Effect of Ethanol on BTEX Biodegradation in Aerobic Aquifer Systems

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Ethanol can affect the biodegradation of gasoline hydrocarbons in groundwater. High concentrations of ethanol can be toxic to subsurface microorganisms that are otherwise capable of degrading hydrocarbons, such as benzene, toluene, ethylbenzene and xylenes (BTEX). At lower concentrations, ethanol may hinder BTEX degradation through substrate competition and the depletion of inorganic nutrients (e.g., nitrogen and phosphorus), oxygen and other electron acceptors needed for BTEX degradation. A series of laboratory experiments were designed to study the effect of ethanol on aquifer microorganisms and on aerobic BTEX biodegradation.

A microcosm experiment was conducted to investigate the effect of ethanol on the biodegradation of BTEX. Microcosms were set up with Borden aquifer material and groundwater in which oxygen and nutrients were not limited. These microcosms contained BTEX in combination with a range of ethanol concentrations. Under these favourable conditions, the presence of ethanol up to concentrations of 1.9% (v/v) (equivalent to 15000 mg/L) caused little inhibition of BTEX degradation.

Further experiments were conducted to study the antimicrobial effects of higher concentrations of ethanol. Following exposure to ethanol concentrations of 25% (v/v) or higher, microbial activity and survival was significantly diminished. Results suggest that a high concentration ethanol slug will have a major impact on the microbial community but that there would likely be potential for recovery.

The recovery potential was examined further in laboratory column experiments designed to simulate a dynamic field situation where a high ethanol pulse is followed by a BTEX plume. These column experiments were conducted with Borden aquifer material and groundwater under aerobic conditions. The concentration of the ethanol pulse was 25% (v/v), which was expected to significantly alter the microbial population without destroying it. Following the ethanol exposure, groundwater and BTEX were allowed to flow through one column to simulate the reinoculation of microorganisms from upgradient groundwater advecting into the contaminated zone. The other column was fed with sterile groundwater and BTEX to evaluate the regeneration of within-column microorganisms that survived the ethanol exposure. Recovery in both columns was rapid. Unfortunately, during

the recovery phase, sterility of the influent groundwater could not be maintained. As a result, recovery by regeneration could not be evaluated. Nonetheless, it is evident that recovery in terms of aerobic BTEX biodegradation was significant under the conditions of the column experiment.

Ethanol did not appear to pose a long-term impact on BTEX biodegradation when oxygen and nutrients were in excess. In field situations, nutrients and electron acceptors may be limited; however, ethanol toxicity is not likely to cause a prolonged inhibition of BTEX biodegradation.

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Chapter 1
Introduction

1.1 Background

1.1.1 Ethanol as a Fuel Oxygenate

Ethanol is added to gasoline as an oxygenate to reduce air pollution. The oxygen content of ethanol improves the octane rating of gasoline, resulting in cleaner, more efficient fuel usage (Rice, 1999). The Government of Ontario has regulated the use of ethanol as a fuel oxygenate (Ontario Ministry of the Environment, 2006). The mandate called for gasoline sold in Ontario to contain an annual average of at least 5% ethanol as of January 2007. Standard vehicles can operate under warranty on gasoline with up to 10% ethanol (American Coalition for Ethanol, 2007). In December 2006, Environment Canada announced that it would develop regulations that would require an annual average renewable content of 5% in gasoline by 2010 and ethanol will be among the options under consideration (Canada Gazette, 2006). Gasoline with up to 10% ethanol (E10) is widely used in the U.S., and the manufacturing of flexible fuel vehicles (vehicles that can run on ethanol, gasoline or any mixture of the two) has prompted an increase in the availability of E85 (gasoline containing up to 85% ethanol) (Renewable Fuels Association, 2007).

The increased use of ethanol as a fuel additive means that there is a greater likelihood for ethanol to be associated with gasoline contaminated sites. Low volume releases of E10 and E85 to the subsurface can occur from leaking underground storage tanks at gasoline service stations. High volume releases may occur as spills of denatured ethanol during transportation (typically by rail, but also by truck) and from above ground storage tanks. Spills of denatured ethanol at distribution facilities are likely to impact soils that are already contaminated with gasoline (Rice et al., 1999).

The monoaromatic hydrocarbons benzene, toluene, ethylbenzene and the three isomers of xylene (*p*-xylene, *m*-xylene, *o*-xylene) (altogether termed BTEX) are major components of gasoline. For example, the gasoline used in this study contains approximately 22 wt% of

BTEX (Appendix B). All of the BTEX compounds are of concern as groundwater contaminants due to their high aqueous solubility compared to other fuel components and their toxicity, particularly benzene, which is known to be a human carcinogen (Boelsterli, 2003). Some physical and chemical properties of ethanol and BTEX compounds are listed in Table 1.1.

Table 1.1 Key physical and chemical properties of ethanol and BTEX compounds (adapted from Diaz and Drogos, 2002).

Property	**EtOH**	**B**	**T**	**E**	***p*-X**	***m*-X**	***o*-X**
Chemical Formula	C_2H_5OH	C_6H_6	C_7H_8	C_8H_{10}	C_8H_{10}	C_8H_{10}	C_8H_{10}
Molecular Weight	46.07	78.11	92.14	106.17	106.17	106.17	106.17
Liquid Density (g/cm^3 @ 20°C)	0.789	0.88	0.87	0.87	0.86	0.88	0.88
Water Solubility (mg/L)	Miscible	1780–1791	535	161	156	146	175
Vapor Pressure (mm Hg @ 25°C)	44–56.5	76–95.19	28.4	9.5	8.7	8.3	6.6
log Koc (@ 25°C)	0.2–1.21	1.1–2.5	1.56–2.25	1.98–3.04	2.05–3.08	2.04–3.15	1.68–1.83
log Kow (@ 25°C)	(-0.31)–(-0.16)	1.56–2.15	2.11–2.80	2.68–3.26	3.08–3.29	3.09–3.37	2.77–3.12
Henry's Law Constant (dimensionless) (@ 25°C)	0.00021–0.000257	0.2219	0.2428	0.345	0.3139	0.3139	0.2084

Aside from anthropogenic sources of contamination, ethanol and BTEX have a natural origin. Thus, native soil microorganisms capable of metabolizing ethanol and/or BTEX are ubiquitous (Atlas and Philp, 2005). Under the appropriate conditions, both ethanol and BTEX can be easily degraded in aerobic and anaerobic environments (Malcolm Pirnie, 1998). Ethanol is oxidized aerobically through the tricarboxylic acid (TCA) cycle, producing several metabolic intermediates. Alcohol dehydrogenase and acetaldehyde dehydrogenase enzymes are involved in the oxidation of ethanol to acetyl aldehyde and acetyl-CoA. Acetyl-CoA is oxidized to the final product, CO_2 (Alvarez and Hunt, 2002). Anaerobic ethanol biodegradation yields products such as organic acids, alcohols, CO_2, CH_4, and H_2 gas (Alvarez and Hunt, 2002). The metabolic intermediates and products of aerobic and anaerobic ethanol biodegradation are non-toxic (Alvarez and Hunt, 2002).

The biodegradation and transport of BTEX in groundwater has been studied extensively and is considerably well understood (Barker et al., 1987; Lovely, 1997; Salanitro, 1993). However, much less is known about the effect of ethanol on the biodegradation of BTEX and further research is needed to gain a better understanding of the impact of ethanol-amended gasoline on groundwater quality. Potential effects of ethanol on BTEX biodegradation that have been discussed previously in the literature relate to toxicity to aquifer microorganisms, preferential ethanol degradation (and the resulting depletion of nutrients and electron acceptors), and alterations of the microbial community makeup. These interactions are discussed below. Possible implications of these impacts are reduced natural attenuation of gasoline contamination and longer BTEX plumes. These effects have been observed in the field for benzene. Mackay et al. (2006) studied benzene, toluene and *o*-xylene (BT*o*X) and found a 95% reduction in the rates of ethanol-impacted B and *o*-X degradation compared to the ethanol-free case. The impact of ethanol on toluene was less substantial (only 50% reduction). A statistical analysis was performed by Ruiz-Aguilar et al. (2003) to compare benzene and toluene plume lengths for ethanol-free and ethanol amended gasoline contaminated sites. Benzene plumes with ethanol were significantly longer than those without ethanol, whereas toluene plume lengths with and without ethanol were not statistically different.

1.1.1.1 Toxicity of Ethanol

Ethanol is well documented as a disinfectant that is most effective at concentrations between 60% and 90% by volume (Ali et al., 1991). In general, ethanol concentrations of 10-15% are considered toxic to most vegetative microorganisms, resulting in immediate inactivation, and growth inhibition is reported for 1-10% ethanol (Ingram and Buttke, 1984). However, research on the toxic effects of ethanol on aquifer microorganisms is limited. Concentrations higher than 40000 mg/L (~5.1% v/v) were reported to be toxic in aquifer microcosms, as indicated by complete lack of oxygen consumption (Hunt et al., 1997). In another aquifer microcosm study, microbial growth and activity was not detected for ethanol concentrations greater than 5% (v/v) (Araújo, 2000). Based on these findings, ethanol toxicity may be an issue for large volume spills of denatured ethanol.

1.1.1.2 Substrate Competition

Ethanol can be degraded by microbial enzymes that are constitutive (i.e., enzymes that are always synthesized regardless of environmental conditions). However, the enzymes responsible for BTEX degradation are generally inducible (i.e., synthesis is stimulated by the presence of some substrate) (Alvarez and Hunt, 2002). The presence of an easily degradable substrate such as ethanol may suppress the synthesis of enzymes required for BTEX degradation (Alexander, 1994). This diauxic effect has the potential to inhibit BTEX biodegradation at sites contaminated with ethanol amended gasoline. Alternatively, microbial growth during ethanol degradation may result in an increase in the number of microorganisms capable of degrading BTEX. However, the preferential biodegradation of ethanol is likely to consume nutrients and electron acceptors that are needed for BTEX degradation.

During the biodegradation of ethanol and BTEX, microorganisms utilize electron acceptors to oxidize the contaminants. Electron acceptor utilization occurs sequentially according to oxidation potential, beginning with oxygen, the most energetically favourable. Under anaerobic conditions, electron acceptors are utilized in the following order: nitrate, ferric iron, sulfate and carbon dioxide (Stumm and Morgan, 1996). Ethanol has a relatively high biological oxygen demand and preferential degradation of ethanol may result in oxygen depletion in aerobic systems. Ethanol degradation will also deplete electron acceptors in anaerobic systems. A possible consequence of the preferential consumption of electron acceptors during ethanol degradation is a deficiency in the supply required for BTEX degradation.

Microorganisms use carbon (C) and inorganic nutrients such as nitrogen (N) and phosphorus (P) in certain ratios depending on the C:N:P ratio in their biomass. The C:N:P ratio of most soil bacteria is roughly 31:5:1 (Paul and Clark, 1996). Nitrogen and phosphorus are rarely limiting in uncontaminated aquifers. However, contamination by an organic compound such as ethanol or BTEX significantly increases the carbon content and is likely to result in inorganic nutrient limitation (Alexander, 1994). Preferential ethanol degradation could exhaust the nutrient supply of an aquifer environment.

Several laboratory studies have demonstrated that ethanol may impede the biodegradation of BTEX (Corseuil et al., 1998; Da Silva and Alvarez, 2002; Hunt et al., 1997; Lenczewski et al., 2007). Reports of preferential ethanol degradation, resulting in a lag in BTEX biodegradation until ethanol disappeared (Corseuil et al, 1998; Deeb et al., 2002; Hunt et al., 1997), suggest that BTEX could persist in the subsurface without significant biodegradation until most of the ethanol is removed.

1.1.1.3 Microbial Community Structure

It is unknown whether preferential biodegradation of ethanol will exert a selective pressure on aquifer microbial communities. It is conceivable that ethanol may shift microbial communities away from BTEX degraders and towards ethanol degraders. Alternatively, ethanol may stimulate the growth of populations capable of BTEX degradation.

Although many studies of microbial community profiles during petroleum hydrocarbon contamination and remediation have been conducted (Greene and Voordouw, 2004; Juck et al., 2000; MacNaughton et al., 1999), studies of microbial communities involved in biodegradation of ethanol-amended gasoline are sparse. Feris et al. (2004) reported that the microbial communities in microcosms containing ethanol and BTEX were significantly different from those inhabiting BTEX only microcosms. This shift in populations was rapid and sustained, suggesting that ethanol may have lasting effects on the local microbial community structure.

Though few studies have been done, advances in molecular biology techniques enable researchers to observe the influence of ethanol and ethanol amended gasoline on microbial community structure. DNA-based molecular tools for analyzing microbial communities are now widely available. Community profiles and perturbations can be monitored using methods such as denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP) analysis.

1.1.2 Field Studies at CFB Borden

Field experiments involving oxygenated gasoline have been conducted by other researchers in the unconfined sandy aquifer at Canadian Forces Base (CFB) Borden, located

approximately 100 km NW of Toronto. The aquifer consists of clean, well-sorted, fine- to medium-grained sand with low organic carbon and clay contents (Mackay et al., 1986). Some general hydrogeological properties of the aquifer material and general groundwater characteristics are listed in Table 1.2. The depth of the aquifer is 9 m (Mackay et al., 1986) and the water table varies seasonally from 0.5 m to 1.65 m below ground surface (Baker, 2002).

Table 1.2 General hydrogeological properties of Borden aquifer material and characteristics of Borden groundwater (adapted from Mackay et al., 1986).

Hydrogeological Properties	
Bulk density (ρ_b)	1.81 g/cm^3
Solids density (ρ_s)	2.71 g/cm^3
Porosity (η)	0.33
Organic carbon content (f_{oc})	0.02%
Groundwater Characteristics	
Ca^{2+}	50-100 mg/L
Mg^{2+}	2.4-6.1 mg/L
Na^{+}	0.9-2.0 mg/L
K^{+}	0.1-1.2 mg/L
Alkalinity (as $CaCO_3$)	100-250 mg/L
Cl^{-}	1-3 mg/L
SO_4^{2-}	10-30 mg/L
NO_3^{-}	<0.6 mg/L
TDS	380-500 mg/L
DOC	<0.7 mg/L
DO	0-8.5 mg/L
Temperature	6-15 °C
pH	7.3-7.9

Experiments conducted by Hubbard et al. (1994) involved methanol rather than ethanol, but it is assumed here that they behave similarly. Methanol (average concentration of 7000 mg/L) apparently enhanced the persistence of B, E and *p*-X. It was believed that competition for electron acceptors was the likely cause of this enhancement of BTEX persistence due to methanol. Mocanu (2007) emplaced residual sources of ethanol amended gasolines with 10% ethanol (E10) or 95% ethanol (E95), and ethanol-free gasoline below the water table in the Borden aquifer, and then monitored the BTX plumes emanating from the three sources. He found that benzene and toluene were more persistent in the E95 plume (where ethanol concentrations up to 12800 mg/L were noted), suggesting ethanol limited their biodegradation. Further studies are planned to simulate a spill of E95 to a site

previously contaminated by gasoline (Freitas, in prog.). The effects of ethanol on the gasoline source zone and contaminant transport in the unsaturated zone, capillary fringe and dissolved plumes will be evaluated. Laboratory experiments to supplement the intended field research are presented in this thesis, and focus on the effects of ethanol on the biodegradation of BTEX in static microcosm and dynamic column experiments with CFB Borden aquifer material and groundwater.

1.2 Objectives

The purpose of this research was to gain further insight into the effect of ethanol on aquifer microorganisms and on BTEX biodegradation in an aquifer environment. Three major laboratory investigations were conducted and the objectives were as follows:

1) To determine if the presence of ethanol influences the biodegradation of BTEX. Static aerobic microcosm experiments were conducted with pristine aquifer material and groundwater from CFB Borden. This investigation is outlined in Chapter 2.

2) To determine the toxic effect of higher concentrations of ethanol on the microbial community. Static inhibition experiments were conducted in which the activity and growth of aquifer microorganisms were measured following exposure to ethanol concentrations greater than 25% (v/v). These experiments are presented in Chapter 3.

3) To determine the effect of ethanol on an aquifer microbial community and its ability to degrade BTEX following ethanol exposure, and to investigate the recovery process of the microbial community following exposure to an inhibitory ethanol concentration. In dynamic column experiments, the recovery of aquifer populations in terms of BTEX degradation was observed following exposure to 25% (v/v) ethanol. The microbial community was analyzed before and after the ethanol exposure and after the recovery of BTEX degradation. This experiment is outlined in Chapter 4.

Finally, implications of this research are presented in Chapter 5.

Chapter 2
Microcosm Experiment

2.1 Objective and Approach

A laboratory experiment was conducted to investigate the effect of ethanol on the biodegradation of BTEX. The objective of the experiment was to determine if the presence of ethanol influences BTEX biodegradation.

The microcosm approach was selected as a laboratory model of the aquifer environment. The microcosms of this experiment were sealed bottles containing aquifer material and groundwater. Although it is impossible to replicate field conditions within a closed system, microcosms can be used to examine trends and processes analogous to those in the field (Krimsky et al., 1995). The controlled environment permits the manipulation of experimental conditions.

In this experiment, conditions favourable to biotransformation of the test compounds were selected, including a temperature of 22 °C, plenty of inorganic nutrients and excess oxygen. Favourable conditions were chosen to simplify the study of substrate interaction and biodegradation processes without the interference of nutrient and oxygen limitations. Other microcosm studies conducted with ethanol (or methanol) and BTEX involved no nutrient amendment (Hubbard, 1992), unlimited oxygen (Deeb et al., 2002; Hubbard, 1992; Hunt et al., 1997), oxygen-limited conditions (Araújo, 2000; Hubbard, 1992; Hunt et al., 1997), and anaerobic conditions (Chen et al., in press; Corseuil et al., 1998), and these provided additional insight into nutrient and oxygen limitation impacts.

Microcosms were treated with various combinations of ethanol and BTEX, and the biotransformation of these test compounds was measured by their disappearance. Sterilized and poisoned treatments served as abiotic controls.

Throughout this thesis, the term “active” refers to the presence of microorganisms and the term “sterile” refers to a lack of living organisms. The terms are generally used to describe the condition of aquifer material or groundwater in the experiments. The term “amended” is used to describe the addition of a substance.

2.2 Methods and Materials

2.2.1 Microcosm Design and Setup

Microcosms were treated with BTEX in combination with a range of ethanol concentrations to investigate the effect of ethanol on BTEX biodegradation. A summary of the experimental treatments is listed in Table 2.1. Initial aqueous ethanol concentrations were 500 mg/L, 5000 mg/L and 15000 mg/L. When BTEX was added, the nominal aqueous concentration was 15 mg/L. Each treatment was prepared in duplicate. Sterile controls were prepared (also in duplicate) for each treatment to consider loss of compounds due to abiotic processes. Therefore, each treatment series consisted of four bottles – two active and two sterile.

The ethanol concentration of 500 mg/L was chosen because, based on previous studies (Corseuil et al., 1998; Hunt et al., 1997), it was expected that all the ethanol would degrade before BTEX degradation would begin. 5000 mg/L and 15000 mg/L are close to the maximum concentrations reported at monitoring fences 15.5 m and 3.5 m downgradient of an E95 source during the field experiment conducted at Borden by Mocanu (2007).

Table 2.1 Summary of experimental treatments for the microcosm study.

Treatment Series	Ethanol Concentration (mg/L)	BTEX Concentration (mg/L)
Series A	500	---
Series B	5000	---
Series C	15000	---
Series D	---	15
Series E	500	15
Series F	5000	15
Series G	15000	15

Cores of pristine aquifer material for use in the laboratory experiments were collected from the Sand Pit area at CFB Borden in June 2005 using 2 cm diameter by 1.5 m long aluminum tubes. Tube ends were covered with aluminum foil before plastic end caps were added to seal in the core material. Cores were collected over the 1.5 m to 3.0 m depth interval. All cores were stored at 4 °C upon arrival to the University of Waterloo until required for use. To prepare the material for experiments, cores were pared inside a laminar clean bench. Material in contact with core barrel walls was discarded or sterilized for use in

sterile controls. The aquifer material used for testing was removed from the centre of each core and mixed thoroughly. The bulk mass density of the prepared aquifer material was measured in the lab to be 2.2 g/cm^3.

Groundwater for use in laboratory experiments was collected at a depth of approximately 4 m from a well close to the core recovery location. Approximately 20 L of water was purged from the well before it was collected into sterile glass carboys. The carboys were stored at 4 °C upon arrival to the laboratory.

The microcosms were assembled within a laminar flow clean bench and all equipment was previously sterilized. Microcosms were designed for repeated sampling in 250 mL glass bottles with screw-cap Mininert™ valves. Each bottle was supplied with 40 g (wet weight) of aquifer material and an appropriate volume of groundwater to result in a final liquid volume of 100 mL. Considering the aquifer bulk density of 2.2 g/cm^3, the resulting headspace in each bottle was approximately 132 mL. Prior to the addition of groundwater, the aquifer material in bottles designated as sterile controls was sterilized by autoclaving for one hour on three consecutive days. Sterile controls were also treated with 2 mL of a 10% sodium azide solution to prevent microbial activity.

Microcosms were amended with inorganic nutrients at the start of the experiment by the addition of 15 mL sterile modified Bushnell-Haas medium (MBH) (Mueller et al., 1991). The concentration of each added nutrient in the microcosms initially was as follows: 150 mg/L each K_2HPO_4, KH_2PO_4, NH_4NO_3, 30 mg/L $MgSO_4 \cdot 7H_2O$, 3 mg/L $CaCl_2 \cdot 2H_2O$ and 1.2 mg/L $FeCl_3 \cdot 6H_2O$. Additional MBH (5 mL aliquots) was periodically added to selected bottles later in the experiment (Days 18 and 42).

Based on stoichiometric calculations (Appendix A), the theoretical oxygen demand required for mineralization of ethanol in this experiment is greater than the oxygen available from air in the headspace and dissolved in the water. In an attempt to maintain aerobic conditions throughout the experiment, the microcosm headspaces were purged with pure oxygen for approximately 5 minutes prior to the addition of ethanol and BTEX. Theoretically, the volume of pure oxygen in the headspace (approximately 132 mL), is sufficient for the degradation of 837 mg/L ethanol. Therefore, the amount of oxygen added at the beginning of the experiment was sufficient to maintain aerobic conditions in Series A,

D and E. Periodic addition of pure oxygen was made for the remaining series, where oxygen would become limited. This involved the addition of 10-40 mL pure oxygen (typically 20 mL) every 1-3 days.

A few extra microcosms were prepared and four of these were sacrificed after equilibration on Day 1 to measure the dissolved oxygen (DO) concentration of the water. The initial DO of the extra bottles were 36.0 mg/L, 35.5 mg/L, 34.6 mg/L and 35.5 mg/L at 22 °C.

Microcosms requiring ethanol were spiked with pure filter-sterilized ethanol (ACS grade, 99.9%). Ethanol was filter-sterilized using a syringe filter with 0.45 μm nylon membrane filters. Appropriate volumes of ethanol were added to the bottles via syringe through the Mininert™ valve.

The volatile BTEX compounds were the last addition to the microcosms. The BTEX stock solution was prepared by saturating groundwater with gasoline following the method of Brookman et al. (1985). The gasoline was API 91-1 gasoline provided by the American Petroleum Institute, which was used in the field experiments at Borden by Mocanu (2007). The composition of the gasoline is given in Appendix B. The gasoline-saturated groundwater was prepared in a glass separatory funnel by combining 1L sterile groundwater with 100 mL gasoline. The mixture was shaken vigorously for 5 minutes and allowed to settle to permit the phases to separate. The mixing procedure was carried out a total of three times. After the mixture was allowed to equilibrate overnight, the gasoline-saturated groundwater was collected. Prior to use in the microcosms, the BTEX composition of the gasoline-saturated water was analyzed in triplicate by gas chromatography and the results are displayed in Table 2.2.

Table 2.2 BTEX composition of the gasoline-saturated water.

Compound	Mean Concentration (mg/L)	Mass Fraction
Benzene	29.6	0.29
Toluene	47.3	0.46
Ethylbenzene	6.2	0.06
p+*m*-Xylene	13.0	0.13
o-Xylene	5.7	0.06

Gasoline-saturated water (13 mL) was added to the bottles via syringe through the Mininert™ valve, resulting in a measured initial aqueous concentration of 13.2 mg/L, close to the target concentration of 15 mg/L.

The microcosms were incubated in the dark at room temperature and mixed every 1-3 days to promote equilibrium of the air and water phases.

2.2.2 Sampling and Analysis

Biodegradation within the microcosms was monitored by measuring ethanol and BTEX loss by GC analysis periodically over 67 days. The microcosms were allowed to equilibrate following test initiation and sampling began on Day 1.

2.2.2.1 BTEX

BTEX concentrations were measured by headspace analysis using a Shimadzu GC-9A gas chromatograph (GC). The GC was equipped with a flame ionization detector and 0.32 mm x 60 m Supelcowax-10 capillary column with a 0.5 μm stationary phase of Carbowax-20. The column temperature was 105 °C and the injector temperature was 200 °C. The GC was calibrated with external standards and the calibration was checked each sampling day using prepared standards.

Microcosms were mixed prior to sampling to promote phase equilibrium. Headspace samples (500 μL) were withdrawn through the Mininert™ valve with a gas-tight syringe and injected into the GC. The detection limits for the BTEX compounds in the gas phase using this method range from 0.4 to 1.6 μg/L. Headspace BTEX concentrations were converted to aqueous concentrations using Henry's Law. All results are reported as aqueous phase concentrations.

2.2.2.2 Ethanol

Ethanol concentrations were measured by direct aqueous injection using a Hewlett Packard 5890 GC equipped with a flame ionization detector and a 0.125 in. x 10 ft. column packed with 3% SP1500 on Carbopack B (80/100 mesh). The oven, injector and detector temperatures of the GC were 115, 115 and 230 °C, respectively. The GC was calibrated with

external standards and the calibration was checked each sampling day using prepared standards.

Approximately 0.5 mL samples of the aqueous phase were removed through the Mininert™ valve using a glass syringe and transferred to a 2 mL glass autosampler vial. Samples not requiring dilution were preserved with 0.01 mL of 10% sodium azide solution to prevent further microbial activity. The majority of the samples required dilution with deionized water to within the detection range of the GC (0.05 to 200 mg/L). In this case, the dilution water was amended with sodium azide at a rate of 2-4 mL of 10% sodium azide solution per 100 mL water. For the first two sampling events (Day 1 and Day 2 AM), sodium azide was mistakenly not used. Samples that could not be immediately analyzed were stored in the refrigerator for up to 48 hours.

