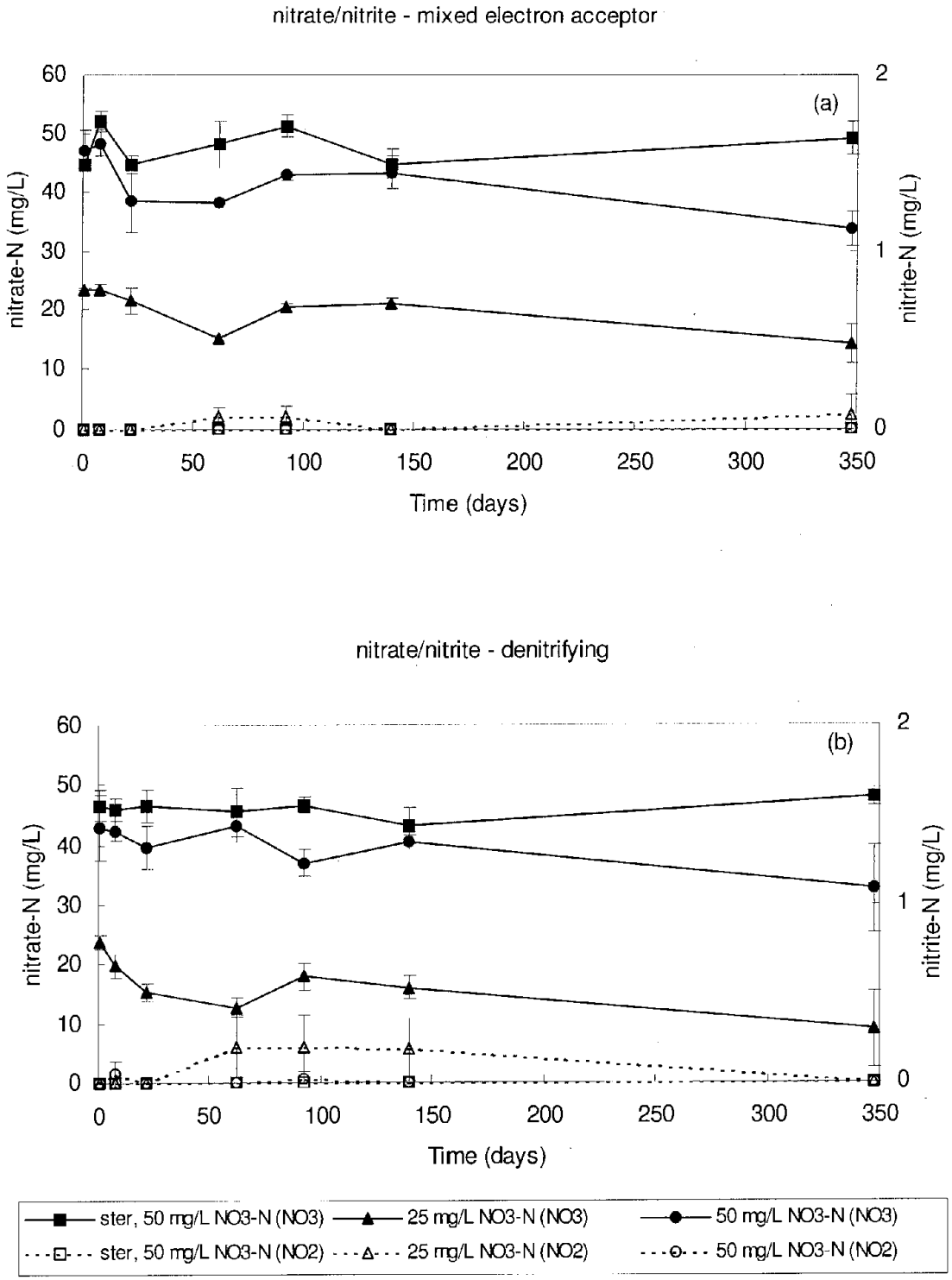


FIGURE 4-9 Nitrate and nitrite in (a) mixed electron acceptor and (b) denitrifying microcosms. Solid symbols and lines = NO_3^--N; open symbols and dashed lines = NO_2^--N. Plotted values are the mean ± s.d. of three replicate microcosms.

intermediates, which is roughly similar to the NO_3^- that would be required to support mineralization of the equivalent amount of parent TEX compounds, and compare the estimated demand to observed losses. This calculation indicates that total mineralization of the ~1665 Fg/L TEX depleted in the mixed electron-acceptor microcosms between days 1 and 8 would require roughly 8 mg/L NO_3^- as an electron acceptor, if complete denitrification to N_2 is assumed. However, no NO_3^- depletion was measured in these microcosms over this interval, nor was NO_2^- detected on day 8 (Figure 4-9a). Alternatively, the mass of O_2 in these microcosms appeared to be adequate for complete mineralization of 1665 µg/L TEX. Assuming no biomass production, the aerobic reaction is

$$C_{47}H_{40} + 57\,O_2 \rightarrow 47\,CO_2 + 20\,H_2O \qquad 4\text{-}1$$

Equation 4-1 shows that the mass of O_2 in a 4-ml air headspace (ca. 1.2 mg) was sufficient to oxidize roughly 400 µg TEX, which exceeds the observed losses of 76 µg TEX (1665 µg/L x 0.046 L) over this period. The O_2 mass would be adequate even if it was conservatively assumed that ca. 75% of the O_2 was consumed by day 1 (Figure 4-9). These calculations demonstrate that the sparing effect does not have to be invoked to explain the BTEX data, which is consistent with the apparent lack of early NO_3^- utilization. However, it remains unclear why in this experiment early mass losses appeared to be more extensive in the mixed electron-acceptor microcosms.

Results and Discussion (ii). This experimental design was repeated (without the anaerobic, NO_3^--free microcosms), with 25 mg/L NO_3-N and 10 mg/L NO_3-N, and core material that proved to be more active under denitrifying conditions. For brevity, results for the active, 25 mg/L NO_3-N condition only are shown (Figures 4-10 and 4-11). Trends in this experiment followed those of the experiment described in the previous section (i.e., rapid aerobic TEX degradation followed by markedly slower, more restricted hydrocarbon losses at the expense of NO_3^-), but in this experiment early aerobic losses were similar in the microaerophilic and microaerophilic / NO_3^- microcosms (Figures 4-10b and 4-10c). In addition, stronger activity was observed under denitrifying conditions, which was initiated within less than 20 days (Figure 4-10a). Dissolved oxygen was depleted rapidly over the first 7 days in those microcosms where it was available, and a low-level plateau (<0.5 mg/L), equivalent in the microaerophilic and denitrifying microcosms, was maintained thereafter (Figure 4-11a).

The zero-order rates of NO_3-N depletion between days 1 and 68 were 0.15 and 0.13 mg/L/day for the denitrifying and microaerophilic / 25 mg/L NO_3-N microcosms, respectively. Nitrate loss continued thereafter, but at a much slower rate (0.03 and 0.02 mg/L/day) (Figure 4-11b). Peak NO_2^- accumulation was observed over the day 20-36 interval (Figure 4-11b) although NO_2^- levels never exceeded about 2 mg/L, suggesting that further denitrification to gaseous products occurred. The similarity of NO_3^- / NO_2^- behavior under mixed electron-acceptor and strictly denitrifying conditions indicates that denitrifying activity was not particularly influenced by the initial presence of microaerophilic O_2.

In this experiment toluene degradation was detected in the microaerophilic microcosms between days 20 and 166, i.e., after cessation of aerobic biotransformation (Figure 4-10c). This activity probably occurred under SO_4^{2-}-reducing conditions, with the Borden groundwater serving as the source of SO_4^{2-}. A sulfide odor was noted in these microcosms on days 68 and 166, and the solids were noticeably grayish. Analysis of water from the microcosms sacrificed on day 166 showed that the mean SO_4^{2-} concentration was 0.18 mg/L (s.d. = 0.32) in the three microaerophilic, NO_3^--free microcosms, whereas SO_4^{2-} averaged 12.9 mg/L (s.d. = 0.54) in all other microcosms (n = 18). In addition, D.O. was below detection limits in some of these microcosms (Figure 4-11a), which was rarely the case in NO_3^--amended microcosms. Toluene biotransformation under SO_4^{2-}-reducing conditions has been observed in other studies (Beller et al., 1992).

The mass losses that were observed in this experiment under anaerobic NO_3^--reducing conditions were larger than in any other laboratory experiment performed in this study. Nonetheless, the total BTEX losses observed after 166 days still did not exceed 60% of the initial concentration of ca. 13 mg/L under microaerophilic conditions (with or without NO_3^-), and 45% under strictly denitrifying conditions. *m*-xylene and ethylbenzene were the only compounds that (sometimes) degraded to concentrations below detection levels. For example, in microaerophilic / 25 mg/L NO_3-N microcosms, BTEX remaining on day 166 (expressed relative to the day 1 concentration) included 74% of the benzene, 2% of the toluene, 0% of the

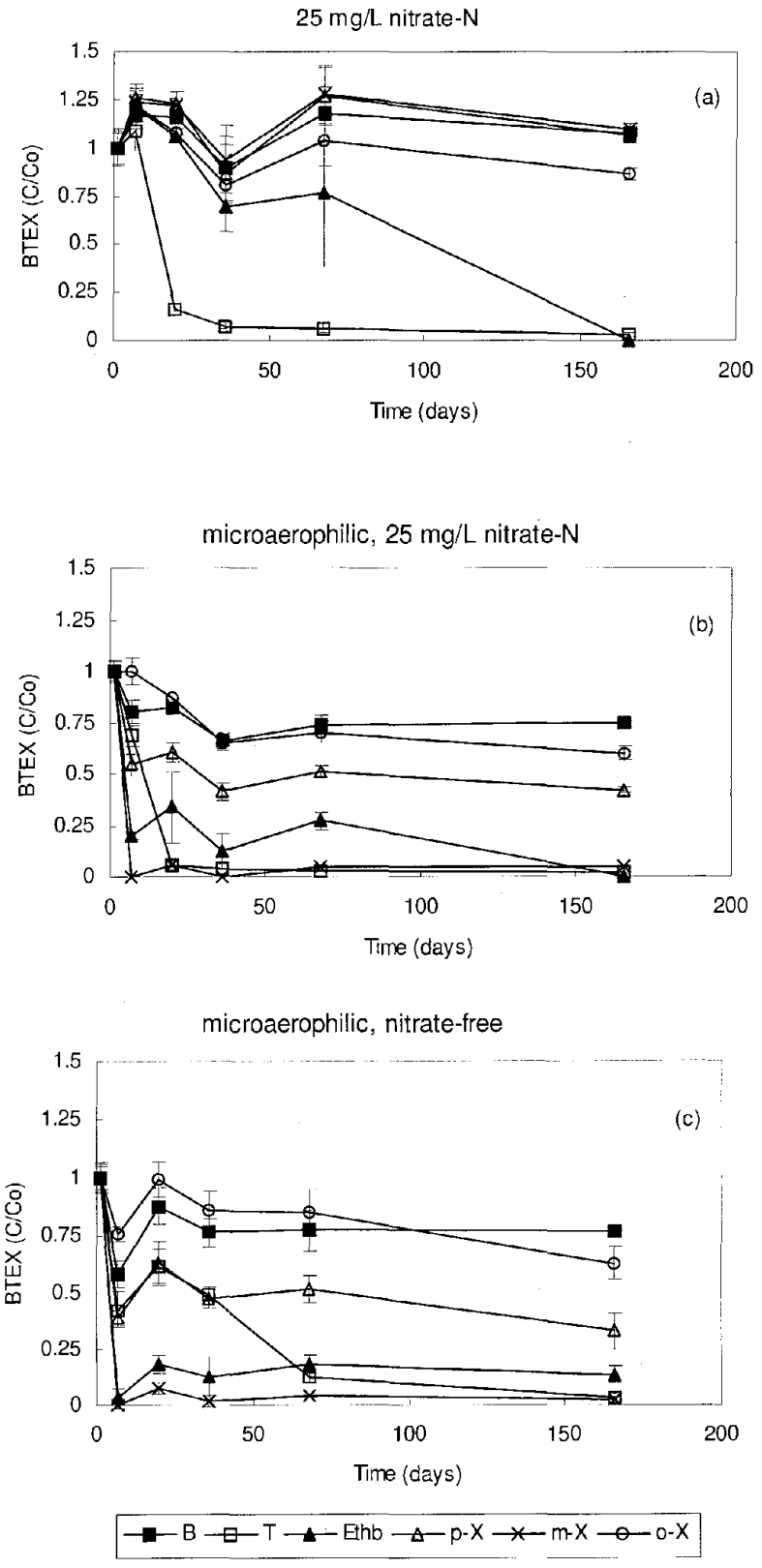
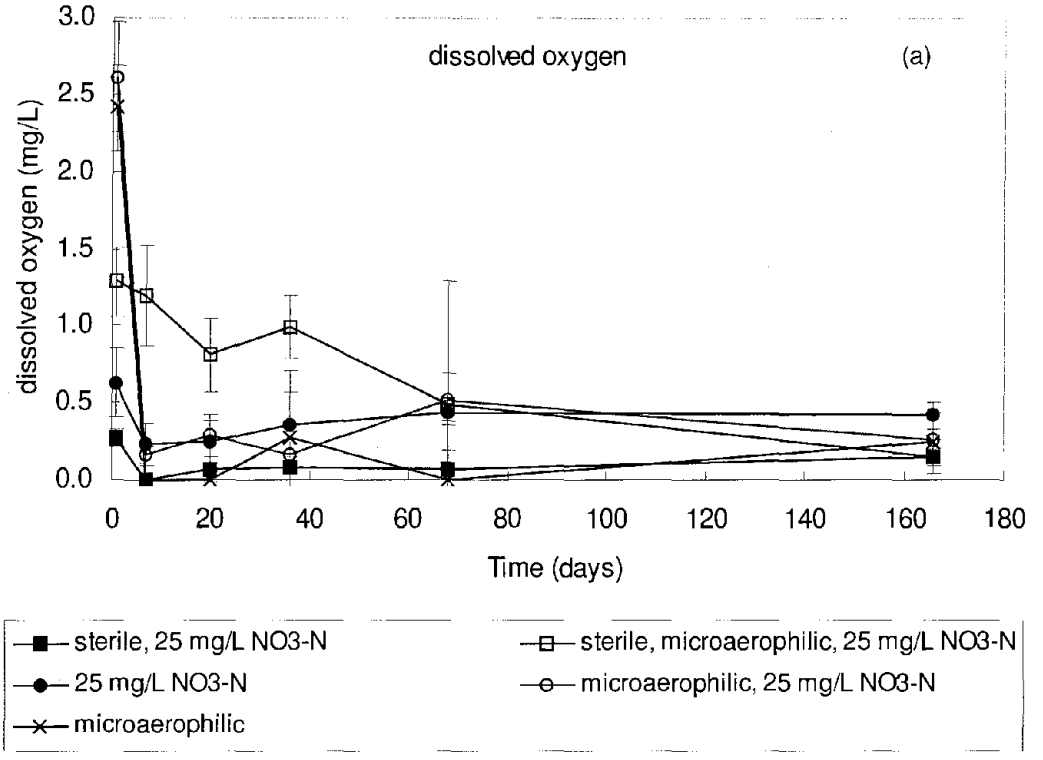


FIGURE 4-10 BTEX biotransformation in denitrifying, mixed electron-acceptor, and microaerophilic Borden microcosms - normalized BTEX. Plotted values are the mean ± s.d. of three replicate microcosms.



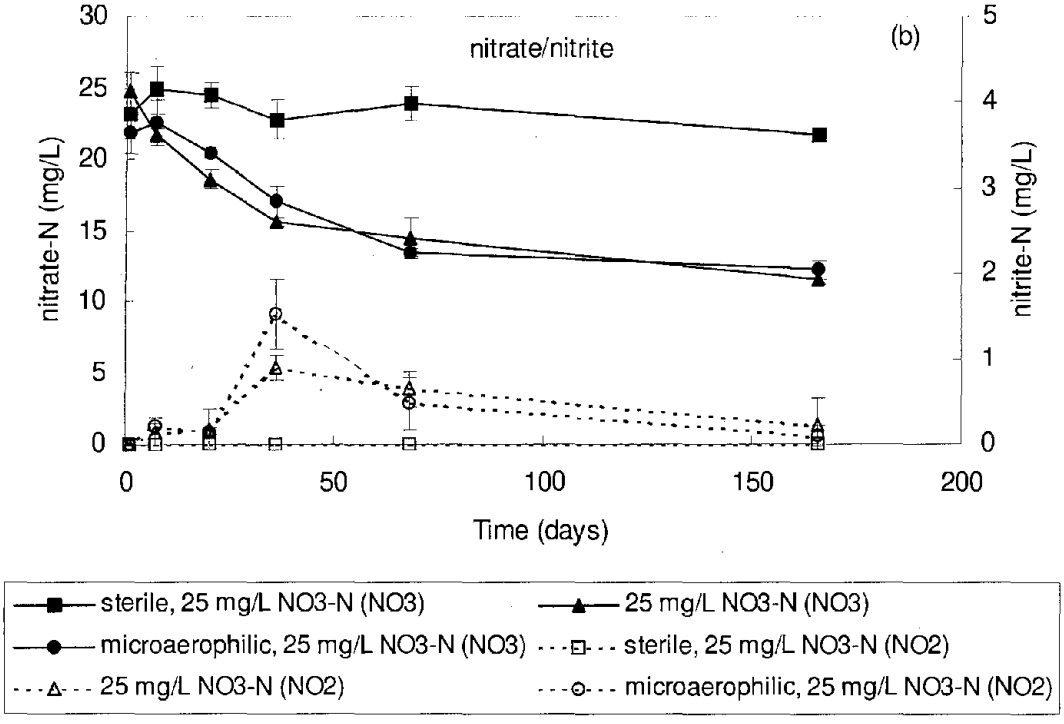


FIGURE 4-11 BTEX biotransformation in denitrifying, mixed electron-acceptor, and microaerophilic Borden microcosms: (a) dissolved O_2, (b) NO_3^- and NO_2^-. In (b), solid symbols and lines = NO_3^--N; open symbols and dashed lines = NO_2^--N. Plotted values are the mean ± s.d. of three replicate microcosms.

ethylbenzene, 5% of the *m*-xylene, 42% of the *p*-xylene, and 60% of the *o*-xylene. In contrast, the Eglin AFB and Park City materials (see Section 4.1.2) reduced the total BTEX content by about 60-65% in 57 days or less under denitrifying conditions, and all compounds but benzene (88-92% remaining) and *o*-xylene (17% (Eglin), 48% (Park City) remaining) were biotransformed to undetectable levels.

Generally, the microcosm data obtained in these two mixed electron-acceptor experiments, and in the others performed in this study (Experiments 4 and 6), were most consistent with the sequential utilization of O_2 and NO_3^-: rapid aerobic BTEX biotransformation was typically followed by more-limited biotransformation under denitrifying conditions over longer time periods. These data indicated therefore that over relatively-long (i.e., several month) incubation periods, total mass losses were enhanced in the presence of mixed electron-acceptors relative to losses under microaerophilic and anaerobic, denitrifying conditions. However, microaerophilic O_2 did not facilitate major benzene losses. The apparent enhanced early BTEX losses observed under mixed electron-acceptor conditions in Experiment (i) of this section were not observed when the experiment was repeated. This lack of replicability in conjunction with other laboratory and field evidence obtained in this study suggest that the sparing effect, if present, was not an important process in this aquifer material.

4.1.4 Experiment 4: Effect of BTEXTMB Concentration Under Microaerophilic Oxygen/Nitrate Conditions

After the gasoline was spilled at the field site, it became evident that the entire volume of the treatment cells would be exposed to pure-phase gasoline and/or high dissolved-phase concentrations of the soluble aromatic hydrocarbons. A microcosm experiment was therefore designed to evaluate the effect of dissolved concentrations on the extent of biotransformation under conditions similar to the field system, i.e., gasoline-saturated groundwater plus NO_3^- and microaerophilic O_2. This experiment utilized API 91-01 gasoline (Appendix A) rather than a locally-purchased gasoline, and the larger suite of aromatic hydrocarbons (i.e., BTEXTMB) was quantified. To simplify the experimental system, particularly the determination of aromatic-hydrocarbon mass loss, a residual gasoline phase was not present in these microcosms.

Design 1 microcosms were set up with pristine Borden sand from cores collected near the field site. A 2-ml air headspace was present in each vial to create microaerophilic conditions. Three treatment groups were used to evaluate the effect of concentration: high-BTEXTMB concentration (gasoline-saturated groundwater), low-BTEXTMB concentration (10x dilution of gasoline-saturated groundwater), and sterile low-BTEXTMB-concentration controls. For each treatment group multiple replicate microcosms were sampled on days 0, 42, and 137 for BTEXTMB, dissolved O_2 (modified Winkler method), NO_3^-, and NO_2^-. The design is summarized in Table 4-8.

This experiment was set up with multiple replicates so that differences between means of treatment groups could be tested statistically for significance. Because substantial variability in the extent of biotransformation is often encountered in Borden-sand microcosms, a large number of replicates was prepared for each treatment group to give the test adequate statistical power. However, concentrations behaved anomalously in these microcosms, increasing between days 0 and 42, which prevented a rigorous statistical analysis of the mass-loss data. Nonetheless, the experiment still provided useful information, as losses relative to controls were observed in the low-BTEXTMB-concentration microcosms, and NO_3^- data indicated that denitrifiers were inhibited in the high-BTEXTMB microcosms.

Table 4-8. *Design Summary, Experiment 4*

Treatment (Initial BTEXTMB Concentration)	Replicates	Sampling Events
Active, 110 mg/L BTEXTMB (gas.-sat. water), 2-ml air headspace, 25 mg/L NO_3^--N, N,P	8 active	3
Active, 11 mg/L BTEXTMB (10x dil.), 2-ml air headspace, 25 mg/L NO_3^--N, N,P	8 active	3
Sterile, 11 mg/L BTEXTMB (10x dil.), 2-ml air headspace, 25 mg/L NO_3^--N, N,P	8 sterile	3

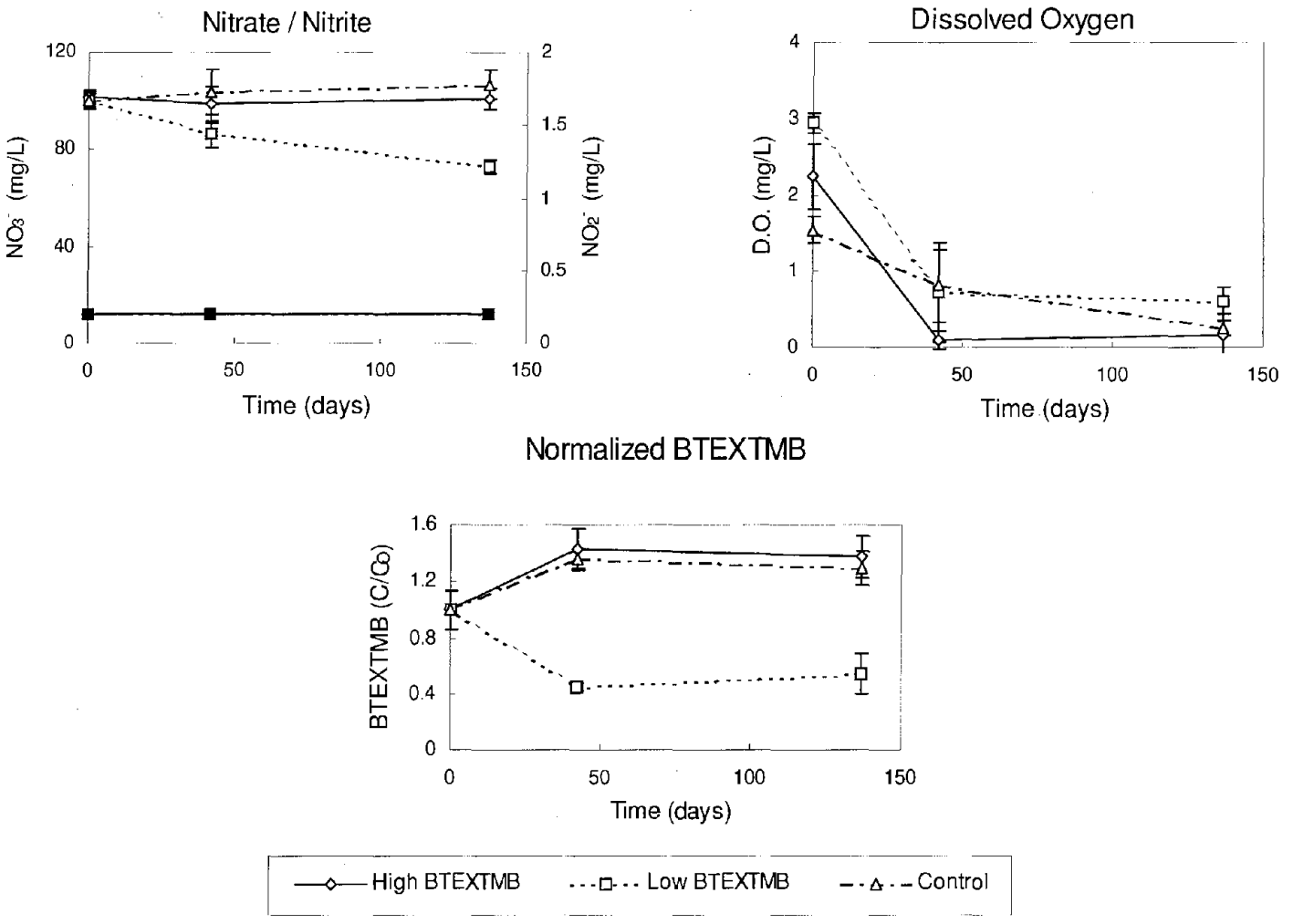


FIGURE 4-12 Nitrate, NO_2^-, D.O., and normalized total BTEXTMB concentrations in Experiment 4 microcosms. High BTEXTMB: gasoline-saturated (110 mg/L); Low BTEXTMB and Control: 10x dilution (11 mg/L). The detection limit value (0.2 mg/L) plotted for not-detected NO_2^- samples (solid squares). Plotted values are the mean ± s.d. of three replicate microcosms.

Table 4-9. *Percent of Individual Aromatic Hydrocarbons Remaining in Active, Low-BTEXTMB Concentration Microcosms Relative to Sterile Controls. Microcosms were Amended with an Air Headspace and NO_3^-, and Contained Pristine Aquifer Material.*

Compound	Day 137 % Remaining (s.d.)[1]
Benzene	86 (14)
Toluene	10 (12)
Ethylbenzene	28 (56)
m+p-Xylene	24 (10)
o-Xylene	76 (15)
1,3,5-Trimethylbenzene	72 (38)
1,2,4-Trimethylbenzene	7 (10)
1,2,3-Trimethylbenzene	86 (52)
Naphthalene	43 (59)

[1] Percent remaining calculated from $\frac{(c/c_o)_{Active}}{(c/c_o)_{Control}} * 100$ where C_o is the concentration on day 0.

Standard deviation of percent remaining calculated using Equation 5-2.

Results and Discussion. BTEXTMB losses were not evident over 137 days of incubation in the high-BTEXTMB or sterile controls microcosms (Figure 4-12). On the other hand, mass losses were observed in the low BTEXTMB microcosms (Figure 4-12). In terms of individual compounds, toluene, ethylbenzene, *m+p* xylenes, 1,2,4-trimethylbenzene, and naphthalene concentrations all declined relative to controls by day 137 (Table 4-9). The remaining aromatic hydrocarbons appeared recalcitrant; the slightly lower concentrations may be attributed to aerobic biotransformation, to greater sorption in the active microcosms relative to the autoclaved controls, or a combination of the two processes.

By day 42, O_2 was depleted to a threshold concentration in the aqueous phases of both active treatments. Unfortunately, the O_2 concentrations in the control microcosms appeared anomalous, possibly from interferences between the Winkler reagents and the sodium azide. Subsequent controls analyzed with the dissolved O_2 meter indicated that the initial dissolved O_2 concentration in microcosms with a 2-ml air headspace was about 4.5 mg/L, declining asymptotically to below 1 mg/L over a four-month period (see Section 4.2.1; Figure 4-15). Assuming similar initial concentrations in the active microcosms, roughly half of the dissolved O_2 was consumed within the first several hours of the experiment (Figure 4-12). As in other laboratory experiments performed for this study, low concentrations (0.1-0.5 mg/L) of dissolved O_2 appeared to persist over the incubation period in the active microcosms. Nitrate also declined in the low-BTEXTMB microcosms relative to the control and high-BTEXTMB microcosms, but accumulation of detectable concentrations of NO_2^- did not occur (Figure 4-12).

Mineralization reaction stoichiometries suggest that the mass of O_2 in the low-BTEXTMB microcosms was sufficient to account for the observed mass losses. Assuming total BTEXTMB was aerobically mineralized to CO_2 and there was no assimilation of C by microbial cells, the mineralization reaction is

$$C_{82}H_{98} + 106.5\ O_2 \rightarrow 82\ CO_2 + 49\ H_2O \qquad 4\text{-}2$$

Based on Equation 4-2, the mass of O_2 in the headspace (0.6 mg O_2) would be sufficient to oxidize 0.2 mg BTEXTMB, which is greater than the observed total BTEXTMB mass loss of approximately 0.17 mg on day 137. The continued utilization of NO_3^- after O_2 depletion in these microcosms indicates that some of this mass loss may have occurred under denitrifying conditions, although it is conceivable that natural organic matter or other dissolved constituents in the gasoline-contacted water served as the carbon source. In the high BTEXTMB microcosms, the mass of O_2 was quite low relative to the mass of dissolved organics. Therefore, although small aerobic losses of aromatics may have occurred, depletion relative to the day 0 sampling event could not be observed because concentrations increased between days 0 and 42. Consequently, the effect of microaerophilic O_2 was unclear. On the other hand, in comparison to low-BTEXTMB microcosms, the lack of NO_3^- depletion after O_2 consumption clearly indicated that denitrifiers were not active in the high BTEXTMB microcosms.

Despite the unexplained BTEXTMB concentration trends, this experiment provided information useful for interpreting *in situ* behavior. Aromatic-hydrocarbon and NO_3^- utilization trends indicated that biotransformation of labile compounds such as toluene and uptake of NO_3^- were negligible in the presence of high dissolved-phase BTEXTMB concentrations under mixed microaerophilic / NO_3^- conditions. This experiment indicated therefore that near the source area, aqueous concentrations were high enough to be inhibitory to an unacclimatized denitrifying population in the Borden aquifer (i.e., NO_3^--based bioremediation would not be effective in this aquifer near the source area). However, as discussed further in Chapter 5, NO_3^- reduction was observed in the field, suggesting that an acclimatized population did develop with exposure to gasoline hydrocarbons. Nitrate depletion was also observed in the laboratory using contaminated core material extracted from the Nitrate Cell (Section 4.2).

4.1.5 Experiment 5: Effect of Oxygen Concentration Under High BTEXTMB Concentration Conditions

Because the small amount of dissolved O_2 added to the Experiment 4 microcosms appeared to be utilized rapidly, biotransformation in the high BTEXTMB microcosms may have been O_2 limited. To address this issue, an additional experiment was performed to determine if the aromatic hydrocarbons would biotransform at near-source concentrations under fully-aerobic conditions, in both the presence and absence of NO_3^-.

Design 1 microcosms were set up with pristine Borden sand. All microcosms in this experiment were prepared with groundwater saturated with API 91-01 gasoline. Each microcosm contained a 4-ml headspace purged with pure O_2 rather than air to obtain a "high" initial dissolved O_2 concentration (and total O_2 mass) to drive aerobic reactions. Three treatment groups were used to evaluate biotransformation of high BTEXTMB concentrations under aerobic conditions: aerobic / NO_3^-, aerobic only, and aerobic sterile controls. Microcosms were incubated at room temperature in a laboratory cupboard. Five replicates from each treatment group were sampled on days 0, 42, 73, and 163. Groundwater was analyzed for BTEXTMB, dissolved O_2 (modified Winkler method), and NO_3^-/NO_2^-, when applicable. The experimental design is summarized in Table 4-10.

Results and Discussion. In terms of mean concentrations, total BTEXTMB losses were observed in both active treatment groups (aerobic / NO_3^- and aerobic only) relative to the sterile controls (Figure 4-13). Mass losses were slightly more extensive in the mixed electron-acceptor microcosms. However, concentrations in individual replicates were extremely variable, particularly in the active microcosms (Figure 4-14). Figure 4-13 also shows that NO_3^- utilization and NO_2^- production were low, and that dissolved O_2 concentrations declined to about 8 mg/L by day 42 and remained steady for the remainder of the experiment. In general, there was no preferential utilization of individual compounds (data not shown); in microcosms that experienced mass loss, all compounds had roughly the same proportional loss relative to initial concentrations.

The extremely large variability in the extent of mass loss in the active microcosms was unexpected. The data suggest either patchy microbial activity and/or biomass, or abiotic losses (e.g., leakage) in individual replicates, or some combination of the two. The lower variability in control microcosms (Figure 4-14) supports the former explanation. It is difficult, however, to fully explain these results without invoking either microcosm leakage or an unknown oxidation reaction. If the observed BTEXTMB mass loss resulted from aerobic oxidation, then from the stoichiometry of the mineralization reaction given in Equation 4-2 (106.5 moles O_2 per mole BTEXTMB), dissolved O_2 should have been completely consumed in microcosms with extensive mass loss. Complete utilization of O_2 was not observed (Figure 4-13); during this experiment depleted dissolved O_2 was observed in only two microcosms. It is conceivable that the added inorganic nutrients were insufficient to support full consumption of added O_2, but corresponding BTEXTMB mass losses should have been low as well. In fact, if CO_2 was the major oxidation product, then from Equation 4-2 the O_2 supplied to the microcosms was sufficient to mineralize only 14 % of the total BTEXTMB. Therefore, unless O_2 was leaking into microcosms, or mineralization was incomplete (which would not be expected with excess O_2), aerobic biotransformation cannot adequately explain these results. It is also possible that a currently undefined oxidation reaction was responsible for the mass loss. In some microcosms, an orange precipitate was observed on the surface of the aquifer sediment, but there was no clear correspondence to low BTEXTMB concentrations. The formation of a precipitate was unusual, but the reaction may have been unrelated to the observed aromatic hydrocarbon depletion (Millette et al., 1998).