2.2.2.3 Dissolved Oxygen

Dissolved oxygen (DO) concentrations were measured occasionally to ensure microcosm conditions remained aerobic. DO analysis was performed on the same aqueous sample collected for ethanol prior to preservation and ethanol analysis. DO measurements were made immediately after retrieval using a DO probe (Microelectrodes, Inc., Model MI-730). The electrode was calibrated each sampling day prior to use using a 0% O_2 standard (2% sodium sulfite) and a 21% O_2 standard (air-sparged deionized water). Any addition of oxygen to microcosms was done after sampling.

2.2.2.4 Microbiology

Restriction fragment length polymorphism (RFLP) analysis was used to detect changes in the microbial communities before and after ethanol and BTEX exposure and biotransformation. RFLP is a molecular-based technique that involves digestion with particular restriction enzymes to cut PCR-amplified DNA. The resulting fragments are separated according to length by agarose gel electrophoresis. The fragments migrate through the gel at different rates depending on their size, creating a banding pattern. Differences in microbial communities are represented by variations in fragment banding patterns.

Using RFLP analysis, a comparison was made between the microbial community of the aquifer material initially used in the microcosm experiment (Day 0) with the community of a Series F microcosm treated with 5000 mg/L Ethanol + 15 mg/L BTEX (Day 79).

The initial (Day 0) sample was collected during the soil allocation. On Day 79, one active replicate of Series F was sacrificed for a microbiology sample. The aquifer material was removed using a sterile spatula and drained using sterile filter paper set in a glass funnel. The aquifer material was allowed to drain, covered with sterile aluminum foil, for 5 days. After 5 days, the sample was collected in sterile Whirlpack bags and stored in a -80 °C freezer until analyzed. The filtrate was discarded.

The RFLP analysis and all preparatory steps were conducted following a method modified from Whyte and Greer (2005). Details of the method are provided in Appendix C. For each sample, the total community DNA was extracted from 1 g (wet weight) subsamples using the UltraClean Soil DNA Isolation Kit (MO Bio Laboratories, Inc.). The polymerase chain reaction (PCR) was used to amplify bacterial 16S rRNA genes from the total DNA with the following primers: primer 1, 5′-GAG TTT GAT CMT GGC TCA G-3′ (M=A+C), and primer 2, 5′-ACG GYT ACC TTG TTA CGA CTT-3′ (Y=C+T). 16S rRNA genes are targeted because they are present in all organisms and are excellent biomarkers for microorganisms (Moyer et al, 1994).

The DNA present in the Day 0 samples was too low to be detected, even after PCR, and required nested PCR to obtain a product. Nested PCR involves performing a second round of PCR using the product of the first reaction as the amplification target (van Elsas and Boersma, 2004). The PCR product of the Day 79 sample and the nested PCR product of the Day 0 sample were cleaned to remove PCR-related contaminants using the MinElute PCR Purification Kit (Qiagen, Inc.).

The purified PCR products were digested with the restriction enzyme TaqI at 65 °C for 1 hour. The resulting digests were analyzed using agarose (2%) gel electrophoresis and the banding patterns were visualized under UV light following ethidium bromide staining.

2.3 Results and Discussion

2.3.1 Controls

Each series had its own set of duplicate sterile controls. Sterile control microcosms appeared to remain microbiologically inactive and concentration variation among duplicates was generally less than 4%.

Ethanol concentrations in the sterile controls were fairly stable. However, concentrations of the volatile BTEX compounds in the controls gradually decreased. The loss of volatiles is probably due to leakage from bottles during incubation and headspace sampling. This is supported by the observation that the most volatile compounds generally appeared to experience the greatest losses within each series. For example, in Series F control microcosms, the concentrations of ethylbenzene, *p*-xylene and *m*-xylene, which have larger Henry's Law constants than benzene, toluene, and *o*-xylene, show the greatest losses.

In order to correct for the abiotic loss of compounds due to volatilization and leakage, values for active microcosms are reported relative to values for sterile controls of the same treatment series. Normalizing data to sterile controls also reduces the effect of analytical bias and temperature fluctuations between sampling periods.

2.3.2 Ethanol

The averages of duplicate microcosms containing ethanol are reported relative to sterile control concentrations (Figure 2.1). Raw data are provided in Appendix D. Ethanol degraded for each treatment series without a significant acclimation period. The average final concentration of ethanol in each treatment is listed in Table 2.3. For microcosms with the same concentration of ethanol, the presence of BTEX did not seem to affect ethanol degradation.

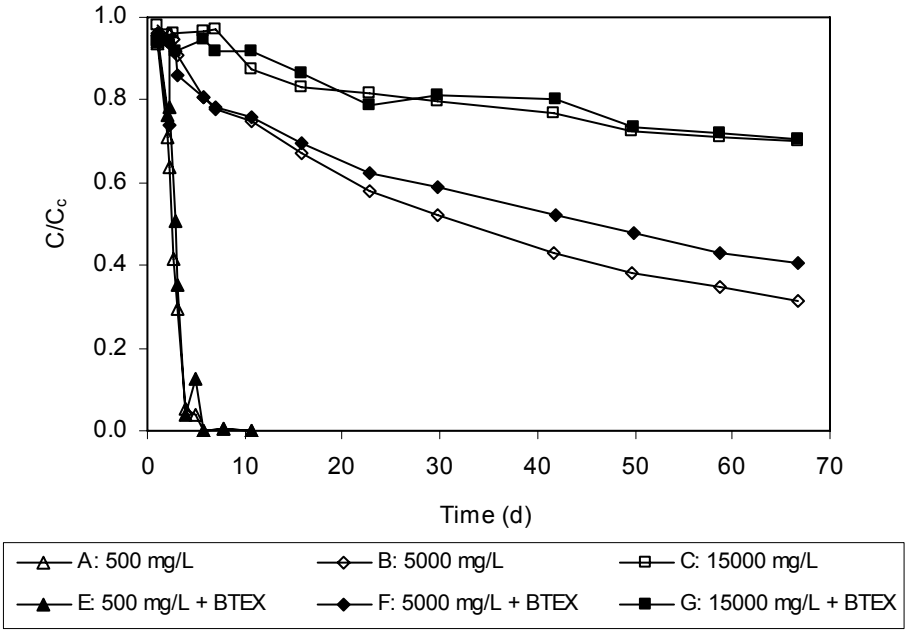

Figure 2.1 Ethanol degradation in microcosms with various concentrations of ethanol, with and without BTEX added. Concentrations of ethanol (C) are expressed relative to sterile control concentrations (C_c) of the same treatment series measured at the same time. All values are the mean of duplicate microcosms.

Table 2.3 Initial and final ethanol concentrations (reported as the mean of duplicate microcosms) and apparent first order rate constants of ethanol loss.

Series	Treatment	Ethanol Concentration (mg/L)		Apparent First Order Degradation Rate Constant (d^{-1})
		Initial (Day 1)	Final (Day 67)	
A	500 mg/L EtOH	443	0	0.83
B	5000 mg/L EtOH	4412	1670	0.02
C	15000 mg/L EtOH	12984	11201	0.007
D	BTEX	—	—	—
E	500 mg/L EtOH + BTEX	446	0.2	0.54
F	5000 mg/L EtOH + BTEX	4412	2170	0.02
G	15000 mg/L EtOH + BTEX	13034	11191	0.005

The constants for the apparent first-order rates of biotransformation were calculated using the natural logarithm of sterile control-corrected concentrations (Table 2.3). Corrections were made by adding the mean concentration loss of the sterile controls to the mean active concentration for each sampling time. The ethanol concentration data followed a first-order rate model, represented by high correlation coefficient values ($R^2 = 0.93 – 0.98$). Rate constants for the loss of ethanol decreased by approximately an order of magnitude as the initial ethanol concentrations increased from 500 to 5000 to 15000 mg/L. This could indicate some inhibition of microbial activity at higher ethanol concentrations, however even at the highest concentration (15000 mg/L), ethanol does not appear to be sufficiently toxic to prohibit ethanol biotransformation. In a similar microcosm study (Araújo, 2000), ethanol loss was also observed for microcosms with initial ethanol concentrations of 3945 mg/L (0.5% v/v) and 11835 mg/L (1.5% v/v). However, unlike the observations presented here, Araújo found that the initial ethanol concentration was not a significant factor in ethanol degradation.

Differences could be seen between the active and control systems throughout the experiment. The aqueous phase of the active microcosms containing ethanol began to develop a cloudy appearance on day 3 and remained cloudy throughout the experiment. This turbidity was interpreted to signify microbial growth. By day 17, the cloudiness was more noticeable and the degree of cloudiness increased with ethanol concentration. It also appeared that nutrient addition on day 18 supported additional turbidity in the same microcosms. Meanwhile the liquid of the controls and "BTEX only" bottles remained clear.

Gas production in the microcosms was observed as positive pressure in bottles containing 5000 mg/L and 15000 mg/L (Series B, C, F and G). The gas was likely CO_2 produced by the aerobic mineralization of ethanol and BTEX, although the gas was not analyzed. Positive pressure during the aqueous sampling of the microcosms led to gas loss, but the amount of gas lost was not quantified.

The evidence of microbial growth and activity represented by turbidity and gas production in microcosms containing 5000 mg/L and 15000 mg/L ethanol supports the observed loss of large amounts of ethanol.

2.3.3 BTEX

The results for microcosms containing BTEX are also reported relative to sterile control concentrations and are given as the average of duplicate microcosms (Figure 2.2). In general, the normalized concentrations of duplicates differed by less than 15%. Raw data are provided in Appendix D. For all treatments, ethylbenzene disappeared first and the xylene isomers were the most persistent. The presence of ethanol appeared to have only a slight effect on the biotransformation of benzene, toluene and ethylbenzene; in fact the apparent first order rate constants of these monoaromatics were slightly greater in the presence of 500 mg/L and 5000 mg/L ethanol compared to the no ethanol case (Table 2.4). At a given concentration, a greater first order rate constant corresponds to a faster rate of degradation. In the presence of the highest concentration of ethanol (15000 mg/L), B, T and E persisted a little longer and had the lowest rate constants. Only for the highest ethanol concentration did there appear to be a short acclimation phase (2-3 days) before degradation of these aromatics began. In these cases, first-order rate constants were calculated from the curve following the acclimation phase. Benzene was typical and results for that compound are shown in Figure 2.3. In the presence of the highest concentration of ethanol (15000 mg/L) benzene did degrade more slowly, but it still degraded within 10 days. These results are in contrast to research reported by Corseuil et al. (1998), Da Silva and Alvarez (2002), and Hunt et al. (1997), in which BTEX degradation was inhibited by the presence of ethanol at concentrations ranging from 20 to 2000 mg/L. Under strictly anaerobic conditions, Chen et al. (in press) observed the inhibition of BTEX degradation by 500 mg/L and 5000 mg/L ethanol, despite an attempt to maintain electron acceptors and nutrients in excess.

Ethanol had a more significant impact on the degradation of the xylene isomers (Figure 2.2 and Table 2.4). This effect was not apparent for the lowest concentration of ethanol (500 mg/L), however all three xylene isomers were more persistent in the two highest concentrations of ethanol (5000 mg/L and 15000 mg/L). For each isomer, the degradation response to 5000 mg/L ethanol was similar to that for 15000 mg/L ethanol. *p*-Xylene was the least affected of the isomers (Figures 2.2 and 2.4). *m*-Xylene and *o*-xylene were still present after 67 days of incubation.

The *p*-xylene plateau may be an indication of nutrient limitation in these microcosms. Further indication of nutrient limitation may be inferred from the response of all the xylene isomers after nutrient addition. Extra nutrients (5 mL MBH) were added to active microcosms with 5000 mg/L and 15000 mg/L ethanol (Series B, C, F and G) on days 18 and 42. These events are denoted by arrows in Figures 2.2 and 2.4. The nutrient addition appears to be followed by a slightly increased rate of xylene degradation. Nutrient addition also appears to increase the ethanol degradation in the treatment with 15000 mg/L ethanol + BTEX (Figure 2.2).

Unlike *m*- and *o*-xylene, *p*-xylene experienced rapid degradation initially (or just following an acclimation phase) in microcosms with the two highest concentrations of ethanol (5000 mg/L and 15000 mg/L). At some point between days 3-9, *p*-xylene degradation decreased significantly to a relative plateau and continued to degrade slowly

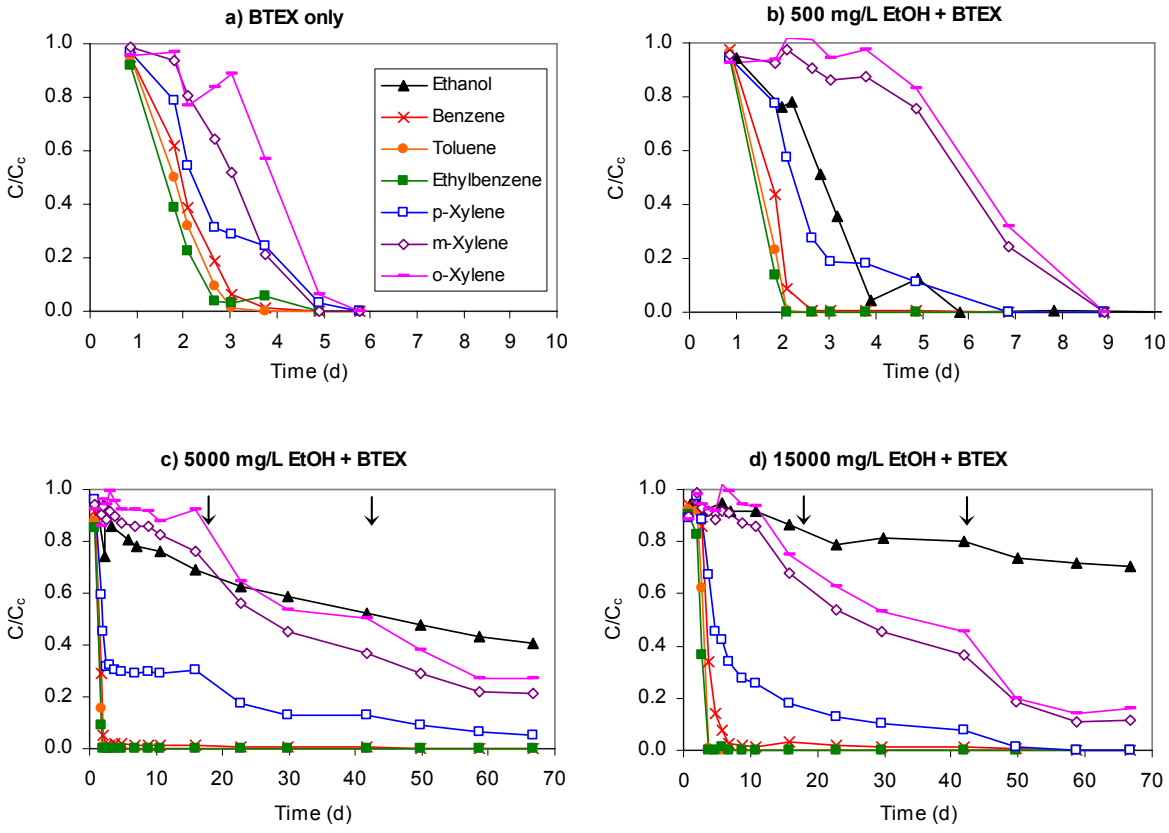

Figure 2.2 Degradation of ethanol and BTEX in microcosms. Concentrations (C) are expressed relative to sterile control concentrations (C_c) of the same treatment series measured at the same time. In c and d additional nutrients were added on days 18 and 42 (denoted by arrows).

throughout the rest of the experiment. For these two cases, apparent first order rate constants were calculated separately for the initial fast degradation phase and for the final slower degradation phase. The curves of the final plateau phase did not fit the first order rate model well (R^2 = 0.63 and 0.83).

In several previous studies (Deeb et al., 2002; Corseuil et al., 1998; Hunt et al., 1997), ethanol was apparently preferentially degraded. In this experiment however, microorganisms biodegraded ethanol and BTEX simultaneously. Additionally, the similar response of ethanol and xylene to nutrient addition suggests that neither ethanol-degraders nor xylene-degraders were dominating completely the competition for nutrients.

Table 2.4 Apparent first order rate constants of BTEX loss in microcosms (reported as the mean of duplicate microcosms), calculated from the curve following the acclimation phase. The correlation coefficient (R^2) represents the goodness of fit of data to the first-order rate model.

Compound	Series	Treatment	Acclimation Phase (d)	R^2	Apparent First Order Degradation Rate Constant (d^{-1})
Benzene	D	BTEX only	0	0.96	0.82
	E	500 mg/L EtOH + BTEX	0	0.93	0.97
	F	5000 mg/L EtOH + BTEX	0	0.94	1.29
	G	15000 mg/L EtOH + BTEX	<3	0.94	0.44
Toluene	D	BTEX only	0	0.95	1.08
	E	500 mg/L EtOH + BTEX	0	0.93	1.47
	F	5000 mg/L EtOH + BTEX	0	0.94	1.60
	G	15000 mg/L EtOH + BTEX	<2	0.95	0.77
Ethylbenzene	D	BTEX only	0	0.93	0.96
	E	500 mg/L EtOH + BTEX	0	0.98	1.48
	F	5000 mg/L EtOH + BTEX	0	0.99	1.68
	G	15000 mg/L EtOH + BTEX	<2	1.00	0.62
p-Xylene	D	BTEX only	0	0.93	0.39
	E	500 mg/L EtOH + BTEX	0	0.92	0.39
	F	5000 mg/L EtOH + BTEX	0	0.99[a] 0.63[b]	0.52[a] 0.01[b]
	G	15000 mg/L EtOH + BTEX	<3	0.99[a] 0.83[b]	0.18[a] 0.01[b]
m-Xylene	D	BTEX only	0	0.98	0.62
	E	500 mg/L EtOH + BTEX	<4	0.99	0.27
	F	5000 mg/L EtOH + BTEX	<9	0.97	0.02
	G	15000 mg/L EtOH + BTEX	<11	0.96	0.01
o-Xylene	D	BTEX only	<3	0.98	0.75
	E	500 mg/L EtOH + BTEX	<4	0.96	0.32
	F	5000 mg/L EtOH + BTEX	<16	0.90	0.02
	G	15000 mg/L EtOH + BTEX	<11	0.96	0.01

[a] Initial rate constant, calculated from the curve following the acclimation phase (if applicable) to a plateau
[b] Final rate constant, calculated for the plateau phase following initial phase

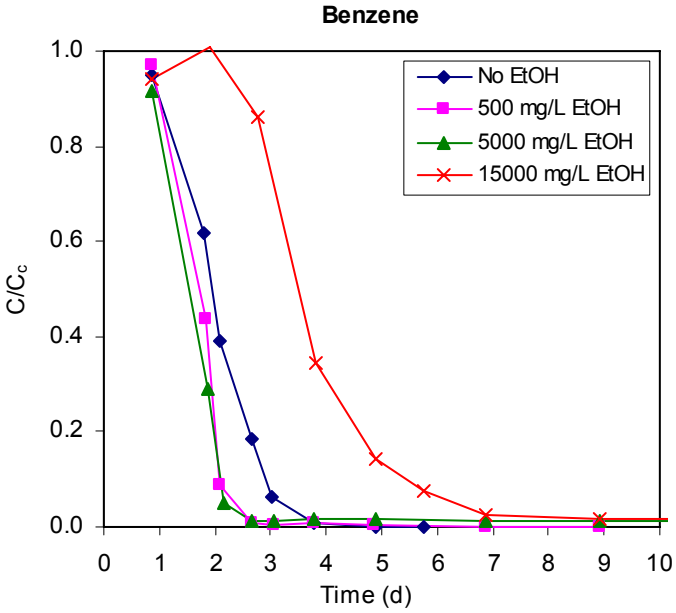

Figure 2.3 Degradation of benzene in microcosms with and without ethanol. Benzene concentrations (C) are expressed relative to sterile control concentrations (C_c) of the same treatment series measured at the same time.

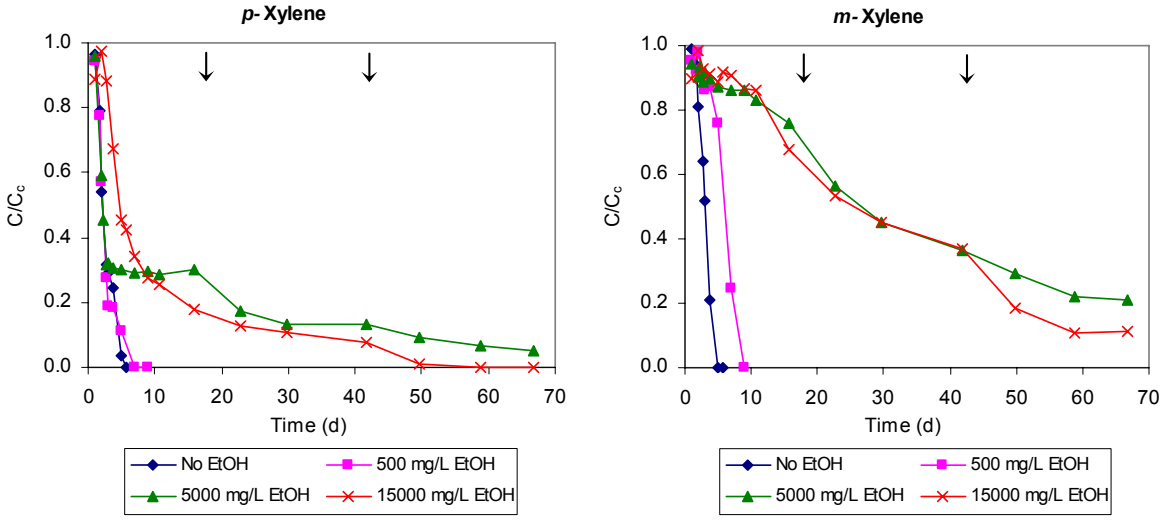

Figure 2.4 Degradation of *p*-xylene and *m*-xylene in microcosms with and without ethanol. Xylene isomer concentrations (C) are expressed relative to sterile control concentrations (C_c) of the same treatment series measured at the same time. Additional nutrients were added on days 18 and 42 (denoted by arrows).

2.3.4 Dissolved Oxygen

Dissolved oxygen concentrations measured throughout the experiment are reported as averages of duplicate microcosms (Table 2.5). Sterile control microcosms remained supersaturated with oxygen. Oxygen was expected to be limited in microcosms containing ethanol concentrations greater than 837 mg/L so oxygen was frequently added to microcosms containing 5000 mg/L and 15000 mg/L (Series B, C, F and G). The lowest dissolved oxygen concentrations measured in these microcosms were between 4.6 and 6.4 mg/L on Day 23. The dissolved oxygen results suggest that aerobic conditions were maintained in the aqueous phase throughout the experiment. However, since the microcosms were not continually mixed, biological activity probably led to localized anoxia in the sediment. It is also possible that anaerobic conditions may have occurred between dissolved oxygen measurements and oxygen addition.

Table 2.5 Dissolved oxygen concentrations reported as microcosm duplicate averages.

		Dissolved oxygen concentration (mg/L)							
Series		Day 11	Day 16	Day 23	Day 30	Day 42	Day 50	Day 59	Day 67
A	control	32.9	—	—	—	—	—	—	—
	active	16.7	—	—	—	—	—	—	—
B	control	33.4	36.4	38.1	33.5	32.1	36.0	27.3	29.2
	active	4.3	7.0	6.0	7.8	18.4	8.0	11.1	18.0
C	control	33.0	28.4	38.7	31.5	31.2	35.2	26.2	28.0
	active	5.2	10.4	6.4	16.6	20.2	20.0	17.7	17.4
D	control	33.4	—	—	—	—	—	—	—
	active	30.3	—	—	—	—	—	—	—
E	control	31.8	—	—	—	—	—	—	—
	active	15.6	—	—	—	—	—	—	—
F	control	33.1	33.7	36.2	31.5	25.2	31.1	25.5	27.7
	active	5.4	6.4	4.6	8.6	22.8	6.7	18.1	22.8
G	control	33.0	30.3	34.2	32.0	29.2	30.9	23.4	25.8
	active	6.4	6.8	4.7	9.9	16.7	8.9	12.9	21.6

'—' = not measured

On day 26 a grey colour began to develop in the liquid phase of microcosms containing 5000 mg/L and 15000 mg/L ethanol (Series B, C, F and G), and a black precipitate, likely iron sulfide, began to accumulate within the aquifer material. This condition, which can be seen in Figure 2.5, is a strong indication of sulfate reduction under anaerobic conditions. This emphasizes the possibility that oxygen was limited in these microcosms despite efforts to provide a surplus of oxygen.

Based on mass balance estimates, enough oxygen was added to provide a surplus of oxygen for the microcosms with 5000 mg/L ethanol. Over the 67-day experiment, a total of 951 mL of oxygen was provided to microcosms containing 5000 mg/L and 15000 mg/L ethanol (132 mL O_2 initially plus 819 mL O_2 throughout the experiment). Stoichiometrically, this volume of oxygen is sufficient to mineralize 0.0130 moles of ethanol, or enough for a microcosm containing 5972 mg/L ethanol (oxygen requirement for 15 mg/L BTEX was comparatively small and was considered negligible). However, the apparent oxygen utilization exceeds that required for the observed ethanol loss, so it appears that not all of the oxygen provided was used for ethanol degradation. This observation, combined with apparent anaerobic conditions, leads to the suspicion that oxygen was lost from the system. It is possible that oxygen escaped during aqueous sampling due to the positive pressure of gas build-up.

Figure 2.5 Photograph of microcosms from treatment series G (15000 mg/L ethanol + BTEX) at the end of the experiment (Day 67). Note the cloudy grey appearance of the liquid phase and the black precipitate in the aquifer material of active microcosms.

2.3.5 Microbiology

The results of the microbial community analysis using RFLP are given in Figure 2.6. Uncut DNA of each sample (approximately 1500 base pairs (bp) in size) was run in lanes 2 and 3 of the gel. The band representing the initial aquifer material sample (Lane 2) is much weaker than the Day 79 Series F microcosm sample band (Lane 3) because there were fewer microorganisms, and therefore less DNA, in the initial sample. It was difficult to recover enough DNA from the initial sample to be detected, even after PCR amplification.

The TaqI restriction enzyme digestion of the two samples resulted in the banding patterns in Lanes 4 and 5. Differences in microbial communities are represented by variations in fragment banding patterns. Although a few bands are visible in both samples (e.g., approximately 900 bp and 600 bp in size), the overall banding patterns of the two samples are different. In other words, a different community of microorganisms was supported by the conditions of the microcosm with 5000 mg/L ethanol + BTEX than was present in the sample initially. The change is likely a shift from the indigenous aquifer community towards more ethanol- and BTEX-degrading microorganisms.

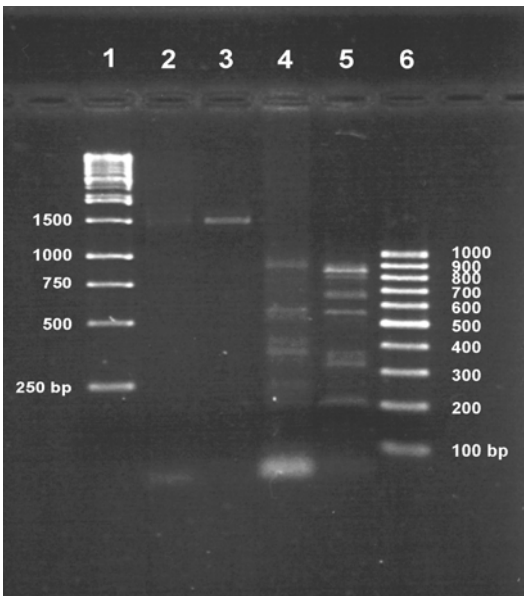

Figure 2.6 RFLP patterns of 16S rRNA genes created by digestion with TaqI restriction endonuclease. Lane 1, 1 kb DNA ladder; Lane 2, uncut initial aquifer material sample; Lane 3, uncut aquifer material sample from Day 79 Series F microcosm (5000 mg/L EtOH + BTEX); Lane 4, digested initial aquifer material sample; Lane 5, digested aquifer material sample from Day 79 Series F microcosm; Lane 6, 100 bp DNA ladder.

In the current research initiative, TaqI was the only restriction enzyme used, however other restriction enzymes can be used for RFLP analysis. Different restriction enzymes will cut at different sites of the DNA, resulting in fragment banding patterns different from those observed here with TaqI. Ideally, a few different enzymes are used for a more thorough community analysis, especially when communities are complex. However, the single enzyme used in the study was sufficient to detect differences in the banding pattern, or "fingerprint" of the microcosm community compared to the initial aquifer community.

Another useful community analysis would be to compare the final communities of different treatments, particularly treatments with and without ethanol. In a community analysis of microcosms treated with BTEX + MTBE and BTEX + MTBE + ethanol, Feris et al. (2004) reported that the communities in microcosms with ethanol were significantly different from those without ethanol.

2.4 Summary of Conclusions

Simultaneous degradation of ethanol and BTEX occurred in the microcosm experiments. This was in contrast to the preferential degradation of ethanol reported in previous microcosm studies (Deeb et al., 2002; Corseuil et al., 1998; Hunt et al., 1997). Ethanol did not greatly inhibit BTE degradation but ethanol at concentrations of 5000 mg/L and 15000 mg/L slowed degradation of the xylene isomers. The highest concentration of ethanol (15000 mg/L) did not completely inhibit microbial activity in the microcosms.

Chapter 3
Inhibition Experiment

3.1 Objective and Approach

Ethanol concentrations up to 15000 mg/L did not completely inhibit microbial activity in the microcosm experiment presented in Chapter 2. The objective of further experiments was to determine the effect of higher concentrations of ethanol on microbial populations in terms of microbial activity and BTEX biodegradation.

The experiments involved the static exposure of aquifer-associated microbes to concentrations of ethanol between 25 and 75% (v/v) (equivalent to 197250 and 591750 mg/L). Ethanol concentrations in this range may occur in the subsurface for short durations near the source of a large-scale E95 spill. The experimental exposure duration of 48-72 hours was intended to resemble exposure to a high concentration short-term ethanol spill. Following exposure, the residual ethanol was removed by washing the aquifer material with water, and the resulting microbial population of the washed material was assessed.

3.2 Methods and Materials

3.2.1 Inhibition Experiment 1

3.2.1.1 Ethanol Exposure and Removal

The ethanol exposure phase of the study was conducted in microcosms. A summary of the experimental treatments is listed in Table 3.1. Tested ethanol concentrations were 0% (Series C) and 32% (v/v) (equivalent to 250000 mg/L) (Series D). Sterile controls with 0% ethanol (Series A) were prepared to measure abiotic processes and to evaluate the risk of contamination during the washing procedure. Unwashed controls (Series B) were prepared to determine the effect of washing on the microbial population. Each treatment was prepared in duplicate except for Series C; Series C consisted of three sets of duplicates, two of which were designated for additional treatment in the subsequent dehydrogenase activity assay only.

Table 3.1 Summary of experimental treatments for the Inhibition Experiment 1.

Treatment Series	Description
Series A	Sterile control (0% ethanol)
Series B	Unwashed control (0% ethanol)
Series C	0% ethanol
Series D	32% (v/v) ethanol (250000 mg/L)

Microcosm assembly and ethanol exposure initiation were conducted in a laminar flow clean bench with sterile equipment. Microcosms were composed of 250 mL bottles containing 45 g (wet weight) of aquifer material plus 30 mL sterile deionized water or appropriate ethanol solution. Aquifer material was collected and prepared as described in Section 2.2.1. Aqueous ethanol solutions were prepared with filter-sterilized ethanol (ACS grade, 99.9%; filtered through 0.45 μm nylon syringe filter) and diluted with sterile deionized water. Prior to the addition of liquid to the microcosms, the sterile controls were autoclaved for one hour on three consecutive days. Microcosms were sealed with Teflon-lined screw caps and mixed by shaking for one minute. The static ethanol exposure period was 48 hours in the dark at room temperature.

After 48 h, the exposure solutions were removed by washing the aquifer material with deionized water. The washing procedure was conducted aseptically in a laminar flow cabinet with sterile equipment. The contents of each microcosm bottle were transferred to a 250 mL centrifuge tube and centrifuged at 7400 x g for 10 minutes to separate the aquifer material and microbes from the liquid phase. The supernatant was carefully withdrawn using sterile Pasteur pipettes to remove as much liquid as possible without disturbing the sediment. Residual ethanol was removed from each sediment, with the exception of the unwashed control (Series B), by conducting the following procedure twice: 1) add 50 mL sterile water and mix, 2) centrifuge, and 3) remove supernatant.

Ethanol concentrations were measured by gas chromatography (GC) following the same method as in Section 2.2.2.2. Samples were collected for ethanol analysis from Series D microcosms at the beginning of the exposure (3 hours after ethanol addition) and after each wash. The samples were diluted with sodium azide treated deionized water to within the detection range of the GC method (0.05 to 200 mg/L). Diluted solutions were transferred to 2 mL glass autosampler vials and analyzed immediately by GC.

The washed aquifer material was subsampled in the laminar flow clean bench and tested to determine the following:

1) dehydrogenase activity
2) number of viable microorganisms
3) toluene biodegradation potential

3.2.1.2 Dehydrogenase Activity

Dehydrogenases are respiratory enzymes produced by microorganisms and dehydrogenase activity is a measure of the general microbial activity in a soil (Friedel et al., 1993). One method of measuring the dehydrogenase activity in a soil sample is the 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) assay (Trevors et al., 1982). Water soluble INT is added to the sample as an alternative electron acceptor and microbial dehydrogenases reduce the INT to form INT-formazan. INT-formazan is a red, water insoluble compound, which can be extracted with methanol and measured by spectrophotometry.

Dehydrogenase activity was measured based on the INT assay described by Trevors et al. (1982). Subsamples (11 g wet weight) from each treatment series A-D were allocated to sterile 40 mL glass vials. The vials were amended with 0.5 mL modified Bushnell-Haas inorganic nutrient medium (MBH) and 0.5 mL of 1% (w/v) yeast extract as a metabolizable substrate. Each vial received 1.5 mL of a filter sterilized 0.4% (w/v) INT solution. The INT solution was sterilized using a syringe filter with cellulose nitrate membrane filters (0.22 μm pore size).

Three duplicate sets of Treatment Series C samples were analyzed by the INT test. One set (labeled "0% EtOH") was treated as described above. A second set (labeled "unamended") received 1 mL of sterile deionized water in place of MBH and yeast extract, in order to measure endogenous microbial activity. The third set (labeled "blank") was prepared without INT to serve as a blank control of the methanolic extract during spectrophotometric analysis. Each of the slurries was mixed and then sealed with Teflon-lined screw caps and incubated in the dark at room temperature.

At each sampling event, a flame-sterilized metal spatula was used to mix the slurry and aseptically transfer approximately 0.5 g of aquifer slurry to a test tube. A total of 5 mL methanol was used to extract the INT-formazan from the sample in two increments, mixing for one minute using a vortex mixer after each methanol addition. The methanolic slurry was poured through a pre-weighed Whatman No. 5 filter paper. The solids remaining on the filter were dried overnight at 100 °C and weighed to determine the dry weight of aquifer material extracted.

The filtrate was transferred to a microcentrifuge tube and centrifuged briefly to prevent interference by small particles during spectrophotometric analysis. The absorbance of the supernatant was measured by spectrophotometer (Thermo Spectronic Genesys 20) at a wavelength of 480 nm using a blank control to zero the instrument. The absorbance readings were converted to units of μg /mL INT-formazan using a standard curve (see Appendix E for INT-formazan standard curve). Dehydrogenase activity was reported as μg INT-formazan/g dry weight aquifer solids.

3.2.1.3 Enumeration of Microorganisms

The number of aerobic, viable, heterotrophic bacteria present in untreated and ethanol-exposed aquifer material was estimated using the plate count technique (APHA, 1995). Microorganisms were extracted from 10 g (wet weight) subsamples of the washed material by dilution in 90 mL of 0.1% sodium pyrophosphate followed by shaking for 10 min. on a rotary shaker at approximately 350 rpm. The extracted samples (10^{-1}) were serially diluted by orders of magnitude down to 10^{-7} in phosphate buffered water (APHA, 1995). Aliquots (0.1 mL) of each dilution were plated in triplicate onto pre-dried R2A agar using the spread plate technique. In addition, 1.0 mL aliquots were plated for the 10^{-1} dilution to yield plates representing a 10^{-1} dilution. Plates were inverted and incubated at room temperature. Colonies were counted after 7 days and counts were reported as the number of colony forming units (CFU)/g dry weight aquifer material. Colony counts were also conducted after 14 and 28 days of incubation, but were not considerably different from those after 7 days.

3.2.1.4 Toluene Biodegradation

A microcosm experiment was conducted to measure the capability of the post-ethanol microbial population to subsequently biodegrade hydrocarbons. For simplification, toluene was selected as a representative monoaromatic hydrocarbon. Subsamples (16 g) of the washed material were added to 100 mL bottles with 40 mL of toluene- and MBH-amended groundwater. The groundwater solution was prepared by adding MBH medium (25 mL/L) and sterilizing before adding toluene (6 mg/L). The microcosms were incubated in the dark at room temperature and toluene degradation was monitored. Toluene concentration was measured in headspace samples and analyzed by GC following the same method as in Section 2.2.2.1.

After toluene loss was observed in Series A (sterile control) on Day 1, sodium azide (0.8 mL of a 10% solution) was added to Series A bottles. Further toluene loss was observed in these microcosms and was assumed to be due to gas leakage from the bottles. The loss of toluene from the sterile controls indicated a problem with the experiment and the results are presented in Appendix F. The results were made difficult to interpret by the unexpected loss of toluene in the sterile controls. However, it was clear that the ethanol-exposed bottles behaved similarly to the sterile controls and differently from the unexposed samples. The sterile controls and ethanol-exposed samples showed gradual toluene loss over 20 days while the unexposed samples showed complete loss within 2 days (Figure F.1 in Appendix F). Despite the questionable toluene loss in the sterile controls, it appears that ethanol exposure had some effect on subsequent toluene degradation.

3.2.2 Inhibition Experiment 2

The inhibitory effect of a greater range of ethanol concentrations was studied in a second experiment. The ethanol exposure system and washing procedure were modified from Experiment 1 following an Exposure and Washing Method Investigation (Appendix G).

3.2.2.1 Ethanol Exposure and Removal

The method for this experiment was similar to Experiment 1 (Section 3.2.1) with differences highlighted in this section.

A summary of the experimental treatments is listed in Table 3.2. Tested ethanol concentrations ranged from 0 to 75% v/v (equivalent to 591750 mg/L). Each treatment was prepared in duplicate. Sterile controls were prepared for each treatment. Unwashed controls were prepared for the 0% ethanol treatment only (Series A).

Table 3.2 Summary of experimental conditions for the Inhibition Experiment 2.

Treatment Series	Ethanol Concentration (% v/v)	Ethanol Concentration (mg/L)
Series A	0%	0
Series B	25%	197250
Series C	50%	394500
Series D	75%	591750

For the second experiment, exposure microcosms were modified to minimize headspace and were composed of 23 mL vials with Teflon-lined screw caps containing 20 g (wet weight) aquifer material and 12 mL aqueous ethanol solution. The solids:liquid ratio was similar to Experiment 1, however the headspace was only approximately 2 mL (compared to approximately 200 mL in Experiment 2) considering an aquifer bulk mass density of 2.2 g/cm^3. The static exposure period was lengthened to 72 hours for ease of scheduling, but was not expected to have a significant impact on the results.

The number of washes was increased to minimize the transfer of residual ethanol to the subsequent tests. Based on the Washing Method Investigation outlined in Appendix G, four washes of 230 mL volumes would have been sufficient to reduce the residual ethanol in each treatment to less than 2 mg/L. In practice, 230 mL volumes were used for the first two washes, but due to leakage during centrifugation, the volumes were reduced to 150 mL for three final washes (total of five washes).

Ethanol samples were collected at the beginning of the exposure and after the final (fifth) wash. Sample collection and analysis was conducted following the same method as in Experiment 1.

3.2.2.2 Enumeration of Microorgansisms

Enumeration of viable microorganisms in the washed material was conducted using the aerobic plate count method (Section 3.2.1.3).

3.3 Results and Discussion

3.3.1 Ethanol Exposure and Removal

Initial ethanol concentrations measured in Experiment 1 (Table 3.3) were lower than the target concentration of 250000 mg/L by an average of 6% and dilution was believed to be responsible. Considering an average measured water content of the aquifer material (θ) of 18%, the water in the aquifer material can account for a 21% dilution of the initial ethanol concentration in Experiment 1.

Ethanol concentrations measured in Experiment 2 are reported as duplicate averages (Table 3.4). Initial ethanol concentrations in Experiment 2 were 22.5% lower than target concentrations, on average. This loss corresponds well to the anticipated 23% dilution by water in the aquifer material.

Table 3.3 Ethanol concentrations of the exposure solutions and after washing in Experiment 1.

		Ethanol Concentration (mg/L)			
Series	Replicate	Target	Measured Initial	Measured After Wash 1	Measured After Wash 2
D	1	250000	241000	31200	5110
D	2	250000	228000	30000	4930

Table 3.4 Ethanol concentrations of the exposure solutions and after the final wash in Experiment 2.

		Ethanol Concentration (mg/L)		
Series		Target	Measured Initial	Measured After Wash 5
B	sterile	197250	147000	1.1
	active	197250	160000	1.1
C	sterile	394500	263000	1.5
	active	394500	320000	0.6
D	sterile	591750	474000	1.3
	active	591750	482000	2.9

The purpose of washing the aquifer material was to remove the ethanol so that the effect of ethanol exposure could be observed in subsequent tests without the interference of residual ethanol. The washing procedure in Experiment 1 removed 98% of the ethanol (87% and 84% in washing steps 1 and 2, respectively). The residual ethanol present in each replicate after washing was greater than desired (5110 and 4930 mg/L). Calculated average concentrations at the start of the INT assay and toluene degradation test were 2500 mg/L and 400 mg/L, respectively. The average concentration in the first dilution (10^{-1}) of the plate count test was calculated as 120 mg/L. The residual ethanol concentrations estimated to be present at the outset of the various test assays were greater than desired and could conceivably influence assay results. Consequently, the washing procedure was improved for the second experiment.

The average residual ethanol concentration after five washes in Experiment 2 was <3 mg/L, resulting in a maximum concentration <0.2 mg/L in the first dilution (10^{-1}) of the plate count assay.

3.3.2 Dehydrogenase Activity

Results of the dehydrogenase activity assay are reported as μg INT-formazan/g dry weight aquifer material (Figure 3.1). The lack of INT-formazan production in sterile controls (Series A) suggests that sterility was likely maintained during the washing procedure. Endogenous microbial activity was considered insignificant due to a lack of INT-formazan production in the unamended controls. The INT-formazan production in the ethanol-free washed and unwashed treatments were not significantly different, indicating that dehydrogenase activity was not affected by the two washes.

Dehydrogenase activity detected in the ethanol-free active treatment reached an average of 365 μg INT-formazan/g aquifer material. A decreasing trend in INT-formazan concentration between Days 19 and 29 was observed for several treatments, and is regarded to be due to uncertainties in the test method, as it is unlikely for INT-formazan to disappear from the system. No significant INT-formazan production was observed in the 250000 mg/L (32%) ethanol treatments series as of Day 9. After Day 9, one of the replicates continued to show no activity (even up to 200 days), whereas the other showed evidence of quite high

activity on Day 30. This response indicates that 250000 mg/L ethanol was effective at disrupting the microbial activity but that there is some potential for survivors.

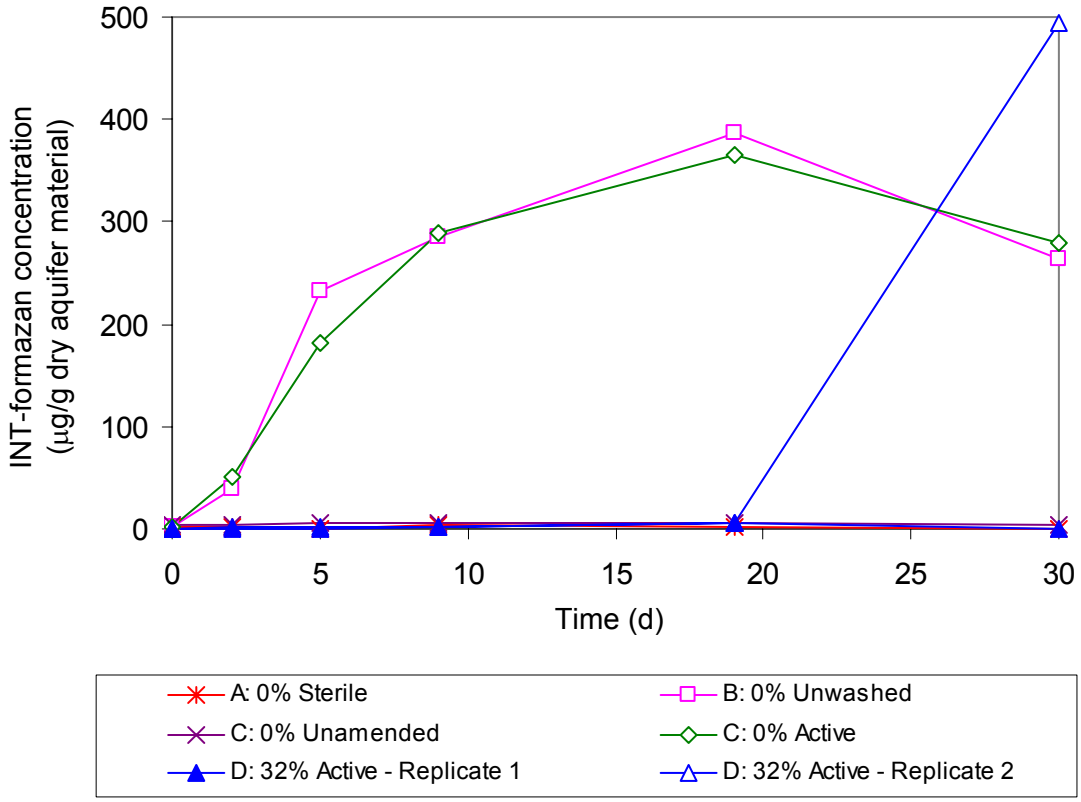

Figure 3.1 Dehydrogenase activity (estimated as INT-formazan production) of aquifer microorganisms following exposure to ethanol.

3.3.3 Enumeration of Microorgansisms

Enumeration of viable microorganisms in the sterile treatments of both experiments support the inference from the dehydrogenase assay that sterility was likely maintained during the washing procedure (Tables 3.5 and 3.6).

Counts for the ethanol-free treatment after two washes (Experiment 1; Table 3.5) and after five washes (Experiment 2; Table 3.6) were approximately 30% and 50% less, respectively, than similar unwashed treatments. The differences indicate that a considerable number of cells were lost during washing; therefore all reported counts are likely to be underestimated. However, the number of cells lost during washing is small relative to the number of cells affected by ethanol exposure.

Table 3.5 Colony forming units in aquifer material after two washes in Experiment 1.

Series	Ethanol Concentration (% v/v)		CFU/g dry weight aquifer material	Standard Deviation
A	0%	Sterile	<1.3	–
B	0%	Unwashed	1.9×10^5	1.5×10^4
C	0%	Active	1.3×10^5	4.3×10^4
D	32%	Active	4.1×10^1 *	1.9×10^1

* estimates due to interference by spreaders

Table 3.6 Colony forming units in aquifer material after five washes in Experiment 2.

Series	Ethanol Concentration (% v/v)		CFU/g dry weight aquifer material	Standard Deviation
A	0%	Sterile	<1.3	–
		Unwashed	1.3×10^5	2.2×10^4
		Active	6.6×10^4	4.1×10^4
B	25%	Sterile	<1.3	–
		Active	4.9×10^1 *	2.7×10^1
C	50%	Sterile	<1.3	–
		Active	<10 *	–
D	75%	Sterile	<1.3	–
		Active	<10 *	–

* estimates due to interference by spreaders

Exposure to ethanol resulted in very few colonies for 25% and 32% ethanol, and almost no colonies for 50% and 75% ethanol. Exposure to all concentrations of ethanol resulted in >99.9% fewer colonies than were counted in aquifer material not exposed to ethanol. Spreading colonies observed in some plates of the least-diluted sample (10^{-1}) introduced an additional source of error. The larger sample volume used to inoculate these plates (1.0 mL instead of 0.1 mL) exceeded the amount which could be absorbed by the agar, resulting in a film of water. If spreading colonies develop within the film of water, they tend to cover part or all of the plate and interfere with counting. Since it is difficult to say whether spreading is attributed to one colony or many, counts obtained from these plates were questionable. In future experiments, I would recommend dividing the volume into three plates (each plate receiving <0.4 mL) and obtaining the result from a combined count. Nonetheless, it is clear that the number of culturable microorganisms was drastically reduced following exposure to ≥25% ethanol. Results also indicate that at least a few microorganisms are likely to survive exposure to 25% and 32% ethanol concentrations.

Overall, the colonies observed on the plates were similar for each sample tested. Colonies appeared round and elevated with a smooth surface; a few were orange but the majority were white. A culture independent method for quantifying microbial populations is recommended, since most (90-99.9%) soil bacteria are not culturable by standard laboratory practices (Torsvik et al., 1998). In addition, a community comparison using molecular techniques (e.g., RFLP or denaturing gradient gel electrophoresis) would be useful to detect changes in the microbial community following ethanol exposure.

3.4 Summary of Conclusions

Overall, the results of the inhibition experiments suggest that concentrations of ethanol 25% (v/v) and higher will have a major impact on an aquifer microbial population in terms of both numbers and microbial activity, but that there is potential for recovery.

Chapter 4
Column Experiment

4.1 Objective and Approach

The objectives of the experiment were to determine the effect that ethanol has on an aquifer microbial community and its ability to degrade BTEX following ethanol exposure, and to observe the recovery process of the microbial community following exposure to an inhibitory ethanol concentration. To meet the objectives, a laboratory column experiment was designed to simulate a dynamic field situation where a pulse of an inhibitory ethanol concentration is followed by a BTEX plume.

The experiment was conducted at room temperature under aerobic conditions with excess oxygen. Experimental conditions were similar to the microcosm experiment (Chapter 2). Favourable conditions were chosen to simplify the study of substrate interaction and biodegradation processes without the interference of nutrient and oxygen limitations.

Initially two columns (Columns A and B) were packed with aquifer material from Borden and fed BTEX to establish an active BTEX-degrading microbial community within the column. Once degradation was observed, BTEX flow was halted and a 72 hour ethanol exposure began. The concentration of the ethanol pulse was 25% (v/v), which was expected to significantly alter the microbial population without destroying it, based on the results of the inhibition experiment (Chapter 3).

Following the ethanol exposure, sterile groundwater was fed to the columns to flush out the ethanol before BTEX flow resumed. The purpose of the flushing phase was to prevent ethanol degradation and oxygen exhaustion during the final BTEX phase. The resulting microbial community and subsequent BTEX degradation were assessed to observe the recovery process following ethanol exposure. One column (Column A) was fed with groundwater and BTEX to simulate the reinoculation of an ethanol-exposed aquifer segment with microorganisms from upgradient in the aquifer. The other column (Column B) was fed with sterile groundwater and BTEX to determine if the within-column microorganisms that survived the ethanol exposure could reproduce and return to detectable BTEX-degradation

without the importation of new microorganisms. This process is referred to in this study as regeneration. The stages of the experiment are summarized in Table 4.1.

The experiment was run twice. In the first experiment, sterility of the groundwater fed to Column B could not be maintained during the recovery phase. The experimental protocol was improved to minimize contamination in Experiment 2 and the second experiment included the addition of a sterile control column (Column C). The sterile control was intended to provide insight to the results if contamination reoccurred in the second experiment. A summary of the experimental treatments for each column are listed in Table 4.2.

Microbial community analyses were performed to investigate community changes related to ethanol exposure and recovery.

Table 4.1 Stages of the column experiment.

	Description	**Purpose**
Stage 1	Initial BTEX	Establish BTEX degradation.
Stage 2	Ethanol Exposure	Microbial inhibition expected, with potential for recovery.
Stage 3	Sterile Groundwater	Flush out ethanol.
Stage 4	Final BTEX (Recovery Phase)	Determine BTEX degradation capability. Observe recovery process.

Table 4.2 Summary of experimental treatments for the column study.

	Column A (reinoculation)	Column B (regeneration)	Column C[1] (sterile control)
Sand	ACTIVE[2]	ACTIVE[2]	STERILE[2]
Stage 1: Initial BTEX	ACTIVE	ACTIVE	STERILE
Stage 4: Final BTEX	ACTIVE	STERILE	STERILE

[1] Column C was present only in Experiment 2.
[2] Active and sterile refer to the state of the groundwater fed to Columns A and B in the stages indicated. Column C contained autoclaved aquifer material and was fed sterilized groundwater.

4.2 Methods and Materials

4.2.1 Design and Setup

4.2.1.1 Column Packing

The columns were constructed of stainless steel. The two original columns (Columns A and B) were 12 cm long with an inner diameter of 10.4 cm. The sterile control column (Column C) that was added later was 15 cm long with an inner diameter of 10.4 cm. Each column was capped with Teflon end plates and sealed with two Viton O-rings. One of the columns is shown in Figure 4.1a.