On the final day of the experiment, microcosms were drained of fluid and put aside for additional work designed to determine whether biotransformation was responsible for the observed aromatic hydrocarbon losses. An aliquot of aquifer material was removed from nine microcosms for enumeration, and then all active microcosms were reamended with 25-ml gasoline-saturated groundwater, NO_3^- where required, nutrients, and sealed with mininert™ valves. Control microcosms received amendments and additional sodium azide solution. Aquifer material was removed from microcosms and enumerated for aerobic

Table 4-10. *Design Summary, Experiment 5*

Treatment (Electron-Acceptor Regime)	Replicates	Sampling Events
Active, 4-ml pure O_2 headspace, 70 mg/L BTEXTMB (gas. sat.), 25 mg/L NO_3^--N, N,P	5 active	4
Active, 4-ml pure O_2 headspace, 70 mg/L BTEXTMB (gas. sat.), N,P	5 active	4
Sterile, 4-ml pure O_2 headspace, 70 mg/L BTEXTMB (gas. sat.), N,P	5 sterile	4

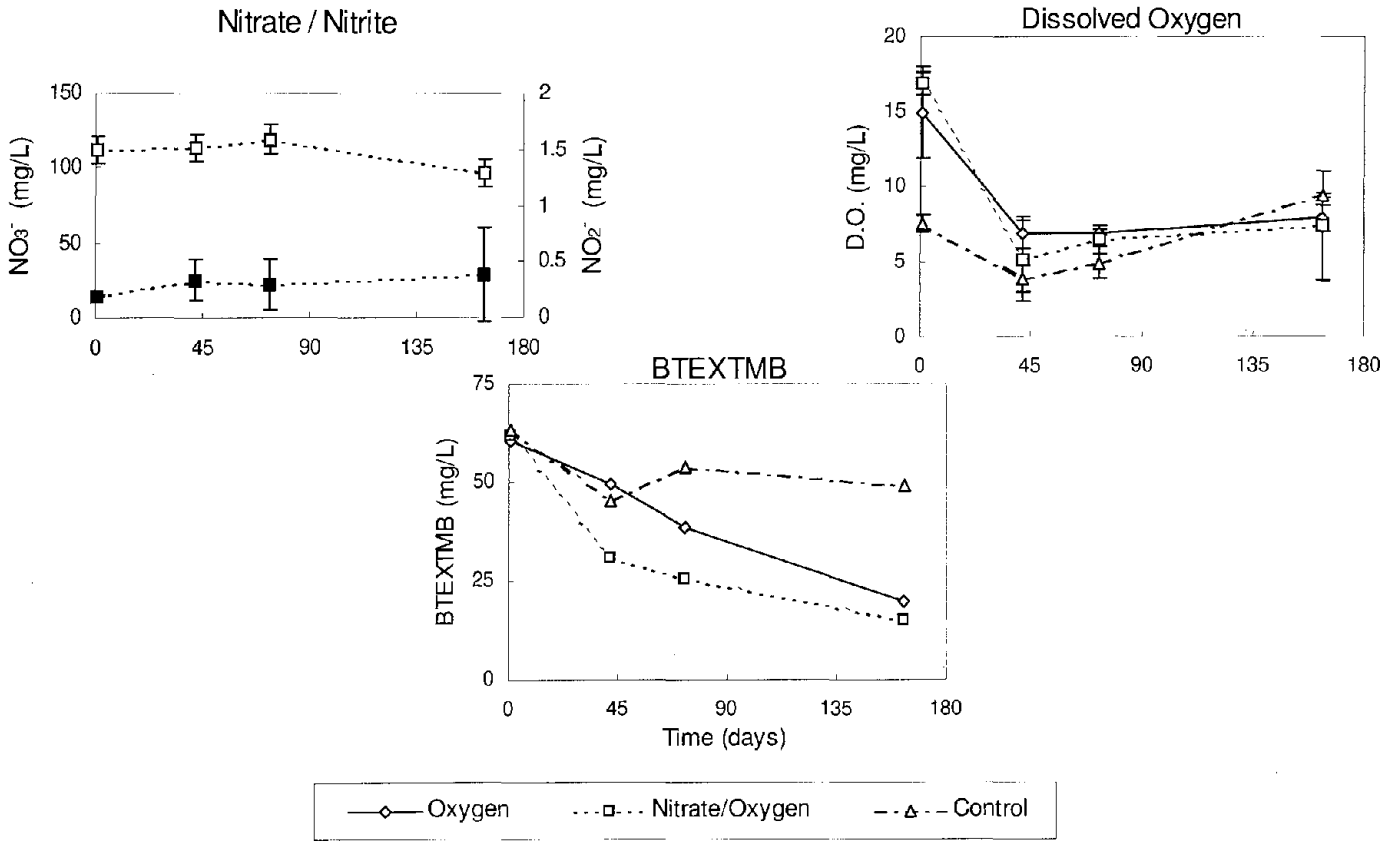


FIGURE 4-13 Nitrate, NO_2^-, D.O., and total BTEXTMB concentrations in Experiment 5 microcosms. The detection limit value (0.2 mg/L) plotted for not-detected NO_2^- samples (solid squares). Plotted values are mean ± s.d. of replicate microcosms. For clarity BTEXTMB error bars not plotted.

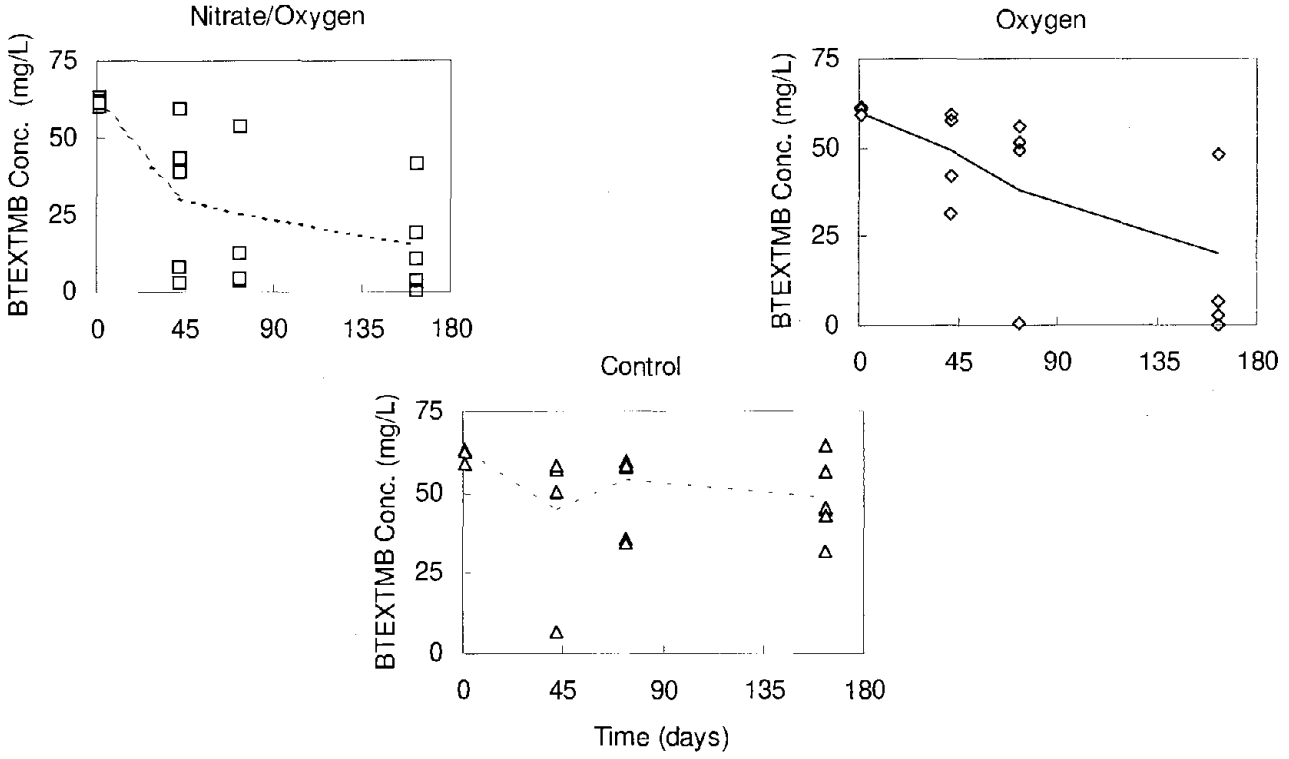


FIGURE 4-14 BTEXTMB concentrations in individual replicates for each of the treatment groups in Experiment 5. Lines connect mean values for each sampling event.

heterotrophs and benzene-toluene degraders using methods described in Section 3.5.1. Reamended microcosms were then sampled periodically over the following 23 days for headspace BTEX. Overall, these headspace BTEX results agreed fairly well with concentration data obtained on day 163. The individual microcosms with low aromatic-hydrocarbon concentrations on day 163 underwent mass loss relative to controls during the 23 day post-experiment incubation period. Similarly, microcosms with high concentrations on day 163 did not lose mass relative to controls. The enumeration results for the control and O_2-only microcosms also agreed quite well with the mass-loss data; low numbers were present in the microcosms with high BTEXTMB concentrations on day 163, and little mass loss during the post-experiment incubation, and vice versa. These data are summarized in Table 4-11. This may be evidence that the experimental conditions (i.e., concentrations of parent compounds or metabolites) were toxic to the microbial community in some replicates, but not in others. Enumeration results were more variable for the NO_3^-/O_2 treatment group.

The relatively rapid uptake after reamendment suggested that microbial activity was responsible for the low aromatic-hydrocarbon concentrations observed on day 163. This in turn suggested that there was potential for aerobic biotransformation of high concentrations of dissolved-phase BTEXTMB in pristine Borden aquifer material. Because of conflicting dissolved-O_2 data, however, and variability in the BTEXTMB concentrations, the possibility of experimental artifact (i.e., abiotic losses) cannot be ruled out. If microbial activity was responsible for the observed mass loss in these microcosms, the distribution of populations tolerant of high concentrations of aromatic hydrocarbons was patchy in this pristine material. This is consistent with the results of Butler et al. (1997) who found that microbial communities were very localized in the Borden aquifer. Distinct populations with varying metabolic capabilities were observed within a <5.5 m^2 area. In contrast, the large and consistent O_2 demand observed in gasoline-contaminated aquifer material extracted from the Nitrate Cell (Section 4.2) indicated that an acclimated population had developed in and immediately below the gasoline source area.

4.2 Microcosm Experiments: Gasoline-Contaminated Borden Sand

4.2.1 Experiment 6: Extent of Biotransformation Under Various Substrate and Mixed Electron-Acceptor Conditions

The purpose of this experiment was to investigate the effects of *in situ* gasoline exposure and the presence of other gasoline hydrocarbons on the ability of the indigenous bacteria to biotransform the aromatic hydrocarbons. When cores were collected in July, 1997 from the Nitrate Cell, the aquifer material had been exposed to gasoline (dissolved- and/or residual-phase) for 19 months, and to NO_3^- for 14 months.

Design 1 microcosms were set up with core material extracted from the Nitrate Cell. Core from a depth interval of approximately 80 to 180 cm bgs was used in the experiment. Before dispensing to microcosms, most of the residual gasoline phase was removed by saturating the aquifer material with sterile water, gently stirring the resulting slurry, and then draining the liquid phase. This was done to remove as much of the existing aqueous aromatics and residual gasoline as possible, with minimal disturbance to the microbial population. Analysis of the washed material indicated that this procedure was successful in lowering aqueous concentrations of benzene, toluene, and ethylbenzene, but mg/L concentrations of the less-soluble aromatics remained. This indicates that the washing procedure did not completely remove the residual gasoline phase, which contained substantial concentrations of these constituents at the end of the field experiment. With the exception of one low-substrate-concentration treatment group, all microcosms were set up with gasoline-saturated groundwater to reflect field conditions. Because a residual phase was still present, initial concentrations of the xylene isomers, trimethylbenzene isomers, and naphthalene were near gasoline-saturated concentrations in the low-concentration (10x dilution) microcosms; as a result the measured day 1 total BTEXTMB concentration was 19.5 mg/L (see Figure 4-16c).

Five treatment groups were used to isolate the effects of electron-acceptor regime and BTEXTMB concentration in contaminated aquifer material: microaerophilic / NO_3^-, aerobic / NO_3^-, microaerophilic /NO_3^- plus low-BTEXTMB (10x dilution of gasoline-saturated water), unamended (no O_2 / NO_3^-), and a microaerophilic / NO_3^- sterile control. All microcosms except in the low-BTEXTMB group were prepared with gasoline-saturated water and O_2 was added to headspaces as described previously. The

Table 4-11. *Microbial Enumerations and Hydrocarbon Degrading Activity for Nine Replicates Selected for Reamendment after Day 163.*

Replicate	Day 163 Total BTEXTMB Concentration (mg/L)	Heterotrophic Plate Count (CFU/g wet wt.) (s.d.)	Benzene-Toluene Degraders (MPN/g wet wt.)	Hydrocarbon-Degrading Activity After Reamendment
Control A	65.0	n.d.[1]	n.d.	(-)
Control B	32.0	5.7×10^2 (3.7×10^2)	n.d.	(-)
Control E	56.4	n.d.	n.d.	(-)
O_2 A	0.0	9.0×10^7 (1.0×10^7)	2.0×10^3	(+)
O_2 C	47.9	4.0×10^5 (4.8×10^4)	23	(-)
O_2 E	2.6	2.7×10^6 (7.3×10^4)	4.3×10^2	(+)
O_2 / NO_3^- A	41.6	1.3×10^6 (9.5×10^4)	2.1×10^2	(+)
O_2 / NO_3^- C	19.3	1.5×10^6 (1.6×10^5)	1.5×10^3	(+)
O_2 / NO_3^- E	3.6	4.3×10^7 (2.6×10^6)	43	(+)

[1] n.d. - none detected. Determination of hydrocarbon degrading activity after reamendment based on observed compound losses.

Table 4-12. *Design Summary, Experiment 6*

Treatment (Electron Acceptor Regime, BTEXTMB Concentration)	Replicates	Expected Sampling Events
Active, 100 mg/L BTEXTMB (gas. sat.), 2-ml air headspace, 25 mg/L NO_3^--N, N,P	3	4
Active, 100 mg/L BTEXTMB (gas. sat.), 4-ml pure O_2 headspace, 25 mg/L NO_3^--N, N,P	3	4
Active, 10 mg/L BTEXTMB (10x dil.), 2-ml air headspace, 25 mg/L NO_3^--N, N,P	3	4
Active, 100 mg/L BTEXTMB gas. sat.), N,P	3	4
Sterile, 100 mg/L BTEXTMB (gas. sat.), 2-ml air headspace, 25 mg/L NO_3^--N, N,P	3 sterile	4
Aqueous Sterile (no aquifer material), 90 mg/L BTEXTMB (neat), 4-ml pure O_2 headspace	3 sterile	4
Aqueous Sterile (no aquifer material), 90 mg/L BTEXTMB (neat), 2-ml air headspace	3 sterile	4

microaerophilic (low O_2) microcosms were incubated in the anaerobic chamber, and the aerobic (high-O_2) microcosms in a laboratory cupboard. Groundwater was analyzed for BTEXTMB, dissolved O_2 (with the O_2 meter), NO_3^-, and NO_2^-. Extra replicates of each treatment group were also prepared for a denitrification assay (acetylene block). These microcosms were analyzed for N_2O in addition to the other parameters. The design is summarized in Table 4-12.

In response to rapid dissolved O_2 and NO_3^- utilization in the aerobic / NO_3^- microcosms, several modifications were made to the experimental design. On day 82, NO_3^- was respiked into the aerobic/NO_3^- microcosms to determine if rapid NO_3^- utilization would continue under anaerobic conditions. Microcosms were amended and subsequently incubated inside the anaerobic chamber to maintain anoxic conditions. At the same time, duplicate microcosms from all treatments except the unamended group were spiked with acetylene and sampled after 15 days of incubation. Finally, on day 154, the headspaces of all remaining microcosms except the unamended group were flushed with pure O_2 to determine if biotransformation of aromatic hydrocarbons would be stimulated under aerobic conditions. To accommodate these changes, microcosms were sacrificed and sampled more frequently over a 173-day incubation period. Microcosms were sampled in triplicate for the first three sampling events, and in duplicate or singly thereafter.

Aqueous (no aquifer material) control microcosms designed to investigate abiotic losses of dissolved O_2 and aromatic hydrocarbons were also prepared (Section 3.2.1) and incubated with other Experiment 6 microcosms (Table 4-12). Microcosms were sampled and analyzed for aromatic hydrocarbons and dissolved O_2 (using the O_2 meter) periodically between days 0 and 159.

Results and Discussion. The aromatic hydrocarbons in the unamended control microcosms did not decline relative to the sterile controls; other microbial populations (e.g., Fe or SO_4^{2-} reducers) were therefore not degrading aromatic-hydrocarbons at a detectable rate. For brevity, unamended microcosm results are not shown or discussed further.

The aqueous sterile control microcosms were sampled on day 0, several hours after preparation; measured concentrations showed that the 2-ml air and 4-ml pure O_2 headspaces provided 4.9 mg/L and 26.2 mg/L initial dissolved O_2, respectively. Diffusive losses of dissolved O_2 occurred during storage both inside (microaerophilic controls) and outside (aerobic controls) the anaerobic chamber, as microcosms equilibrated with the external atmosphere (Figure 4-15). The rate of loss was, however, much lower than in active microcosms. The agreement between the microaerophilic controls with and without aquifer

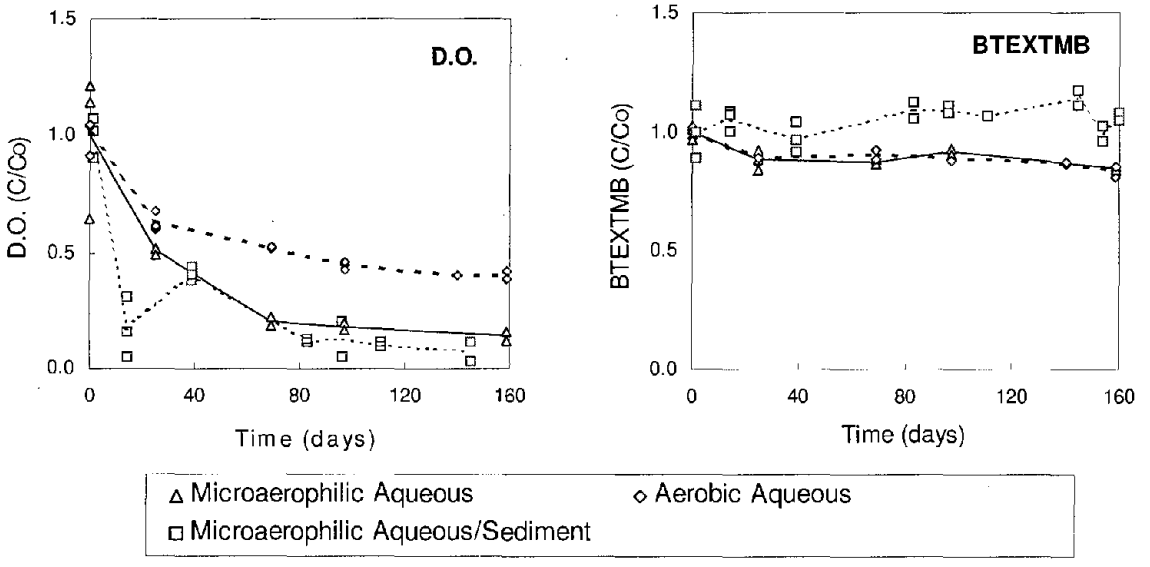


FIGURE 4-15 Normalized D.O. and total BTEXTMB concentrations in sterile microcosms with and without gasoline-contaminated aquifer material from the Nitrate Cell (Experiment 6). Lines connect single values or means of duplicate and triplicate replicates.

material indicated that the abiotic O_2 demand of the contaminated aquifer material was quite low. This is not surprising because the redox potential in the Nitrate Cell was buffered by the continuous presence of NO_3^-, and consequently, significant quantities of reduced inorganic species such as Fe and Mn that are capable of reacting abiotically with O_2 were not produced. In contrast to O_2, diffusive loss of dissolved BTEXTMB in both aqueous and conventional (i.e., containing aquifer material) sterile controls was low (Figure 4-15).

Initial BTEXTMB losses relative to controls were observed between preparation and the first sampling event (24 hours) for all active microcosms, and the extent of consumption was roughly proportional to the mass of O_2 in the microcosm (Figures 4-16a, 4-16b, and 4-16c). For example, the largest decline during early time occurred in the microcosms amended with pure O_2. In the high-BTEXTMB-concentration microcosms amended with microaerophilic O_2 and NO_3^-, which is the best analogy to the Nitrate Cell, the close agreement between active and sterile-control BTEXTMB concentrations shows that the microaerophilic O_2 had only a minor effect on mass loss (Figure 4-16b). This result is consistent with field observations (Chapter 5).

Once O_2 was depleted, biotransformation losses of the aromatic hydrocarbons were minor. Percentage losses of individual compounds for the two microaerophilic / NO_3^- treatment groups (high- and low-BTEXTMB) are shown on Table 4-13. It should be noted that because the microaerophilic O_2 was essentially consumed by day 1, and losses were calculated relative to day 1 concentrations, depletion of aromatics between days 1 and 145 occurred primarily under anaerobic conditions, possibly at the expense of NO_3^-. Under O_2-depleted conditions, only toluene, and possibly ethylbenzene declined in low-BTEXTMB microcosms. After O_2 was added on day 154, losses of benzene, toluene, ethylbenzene, *m+p*-xylenes, 1,2,4-trimethylbenzene, and to a lesser extent, *o*-xylene, were observed in the microcosms amended with a 10x dilution of gasoline-saturated water. In high-BTEXTMB microcosms, on the other hand, only benzene, toluene, and ethylbenzene concentrations declined after O_2 reamendment. 1,3,5-trimethylbenzene, 1,2,3-trimethylbenzene, and naphthalene appeared recalcitrant under these incubation conditions. It is unclear why the observed trimethylbenzene isomers and naphthalene ratios are higher on day 173 relative to day 145. One plausible explanation is that the concentrations in the sterile controls declined more than in the active treatments when the microcosms were opened on day 154 to replenish headspaces with O_2. This most likely resulted from the lack of a residual gasoline phase in the controls, which acted as a reservoir for the relatively insoluble aromatics in the active microcosms. Consequently, it is possible that although the relatively insoluble aromatics appeared recalcitrant, the presence of a residual phase may have obscured minor removal from the aqueous phase.

In the aerobic / NO_3^- microcosms, the initial dissolved O_2 (ca. 26 mg/L, assumed from aqueous sterile controls) was rapidly consumed (Figure 4-16a). It should be noted that the complete mass of O_2 in these microcosms was probably not consumed within 24 hours; after the initial rapid consumption of the O_2 dissolved in the aqueous phase, the rate of consumption may have been controlled by diffusion across the water-headspace interface. In both of the microaerophilic treatment groups, the dissolved O_2 concentration also dropped rapidly, from an initial concentration of 4.9 mg/L (assumed from sterile control microcosms) to a threshold concentration within 24 hours. When microcosms were reamended with pure O_2 on day 154, rapid BTEXTMB and dissolved O_2 depletion were again observed in all active microcosms (Figures 4-16a, 4-16b, and 4-16c). Overall, these responses showed that an acclimated aerobic population was capable of producing a large O_2 demand in the contaminated aquifer material relative to the pristine material, and that dissolved O_2 was required for biotransformation of the aromatic hydrocarbons (with possible exceptions of toluene and ethylbenzene).

The utilization of NO_3^- varied depending on the treatment group. The one similarity was that all three active groups lost some NO_3^- relative to the sterile-control group during the first 24 hours of the experiment when dissolved O_2 was present. The greatest losses occurred in the aerobic microcosms, where NO_3^- was completely consumed by day 14 (Figure 4-16a). The rapid consumption of NO_3^- in these microcosms was surprising because such rapid losses had not been observed in the Nitrate Cell. One possible explanation is that the NO_3^- was being utilized as an assimilatory nitrogen source during aerobic biotransformation; because the mass of O_2 and available hydrocarbons in these microcosms was large, the mass of NH_4-N may have been insufficient to meet the assimilatory N demand. As a consequence,

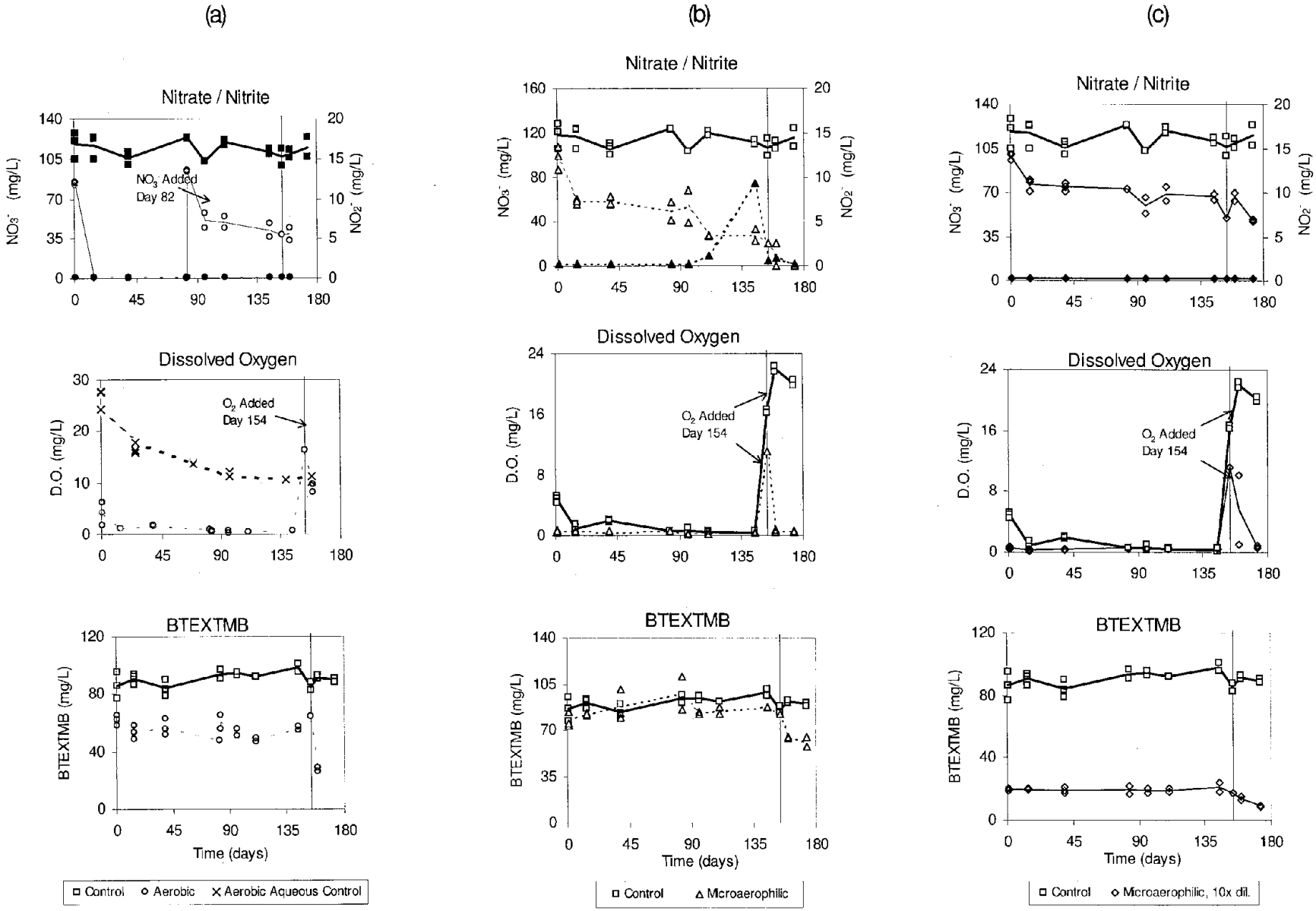


FIGURE 4-16 Nitrate, NO_2^-, D.O., and total BTEXTMB concentrations in active and sterile control microcosms with gasoline-contaminated aquifer material from the Nitrate Cell (Experiment 6) (a) Aerobic (high O_2) treatment (b) Microaerophilic (low-O_2) treatment (c) Microaerophilic, 10x BTEXTMB dilution treatment. Oxygen added to all microcosms on day 154 except high-O2 aqueous control plotted on (a). Nitrite not detected in sterile controls; for clarity, data not shown on plots. Nitrite in active microcosms plotted as solid symbols. Lines connect single values or means of duplicate and triplicate replicates.

Table 4-13. *Percent of Individual Aromatic Hydrocarbons Remaining in Active, Low- and High-BTEXTMB Concentration Microcosms Relative to Sterile Controls. Microcosms were Amended Initially with an Air Headspace and NO_3^-, and Contained Gasoline-Contaminated Aquifer Material from the Nitrate Cell.*

Compound	% Remaining Relative to Sterile Controls [1]			
	10x Dilution of Gasoline-Saturated Water (2-ml Air Headspace)		Gasoline-Saturated Water (2-ml Air Headspace)	
	Day 145 (O_2 depleted)	**Day 173** (19 days after pure-O_2 addition)	**Day 145** (O_2 depleted)	**Day 173** (19 days after pure-O_2 addition)
Benzene	83	28	97	80
Toluene	50	0.7	101	55
Ethylbenzene	77	2	106	21
m+p-xylene	101	38	98	109
o-xylene	104	83	96	105
1,3,5-Trimethylbenzene	106	130	90	127
1,2,4-Trimethylbenzene	105	51	89	124
1,2,3-Trimethylbenzene	104	122	87	120
Naphthalene	98	110	84	125

[1] Percent remaining for a given sampling event calculated from $\frac{(C/C_0)_{Active}}{(C/C_0)_{Control}} * 100$ where C_o is mean concentration on day 1.

Table 4-14. *Dissolved Nitrous Oxide Concentrations in Selected Microcosms Containing Contaminated Aquifer Material from the Nitrate Cell. Nitrate was Present in All Microcosms when Analyzed. Acetylene was Added on Day 82, 15 Days Prior to Analysis.*

Individual Microcosm	Acetylene Added	Dissolved N_2O (mg/L)
Aerobic, gas.-sat.	(+)	5.45
Aerobic, gas.-sat.	(+)	9.64
Aerobic, gas.-sat.	(-)	<0.45
Microaerophilic, gas.-sat.	(+)	<0.45
Microaerophilic, gas.-sat.	(+)	<0.45
Microaerophilic, gas.-sat.	(-)	<0.45
Microaerophilic, 10x dil.	(+)	0.39
Microaerophilic, 10x dil.	(+)	1.00
Microaerophilic, 10x dil.	(-)	<0.45
Sterile Control	(+)	<0.45
Sterile Control	(+)	<0.45

inorganic nutrients did not limit the consumption of this large mass of O_2. When NO_3^- was replenished on day 82, additional NO_3^- losses were observed under anaerobic conditions, but the rate was lower (Figure 4-16a). The production of N_2O in acetylene-amended microcosms between days 82 and 97 suggested that denitrification was occurring in these microcosms under anaerobic conditions (Table 4-14).

Nitrate utilization was also observed in the microaerophilic / NO_3^-, high-BTEXTMB microcosms (Figure 4-16b), the condition most similar to the field treatment cell. A comparison of these results with those of Experiments 4 and 5 clearly indicates that the extent of NO_3^- utilization in the presence of high aqueous concentrations of BTEXTMB increased after prolonged exposure to contamination. Although NO_3^- declined under O_2-depleted conditions, however, utilization of the substrates that are typically labile under denitrifying conditions (toluene or ethylbenzene) was not evident. Assuming toluene was mineralized in a denitrification reaction, and there was no assimilation of C and N by microbial cells, mass loss is governed by

$$C_7H_8 + 7.2H^+ + 7.2NO_3^- \rightarrow 3.6N_2 + CO_2 + 7.6H_2O \qquad 4\text{-}3$$

Consequently, using an initial toluene concentration of 40 mg/L (2 mg initial mass), consumption of 25 mg/L NO_3^--N (5 mg NO_3^- initial mass) would have produced an observable decrease in toluene mass of about 50%. Therefore, although denitrifying activity in gasoline-contaminated aquifer material was apparently not inhibited, the labile aromatic hydrocarbons were either recalcitrant or utilized at a very low rate. This may reflect a preference for other organic substrates such as other gasoline hydrocarbons, as observed in other studies with hydrocarbon-contaminated aquifer material (Hutchins et al., 1991a). Alternatively, it is possible that NO_3^- was used to satisfy an N demand for an undefined, anaerobic reaction (e.g., fermentation reactions).

The specific denitrifying pathway responsible for the NO_3^- utilization in these microcosms is unclear because although NO_2^- accumulation was evident in the microaerophilic / NO_3^-, high BTEXTMB microcosms after day 82, N_2O was not present in the duplicate microcosms amended with acetylene (Table 4-14). These NO_3^- data yielded a zero-order (linear) depletion rate of 0.43 mg/L/d, which is within the range of rates measured in the field treatment cell (see Section 5.1.2). In contrast, in the microaerophilic / NO_3^-, low-BTEXTMB group, there was less NO_3^- utilization, and no observable NO_2^- accumulation (Figure 4-16c), but there were minor losses or toluene and ethylbenzene under O_2-depleted conditions (Table 4-13). The reasons for the differences in the extent of NO_3^- utilization in these microaerophilic microcosms were not evident from the data collected in this experiment.

4.3 Microbial Characterization Results

Microbial enumerations and activity measurements were used as additional indirect lines of evidence for *in situ* biotransformation of gasoline hydrocarbons. Patterns of microbial activity and numbers, in conjunction with other lines of evidence, have been used in other studies to document biotransformation in contaminated aquifers (Harvey et al., 1984; Song and Bartha, 1990; Madsen et al., 1991). In this study, we compared numbers and activity in pristine Borden aquifer material with material extracted from the treatment cells (19 month exposure to dissolved- and /or residual-gasoline). A detailed characterization of the microbial populations of the Borden aquifer, including changes that occurred in response to hydrocarbon contamination, is provided by Butler et al. (1997).

4.3.1 Pristine Aquifer Material

Enumerations. Several cores collected near the field site for use in laboratory experiments were enumerated to determine the numbers of aerobic heterotrophs and denitrifiers in background (pristine) Borden aquifer material (Table 4-15). In all cases, core material from the shallow saturated zone (depths less than two meters below the water table) was used. The majority of the cores were collected in 1993 or 1994, near the beginning of this study, and enumerated when used for an experiment. Core 2-2 was collected in June, 1997, and enumerated in September, 1997. All cores were collected in the northeast corner of the sand pit, within about 100 m of the treatment cells. Results indicate that the age of the core had no consistent effect on the numbers of culturable organisms or the capacity to biotransform TEX under denitrifying conditions (Table 4-15).

Table 4-15. *Microbial Enumerations of Borden Cores*

Core I.D.	Laboratory Experiment	Heterotrophic Plate Counts (CFU/g dry wt)	Denitrifiers (MPN/g dry wt)	Approx. Core Age (months)[1]	TEX Degradation with NO_3^-
#7-93	Microcosm 1	6.2×10^4	1.7×10^4	6	(+)
#5-93	Microcosm 2	1.1×10^5	2.8×10^2	8	(+) (1 of 3)
#4-93	ETS Activity	1.2×10^5	2.5×10^3	11	(-)
#2-94	None	1.25×10^6	2.7×10^5	1	(+)
#6-93	Microcosm 3(i)	n.t.[2]	n.t.	8	(-)[3]
#2-93	None	5.8×10^5	1.2×10^6	13	(+)
#1-94/#3-94	Microcosm 3(ii)	1.1×10^6	4.9×10^4	2	(+)
	Microcosm 6				

[1] months of storage at 4°C before use.
[2] n.t. not tested.
[3] a few replicates (+) after ca. 1 year incubation

Counts of viable, aerobic heterotrophs in pristine aquifer material varied by about two orders of magnitude (Table 4-15). HPCs ranged from 6×10^4 CFU/g (colony forming units per gram) to 1×10^6 CFU/g. Counts from these cores were consistent with other studies of pristine Borden aquifer material (Butler et al., 1997; Barbaro et al., 1994). Using a much larger set of data, Barbaro et al. (1994) found that the numbers of aerobic, culturable microorganisms in the upper 2 m of the aquifer varied by more than six orders of magnitude, and were correlated strongly with depth and *in situ* dissolved-O_2 concentration. Denitrifier numbers were more variable than aerobic heterotrophs in these cores, with a range from 3×10^2 MPN/g (most probable number per gram) to 1×10^6 MPN/g. As noted by Butler et al. (1997), the magnitudes of the aerobic heterotroph and denitrifier counts are similar enough to suggest that a substantial fraction of the population in pristine material has the capacity to denitrify. Overall, the numbers of culturable microorganisms in Borden aquifer material were consistent with numbers obtained in other shallow, sandy aquifers (e.g., Beloin et al., 1988).

Activity Assays. The potential for gasoline-saturated groundwater to inhibit the activity of the Borden microbiota was assessed using the ETS assay, which is based on the reduction of INT to INT-formazan by microbial activity (Section 3.4.2). The solid:liquid ratio in these systems (10 g:3 ml) differed from that in the microcosm systems (20 g:50-55 ml). Based on a gasoline-saturated water concentration of 130 mg/L total BTEX, the BTEX content of the liquid phase in these INT systems was approximately 43 mg/L (gasoline-saturated water) or 4.3 mg/L (10x dilution of gasoline-saturated water). The experimental design is summarized in Table 4-16. The endogenous activity of pristine aquifer material is too low to be detected by the ETS assay (Butler et al., 1997), so the effect of added gasoline-saturated groundwater alone, or in the presence of R2A broth, as a nutrient source, was investigated. It should be noted that this experiment was conducted at natural aquifer temperature (10°C), rather than at room temperature, as was necessary for most of the other laboratory experiments (Section 3.2.1).

The presence of the 43 mg/L BTEX water slowed, but did not prevent INT-formazan accumulation in aerobically-incubated, R2A-containing, Borden aquifer material (Figure 4-17). On the other hand, the more dilute contaminated water had little effect on INT-formazan accumulation, and may have even stimulated ETS activity in the presence of R2A to a small degree. No ETS activity was detectable over 37 days in the presence of 43 mg/L BTEX alone, but there was a small amount of activity up to day 17 in the 4.3 mg/L BTEX-amended material. The results in the presence of R2A indicate that the higher level of water-soluble gasoline components caused some inhibitory effect but did not severely impair aerobic microbial activity. On this basis, one might expect an eventual onset of metabolic activity in the vials with 43 mg/L BTEX-containing water but no R2A, but this was not observed (Figure 4-17). Such activity may have been delayed (i.e., lag >37 days), or may have been largely suppressed because of a lack of

Table 4-16. *Experimental Design: ETS Activity in Pristine Aquifer Material*

Treatment[1]	Hypovial Contents
Blank	Borden aquifer solids, water
Active	Borden aquifer solids, INT, water
R2A	Borden aquifer solids, INT, R2A, water
Gas	Borden aquifer solids, INT, 1 ml gasoline-saturated water, water
Gas + R2A	Borden aquifer solids, INT, 1 ml gasoline-saturated water, R2A
1/10 gas	Borden aquifer solids, INT, 0.1 ml gasoline-saturated water, water
1/10 gas + R2A	Borden aquifer solids, INT, 0.1 ml gasoline-saturated water, R2A

[1] Each treatment was prepared in duplicate. Unleaded gasoline was obtained from a local source.

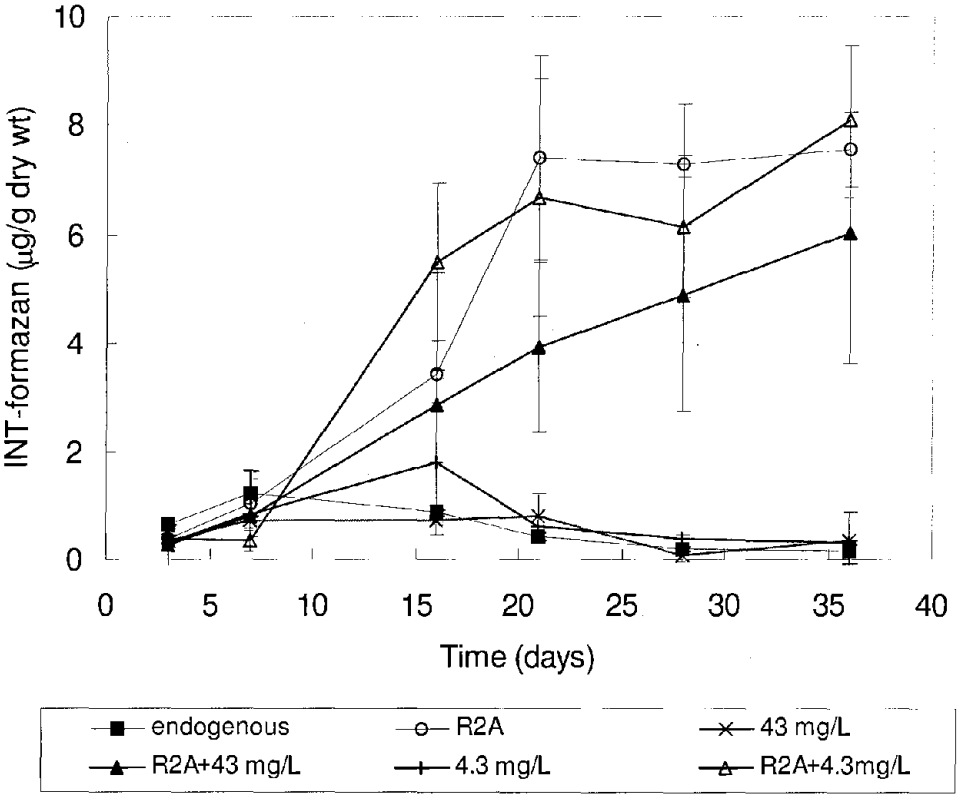


FIGURE 4-17 Effect of gasoline-contaminated groundwater on ETS activity in pristine Borden material. Plotted values are the mean ± s.d. of two replicates.

inorganic nutrients (N, P) in the Borden sand. It is also possible that the 43 mg/L BTEX concentration was more stressful to the indigenous population when R2A medium was not available. Substrates in the R2A (e.g., glucose, casamino acids, yeast extract, peptone, starch) are easily metabolized, and protective components (e.g., pyruvate, peptone, phosphate buffer) are present as well. This could be considerably more supportive than the fairly high levels of potentially-toxic BTEX substrates. We have readily developed BTEX-degrading enrichment cultures capable of metabolizing ≥50 mg/L BTEX from Borden aquifer material, but this was done in a stepwise manner, which allowed for adaptation to progressively higher BTEX concentrations.

4.3.2. Gasoline-Contaminated Aquifer Material

Enumerations. To compare pristine and gasoline-contaminated aquifer material, aquifer material from cores extracted aseptically from the treatment cells (July, 1997) was also enumerated for viable, aerobic heterotrophs and denitrifiers, as well as for aerobic benzene-toluene degraders. Two aquifer samples were enumerated from each cell: A shallow sample from the 50 to 80 cm bgs interval that contained a residual gasoline phase, and a deep sample from 155 to 190 cm bgs interval that had been exposed to high dissolved-phase concentrations but not to gasoline. The results are summarized on Figure 4-18. For comparison with pristine material, the sample from Core 2-2, which was prepared and incubated with the contaminated samples, is also plotted on this figure. Viable, aerobic heterotrophs ranged from 5 x 10^4 CFU/g (shallow Control Cell) to 10^7 CFU/g (deep Nitrate Cell), with Core 2-2 falling within this range. Counts in the pristine sample were greater than in three of the four contaminated samples (Figure 4-18). However, a response to gasoline contamination was indicated by clearly-discernable differences in colony types between the pristine- and contaminated-sample plates (data not shown). Denitrifiers ranged from 4 x 10^3 MPN/g in the pristine sample to >2 x 10^6 MPN/g in the deep sample from the Nitrate Cell. Samples from the Nitrate Cell contained the highest numbers of denitrifiers. Benzene-toluene degraders were less prolific, with numbers ranging from 4 x 10^1 MPN/g in the pristine sample to 7.5 x 10^3 MPN/g in the deep sample from the Nitrate Cell.

Overall the data collected here indicate that the numbers of culturable microorganisms in the samples extracted from the treatment cells were higher than the pristine sample from Core 2-2. Both denitrifiers and benzene-toluene degraders were higher by up to two orders of magnitude, but differences in aerobic heterotrophs were less pronounced. However, it should be noted that the number of samples collected from the gasoline-contaminated areas was too small to characterize the variability within these areas, which would be required for a meaningful statistical comparison. If one considers all of the enumeration results in pristine aquifer material (Table 4-15), numbers of aerobic heterotrophs and denitrifiers are quite variable, and it is less clear that numbers in contaminated areas are elevated. Similar numbers are

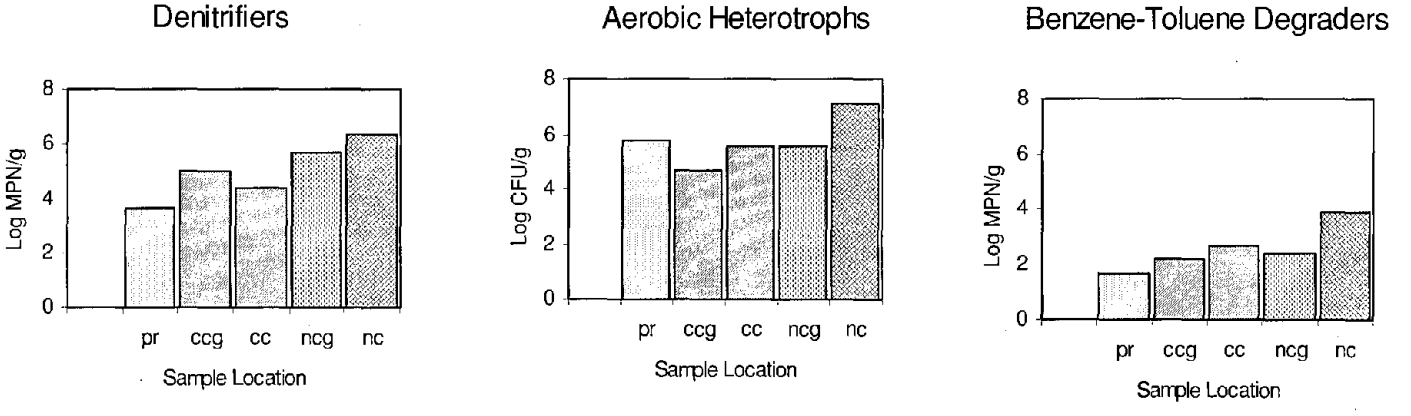


FIGURE 4-18 Numbers of denitrifiers, aerobic heterotrophs, and benzene-toluene degraders in pristine and contaminated aquifer material. pr: pristine (Core 2-2); ccg and ncg: samples from gasoline-contaminated zones in Control and Nitrate Cells, respectively; cc and nc: samples from below gasoline-contaminated zones in Control and Nitrate Cells, respectively.

Table 4-17. *Experimental Design: ETS Activity in Pristine and Contaminated Aquifer Material*

Treatment[1]	Hypovial Contents
Blank	Borden aquifer solids, water, N,P
Sterile	Autoclaved Borden aquifer solids, INT, 0.5 ml sodium azide, water N,P
Unamended	Borden aquifer solids, INT, water, N,P
Positive	Borden aquifer solids, INT, 0.5 ml glucose, water, N,P
Gasoline-saturated	Borden aquifer solids, INT, 1 ml gasoline-saturated water, water, N,P
1/10 gasoline-saturated	Borden aquifer solids, INT, 1 ml 10x dilution of gasoline-saturated water, water, N,P

[1] Each treatment was prepared in duplicate. Gasoline: API 91-01.

consistent with electron-acceptor and organic substrate uptake data which suggested that electron-acceptor flushing did not stimulate the growth of a large *in situ* population.

Activity Assays. A second ETS assay was performed to compare aerobic ETS activity in pristine and contaminated aquifer material. The treatment groups are summarized in Table 4-17. As in the other ETS assay, because of dilution with other fluid, the actual dissolved hydrocarbon concentrations in the vials were roughly half the gasoline-contacted concentrations (ca. 50 mg/L and 5 mg/L total aromatics). For consistency with the enumerations, these vials were incubated at room temperature. Microbial activity was assayed in samples of pristine aquifer material (Core 2-2), the zones below the gasoline contamination (155-190 cm bgs) in both treatment cells, and the gasoline-contaminated zone (80-140 cm bgs) of the Nitrate Cell.

Blank-corrected ETS activity is shown in Figure 4-19. For clarity data are plotted as means of duplicate determinations, but for some samples there was substantial variability between duplicates. Trends based on means provided, therefore, only an approximate measure of differences in activity. Activity varied by both sample location and amendment. The largest accumulation of INT-formazan occurred in the deep sample from the Nitrate Cell when gasoline-saturated water was supplied as a carbon source (Figure 4-19). Activity in the deep sample from the Control Cell was similar, except that formazan accumulation in the gasoline-saturated vials was substantially lower. In both of these samples, substantial activity also occurred in unamended vials, which probably reflects utilization of existing gasoline hydrocarbons. In the Control Cell, activity in the positive (glucose-amended) treatment was similar to the unamended treatment, but in the Nitrate Cell, it was much greater. It is not clear why the positive controls behaved differently. Activity was generally lower in the pristine sample, but surprisingly, accumulations over the 28-day incubation in the treatments amended with gasoline-saturated water were greater than those in the positive control which contained an easily-utilized carbon source. Again, as in the Control Cell, it is not clear why the activity in the glucose-amended vials was low. The lowest activities occurred in the sample collected from the gasoline-contaminated zone in the Nitrate Cell. Therefore, the gasoline phase seemed to inhibit ETS activity somewhat relative to the other contaminated sample locations included in this experiment.

Overall, this assay indicated that the greatest potential for aerobic biotransformation of gasoline-saturated water existed in the lower samples collected from the treatment cells, with highest ETS activity observed in the Nitrate Cell. Activity was relatively low in the presence of a gasoline phase relative to these lower depths. While activity measured by this assay was low in the presence of a gasoline phase relative to nearby locations, there was other evidence that the aerobic population, and hence the potential for aerobic biotransformation of aromatic hydrocarbons, was not completely inhibited within the gasoline source area (Experiment 6).

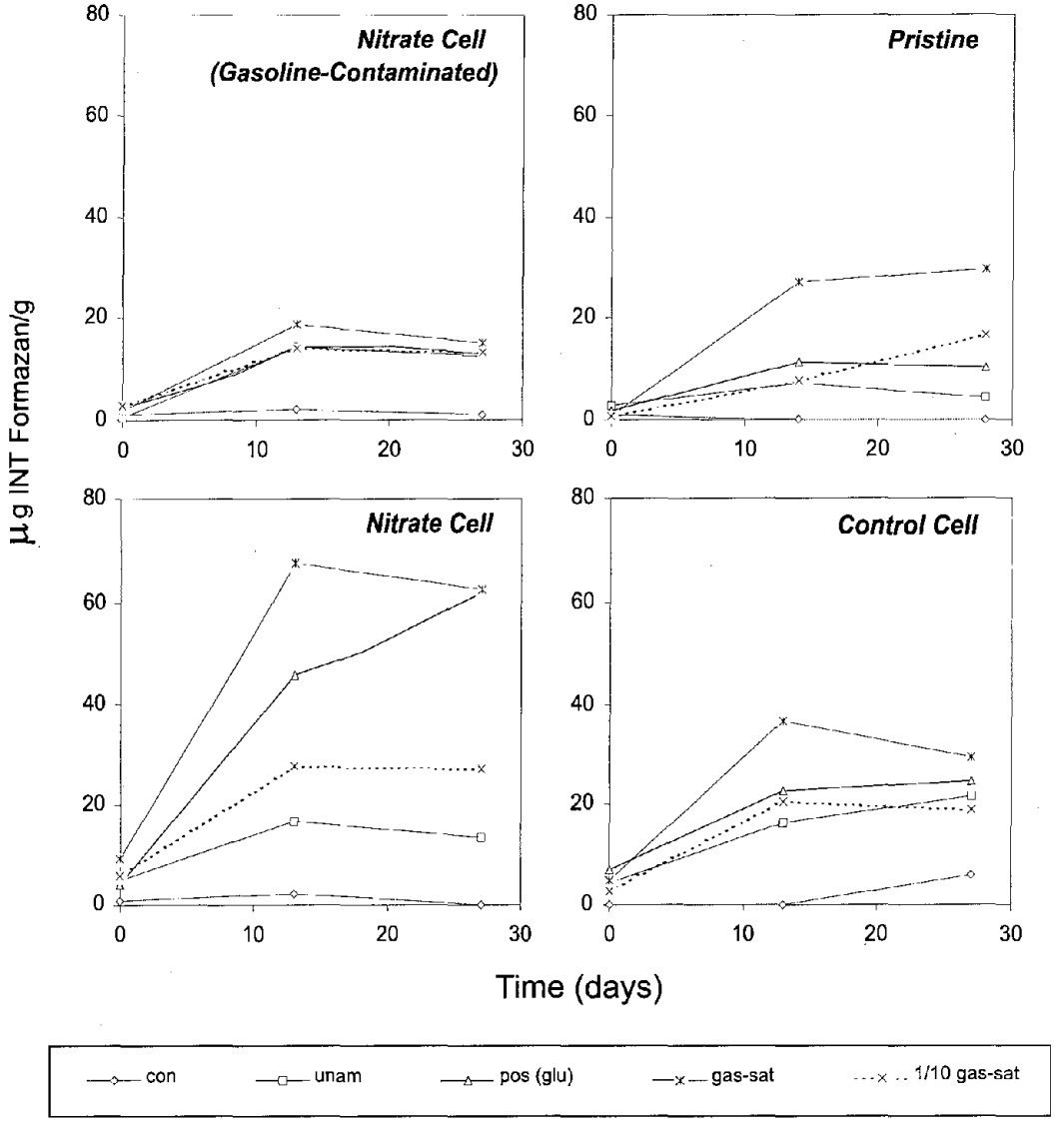


FIGURE 4-19 Accumulation of INT formazan as a measure of ETS activity in pristine and contaminated aquifer material. Results shown in bottom two graphs from below gasoline-contaminated zones. con: sterile control; unam: no carbon source added; pos: glucose added; gas-sat: gasoline-saturated water added; 1/10 gas-sat: 10x dilution of gasoline-saturated water added. Means of two replicates plotted.

4.4 Discussion and Conclusions

These laboratory results indicated that the microbial populations capable of biotransforming high concentrations of BTEXTMB were patchily distributed in pristine aquifer material. This is not surprising given the oligotrophic conditions in the Borden aquifer (Butler et al., 1997). It appeared that with *in situ* exposure, the population in the Borden aquifer acclimatized to the gasoline phase and associated high aqueous concentrations of BTEXTMB. Acclimation periods are typically attributed to factors such as enzyme induction, genetic change, diauxie, or selective enrichment and growth of organisms capable of degrading hydrocarbon constituents (Chapelle, 1993; Leahy and Colwell, 1990). We observed distinct differences in the uptake of O_2 and NO_3^-, and the utilization of the aromatic hydrocarbons between pristine and contaminated (19 month exposure) aquifer material. Although the number of replicates was insufficient for statistical comparisons, the data suggested that numbers of heterotrophs, denitrifiers, and benzene-toluene degraders in exposed Borden aquifer material did not increase dramatically (<2 orders of magnitude) relative to pristine material. However, on the basis of the follow-up microcosm study, aromatic-hydrocarbon degrading activity was much more robust in the exposed aquifer material. There

were also indications (e.g., ETS activity) that microbial activity was suppressed somewhat in the presence of a gasoline phase relative to less-contaminated regions of the treatment cells, but the O_2 demand observed in gasoline-contaminated aquifer material was large, suggesting that stimulating biotransformation in the residual gasoline source area with O_2 would be viable after prolonged exposure.

In the preliminary experiments with low concentrations of benzene, toluene, ethylbenzene, and the xylene isomers, mass loss under aerobic and denitrifying conditions was generally consistent with previous studies (Barbaro et al., 1992; Major et al., 1988; Barker et al., 1987). All of these compounds were degradable under aerobic conditions, but microbial activity was limited by inorganic nutrients. Previous studies have also shown that the activity of microorganisms in the Borden aquifer is nitrogen-limited under aerobic conditions (Barbaro et al., 1994). In the presence of O_2 *m*-xylene was typically utilized first followed by the other aromatic compounds. Under anaerobic, denitrifying conditions, both toluene and ethylbenzene biodegraded most consistently, while the other aromatic compounds appeared to be recalcitrant. We observed no nutrient limitations under anaerobic conditions. Under denitrifying conditions, mass losses were generally small and the minor assimilatory requirement for N may have been satisfied by NO_3^-, eliminating the need for an additional source of supplied N. A concentration of 25 mg/L NO_3^--N was found to be adequate to support aromatic-hydrocarbon mass loss under denitrifying conditions over the incubation periods used in this study.

Overall the laboratory experiments indicated that the effect of microaerophilic dissolved O_2 concentrations depended primarily on the concentrations of the aromatic hydrocarbons and other carbon compounds in the system. When the concentration of total aromatic hydrocarbons was low (i.e., on the order of 10-15 mg/L) and there were no other sources of labile carbon (pristine aquifer material), the mass of O_2 in a microcosm was fairly large relative to the mass of carbon. For example, under these conditions, the mass of O_2 derived from an air headspace was sufficient to mineralize about 50% of the mass of aromatic hydrocarbons in a microcosm. In these low-carbon systems, we did observe more extensive mass losses in the presence of microaerophilic O_2 relative to losses under anaerobic, NO_3^--reducing conditions (e.g., Figures 4-6 and 4-7). Notably, however, benzene losses were minimal in microaerophilic microcosms.

In gasoline-contaminated microcosms, on the other hand (Experiment 6), less extensive losses of the aromatics were observed in the presence of microaerophilic O_2, even when aqueous hydrocarbon concentrations were low (10x dilution of gasoline-saturated water). This apparently was the result of O_2 consumption by microorganisms growing on other carbon compounds. Abiotic O_2 consumption in laboratory microcosms appeared minor. Other carbon compounds that may have been labile include non-target dissolved gasoline constituents such as phenolic compounds (see Table 5-2) and various C_4 through C_7 straight-chained aliphatic compounds which have relatively high aqueous solubilities, or various insoluble hydrocarbons retained in the gasoline phase. Biosurfactant-producing bacteria capable of assimilating insoluble hydrocarbons have been isolated in previous studies from petroleum products (Marin et al., 1996). When the concentrations of dissolved aromatic hydrocarbons were increased to gasoline-saturated concentrations to reflect field conditions, microaerophilic O_2 had no observable effect. Under these conditions, the mass of O_2 was apparently too low to observe any losses even if the aromatic hydrocarbons were the preferred substrates. Therefore, although there was an observable effect under favorable conditions (i.e., low substrate concentrations in pristine aquifer material), microaerophilic O_2 was not effective in enhancing the removal of recalcitrant compounds under conditions similar to those established in the field. These laboratory data suggest, therefore, that the addition of microaerophilic O_2 for enhanced bioremediation may be more effective in locations downgradient of the source. This conclusion is consistent with observations from the field experiment.

Complete utilization of microaerophilic O_2 was not observed in laboratory microcosms. A threshold dissolved O_2 concentration of 0.1-0.5 mg/L persisted in active microcosms incubated in the anaerobic chamber. It is unclear whether this threshold resulted from positive sampling bias (i.e., from removal of the microcosms from the anaerobic chamber, or sampling procedures), or was representative of the microcosm liquid. In contrast to the field cells, where threshold concentrations were also observed (Chapter 5), laboratory samples were not subjected to negative pressure prior to sampling and steps were taken to minimize contamination with atmospheric O_2 prior to measurement (Appendix C). Nonetheless, in this study, it was assumed that the threshold represented the lowest measurable concentration, and

microcosms with O_2 concentrations near this level were considered to be "O_2-depleted". Assuming, on the other hand, that this residual O_2 was not an experimental artifact, the data suggest that microbial consumption was very slow relative to the lengths of the incubation periods. Persistence could have been related to slow kinetic uptake at these concentrations (Section 1.3.1). The effect, if any, on the denitrifier population could not be ascertained. The presence or absence of a threshold concentration, while of interest for understanding the fate of supplied O_2, did not appear to be critical in the assessment of the effect of microaerophilic O_2 in the laboratory experiments.

Although one of the goals of this study was to evaluate nitrate-based biotransformation, the laboratory experiments clearly showed that aromatic-hydrocarbon mass losses were minor unless O_2 was present. In pristine aquifer material, NO_3^- utilization was observed under anaerobic conditions, but only when aromatic-hydrocarbon concentrations were low (10x dilution of gasoline-contaminated groundwater). Under these conditions, mass losses were limited to toluene, ethylbenzene, and less consistently, *m*-xylene. When aqueous concentrations were increased to gasoline-saturated levels (Experiment 4), negligible NO_3^- uptake suggested that denitrifying activity was inhibited in the pristine aquifer material. Prolonged (19 months) *in situ* exposure to gasoline hydrocarbons and NO_3^- did lead to increased NO_3^- utilization under anaerobic conditions, but not to increased mass loss of labile aromatic compounds. The microcosm experiment with gasoline-contaminated aquifer material extracted from the Nitrate Cell (Experiment 6) suggested that, although NO_3^--reducing activity was not inhibited, the labile aromatic compounds were not preferred substrates in this carbon-rich environment. Moreover, the rate and extent of NO_3^--utilization was quite variable among the different treatment groups in this experiment. The inconsistent and often weak NO_3^--reducing activity appears to be characteristic of the Borden aquifer. In contrast, benzene, toluene, ethylbenzene, *m*-xylene, *p*-xylene, 1,2,4-trimethylbenzene, and naphthalene were observed to biotransform rapidly at the expense of O_2 in the gasoline-contaminated material. *o*-Xylene and the other trimethylbenzene isomers may also have been degrading at lower rates, but they were effectively recalcitrant because of rapid O_2 consumption in other reactions.

Nitrate may have been involved in both assimilatory and dissimilatory reactions. Indirect evidence of assimilatory reduction in gasoline-contaminated material was provided by the rapid utilization of NO_3^- in the presence of a large mass of O_2 (Figure 4-16a) If this NO_3^- utilization had resulted from denitrifying activity following significant aerobic growth of facultative microorganisms, then rapid utilization of NO_3^- should have continued under anaerobic conditions when NO_3^- was replenished. Subsequent rapid uptake was not observed. Utilization of NO_3^- as a source of N for O_2-driven reactions can be considered a positive aspect of an O_2 / NO_3^- mixture, but under microaerophilic conditions the effect would be limited. Under O_2-depleted conditions, the intermittent accumulation of NO_2^- as well as the production of N_2O in acetylene-blocked microcosms suggested that dissimilatory NO_3^- reduction had been induced, but these data were variable and the specific nitrate-reducing pathway (e.g., denitrification) remained poorly defined in some experiments.

Patterns of BTEX, O_2, and NO_3^- / NO_2^- concentrations suggested that O_2 and NO_3^- were used sequentially under mixed electron-acceptor conditions; most BTEX biotransformation occurred early, likely at the expense of microaerophilic O_2; additional TEX losses occurred later under mixed electron-acceptor, and denitrifying conditions. In contrast to other published studies (e.g., Hutchins, 1991a), our experiments provided no clear evidence that low levels of O_2 were facilitating the transformation of recalcitrant compounds such as benzene under anaerobic, NO_3^--reducing conditions. Our experiments with low substrate concentrations did show, however, that mass losses of labile compounds in mixed microaerophilic / NO_3^- microcosms exceeded losses in comparable microaerophilic only and anaerobic, denitrifying microcosms. This indicated that denitrifying activity commenced after O_2 depletion with no apparent lag period, and that the extent of biotransformation was maximized by the presence of two electron acceptors. Unfortunately, in the Borden aquifer this mixed electron-acceptor-driven oxidation appeared to be limited to low-carbon conditions. In experiments with gasoline-contaminated aquifer material, aromatic-hydrocarbon mass loss was either very small or negligible under mixed microaerophilic / NO_3^- conditions. In the high-BTEXTMB microcosms, the mass of O_2 was apparently too small to observe (i.e., not detectable above experimental variability) any aromatic-hydrocarbon losses, and the denitrifying population did not appear to utilize those compounds that are typically labile at lower concentrations. These results are broadly consistent with those obtained *in situ*.

CHAPTER 5. FIELD EXPERIMENTS

5.1 Overview of Results

5.1.1 Flow Characteristics

All breakthrough curves (BTCs) within the pea gravel layers (60 cm bgs) reached a relative concentration near C/C_0=1 within 12 hours of the beginning of the July, 1996 tracer test (Appendix B). Because the pea-gravel layers extended to the sheet-piling walls, it likely that injected water spread horizontally to the edges of the cells throughout the flushing experiments, providing a uniform initial distribution of NO_3^- and dissolved O_2, although the apparent rapid consumption of O_2 may have restricted its initial distribution to the region around the injection well.

Breakthrough data from piezometer ports at two depths, 120 and 180 cm bgs, were used to calculate dispersivities and groundwater velocities (Appendix B). As the tracer front migrated downward through the cells, spatial groundwater velocity fluctuations were observed, but tracer broke through all monitored ports; this indicated that there were no large regions being bypassed by the injected fluid. Velocity fluctuations in individual flow tubes were caused by spatial variability in the hydraulic gradient (i.e., higher velocities above the extraction well), in aquifer properties such as hydraulic conductivity (heterogeneities), and possibly in gasoline content. Average linear groundwater velocities, determined from fitting a one-dimensional advection-dispersion equation to the tracer BTCs, ranged from 0.6 to 1.2 cm/hr in the Control Cell, and 0.4 to 1.1 cm/hr in the Nitrate Cell (see Table B-2).

The velocities measured from breakthrough data can be compared to the expected velocity under steady flow conditions. Using a porosity of 0.33, a 200 ml/min target injection rate yields an expected velocity of 0.9 cm/hr, which is consistent with the breakthrough data. Accordingly, the mean injection flow rates were used in advective mass-flux calculations to estimate the masses of dissolved constituents added and removed from the cells under flushing conditions. The good agreement between injection rates and breakthrough data also indicated that there was no short-circuiting of injected water along the walls of the piezometers.

5.1.2 Dissolved Oxygen and Nitrate

Dissolved Oxygen. Dissolved O_2 depletion was observed in both treatment cells. The mean injection dissolved O_2 concentrations were 2.3±1.8 mg/L (n=80) and 2.3±1.7 mg/L (n=81) for the Nitrate and Control Cells, respectively, where the variability is expressed as a standard deviation. These means include the initial seven days of the experiment when fully-oxygenated water was injected into the cells. If the measurements from the first week are excluded, mean injection concentrations fall to 1.9±0.9 mg/L and 2.0±1.0 mg/L for the Nitrate and Control Cells, respectively. The mean concentrations obtained from the extraction-well sampling ports were 0.30±0.14 mg/L (Nitrate Cell) and 0.27±0.26 mg/L (Control Cell). Measured concentrations are shown on Figure 5-1, where a mean value is plotted when more than one sample was collected in a 24 hr period.

Dissolved O_2 was depleted rapidly to a non-zero threshold concentration in both cells (Figure 5-2). Concentrations in samples from 60-cm ports were at the threshold, and additional uptake between 60- and 180-cm depths was not observed. Therefore, the majority of the dissolved O_2 was utilized within hours of injection. The threshold concentration varied from about 0.4 mg/L to 1 mg/L, depending on the location, and averaged around 0.75 mg/L at a given depth interval (Figure 5-2). Non-zero threshold concentrations were also measured during both the static and 24-day flushing periods that followed the initial flushing experiment (data not shown). It appears, based on rapid rates of uptake in active laboratory microcosms relative to controls (e.g., Experiment 6), that O_2 was utilized by microbial activity,

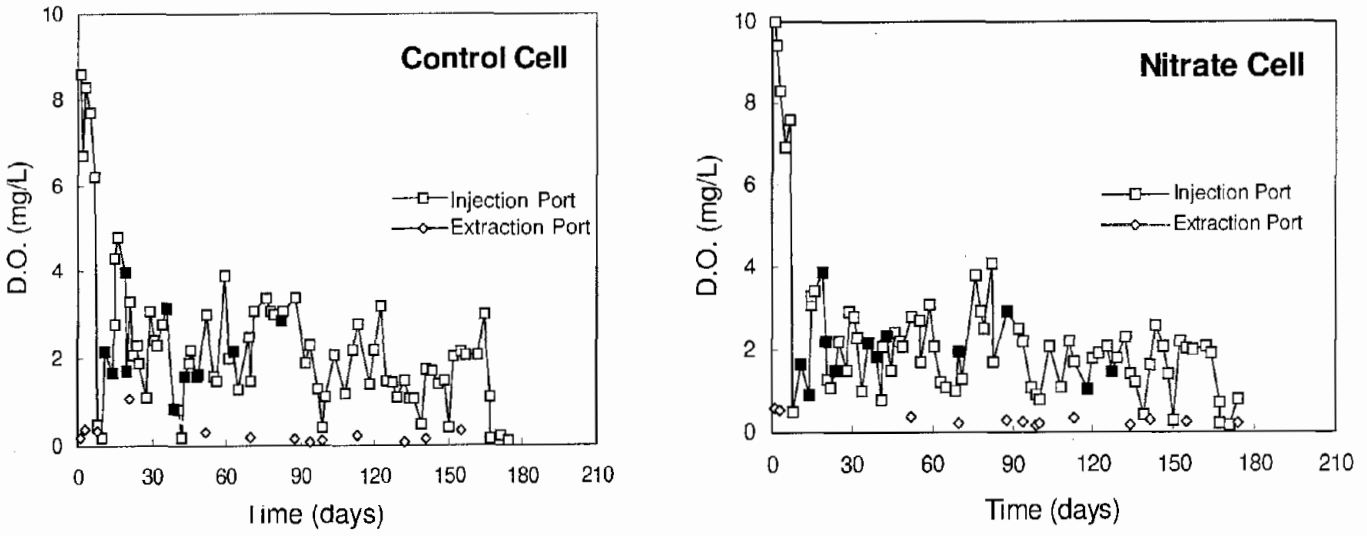


FIGURE 5-1 Injection and extraction D.O. concentrations. Mean values plotted when more than one sample collected within 24-hr period (solid squares).