Fine and coarse stainless steel mesh screens were used at each end to separate the end plates from the packed column material. The fine screen was placed on the packed sand to prevent the loss of sediment from the column. The coarse screen was placed against the endplate to assist the endplate groove pattern (Figure 4.1b) in distributing influent solutions uniformly across the base of the column. Sampling ports located along the length of the column were plugged, as they were not used in this study.

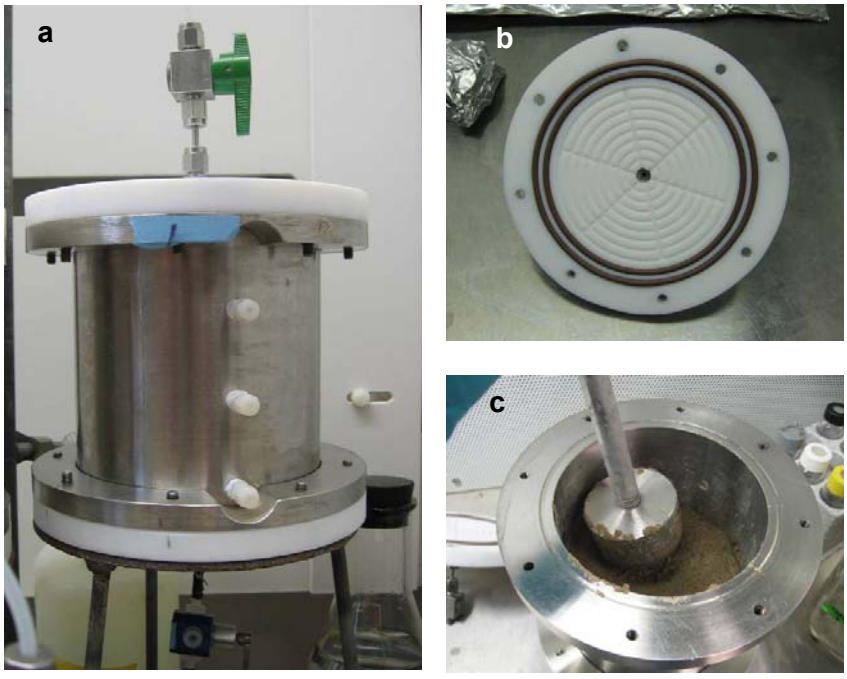

Figure 4.1 Photographs of a) an assembled column, b) the underside of an endplate showing the concentric grooves and Viton O-rings, and c) column packing.

The columns were packed with aquifer material from Borden (see Section 2.2.1 for details of collection and treatment prior to use). All column packing was done in a laminar flow clean bench using sterile equipment. Prior to packing, the two original columns and endplates were rinsed with methanol and allowed to dry overnight in the laminar flow clean bench. The bottom end plates and screens were secured and the columns were packed from the bottom upwards. The material was added wet in one tablespoon increments. After the addition of each tablespoon, the material was compacted using a 5 cm diameter stainless steel tamper (Figure 4.1c). After every 4-6 tablespoons, the material was encouraged to settle by striking the column sides with a heavy rubber mallet. Because the material was added wet, a pool of water developed at the top of the columns. This water was removed using sterile pipettes and the resulting space was packed with more sand. In future experiments, I would recommend leaving the bottom port open during packing to allow the material to drain. After packing was complete, the screens and top plate were secured. It should be noted that Columns A and B were not repacked for Experiment 2, despite being subjected to different treatments during Experiment 1.

The sterile column that was added for the second experiment, and its end plates, were sterilized by steaming for one hour, then wiping the surfaces with methanol. The aquifer material used in the sterile control column was sterilized by autoclaving for one hour on three consecutive days. The sterile control column was packed in the same manner as the others but the material was relatively dry from the autoclaving protocol.

After packing, columns were flushed with carbon dioxide (filtered through a 0.1 μm pore sized gas filter) for 2 hours to displace entrapped air. The two original columns were wetted using groundwater in an upward flow at a rate of 0.5 mL/min using a peristaltic pump and sterilized Teflon tubing with an inner diameter of 1.6 mm. These two columns were wetted for three days prior to tracer testing.

4.2.1.2 Tracer Test

A bromide tracer test was performed on each of the two original columns (Columns A and B) but not on the sterile control column (Column C). Tracer tests were conducted using groundwater amended with sodium bromide to a concentration of 70 mg/L bromide ion (Br^-),

equivalent to 90 mg/L sodium bromide solution. A pumping rate of 0.5 mL/min allowed for 10 mL samples of effluent to be collected over 20 minute intervals. Bromide concentrations were determined by measuring electrical potential using a bromide electrode and converting to concentration units using a standard curve (see Appendix H for bromide standard curve).

The breakthrough data were fit to the simplified Ogata-Banks solution for one-dimensional flow with advection and dispersion (Domenico and Schwartz, 1998) (see Appendix H for breakthrough curve and calculations). From the breakthrough curve, the dispersion coefficient was estimated to be 0.003 cm^2/h, and the effective porosity was estimated to be 0.32, yielding a pore velocity of 1.1 cm/h. One pore volume was displaced every 11 h.

4.2.1.3 Setup

A photograph and a schematic of the column experiment set up are displayed in Figures 4.2 and 4.3, respectively. The figures specifically represent the setup for Stages 1 and 4 of the second experiment. All tubing that was used was 1.6 mm inner diameter Teflon tubing with Viton connector tubing and stainless steel Swagelok fittings. To control flow rates, peristaltic pumps were used with 2.06 mm inner diameter Viton pump tubing. All tubing and fittings were sterilized prior to use in each stage.

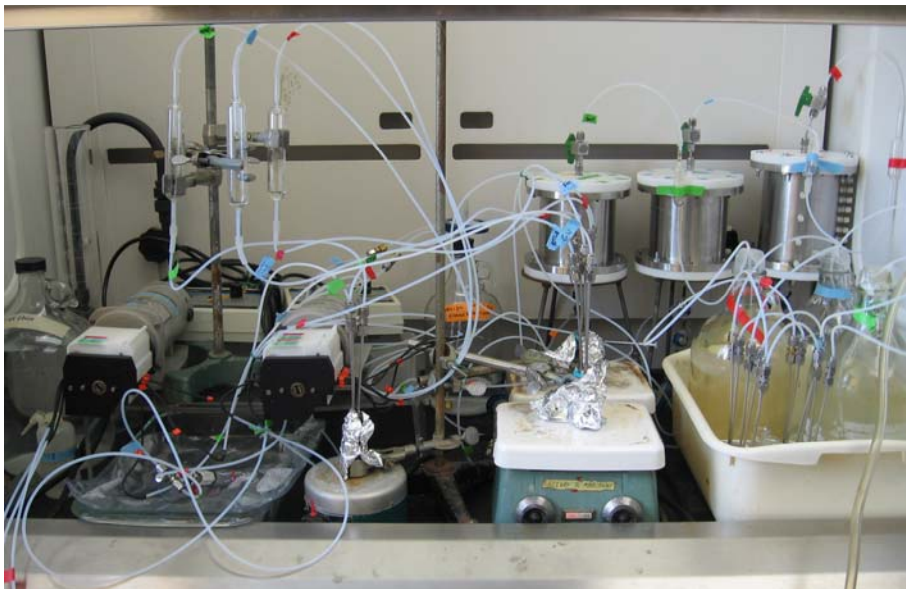

Figure 4.2 Photograph of the column experiment setup for Stages 1 and 4 of the second experiment.

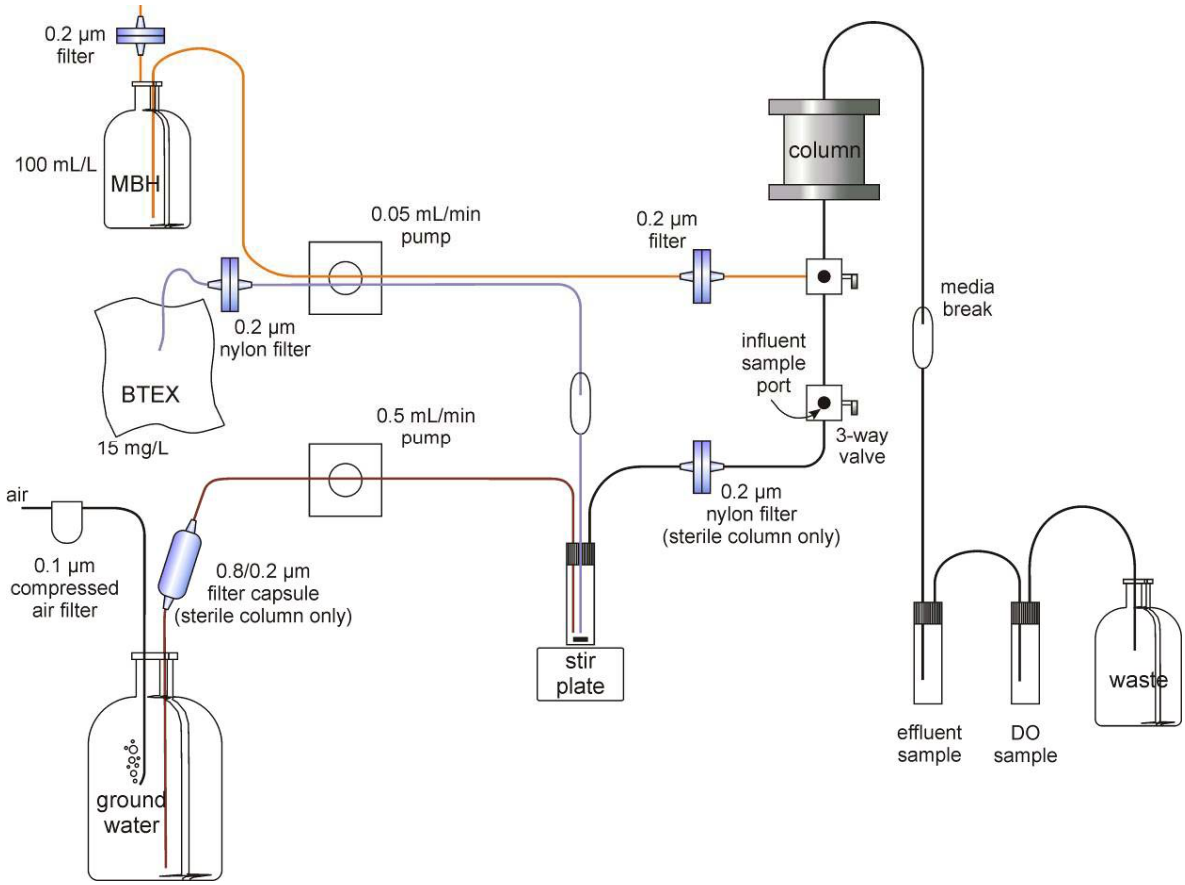

Figure 4.3 Column experiment setup for Stages 1 and 4 of the second experiment.

4.2.1.4 Groundwater (Stages 1, 3 and 4)

During column operation, groundwater was stored in 10 or 20 L glass carboys. For columns requiring sterile groundwater, groundwater was autoclaved. In the second experiment, groundwater was filtered prior to autoclaving with 0.22 μm cellulose nitrate membranes to remove particles and bacteria that may clog in-line filters.

Active and sterile groundwater source carboys were constantly aerated with filtered air to ensure oxygen saturation. The pore size of the air filter in the first experiment was 0.3 μm, which was upgraded to 0.1 μm for the second experiment.

Active and sterile groundwater were pumped separately at a rate of 0.5 mL/min. As an extra precaution in the second experiment, sterile groundwater was filtered further as it was pumped out of the source carboy using an in-line filter capsule with a 0.2 μm Supor membrane and a 0.8 μm prefilter. A 0.22 μm filter was also added to the sterile groundwater lines between the mixing chamber and the column influent port.

4.2.1.5 BTEX (Stages 1 and 4)

The source of BTEX to the columns was prepared from a neat stock of BTEX with relative proportions of BTEX compounds similar to the gasoline-saturated water used in the microcosom experiment (Chapter 2). The neat stock was diluted with sterile groundwater to produce an aqueous BTEX solution with a target concentration of 15 mg/L. The aqueous solution was stored in collapsible 4L Tedlar bags to avoid volatilization as the solution was used. The bags were sterilized prior to use by rinsing several times with methanol and drying with filter-sterilized air using a dull cannula. Only one BTEX bag was connected to the system at a time during operation and was replaced as necessary.

The BTEX and groundwater were pumped separately to minimize premature BTEX degradation within the lines, particularly when active groundwater was used. BTEX was pumped from the bag at a rate of 0.05 mL/min. The ratio of the pumping rates of BTEX to groundwater was 1:10, and the solutions were mixed to dilute BTEX further to a target concentration of 1.5 mg/L before entering the column. The mixing chamber consisted of a 25 mL glass flow-through vial equipped with a stainless steel sampling head sealed with a Viton O-ring. The chamber was covered with foil to prevent photodegradation. The

solutions were gently mixed on a stirplate using a magnetic stir bar, and then pumped into the column. A 3-way valve situated before the column input served as a sampling port for collecting influent solution for analysis.

A media break was located in each line between the BTEX source bag and the mixing chamber to prevent source bag contamination due to backflow. In the second experiment, extra precautions taken to minimize contamination included filtering the neat BTEX stock with a 0.22 μm sterile nylon filter and filtering the aqueous BTEX stock in-line between the source bag and the mixing chamber with a 0.22 μm nylon filter.

4.2.1.6 Nutrients (Stages 1 and 4)

Inorganic nutrients were initially omitted from the experiment to determine if BTEX degradation could be established in Stage 1 without nutrient amendment. Little degradation occurred so it appeared that nutrients were necessary. Therefore, beginning on Day 21 of Experiment 1, modified Bushnell-Haas medium (MBH; refer to Section 2.2.1) was added to the groundwater source carboy at a rate of 10 mL/L. This method resulted in excessive BTEX degradation within the lines and mixing chambers before the column. Consequently, a different approach involving a separate MBH source bottle was introduced on Day 30 with separate MBH lines delivering nutrients immediately before the column (after the influent sample port). A 100 mL/L MBH source solution was prepared and pumped at a rate one tenth of the groundwater pumping rate for a dilution to a final application rate of approximately 10 mL/L. The MBH source bottle approach was also used for the second experiment with the addition of sterile 0.22 μm filters in the MBH lines before the column and in the bottle neck to filter air that entered the source bottle headspace as the solution was pumped out.

4.2.1.7 Ethanol (Stage 2 and 3)

Pure ethanol was filter-sterilized with 0.22 μm nylon membrane filters and diluted with sterile groundwater to a concentration of 25% (v/v), equivalent to 197250 mg/L.

During Stage 2, 25% (v/v) ethanol was pumped into the columns at a rate of 0.5 mL/min until the ethanol concentration of the effluent was equal to the input

concentration (approximately 19 hours). The column ports were closed to allow static ethanol exposure for approximately 53 hours, resulting in a total exposure of 72 hours.

During Stage 3, sterile groundwater was pumped to all columns at a rate of 0.5 mL/min until ethanol was removed.

In the second experiment, 0.22 μm nylon filters were installed in-line before the column inlet for Stages 2 and 3 to reduce the risk of contamination.

4.2.1.8 Effluent (Stages 1 and 4)

Column effluent was collected in a series of two flow-through sample vials. The first was used for BTEX analysis and the second was used for dissolved oxygen analysis. The overflow was collected in a waste jug. The volumes of the samples and waste were measured to determine the discharge rate. The average measured discharge rate was 0.59 mL/min.

For the second experiment, a media break was added to the effluent lines before the sample vials to prevent contamination of the column due to backflow from the effluent sample and waste system.

4.2.2 Sampling and Analysis

4.2.2.1 BTEX and Dissolved Oxygen (Stages 1 and 4)

During Stages 1 and 4, column influent and effluent were sampled to monitor BTEX degradation.

Influent samples were collected in 60 mL glass syringes which connected to a luer-lok adapter at the influent sampling port. The syringe was allowed to fill at the normal operating flow rate so as not to disturb the mixing ratio of groundwater and BTEX. A portion of the influent sample was reserved for BTEX analysis in 25 mL (or 40 mL) glass vials with screw caps and Teflon-lined septa. Samples for BTEX analysis were stored without headspace and preserved with 0.25 mL (or 0.4 mL for 40 mL samples) of 10% sodium azide solution. The samples were stored in the refrigerator until analyzed (up to 5 days). The remaining influent sample (approximately 20-25 mL) was analyzed for dissolved oxygen.

Effluent samples were collected from two 25 mL (or 40 mL) flow-through glass sample vials. The second vial in sequence was disconnected first and immediately analyzed for dissolved oxygen to minimize oxygen addition to the sample. The first vial was then disconnected and the sample was preserved with 0.25 mL (or 0.4 mL for 40 mL samples) of 10% sodium azide solution and stored without headspace in the refrigerator until analyzed (up to 5 days). Effluent samples were collected before influent samples so that the effluent samples would not be disturbed by the stop of flow to the columns during influent sampling.

The dissolved oxygen (DO) samples were analyzed immediately after collection using a DO probe. A single point calibration was performed each sampling day prior to use using a water-saturated air calibration sleeve.

Aqueous BTEX concentrations were determined with a solvent extraction technique (modified from Henderson et al., 1976). Samples (19 mL) were extracted with 1 mL of dichloromethane, and the extract was analyzed using a Hewlett Packard 5890 gas chromatograph (GC). The GC was equipped with a splitless injection port, DB5 capillary column and a flame ionization detector. The GC was calibrated with external standards and the calibration was checked each sampling day using prepared standards. The detection limits for the BTEX compounds using this method range from 1.1 μg /L to 2.3 μg /L.

4.2.2.2 Ethanol (Stages 2 and 3)

Ethanol concentrations were measured by GC following the same method as in Section 2.2.2.2. Samples were collected for ethanol analysis during the Stage 2 ethanol exposure period to ensure both influent and effluent concentrations were 25% before static exposure began. Influent samples were collected directly from the 25% ethanol source stock solution. Effluent samples were collected directly from the end of the output tubing. The samples were diluted with sodium azide treated deionized water to within the detection range of the GC (0.05 to 200 mg/L). Diluted solutions were transferred to 2 mL glass autosampler vials and analyzed immediately by GC.

During the ethanol removal stage (Stage 3), sterile groundwater was fed to the columns and output samples were collected to monitor the concentration of ethanol in the effluent. Once the effluent concentrations were below detection (<0.05 mg/L), the final BTEX

treatment phase (Stage 4) could begin. For both experiments, five days were required for Stage 3.

4.2.2.3 Sterility Checks

The sterility of column influent solutions was monitored throughout the experiments (occasionally in Experiment 1 and regularly in Experiment 2). Samples were collected from the influent sample ports using 1 mL sterile syringes and plated on R2A agar. Plates were incubated for one week at room temperature. In the second experiment, the sterility of each batch of source solution (groundwater, BTEX, nutrients and ethanol) was also checked using the same method.

4.2.2.4 Microbiology

4.2.2.4.1 Sampling for Microbiology

A sample of the pristine aquifer material was reserved at the time of column packing. Aquifer material from the columns was collected before and after the 72 hour ethanol exposure and at the end of the final experiment. Sampling was conducted in a laminar flow clean bench using sterile equipment. The columns were closed off, the bottom end plates were removed and approximately 6 g of material was collected from the inlet of each column, where much of the microbial activity was expected to occur. Samples were removed with a spatula, transferred to sterile tubes and stored in a -80 °C freezer until analyzed by denaturing gradient gel electrophoresis (DGGE). The excavated material was replaced with sterile material (autoclaved for one hour on three consecutive days) then the columns were reassembled.

At the end of the final experiment, aquifer material was collected from the centre of the columns. The material of 1 cm thickness lining the column walls was discarded. The 12 cm length of material in each column was separated into three sections: 1 cm bottom sample, 3 cm middle sample, and 8 cm top sample. The extra 3 cm at the top of the 15 cm long Column C was discarded.

Approximately 6 g of the bottom samples were stored in sterile tubes in a -80 °C freezer for DGGE analysis. The remaining bottom samples plus the middle and top samples were reserved for most probable number (MPN) analysis.

4.2.2.4.2 Enumeration of BTEX Degraders – MPN Test

Microbial enumeration of the BTEX degrading population was performed using a three-tube MPN analysis. The MPN test is a statistical method used to estimate the number of bacteria in a sample based on the number of tubes in a dilution series showing evidence of growth (Mayou, 1976). Samples analyzed by this method were the pristine aquifer material and samples from the bottom, middle and top of each column at the end of the second experiment.

Microorganisms were extracted from 10 g samples of aquifer material by dilution in 90 mL of 0.1% sodium pyrophosphate followed by shaking for 10 minutes on a rotary shaker at approximately 350 rpm. Extracted samples were serially diluted by orders of magnitude down to 10^{-7} in phosphate buffered water. Each dilution supplied the inoculum (1 mL) to three 25 mL tubes containing 18 mL of BTEX-containing medium. The BTEX medium was prepared by adding 85 μL of neat BTEX stock to 4.3 L of MBH nutrient media and the actual concentration of the solution was measured as 16.5 mg/L. Sixteen non-inoculated controls were prepared – eight at the start and eight at the end of the test setup. Following a three week incubation period in the dark at room temperature, BTEX concentrations in the tubes were analyzed by GC. Tubes were ranked as positive or negative for BTEX degradation, where ≥50% loss of BTEX was considered positive. The most probable number of BTEX degraders, including 95% confidence intervals, was determined from a three-tube statistical table (Mayou, 1976).

4.2.2.4.3 Microbial Community Analysis – DGGE Test

Denaturing gradient gel electrophoresis (DGGE) was used to analyze the microbial community at different stages of the experiments and to observe shifts in the microbial community structure. DGGE is a molecular technique based on the electrophoresis of PCR-amplified DNA fragments of the 16S rRNA gene (Muyzer et al., 1993). DNA from various organisms is separated on a polyacrylamide gel with an increasing gradient of denaturants. As the DNA fragments are subjected to denaturing conditions, the double-stranded DNA

partially melts depending on its sequence composition, and migration through the gel is retarded. The exact base sequence of the 16S rRNA gene will differ between organisms, affecting migration through the gel and permitting separation of the DNA. The resulting banding patterns can be used to compare microbial communities. The number and intensity of the bands also provides a measure of the genetic diversity of a community (Kirk et al., 2004). Individual bands can be extracted and sequenced to identify organisms in the community based on known bacterial sequences in public databases (Haines et al., 2002).

The DGGE analysis was performed by Microbial Insights, Inc. (Rockford TN). Only the most prominent bands of each sample (maximum of 6) were excised and sequenced. Sequence identification was based on comparison to the Ribosomal Database Project (RDP) (Maidak et al., 2001).

4.3 Results and Discussion

4.3.1 BTEX

BTEX results for Columns A and B in Experiment 1 are displayed in Figure 4.4. The first 21 days of Stage 1 were nutrient-free and a steady loss of BTEX in the columns (observed as the difference between influent and effluent concentrations) may indicate either a small degree of biodegradation or abiotic loss. There was no sterile control to verify abiotic loss.

A steady decline in influent BTEX concentrations suggested that BTEX loss was occurring in the BTEX source bag. The occasional sharp increase in influent concentration (e.g., after Day 14, 30 and 56) coincides with the replacement of depleted BTEX source bags.

Between Days 21 and 30, both influent and effluent BTEX concentrations reached zero. During this time, nutrients were added directly to the groundwater source so it is assumed that influent concentrations of zero were the result of premature degradation in the lines and mixing chamber before entering the column.

After the nutrient addition technique was changed and the separate nutrient source was instituted on Day 30, the influent concentrations returned to acceptable values and complete BTEX degradation was established within both columns prior to ethanol exposure.

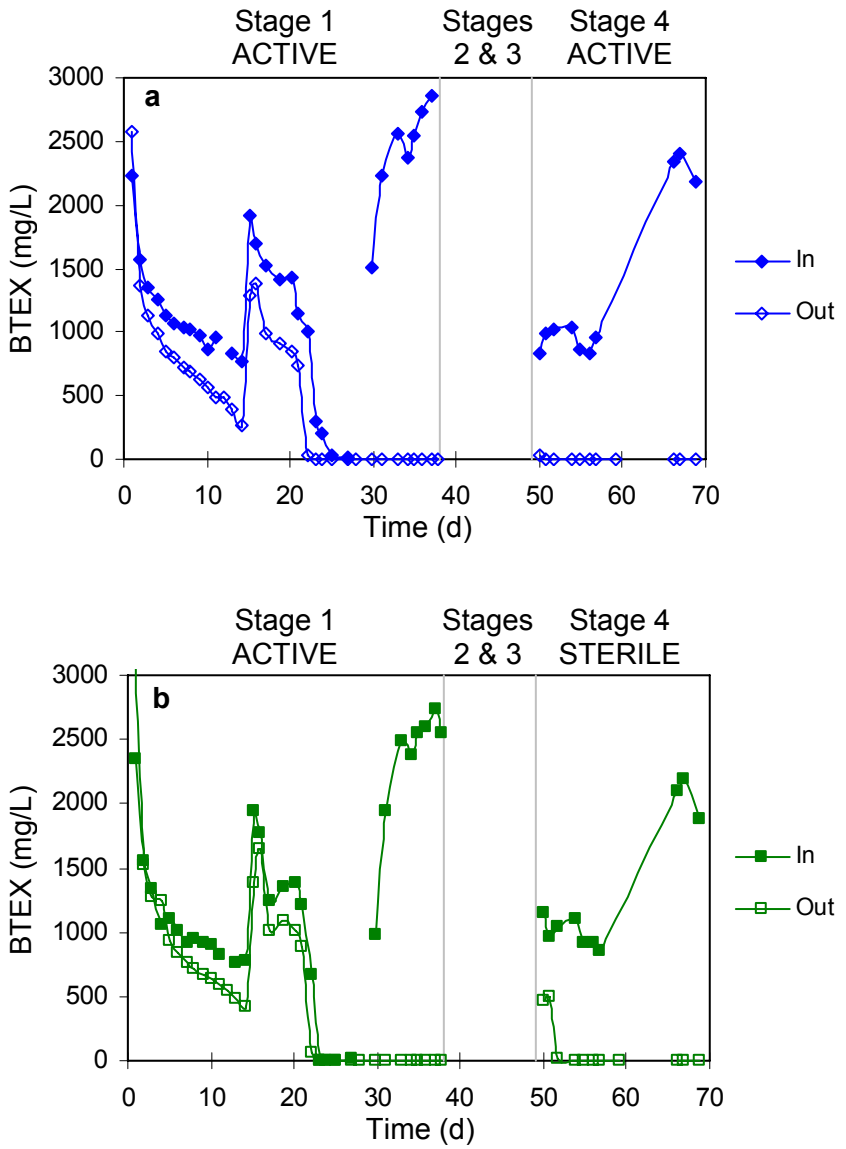

Figure 4.4 Influent and effluent BTEX concentrations in Experiment 1 for a) Column A and b) Column B.