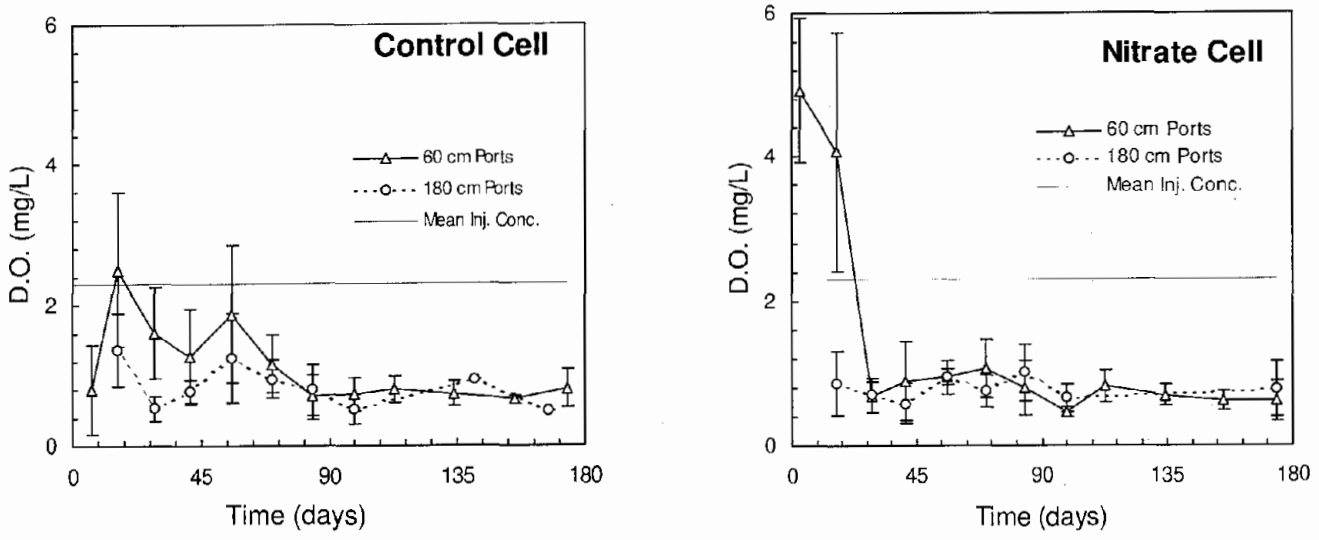


FIGURE 5-2 D.O. concentrations at 60- and 180-cm bgs ports during the 174-day flushing experiment. Plotted values at each depth are means and standard deviations from five piezometer ports.

but given the detection of dissolved Mn in water extracted from the supply well (Table 2-1), it is possible that in the field some of the O_2 was utilized in abiotic reactions as well.

The observed threshold concentrations obtained from the piezometers may not represent *in situ* dissolved-O_2 concentrations. One possible source of positive bias is the sampling procedure. A small amount of O_2 could have been incorporated into the flowing stream of groundwater during sampling under suction. To further investigate this possibility, similar sampling techniques were used to measure the dissolved O_2 concentrations from bundle piezometers in the monitoring grid used by Barbaro et al. (1992). Mean concentrations of duplicate samples from seven piezometer ports in the anaerobic, landfill leachate plume ranged from 0.41 mg/L to 0.9 mg/L. This suggested that contamination with atmospheric O_2 occurred during sampling. On the other hand, vertical profiles from center piezometers indicated that dissolved O_2 concentrations decreased progressively during the flushing experiments (Figure 5-3). Concentrations lower than the threshold values discussed above were obtained on the last sampling date when the cells were static, suggesting that it was possible to measure lower O_2 concentrations from these ports. Because of these inconsistencies, the dissolved O_2 data obtained from the multilevel piezometers are considered semi-quantitative; they showed that dissolved O_2 was essentially removed, but could not be used to define the actual residual concentration.

Nitrate. The mean injection NO_3^- concentration from the initial 174-day flushing experiment was 116±33 mg/L (n=25). The mean concentration from the extraction well port was 82±23 mg/L (n=13). Because there were two target injection concentrations (Figure 5-4), the means and standard deviations were calculated as described in Section 3.5.3 for injection rates. Nitrate was not detected in the Control-Cell injection water (n=3).

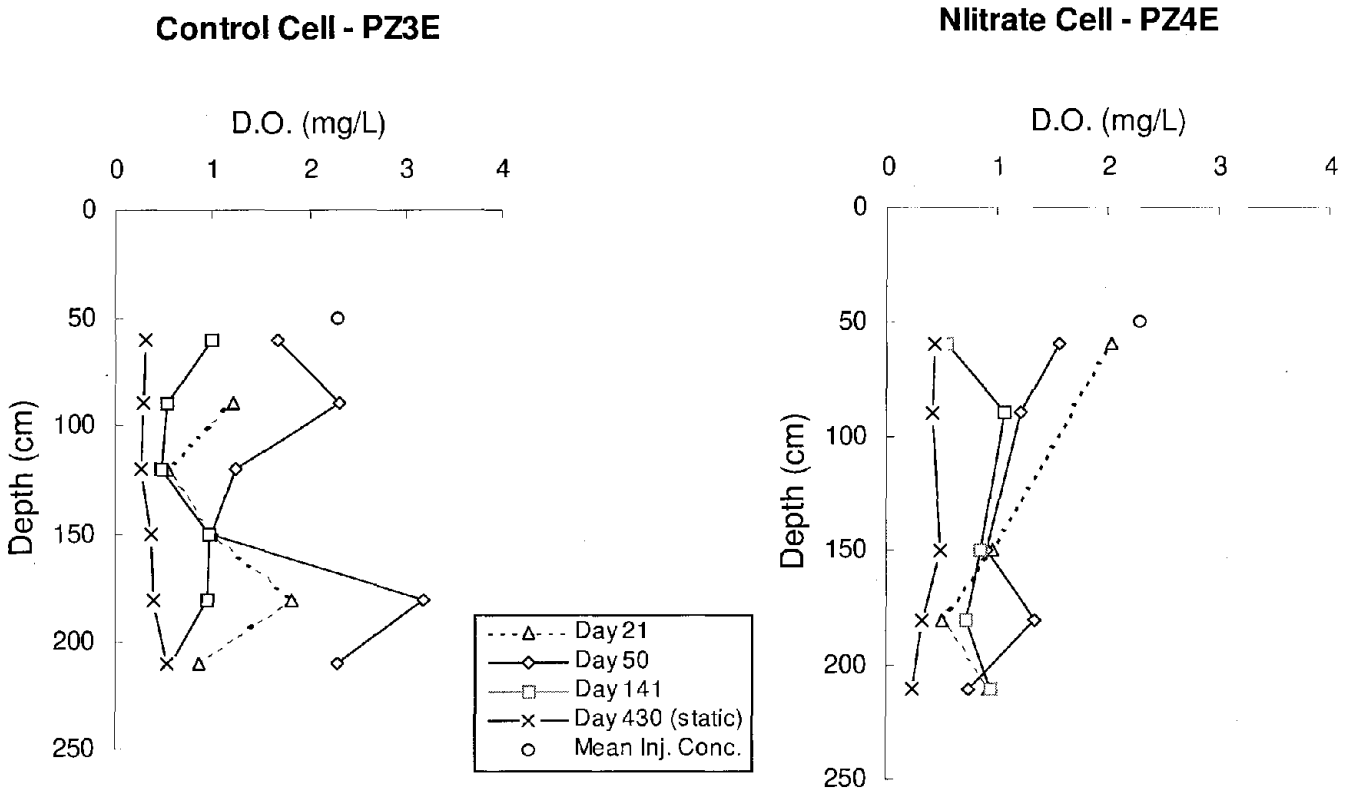


FIGURE 5-3 Vertical profiles of D.O. at various times during and after the 174-day flushing experiment. Data collected from center piezometers.

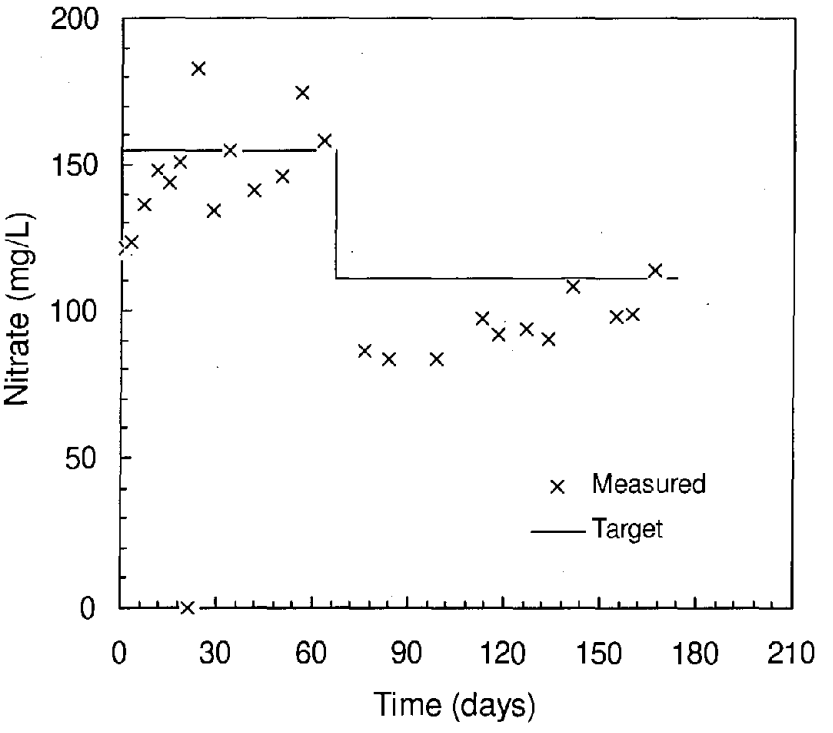


FIGURE 5-4 Injection NO_3^- concentrations.

Consumption of NO_3^- under flushing conditions was relatively low. Similar to dissolved O_2, NO_3^- depletion during the 174-day experiment appeared to occur rapidly after injection into the cell (Figure 5-5). Mean concentrations from 60-cm ports were below injection concentrations, but additional losses between 60 and 180 cm depths appeared to be quite small (Figure 5-5). Similar behavior was observed during the 24-day flushing experiment. The addition of the MBH medium during this 24-day period did not result in an observable increase in the rate of NO_3^- utilization. This result is consistent with laboratory experiments (Chapter 4), which show that the sluggish NO_3^--reduction typical of the Borden aquifer does not result from nutrient limitations. The amount of NO_3^- that was utilized during the 174-day flushing experiment is calculated in Section 5.2.2.

To determine if NO_3^- utilization would be observed during a longer residence time, a conservative tracer (bromide) was pumped into the Nitrate Cell during the final week of the 174-day flushing experiment. Three ports from the 60-cm depth and three from the 180-cm depth were then sampled over the following 138 days when the cells were static to observe the extent of anaerobic NO_3^- depletion relative to Br^-. However, depletion of both Br^- and NO_3^- was observed, particularly at the lower depth where dilution with underlying groundwater may have occurred in response to a rising water table. To account for depletion of the tracer, Br^- concentrations from each sampling port were corrected back to the initial concentration. These correction factors were then applied to the corresponding NO_3^- value to obtain a corrected concentration for each sampling event. A mean corrected NO_3^- concentration was then calculated from the three ports at each depth for each sampling event (Figure 5-6). While there is substantial scatter in the data, this figure indicates that there were losses of NO_3^- relative to Br^-, with greater losses at the lower depth. If depletion is described with a zero-order (linear) model, depletion rates of 0.20 mg/L/d (60 cm) and 0.67 mg/L/d (180 cm) are obtained. Given the scatter in the data, a first-order model could also be fit reasonably well to the data. These fits yielded first-order rate constants of k=0.002/d (60 cm) and k=0.02/d (180 cm). As discussed further in the following sections, these trends have been attributed to biological activity.

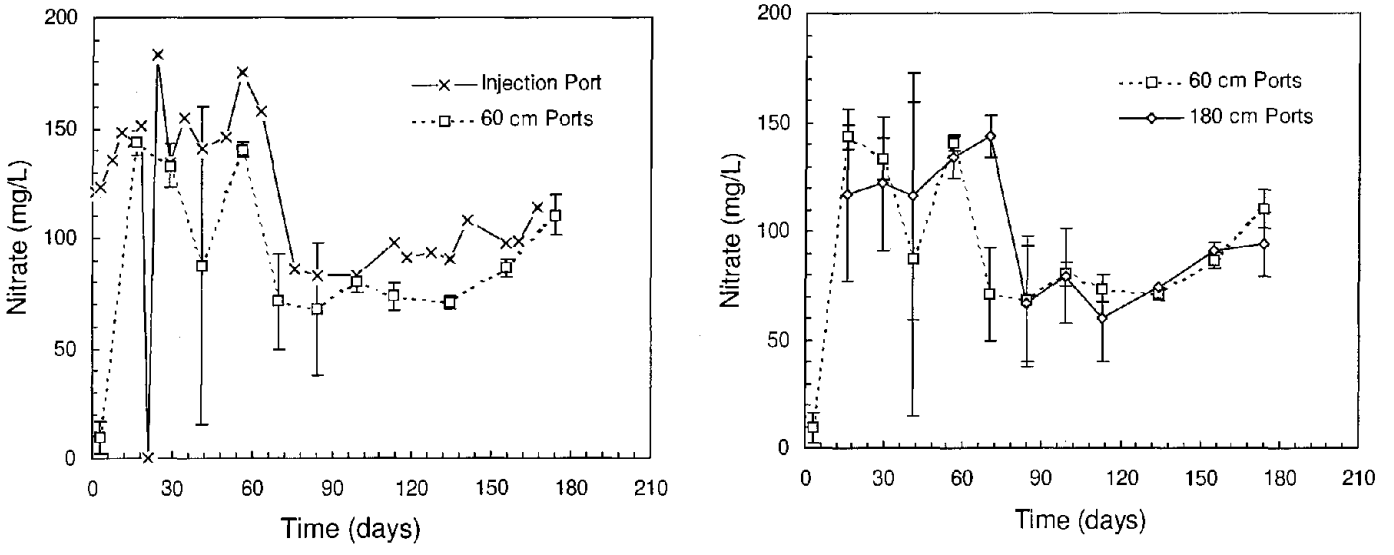


FIGURE 5-5 Nitrate concentrations in injection water and 60- and 180-cm bgs ports during the 174-day flushing experiment. Plotted values for 60- and 180-cm depths are means and standard deviations from five piezometer ports.

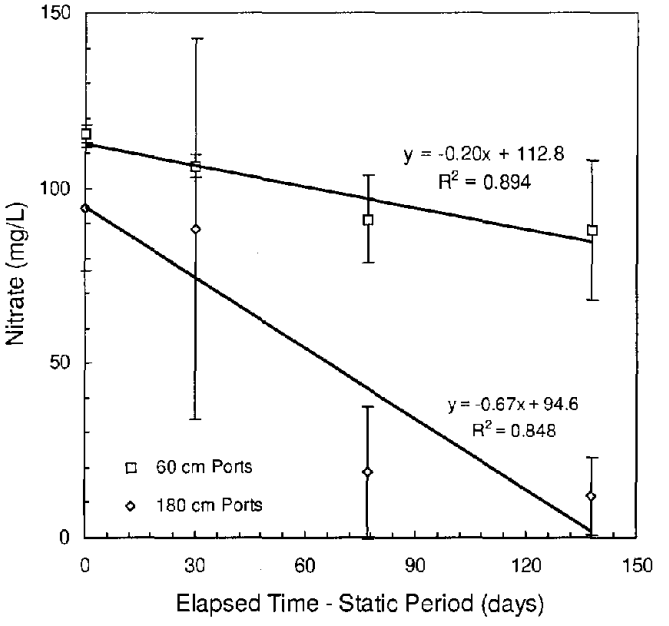


FIGURE 5-6 Nitrate concentrations during the static period between flushing experiments. Bromide was used to correct NO_3^- concentrations as discussed in text. Plotted values at each depth are means and standard deviations from three piezometer ports.

5.1.3 Organics

Dissolved-Phase. As shown by effluent data, dissolved aromatic hydrocarbons concentrations were quite high in both cells (Figure 5-7). By the end of the flushing experiments, concentrations ranged from about 1 mg/L for benzene to 20 mg/L for toluene. With the exception of benzene and toluene, final concentrations in effluent water were similar to their respective concentrations in gasoline-saturated water, as measured in laboratory equilibration experiments with fresh API 91-01 (see Table A-4). Benzene and toluene concentrations declined to ca. 5% and 50% of initial gasoline-saturated values, respectively, reflecting rapid changes in the mole fractions of these constituents. In contrast, after about 2 months of flushing, concentrations of the less soluble compounds (i.e., xylenes, trimethylbenzenes and naphthalene) reached initial saturated values and remained at these levels throughout the experiment. The reasons for the relatively slow approach to equilibrium concentrations are not clear; the best explanation may be substantial preferential flow around the most heavily contaminated regions of the treatment cells during the early stages of flushing. These trends are shown for the Nitrate Cell on Figure 5-8.

Relatively rapid depletion of the more soluble compounds is consistent with the dissolution of a multi-component organic liquid. As indicated by these dissolved-phase results, the gasoline phase was nearly depleted in benzene, but not in the less-soluble aromatics, by the end of the experiment (Figures 5-7 and 5-9). The rapid depletion of benzene was expected on the basis of its high solubility, and was consistent with equilibrium partitioning between the gasoline and mobile groundwater. The vertical profiles shown in Figure 5-9 suggest that benzene was depleted most rapidly from the top of the gasoline-contaminated region, and that the depleted zone propagated downward as flushing progressed. Other relatively-soluble compounds such as toluene showed similar trends. These temporal trends probably represent the downward propagation of a dissolution front through the gasoline-contaminated region. The relatively rapid removal of the soluble constituents is also evident from 180-cm BTCs (Figures 5-10 and 5-11).

The temporal and spatial patterns of dissolved-phase concentrations were also quite similar between cells (Figures 5-7 through 5-11). Based on the 180-cm BTCs, there were no major differences between the cells in the patterns of dissolved-phased aromatic hydrocarbons (Figures 5-10 and 5-11). For example, enhanced removal of toluene, the most labile compound under denitrifying conditions, was not evident in the Nitrate Cell relative to the Control Cell. Similarities between the cells are also apparent in the extraction-well BTCs (Figure 5-7), which represent volume-averaged concentrations exiting the cells. The close similarity of the Nitrate- and Control-Cell breakthrough curves is additional evidence that a common, abiotic transport process such as dissolution was the dominant mass removal mechanism.

Concentrations of aromatic hydrocarbons did not decline appreciably in either cell during the static period between flushing experiments. This includes compounds such as toluene and ethylbenzene that are typically labile under anaerobic, denitrifying conditions (Chapter 4). The apparent lack of aromatic-hydrocarbon utilization in the Nitrate Cell is important because NO_3^- depletion was observed during this period. Figure 5-12 shows toluene, ethylbenzene, and NO_3^- from one of the piezometers (PZ4A) that was sampled during the static period. Concentrations were corrected for declining Br^- as described in Section 5.1.2. These plots suggest that if NO_3^- was being used by denitrifying bacteria as an electron acceptor, the labile aromatic hydrocarbons did not serve as electron donors.

Core Extracts. The distribution and quantity of aromatic hydrocarbons remaining in the cells after the flushing experiments were completed was estimated from core extract samples. Concentrations of total BTEXTMB ranged from below detection limits to about 6,000 mg/kg of aquifer material (Figures 5-13 and 5-14). Patterns between cells were remarkably similar. Peak concentrations show that the gasoline-contaminated zones were located within the 50 to 150 cm bgs depth interval. This pattern is consistent with the dissolved-phase profiles shown in Figures 3-5, 3-6, and 5-9. With the exception of cores 3K and 4L, located near the centers of the cells where the emplaced gasoline was deeper, all cores transected the entire gasoline-contaminated interval. There was very little difference between cells in the spatial patterns of BTEXTMB. Highest concentrations of residual aromatics were present in the center of the cells and in the upper right quadrants (Figures 5-13 and 5-14). As with the dissolved phase, concentrations of individual compounds in the residual gasoline phase were broadly consistent with trends expected to result from gasoline dissolution, i.e., the residual gasoline was most depleted in soluble compounds such as benzene and toluene.

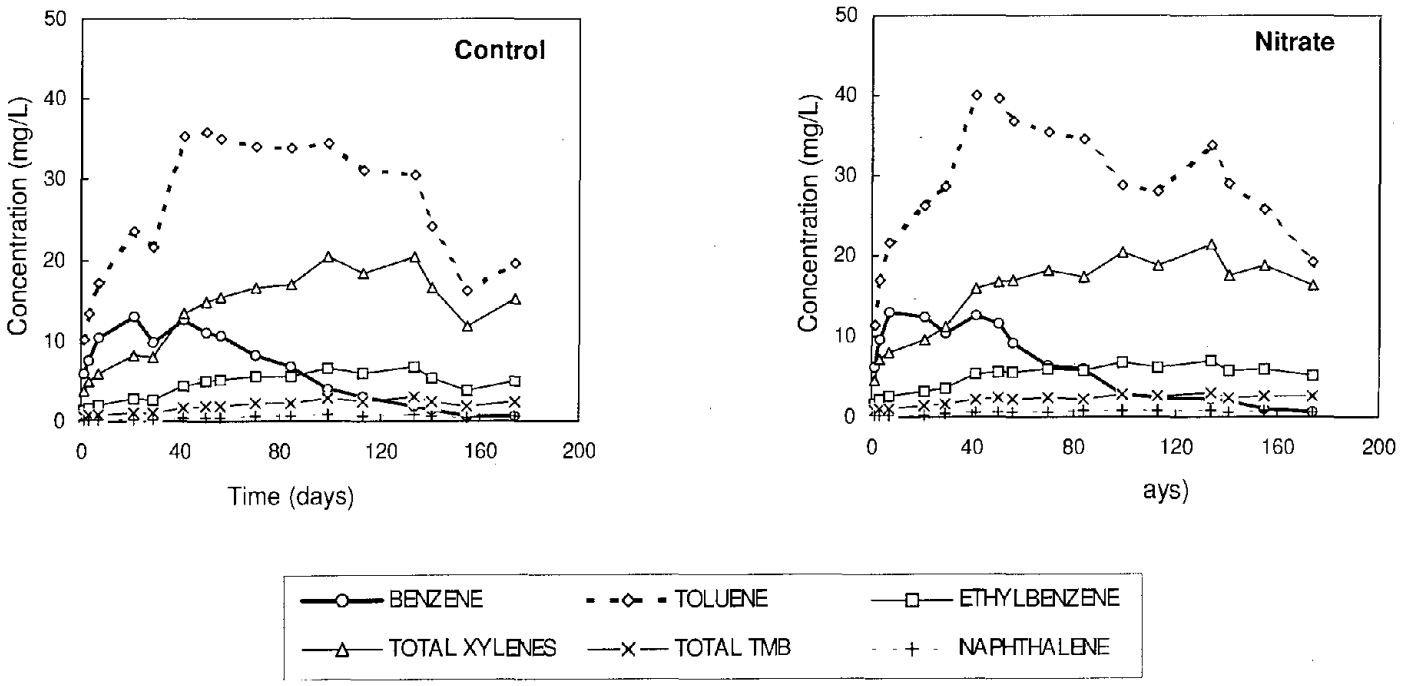


FIGURE 5-7 Concentrations of dissolved aromatic hydrocarbons in samples collected from extraction-well ports.

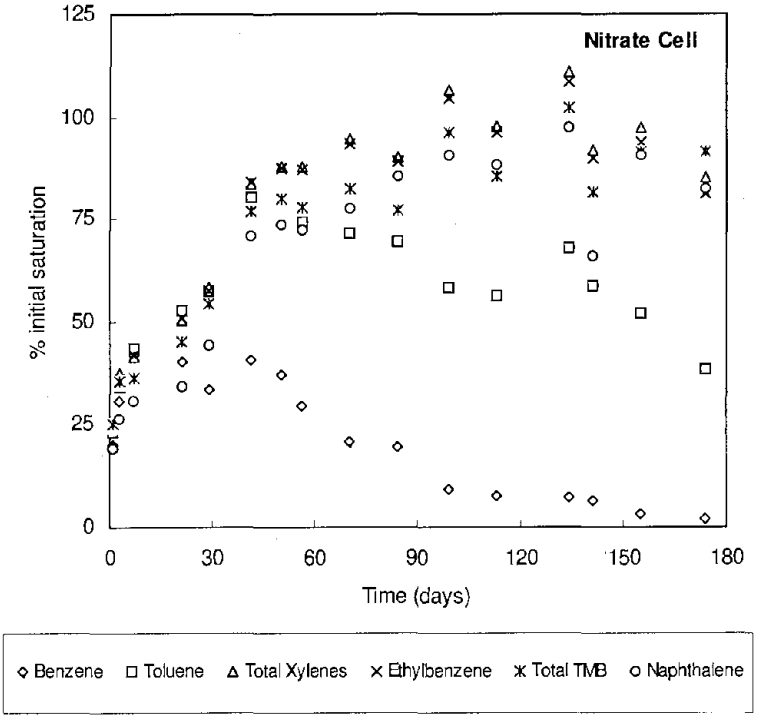


FIGURE 5-8 Concentrations of aromatic hydrocarbons in samples collected from the extraction-well expressed as percentages of concentrations in water equilibrated with fresh API 90-01 gasoline.

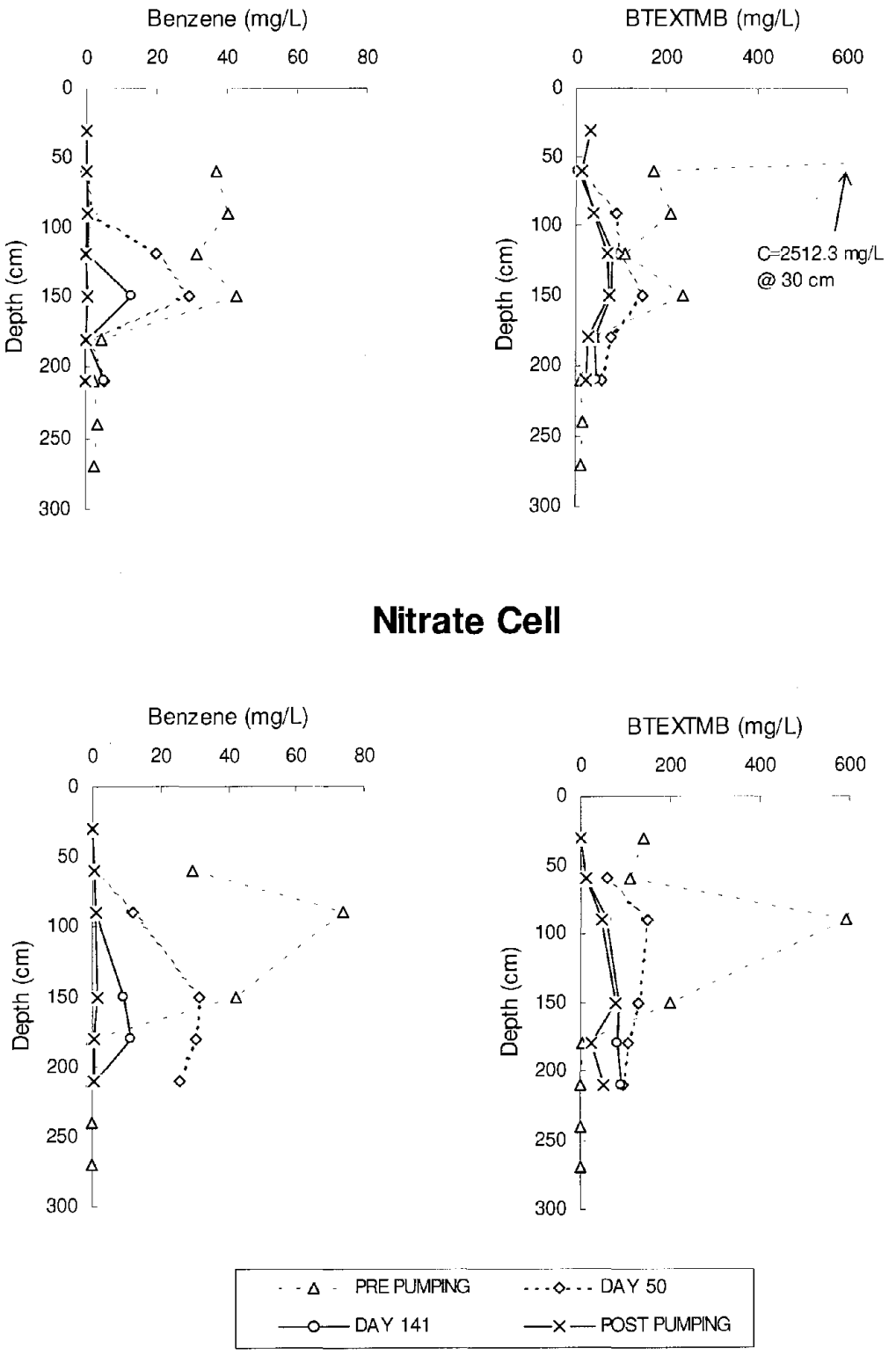


FIGURE 5-9 Vertical profiles of benzene and total BTEXTMB before, during, and after the flushing experiments. Data collected from center piezometer.

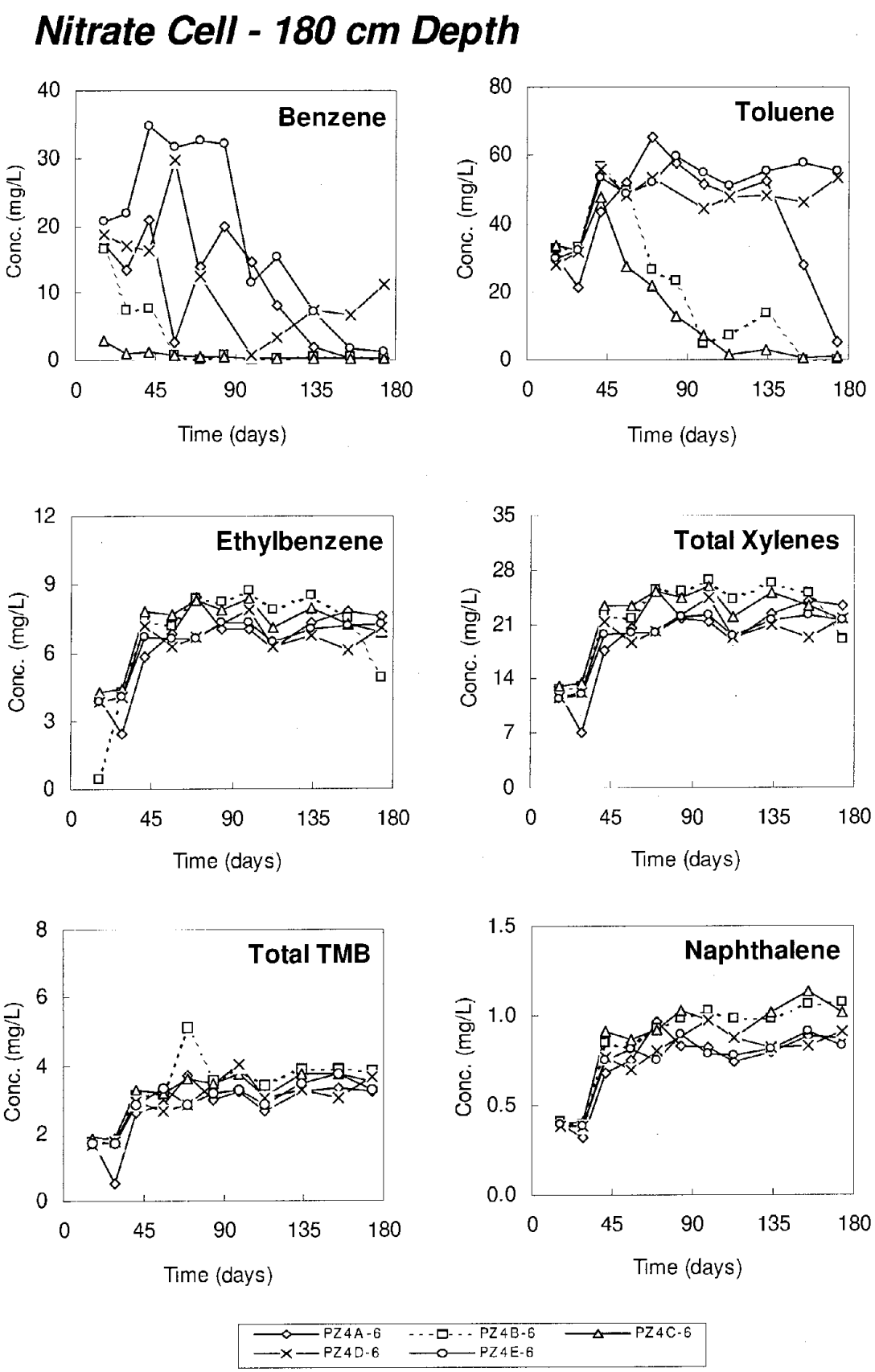


FIGURE 5-10 Concentrations of dissolved aromatic hydrocarbons at individual 180-cm bgs ports in the Nitrate Cell during the 174-day flushing experiment.