During Stage 4, after ethanol exposure, complete recovery of BTEX degradation was observed within two days in Column A and four days in Column B. However, after one day of Stage 4 treatment, influent contamination was observed during R2A plate sterility checks. It was confirmed through testing of the contaminated influent that the microbial population present was capable of BTEX degradation (data not shown). The experiment was deemed flawed and improvements were made for the second experiment.

For Experiment 2, BTEX degradation in Stage 1 for Columns A and B was easily re-established (Figure 4.5). It is likely that active BTEX-degraders were still populating the columns from Experiment 1.

The difference between influent and effluent concentrations in the sterile column C was consistent during Stage 1, and the loss was interpreted to be abiotic. Abiotic loss may be attributed to sorption to aquifer material in the columns; however, the low organic carbon content of Borden sand suggests that sorption would be minimal. The loss is more likely due to volatilization into the headspace of the media breaks added for the second experiment between the column outlet and the effluent sampling system.

Influent concentrations were more stable throughout Experiment 2, suggesting that the in-line filters and extra efforts to prevent contamination in the BTEX source bag and lines were beneficial. One unusually high influent concentration was measured during Stage 4 (Day 20) for Column B. This anomaly is attributed to equipment error, likely due to difficulties with leaking and clogging filters during sampling.

During Stage 4, following ethanol exposure, recovery (in terms of BTEX degradation) was rapid in both Columns A and B. Of the individual BTEX compounds, ethylbenzene was the first to disappear in each column and benzene was the last to degrade in each column. Complete BTEX degradation resumed within 5 days for the column receiving active groundwater (Column A). The column receiving sterile groundwater (Column B) recovered slower than active Column A, but still returned to complete BTEX degradation within 10 days. However, as in Experiment 1, influent sterility could not be maintained past the second day of Stage 4. As a result, recovery by regeneration could not be evaluated.

The BTEX degradation response in the sterile Column C during Stage 4 was similar to Columns A and B, implying that the column was in fact not sterile. The lack of sterility in Column C may be due to influent contamination (which was detected after the second day of Stage 4) or to microbial survivorship within the column. After 10 days, approximately 94% of the BTEX was being degraded.

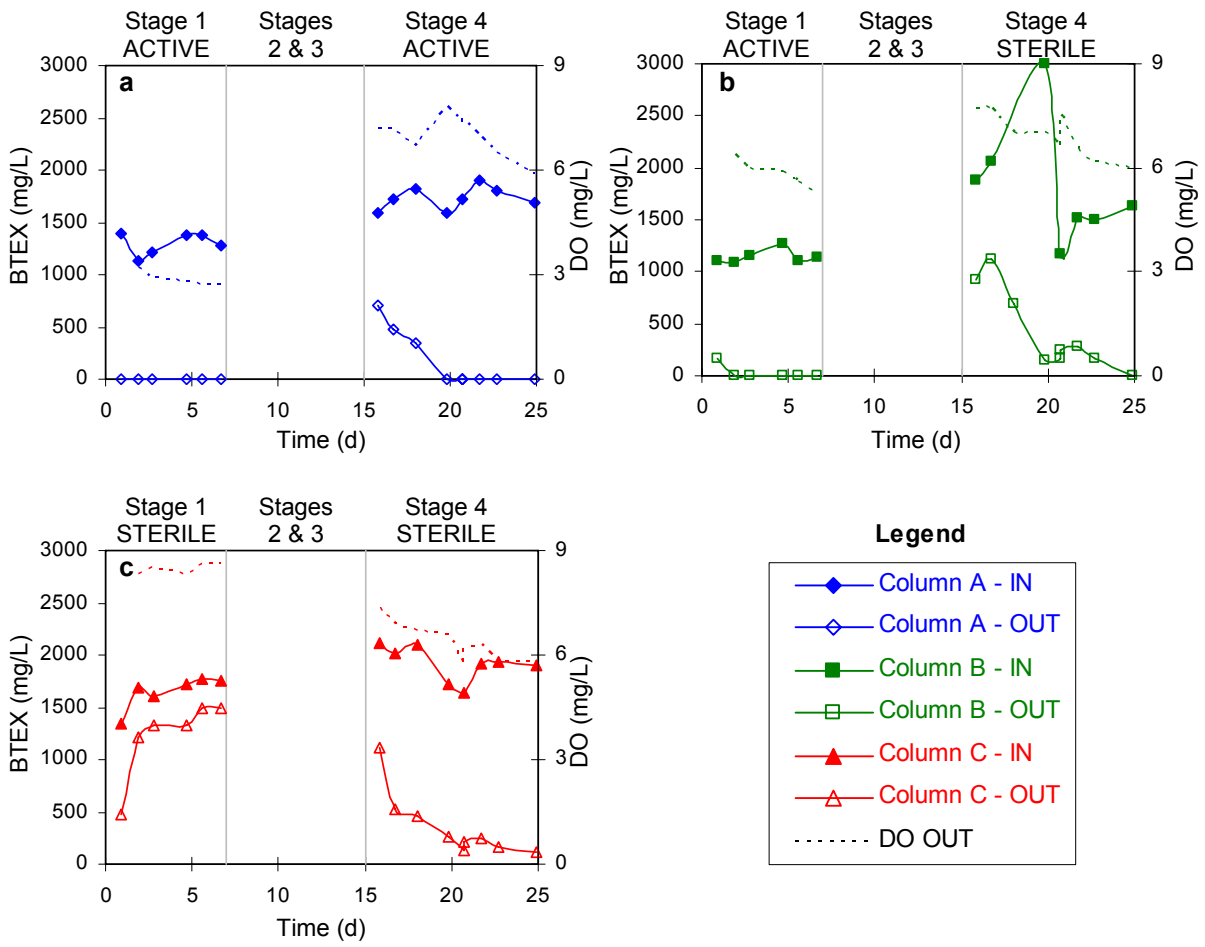

Figure 4.5 Influent and effluent BTEX concentrations and effluent dissolved oxygen concentrations in Experiment 2 for a) Column A, b) Column B, and c) Column C.

Plates of the contaminated influents on R2A agar (Figure 4.6) suggest that the overall culturable populations of each influent appear different, based on the colonies formed. All colonies were round and elevated with a smooth surface. White colonies developed on the plates of all three influents; however, Column B plates also revealed yellow and orange colonies. Column C plates consisted mainly of white and yellow colonies with one or two orange colonies per plate. The colonies were not unusual for groundwater samples (Butler, personal communication, 2007). The fact that Columns B and C appear slightly different, despite having the same groundwater source, may suggest contamination of the lines or influent sampling ports.

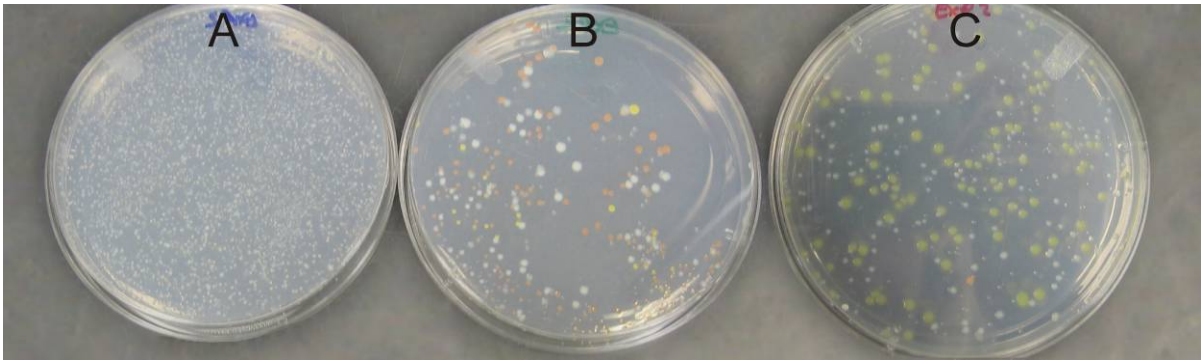

Figure 4.6 Examples of colonies formed from influent solutions for Column A, Column B, and Column C. Samples were collected on Day 20 of Experiment 2 and colonies were grown on R2A agar during an incubation of one week at room temperature.

The rapid recovery of BTEX degradation reflects the ubiquity of BTEX degrading microorganisms in the subsurface environment. It may also imply that under favourable conditions, the microbial community retains a significant degree of viability during 25% ethanol exposure. Faster-than-expected recovery of a waste-water treatment process was observed by Speece et al. (1986) when the toxicity of a wide range of toxicants was studied. Although ethanol was not included in the study, they found a similar generic response pattern which was typical for a variety of toxicants.

Dissolved oxygen concentrations measured throughout the experiments suggest that aerobic conditions were maintained. In Experiment 2, the DO of the influent was always close to saturation, with an average of 8.7 mg/L for all columns. Effluent DO concentration decreased concurrently with BTEX loss (Figure 4.5), but in no case was the oxygen consumption stoichiometrically sufficient to account for BTEX mineralization. Assuming partial biodegradation, an attempt was made to measure breakdown products in the effluent using a method modified from Cozzarelli et al. (1994), however, none were detected (detection limit approximately 50-100 μg /L (Chatten, personal communication, 2007)). It is also reasonable to assume that a significant proportion of the carbon from BTEX was transformed to microbial biomass, instead of being converted to mineralization products. The oxygen needed for incorporation of carbon into biomass is likely less than that required for mineralization. In general, however, it is difficult to predict the percentage of carbon substrate incorporated into biomass as it depends on the organisms involved and is quite variable (Alexander, 1994).

Microbial activity within the sterile Column C was confirmed in a most probable number (MPN) test (Figure 4.7). The numbers of BTEX degraders estimated in all three columns at the end of the experiment were comparable and ranged from 1-3 orders of magnitude greater than the initial population. The 95% confidence intervals for each point were characterized by roughly 10-fold margins about the MPN. Complete MPN results are provided in Appendix I.

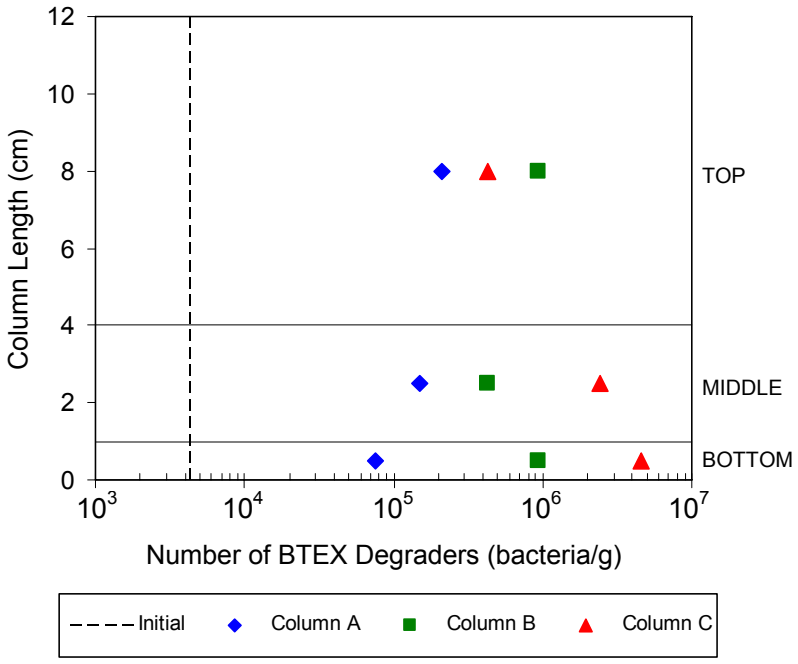

Figure 4.7 Most probable number values for BTEX degrading bacteria at the end of Experiment 2. Samples were collected from the bottom, middle and top of each column. Dashed line (labeled as "initial") represents the MPN value in the pristine aquifer material used to pack the columns.

4.3.2 Microbial Community Analysis

The results of the microbial community analysis using DGGE are given in Figure 4.8. Each banding pattern provides a profile of the community of that sample. Visible bands represent organisms that constitute at least 1-2% of the total bacterial community (Muyzer et al., 1993) and band intensity is an indication of an organism's dominance.

The bands labeled in Figure 4.8 were excised and sequenced for further analysis. The sequences were identified by matching to reference sequences provided in databases, and similarity indices denote the adequacy of matches. The range of reported similarity indices was 0.701 to 1.00, where indices above 0.900 are considered excellent and 0.700-0.800 are

good. Based on information provided by Microbial Insights, the bands were tentatively identified as listed in Table 4.3. When interpreting DGGE profiles, it should be kept in mind that one band may not necessarily represent one organism type. One band may originate from several different DNA sequences which happen to have similar denaturing behaviour (Gelsomino et al., 1999). However, each band is matched with only one sequence. Therefore, for this study, each band that was analyzed was identified as only one bacterial type. Complete DGGE results are provided in Appendix J.

All sequences identified were gram-negative, aerobic bacteria commonly found in soil. The communities consisted almost entirely of α- and β-*Proteobacteria*, and many of the bacteria identified are capable of hydrocarbon degradation or have been associated with hydrocarbon contaminated sites (Cavalca et al., 2004; Fahy et al., 2006; Fredrikson et al., 1995; Hendrickx et al., 2005; Kane et al., 2007; MacNaughton et al., 1999; Röling et al., 2002).

There were fewer bands visible in the initial pristine sand sample 1 (11 ± 1) than in the column samples 4-9, where the number of bands ranged from 14 ± 1 in sample 6 to 16 ± 1 in sample 4.

The banding patterns of samples 4 and 5 taken after the Stage 1 BTEX treatment, just before ethanol exposure began, are quite different from the initial pristine aquifer material sample 1. Little was revealed about the identity of the original community except that it contained *Hydrogenophaga spp*. The Stage 1 BTEX treatment resulted in more prominent bands and favoured the selection of three dominant organisms in each column. The profiles of the two columns after the Stage 1 BTEX treatment (samples 4 and 5) differ in some of the minor bands and some of the dominant bacteria (4.1 *Erythrobacter spp*. contrasting 5.1 *Chitinophaga spp*.), but have some similarities. Some of the minor bands are similar and both communities include a dominant *Sphingomonas spp*. Although both columns were treated the same for Stage 1 of Experiment 2, community differences at this point may be attributed to the previous difference in treatments during Experiment 1.

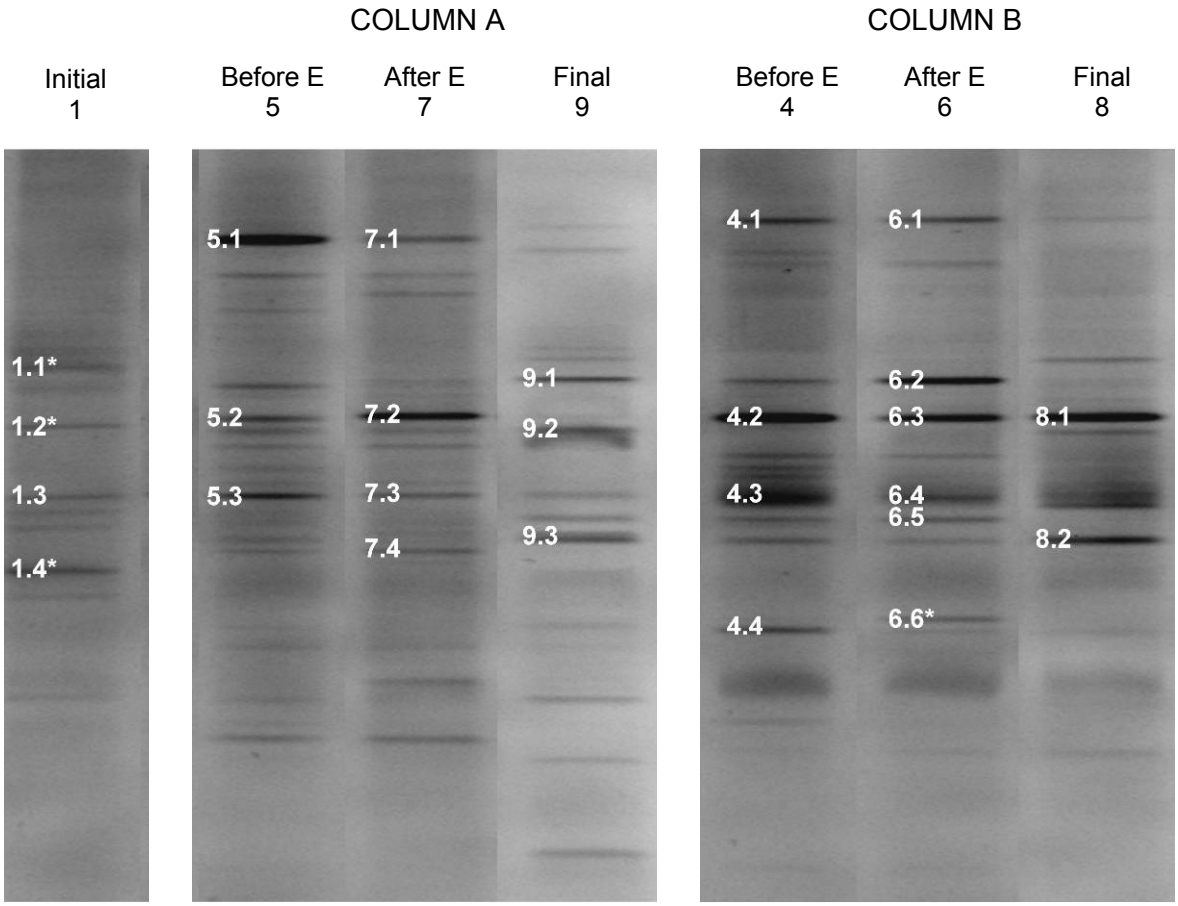

Figure 4.8 DGGE profiles of amplified 16S rRNA gene fragments from Column Experiment 2. Sample (1) is pristine Borden aquifer material. Samples 5, 7 and 9 are from Column A: (5) between Stages 1 and 2, (7) between Stages 2 and 3, and (9) after Stage 4. Samples 4, 6 and 8 are from Column B: (4) between Stages 1 and 2, (6) between Stages 2 and 3, and (8) after Stage 4. All labeled bands were excised and sequenced and correspond to the list in Table 4.3. Bands marked with an asterisk were not identified because they failed to match any reference sequence.

Table 4.3 Sequence identities of the dominant DGGE bands appearing in Figure 4.8.

Band	**Phylogenetic identification**
1.3, 6.4	*Hydrogenophaga spp.*
4.1, 4.2	*Erythrobacter spp.*
4.3, 5.2, 5.3, 6.3, 6.5, 8.1, 9.2	*Sphingomonas spp.*
4.4	*Bradyrhizobium spp.*
5.1, 7.1	*Chitinophaga spp.*
6.1, 7.3	*Methylibium spp.*
6.2, 9.1	*Acidovorax spp.*
7.2	*Zoogloea spp.*
7.4	*Nitrospira spp.*
8.2	*Caulobacteraceae (family)*
9.3	*Caulobacter spp.*

For both columns there appear to be similarities in the banding patterns before and after ethanol, with the major difference being a change in the intensity of some of the bands. For example, bands 6.2 and 7.2 are darker than their corresponding bands in samples 4 and 5, respectively. Bands 6.1 and 6.4 became fainter than similar bands in sample 4, and bands 7.1 and 7.3 were less intense than bands 5.1 and 5.3. Based on the profiles before and after ethanol exposure, it is difficult to determine whether 25% ethanol was sufficient to reduce the populations as severely as expected (based on the results from the inhibition experiment). Perhaps under the conditions of the column experiment, the inhibitory effect of 25% ethanol was less severe than was inferred from the inhibition experiments in Chapter 3. A possible explanation for this discrepancy is related to ethanol dosage. The ratio of aquifer material to 25% ethanol solution was approximately 1:0.3 for the column experiment (based on porosity), and 1:0.6 for the inhibition experiment. The columns received about half of the dose of ethanol per gram of aquifer material compared to the inhibition experiment. Furthermore, the number of microbes present in the column at the start of the ethanol exposure was much greater than that in the initial pristine aquifer material used in the inhibition experiment (as indicated by the MPN results in this chapter). Therefore, the dosage of ethanol per microbial cell is expected to have been much lower in the column experiment, and may have diminished the toxic response compared to the inhibition experiment. It must also be considered that DGGE is not a quantitative technique, and band intensity may only reveal relative differences between the number of certain bacterial types within the community. It is recommended that conclusions about the number of total microbes before and after ethanol exposure be drawn from a quantitative analysis, such as quantitative real-time PCR, MPN or plate count estimates.

The overall community profiles of each column were significantly altered during the Stage 4 final BTEX treatment. That is to say the patterns of samples 8 and 9 are quite different from the patterns of 6 and 7, respectively.

Although the two columns were treated differently during Stage 4, the two final communities display some commonalities, particularly with respect to the dominant constituents. Bands 8.1 and 9.2 were both identified as *Sphingomonas spp*., and 8.2 and 9.3 are both *Caulobacteraceae*-affiliated bacteria. It appears that during Stage 4, regardless of

the treatment, the community did not return to the pre-ethanol BTEX-degrading community, but was similarly effective at BTEX degradation.

4.4 Summary of Conclusions

The experimental system of the column experiment was difficult to keep sterile; therefore recovery of surviving microorganisms by regeneration could not be evaluated. The recovery of aerobic BTEX biodegradation was fast in all columns, although it was faster for the column receiving active groundwater than for the column receiving sterile groundwater. Ethanol at a concentration of 25% did not appear to cause a major, long-term change in BTEX biodegradation potential. The microbial community of each column was altered after exposure to 25% ethanol and the resulting population had the ability to degrade BTEX.

Chapter 5
Implications of the Research

The laboratory experiments that compose this research effort were designed to investigate some principles of the effect of ethanol on BTEX biodegradation and were not intended to serve as field analogues. The experimental conditions, which included excess oxygen and nutrients, are not conditions realistically expected to be encountered in most field situations. In addition, behaviour in the field will be site-specific. However, the expectation is that the results may provide some insight into potential field performance.

In microcosm experiments (Chapter 2), ethanol concentrations ≤15000 mg/L (1.9% v/v) had a minimal impact on benzene, toluene and ethylbenzene degradation, but xylene isomers were more persistent in the presence of 5000 mg/L and 15000 mg/L ethanol. Based on the results of the microcosm experiment, it appears that ethanol concentrations up to 15000 mg/L are unlikely to inhibit subsurface microbial activity in terms of aerobic BTE degradation. These conclusions are contrary to those from studies conducted under oxygen-limited conditions (Araújo, 2000; Hunt et al., 1997), where BTEX degradation was inhibited by ethanol concentrations less than 15000 mg/L. Therefore oxygen limitations in the field are expected to influence the response.

Several microcosm studies reported that ethanol was preferentially degraded before BTEX (Deeb et al., 2002; Corseuil et al., 1998; Hunt et al., 1997), but results here suggest the potential for simultaneous degradation of ethanol and BTEX to occur. In the microcosms containing 5000 mg/L and 15000 mg/L, nutrient addition was followed by a slightly increased rate of both ethanol and xylene degradation. This response suggests that ethanol-degraders and xylene-degraders were equitably exploiting nutrients. At a field scale, this impartiality could be beneficial for remediation; as long as an electron acceptor and nutrient supply is ensured, the ethanol and BTEX may be expected to degrade simultaneously.

Higher concentrations of ethanol are likely to have a significant impact on microbial populations in the subsurface. Although concentrations between 25% and 75% (v/v) are expected to be toxic to aquifer microorganisms, the potential for complete sterilization of the affected area is low. In inhibition experiments (Chapter 3), ethanol concentrations of 25%

and 32% resulted in drastic reductions in the number of bacteria present and concentrations of 50% and 75% had an even more severe impact on the numbers of bacteria. For each concentration, the number of colonies grown on R2A plates was <50 CFU/g dry weight aquifer material, whereas ethanol-free samples yielded 1.3 x 10^5 CFU/g dry weight aquifer material. Microbial activity was disrupted by 32% ethanol and recovery was observed for one duplicate. The results suggest that ethanol concentrations of 25%-75% are likely to cause significant short-term damage to aquifer microbial populations and activity. They also suggest that there is potential for a few survivors and subsequent recovery of microbial activity by regeneration of the surviving population. This suggests that microbial activity can be reestablished in the field once ethanol levels in an area are reduced by advection and dispersion to less inhibitory concentrations.

The recovery response following ethanol exposure was observed in the column experiment (Chapter 4). Recovery in terms of aerobic BTEX degradation after exposure to 25% ethanol was rapid (less than 10 days). Although recovery by regeneration could not be evaluated due to sterility issues, it was evident that the overall recovery was significant. In a field situation, even if sufficient oxygen and nutrients were provided, the reestablishment of aerobic BTEX-biodegrading microbial activity is expected to be slower than in the column experiments due to slower groundwater flow rates and colder temperatures.

The recovered community composition was significantly different from the community before ethanol exposure even though both communities were capable of BTEX degradation and this may reflect the ubiquity of aerobic BTEX-degrading microorganisms in the environment. In the field, a BTEX-degrading community that is disturbed by a high concentration of ethanol may be replaced by a new, different community from infiltrating groundwater. The new community is likely to be different from the original community, but BTEX degraders are so ubiquitous that the new community may be similarly effective at BTEX degradation.

Mocanu (2007) conducted a field experiment at CFB Borden where sources of E10 and E95 were emplaced below the water table. Although the overall field data have some uncertainty, maximum ethanol concentrations of 2370 mg/L and 12800 mg/L were measured approximately 3.5 m downgradient of the E10 and E95 sources, respectively. While higher

concentrations could have occurred locally, these concentrations are unlikely to have a great effect on microbial activity based on the research presented here.

Ethanol has the potential to inhibit BTEX biodegradation and alter microbial populations, but even at high concentrations, the impact to BTEX biodegradation may be short-term. Nutrient and electron acceptor limitations will presumably occur in field situations; however, this research may apply to spill scenarios in the capillary fringe or unsaturated zone of nutrient rich soils. Nonetheless, this research indicates that ethanol toxicity is not likely to cause prolonged inhibition of BTEX biodegradation for ethanol-amended gasoline spills at most sites. The most severe impact is likely to be the depletion of nutrients and electron acceptors used by the microbial community during biodegradation.

Chapter 6
Recommendations

As a result of this research, the following recommendations are made to direct future research and to improve similar experiments:

1) Since oxygen is likely to be limited at ethanol-amended gasoline spill sites, similar research should be conducted under anaerobic conditions to study the effect of ethanol on anaerobic BTEX biodegradation and on the anaerobic microbial community.

2) Prior to undertaking a column experiment requiring sterile conditions, some procedures should be improved to further minimize contamination. Suggestions include:

    a) Sterilization by gamma irradiation. An advantage of gamma irradiation is items may be pre-sealed before sterilization because of penetrating gamma rays. The use of gamma irradiation to sterilize the columns would allow for a control column to be packed with aquifer material prior to sterilization.

    b) Develop an aseptic procedure for column influent sample collection.

    c) Specific to my experiment, the duration of the sterile groundwater phase to flush out residual ethanol from the column (Stage 3) should be decreased. The experimental system can only be expected to remain sterile for a limited time. The pumping rate of sterile groundwater to the columns may be increased during this stage in order to minimize the duration.

3) A more comprehensive microbial community analysis should be undertaken. The molecular techniques used in this research are capable of detecting community perturbations (RFLP) and analyzing community composition (DGGE). In order to further study the effect of ethanol on aquifer microbial communities and on BTEX biodegradation, stable isotope probing may be used to determine which organisms are responsible for the biodegradation of ethanol and BTEX.

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Appendix A
Oxygen Demand Calculations

The following oxygen demand calculations were performed to provide insight into oxygen limitations in the microcosm experiment (Chapter 2).

A.1 Oxygen Demand from Ethanol – Air in Headspace

Microcosm headspace volume = 132 mL
Volume of O_2 in headspace from air = (132 mL)(0.21) = 27.7 mL

Based on the Gas Law, the number of moles of O_2 in headspace (n) was calculated:

$$PV = nRT \qquad n_{O_2 avail} = \frac{PV}{RT} = \frac{(1atm)(0.0277L)}{(0.0821L \cdot atm \cdot mol^{-1}K^{-1})(295K)} = 0.00114mol$$

Based on the stoichiometry of the mineralization of ethanol:

$$CH_3CH_2OH + 3O_2 \rightarrow 2CO_2 + 3H_2O$$

three moles of O_2 are required to mineralize one mole of ethanol.

For Series A and E (500 mg/L; the minimum concentration of ethanol used in the experiment), assuming no partitioning from the aqueous phase, and based on the molecular weight of ethanol, the number of moles of ethanol in the aqueous phase of the microcosms was calculated:

$$n_{EtOH} = \frac{(500mg/L)(0.100L)}{(46.07g \cdot mol^{-1})} \times \frac{g}{1000mg} = 0.00109mol$$

Hence, the mineralization of 500 mg/L ethanol in the microcosms requires:

$$n_{O_2 req'd} = 0.00109mol \cdot EtOH\left(\frac{3mol \cdot O_2}{1mol \cdot EtOH}\right) = 0.00326mol$$

The O_2 required (0.00326 mol) is greater than the O_2 available (0.00114 mol).

Therefore, the theoretical oxygen demand required for mineralization of ≥500 mg/L ethanol (Series A, B, C, E, F and G) was greater than the oxygen available from air in the headspace. So microcosm headspaces were purged with pure oxygen (see Part A.2). The oxygen demand from 15 mg/L BTEX was considered negligible (see Part A.3).

A.2 Oxygen Demand from Ethanol – Pure Oxygen in Headspace

Volume of pure O_2 in headspace = microcosm headspace volume = 132 mL
Based on the Gas Law, the number of moles of O_2 in headspace (n) was calculated:

$$PV = nRT \qquad n_{O_2 avail} = \frac{PV}{RT} = \frac{(1atm)(0.132L)}{(0.0821L \cdot atm \cdot mol^{-1}K^{-1})(295K)} = 0.00545mol$$

Based on the stoichiometry of the mineralization of ethanol (see Part A.1), the maximum number of moles of ethanol (and mass and concentration) that can be mineralized in the microcosm with pure O_2 in the headspace is:

$$n_{EtOH} = 0.00545 mol \cdot O_2 \left(\frac{1 mol \cdot EtOH}{3 mol \cdot O_2} \right) = 0.00182 mol$$

$$M_{EtOH} = (0.00182 mol)(46.07 g \cdot mol^{-1}) \times \frac{1000 mg}{g} = 83.7 mg$$

$$C_{EtOH} = \frac{83.7 mg}{0.100 L} = 837 mg / L$$

Therefore, purging the microcosm headspace was sufficient to provide unlimited oxygen for the degradation of 500 mg/L ethanol (Series A and E), but not for 5000 or 15000 mg/L (Series B, C, F and G). The oxygen demand from 15 mg/L BTEX was considered negligible (see Part A.3).

A.3 Oxygen Demand from BTEX

Based on the stoichiometry of the mineralization of benzene (representing all BTEX compounds):

$$C_6H_6 + 7.5O_2 \rightarrow 6CO_2 + 3H_2O$$

7.5 moles of O_2 are required to mineralize one mole of BTEX.

For Series D-G, containing 15mg/L BTEX, assuming no partitioning from the aqueous phase, and based on the molecular weight of benzene, the number of moles of BTEX in the aqueous phase of the microcosms was calculated:

$$n_{BTEX} = \frac{(15 mg / L)(0.100 L)}{(78 g \cdot mol^{-1})} \times \frac{g}{1000 mg} = 0.000019 mol$$

Hence, the mineralization of 15 mg/L BTEX in the microcosms requires:

$$n_{O_2 req'd} = 0.000019 mol \cdot BTEX \left(\frac{7.5 mol \cdot O_2}{1 mol \cdot BTEX} \right) = 0.00014 mol$$

Therefore the O_2 required for mineralization of 15 mg/L in Series D-G is small compared to the oxygen required for the mineralization of ≥500 mg/L ethanol.

Appendix B
Composition of API 91-1 Gasoline

CONSCI

American Petroleum Institute
1220 L Street, Northwest
Washington, D.C. 20005

November 29, 1991

Attn: Chris Sexsmith

Invoice #: 97346
P.O. #: 76520

Certificate #: 11105008
Sample ID: Unleaded Gas(API 91-1),10/24/91
Date Received: November 5, 1991

CERTIFICATE OF ANALYSIS

Component Name	WT %	LV %
Propane	0.01	0.01
Isobutane	0.14	0.19
n-Butane	4.88	6.24
1,2-Butadiene	0.03	0.03
3-Methyl-1-butene	0.03	0.04
Isopentane	4.51	5.39
1-Pentene	0.63	0.73
n-Pentane	3.61	4.27
2-Methyl-1,3-butadiene	0.01	0.01
trans-2-Pentene	0.73	0.83
cis-2-Pentene	0.43	0.49
2-Methyl-2-butene	1.21	1.35
2,2-Dimethylbutane + Cyclopentadiene	1.01	1.15
Cyclopentene	0.17	0.16
4-Methyl-1-pentene	0.06	0.07
3-Methyl-1-pentene	0.08	0.09
Cyclopentane	0.23	0.23
2,3-Dimethylbutane	1.65	1.85
4-Methyl-cis-2-pentene	0.06	0.07
2-Methylpentane	5.52	6.27
4-Methyl-trans-2-pentene	0.04	0.04
3-Methylpentane	3.12	3.48
2-Methyl-1-pentene	0.28	0.31
1-Hexene	0.12	0.13

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CERTIFICATE OF ANALYSIS

Component Name	WT %	LV %
n-Hexane + 2-Ethyl-1-butene	2.65	2.98
trans-3-Hexene	0.18	0.20
trans-2-Hexene	0.38	0.42
3-Methylcyclopentene	0.42	0.41
3-Methyl-cis-2-pentene	0.11	0.12
4-Methylcyclopentene	0.26	0.25
3-Methyl-trans-2-pentene	0.05	0.05
cis-2-Hexene	0.21	0.23
2,2-Dimethylpentane	0.43	0.47
Methylcyclopentane	1.11	1.10
2,4-Dimethylpentane	0.69	0.76
C6 Olefin,s	0.13	0.14
1-Methylcyclopentene	0.38	0.36
C7 Olefin	0.01	0.01
Benzene	1.22	1.03
3,3-Dimethylpentane + C7 olefin	0.14	0.15
Cyclohexane + C7 olefin	0.28	0.27
C7 Cyclo-olefin/diolefin	0.06	0.06
C7 Cyclo-olefin/diolefin	0.02	0.02
C7 Cyclo-olefin/diolefin	0.05	0.05
C7 Olefin	0.12	0.13
2-Methylhexane	1.63	1.78
2,3-Dimethylpentane + C7 olefin	1.30	1.39
1,1-Dimethylcyclopentane	0.04	0.04
C7 Cyclo-olefin/diolefin	0.05	0.05
3-Methylhexane	1.70	1.84
C7 Olefin	0.04	0.04
t-1,3-Dimethylcyclopentane	0.32	0.32
c-1,3-Dimethylcyclopentane	0.28	0.28
3-Ethylpentane + C7 olefin	0.47	0.50
t-1,2-Dimethylcyclopentane	1.76	1.74
2,2,4-Trimethylpentane + C7 olefin	0.21	0.23
C7 Olefin	0.11	0.12
n-Heptane	1.30	1.41
C7 Olefin	0.05	0.05
C7 Olefin	0.24	0.25
C7 Olefin	0.11	0.12
C7 Olefin	0.12	0.13
C7 Olefin	0.06	0.06
C7 Olefin	0.13	0.14
C8 Olefin	0.09	0.09

CERTIFICATE OF ANALYSIS

Component Name	WT %	LV %
C8 Olefin	0.09	0.09
C8 Olefin (2)	0.08	0.08
C8 Olefin	0.05	0.05
c-1,2-Dimethylcyclopentane	0.18	0.17
Methylcyclohexane	0.43	0.42
Ethylcyclopentane	0.46	0.46
2,2,3-Trimethylpentane + C8 olefin + 2,4-Dimethylpentane	0.55	0.58
C7 Cyclo-olefin/diolefin	0.12	0.12
C8 Olefin	0.06	0.06
1,2,3-Trimethylcyclopentane + C8 Cyclo-olefin/diolefin	0.10	0.10
C8 Cyclo-olefin/diolefin	0.02	0.02
2,3,4-Trimethylpentane + C8 olefin	0.73	0.75
Toluene + C8 olefin	7.68	6.58
2,3-Dimethylhexane + C8 olefin	0.41	0.43
C8 Olefin	0.11	0.11
C8 Olefin	0.07	0.07
2-Methylheptane	0.85	0.90
4-Methylheptane	0.36	0.38
C8 olefins	0.14	0.14
3-Methylheptane	0.02	0.02
3-Ethylhexane + C8 olefin	1.21	1.26
C8 Naphthene + C8 olefin	0.14	0.14
C8 Paraffin + C8 olefin	0.24	0.23
C8 Naphthene + C8 olefin	0.16	0.16
C8 Naphthene + C8 olefin	0.13	0.13
C8 Naphthene	0.09	0.09
C8 Naphthene	0.13	0.13
n-Octane + C8 olefin	0.65	0.69
t-1,2-Dimethylcyclohexane	0.12	0.11
C8 Olefin	0.05	0.05
C9 Naphthene	0.21	0.20
C9 Paraffin + C8 olefin	0.05	0.05
C8 Olefin	0.06	0.06
C9 Paraffin	0.19	0.19
c-1,2-Dimethylcyclohexane + C9 olefin	0.05	0.05
C9 Naphthene	0.14	0.13
C9 Paraffin	0.03	0.03
C9 Naphthene	0.06	0.06
C9 Naphthene + C9 olefin	0.25	0.24
C9 Naphthene	0.03	0.03
C9 Naphthene	0.02	0.02

CERTIFICATE OF ANALYSIS

Component Name	WT %	LV %
Ethylbenzene	3.37	2.89
C9 Naphthene	0.03	0.03
C8 Naphthene	0.01	0.01
m-Xylene + C9 paraffin	5.31	4.57
p-Xylene + C9 paraffin	2.13	1.84
C9 Olefins + C9 Paraffins	0.06	0.06
2-Methyloctane	0.28	0.29
4-Methyloctane	0.32	0.33
C9 Naphthene	0.02	0.02
C9 Olefin	0.01	0.01
3-Ethylheptane	0.07	0.07
3-Methyloctane	0.34	0.35
o-Xylene	2.64	2.23
C10 Naphthene	0.04	0.04
C9 Naphthene + C9 olefin	0.14	0.13
C10 Naphthene + C9 olefin	0.02	0.02
C9 Naphthene	0.07	0.07
C10 Naphthene + C9 olefin	0.03	0.03
C9 Olefin	0.03	0.03
n-Nonane	0.24	0.25
C9 Naphthene	0.02	0.02
C9 Naphthene	0.02	0.02
Isopropylbenzene + C9 Naphthene	0.22	0.19
C9 Naphthene	0.03	0.03
C10 Olefin	0.05	0.05
C10 Olefin	0.01	0.01
C10 Paraffin	0.02	0.02
C10 Olefin	0.05	0.05
Naphthene	0.02	0.02
C10 Naphthene	0.01	0.01
C10 Naphthene	0.03	0.03
Naphthene	0.02	0.02
C10 Naphthene	0.03	0.03
C10 Paraffin	0.06	0.06
n-Propylbenzene	0.75	0.65
1-Methyl-3-ethylbenzene	2.34	2.01
1-Methyl-4-ethylbenzene	1.06	1.07
C10 Paraffin	0.03	0.03
1,3,5-Trimethylbenzene + C10 paraffin	1.10	0.94
4-Methylnonane	0.11	0.11
2-Methylnonane	0.15	0.15
1-Methyl-2-ethylbenzene	0.72	0.61
3-Ethyloctane	0.12	0.12

CERTIFICATE OF ANALYSIS

Component Name	WT %	LV %
1,2,4-Trimethylbenzene	3.37	2.86
Unidentified C10's	0.06	0.06
n-Decane	0.09	0.09
1-Methyl-3-isopropylbenzene	0.06	0.05
1,2,3-Trimethylbenzene	0.68	0.57
1-Methyl-4-isopropylbenzene	0.02	0.02
C11 Paraffin	0.02	0.02
Indan	0.40	0.31
C11 Paraffin	0.05	0.05
Olefin	0.01	0.01
C11 Paraffin	0.03	0.03
1,3-Diethylbenzene	0.22	0.19
1-Methyl-3-n-propylbenzene	0.48	0.41
1-Methyl-4-n-propylbenzene	0.28	0.24
n-Butylbenzene	0.14	0.12
1,2-Diethylbenzene	0.44	0.37
1,3-Dimethyl-5-ethylbenzene + 1,4-Diethylbenzene	0.04	0.03
C11 Paraffin	0.01	0.01
1-Methyl-2-n-propylbenzene	0.18	0.15
C11 Paraffin	0.04	0.04
C11 Paraffin	0.06	0.06
1,4-Dimethyl-2-ethylbenzene	0.28	0.24
1,3-Dimethyl-4-ethylbenzene	0.25	0.21
C11 Paraffin	0.13	0.13
1,2-Dimethyl-4-ethylbenzene + C10 indan	0.62	0.53
Unidentified C11's	0.12	0.12
1,2-Dimethyl-3-ethylbenzene	0.15	0.12
n-Undecane	0.06	0.06
1,2,4,5-Tetramethylbenzene	0.24	0.20
1,2,3,5-Tetramethylbenzene	0.33	0.28
C12 Paraffins	0.37	0.36
C11 Aromatics	0.32	0.27
C10/C11 Indans	0.09	0.07
Naphthalene	0.53	0.34
Unidentified C12's	0.19	0.19
n-Dodecane	0.05	0.05
C13 Paraffins	0.19	0.18
C12 Aromatics	1.06	0.88
C12 Indans	0.54	0.42
Methylnaphthalenes	0.69	0.45
Heavies	0.99	0.89
	100.00	100.00

Hydrocarbon Summary	WT %	LV %
n-Paraffins	13.58	16.04
Isoparaffins	27.43	30.34
Naphthenes	6.84	6.72
Aromatics	39.05	33.17
Olefins	11.74	12.47
Unknowns	1.36	1.26
Total	100.00	100.00

Reid Vapor Press.,PSI	8.5
MTBE,vl%	< 0.10
Methanol,vl%	< 0.10
Benzene,vl%	1.03
Bromine #	24
Research Octane No.	92.2
Motor Octane No.	82.5
R + M /2	87.4
Total Sulfur,ppm/wt	295
Carbon wt%	85.95
Hydrogen,wt%	13.00
Nitrogen,wt%	0.0083
Oxygen,wt%	0.74
Molecular Weight,(Calculated)	94.3

D-86 Distilation	
IBP/5	98/124
10/20	136/158
30/40	178/201
50/60	224/250
70/80	272/304
90/95	342/376
End Point	422
Recovered	98.0
Residue	1.0
Loss	1.0

Ben Miller

BHM/ar

Appendix C
Restriction Fragment Length Polymorphism (RFLP) Method

C.1 Extraction and purification of total community DNA

Materials
Aquifer material sample
Mo Bio Ultra Clean™ soil DNA kit materials
balance, small spatula
disposable gloves
jar of ethanol for flame sterilization
Bead Beater unit
P1000, P200 micropipettes, tips
Microcentrifuge
Ice and ice bucket

Procedure
1) Use a flame-sterilized spatula to weigh out 1.0 g of aquifer material.
2) Add the aquifer material to a 2-mL Bead Solution tube provided in the Mo Bio UltraClean Soil DNA Isolation Kit. Extract and purify the total community DNA from the aquifer sample following the kit protocol provided by Mo Bio (www.mobio.com).
3) Keep extracted DNA on ice. If DNA cannot be analyzed immediately store in a -20 °C freezer to avoid DNA degradation.

C.2 Gel electrophoresis of total community DNA

Materials
aquifer community DNA extract, approximately 50 µL per sample
gel electrophoresis unit, with dams and gel comb, power supply
10X TBE stock buffer
Graduated cylinders, 50 and 100 mL
Agarose, scale, weigh papers, spatula
125-mL flask
Heat/stir plate
P20 and P10 micropipettes with sterile tips
lambda HindIII DNA ladder (500 ng in 10 µL)
10 X gel loading buffer
ethidium bromide staining solution
dH_2O destain
UV light box with protective cover
Polaroid camera, type 55 film
latex gloves

Procedure
1) Assemble the gel electrophoresis unit and position the comb.
2) Using a graduated cylinder, prepare 350 mL of 1X TBE from the 10X TBE stock. Cover with Parafilm and invert the cylinder to mix.

3) Prepare a 40 mL 0.8% agarose gel. Weigh out 0.32 g agarose and add it to a 125 mL Erlenmeyer flask Measure out 40 mL of your 1X TBE and add this to the flask. While stirring, heat the solution to boiling. Cool the agarose to touch before pouring it into the gel rig. After pouring, allow the agarose to solidify (approximately 20 minutes).
4) Add a small amount of 1X TBE buffer to the gel rig and carefully remove the comb and dams. Add the remaining 1X TBE buffer until the gel is covered by ~3 mm of buffer.
5) Transfer 10 µL of aquifer DNA extract to a 1.5 mL microcentrifuge tube, add 1 µL of gel loading buffer, mix by pipetting. Pop-spin the mixture in the microcentrifuge, to collect the sample at the bottom of the tube. Keep your remaining soil/water DNA extract on ice, to inhibit degradation.
6) Transfer the total mixed volume of 11 µL to one of the lanes on the gel. Load 10 µL (500 ng) of the Lambda HindIII DNA ladder to the first and last lane of the agarose gel.
7) When all the samples have been loaded onto the gel, replace the lid, attach the appropriate electrodes into the power supply unit and turn on the power supply. Run the gel at approximately 110 V for 45 min to one hour, or until the first dye front is about 10-15 mm from the end of the gel.
8) Turn off the power supply and remove the electrodes. Remove the lid of the electrophoresis unit and take the gel to the staining station. Carefully transfer the gel to a large weighboat, then add enough ethidium bromide staining solution to cover the gel. Stain the gel for 10 minutes.
9) Carefully pour the ethidium bromide solution back into the container, then add dH_2O to cover the gel. De-stain the gel for 5 to 10 minutes.
10) Carefully transfer the gel onto the UV light box, close the lid, and turn on the UV light source to observe the gel.
11) Look first at the Lambda HindIII DNA ladder to ensure that the electrophoresis has been properly performed. Then in the appropriate lane look for the presence of a thin, weak chromosomal band that has run to just above the 23-kb marker. You may also observe a smear of sheared or degraded DNA in the lane. Or you may have not extracted enough DNA to be visually detected by this method.
12) Take a photo of the gel to document the gel electrophoresis, to allow you to estimate the amount of DNA required for your PCR.
13) Discard gel in an ethidium bromide waste container.

C.3 Polymerase chain reaction (PCR) amplification of the 16S rRNA gene

Materials

aquifer community DNA extract
PCR primers – 16S rDNA for Bacteria as follows:
Primer 1 - 5' GAG TTT GAT CMT GGC TCA G 3' (M = A + C)
Primer 2 - 5' ACG GYT ACC TTG TTA CGA CTT 3' (Y = C + T)
ice in ice bucket
PCR thermocycler machine
P20 and P10 micropipettes with sterile tips
200-µL PCR microcentrifuge tubes
DNA extracts of positive control strain, *E. coli*
Sterile distilled water for dilution and negative control

Master Mix: Prepare as follows (volumes are per reaction):
14.5 µL sterile distilled water
5.0 µL 10X PCR buffer (from Roche; 200 mM Tris-Cl pH 8.4, 200 mM KCl)
1.0 µL dNTP solution (10 mM each of dATP, dCTP, dGTP, dTTP)
1.25 µL primer 1 @ 10 pmol/µL
1.25 µL primer 2 @ 10 pmol/µL
2.0 µL Taq DNA polymerase (1U/µL)

Protocol – PCR Amplification

1) Obtain a bucket of ice. It is important to keep all tubes on ice at all times. When adding aliquots of fluid to a tube, mix tube contents by vortexing briefly (2-3 sec) then place the tube back on ice.
2) Prepare PCR tubes in duplicate
3) Add 25 µL of the master mix to all tubes.
4) To the positive control tube, add 25.0 µL of the positive control DNA.
5) To the negative control tube, add 25.0 µL of sterile distilled water (DNA-free).
6) Add the appropriate volume of DNA extract to each of the sample tubes. **Note:** the exact volume of extract to be used will be determined based on results of the gel electrophoresis conducted in Part C.2 (above). Also add an appropriate amount of sterile PCR water to each tube, so that all tubes contain a final volume of 25 µL.
7) Put the reaction mixtures in the PCR thermocycler machine carry out the following PCR amplification program:
    1. 94°C, 2 min, 1 cycle
    2. 94°C, 45 sec
    3. 58°C, 30 sec
    4. 72°C, 1 min
    5. go to 2.; 30 cycles
    6. 72°C, 10 min
    7. hold at 10°C

    where 94 °C is the denaturing temperature, 58 °C the annealing temperature, and 72 °C the template elongation temperature.

C.4 Gel electrophoresis of the PCR products

Materials

PCR-DNA product
gel electrophoresis unit, with dams and gel comb, power supply
10X TBE stock buffer
Graduated cylinders, 50 and 100 mL
Agarose, scale, weigh papers, spatula
125-mL flask
Heat/stir plate
P20 and P10 micropipettes with sterile tips
Marker DNA, 1 kb ladder and 100 bp ladder (MBI Fermentas)
10 X gel loading buffer
ethidium bromide staining solution
UV light box with protective cover
Polaroid camera, type 55 film
latex gloves

Procedure

1) Prepare a 40 mL 2% agarose gel following the same method as in Part C.2. This time, weigh out 0.8 g agarose.
2) Transfer 15 µL of each PCR-DNA product, positive *E.coli* control, and negative control to a 1.5 mL microcentrifuge tube, add 1.5 µL of gel loading buffer, mix by pipetting. Pop-spin each mixture in the microcentrifuge.
3) Transfer each total mixed volume of 16.5 µL to the lanes on the gel. Load 10 µL (500 ng) of the 1 kb DNA ladder to the first lane and 10 µL (500 ng) of the 100 bp DNA ladder to the last lane of the agarose gel.
4) Run, stain, and photograph the gel following the same method as in Part C.2.
5) If you obtained a satisfactory signal from the PCR-DNA product, continue purification of the product in preparation for restriction analysis. If the PCR-DNA product cannot be further analyzed immediately, it may be stored in a -20°C freezer to avoid DNA degradation

C.5 Extraction and purification of total community DNA

Materials

PCR-DNA product
Qiagen MinElute PCR Purification Kit
P1000, P10 micropipettes with sterile tips
sterile 1.5 mL microcentrifuge tubes
microcentrifuge

Procedure

1) Clean each PCR-DNA product following the MinElute PCR Purification Kit protocol provided by Qiagen (www1.qiagen.com). You will end up with approximately 9 µL of purified PCR aquifer DNA.
2) Remove a 1 µL volume from each purified PCR aquifer DNA sample, and place into a new sterile microcentrifuge tube. Add 1 µL of 10X loading dye and 8.0 µL of sterile distilled water. Store on ice. These samples of uncut, cleaned DNA will be run on an agarose gel in parallel with restriction enzyme-digested samples (Part C.7).
3) Immediately continue on with the restriction analysis (Part C.6) using the ~8-µL volumes of cleaned PCR products that remain.

C.6 Restriction fragment length polymorphism (RFLP) analysis using Taq I restriction enzyme

Different restriction enzymes can be used for RFLP analysis when first examining a new set of samples, it is common to try a number of enzymes. We will use a single enzyme, Taq I, which recognizes and cuts at the following sites:

```
      ↓
5'  -T C G A-  3'
3'  -A G C T-  5'
        ↑
```

Materials
purified PCR-DNA samples (including *E. coli* control)
sterile water
Taq I, 10X Taq I buffer (MBI Fermentas)
P10 pipetman with sterile tips
Block heater set to 65 ºC, thermometer
10X loading dye (for subsequent gel electrophoresis)

Procedure
1) Set up the following restriction digest in 1.5 mL microcentrifuge tubes for each purified PCR-DNA sample:

   7.5 µL purified PCR-DNA
   5 µL sterile distilled water
   1.5 µL 10X Taq I restriction endonuclease buffer, (MBI Fermentas)
   1 µL Taq I restriction endonuclease, (MBI Fermentas), **add last**

2) Mix by pipetting gently up and down. Pop spin the mixed tube for a few seconds if there are drops of liquid up the side of the tube.
3) Incubate for 1 hour at 65ºC. When the incubation time is over add 1.5 µL of the 10X loading dye to each digest, pop spin, and store the samples on ice until ready to load the agarose gel.