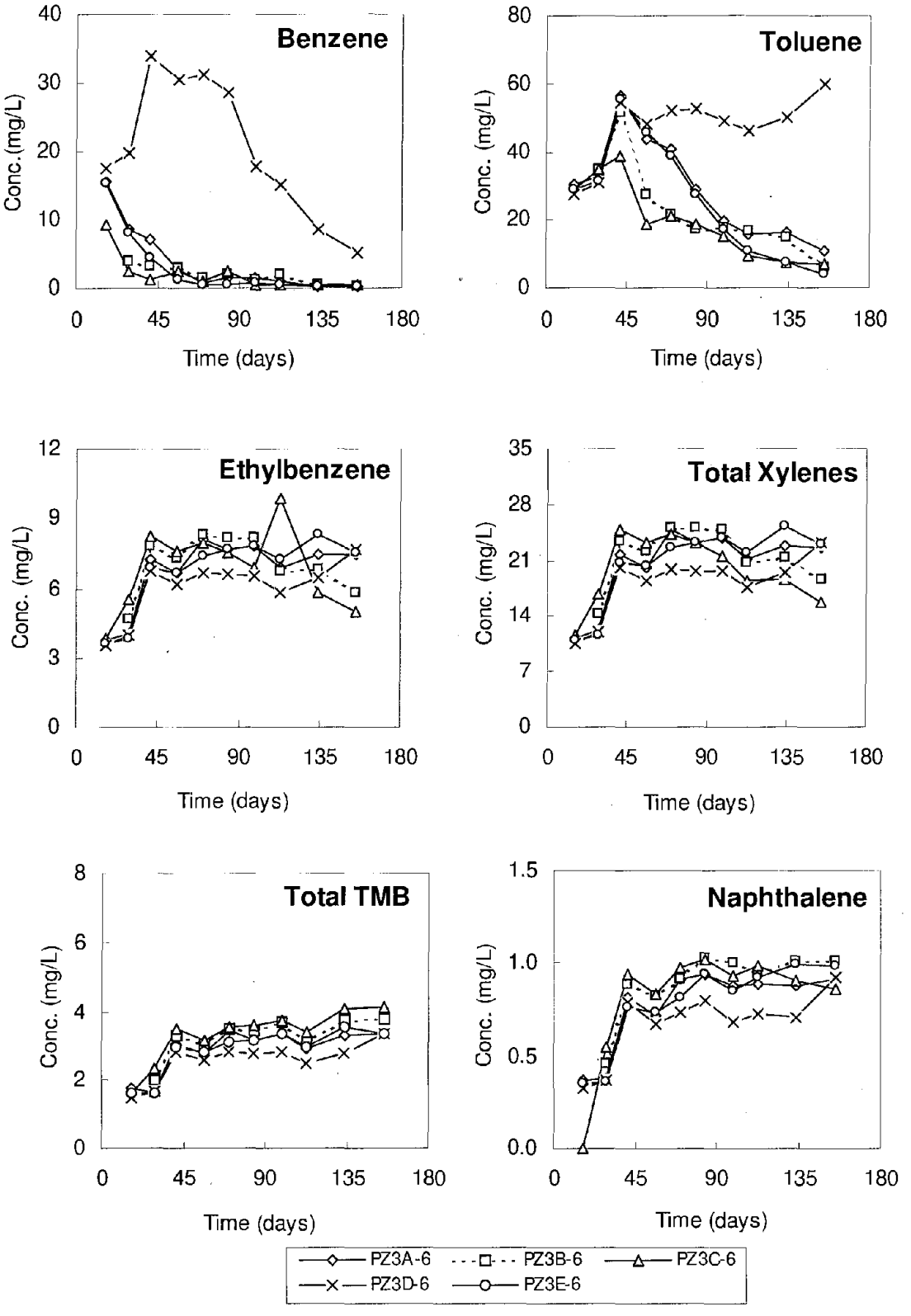


FIGURE 5-11 Concentrations of dissolved aromatic hydrocarbons at individual 180-cm bgs ports in the Control Cell during the 174-day flushing experiment.

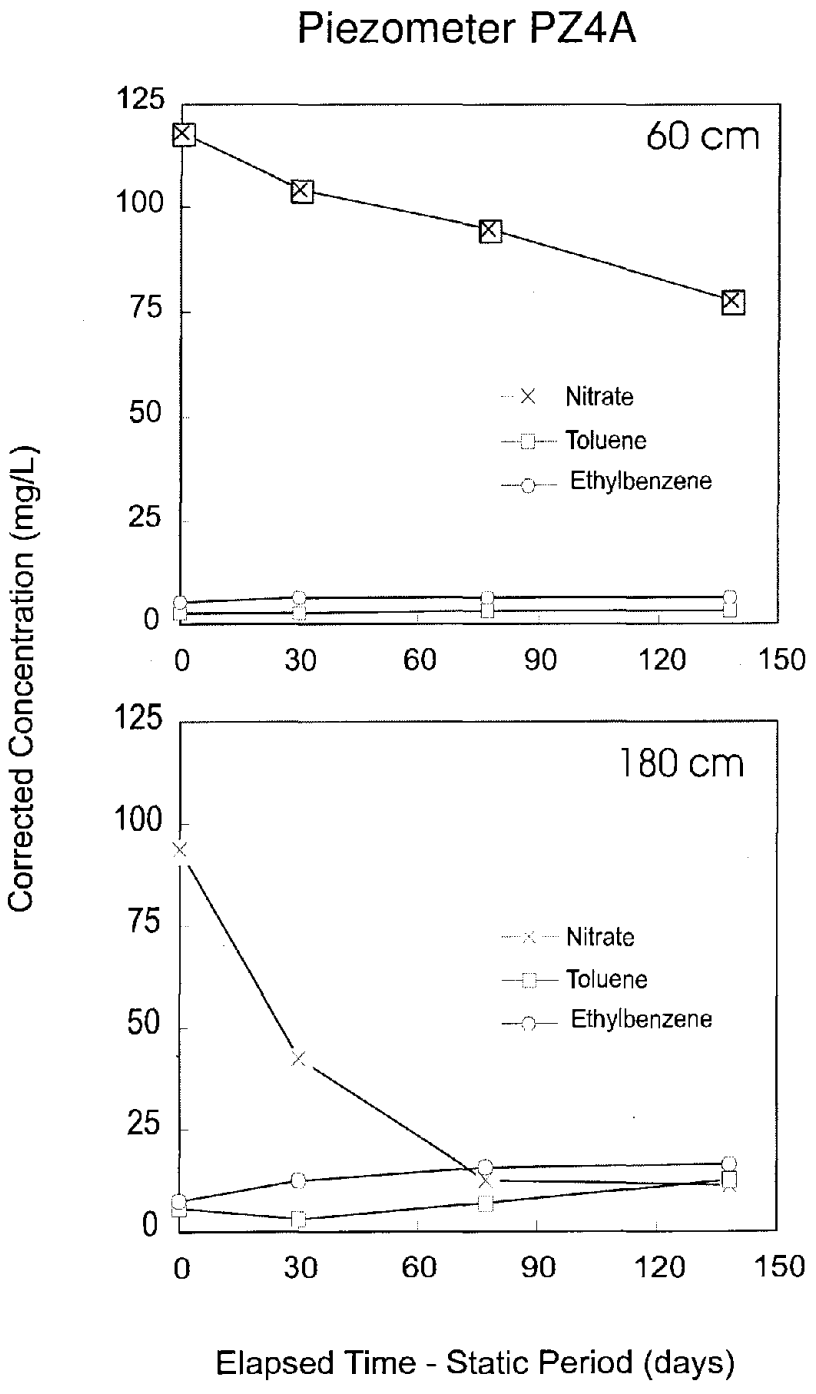


FIGURE 5-12 Corrected toluene, ethylbenzene, and NO_3^- concentrations from a selected piezometer in the Nitrate Cell that was sampled during the static period. Upper graph: 60-cm bgs port; Lower graph:180-cm bgs port. Bromide was used to correct concentrations as discussed in text.

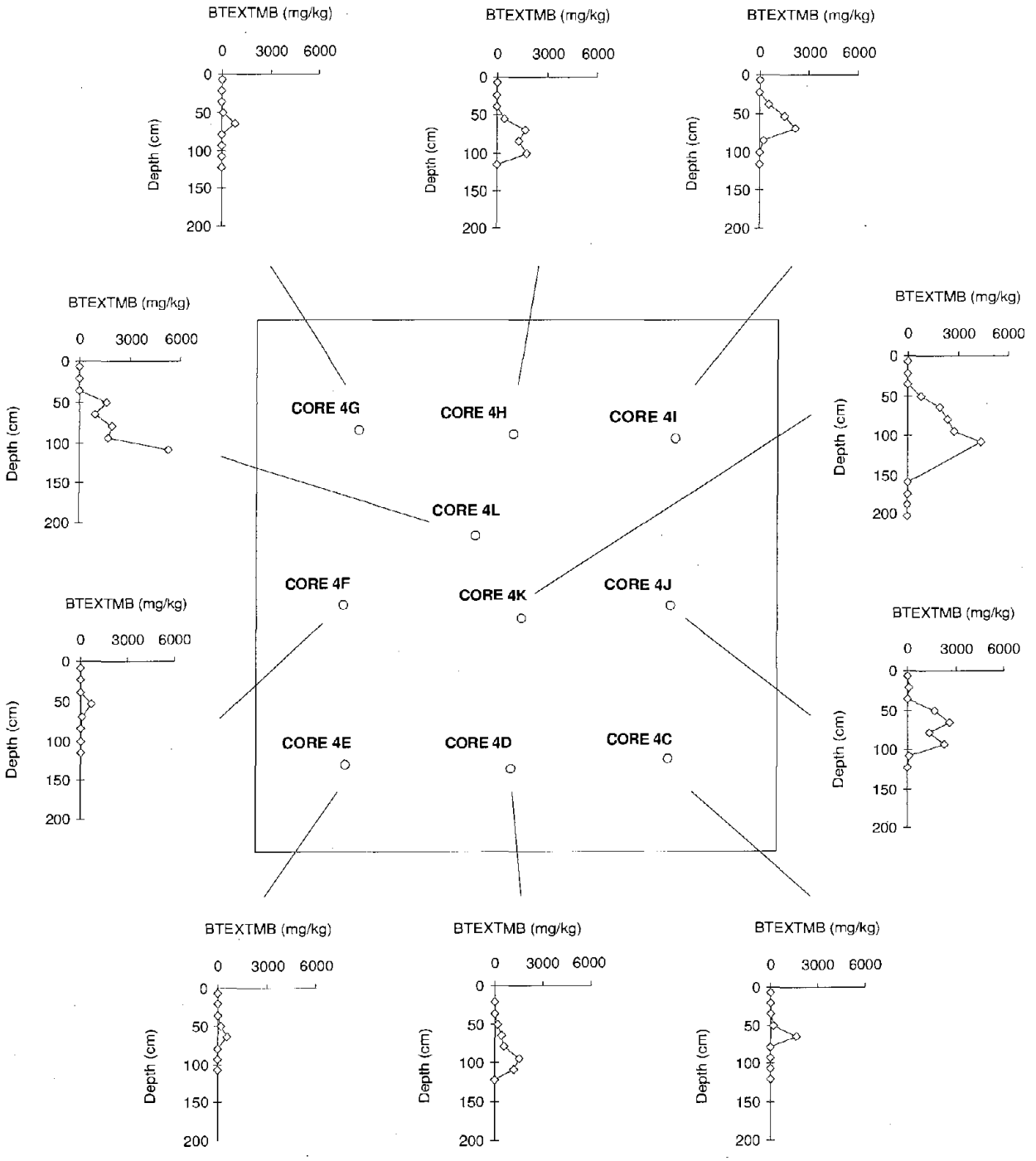


FIGURE 5-13 Vertical profiles of residual BTEXTMB in the Nitrate Cell. Concentrations obtained from field methanol extraction of core subsamples. Cores were collected during August, 1997. Surveyed core locations also shown.

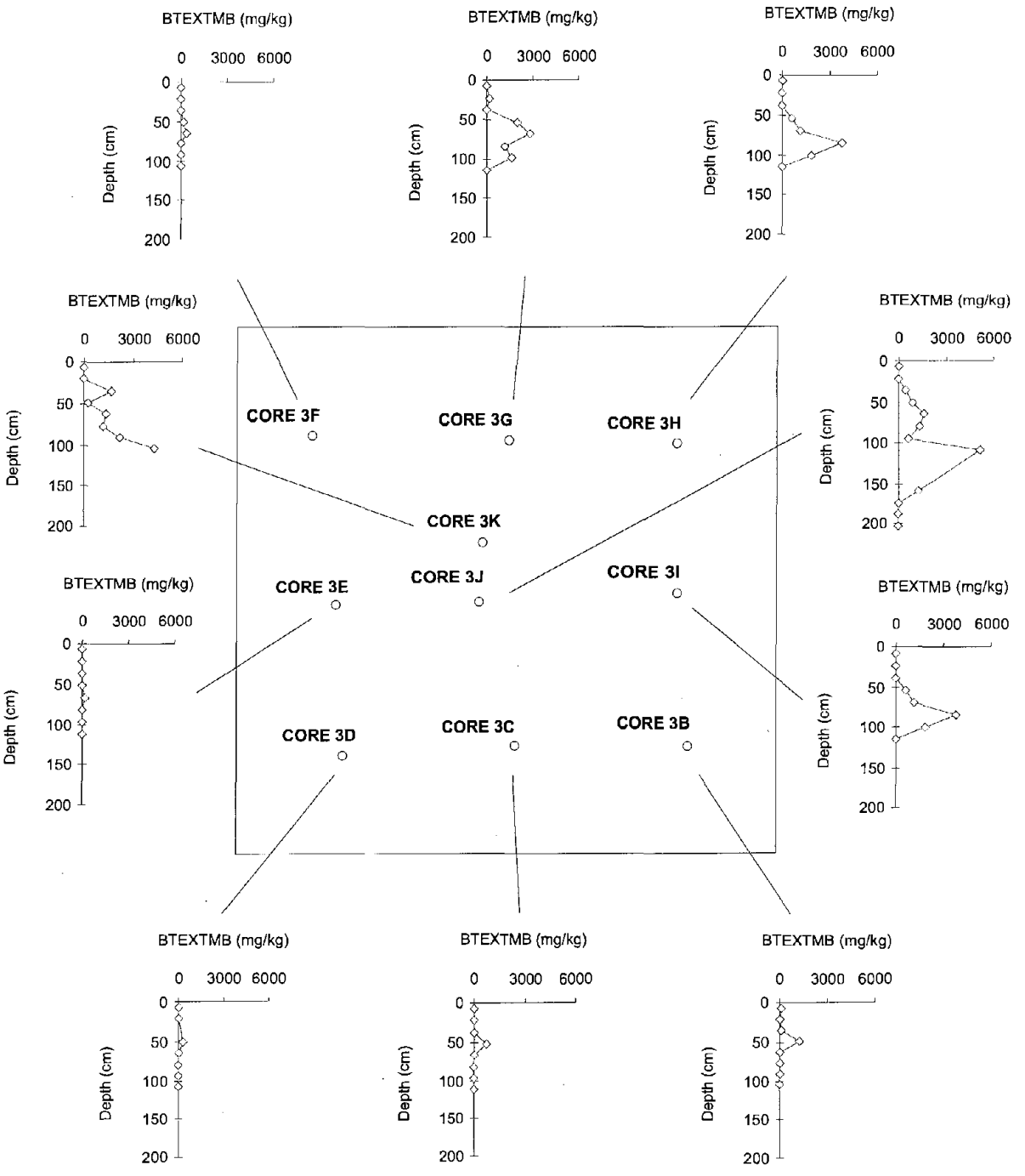


FIGURE 5-14 Vertical profiles of residual BTEXTMB in the Control Cell. Concentrations obtained from field methanol extraction of core subsamples. Cores were collected during August, 1997. Surveyed core locations also shown.

With the exception of Core 4I which appeared to be depleted in alkanes, the analysis of core extracts for gasoline component classes did not reveal substantial depletion of any hydrocarbon component group relative to the fresh gasoline standard and sample spiked with fresh API 91-01 (Table 5-1). This was consistent with the short duration of the flushing experiments and the low solubilities of the majority of the gasoline constituents.

5.1.4 Nitrite Production

Nitrite was frequently present in groundwater samples collected from the Nitrate Cell. Nitrite was never detected in samples of injection water spiked with NO_3^-, but was present in 70% of the groundwater samples (n=189), collected under both flushing and static conditions, that contained NO_3^- (data not shown). Concentrations were typically less than 1 mg/L as NO_2^-, but there were numerous occurrences between 1-10 mg/L, and the highest measured concentration was 17.2 mg/L as NO_2^-. In general, NO_2^- was detected more frequently and at higher concentrations in samples collected from lower depth intervals. The presence of NO_2^- is considered evidence of dissimilatory NO_3^- reduction (Mikesell et al., 1993). Nitrate can be reduced to N_2 abiotically in the presence of Fe^{2+}, but because the reaction rate is negligible unless pH is alkaline (i.e., >7), and a catalyst such as Cu is present (Buresh and Moraghan, 1976), it has been discounted as an important mechanism here.

5.1.5 Metabolite Production

Additional evidence of microbial activity was obtained from metabolite production (Table 5-2). It is recognized that these samples were collected after the flushing experiments were completed, and the MBH medium had been pumped into the cells, and therefore may not be representative of earlier flushing conditions when the majority of the electron-acceptor uptake and aromatic-hydrocarbon mass removal occurred. However, because the response (i.e., changes in electron-acceptor and hydrocarbon utilization) to the MBH solution was negligible, and concentrations of other redox-sensitive species were similar to those measured during flushing conditions (Table 5-3), we have assumed that conditions at the time of metabolite sampling were representative of this earlier period.

The sample of fresh API 91-01 gasoline did not contain any aromatic or aliphatic acids. The supply well was sampled on two occasions, and several C_3 through C_5 aliphatic acids as well as benzoic acid were present in the supply water. This may indicate that a plume of partially-oxidized hydrocarbon constituents from the upgradient treatment mound had reached the supply well by the end of the experiment. Alternatively, short-chained aliphatic acids can be formed from the fermentation of natural organic matter in low-O_2 environments (Thurman, 1985; McMahon and Chapelle, 1991). Concentrations of the constituents detected in supply water were generally higher in the treatment cells, suggesting additional production within the cells. For example, although benzoic acid was detected at 5 µg/L in the supply well, it was present at concentrations up to 41 µg/L (PZ4E-3) in the Nitrate Cell, and 21 µg/L (PZ3E-3) in the Control Cell.

Table 5-1. *Hydrocarbon Component Classes in API 91-01 Gasoline and Gasoline-Contaminated Core Extract Samples. Core Samples Collected July, 1997. All Results Expressed in Weight Percent.*

			CONTROL CELL			NITRATE CELL		
	API 91-01 Gasoline	Gasoline Spike	Core 3J	Core 3H	Core 3K	Core 4I	Core 4L	Core 4K
Class Depth (cm bgs):			80	69	50	70	65	65
Alkanes	46.8	40.7	43.0	44.2	48.0	30.6	46.6	42.8
Aromatics	35.3	44.2	39.9	38.4	34.0	61.1	34.9	40.0
Bicycloalkanes	0.1	<0.1	n.d.[1]	n.d.	n.d.	n.d.	n.d.	n.d.
Naphthenes	7.9	5.7	7.1	7.3	7.0	3.5	8.0	6.9
Olefins	6.6	5.6	5.4	5.7	5.5	0.7	5.3	5.9
PNA	0.5	0.2	0.5	0.7	0.7	nd	1.1	0.8
Other	2.9	3.6	4.1	3.8	4.8	4.1	4.1	3.7

[1] n.d. - not detected.

Table 5-2. *Detected Organic Acids and Phenols in API 91-01 Gasoline and Groundwater. All Concentrations in μg/L.*

				NITRATE CELL				CONTROL CELL			
Collection Date[1]:	NA	6/12/97	7/30/97	6/12/97	7/30/97	6/12/97	7/30/97	6/12/97	7/30/97	6/12/97	7/30/97
Location:	Gasoline	Supply Well	Supply Well	PZ4E-2	PZ4E-3	PZ4E-6	PZ4E-6	PZ3E-2	PZ3E-3	PZ3E-6	PZ3E-6
Compounds Depth (cm bgs):				60	90	180	180	60	90	180	180
Acetic acid		41		51		*		25		8	
Propanoic acid	*[2]		7		6		3		7		**
2-methylpropanoic acid	**[3]	*	**	32	**	*	**	50	11	1	**
3,3-dimethylpropanoic acid	*		**		10		5		5		5
trimethylacetic acid	**	*	14	*	22	*	16	*	26	14	37
butyric acid	**	2	4	66	**	1	**	10	10	*	**
2-methylbutyric acid	*	*	3	36	**	*	**	83	16	*	**
3-methylbutyric acid	**	*	4	48	**	*	**	234	34	*	**
3,3-dimethylbutyric acid		59		26		60		41		42	
pentanoic acid	**	*	3	75	**	11	**	23	3	80	**
hexanoic acid	*	*	5	16	7	3	5	*	9	*	
2-ethylhexanoic acid	**	*	**	*	**	*	**	*	*	2	**
2-methylhexanoic acid	*		*		*		**		8		10
heptanoic acid	**	*	**	14	**	*	**	*	**	*	**
benzoic acid	*	*	5	22	41	*	41	16	21	*	11
phenylacetic acid	**	*	*	71	7	*	12	126	*	*	**
o-methylbenzoic acid	*	*	*	218	8	3	21	*	*	*	**
m-methylbenzoic acid	*	*	*	83	17	*	5	*	28	*	7
m-tolyacetic acid	*	*	*	*	**	*	*	*	*	*	*
p-tolyacetic acid	*	*	*	*	6	*	4	*	*	*	**
p-methylbenzoic acid	*	*	*	*	5	*	8	*	*	*	**
2,6-dimethylbenzoic acid	*	*	*	26	*	*	*	*	*	*	*
2,5-dimethylbenzoic acid	*	*	*	409	16	3	11	*	**	*	**
3,5-dimethylbenzoic acid	**	*	*	29	5	*	**	*	**	*	*
2,4-dimethylbenzoic acid	**	*	*	37	6	*	4	*	**	*	**
decanoic acid	*	*	**	18	**	*	**	*	3	*	**
4-ethylbenzoic acid	*	*	*	*	**	*	**	*	**	*	**
2,4,6-trimethylbenzoic acid	*	*	*	99	4	*	**	*	**	*	**
3,4-dimethylbenzoic acid	*	*	*	14	6	*	5	*	**	*	**
2,4,5-trimethylbenzoic acid	*		*	53/57	7		4		*		**
Phenol	44,000		**		4		**		4		**
o-cresol	53,800		**		7		**		**		**
m-cresol	37,600		**		5		**		**		**
p-cresol	19,000		**		**		**		**		**
o-ethylphenol	9,600		*		**		**		**		*
2,6-dimethylphenol	1,400		*		**		**		*		**
2,5-dimethylphenol	10,700		*		**		**		*		**
2,4-dimethylphenol	5,600		*		**		*		**		*
3,5-dimethylphenol + m-ethylphenol	24,900		*		9		*		**		*
2,3-dimethylphenol	5,500		*		**		**		**		*
p-ethylphenol	6,000		*		*		**		*		*
3,4-dimethylphenol	4,700		*		**		**		*		**

[1] Samples collected on 6/12/97 analyzed by National Center for Integrated Bioremediation and Development, University of Michigan, Ann Arbor, MI. Samples collected on 7/30/97 and API 91-01 gasoline sample analyzed by National Risk Management Research Laboratory, U.S. EPA, Ada, OK.

[2] * Not found.

[3] ** Concentration of extract was below lowest calibration standard (3 ug/L).

Table 5-3. *Concentrations in mg/L of Selected Redox-Sensitive Constituents in the Experimental Cells and Injection Water. Concentrations Obtained During the Flushing Periods are Expressed as a Range of Measured Values. Concentrations from 3/22/97 and 6/12/97 Sampling Dates are from a Single Piezometer Sample in Each Cell. All Samples in Cells Collected from Various Ports between 60- and 180-cm Depths.*

	Injection Water		Nitrate Cell			Control Cell		
Sampling Interval:	(5/96 - 11/96)	(5/97)	(5/96 - 11/96)	(3/97)	(6/97)	(5/96 - 11/96)	(3/97)	(6/97)
# Days:	174	24	174 flushing	1 static	1 static	174 flushing	1 static	1 static
pH	6.84 - 7.48 [(3)1]	7.56	n.a.[2]	n.a.	n.a.	n.a.	n.a.	n.a.
Alkalinity ($CaCO_3$)	252 - 269 [(3)]	n.a.	248 - 267 [(8)]	n.a.	n.a.	252 - 277 [(8)]	n.a.	n.a.
Sulfate	2.05 - 9.07 [(7)]	4.04	3.13 - 9.47 [(13)]	1.70	4.14	2.65 - 10.6 [(16)]	2.23	4.78
Sulfide	n.d.[3]	n.a.	n.d. [(2)]	n.d.	n.d.	n.d. [(4)]	n.d.	n.d.
Total Iron	n.d. - 0.29 [(8)]	2.37	n.d. - 0.12 [(13)]	0.28	0.14	n.d. - 0.10 [(16)]	0.34	1.30
Methane	1.04	n.a.	n.a.	1.47	0.46	0.24	0.94	0.088
Nitrate	n.d. - 175 [(25),4]	100 - 123 [(5),4]	n.d. - 160 [(189)]	10.6	88.9	n.d. [(13)]	n.a.	n.a.
Nitrite	n.d. [(25),4]	n.d. [(5),4]	n.d. - 17.2 [(189)]	0.68	0.7	n.d. [(13)]	n.a.	n.a.
Dissolved Oxygen	0.2 - 10.0 [(161)]	3.9 - 5.6 [(8)]		0.46	0.09		0.52	0.16

[1] (n) - Number of samples collected during the indicated period.
[2] n.a. - Not analyzed.
[3] n.d. - Not detected. Detection limits provided in text.
[4] Nitrate Cell only.

Additional aliphatic and aromatic acids that were not detected in supply water or fresh gasoline were present in the treatment cells (Table 5-2). Groundwater in the Nitrate Cell contained alkyl-substituted benzoic acids at concentrations up to ca. 400 µg/L for 2,5-dimethylbenzoic acid. These compounds are structurally similar to the aromatic-hydrocarbon fraction of the gasoline, and although it could not be verified in this experimental system, their presence in the treatment cells suggests that they were products of parent compounds in API 91-01. In other field studies alkyl-substituted benzoic acids have been related to alkylbenzene parent compounds (Cozzarelli et al., 1990, 1994). In the laboratory ^{14}C mineralization experiment conducted in this study, unidentified intermediates of toluene biotransformation were produced in anaerobic microcosms amended with NO_3^-. Toluene biodegraded in two of three replicates amended with NO_3^-, but not in unamended (no NO_3^- added) or sterile controls (data not shown). By day 20, about 50% of the total radiolabel was recovered in oxidized products, of which 50% was in a nonvolatile intermediate fraction and 50% was CO_2. By the end of the experiment on day 63, 100% of the radiolabel was transformed to oxidized products in these microcosms, with a substantial fraction, ca. 10%, still present as aqueous nonvolatile intermediates. Although these compounds were not identified, they suggest that the potential exists for the formation of persistent intermediates under NO_3^--reducing conditions. This result is consistent with the more-detailed laboratory study of Evans et al. (1992).

The suite of compounds detected in the Control Cell was somewhat different. With the exception of *m*-methylbenzoic acid, samples from the Control Cell did not contain aromatic acids. This may represent lack of production of these compounds, or relatively rapid turnover to other oxidized constituents (Grbić-Galić and Vogel, 1987). There were, however, elevated concentrations of short-chained aliphatic acids (Table 5-2). The origin of these partially-oxidized compounds is less clear because of the numerous potential parent compounds in the gasoline.

The presence of low molecular weight organic acids derived from gasoline hydrocarbons demonstrates that, by the end of the flushing experiment, there was microbial activity in the treatment cells. The number of samples collected in this study was insufficient to determine whether these compounds resulted from aerobic or anaerobic transformations of gasoline constituents. However, production and persistence of these compounds has been observed frequently in anaerobic environments (Cozzarelli et al., 1990 (Fe-reducing and methanogenic); Kuhn et al., 1988 (NO_3^--reducing); Grbić-Galić and Vogel, 1987 (methanogenic)). In the Nitrate Cell, the redox potential of the system was probably buffered by the continuous presence of NO_3^- (Kehew and Passero, 1990). This suggests that acid production was most likely associated with NO_3^--reducing and/or O_2-reducing activity. In the Control Cell, the dominant terminal electron-accepting process is not known. Nitrate was not present, and periodic sampling for other electron acceptors did not provide evidence of substantial Fe-reducing, SO_4^{2-}-reducing, or methanogenic activity. As shown in Table 5-3, ranges of SO_4^{2-} and total dissolved Fe in the Control Cell were similar to the supply water, as well as to the Nitrate Cell. Methane was present in both cells, but was also detected once in the supply-well water. The peat layer encountered at 2.8 m rather than gasoline hydrocarbons may have been the source of the CH_4 in these samples.

5.2 Mass Balance Calculations

Mass balances were performed on both added electron-acceptors and the aromatic hydrocarbons. To simplify the analysis, it was assumed that flow in the cells was at steady state over the 174-day flushing period, so that the injection rate could be used to estimate mass fluxes both into and out of the cells, as required. In addition the volumetric flow rates were assumed constant in space (i.e., the treatment cells were treated as one-dimensional columns) and time (i.e., mean injection rates were used in calculations). These assumptions were supported by the continuous injection and constant upper and lower head conditions maintained during the flushing experiments, as well as the Br^- tracer-test results. For all constituents, the mass advected across the horizontal plane located 20 cm above the top of the extraction well screen (180 cm bgs) was used as the most reliable measure of the mass "extracted" from the cells. For most of the mass-balance calculations, data from the extraction wells were not used because concentrations may have been affected by cyclical pumping rates and unquantified dilution with underlying groundwater.

5.2.1 Dissolved Oxygen

Because of the difficulties associated with obtaining reliable results from the multilevel piezometers, a rigorous mass balance was not performed on the dissolved O_2. However, the mass injected into each cell was estimated using a simple mass flux calculation. In this study, mass flux was calculated for the entire 2m x 2m area rather than a unit area; mass flux (QC) therefore had units of [M/T]. The mass injected into each cell was obtained by integrating the advective mass flux over the 174-day injection period:

$$M = \int_0^t F dt = \int_0^t Q(t)C(t)dt = \overline{Q}\,\overline{C}t \qquad 5\text{-}1$$

where $\overline{Q}\left[L^3/T\right]$ and $\overline{C}\left[M/L^3\right]$ are the mean volumetric injection rates and dissolved O_2 concentrations, respectively, and M is the mass. The propagated error in this mass estimate was calculated from:

$$s_M^2 = M^2\left(\frac{s_{\overline{C}}^2}{\overline{C}^2} + \frac{s_{\overline{Q}}^2}{\overline{Q}^2}\right) \qquad 5\text{-}2$$

where s_M^2, $s_{\overline{C}}^2$, and $s_{\overline{Q}}^2$ are the variances of the respective means (Bevington, 1969). Using the mean injection rates and dissolved O_2 concentrations provided in Sections 3.5.3 and 5.1.2, respectively, the

masses of O_2 injected into the cells over the 174-day flushing experiment were 143±115 g (Nitrate Cell) and 136±100 g (Control Cell). To calculate the additional masses pumped into the cells during the 24-day flushing experiment, mean injection concentrations (4.6±1.3 mg/L (n=5) and 4.5±1.4 mg/L (n=5) for the Nitrate and Control Cells, respectively) were multiplied by the injection rate (200 ml/min, based on one measurement in each treatment cell), and the error was calculated from Equation 5-2. Resulting masses of O_2 injected into the cells during this period were 32±9 g (Nitrate Cell) and 31±10 g (Control Cell).

Although the magnitude of the trapped air phase was not measured, it is possible to estimate the size of this O_2 reservoir. Assuming that the volumetric residual air content was in the range of 5-10% (R.W. Gillham, personal communication), a dewatered volume of 4.4 m^3 (2m x 2m x 1.1m height), and an oxygen gas density of 1.43 g/L (standard temperature and pressure) (Hillel, 1982), the initial mass of O_2 could range from 60-120 g per cell. Based on the assumptions given above, the mass of O_2 derived from trapped air was roughly 50-100% of the mean injected O_2 masses, and therefore probably constituted a significant component of the total O_2 budget. Considering both sources of O_2, the total mass available for reactions may have been as high as ca. 300 g per cell.

5.2.2 Nitrate

The NO_3^- mass loss during the 174-day flushing experiment was estimated by comparing the NO_3^- masses injected into and extracted from the Nitrate Cell. Data collected during the 24-day flushing period were insufficient to perform a mass balance, but qualitatively, the rate of NO_3^- utilization, as indicated by concentration differences, was similar. Mass loss was obtained by difference from $M_{loss} = M_{injected} - M_{extracted}$. Injected and extracted masses were calculated by integrating the advective mass flux into and out of the cell, respectively, over the 174 day flushing experiment:

$$M = \int_0^t F\,dt = \int_0^t Q(t)C(t)\,dt = \overline{Q}\int_0^t C(t)\,dt \qquad 5\text{-}3$$

where,

F = Mass flux [M/T],

$\overline{Q}$ = Mean injection rate [L^3/T],

C = NO_3^- concentration at time t [M/L^3],

t = Time [T]

Because the product QC was not available for every sampling event, masses were calculated using the mean injection rate ($\overline{Q}$) over the entire flushing period. The integral Cdt was evaluated numerically with a FORTRAN code adapted from Bevington (1969). To calculate the mass added to the cell, the injection NO_3^- concentration vs. time curve was integrated (Figure 5-5), and then multiplied by the mean injection rate. To calculate the mass advected across the 180 cm bgs plane (extracted mass), the NO_3^- BTCs from each of the ports at this depth were integrated with respect to time, and then a mean value for this integral was calculated. This quantity was then multiplied by the mean injection rate to calculate the NO_3^- mass. Equation 5-2 was used to calculate the propagated error in the mass estimate. As shown in Table 5-4, 7,130±390 g were pumped into the cell during the 174-day flushing experiment, and 6,240±1,160 g were removed. By difference, the calculated mass loss for this period was 890±1,220 g, where the propagated error, expressed as a standard deviation, was calculated from $s^2_{M_{LOSS}} = s^2_{M_{IN}} + s^2_{M_{OUT}}$ (Bevington, 1969). The large error in the mass-loss estimate arises from the substantial spatial variability associated with the amount of mass flushed from the cell. During the following 24-day flushing experiment (mean injection concentration 116.4±10.2 mg/L (n=5)), an additional 802±70 g NO_3^- was pumped into the cell, for a total injected mass of approximately 7.9 kg NO_3^-. Because of sparse data, the mass extracted from the cell during this period was not calculated, but concentrations at the 180-cm depth were similar to those measured during the 174-day experiment.

Based on the mass balance there was a small utilization of NO_3^- under flushing conditions. The estimated mass loss of 890 g represents only 12% of the NO_3^- pumped into the cell over the 174-day

flushing experiment. Because there was substantial experimental error, it is possible that the calculated NO_3^- loss was an experimental artifact. The frequent detection of NO_2^-, however, suggests that at least some of the observed NO_3^- utilization was real (i.e., due to biological NO_3^- reduction).

5.2.3 BTEXTMB

The goal of the mass balance was to estimate the extent of aromatic-hydrocarbon mass loss in each treatment cell. BTEXTMB mass losses were calculated by comparing the initial mass of a constituent in the gasoline to the sum of the mass flushed by advection plus the mass remaining in the gasoline-contaminated zone. The sorbed mass was negligible relative to the other terms in the mass-balance equation, and therefore, was not explicitly considered here. Because it was not feasible to core the cells repeatedly, cores were not collected prior to the flushing experiment to determine the initial mass. This mass balance therefore represents total losses that were incurred over the entire 19-month period from the gasoline spill to final core collection. Volatilization losses were minimized by cell construction, but any losses by this mechanism would be included in the M_{LOSS} term. Total mass loss was obtained by difference,

$$M_{LOSS} = M_i - (M_a + M_r) \qquad 5\text{-}4$$

where,

M_{LOSS} = Mass loss over time period of interest,

M_i = Initial mass in gasoline,

M_a = Mass removed from flushing, and

M_r = Mass remaining in the gasoline-contaminated zone.

For a given constituent, the initial mass, M_i, in 70 liters of fresh gasoline was calculated from weight fraction of the constituent and the density of the gasoline (see Table A-2). As discussed in Appendix A, the weight-fraction data were found to be representative of the composition of fresh API 91-01. Initial masses are shown in Table 5-5. Relative to other components of the mass balance, the uncertainty in the initial mass was probably small.

Estimates of the mass flushed from the cells were obtained for both the 174-day and the following 24-day flushing experiments. These masses were then added to obtain total flushed mass, M_a, for each constituent (Table 5-5). To determine the mass advected across the 180-cm bgs plane during the 174-day experiment (extracted mass), the approach described above for NO_3^- was followed. It should be noted that data points were added to each of the piezometer BTCs to close some existing gaps. Because samples were not collected at 180 cm until day 16, concentrations from this sampling event were extrapolated to day 7. An additional data point was also added to Control-Cell BTCs; concentrations from day 155, the last sampling event in this cell, were extrapolated to day 174. These changes were

Table 5-4. *Nitrate Mass Balance Results for 174-Day Flushing Experiment.*

	Integrated Breakthrough Curve		Injection Rate		Mass	
	(mg/L*d)	(s.d.)[1]	(L/d)	(s.d.)	(g)	(s.d.)
INJECTED	19791.4[2]		360	(20.0)	7,130	(390)
EXTRACTED	17,323.4[3]	(3,075.7)	360	(20.0)	6,240	(1,160)
MASS LOSS					890	(1,220)
MASS RECOVERED (%)					88	

[1] s.d. - standard deviation.
[2] Injection concentration vs. time record.
[3] Mean of integrated breakthrough curves from 180-cm ports.

Table 5-5. *Aromatic Hydrocarbon Mass Balance*

CONTROL CELL (mass in grams)

	Initial Mass	Residual Mass	Flushed Mass 1996	(s.d.)[1]	1997	(s.d.)	Total Recovered Mass	Mass Loss	Initial Mass Recovered (%)	Initial Mass Flushed (%)	Initial Mass Remaining in Gasoline (%)
Benzene	640	80	342	(420)	3	(0.8)	425	216	66	54	13
Toluene	4,000	1,460	1,620	(696)	136	(12)	3,216	784	80	44	37
Ethylbenzene	1,760	1,030	385	(41)	35	(1)	1,450	310	82	24	59
m+p - Xylene	3,880	2,330	820	(93)	75	(2)	3,225	655	83	23	60
o-Xylene	1,380	810	339	(39)	30	(2)	1,179	201	85	27	59
135 TMB	570	430	33	(4)	3	(0.1)	466	105	82	6	75
124 TMB	1,760	1,150	111	(15)	11	(0.4)	1,272	488	72	7	65
123 TMB	360	260	32	(4)	3	(0.08)	295	65	82	10	72
Naphthalene	280	240	46	(6)	3	(0.9)	289	-9	103	17	86
Total BTEXTMB	14,630	7,760	3,727	(1,081)	298	(19)	11,785	2,845	81	28	53

NITRATE CELL (mass in grams)

	Initial Mass	Residual Mass	Flushed Mass 1996	(s.d.)	1997	(s.d.)	Total Recovered Mass	Mass Loss	Initial Mass Recovered (%)	Initial Mass Flushed (%)	Initial Mass Remaining in Gasoline (%)
Benzene	640	80	540	(431)	4	(0.3)	624	16	98	85	13
Toluene	4,000	1,410	2,166	(937)	136	(9)	3,712	288	93	58	35
Ethylbenzene	1,760	940	403	(30)	36	(0.6)	1,379	381	78	25	53
m+p - Xylene	3,880	2,180	883	(88)	80	(1)	3,143	737	81	25	56
o-Xylene	1,380	750	370	(28)	32	(0.2)	1,152	228	83	29	54
135 TMB	570	410	36	(6)	3	(0.2)	449	121	79	7	72
124 TMB	1,760	1,090	117	(10)	11	(0.6)	1,218	542	69	7	62
123 TMB	360	240	33	(3)	3	(0.2)	276	84	77	10	67
Naphthalene	280	210	49	(5)	4	(1)	263	17	94	19	75
Total BTEXTMB	14,630	7,270	4,598	(1,248)	309	(8)	12,177	2,454	83	34	50

[1] s.d. - standard deviation.

supported by the tracer test, which showed sharp arrival of fronts at 180 cm within seven days, and the extraction-well BTCs, which confirmed that extrapolated concentrations were reasonable. To calculate the small additional mass removed during the 24-day pumping period, fewer data were available, and the simpler approach described in Section 5.2.1 for dissolved O_2 was followed. For each constituent, a mean concentration was calculated from extraction-well samples (n=3). This mean was then multiplied by the injection rate (200 ml/min) to obtain a mass estimate, and the error was again calculated from Equation 5-2. Proportionally the largest errors were associated with benzene and toluene, which had large spatial variations in breakthrough behavior.

The mass remaining in the cells, M_r, was estimated from concentrations in core-extract samples. To obtain a mass estimate for the entire cell from discrete samples, an approach similar in principle to Freyberg (1986) was followed. As discussed in detail in Freyberg (1986), an estimate of the mass in a volume of interest can be obtained by integrating the concentration distribution:

$$M_r = \int_{y_1}^{y_2}\int_{x_1}^{x_2} C_z(x,y)\,dx\,dy \qquad 5\text{-}5$$

where C_z is obtained from

$$C_z = \int_{z_1}^{z_2} C(x,y,z)\,dz \qquad 5\text{-}6$$

The concentration, C_z, is expressed above in terms of mass per unit volume of porous medium. The limits of integration in Equation 5-5 correspond to the cell dimensions (2m by 2m), and in Equation 5-6 to the vertical length of the core.

The mass of each constituent was determined by first transforming concentrations in mg/kg to g/m^3 aquifer material using a wet bulk density of 2.15 g/cm^3, calculated from the well-characterized dry bulk density of Borden sand of 1.82 g/cm^3 (Ball et al., 1990), and a fully-saturated porosity of 0.33 (Mackay et al., 1986). At each core location, concentration profiles (Figures 5-13 and 5-14) were then vertically integrated by multiplying each concentration by the appropriate depth interval, Δz, yielding an integrated concentration, C_z, in g/m^2. Kriging was then used to interpolate vertically-integrated data from all of the core locations within a cell onto a regular grid. A linear model of the observed variogram was used to determine the weighting factors in the kriging matrix. Concentration contours of the kriged grid data agreed very well with hand-drawn contours based on linear interpolations between core locations. The GEOSOFT Mapping and Processing System (Geosoft Inc., 1994) was used to calculate the variogram and krige the data. An areal integration of the kriged surface (concentration in g/m^2 x area in m^2) was then performed with GEOSOFT to obtain the total mass of the constituent in grams.

Results are summarized in the second column of Table 5-5. Several assumptions were made in arriving at these residual mass estimates. Because core recoveries were less than 100% of the core run, it was necessary to assign depth intervals to the recovered aquifer material. The calculations in Table 5-5 assume that compaction of the aquifer material inside the core barrel was minimal (reasonable for a sand deposit), and that, because of increasing frictional resistance inside the core barrel as the run proceeded, the missing interval was from the bottom of the core run. Consequently, a core from ground surface with 100 cm recovery was taken to represent the 0 to 100 cm depth interval. Second, the magnitude of the wet bulk density (2.15 g/cm^3) was calculated on the assumption that the sub-samples were fully saturated with water. On the basis of the rapid extraction and capping of the cores, and visual observations while coring, the assumption of full saturation appeared reasonable. Calculations were also simplified by treating the bulk density as a spatially-uniform parameter, and ignoring the small effect of residual gasoline on the magnitude of the bulk density. The value of the bulk density used here may have resulted in a systematic overestimation or underestimation of the mass estimate. Although the net bias would affect the absolute magnitude of the mass loss, it would apply to both cells, and relative differences between cells could still be determined.

In addition, the mass calculation required assigning numerical values to the samples with concentrations below method detection limits. A sensitivity analysis showed that, with the exception of benzene and naphthalene, calculations were not sensitive to the value used for the detection limit; mass estimates varied by less than one percent when concentrations in not-detected samples were varied from zero to the value of the detection limit. For benzene and naphthalene, which had higher proportions of samples below detection limits, mass estimates increased by more than 10% when the detection-limit-value was used in the calculation. However, in the absence of a gasoline phase, the detection-limit value overestimated the total mass that would be present in the sorbed and dissolved phases. Because most of the samples were from locations that did not contain gasoline, concentrations of all constituents in samples below detection limits were assumed to be zero in the calculation of residual mass presented here. This may have resulted in a small underestimation of the residual mass. Finally, it was necessary to estimate the lengths of the contaminated intervals of the lowest samples in cores 4L and 3K. These cores did not straddle the contaminated zone, so the base of the lowest interval was undefined. In both cells, the lengths were chosen to be consistent with adjacent cores, 4K and 3J, which had similar vertical concentration profiles (Figures 5-13 and 5-14).

These assumptions and process of creating smooth concentration distributions from discrete data probably lead to substantial uncertainty in the residual mass estimates. It was not possible, however, to determine the magnitude of this uncertainty quantitatively. Accordingly, the error in the total amount of recovered mass is not given on Table 5-5, but based on error propagation considerations, its magnitude is at least as large as the uncertainty in the flushed mass. These mass balance calculations show therefore that even under highly-controlled experimental conditions, the experimental variability and resulting uncertainty in the estimates of aromatic-hydrocarbon and electron-acceptor utilization can be difficult to control.

Nonetheless, with due consideration of the uncertainty associated with these field data, the mass balance provided reasonable qualitative results. As a percentage of initial mass, the gasoline phase in both cells was relatively depleted in the most soluble compounds such as benzene and toluene (despite the high initial mass of toluene in API 91-01), and the majority of relatively insoluble compounds such as naphthalene remained in the residual mass fraction. Similarly, the percentage of initial mass flushed from the cells corresponded fairly well to the effective solubility of the compound. However, there were differences between aqueous concentrations at the end of the experiment and the mass remaining in the residual phase for most aromatic compounds. For example, as shown on Figure 5-8, by the end of the experiment, aqueous concentrations of benzene, toluene, ethylbenzene, total xylenes, and total trimethylbenzenes in the Nitrate Cell had dropped to 1.5%, 39%, 82%, 85%, and 92% of initial gasoline-saturated values measured in the laboratory, respectively, while masses remaining in the gasoline phase declined to 13%, 35%, 53%, 56%, and 65% of initial masses. Assuming equilibrium partitioning, which is likely in this experimental system, the changes in aqueous concentration are directly proportional to changes in mole fraction, and should be roughly equal to changes in residual mass. The observed differences may reflect negative bias in the estimation of residual mass. A dissolution model is currently being developed to evaluate quantitatively the relationship between aqueous concentrations and masses in the residual gasoline phase.

In general, more mass was flushed from the Nitrate Cell than the Control Cell, and residual masses were slightly higher in the Control Cell. Total recovered masses ranged from a low of 66% (benzene, Control Cell) to a high of 103% (naphthalene, Control Cell). In terms of total BTEXTMB, recovered masses were 81% (Control) and 83% (Nitrate) of initial mass.

It was not possible to conclude that mass loss was enhanced in one cell relative to the other. This conclusion could be made only if a large difference between cells was observed. For instance, if depletion of a labile compound such as toluene was much more extensive in the Nitrate Cell than the Control Cell, then this could reasonably be attributed to biological activity. Moreover, it is important to note that this approach does not provide a direct estimate of biotransformation mass loss. The mass loss term, M_{LOSS}, incorporates all mass removal mechanisms, which may include losses from volatilization and cell flooding. Therefore, even though benzene and toluene mass losses were larger in the Control Cell, the differences between cells could conceivably have resulted from abiotic processes.

5.3 *Discussion and Conclusions*

Despite the uncertainty associated with the low-concentration O_2 data, Figure 5-1 clearly shows rapid depletion of the majority of the O_2 injected into the treatment cells. After about 15 days in the Nitrate Cell and 60 days in the Control Cell, groundwater collected from the pea-gravel layer (60-cm bgs sampling ports) was typically at the threshold O_2 concentration; depletion therefore occurred within hours of injection. Downward migration of dissolved O_2 probably did not exceed ca. 10 cm. The same pattern was observed when the injection dissolved-O_2 concentration was increased to approximately 5 mg/L during the 24-day flushing period the following spring. These results are consistent with the rapid uptake of dissolved O_2 in laboratory microcosms that contained contaminated aquifer material. In those experiments aromatic hydrocarbon losses were evident under fully-aerobic conditions. The extent of microaerophilic O_2 utilization by other gasoline hydrocarbons or abiotic reactions is not known.

In contrast to rapid dissolved-O_2 utilization, NO_3^- uptake was relatively low. Most of the NO_3^- depletion appeared to occur initially in the presence of dissolved O_2, possibly to satisfy an assimilatory nitrogen requirement (Bazylinski and Blakemore, 1983). Previous studies have shown that the activity of microorganisms in this aquifer is nitrogen-limited under aerobic conditions (Barbaro et al., 1994).

Additional NO_3^- utilization also occurred under O_2-depleted conditions, but rates were too low to observe much depletion during the nine-day flushing residence time. The concentration profile at 180 cm showed that about five months were required to deplete the added NO_3^- under static conditions (i.e., a zero-order rate of 0.67 mg NO_3^-/L/d). An inhibitory effect associated with the gasoline phase may account for the lower rate (0.2 mg/L/d) observed during this period at the 60-cm depth interval. Toxicity effects in a microbial community adapted to hydrocarbons have been observed in other studies. Dibble and Bartha (1979) observed declining microbial activity with increasing concentrations of oil sludge. Their microbial community was already adapted to the hydrocarbon mixture; toxicity was manifested as a lower rate of substrate utilization. On the other hand, these rates are similar to the rate observed by Barbaro et al. (1992) during biotransformation of aromatic hydrocarbons and possibly other carbon sources in the Borden landfill leachate plume, and the laboratory experiments performed in this study in which NO_3^- uptake was observed. On the basis of the lack of response to the addition of MBH medium, it appears that anaerobic, denitrifying activity was not limited by inorganic nutrients.

Given the low rate of utilization and the nine-day residence time in the cell, NO_3^--reducing reactions in the absence of dissolved O_2 did not appear to be important within the Nitrate Cell. However, to evaluate the potential of nitrate-based biotransformation in our experimental system, the specific denitrifying pathway was of some interest. The production of NO_2^- and apparent lack of other terminal electron-accepting processes (e.g., methanogenesis) in the treatment cell suggested that NO_3^- was utilized primarily as an electron-acceptor in a dissimilatory reaction, rather than as a nitrogen source for cell protein. The specific denitrifying pathway, denitrification to N_2O or nitrogen gas or dissimilatory reduction of NO_3^- to ammonium (NH_4^+) (DRNA), was not defined *in situ*. Denitrification is generally assumed to be the predominant NO_3^--reducing mechanism in carbon-limited environments, while DRNA may be favored under NO_3^--limited conditions (i.e., in carbon-rich, anoxic environments) (Korom, 1992; Tiedje et al., 1982). DRNA has been identified as an important mechanism in contaminated aquifers (Bulger et al., 1989), but there are no reported cases of aromatic hydrocarbon utilization by this pathway.

The major electron-donor was assumed to be carbon, but autotrophic microbial denitrification coupled to the reduction of sulfide minerals such as pyrite (Aravena and Robertson, 1998) and reduced Fe (Korom, 1992; Postma et al., 1991) have been reported. Sulfide minerals have not been identified in the Borden aquifer, but field observations indicate that greater quantities of reduced sulfur are present in proximity to the peat layer. Assuming carbon was the major donor in the Nitrate Cell, both the laboratory and field results indicated that other carbon sources were utilized in preference to the aromatic hydrocarbons under NO_3^--reducing conditions in this highly-contaminated experimental system. This conclusion is supported by the laboratory experiments performed in this study, and is consistent with other experiments using hydrocarbon-contaminated aquifer material (Hutchins et al., 1991a).

The mass balance results and hypothetical reactions were used to estimate the maximum mineralization that could occur under aerobic conditions. If the observed dissolved-O_2 threshold concentration was representative of *in situ* conditions, then only about 50-75% of the O_2 supplied to the aquifer for microaerophilic reactions was utilized. Even if all of the available O_2 was utilized, however, the mass of O_2 added to the cells was probably insufficient to stimulate an observable removal of recalcitrant compounds such as benzene. For example, if aerobic benzene biotransformation is assumed to proceed completely to CO_2 with no assimilation of C by microbial cells, the mineralization reaction is

$$C_6H_6 + 7.5\,O_2 \rightarrow 6\,CO_2 + 3\,H_2O \qquad 5\text{-}7$$

From the stoichiometry of this reaction, 3.1g of O_2 are required to oxidize 1g of benzene. Assuming that all of the injected and residual O_2 (ca. 300 g) in a given cell was used to mineralize benzene, then 97 grams or 15% of the 640 grams of benzene added to the cell would be removed. This is clearly an overestimate because O_2 would likely be utilized in the oxidation of other carbon compounds as well, including other aromatic hydrocarbons (Chapter 4). More realistically, if only a fraction (for example, one quarter) of the available O_2 was utilized for benzene oxidation, then only 24 grams of benzene would be removed, which is well within the experimental error of the mass balance. Assuming all of the available O_2 was consumed by the aromatic hydrocarbons (i.e., none was consumed in reactions with other gasoline hydrocarbons), 300 g O_2 would oxidize only 95 g total BTEXTMB to CO_2. Based on these assumed reactions, the calculated mass loss was very small relative to the initial mass in a given cell.

Therefore, if the addition of microaerophilic O_2 did contribute to a reduction in the flux of soluble compounds from the source area, the effect would have been very small in relation to the size of the carbon pool.

The NO_3^- mass balance was used in a similar manner to estimate the maximum mass loss from mineralization that could occur under NO_3^--reducing conditions. Although most of the NO_3^- consumed in the NO_3^--amended cell may have been utilized as an N source during aerobic biotransformation, and aromatic-hydrocarbon uptake under NO_3^--reducing conditions appeared low, some biotransformation of the more labile compounds may have occurred. These losses would have been difficult to detect in the experimental system. For example, assuming toluene is mineralized to CO_2 by a denitrification reaction and there is no C or N assimilation by microbial cells (Equation 4-3), 446.4 g NO_3^- are required to oxidize 92.1 g toluene. Stoichiometries of the other NO_3^--reducing reactions (NO_2^- or N_2O end products) that may have occurred yield similar molar ratios. From Equation 5-8, the 890 g of NO_3^- that were utilized over the 174-day flushing experiment (Table 5-4) correspond to about 180 g toluene, or 7.8 % of the flushed mass (2300 g) and 4.5 % of the total mass (4,000 g) of toluene in the cell. This calculation overestimates toluene removal because it is based on the assumptions that all of the NO_3^- was consumed under anaerobic conditions, with toluene as the sole substrate. Nonetheless, the calculated mass loss is still small relative to the size of the toluene pool, and would have been within the uncertainty of the toluene mass balance and difficult to detect.

The detection of metabolites demonstrated that some partial oxidation of gasoline hydrocarbons was occurring in both cells. A transformation to a partially-oxidized intermediate, such as benzoic acid, requires less O_2 per mole of parent compound oxidized. It is conceivable, therefore, that losses of aromatic hydrocarbons from biotransformation were more extensive than indicated by the mineralization reactions. If partial oxidation was occurring, reaction stoichiometries show that mass loss of a given constituent could have been substantial relative to the total mass in the cell. For example, assuming no assimilation of C and N by microbial cells, the following reaction controls the partial oxidation of benzene to phenol (Cozzarelli et al., 1990),

$$2\,C_6H_6 + O_2 \rightarrow 2\,C_6H_6O \qquad 5\text{-}8$$

Equation 5-8 shows that one gram of O_2 would partially oxidize 4.9 g benzene, and if it is assumed that the maximum benefit was gained from microaerophilic O_2 addition (i.e., 100% partial oxidation of benzene), 300 g of O_2 would be sufficient to partially oxidize 1470 g benzene, which exceeds the total benzene mass in the cell. More realistically, if it is arbitrarily assumed that only 25% of the total O_2 mass was consumed for benzene degradation, then 75 g would be available for reaction, which corresponds to a transformation of 367 g benzene to phenol, or approximately 50 % of the initial benzene mass. Using similar reasoning, if toluene was transformed to benzoic acid under NO_3^--reducing conditions (Kuhn et al., 1988), then partial oxidation of toluene could produce substantial toluene losses and concentrations of benzoic acid in the mg/L range.

These calculations suggest that if significant partial oxidation had occurred, biotransformation would clearly have been observable in the treatment cells. However, the low µg/L concentrations of partially-oxidized compounds suggest that these hypothetical reactions were only of minor importance. Alternatively, the metabolites may not have been persistent, in which case additional oxidant would have been consumed to complete the reaction, and the stoichiometries of the mineralization reactions discussed above would be predominant. Regardless of the extent of mineralization, the low consumption of NO_3^- and the presence of multiple carbon sources in combination with a limited mass of O_2 indicate that mass losses of aromatic hydrocarbons from biotransformation were low, and probably much less than the ca. 2500 g total BTEXTMB mass losses obtained from the mass balances (Table 5-5). These observations were confirmed by the followup microcosm experiment. The large mass losses from the mass balance probably resulted from a combination of physical losses and experimental error, with only a minor contribution from biotransformation.

Although there was evidence from metabolite formation and electron-acceptor uptake that *in situ* biotransformation had been stimulated in the gasoline-contaminated zone, the extent of biotransformation of the aromatic hydrocarbons was too limited to significantly affect the formation of a plume. To understand the net result of flushing fluid through a NAPL, consideration must be given to the complex

interactions between dissolution and chemical reactions. Flushing mixes reactants (in this instance, electron acceptors) into the source and associated plume, which is required to enhance mass removal, but also increases the advective flux of dissolved constituents into the aquifer. If the biotransformation rate is low, soluble compounds may be advected from the residually-saturated zone by flushing before significant biotransformation occurs (Malone et al., 1993). Conversely, if the biotransformation rate is fast relative to the dissolution rate, biotransformation can enhance the dissolution rate by increasing the concentration gradient which is the driving force for dissolution, as well as reduce the mass being advected into the plume (Seagren et al., 1993, 1994). In our experimental system, gasoline dissolution was the dominant mass removal mechanism. Concentrations flushed from the cells were consistent with equilibrium partitioning between the gasoline and water phases. Biotransformation appeared therefore to be too minimal to appreciably lower the flux of soluble constituents from the gasoline-contaminated zone.

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6.0 CONCLUSIONS AND IMPLICATIONS

Generally the findings on the degradability of aromatic hydrocarbons in the presence of microaerophilic O_2 and NO_3^- were consistent between laboratory and field experiments: mass losses were minor in the Nitrate Cell as well as in both laboratory microcosm experiments in which conditions similar to the field were established (i.e., BTEXTMB concentrations near gasoline-saturated concentrations and microaerophilic / NO_3^- conditions (Experiments 4 and 6)). As discussed in Chapter 5, however, ascertaining small mass losses in the field was difficult because of the size of the carbon pool and the uncertainty associated with the mass balance. The apparent lack of extensive mass losses in the field was also consistent with the activity and biomass assays performed in the laboratory: Despite strong O_2 consumption when available, numbers and activity in aquifer material extracted from the treatment cells were not indicative of a large hydrocarbon-degrading population. In both the field and the laboratory microcosm experiment with pre-exposed aquifer material (Experiment 6), NO_3^- reduction was observed over time periods on the order of 100 days, but accompanying utilization of labile aromatic compounds was not apparent. The use of nitrate solely as an electron acceptor was equivocal; utilization of some NO_3^- as an N source may have occurred in both instances. In both of these experimental systems, the effect of microaerophilic O_2 was small, although the disparity between static microcosms and dynamic field conditions makes the comparison tenuous. Perhaps most important for remediation, there was agreement on the minor benzene losses in the presence of microaerophilic O_2.

Although under certain conditions the extent of mass loss was maximized by the presence of dual electron acceptors, the bulk of the field and laboratory results indicated that NO_3^- / microaerophilic-O_2 based bioremediation was not an effective source-area remedial technology in this aquifer. Rapid uptake of O_2, observed at both laboratory and field scales, demonstrated that aerobic activity was not inhibited *in situ*, but mass losses were limited under microaerophilic conditions by the small quantity of O_2 available for reaction. Based on laboratory results, the addition of dissolved O_2 may have led to oxidation of compounds that otherwise would have been recalcitrant under anaerobic, denitrifying conditions, but the effect *in situ* was small relative to the mass of gasoline hydrocarbons in the cells. This suggests that the partial oxidation of parent compounds by microaerophilic O_2 was a relatively unimportant process. Consequently, the majority of the mass of recalcitrant compounds (e.g., benzene) was flushed into the aquifer. Moreover, NO_3^- reduction was slow relative to the residence time in the source area, and did not appear to be associated with aromatic-hydrocarbon utilization in the presence of other gasoline constituents. A large denitrifying population capable of rapid aromatic-hydrocarbon biotransformation did not develop in the treatment cell in response to extended exposure to abundant substrate and NO_3^-. Consequently, nitrate-based bioremediation was ineffective for source-area remediation in this experimental system. It is not clear why this population did not develop under the conditions established in the field. Given these observations and the results of the mass balance, there were no apparent advantages associated with the microaerophilic / NO_3^- treatment relative to the unremediated control. Rapid O_2 uptake and aromatic-hydrocarbon utilization in gasoline-contaminated aquifer material (Experiment 6) suggests that a more conventional injection fluid with NO_3^- and air-equilibrated O_2 (7-10 mg/L) would have been more beneficial in this system with a similar level of effort.

These conclusions pertain to specific experimental system evaluated in this study (i.e., a recent gasoline spill flushed for a relatively short period of time and monitored over a short flow path). It is conceivable that this approach, particularly with respect to the effects of microaerophilic O_2, would be more effective during the latter stages of an enhanced bioremediation project when source-area concentrations were lower. Similarly, although NO_3^- utilization was minor over the flow path evaluated here, acclimation resulting in the development of a substantial population capable of degrading TEX may

have occurred with continued exposure. Existing studies have indicated, however, that NO_3^- uptake rates are generally slow relative to O_2 (Section 1.3.2). This suggests that a longer residence time would be needed to fully evaluate the effects of NO_3^- addition. Based on existing data for this aquifer (e.g., Barbaro et al., 1992), it is possible that substantial NO_3^- utilization would have occurred further downgradient (beyond our experimental system) in the anaerobic core of the plume, providing a benefit to an engineered or intrinsic remediation strategy.

Finally, the results reported here are specific to the Borden aquifer; generalization of the results to other sites is inadvisable without verification from site-specific testing. The comparison of aromatic hydrocarbon mass loss in both pristine and exposed Borden sediment with sediment from other petroleum-hydrocarbon-contaminated sites revealed a substantial range in the capabilities of the indigenous denitrifying populations. Similarly, although microaerophilic O_2 was not found to be beneficial in our experimental system, the contribution to *in situ* mass loss could be significant in other circumstances (e.g., lower-carbon environments). For example, mixed electron acceptors could conceivably be effective for downgradient plume control using a reactive wall or other semi-passive remedial technology.

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APPENDIX A: GASOLINE CHARACTERISTICS

APPENDIX A. GASOLINE CHARACTERISTICS

American Petroleum Institute (API) gasoline was used in most of the experiments in this study. This unleaded gasoline, designated 91-01, was used for various toxicological research programs. Consequently, comprehensive analytical work was done by the API in 1991 when the gasoline was formulated to define its composition and physical properties (raw data not shown). Although gasoline may contain more than 1,000 compounds (Brookman et al., 1985), a relatively small group accounts for most of the weight. Approximately 150 compounds and generic compound classes were identified in API 91-01, of which only 25 account for 65% of the gasoline weight (Table A-1). An accurate characterization of the gasoline composition was required to complete the field mass balance, and to predict dissolved aromatic hydrocarbon concentrations. Molar fractions for all identified compounds are compiled in Table A-1. Basic characteristics of the gasoline, including weight fractions of BTEXTMB, are summarized in Table A-2.

Several analyses were performed over the course of this study to verify the accuracy of the API weight fraction data. GC analyses (Appendix C) of known volumes of API 91-01 gasoline in methanol yielded weight fractions of aromatic hydrocarbons that agreed well with the API weight fractions listed in Tables A-1 and A-2. These analyses are summarized in Table A-3. All of the samples analyzed by the Organic Geochemistry Laboratory at the University of Waterloo were taken from an aliquot of API 91-01 stored in laboratory. The August, 1997 sample was submitted and analyzed with the core extract samples collected from the treatment cells. The August, 1997 samples were also analyzed one year later to obtain additional gasoline composition data. Percent differences were less than approximately 20% for all compounds, with the exception of naphthalene (up to 40%).

Aqueous concentrations of BTEXTMB in gasoline-saturated water, measured in laboratory batch equilibrium tests, agreed well with concentrations calculated from Raoult's Law (Table A-4; Figure A-1). Raoult's Law calculations were based on literature solubilities and the mole fractions listed in Table A-1. Calculated concentrations were lower than measured by ca. 20%, possibly because of non-ideal behavior in the gasoline phase (Borden and Kao, 1992). With the exception of naphthalene which behaved anomalously, the reasonable agreement between measured and calculated concentrations indicated not only that the API weight-fraction data were representative, but also that Raoult's Law could be used to describe equilibrium partitioning between the gasoline and groundwater (Eganhouse et al., 1996).