C.7 Gel electrophoresis of the uncut and restriction enzyme digested samples

Materials
gel electrophoresis unit, with dams and gel comb, power supply
10X TBE stock buffer
Graduated cylinders, 50 and 100 mL
Agarose, scale, weigh papers, spatula
125-mL flask
Heat/stir plate
P20 and P10 micropipettes with sterile tips
Marker DNA, 1 kb ladder and 100 bp ladder (MBI Fermentas)
10X gel loading buffer
ethidium bromide staining solution
dH_2O destain
UV light box with protective cover
Polaroid camera, type 55 film
latex gloves

Procedure
1) Prepare a 40 mL 2% agarose gel following the same method as in Part C.2. This time, weigh out 0.8 g agarose.
2) Load all of each Taq I digested PCR-DNA sample, and all of the clean, uncut products into the lanes on the gel. Load 10 µL (500 ng) of the 1 kb DNA ladder to the first lane and 10 µL (500 ng) of the 100 bp DNA ladder to the last lane of the agarose gel.
3) Run, stain, and photograph the gel following the same method as in Part C.2.

Appendix D
Microcosm Experiment Raw Data

Table D.1 Ethanol concentrations in microcosms (Series A, B, C, E, F and G).

	Ethanol Concentration (mg/L)											
	Series A - 500 mg/L				Series B - 5000 mg/L				Series C - 15000 mg/L			
Time (d)	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2
1.0	473.0	472.0	444.0	441.0	4528.8	4630.8	4314.6	4508.4	12958.3	13503.7	12746.2	13220.9
2.0	463.5	458.0	353.0	303.0	4656.3	4559.4	4304.4	4355.4	13422.9	13523.9	12816.9	12928.0
2.2	466.0	458.0	317.0	272.0	4477.8	4503.3	4227.9	4375.8	—	—	—	—
2.7	465.5	454.5	186.3	197.7	4467.6	4375.8	4085.1	4299.3	12928.0	13645.1	12584.6	13018.9
3.1	491.5	485.5	138.5	148.5	4885.8	4921.5	4437.0	4472.7	—	—	—	—
3.8	455.6	449.5	31.2	16.4	—	—	—	—	—	—	—	—
4.8	483.0	447.0	0.5	36.8	—	—	—	—	—	—	—	—
5.7	447.3	443.5	0.9	0.6	3994.3	4424.3	3336.9	3450.2	12195.8	11312.0	11312.0	11362.5
7.8	470.6	453.0	0.0	4.6	4533.9	4478.8	3507.8	3487.4	12887.6	11136.3	11136.3	12207.9
10.7	464.6	466.1	0.0	0.0	4539.0	4562.5	3371.1	3458.3	13063.3	13759.2	11371.6	12130.1
15.7	—	—	—	—	4626.2	4679.8	3105.9	3153.8	13611.8	14219.8	11149.4	11941.2
22.7	—	—	—	—	4526.3	4607.9	2625.0	2689.2	13549.2	14119.8	11190.8	11372.6
29.7	—	—	—	—	4507.4	4520.1	2343.5	2362.3	13847.1	13620.9	10716.1	11132.2
41.7	—	—	—	—	4431.9	4530.3	2042.6	1828.9	13171.4	13615.8	10058.6	10452.5
49.7	—	—	—	—	4560.9	4725.2	1861.0	1689.1	13679.4	14190.5	9767.7	10381.8
58.8	—	—	—	—	5258.1	5360.1	1856.4	1830.9	15927.7	16028.7	11078.7	11552.4
66.8	—	—	—	—	5283.6	5411.1	1611.6	1728.9	15786.3	16170.1	10968.6	11433.2
	Ethanol Concentration (mg/L)											
	Series E - 500 mg/L + BTEX				Series F - 5000 mg/L + BTEX				Series G - 15000 mg/L + BTEX			
Time (d)	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2
1.0	473.5	472.0	441.5	449.5	4528.8	4686.9	4386.0	4437.0	13847.1	13786.5	12887.6	13180.5
2.0	475.0	462.5	361.5	355.5	4549.2	4554.3	3478.2	3253.8	—	—	—	—
2.2	465.5	459.5	365.0	357.0	4503.3	4569.6	4258.5	4253.4	13584.5	13433.0	12645.2	12837.1
2.8	497.0	472.5	260.0	234.0	4569.6	4641.0	4294.2	4181.0	14119.8	14039.0	12824.0	13039.1
3.2	482.7	484.8	192.5	150.0	4830.2	4860.3	4192.7	4146.3	—	—	—	—
3.9	456.6	459.4	26.6	10.8	—	—	—	—	—	—	—	—
4.9	445.8	449.0	83.3	30.2	—	—	—	—	—	—	—	—
5.8	448.3	448.3	1.0	0.1	4425.8	4469.6	3575.1	3593.5	12612.9	13190.6	12143.2	12323.0
7.8	456.2	462.2	1.9	3.8	4551.2	4590.0	3584.8	3560.3	13249.2	13510.8	12191.7	12373.5
10.7	466.0	474.7	0.3	0.0	4451.8	4625.7	3414.5	3476.2	13203.7	13420.9	12058.4	12348.3
15.8	—	—	—	—	4636.9	4770.5	3285.9	3238.5	13783.5	14083.4	12019.0	12120.0
22.8	—	—	—	—	4628.8	4701.2	2952.9	2866.2	14703.6	13918.8	11195.9	11345.3
29.8	—	—	—	—	4636.4	4638.5	2816.2	2651.5	13313.8	13530.0	10823.2	10973.7
41.8	—	—	—	—	4489.0	4557.9	2419.4	2315.9	13271.4	13569.4	10754.5	10781.8
49.9	—	—	—	—	4604.3	4592.6	2248.1	2136.4	13534.0	13847.1	10049.5	10049.5
58.8	—	—	—	—	5416.2	5385.6	2434.7	2223.6	15675.2	16018.6	11413.0	11413.0
66.8	—	—	—	—	5319.3	5344.8	2320.5	2019.6	15887.3	15836.8	11180.7	11200.9

Table D.2 Benzene concentrations in microcosms (Series D, E, F and G)

Time (d)	Benzene Concentration (mg/L)															
	Series D - BTEX only				Series E - 500 mg/L + BTEX				Series F - 5000 mg/L + BTEX				Series G - 15000 mg/L + BTEX			
	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2
0.9	3.08	2.92	2.84	2.85	3.08	3.20	3.19	2.91	3.05	2.88	2.76	2.68	3.37	3.24	3.15	3.06
1.8	2.66	2.63	2.15	1.11	2.68	2.63	0.83	1.48	–	–	–	–	–	–	–	–
1.9	–	–	–	0.85	–	–	–	–	2.69	2.69	1.48	0.06	2.85	2.67	2.79	2.78
2.1	2.73	2.70	1.88	0.23	2.70	2.71	0.03	0.45	2.75	2.75	0.29	0.00	–	–	–	–
2.2	–	–	–	–	–	–	0.01	0.26	–	–	0.22	–	–	–	–	–
2.7	2.71	2.62	0.99	0.00	2.65	2.61	0.00	0.03	2.66	2.69	0.06	0.00	2.76	2.67	2.45	2.21
2.8	–	–	0.78	–	–	–	–	–	–	–	–	–	–	–	–	–
3.0	2.71	2.71	0.35	0.00	2.75	2.74	0.00	0.03	2.84	2.83	0.08	0.00	–	–	–	–
3.1	–	–	0.24	–	–	–	–	–	–	–	–	–	–	–	–	–
3.8	2.74	2.67	0.05	0.00	2.69	2.67	0.00	0.04	2.69	2.75	0.09	0.00	2.91	3.01	1.27	0.76
4.9	2.71	2.66	0.00	0.00	2.65	2.67	0.00	0.02	2.74	2.74	0.09	0.00	2.86	2.82	0.52	0.29
5.8	2.60	2.54	0.00	–	–	–	–	–	–	–	–	–	2.78	2.73	0.37	0.05
6.9	2.57	–	–	–	2.57	2.54	0.00	0.00	2.56	2.59	0.06	0.00	2.66	2.67	0.11	0.02
8.9	–	–	–	–	2.59	2.51	0.00	0.00	2.53	2.54	0.06	0.00	2.63	2.64	0.09	0.01
10.7	–	–	–	–	–	–	–	–	2.55	2.53	0.06	0.00	2.67	2.67	0.08	0.01
15.8	–	–	–	–	–	–	–	–	2.46	2.29	0.05	0.00	2.61	2.50	0.16	0.00
22.7	–	–	–	–	–	–	–	–	2.58	2.57	0.04	0.00	2.70	2.64	0.10	0.00
29.7	–	–	–	–	–	–	–	–	2.48	2.56	0.04	0.00	2.67	2.61	0.05	0.00
41.8	–	–	–	–	–	–	–	–	2.53	2.48	0.03	0.00	2.63	2.54	0.07	0.00
49.7	–	–	–	–	–	–	–	–	2.48	2.47	0.01	0.00	2.50	2.54	0.04	0.00
58.7	–	–	–	–	–	–	–	–	2.56	2.55	0.00	0.00	2.78	2.69	0.00	0.00

Table D.3 Toluene concentrations in microcosms (Series D, E, F and G)

	Toluene Concentration (mg/L)															
	Series D - BTEX only				Series E - 500 mg/L + BTEX				Series F - 5000 mg/L + BTEX				Series G - 15000 mg/L + BTEX			
Time (d)	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2
0.9	4.95	4.69	4.64	4.52	5.03	5.26	5.21	4.73	4.94	4.65	4.32	4.12	5.44	5.17	4.93	4.86
1.8	4.36	4.30	3.16	1.15	4.45	4.37	0.62	1.40	–	–	–	–	–	–	–	–
1.9	–	–	–	0.66	–	–	–	–	4.24	4.18	1.30	0.00	4.06	4.02	3.77	3.61
2.1	4.56	4.51	2.72	0.16	4.46	4.49	0.00	0.05	4.34	4.35	0.03	0.00	–	–	–	–
2.2	–	–	–	–	–	–	0.00	0.00	–	–	0.00	0.00	–	–	–	–
2.7	4.44	4.26	0.82	0.00	4.28	4.25	0.00	0.00	4.23	4.30	0.00	0.00	4.20	4.11	2.92	2.23
2.8	–	–	0.55	–	–	–	–	–	–	–	–	–	–	–	–	–
3.0	4.42	4.46	0.12	0.00	4.47	4.46	0.00	0.00	4.40	4.35	0.00	0.00	–	–	–	–
3.1	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–	–	–
3.8	4.51	4.37	0.00	0.00	4.40	4.34	0.00	0.00	4.17	4.28	0.00	0.00	4.17	4.14	0.04	0.00
4.9	4.36	4.32	0.00	0.00	4.32	4.39	0.00	0.00	4.25	4.30	0.00	0.00	4.20	4.16	0.00	0.00

Table D.4 Ethylbenzene concentrations in microcosms (Series D, E, F and G)

	Ethylbenzene Concentration (mg/L)															
	Series D - BTEX only				Series E - 500 mg/L + BTEX				Series F - 5000 mg/L + BTEX				Series G - 15000 mg/L + BTEX			
Time (d)	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2
0.9	0.54	0.55	0.52	0.48	0.56	0.58	0.57	0.51	0.55	0.51	0.47	0.43	0.63	0.63	0.57	0.56
1.8	0.47	0.47	0.29	0.07	0.49	0.48	0.04	0.09	0.49	0.46	0.09	0.00	0.44	0.45	0.38	0.36
1.9	–	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–	–
2.1	0.59	0.53	0.25	0.00	0.49	0.50	0.00	0.00	0.48	0.48	0.00	0.00	–	–	–	–
2.7	0.49	0.47	0.03	0.00	0.46	0.47	0.00	0.00	0.46	0.48	0.00	0.00	0.47	0.45	0.20	0.13
3.0	0.49	0.49	0.03	0.00	0.48	0.49	0.00	0.00	0.48	0.47	0.00	0.00	–	–	–	–
3.8	0.50	0.48	0.05	0.00	0.49	0.48	0.00	0.00	0.45	0.47	0.00	0.00	0.46	0.46	0.00	0.00

Table D.5 *p*-Xylene concentrations in microcosms (Series D, E, F and G)

Time (d)	*p*-Xylene Concentration (mg/L)															
	Series D - BTEX only				Series E - 500 mg/L + BTEX				Series F - 5000 mg/L + BTEX				Series G - 15000 mg/L + BTEX			
	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2
0.9	0.33	0.38	0.34	0.34	0.36	0.36	0.35	0.33	0.33	0.32	0.31	0.31	0.39	0.41	0.36	0.35
1.8	0.30	0.30	0.27	0.20	0.31	0.30	0.23	0.24	0.33	0.28	0.23	0.14	0.27	0.28	0.27	0.28
2.1	0.42	0.35	0.28	0.14	0.30	0.31	0.15	0.20	0.30	0.30	0.19	0.08	—	—	—	—
2.7	0.31	0.29	0.17	0.02	0.29	0.29	0.07	0.09	0.29	0.30	0.14	0.04	0.29	0.28	0.26	0.24
3.0	0.31	0.30	0.18	0.00	0.30	0.30	0.03	0.08	0.30	0.29	0.15	0.04	—	—	—	—
3.8	0.31	0.29	0.15	0.00	0.30	0.30	0.02	0.09	0.28	0.29	0.14	0.04	0.29	0.29	0.21	0.18
4.9	0.29	0.29	0.02	0.00	0.32	0.31	0.00	0.07	0.29	0.29	0.13	0.04	0.29	0.28	0.15	0.11
5.8	0.28	0.28	0.00	—	—	—	—	—	—	—	—	—	0.27	0.26	0.14	0.09
6.9	0.29	—	—	—	0.28	0.27	0.00	0.00	0.27	0.27	0.12	0.03	0.26	0.26	0.10	0.07
8.9	—	—	—	—	0.30	0.27	0.00	0.00	0.26	0.27	0.12	0.03	0.26	0.25	0.09	0.05
10.7	—	—	—	—	—	—	—	—	0.27	0.26	0.12	0.03	0.25	0.25	0.08	0.05
15.8	—	—	—	—	—	—	—	—	0.25	0.23	0.12	0.02	0.26	0.24	0.06	0.03
22.7	—	—	—	—	—	—	—	—	0.27	0.26	0.08	0.02	0.26	0.25	0.04	0.03
29.7	—	—	—	—	—	—	—	—	0.24	0.25	0.06	0.00	0.24	0.25	0.03	0.02
41.8	—	—	—	—	—	—	—	—	0.24	0.24	0.06	0.00	0.24	0.23	0.02	0.02
49.7	—	—	—	—	—	—	—	—	0.24	0.24	0.04	0.00	0.22	0.23	0.00	0.01
58.7	—	—	—	—	—	—	—	—	0.27	0.25	0.03	0.00	0.26	0.24	0.00	0.00
66.7	—	—	—	—	—	—	—	—	0.26	0.23	0.03	0.00	0.23	0.22	0.00	0.00

Table D.6 *m*-Xylene concentrations in microcosms (Series D, E, F and G)

Time (d)	*m*-Xylene Concentration (mg/L)															
	Series D - BTEX only				Series E - 500 mg/L + BTEX				Series F - 5000 mg/L + BTEX				Series G - 15000 mg/L + BTEX			
	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2
0.9	0.73	0.76	0.74	0.74	0.79	0.82	0.80	0.73	0.75	0.70	0.69	0.69	0.88	0.87	0.79	0.78
1.8	0.65	0.65	0.64	0.59	0.70	0.68	0.64	0.64	0.68	0.63	0.58	0.60	0.59	0.62	0.59	0.61
2.1	0.83	0.74	0.68	0.59	0.67	0.68	0.65	0.67	0.66	0.65	0.63	0.60	—	—	—	—
2.7	0.67	0.63	0.51	0.32	0.62	0.63	0.58	0.55	0.63	0.64	0.58	0.55	0.62	0.60	0.58	0.56
3.0	0.66	0.67	0.52	0.18	0.65	0.66	0.56	0.57	0.65	0.63	0.61	0.56	—	—	—	—
3.8	0.67	0.66	0.28	0.00	0.67	0.64	0.56	0.59	0.61	0.63	0.58	0.53	0.64	0.62	0.59	0.55
4.9	0.64	0.64	0.00	0.00	0.67	0.68	0.49	0.54	0.63	0.63	0.57	0.54	0.62	0.61	0.54	0.55
5.8	0.62	0.60	0.00	—	—	—	—	—	—	—	—	—	0.58	0.57	0.54	0.52
6.9	0.62	—	—	—	0.60	0.60	0.29	0.00	0.58	0.60	0.51	0.50	0.56	0.56	0.52	0.50
8.9	—	—	—	—	0.63	0.59	0.00	0.00	0.55	0.58	0.51	0.47	0.55	0.56	0.49	0.47
10.7	—	—	—	—	—	—	—	—	0.58	0.57	0.49	0.46	0.55	0.54	0.48	0.46
15.8	—	—	—	—	—	—	—	—	0.53	0.50	0.42	0.37	0.55	0.53	0.35	0.38
22.7	—	—	—	—	—	—	—	—	0.58	0.56	0.34	0.31	0.56	0.55	0.27	0.33
29.7	—	—	—	—	—	—	—	—	0.53	0.55	0.28	0.21	0.53	0.55	0.20	0.29
41.8	—	—	—	—	—	—	—	—	0.53	0.53	0.23	0.16	0.53	0.51	0.17	0.21
49.7	—	—	—	—	—	—	—	—	0.53	0.53	0.17	0.14	0.49	0.50	0.03	0.15
58.7	—	—	—	—	—	—	—	—	0.56	0.55	0.13	0.11	0.56	0.52	0.00	0.12
66.7	—	—	—	—	—	—	—	—	0.54	0.50	0.10	0.12	0.50	0.46	0.00	0.11

Table D.7 *o*-Xylene concentrations in microcosms (Series D, E, F and G)

Time (d)	*o*-Xylene Concentration (mg/L)															
	Series D - BTEX only				Series E - 500 mg/L + BTEX				Series F - 5000 mg/L + BTEX				Series G - 15000 mg/L + BTEX			
	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2	Control Rep 1	Control Rep 2	Active Rep 1	Active Rep 2
0.9	0.53	0.63	0.56	0.54	0.59	0.60	0.59	0.50	0.55	0.51	0.49	0.49	0.65	0.70	0.60	0.58
1.8	0.47	0.48	0.47	0.45	0.51	0.50	0.48	0.46	0.56	0.46	0.42	0.45	0.43	0.45	0.42	0.45
2.1	0.75	0.59	0.53	0.51	0.49	0.50	0.50	0.50	0.50	0.48	0.47	0.47	–	–	–	–
2.7	0.50	0.47	0.42	0.39	0.45	0.46	0.50	0.43	0.47	0.48	0.45	0.44	0.46	0.45	0.45	0.41
3.0	0.50	0.50	0.53	0.36	0.48	0.49	0.46	0.45	0.48	0.45	0.47	0.45	–	–	–	–
3.8	0.51	0.48	0.44	0.09	0.49	0.47	0.46	0.48	0.43	0.46	0.44	0.42	0.47	0.46	0.44	0.41
4.9	0.48	0.48	0.06	0.00	0.54	0.51	0.43	0.44	0.47	0.47	0.43	0.44	0.46	0.45	0.41	0.43
5.8	0.46	0.45	0.00	–	–	–	–	–	–	–	–	–	0.42	0.42	0.46	0.41
6.9	0.47	–	–	–	0.44	0.45	0.28	0.00	0.43	0.44	0.40	0.41	0.41	0.42	0.41	0.42
8.9	–	–	–	–	0.51	0.45	0.00	0.00	0.42	0.44	0.40	0.38	0.41	0.42	0.39	0.38
10.7	–	–	–	–	–	–	–	–	0.46	0.42	0.40	0.38	0.40	0.40	0.38	0.38
15.8	–	–	–	–	–	–	–	–	0.40	0.37	0.39	0.32	0.41	0.39	0.28	0.32
22.7	–	–	–	–	–	–	–	–	0.46	0.41	0.30	0.27	0.41	0.41	0.22	0.29
29.7	–	–	–	–	–	–	–	–	0.40	0.41	0.24	0.20	0.40	0.41	0.16	0.27
41.8	–	–	–	–	–	–	–	–	0.40	0.39	0.24	0.16	0.40	0.38	0.14	0.21
49.7	–	–	–	–	–	–	–	–	0.40	0.39	0.17	0.13	0.36	0.38	0.00	0.15
58.7	–	–	–	–	–	–	–	–	0.47	0.41	0.13	0.11	0.42	0.39	0.00	0.12
66.7	–	–	–	–	–	–	–	–	0.44	0.37	0.11	0.11	0.37	0.35	0.00	0.12

Appendix E
INT-Formazan Standard Curve

The INT-formazan standard curve was made using two 100 mg/L stock solutions (Stock A and B) of INT-formazan in methanol. INT-formazan (0.01 g) was dissolved in 100 mL methanol and stirred for 4 hours. The stock solutions were diluted to final concentrations ranging from 2 to 50 mg/L. Absorbance of the diluted solutions was measured in triplicate by spectrophotometer (Thermo Spectronic Genesys 20) at a wavelength of 480 nm against a methanol blank. Absorbance readings (Table E.1) were plotted against INT-formazan concentrations to produce the standard curve (Figure E.1).

Table E.1 INT-formazan standard curve raw data.

INT-Formazan Concentrations (mg/L)	Stock Used	Absorbance (OD_{480})		
		Replicate 1	Replicate 2	Replicate 3
2	Stock A	0.081	0.085	0.082
3	Stock B	0.123	0.125	0.124
5	Stock A	0.212	0.213	0.213
10	Stock A	0.421	0.424	0.425
20	Stock B	0.830	0.834	0.836
30	Stock B	1.213	1.221	1.222
50	Stock A	1.835	1.867	1.874

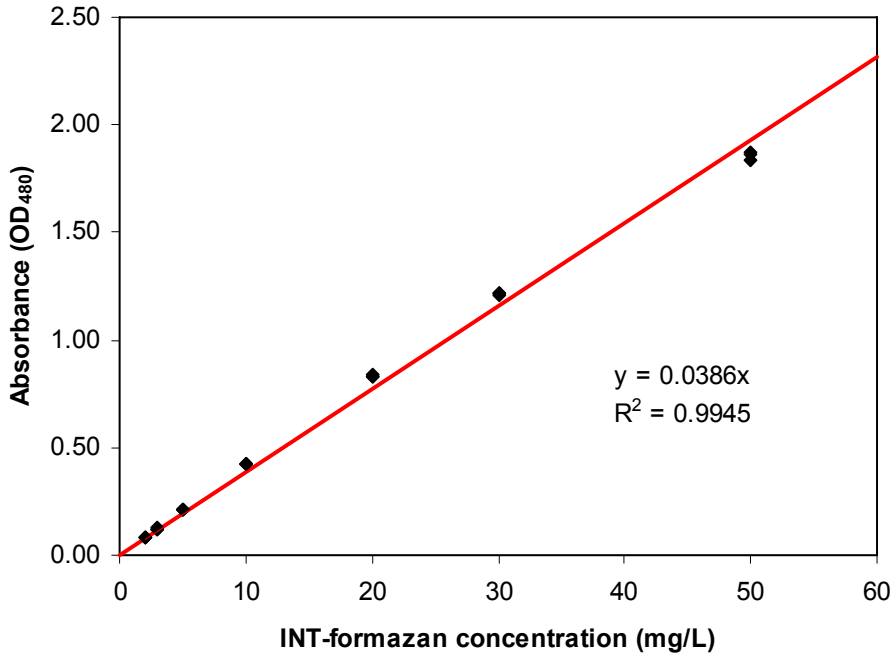


Figure E.1 INT-formazan standard curve

Appendix F
Inhibition Experiment – Toluene Biodegradation

Following the ethanol exposure during the first inhibition experiment (Chapter 3), a microcosm experiment was conducted to measure the capability of the post-ethanol microbial population to subsequently biodegrade hydrocarbons. For simplification, toluene was selected as a representative monoaromatic hydrocarbon. Subsamples (16 g) of the washed material were added to 100 mL bottles with 40 mL of toluene- and MBH-amended groundwater. The groundwater solution was prepared by adding MBH medium (25 mL/L) and sterilizing before adding toluene (6 mg/L). The microcosms were incubated in the dark at room temperature and toluene degradation was monitored. The results of measured toluene concentrations are provided in Table F.1. After toluene loss was observed in Series A (sterile control) on Day 1, sodium azide (0.8 mL of a 10% solution) was added to Series A bottles. Further toluene degradation was observed in these microcosms. The experiment was deemed flawed and the results were inconclusive. The results for toluene degradation are reported as microcosm duplicate averages (Figure F.1).

Table F.1 Aqueous toluene concentrations measured in microcosms.

Time (d)	Toluene Concentration (mg/L)							
	Series A Sterile Control		Series B Unwashed Control		Series C 0% Ethanol		Series D 32% Ethanol	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
0.8	2.3	2.2	1.1	1.3	1.9	1.4	3.2	4.1
0.9	2.0	1.7	0.1	0.2	1.1	0.0	2.7	3.3
1.8	2.3	2.4	0.0	0.0	0.0	0.0	2.5	3.7
1.9	2.5	2.3	0.0	0.0	0.0	0.0	2.2	3.9
4.8	2.3	2.3	0.0	0.0	0.0	0.0	0.4	3.5
6.7	1.9	2.1	0.0	0.0	0.0	0.0	0.3	2.4
8.8	1.7	1.2	0.0	0.0	0.0	0.0	0.2	2.1

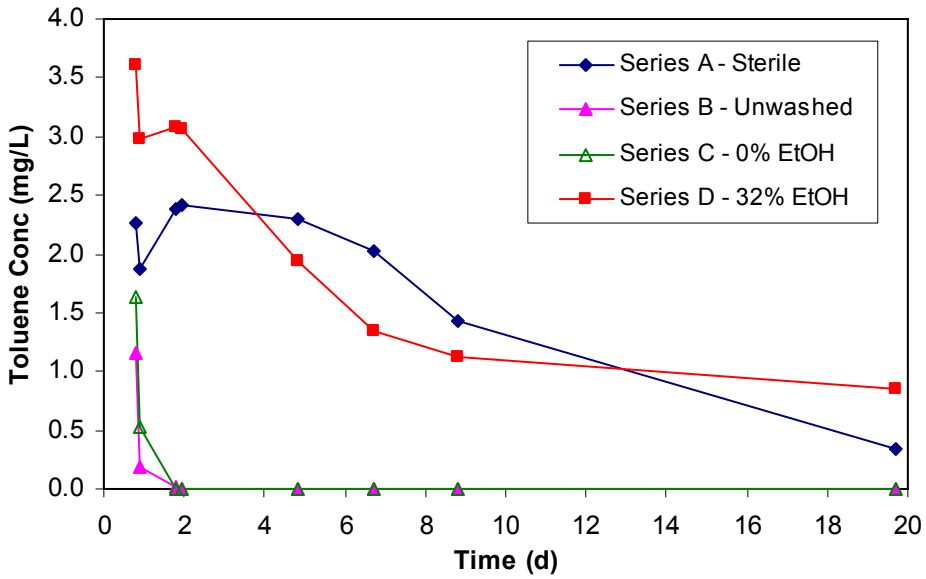


Figure F.1 Toluene loss in microcosms reported as duplicate averages of aqueous concentrations.