TABLE A-1 *Mole Fractions of Identified Compounds in API 91-01 Gasoline. Compound Identification and Weight Fractions Compiled from an Unpublished 1991 Analysis of the Gasoline Performed by the API[1].*

Compound	Carbon	Hydrogen	Wt.%	C x Wt%	H x Wt%	Mol. Wt. (g/mole)	wt%/mol. Wt. (moles/100g)	Mole Fraction
Propane	3	8	0.01	0.03	0.08	44.10	0.0002	0.00021
Isobutane	4	10	0.14	0.56	1.40	58.12	0.0024	0.00227
n-Butane	4	10	4.88	19.52	48.80	58.12	0.0840	0.07896
1,2-Butadiene	4	6	0.03	0.12	0.18	54.09	0.0006	0.00052
3-Methyl-1-butene	5	10	0.03	0.15	0.30	70.13	0.0004	0.00040
Isopentane	5	12	4.51	22.55	54.12	72.15	0.0625	0.05879
1-Pentene	5	10	0.63	3.15	6.30	70.13	0.0090	0.00845
n-Pentane	5	12	3.61	18.05	43.32	72.15	0.0500	0.04705
trans-2-Pentene	5	10	0.73	3.65	7.30	70.13	0.0104	0.00979
cis-2-Pentene	5	10	0.43	2.15	4.30	70.13	0.0061	0.00577
2-Methyl-2-butene	5	10	1.21	6.05	12.10	70.13	0.0173	0.01622
2,2-dimethylbutane	6	14	0.505	3.03	7.07	86.18	0.0059	0.00551
cyclopentadiene	5	6	0.505	2.53	3.03	66.10	0.0076	0.00718
cyclopentene	5	8	0.17	0.85	1.36	68.12	0.0025	0.00235
4-methyl-1-pentene	6	12	0.06	0.36	0.72	84.16	0.0007	0.00067
3-methyl-1-pentene	6	12	0.08	0.48	0.96	84.16	0.0010	0.00089
Cyclopentane	5	10	0.23	1.15	2.30	70.13	0.0033	0.00308
2,3-dimethylbutane	6	14	1.65	9.90	23.10	86.18	0.0191	0.01801
4-methyl-cis-2-pentene	6	12	0.06	0.36	0.72	84.16	0.0007	0.00067
2-methylpentane	6	14	5.52	33.12	77.28	86.18	0.0641	0.06024
4-methyl-trans-2-pentene	6	12	0.04	0.24	0.48	84.16	0.0005	0.00045
3-methylpentane	6	14	3.12	18.72	43.68	86.18	0.0362	0.03405
2-methyl-1-pentene	6	12	0.28	1.68	3.36	84.16	0.0033	0.00313
1-hexene	6	12	0.12	0.72	1.44	84.16	0.0014	0.00134
n-hexane	6	14	2.65	15.90	37.10	86.18	0.0308	0.02892
trans-3-hexene	6	12	0.18	1.08	2.16	84.16	0.0021	0.00201
trans-2-hexene	6	12	0.38	2.28	4.56	84.16	0.0045	0.00425
3-methyl-cis-2-pentene	6	12	0.11	0.66	1.32	84.16	0.0013	0.00123
4-methylcyclopentene	6	10	0.26	1.56	2.60	82.15	0.0032	0.00298
3-methyl-trans-2-pentene	6	12	0.05	0.30	0.60	84.16	0.0006	0.00056
cis-2-hexene	6	12	0.21	1.26	2.52	84.16	0.0025	0.00235
2,2-dimethylpentane	7	16	0.43	3.01	6.88	100.20	0.0043	0.00404
methylcyclopentane	6	12	1.11	6.66	13.32	84.16	0.0132	0.01240
2,4-dimethylpentane	7	16	0.69	4.83	11.04	100.20	0.0069	0.00648
1-methylcyclopentene	6	10	0.38	2.28	3.80	82.15	0.0046	0.00435
C7 olefin	7	14	0.01	0.07	0.14	98.19	0.0001	0.00010
benzene	6	6	1.22	7.32	7.32	78.11	0.0156	0.01469
3,3-dimethylpentane	7	16	0.14	0.98	2.24	100.20	0.0014	0.00131
cyclohexane	6	12	0.28	1.68	3.36	84.16	0.0033	0.00313
C7 cyclo-olefin/diolefin	7	12	0.06	0.42	0.72	96.17	0.0006	0.00059
C7 olefin	7	14	0.12	0.84	1.68	98.19	0.0012	0.00115
2-methylhexane	7	16	1.63	11.41	26.08	100.20	0.0163	0.01530
2,3-dimethylpentane	7	16	1.3	9.10	20.80	100.20	0.0130	0.01220
1,1-dimethylcyclopentane	7	14	0.04	0.28	0.56	98.19	0.0004	0.00038
3-methylhexane	7	16	1.7	11.90	27.20	100.20	0.0170	0.01595
C7 olefin	7	14	0.04	0.28	0.56	98.19	0.0004	0.00038
trans-1,3-dimethylcyclopentane	7	14	0.32	2.24	4.48	98.19	0.0033	0.00306
cis-1,3-dimethylcyclopentane	7	14	0.28	1.96	3.92	98.19	0.0029	0.00268
3-ethylpentane	7	16	0.235	1.65	3.76	100.20	0.0023	0.00221
C7 olefin	7	14	0.235	1.65	3.29	98.19	0.0024	0.00225
trans--1,2-dimethylcyclopentane	7	14	1.76	12.32	24.64	98.19	0.0179	0.01686
2,2,4-trimethylpentane	8	18	0.105	0.84	1.89	114.23	0.0009	0.00086
C7 olefin	7	14	0.105	0.74	1.47	98.19	0.0011	0.00101
C7 olefin	7	14	0.11	0.77	1.54	98.19	0.0011	0.00105
n-heptane	7	16	1.3	9.10	20.80	100.20	0.0130	0.01220
C7 olefin	7	14	0.05	0.35	0.70	98.19	0.0005	0.00048

TABLE A-1. *Continued.*

Compound	Carbon	Hydrogen	Wt.%	C x Wt%	H x Wt%	Mol. Wt. (g/mole)	wt%/mol. Wt. (moles/100g)	Mole Fraction
C7 olefin	7	14	0.11	0.77	1.54	98.19	0.0011	0.00105
C7 olefin	7	14	0.12	0.84	1.68	98.19	0.0012	0.00115
C7 olefin	7	14	0.06	0.42	0.84	98.19	0.0006	0.00057
C7 olefin	7	14	0.13	0.91	1.82	98.19	0.0013	0.00125
C8 olefin	8	16	0.09	0.72	1.44	112.21	0.0008	0.00075
C8 olefin	8	16	0.09	0.72	1.44	112.21	0.0008	0.00075
C8 olefin	8	16	0.08	0.64	1.28	112.21	0.0007	0.00067
C8 olefin	8	16	0.05	0.40	0.80	112.21	0.0004	0.00042
cis-1,2-dimethylcyclopentane	7	14	0.18	1.26	2.52	98.19	0.0018	0.00172
methylcyclopentane	6	12	0.43	2.58	5.16	84.16	0.0051	0.00480
ethylcyclopentane	7	14	0.46	3.22	6.44	98.19	0.0047	0.00441
2,2,3-trimethylpentane	8	18	0.18	1.44	3.24	114.23	0.0016	0.00148
C8 olefin	8	16	0.19	1.52	3.04	112.21	0.0017	0.00159
2,4-dimethylhexane	8	18	0.18	1.44	3.24	114.23	0.0016	0.00148
C8 olefin	8	16	0.06	0.48	0.96	112.21	0.0005	0.00050
1,2,3-trimethylcyclopentane	8	16	0.05	0.40	0.80	112.21	0.0004	0.00042
C8 cyclo-olefin/diolefin	8	14	0.05	0.40	0.70	110.20	0.0005	0.00043
2,3,4-trimethylpentane	8	18	0.365	2.92	6.57	114.23	0.0032	0.00300
C8 olefin	8	16	0.365	2.92	5.84	112.21	0.0033	0.00306
toluene	7	8	7.68	53.76	61.44	92.14	0.0834	0.07839
2,3-dimethylhexane	8	18	0.205	1.64	3.69	114.23	0.0018	0.00169
C8 olefin	8	16	0.205	1.64	3.28	112.21	0.0018	0.00172
2-methylheptane	8	18	0.85	6.80	15.30	114.23	0.0074	0.00700
4-methylheptane	8	18	0.36	2.88	6.48	114.23	0.0032	0.00296
3-methylheptane	8	18	0.02	0.16	0.36	114.23	0.0002	0.00016
3-ethylhexane	8	18	0.605	4.84	10.89	114.23	0.0053	0.00498
C8 olefin	8	16	0.605	4.84	9.68	112.21	0.0054	0.00507
C8 Naphthene	8	16	0.07	0.56	1.12	112.21	0.0006	0.00059
C8 olefin	8	16	0.07	0.56	1.12	112.21	0.0006	0.00059
C8 Naphthene	8	16	0.08	0.64	1.28	112.21	0.0007	0.00067
C8 olefin	8	16	0.08	0.64	1.28	112.21	0.0007	0.00067
C8 Naphthene	8	16	0.065	0.52	1.04	112.21	0.0006	0.00054
C8 olefin	8	16	0.065	0.52	1.04	112.21	0.0006	0.00054
C8 Naphthene	8	16	0.09	0.72	1.44	112.21	0.0008	0.00075
C8 Naphthene	8	16	0.13	1.04	2.08	112.21	0.0012	0.00109
n-octane	8	18	0.65	5.20	11.70	114.23	0.0057	0.00535
trans-1,2-dimethylcyclohexane	8	16	0.12	0.96	1.92	112.21	0.0011	0.00101
C8 olefin	8	16	0.05	0.40	0.80	112.21	0.0004	0.00042
C9 naphthene	9	18	0.21	1.89	3.78	126.24	0.0017	0.00156
C9 paraffin	9	20	0.025	0.23	0.50	128.26	0.0002	0.00018
C8 olefin	8	16	0.025	0.20	0.40	112.21	0.0002	0.00021
C8 olefin	8	16	0.06	0.48	0.96	112.21	0.0005	0.00050
C9 paraffin	9	20	0.19	1.71	3.80	128.26	0.0015	0.00139
cis-1,2-dimethylcyclohexane	8	16	0.025	0.20	0.40	112.21	0.0002	0.00021
C9 olefin	9	18	0.025	0.23	0.45	126.24	0.0002	0.00019
C9 paraffin	9	20	0.03	0.27	0.60	128.26	0.0002	0.00022
C9 naphthene	9	18	0.03	0.27	0.54	126.24	0.0002	0.00022
Ethylbenzene	8	10	3.37	26.96	33.70	106.17	0.0317	0.02985
m-Xylene	8	10	5.31	42.48	53.10	106.17	0.0500	0.04704
p-Xylene	8	10	2.13	17.04	21.30	106.17	0.0201	0.01887
2-methyloctane	9	20	0.28	2.52	5.60	128.26	0.0022	0.00205
4-methyloctane	9	20	0.32	2.88	6.40	128.26	0.0025	0.00235
3-methyloctane	9	20	0.34	3.06	6.80	128.26	0.0027	0.00249
o-Xylene	8	10	2.64	21.12	26.40	106.17	0.0249	0.02339
C10 naphthene	10	20	0.04	0.40	0.80	140.27	0.0003	0.00027
n-nonane	9	20	0.24	2.16	4.80	128.26	0.0019	0.00176
C9 naphthene	9	18	0.02	0.18	0.36	126.24	0.0002	0.00015
isopropylbenzene	9	12	0.22	1.98	2.64	120.19	0.0018	0.00172

TABLE A-1. *Continued.*

Compound	Carbon	Hydrogen	Wt.%	C x Wt%	H x Wt%	Mol. Wt. (g/mole)	wt%/mol. Wt. (moles/100g)	Mole Fraction
C9 naphthene	9	18	0.02	0.18	0.36	126.24	0.0002	0.00015
n-propylbenzene	9	12	0.75	6.75	9.00	120.19	0.0062	0.00587
1-methyl-3-ethyl-benzene	9	12	2.34	21.06	28.08	120.19	0.0195	0.01831
1-methyl-4-ethyl-benzene	9	12	1.06	9.54	12.72	120.19	0.0088	0.00829
C10 paraffin	10	22	0.03	0.30	0.66	142.28	0.0002	0.00020
1,3,5-trimethylbenzene	9	12	1.1	9.90	13.20	120.19	0.0092	0.00861
4-methylnonane	10	22	0.11	1.10	2.42	142.28	0.0008	0.00073
2-methylnonane	10	22	0.15	1.50	3.30	142.28	0.0011	0.00099
1-methyl-2-ethylbenzene	9	12	0.72	6.48	8.64	120.19	0.0060	0.00563
1,2,4-trimethylbenzene	9	12	3.37	30.33	40.44	120.19	0.0280	0.02637
1,2,3-trimethylbenzene	9	12	0.68	6.12	8.16	120.19	0.0057	0.00532
Indane	10	26	0.4	4.00	10.40	146.32	0.0027	0.00257
C11 paraffin	11	24	0.05	0.55	1.20	156.31	0.0003	0.00030
C11 paraffin	11	24	0.22	2.42	5.28	156.31	0.0014	0.00132
1-methyl-3-n-propylbenzene	10	14	0.48	4.80	6.72	134.22	0.0036	0.00336
1-methyl-4-n-propylbenzene	10	14	0.28	2.80	3.92	134.22	0.0021	0.00196
n-butylbenzene	10	14	0.14	1.40	1.96	134.22	0.0010	0.00098
1,2-diethylbenzene	10	14	0.44	4.40	6.16	134.22	0.0033	0.00308
1,3-dimethyl-5-ethylbenzene	10	14	0.02	0.20	0.28	134.22	0.0001	0.00014
1,4-diethylbenzene	10	14	0.02	0.20	0.28	134.22	0.0001	0.00014
C11 paraffin	11	24	0.01	0.11	0.24	156.31	0.0001	0.00006
1,3-dimethyl-4-ethylbenzene	10	14	0.125	1.25	1.75	134.22	0.0009	0.00088
Indane	10	26	0.125	1.25	3.25	146.32	0.0009	0.00080
1,2-dimethyl-4-ethylbenzene	10	14	0.31	3.10	4.34	134.22	0.0023	0.00217
Indane	10	26	0.31	3.10	8.06	146.32	0.0021	0.00199
1,2-dimethyl-3-ethylbenzene	10	14	0.15	1.50	2.10	134.22	0.0011	0.00105
n-undecane	11	24	0.06	0.66	1.44	156.31	0.0004	0.00036
1,2,4,5-tetramethylbenzene	10	14	0.24	2.40	3.36	134.22	0.0018	0.00168
1,2,3,5-tetramethylbenzene	10	14	0.33	3.30	4.62	134.22	0.0025	0.00231
dodecane	12	26	0.05	0.60	1.30	170.34	0.0003	0.00028
Naphthalene	10	8	0.53	5.30	4.24	128.17	0.0041	0.00389
C13 paraffins	13	28	0.19	2.47	5.32	184.36	0.0010	0.00097
C12 aromatics	12	18	1.06	12.72	19.08	162.27	0.0065	0.00614
C12 Indanes	10	26	0.54	5.40	14.04	146.32	0.0037	0.00347
Methylnaphthalenes	11	12	0.69	7.59	8.28	144.22	0.0048	0.00450
Unidentified heavies	12	26	0.99	11.88	25.74	170.34	0.0058	0.00547
unknowns	7	16	4.86	34.02	77.76	100.20	0.0485	0.04561
TOTAL			100.00			94.00[2]	1.0633	1.00000

[1] c8, c9, and c10 naphthene molecular weights taken from Table 2-A Poulson et.al. (1990) Indanes taken from same source. Assumed average group molecular weight. Assumed value near gasoline molecular weight for unknown fraction. Assumed dodecane molecular weight for unknown heavies.

[2] Calculated molecular weight.

Table A-2. *Characteristics of API 91-01 Gasoline*

Molecular Weight	(g/mole)	94.3
Density[1]	(g/cm^3)	0.747
MTBE	(vol %)	<0.10
Methanol	(vol %)	<0.10
HYDROCARBON CLASSES (wt %)		
Paraffins		41.01
Naphthenes		6.84
Aromatics		39.05
Olefins		11.74
Unknowns		1.26
AROMATIC HYDROCARBONS (wt % (mole fraction))		
Benzene		1.22 (0.0147)
Toluene		7.68 (0.0787)
Ethylbenzene		3.37 (0.0300)
m-Xylene		5.31 (0.0472)
p-Xylene		2.13 (0.0189)
o-Xylene		2.64 (0.0235)
1,3,5-Trimethylbenzene		1.10 (0.00864)
1,2,4-Trimethylbenzene		3.37 (0.0265)
1,2,3-Trimethylbenzene		0.68 (0.00534)
Naphthalene		0.53 (0.00390)

[1] Gasoline density measured at University of Waterloo (Oliveira, 1997). All other information from an unpublished 1991 analysis of the gasoline performed by API.

Table A-3. *Comparison of Measured Concentrations of Aromatic Hydrocarbons in API 91-01 Gasoline. All Concentrations Expressed as Weight Percent.*

Compound	API	This Study			
	(1991)	(August, 1997)	(August, 1998)[1]	(August, 1998)	(August, 19
Benzene	1.22	1.28	1.02	0.96	0.97
Toluene	7.68	8.26	8.67	8.31	8.21
Ethylbenzene	3.37	3.48	3.50	3.33	3.30
m+*p*-Xylene	7.44	7.50	8.06	7.66	7.60
o-Xylene	2.64	2.70	2.75	2.61	2.59
1,3,5-Trimethylbenzene	1.10	1.13	1.18	1.12	1.11
1,2,4-Trimethylbenzene	3.37	3.02	3.18	3.01	2.97
1,2,3-Trimethylbenzene	0.68	0.67	0.74	0.70	0.69
Naphthalene	0.53	0.66	0.74	0.70	0.69

[1] Samples analyzed August, 1998 are means of three injections.

Table A-4. *Measured and Calculated Concentrations of Aromatic Hydrocarbons in Water Equilibrated with API 91-01 Gasoline at 10°C.*

Compound	Concentration (mg/L)		
	Measured[1]	Calculated[2]	% Diff[3]
Benzene	30.90	26.25	15.1
Toluene	49.72	42.10	15.3
Ethylbenzene	6.41	5.42	15.3
m+*p* Xylene	13.45	10.59	21.3
o-Xylene	5.79	4.18	27.8
1,3,5-Trimethylbenzene	0.54	0.42	23.2
1,2,4-Trimethylbenzene	1.89	1.56	17.3
1,2,3-Trimethylbenzene	0.51	0.40	20.7
Naphthalene	0.84	0.13	84.1

[1] Measured concentrations are means of four replicates collected from two separatory funnels. Coefficients of variation ranged from 1.3% (toluene) to 11% (naphthalene).

[2] Calculated concentrations are based on Raoult's Law, $C_i = X_i S$, where X is the mole fraction of component i (Table A-1), S is the pure-phase solubility, and C is the calculated concentration. Solubilities were obtained from published sources: benzene 1780 mg/L; toluene 535 mg/L; p-xylene 160 mg/L; o-xylene 178 mg/L; 1,3,5-trimethylbenzene 48 mg/L; 1,2,4-trimethylbenzene 59 mg/L; 1,2,3-trimethylbenzene 75 mg/L; naphthalene 34 mg/L (Source: References cited within Montgomery, J.H. 1996. Groundwater Chemicals Desk Reference, 2nd Ed., CRC-Lewis Publishers, Boca Raton, Fla.).

[3] Percent difference: $\frac{(C_{meas} - C_{calc})}{C_{meas}} * 100$.

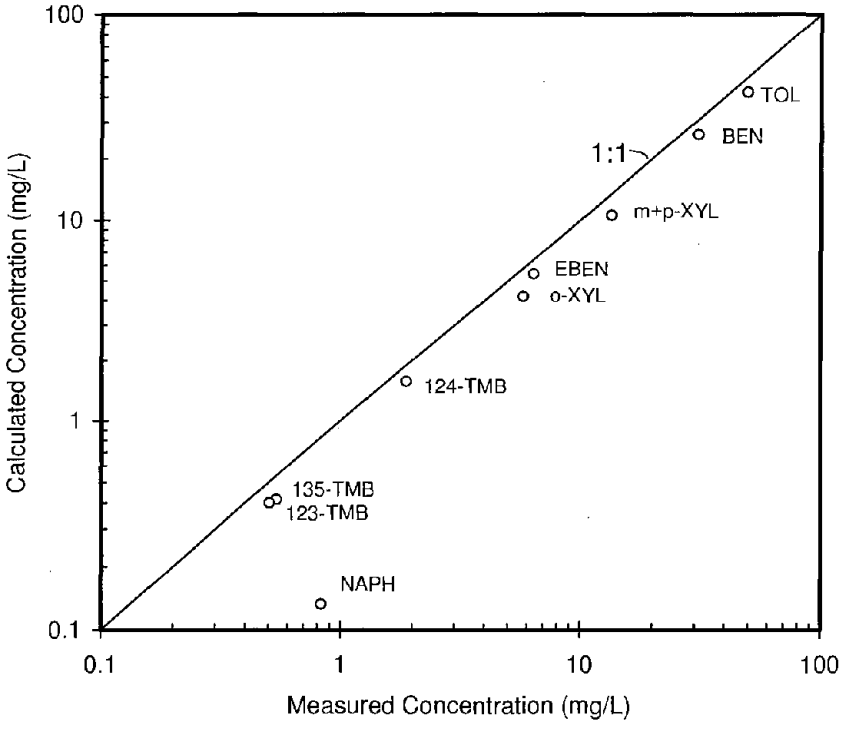

FIGURE A-1 Calculated vs. measured BTEXTMB concentrations in gasoline-saturated water. Concentrations obtained from equilibration of water with fresh API 91-01 gasoline as described in text.

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APPENDIX B: FIELD TRACER TEST

APPENDIX B: FIELD TRACER TEST

B.1 Introduction

Tracer tests were performed in both experimental cells during the month of July, 1996 while the flushing experiment was in progress. The objectives of the test were to confirm that the injected water was distributed uniformly in the cells, and to calculate linear groundwater velocities and longitudinal dispersivities at different locations within the cells. Bromide (Br^-) was used as the conservative tracer.

B.2 Methods

A concentrated Br^- solution was prepared with KBr salt. The solution was pumped into the injection flow lines of both cells. A peristaltic pump (Masterflex L/S Series) equipped with an Ismatec multichannel head was used to feed the stock solution into the flow lines. The pumping rate was calibrated to a rate that yielded a 100x dilution of the concentrated solution. Brominated water was pumped into the cells over a 45 hr period to create a slug input. Injection water to each cell was sampled periodically to determine the mean injection concentrations (C_o).

In addition to injection samples, groundwater samples were collected periodically from all of the 60-cm, 120-cm, and 180-cm bgs piezometer ports, and the extraction-well sampling ports. These data were used to develop relative concentration vs. time breakthrough curves (BTCs) at each sampling location. A plastic 60-cc syringe was used to collect groundwater samples from the piezometers. Prior to collecting a sample, about 20-ml water was removed to clear stagnant water from the piezometer tube and to flush the syringe. Samples from the injection flow lines were collected by holding the sample vial under the flowing stream of water at the tube outlet. Samples from the extraction flow lines were collected from the in-line sampling ports (see Figure 3-6). Once sampling began at a given depth, samples were collected approximately every six hours. A total of 540 samples was collected over a 400 hr period, and stored in plastic scintillation vials for analysis.

Samples were screened on-site with a conductivity meter. These data were used to monitor the position of the tracer slug as the test progressed. The goal was to obtain complete breakthrough curves for each piezometer port and the extraction wells. After the test, samples were returned to the laboratory and analyzed with a bromide-specific electrode (Corning, Model 476128). Samples were prepared for analysis by adding 5 ml ionic strength adjuster (0.2 M KNO_3 solution) to a 5 ml groundwater sample. Standards were prepared from a concentrated KBr stock solution, and diluted to the appropriate concentrations using aerobic Borden groundwater and 5 ml ionic strength adjustor. Each day, standards were run in duplicate or triplicate to generate a standard curve. Sample quantification was based on a linear regression of electrode response (mV) vs. natural log of the standard concentration. To check the accuracy of the Br^- electrode results, a subset of the groundwater samples analyzed on different days with the electrode was submitted to the Water Quality Laboratory for analysis by ion chromatography (see Section 3.6.5). As shown in Table B-1, there was good agreement between methods.

B.3 Results

Injection Br^- concentrations and extraction well BTCs are shown in Figures B-1 and B-2 for the Control and Nitrate Cells, respectively. Breakthrough curves from piezometer ports at the 60-, 120- and 180-cm depths of both experimental cells are shown in Figures B-3 through B-8. Based on eight samples, the average injection concentrations of the Br^- slug, C_o, were 260.1 $\pm$ 9 mg/L and 260.1 $\pm$ 12.4 mg/L for the Nitrate and Control Cells, respectively. As shown on the figures, complete BTCs were obtained for nearly all piezometer ports monitored during the test; in some cases (e.g., PZ3C-4, PZ3D-4), the initial arrival of the tracer slug was missed. The shapes of the BTCs were quite similar at a given depth, and there were no indicators (e.g., very early arrival of tracer) of short-circuiting along piezometer casings.

Linear groundwater velocities and dispersivities were calculated from breakthrough data at all 120-cm and 180-cm piezometer ports, and the extraction-well sampling ports. These parameters were obtained by fitting the one-dimensional advection-dispersion equation to the normalized BTCs. Modeling was done with Wpulsepe (Devlin and Barker, 1996; Sorel et al., 1998). This program uses a simplex optimization routine to find the best fit of the model solution to the data. The model requires only the length of the flowpath, the mean injection concentration, and the concentration vs. time breakthrough data as input. The lengths of the flow paths were calculated assuming the upper head remained at 50-cm bgs during the test (70 cm and 130 cm flow paths for 120- and 180-cm bgs piezometer ports, respectively, and 175 cm to the midpoint of the extraction-well screens). Both velocity and dispersivity are used by the model as fitting parameters. As shown in the figures, the model fit the measured breakthrough data very well at most sampling locations.

Model-calculated velocities and dispersivities are shown in Table B-2. Velocities ranged from 0.45 cm/hr to 1.2 cm/hr and were consistent with a 200 ml/min injection rate. The velocities from piezometer BTCs represent the average rate of flow of the tracer in the individual flow tube intersecting the sample port. If sufficient ports are measured to obtain a representative sample of all of the flow tubes in the domain, the mean should be equal to the volumetric tracer injection rate. For this test, the expected linear velocity of 0.91 cm/hr (assumed porosity of 0.33) did fall within the error of the mean velocities calculated from piezometer breakthrough data in both cells (Table B-2). The velocities at the extraction wells were slightly higher, possibly reflecting the influence of the early arrival of Br^- in the faster flow tubes. Dispersivities were in the millimeter range in the Control Cell, and the millimeter to centimeter range in the Nitrate Cell (Table B-2). The largest dispersivities were measured at the extraction wells. These large dispersivities were caused by an integration of variable tracer concentrations along the 50-cm well screens (Sorel et al., 1998); extraction-well breakthrough curves represent the sum of the relative concentrations in all of the flow tubes in the domain, as well as clean water from the underlying aquifer.

Table B-1. *Comparison of Bromide Concentrations Determined from a Bromide Electrode and Ion Chromatography (IC). Concentrations in mg/L.*

Sample	IC	Electrode
T-24	155.0	154.6
T-41	3.8	4.3
T-50	250.0	246.7
T-84	258.0	260.8
T-108	<0.05	0.6
T-126	7.8	7.8
T-131	44.4	40.9
T-160	222.0	223.5
T-175	0.21	1.1
T-216	289.0	292.8
T-284	1.3	1.5
T-314	62.8	67.1
T-349	293.0	277.8
T-406	87.1	88.5

Bromide Breakthrough Curves - Nitrate Cell

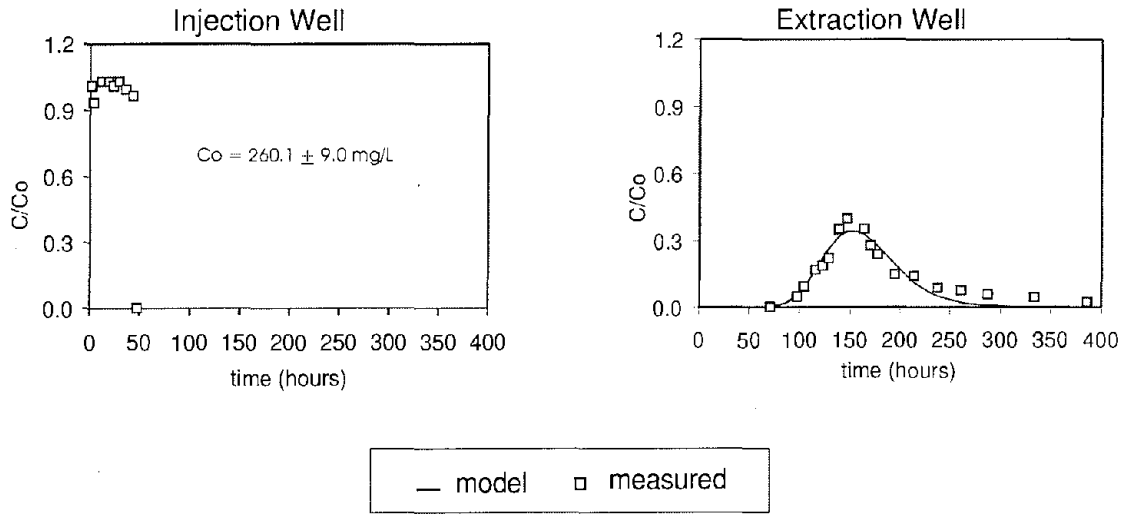

FIGURE B-1 Normalized bromide concentrations in samples of injection and extraction water during bromide tracer test (Nitrate Cell).

Bromide Breakthrough Curves - Control Cell

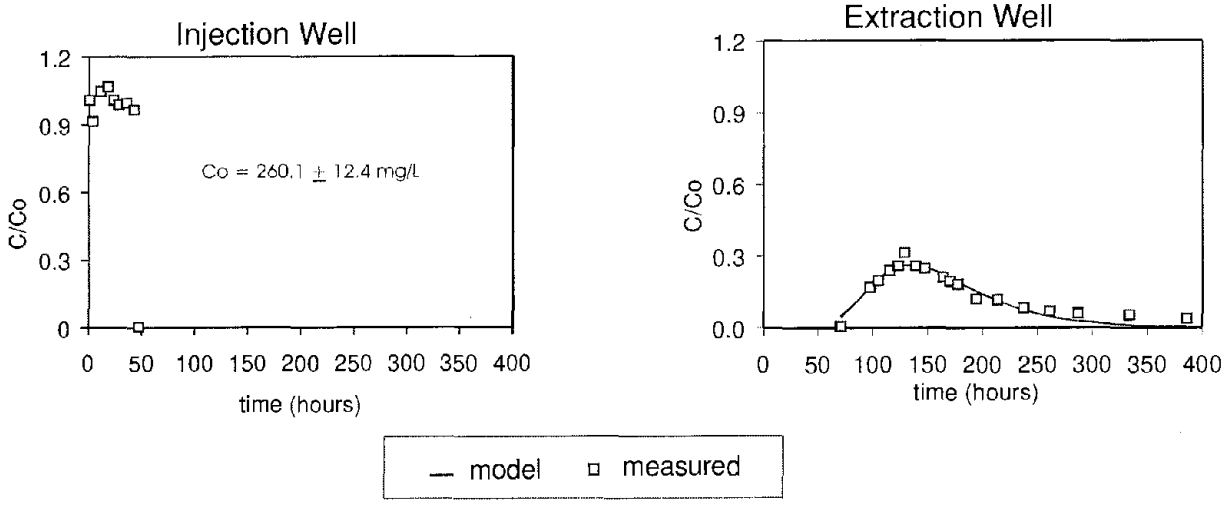

FIGURE B-2 Normalized bromide concentrations in samples of injection and extraction water during bromide tracer test (Control Cell).

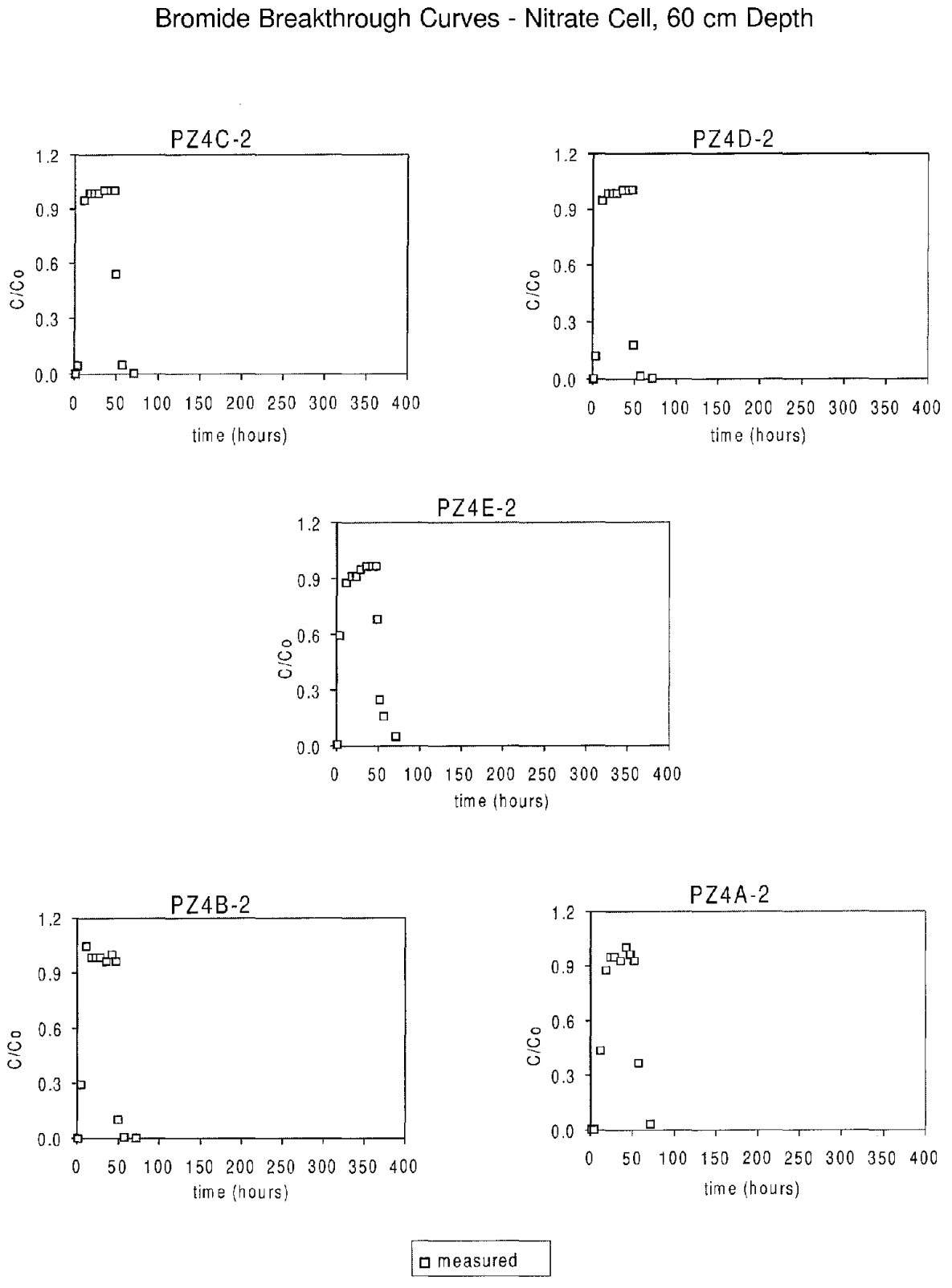

FIGURE B-3 Measured bromide breakthrough curves at 60-cm bgs ports in the Nitrate Cell.

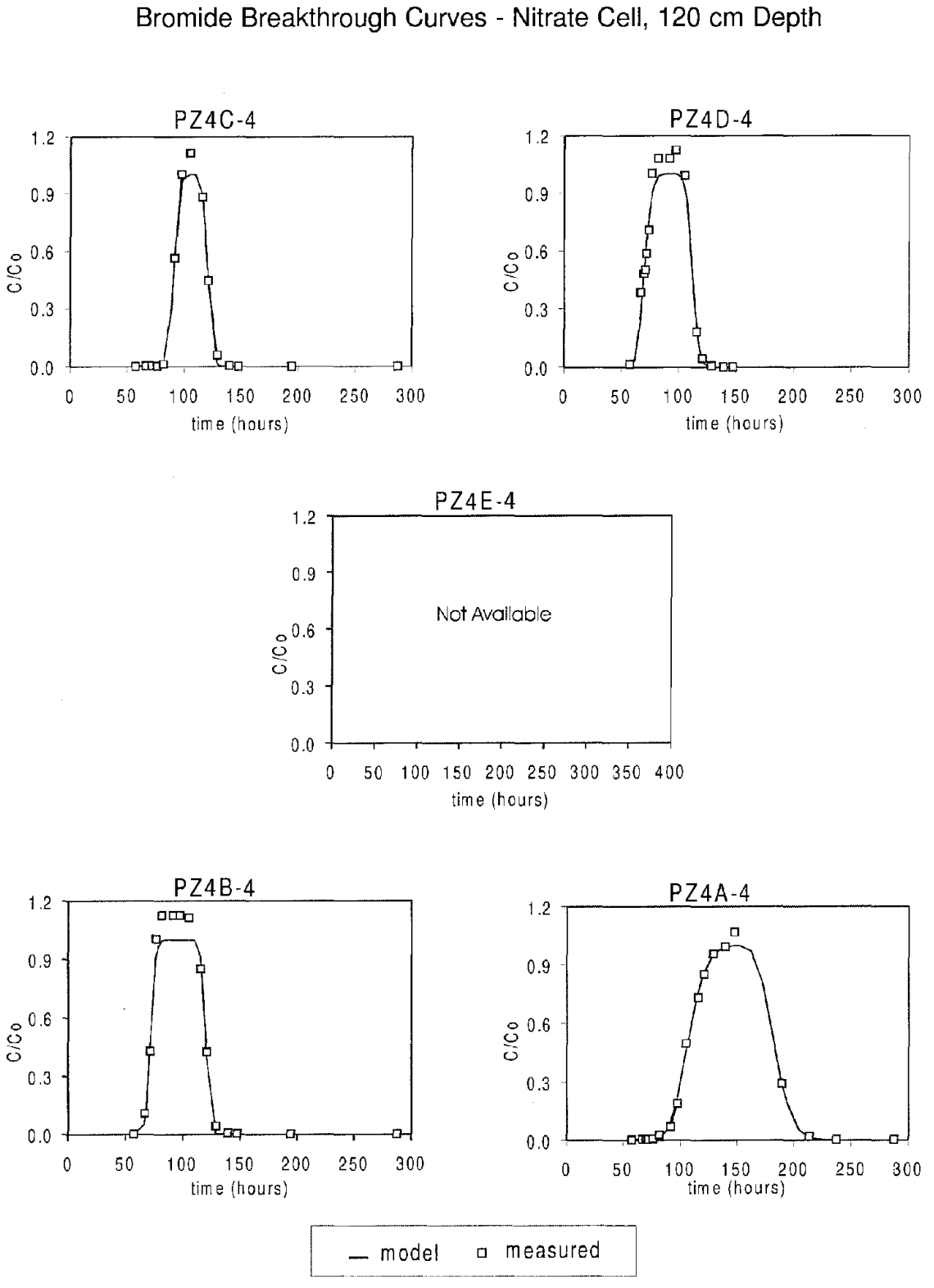

FIGURE B-4 Measured and calculated bromide breakthrough curves at 120-cm bgs ports in the Nitrate Cell. Samples could not be collected from the 120 cm port on the center piezometer (PZ4E-4).

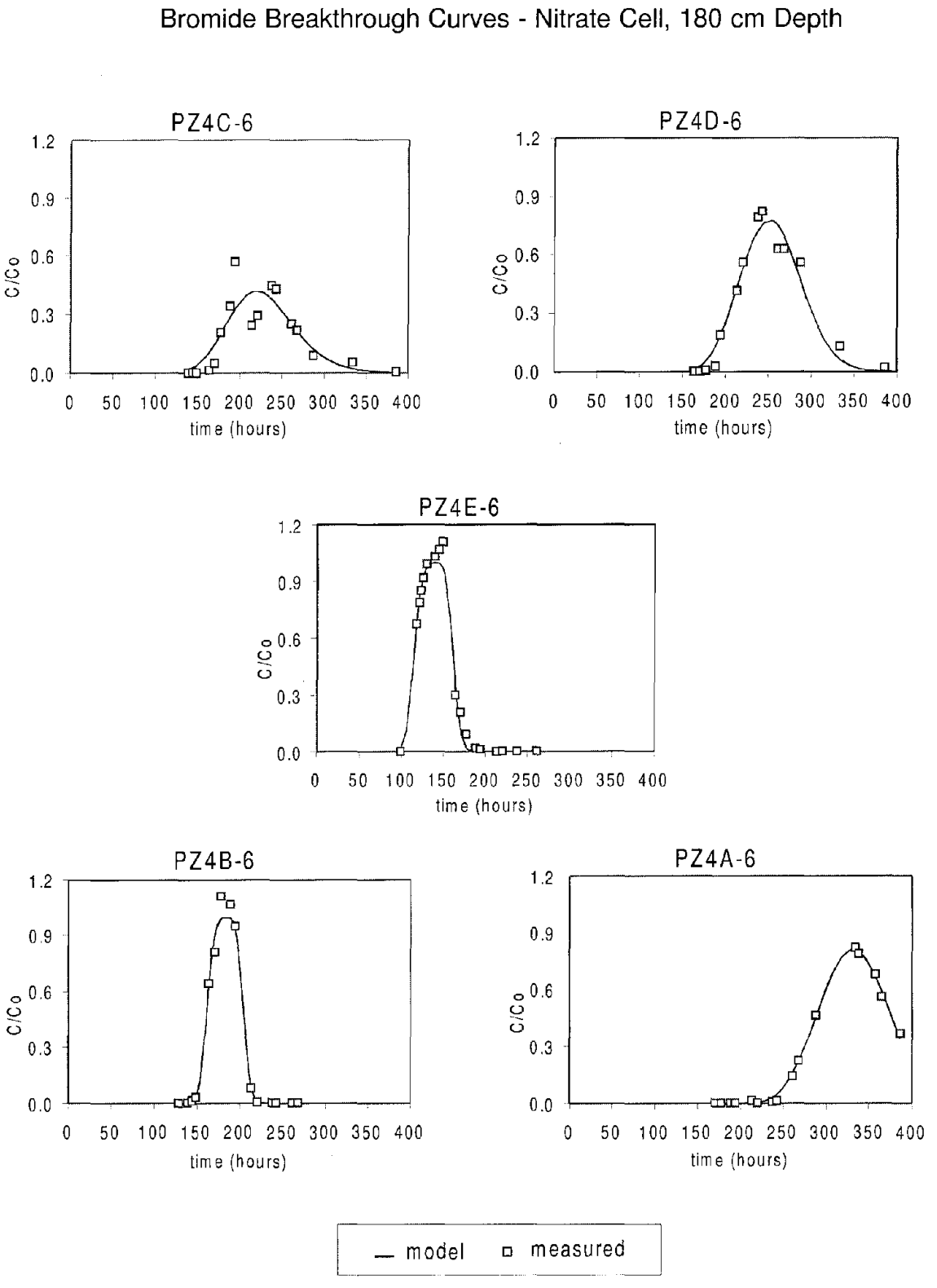

FIGURE B-5 Measured and calculated bromide breakthrough curves at 180-cm bgs ports in the Nitrate Cell.

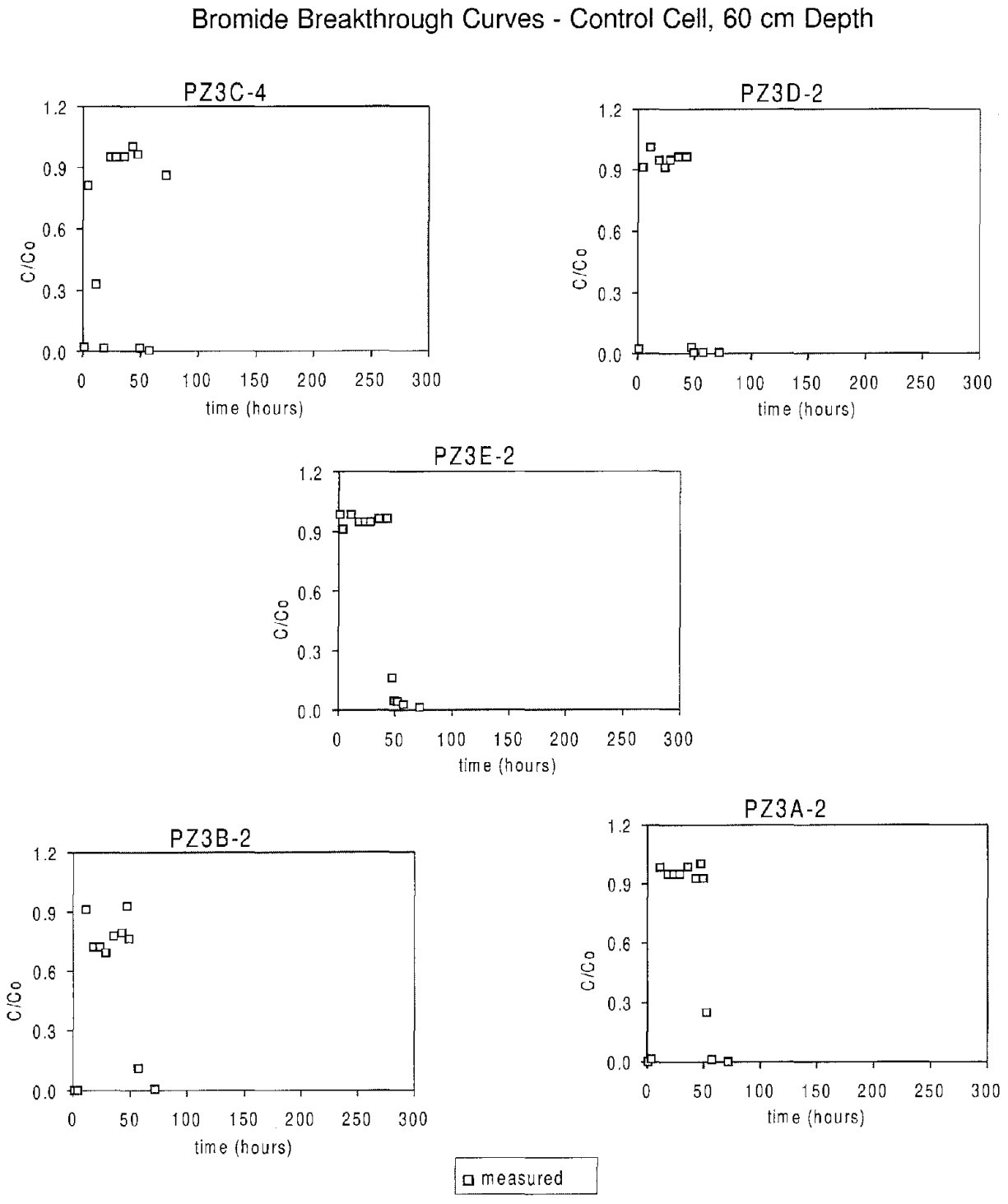

FIGURE B-6 Measured bromide breakthrough curves at 60-cm bgs ports in the Control Cell.

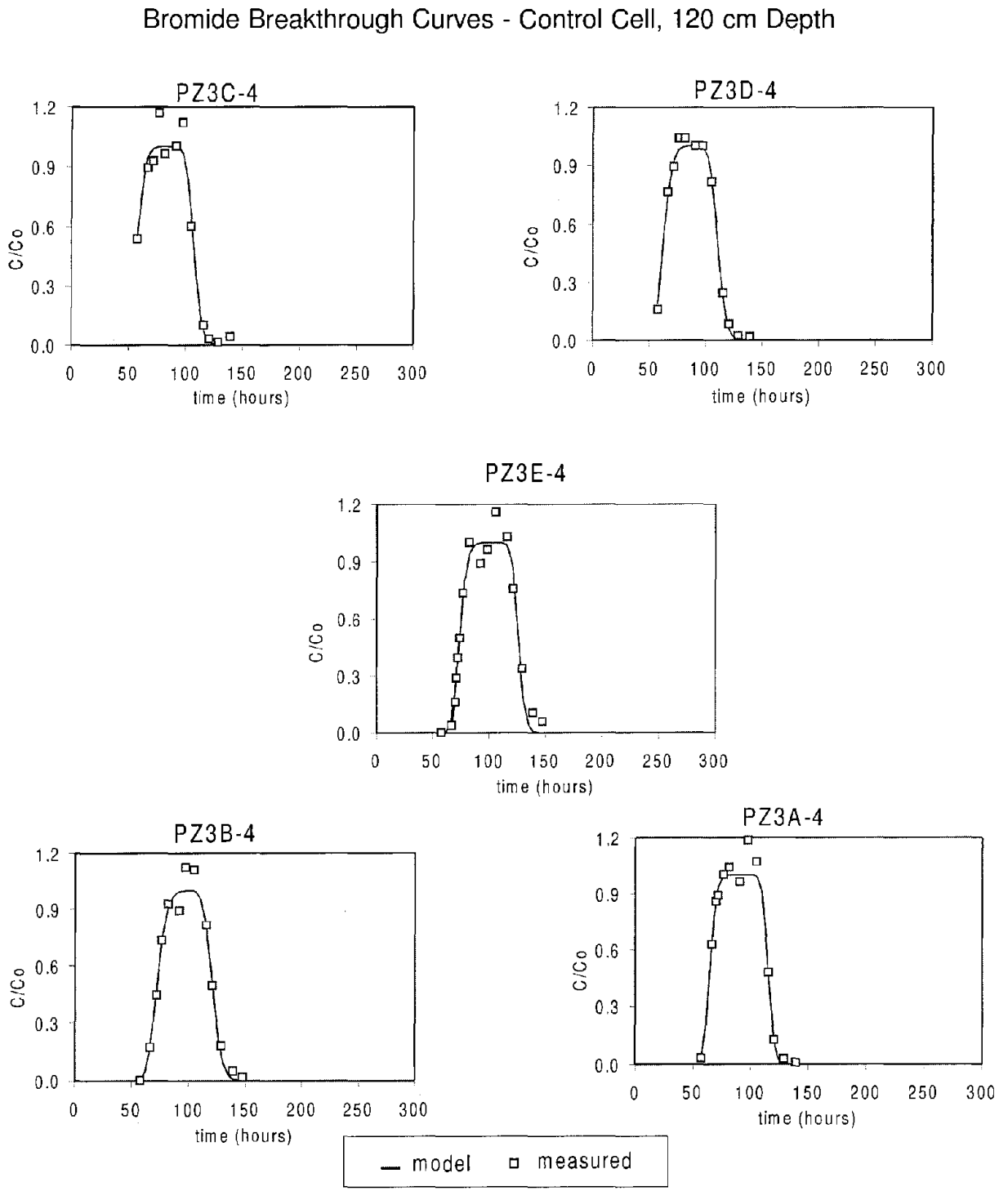

FIGURE B-7 Measured and calculated bromide breakthrough curves at 120-cm bgs ports in the Control Cell.

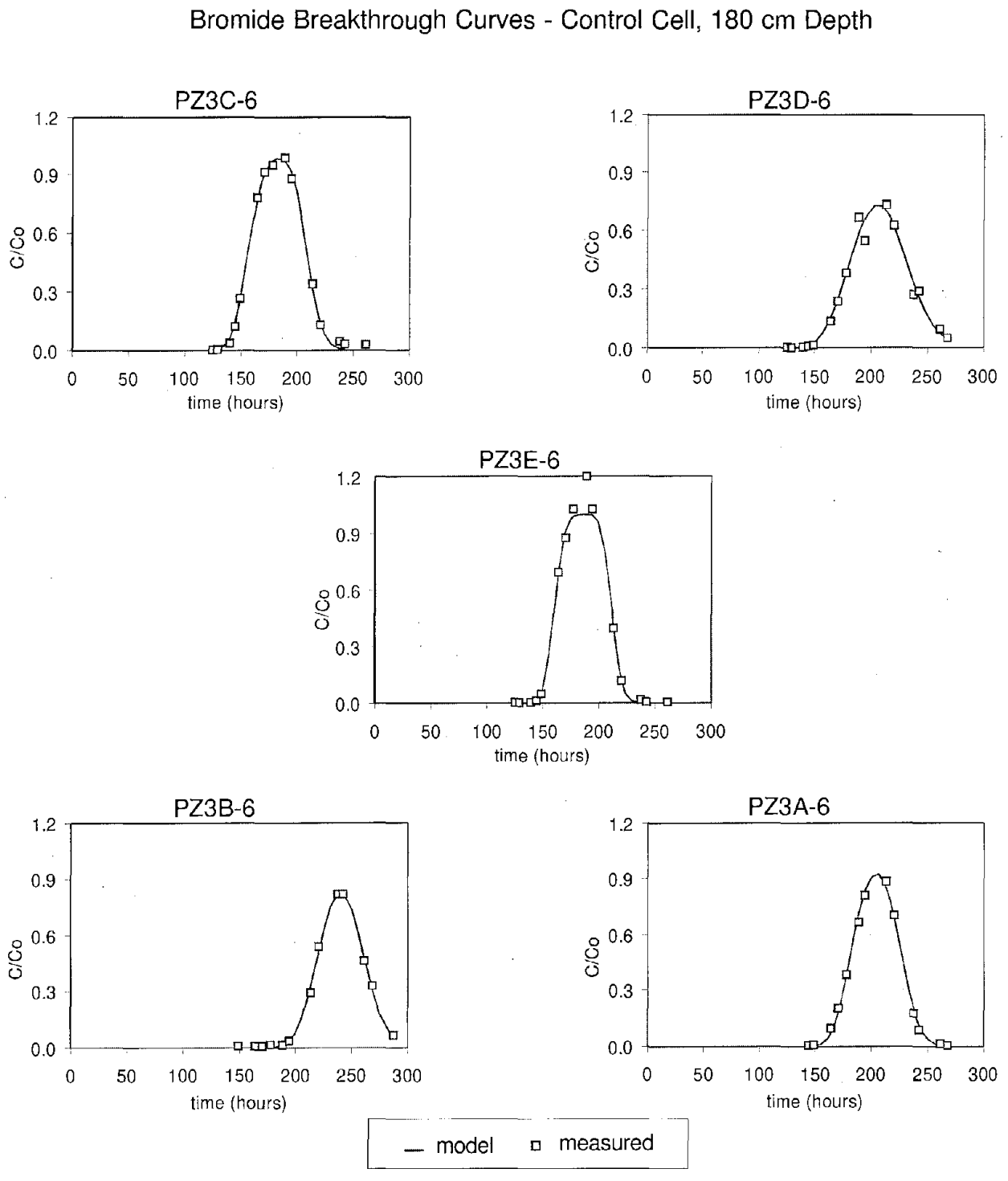

FIGURE B-8 Measured and calculated bromide breakthrough curves at 180-cm bgs ports in the Control Cell.

Table B-2. *Bromide Tracer Test Results. Velocities and Dispersivities Calculated by Fitting Wpulsepe to Bromide Breakthrough Data.*

Location	Vertical Distance[1] (cm)	Velocity (cm/hr)	Dispersivity (cm)
Control Cell			
PZ3A-4	70	1.08	0.17
PZ3B-4	70	0.97	0.31
PZ3C-4	70	1.23	0.35
PZ3D-4	70	1.12	0.33
PZ3E-4	70	0.95	0.17
PZ3A-6	130	0.72	0.32
PZ3B-6	130	0.59	0.30
PZ3C-6	130	0.84	0.32
PZ3D-6	130	0.71	0.81
PZ3E-6	130	0.81	0.13
Extraction Well	175[2]	1.29	13.45
MEAN[3](s.d.)		0.90 (0.2)	0.32 (0.19)
Nitrate Cell			
PZ4A-4	70	0.66	0.46
PZ4B-4	70	0.97	0.08
PZ4C-4	70	0.77	0.06
PZ4D-4	70	1.00	0.17
PZ4A-6	130	0.45	0.75
PZ4B-6	130	0.80	0.11
PZ4C-6	130	0.63	2.13
PZ4D-6	130	0.60	1.08
PZ4E-6	130	1.13	0.26
Extraction Well	175[2]	1.21	5.26
MEAN[3](s.d.)		0.78 (0.22)	0.57 (0.68)

[1] Vertical distances calculated assuming upper head at 50 cm bgs during test.
[2] Distance to center of well screen.
[3] Mean velocities and dispersivities do not include extraction well values.

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APPENDIX C. ANALYTICAL METHODS

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C.1 Sampling Procedures and Analytical Methods - Laboratory Samples

Aromatic Hydrocarbons. For microcosms with mininert valves, a 400 µL sample of the headspace gas was collected with a syringe and injected directly onto a Shimadzu GC-9a gas chromatograph equipped with a flame ionization detector and 60 m Supelcowax-10 capillary column. Each sample was injected manually with the aid of a sample loop. The GC was run isothermally at a column temperature of 105°C, and an injector temperature of 200°C. The method detection limit (MDL) for benzene and toluene was 0.001 mg/L headspace; the MDLs for the other aromatics were not determined, but were expected to be of similar magnitude.

For hypovial microcosms, vials were decrimped and aliquots of liquid quickly transferred with a glass syringe to either 22-ml autosampler vials or 18-ml hypovials, which were then crimp sealed, and analyzed immediately. Samples were collected outside the anaerobic chamber. Either the pentane microextraction technique (described in Section C.2) or headspace GC-PID was used to quantify the aromatic hydrocarbons, depending on the experiment. GC-PID was used for microcosm Experiment 3 (Section 4.1.3), and the pentane microextraction technique for Experiments 4,5, and 6 (Sections 4.1.4, 4.1.5, and 4.2). The method of analysis was not changed within a given experiment.

For headspace GC-PID, 8 ml of fluid was removed from the 22-ml autosampler vials by glass syringe just before analysis. Vials were then resealed and placed in an autosampler. Analysis was conducted on a Hewlett Packard 5890 gas chromatograph equipped with a split injection port, a Varian Genesis headspace autosampler (platen temperature of 75°C), and a photoionization detector (10.2 eV). A 30m x 0.32mm Stabilwax column (0.5 µm film thickness) was used, operating at 65°C under isothermal conditions. Injector and detector temperatures were 150°C, and helium was used as the carrier (3.5 ml/min) and makeup (30 ml/min) gas. Calibration was by the external standard method, using calibration standards prepared by spiking methanolic stocks into organic-free water contained in the same autosampler vials. The MDLs for BTEX compounds ranged from 2-15 µg/L, as determined by the U.S. EPA method (Longbottom and Lichtenberg, 1982). During analyses, check standards were run approximately every ten samples.

Dissolved Oxygen. In the laboratory, the dissolved O_2 concentration in microcosm liquid was determined immediately after organic sample collection. Measurements were typically made within 30 seconds of opening a microcosm, and care was taken to minimize the disturbance of the liquid. Dissolved O_2 determinations were made with either the azide-modified Winkler titration method (APHA, 1985), or a D.O. meter (Microelectrodes, Inc., Model MI-730), depending on the experiment. The analytical method was not changed within a given experiment.

For the Winkler method, 18-ml glass vials were flushed with argon, filled with microcosm liquid, and sealed with a Teflon™-lined septum and aluminum crimp seal. Winkler reagents were then injected directly into the vial using a syringe. The solution in the vial was then titrated with a standardized 0.0025 N sodium thiosulfate solution to determine the dissolved O_2 concentration. The MDL for this sample size was 0.22 mg/L. D.O. values determined with the Winkler method were corrected for the O_2 added to samples as a result of reagent addition. It was observed that the sodium azide used to inhibit microbial activity in control microcosms may have interfered with the analysis, resulting in D.O. concentrations that appeared to be anomalous. Consequently, the D.O. meter was used in the follow-up microcosm study (Experiment 6) to obtain data on initial dissolved O_2 concentrations and abiotic losses in the absence of microbial activity (i.e., sterile controls).

To obtain sample concentrations with the dissolved-O_2 meter a daily two-point calibration (0 to 21% dissolved O_2) was used. The instrument was zeroed with a 2% (w/v) sodium sulfite solution. Measurements were made by inserting the probe directly into the microcosm liquid. To minimize diffusion of atmospheric O_2 into the sample, measurements were made under a stream of argon gas. Although steps were taken to minimize contamination with atmospheric O_2 during this sampling procedure, it could not be determined whether the low concentrations of dissolved O_2 that were typically observed after lengthy incubations in the anaerobic chamber (Chapter 4) resulted from positive sampling bias or were representative of microcosm liquid.

Headspace Oxygen. The oxygen content of the air-filled headspace of certain microcosms was measured using a Fisher/Hamilton gas partitioner (Model 29) equipped with columns of 30% di-2-ethylhexyl-sebacate on 60-80 mesh Chromosorb-P column and molecular sieve 13X in series, and a thermal conductivity detector. Sample was introduced on-column via a sample loop, and analysis was conducted at room temperature, using helium (20 ml/min) as the carrier gas. Calibration was by the external standard method, using commercially-obtained, certified gas mixtures; chromatogram peak heights of standards and samples were compared to determine unknown concentrations.

Nitrate and Nitrite. After collecting samples for organics and dissolved O_2, samples for NO_3^- and NO_2^- analysis were obtained by transferring an additional 15 ml of liquid to 18-ml plastic scintillation vials. These samples were preserved with 30 µL concentrated sulfuric acid and refrigerated until analysis. Nitrate and NO_2^- were determined colorimetrically with the automated cadmium reduction method, using a Technicon Autoanalyzer equipped with a 15 mm tubular flow cell and 550 nm filters. Before analysis, samples were diluted as required, and the pH adjusted with ammonium hydroxide to between 7 and 9. The MDLs were 0.2 mg/L and 0.26 mg/L for NO_2^- and NO_3^-, respectively.

Nitrous Oxide. In design 1 microcosms that received acetylene, N_2O accumulation was measured using a GOW-MAC Series 350 GC equipped with a Thermal Conductivity Detector and a 1.8m, 100-120 mesh Poropak Q column. Samples were run isothermally at 40°C with a helium carrier (17 ml/min). Fifteen ml groundwater samples were collected from microcosms in 30 ml glass syringes. To obtain a gas-phase sample for analysis, syringes received 13-ml helium, and were sealed, shaken 100 times, and allowed to equilibrate for at least 2 hours. A sample loop was then used to inject a 2-ml gas sample onto the GC. Triplicate standards (0.1% and 0.5% N_2O in helium) were run to develop a two-point calibration curve. Partitioning theory was used to calculate the concentration of N_2O in the aqueous phase. The MDL was 0.45 mg/L dissolved N_2O. Acetylene was not quantified, but its presence was confirmed by reviewing chromatograms.

C.2 Analytical Methods - Field Samples

Aromatic Hydrocarbons. Concentrations of aromatic hydrocarbons in all groundwater samples collected in the field (as well as three laboratory experiments) were determined with a pentane micro-extraction procedure. Samples were first extracted by adding 1 ml of pentane containing an internal standard (*m*-fluorotoluene) to 16.5 ml of groundwater and agitating for twenty minutes. Approximately 0.8 ml pentane was then transferred to an autosampler vial for analysis. Samples were run on a Hewlett Packard 5890 GC equipped with an HP7673A autosampler and a 30m x 0.25mm I.D. DB-5 column. The oven temperature program was 35°C for 1 min increasing to 165°C at 13°C/min with a 4 min final hold. The injector and detector temperatures were 200°C and 250°C, respectively. A calibration curve based on three standards was prepared for each sample run, and check standards were run approximately every ten samples. Standards were prepared by weighing known amounts of neat compounds into a known weight of methanol, and then diluting the stock solution into water to obtain a concentration range that bounded expected sample concentrations. Standards were analyzed in triplicate. Results for *m*-xylene and *p*-xylene were reported as a sum because these two compounds co-eluted on this column. The MDLs were 19 µg/L (benzene), 13 µg/L (toluene), 8 µg/L (ethylbenzene, and *o*-xylene), 9 µg/L (m+p xylene, 135-trimethylbenzene, and 124-trimethylbenzene), 6 µg/L (123-trimethylbenzene), and 120 µg/L (naphthalene).

Concentrations of aromatic hydrocarbons in core-extract samples were determined by a direct injection of the extractant onto the GC. Samples were run on a Hewlett Packard HP 5890 GC equipped with an HP7673A autosampler, and a 30m x 0.25mm I.D. DB-5 column. A 3 µL on-column injection of

methanol was performed. The oven temperature program was as follows: 35°C for 5 min increasing to 150°C at 10°C/min with a 5 min final hold. The injector and detector temperatures were 200°C and 250°C, respectively. As for aqueous samples, a calibration curve based on three standards was prepared for each sample run, and check standards were run approximately every ten samples. Standards were prepared by weighing known amounts of neat compounds into a known weight of methanol. The standards were then further diluted in methanol to reach the appropriate concentration range, and analyzed in triplicate. The mass of aquifer material and methanol in each sample was used to express concentrations in mg/ml methanol on a wet-weight basis (mg/kg). The average method detection limits, which varied with methanol and sample mass, were as follows: 2.6 mg/kg for benzene, toluene, ethylbenzene, and *p*+*m*-xylene; 1.3 mg/kg for *o*-xylene and trimethylbenzene isomers; and 3.9 mg/kg for naphthalene.

To measure the concentrations of aromatic hydrocarbons in API 91-01 gasoline, samples were prepared by weighing 15 ml of methanol into pre-weighed 40-ml glass vials with Teflon-faced septa. Approximately 0.5 ml of gasoline was then weighed into the methanol and mixed. Aliquots were then transferred to autosampler vials for analysis. Standards were prepared as described in the previous paragraph. Concentrations were expressed as g analyte/g gasoline or weight percent.

Inorganic Parameters. Inorganic parameters were analyzed by the Water Quality Laboratory at the University of Waterloo. Anions such as SO_4^{2-}, Br^-, NO_3^-, and NO_2^- were analyzed using a Dionex System 2000 Ion Chromatograph equipped with a Dionex AS4A anion exchange column. A daily run of 20 to 50 samples contained 10 to 20 in-house standards. A commercially-prepared standard was run along with in-house standards to maintain standard quality. Samples were reanalyzed if the commercial standard did not come within five percent of its stated value. The method detection limit for all compounds was 0.05 mg/L.

Because the highly-contaminated water samples collected in this study appeared to be damaging the anion exchange column, most of the NO_3^- and NO_2^- samples collected after the first month of the flushing experiment were analyzed colorimetrically using the automated cadmium reduction method. Samples were run on an Alpkem Perstorp Analytical Environmental Flow Solution system. In-house standards were run for calibration and quality control as described above. The method detection limits were 0.4 mg/L (NO_3^-) and 0.2 mg/L (NO_2^-). To verify that these two analytical methods provided consistent results, several batches of NO_3^- samples were analyzed by both methods.

Iron was analyzed on a Varian Model 1475 Atomic Absorption Spectrophotometer. All samples are run in duplicate. As above, samples are run with in-house standards and a commercially-prepared standard. Commercial standards were analyzed every five samples to monitor instrument drift. The method detection limit was 0.05 mg/L.

Dissolved Methane. Water samples for CH_4 analysis were analyzed on a Hewlett Packard 5840A GC equipped with a flame ionization detector and 30 m megabore GS-Q column. Analyses were run isothermally at 100°C with a helium carrier gas (12 ml/min). The detector and injector temperatures were 200°C and 100°C, respectively. To prepare samples for analysis, 15-ml aliquots of groundwater were withdrawn from the sample bottle into a 30-ml glass syringe. An additional 13 ml of helium was added, and the syringe was shaken and allowed to equilibrate for 3 hr. A 5 ml sample of the gas phase was then injected via a 2-ml sample loop. The GC was calibrated in an external standard mode using several concentrations of a commercial gas mixture (Praxair). Henry's Law, the Ideal Gas Law and CH_4 solubility were then used to calculate concentrations in the aqueous phase. The MDL for dissolved CH_4 was 0.1 µg/L.

Metabolites. Descriptions of these analytical methods are provided elsewhere (Barcelona et al., 1995 (NCIBRD Laboratory); Hutchins et al., 1998 (NRMRL)).

APPENDIX D: AQUIFER MONITORING RESULTS

Table D-1. *Environmental Monitoring Downgradient of Wastewater Treatment Mound and Treatment Cells. All Concentations in mg/L.*

	Sample I.D.	Date	Depth (m bgs)	Ben	Tol	Eben	m+p-Xyl	o-Xyl	1,3,5-TMB	1,2,4-TMB	1,2,3-TMB	Naph
Wastewater Treatment												
Mound												
Biopile Well	E-640	10/2/96	0.4 - 1.9	n.d.[1]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	E-641	10/16/96	0.4 - 1.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	E-821	5/27/98	0.4 - 1.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Bundle Piezometer												
BP5-1	E-795	7/9/97	0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP5-1	E-822	5/27/98	0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP5-2	E-796	7/9/97	1.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP5-2	E-823	5/27/98	1.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP5-3	E-797	7/9/97	1.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP5-3	E-824	5/27/98	1.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Treatment Cells												
Bundle Piezometers												
BP1-1	E-635	10/2/96	2.9	n.d.	0.010	n.d.	0.010	n.d.	n.d.	n.d.	n.d.	n.d.
BP1-2	E-636	10/2/96	3.2	n.d.	n.d.	n.d.	0.007	n.d.	n.d.	n.d.	n.d.	n.d.
BP1-3	E-637	10/2/96	3.5	n.d.	n.d.	n.d.	0.007	n.d.	n.d.	n.d.	n.d.	n.d.
BP1-4	E-638	10/2/96	3.8	n.d.	n.d.	n.d.	0.011	n.d.	n.d.	n.d.	n.d.	n.d.
BP1-5	E-639	10/2/96	4.1	n.d.	n.d.	n.d.	0.008	n.d.	n.d.	n.d.	n.d.	n.d.
BP2-1	E-629	10/2/96	2.4	n.d.	0.016	n.d.	0.015	n.d.	n.d.	n.d.	n.d.	n.d.
BP2-2	E-630	10/2/96	2.7	n.d.	0.012	n.d.	0.012	n.d.	n.d.	n.d.	n.d.	n.d.
BP2-3	E-631	10/2/96	3.0	n.d.	0.010	n.d.	0.011	n.d.	n.d.	n.d.	n.d.	n.d.
BP2-4	E-632	10/2/96	3.3	n.d.	0.013	0.016	0.05	0.016	0.013	0.046	0.013	0.074
BP2-4	E-642	10/16/96	3.3	n.d.	0.014	0.009	0.033	0.010	0.009	0.032	0.009	0.026
BP2-5	E-633	10/2/96	3.6	n.d.	0.014	0.010	0.030	0.010	0.015	0.053	0.014	0.027
BP2-6	E-634	10/2/96	3.9	n.d.	0.011	n.d.	0.011	n.d.	n.d.	n.d.	n.d.	n.d.
BP3-4	E-371	7/2/96	3.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP3-4	E-627	10/2/96	3.6	n.d.	0.027	0.008	0.022	0.006	n.d.	n.d.	n.d.	n.d.
BP3-4	E-793	7/9/97	3.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP3-4	E-827	5/27/98	3.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP3-5	E-372	7/2/96	3.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP3-5	E-628	10/2/96	3.9	n.d.	0.024	0.007	0.019	n.d.	n.d.	n.d.	n.d.	n.d.
BP3-5	E-794	7/9/97	3.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP3-5	E-828	5/27/98	3.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP4-5	E-373	7/2/96	3.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP4-5	E-625	10/2/96	3.7	n.d.	0.06	0.015	0.039	0.013	n.d.	0.007	n.d.	n.d.
BP4-5	E-642	10/16/96	3.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP4-5	E-791	7/9/97	3.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP4-5	E-825	5/27/98	3.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP4-6	E-374	7/2/96	4.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP4-6	E-626	10/2/96	4.0	n.d.	0.026	n.d.	0.02	n.d.	n.d.	n.d.	n.d.	n.d.
BP4-6	E-792	7/9/97	4.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BP4-6	E826	5/27/98	4.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[1]n.d. - Not detected. Detection limits provided in Appendix C.

TECHNICAL REPORT DATA

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16. ABSTRACT

This project represents a cooperative effort between the University of Waterloo and the U.S. Environmental Protection Agency. This report summarizes research conducted using both laboratory batch microcosms and field-scale sheet-piling cells to evaluate whether bioremediation of monoaromatic fuel hydrocarbons can be enhanced using mixed rather than single electron acceptors. The studies focused on nitrate for anaerobic bioremediation and oxygen for aerobic bioremediation, and experiments were designed to test the hypothesis that low levels of oxygen may enhance biodegradation of more recalcitrant compounds (such as benzene) under denitrifying conditions. The findings from this project are directly applicable to the field-scale remediation of subsurface environments contaminated by petroleum hydrocarbons.

17. KEY WORDS AND DOCUMENT ANALYSIS

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