Appendix G
Ethanol Exposure and Washing Method Investigation

G.1 Washing Method Investigation

The washing procedure employed in the Inhibition Experiment 1 (Section 3.2.1) resulted in greater than desired residual ethanol concentrations. A washing method investigation was conducted to improve the method. The washing volume was increased from 50 mL to 200 mL and the number of washes was increased from 2 to 5. The volume of ethanol solution used for exposure was decreased from 30 mL to 12 mL while maintaining a similar solids:liquids ratio.

The ethanol exposure was conducted in 100 mL bottles with 20 g (wet weight) aquifer material and 12 mL liquid (ethanol solution). Assuming a bulk mass density of 2.2 g/cm^3 for the aquifer material, the headspace of the exposure system was ~79 mL. Three concentrations of ethanol were tested in duplicate: 25% v/v (197250 mg/L), 50% v/v (394500 mg/L), and 95% v/v (749550 mg/L). Following ethanol exposure, the aquifer material was washed to reduce the residual ethanol concentrations. Washing followed the same procedure as in Inhibition Experiment 1 (Section 3.2.1.1) except that it consisted of 5 washes with 200 mL volumes. Ethanol concentrations were measured after each wash. A summary of the results are provided in Figure G.1.

The investigation showed that four washes of 200 mL volumes were sufficient to reduce the residual ethanol concentration to <1 mg/L and that each wash resulted in an average of ~96% ethanol removal. However, the initial ethanol concentration was consistently lower than the target concentration by ~20%. Several explanations were proposed for this loss and they are listed below. Some of these issues were tested in an additional experiment (Ethanol Exposure Method Investigation – Part G.2).

1) *Source ethanol was less than 100%* – The concentration of the pure source ethanol was measured to verify that the concentration was 100%. For 100% (v/v), the expected concentration of the source ethanol (based on the liquid density for ethanol of 0.789 g/mL) was 789000 mg/L. The average measured concentration of the source ethanol (766600 mg/L) was 3% less than the expected value but was within a reasonable range considering analytical error and error associated with diluting the sample to within the detection range of the analysis (≤ 200 mg/L).
2) *Partitioning into headspace or sorption to solids* – An equilibrium partitioning calculation indicated that only 0.1% of the ethanol is expected to partition into the headspace and only 0.5% of the ethanol is expected to sorb to the aquifer material. Therefore, equilibrium partitioning was not considered significant. Nevertheless, the headspace volume was minimized in the next investigation.

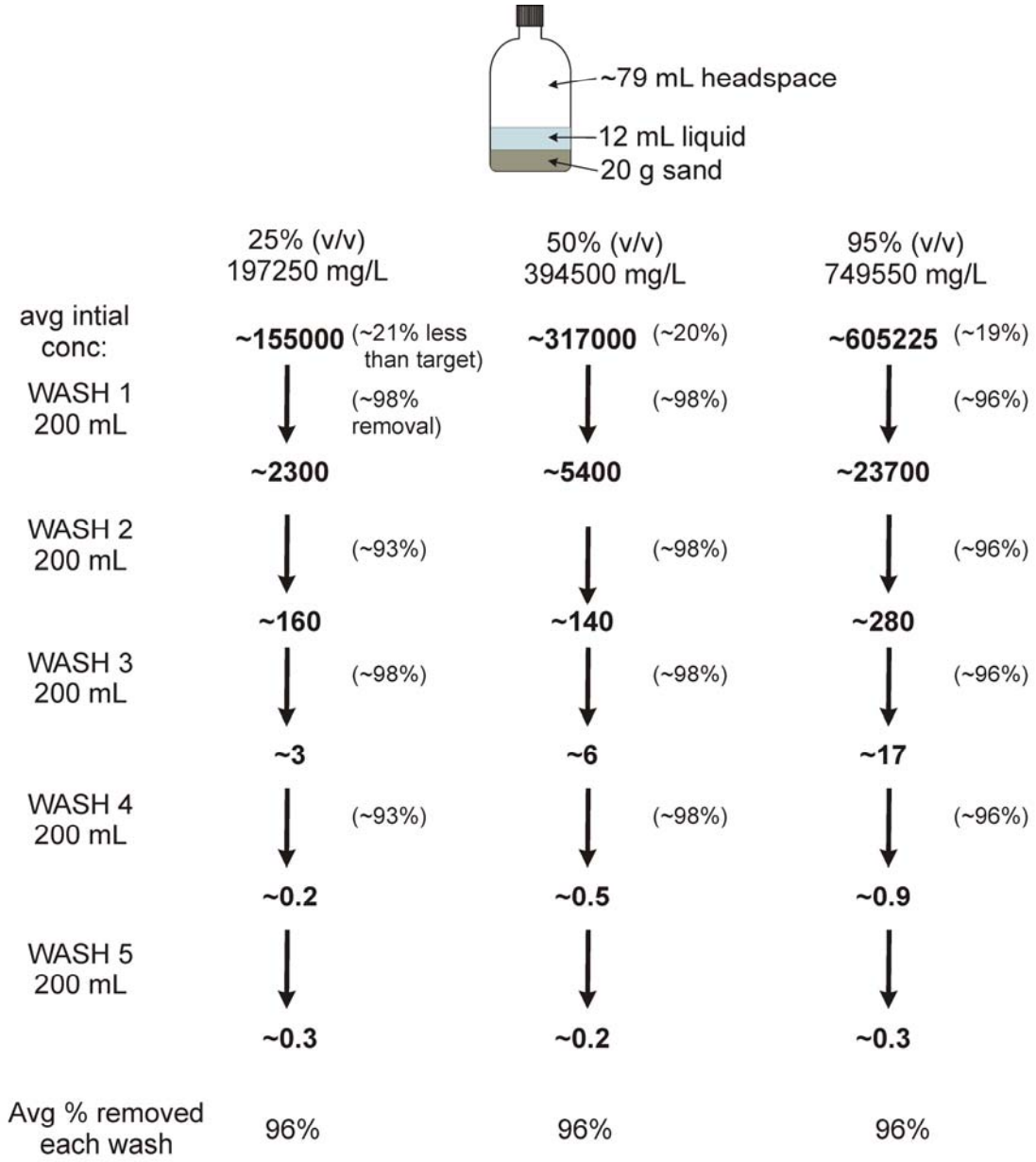


Figure G.1 Set up and results of the Washing Method Investigation.

3) *Ethanol biodegradation during exposure* – An oxygen demand calculation was performed to evaluate this possibility. For a headspace of 79 mL, the O_2 in the headspace should only have been enough to support ~0.4% loss of ethanol to biodegradation. Therefore biodegradation during exposure was not considered significant. Nevertheless, the headspace volume was reduced to ~2 mL in the next investigation. In addition, ethanol concentrations were measured at the start and end of the ethanol exposure period to determine if ethanol was lost during exposure.

4) *Ethanol biodegradation during sampling/dilution for analysis* – The ethanol samples for analytical analysis were preserved with sodium azide, therefore ethanol biodegradation was not expected to occur in the samples. In the next investigation, unpreserved samples and samples preserved with sodium azide were compared to determine if the preservative interferes with the analysis. It was recommended that an alternative preservative be tested, however, this was not conducted in this study.

5) *Dilution of ethanol by the aquifer material pore water* – Based on wet and dry weights of the initial aquifer material, the water content was 18%. So the volume of pore water in the system could be calculated:

$$V_{pw} = (20\ g)(0.18)(1\ mL/g) = 3.6\ mL$$

and the concentration of 197250 mg/L ethanol after dilution by the pore water was estimated:

$$C_{dil} = (197250\ mg/L)(12\ mL) / (12\ mL + 3.6\ mL) = 151731\ mg/L$$

Therefore, in this system, dilution of ethanol by the aquifer pore water can account for ethanol concentrations 23% lower than the concentration of the ethanol solution added. This was tested again in the next investigation by measuring ethanol concentrations for systems with and without aquifer material added.

6) *Sampling/dilution error and analytical error* – Analytical error and error associated with diluting the sample to within the detection range of the analysis (≤ 200 mg/L) are inherent. Although they are considered to influence the results, the effect was not explicitly tested in this study.

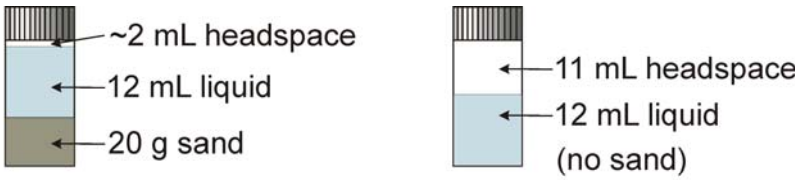
G.2 Ethanol Exposure Method Investigation

The results of the Washing Method Investigation (Part G.1) suggested that ethanol was diluted by the aquifer material pore water during exposure. This theory was tested by comparing the ethanol concentrations of systems with aquifer material (20 g wet weight) and without aquifer material. A 12 mL volume of 25% v/v (197250 mg/L) ethanol solution was added to all systems. Each system was tested in duplicate. The headspace was minimized to reduce the possibility of ethanol partitioning into the gas phase, even though partitioning was considered insignificant (Part G.1). By using 23 mL vials (instead of 100 mL or 250 mL bottles), the headspace was reduced to ~2 mL for systems with aquifer material and 11 mL for systems without aquifer material. Although the possibility of biodegradation of ethanol during exposure was also considered to be low, ethanol concentrations were measured at the start and end of the exposure period to verify this. In addition, the potential interference by sodium azide was tested by comparing ethanol concentrations of preserved and unpreserved samples. The results are summarized in Figure G.2.

Similar ethanol concentrations for preserved and unpreserved samples suggest that ethanol biodegradation during sampling and analysis was not significant and that sodium azide did not interfere with the analysis. Ethanol concentrations at the end of the exposure were not less than those

at the start of the exposure, suggesting that ethanol biodegradation did not occur during the exposure. In fact, the concentrations were slightly higher at the end than at the start, which can be reasonably attributed to error associated with sample dilution to within the detection range of the analysis or to analytical bias.

The results confirm that dilution of the ethanol by aquifer material was occurring. For systems with aquifer material, the measured ethanol concentrations were 15-17% lower than the concentration of the ethanol solution added, whereas there was no considerable difference observed for systems without aquifer material. As in Part G.1, the aquifer material pore water in this system can account for ethanol concentrations 23 % lower than the concentration of the ethanol solution added.



	25% (v/v) 197250 mg/L		25% (v/v) 197250 mg/L	
	unpreserved	Na azide preserved	unpreserved	Na azide preserved
start of 72h exposure:	**~164000** (~17% less than target)	**~164000** (~17%)	**~197000**	**~198000**
end of 72h exposure:	**~167000** (~15% less than target)	**~167000** (~15%)	**~201000**	**~202000**

Figure G.2 Set up and results of the Ethanol Exposure Method Investigation.

G.3 Washing Calculation for Inhibition Experiment 2

The number and volume of washing steps required to reduce the residual concentration of ethanol to <2 mg/L for Inhibition Experiment 2 was estimated.

The Washing Method Investigation (Part G.1) resulted in an average of ~96% ethanol removal from 20 g aquifer material during each 200 mL washing step. This empirical data was used to estimate the volume of residual liquid remaining in the centrifuge tube after a washing step:

$$C_{res}V_{res} = C_{wash}\left(V_{res} + V_{wash}\right)$$

$$V_{res} = \frac{C_{wash}V_{wash}}{C_{res} - C_{wash}}$$

where V_{res} is the volume of residual liquid after removal
C_{res} is the ethanol concentration of the residual liquid after removal
V_{wash} is the volume of sterile water added during the washing step
C_{wash} is the ethanol concentration of the solution after addition of water during the washing step.

and $C_{wash} = 0.04\ C_{res}$ when V_{wash} = 200 mL, based on empirical data in Part G.1.

Therefore, for 200 mL washing volumes, the volume of residual liquid after each washing stage was estimated:

$$V_{res} = \frac{0.04C_{res}V_{wash}}{C_{res} - 0.04C_{res}} = \frac{0.04V_{wash}}{1 - 0.04} = \frac{0.04}{0.96}(200mL) = 8.3mL$$

Assuming that the material is washed consistently so that the residual volume of liquid after washing and removal is always 8.3 mL, the washing efficiency for any washing volume can be calculated.

So, for 230 mL wash volumes:

$$C_{wash} = \frac{C_{res}V_{res}}{V_{res} + V_{wash}} = \left(\frac{8.3mL}{8.3mL + 230mL}\right)C_{res} = 0.035C_{res}$$

Therefore, each 230 mL washing step may be expected to remove ~96.5% ethanol. Based on this information, the concentrations theoretically expected during each washing step of initial concentrations of 25% v/v (197250 mg/L), 50% v/v (394500 mg/L), and 75% v/v (591750 mg/L) were calculated and are presented in Figure G.3.

For Inhibition Experiment 2, each 230 mL wash was anticipated to remove 96.5% of the ethanol and a series of 4 washes was anticipated to yield final residual ethanol concentrations of <2 mg/L. In addition, the aquifer material pore water in this system was expected to dilute the ethanol, resulting in initial concentrations 23% lower than the target concentrations.

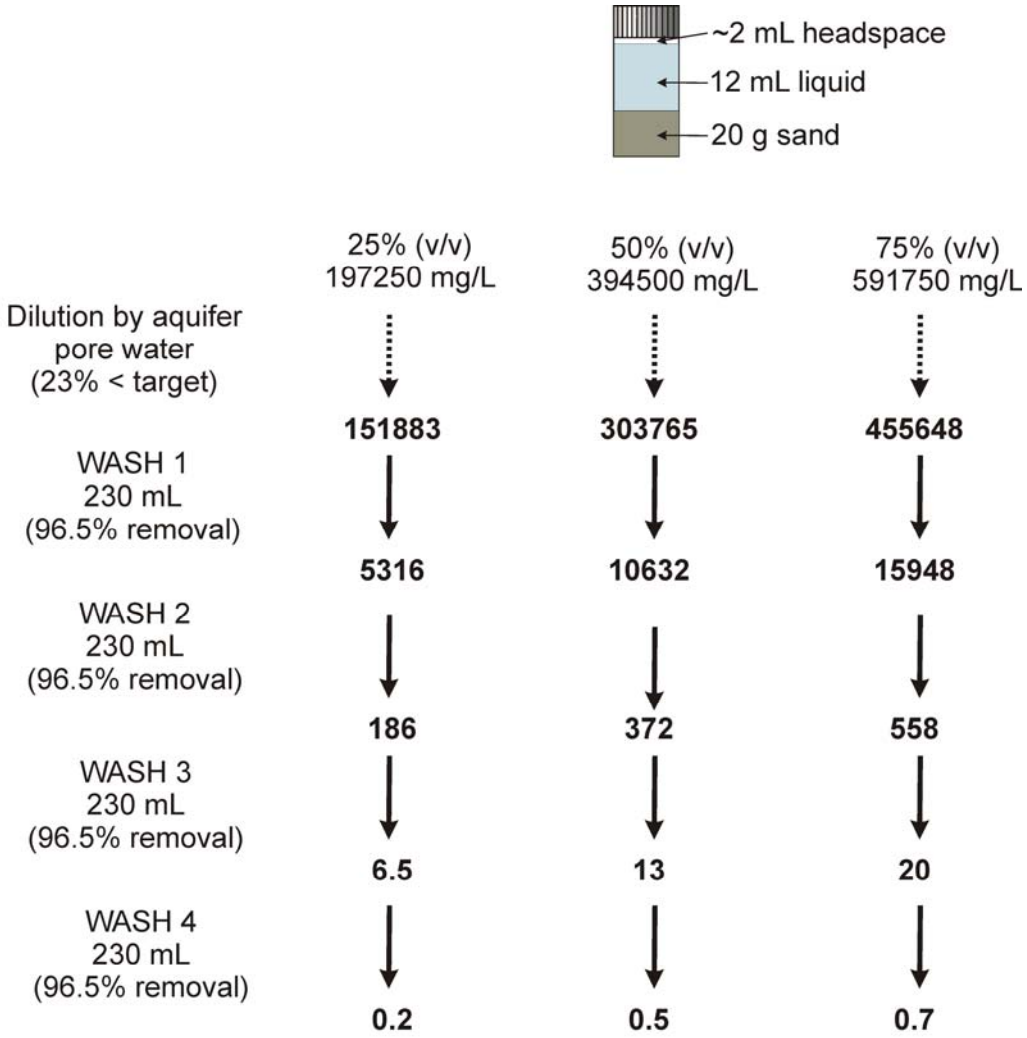


Figure G.3 Theoretical ethanol concentrations expected for Inhibition Experiment 2. Initial dilution of by the aquifer material pore water is expected to result in initial ethanol concentrations ~23% less than the target concentrations. Each 230 mL wash is expected to reduce the ethanol concentration by 96.5%.

Appendix H
Bromide Tracer Test

H.1 Bromide Standard Curve

The bromide standard curve was made using two stock solutions (Stock A and B) of 1000 mg/L bromide ion (Br^-) (prepared as 1288 mg/L NaBr) in deionized water. The stock solutions were diluted to final Br^- concentrations ranging from 2 to 200 mg/L. Electrical potential of the diluted solutions was measured in triplicate (Table H.1) and plotted against Br^- concentration to produce the standard curve (Figure H.1).

Table H.1 Bromide standard curve raw data.

Bromide Concentration (mg/L)	Stock Used	Electrical Potential (mV)		
		Replicate 1	Replicate 2	Replicate 3
2	Stock A	-60.0	-56.3	-65.0
5	Stock B	-80.0	-78.0	-82.8
10	Stock A	-96.3	-95.7	-97.9
25	Stock A	-119.9	-119.8	-120.0
50	Stock B	-137.8	-137.8	-137.9
100	Stock B	-156.0	-154.3	-155.5
200	Stock A	-172.9	-172.8	-173.3

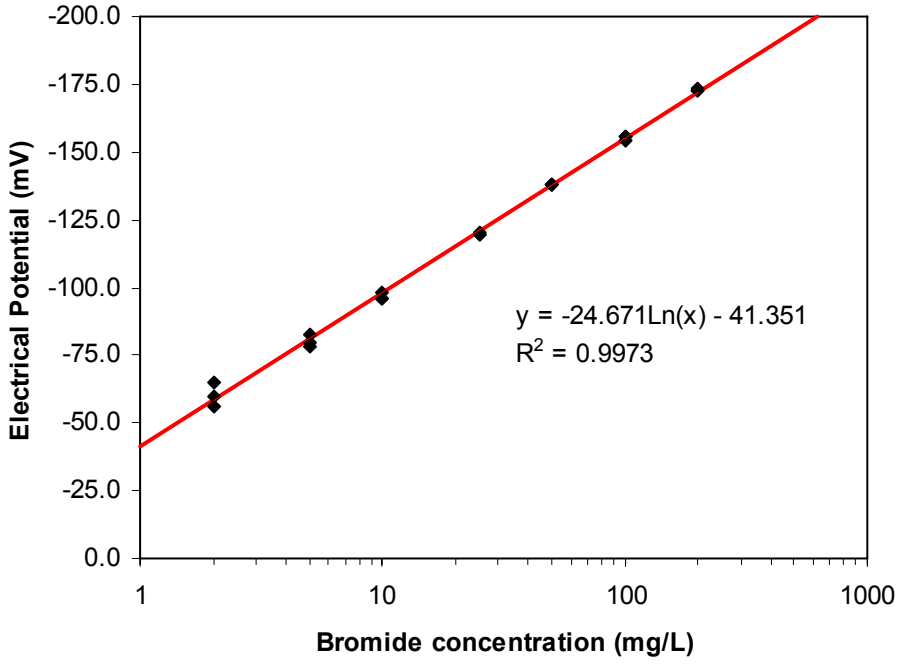


Figure H.1 Bromide standard curve

H.2 Bromide Breakthrough Curve

The tracer tests were conducted for Columns A and B using groundwater amended with sodium bromide to a concentration of 70 mg/L Br^- (90 mg/L NaBr). The pumping rate was 0.5 mL/min and samples of effluent were collected over 20 minute intervals. The breakthrough data were fit to the simplified Ogata-Banks solution for one-dimensional flow with advection and dispersion (Equation H-1; from Domenico and Schwartz, 1998).

$$C(x,t) = \left(\frac{C_0}{2}\right) erfc\left[\frac{(x - vt)}{2(Dt)^{1/2}}\right] \qquad \text{(H-1)}$$

where C is the Br^- concentration, C_0 is the Br^- source concentration, x is the distance from the column inlet, v is the velocity of the Br^- tracer, t is time, and D is the dispersion coefficient.

Dispersion Coefficient

For the best fit of the Ogata-Banks solution to the data, the dispersion coefficient was estimated:

$$D = 3 \times 10^{-7}\, m^2 / \min = 0.003 cm^2 / \min$$

Pore Velocity

For the following known data and from the breakthrough data the tracer velocity (v) was estimated:

Column length (x) = 12 cm

From the breakthrough data, $C/C_0 = 0.5$ at approximately t = 660 min.

$$v = \frac{x}{t} = \frac{12 cm}{660 \min} = 0.018 cm / \min = 1.1 cm / h$$

Porosity

For the following known and measured data and from the calculated velocity the porosity (η) was estimated:

Column radius (r) = 5.2 cm

Discharge rate (Q) = 0.486 mL/min

$$\eta = \frac{Q}{Av} = \frac{Q}{(\pi r^2)v} = \frac{0.486 mL / \min}{\pi (5.2)^2 (0.018 cm / \min)} = 0.32$$

Pore Volume

Based on the length of the column and the pore velocity, the time for one pore volume to be displaced (t_{PV}) was estimated:

$$t_{PV} = \frac{x}{v} = \frac{12 cm}{1.1 cm / h} = 11 h$$

Reference

Domenico, P.A., and F.W. Schwartz. 1998. Physical and Chemical Hydrogeology. John Wiley & Sons, New York, pp. 372-375.

Table H.2 Breakthrough data of the bromide tracer tests for Columns A and B.

Elapsed Time (min)	Column A				Column B			
	Potential (mV)	Br- conc (mg/L)	Measured C/Co	Simplified Ogata Banks C/Co	Potential (mV)	Br- conc (mg/L)	Measured C/Co	Simplified Ogata Banks C/Co
50	0.1	0.19	0.00	0.00	-1.9	0.20	0.00	0.00
70	1.1	0.18	0.00	0.00	1.9	0.17	0.00	0.00
90	1.2	0.18	0.00	0.00	1.7	0.17	0.00	0.00
110	1.6	0.18	0.00	0.00	1.1	0.18	0.00	0.00
130	1.9	0.17	0.00	0.00	1.6	0.18	0.00	0.00
150	1.4	0.18	0.00	0.00	2.1	0.17	0.00	0.00
170	2.2	0.17	0.00	0.00	2.1	0.17	0.00	0.00
190	2.3	0.17	0.00	0.00	2.7	0.17	0.00	0.00
210	1.6	0.18	0.00	0.00	2.0	0.17	0.00	0.00
230	2.1	0.17	0.00	0.00	2.2	0.17	0.00	0.00
250	1.7	0.17	0.00	0.00	1.8	0.17	0.00	0.00
270	2.0	0.17	0.00	0.00	3.0	0.17	0.00	0.00
290	0.4	0.18	0.00	0.00	2.1	0.17	0.00	0.00
310	-1.3	0.20	0.00	0.00	-0.5	0.19	0.00	0.00
330	-1.8	0.20	0.00	0.00	-1.4	0.20	0.00	0.00
350	-5.8	0.24	0.00	0.00	-4.6	0.23	0.00	0.00
370	-12.0	0.30	0.00	0.00	-10.3	0.28	0.00	0.00
390	-19.7	0.42	0.01	0.00	-19.7	0.42	0.01	0.00
410	-30.9	0.65	0.01	0.00	-32.7	0.70	0.01	0.00
430	-45.0	1.16	0.02	0.00	-40.0	0.95	0.01	0.00
450	-56.5	1.85	0.03	0.01	-52.3	1.56	0.02	0.01
470	-67.7	2.91	0.04	0.01	-62.9	2.40	0.03	0.02
490	-77.5	4.33	0.06	0.03	-72.7	3.56	0.05	0.04
510	-85.9	6.08	0.09	0.05	-81.9	5.17	0.07	0.06
530	-94.8	8.73	0.12	0.07	-93.0	8.11	0.12	0.09
550	-99.9	10.73	0.15	0.11	-97.9	9.90	0.14	0.14
570	-106.1	13.80	0.20	0.16	-102.1	11.73	0.17	0.19
590	-110.0	16.16	0.23	0.21	-108.8	15.39	0.22	0.25
610	-115.0	19.79	0.28	0.27	-115.0	19.79	0.28	0.32
630	-119.2	23.46	0.33	0.34	-120.9	25.14	0.36	0.39
650	-124.1	28.62	0.41	0.41	-126.1	31.04	0.44	0.46
670	-128.6	34.35	0.49	0.48	-129.9	36.20	0.51	0.54
690	-131.7	38.95	0.55	0.55	-133.2	41.39	0.59	0.61
710	-134.2	43.10	0.61	0.62	-135.6	45.61	0.65	0.67
730	-136.5	47.31	0.67	0.68	-138.2	50.68	0.72	0.73
750	-138.4	51.10	0.73	0.74	-139.9	54.30	0.77	0.78
770	-141.6	58.17	0.83	0.78	-141.9	58.89	0.84	0.82
790	-142.0	59.12	0.84	0.83	-143.3	62.32	0.89	0.86
810	-143.0	61.57	0.87	0.86	-143.3	62.32	0.89	0.89
830	-144.7	65.96	0.94	0.89	-145.0	66.77	0.95	0.92
850	-145.2	67.31	0.96	0.92				0.94
870	-145.6	68.41	0.97	0.94	-145.9	69.25	0.98	0.95
890	-146.0	69.53	0.99	0.95	-146.2	70.10	1.00	0.96
910	-146.2	70.10	1.00	0.96	-146.3	70.38	1.00	0.97
930	-146.2	70.10	1.00	0.97	-146.3	70.38	1.00	0.98
950	-146.3	70.38	1.00	0.98	-146.3	70.38	1.00	0.99
970	-146.3	70.38	1.00	0.99	-146.4	70.67	1.00	0.99
990	-146.4	70.67	1.00	0.99	-146.3	70.38	1.00	0.99
1010	-146.3	70.38	1.00	0.99	-146.3	70.38	1.00	1.00

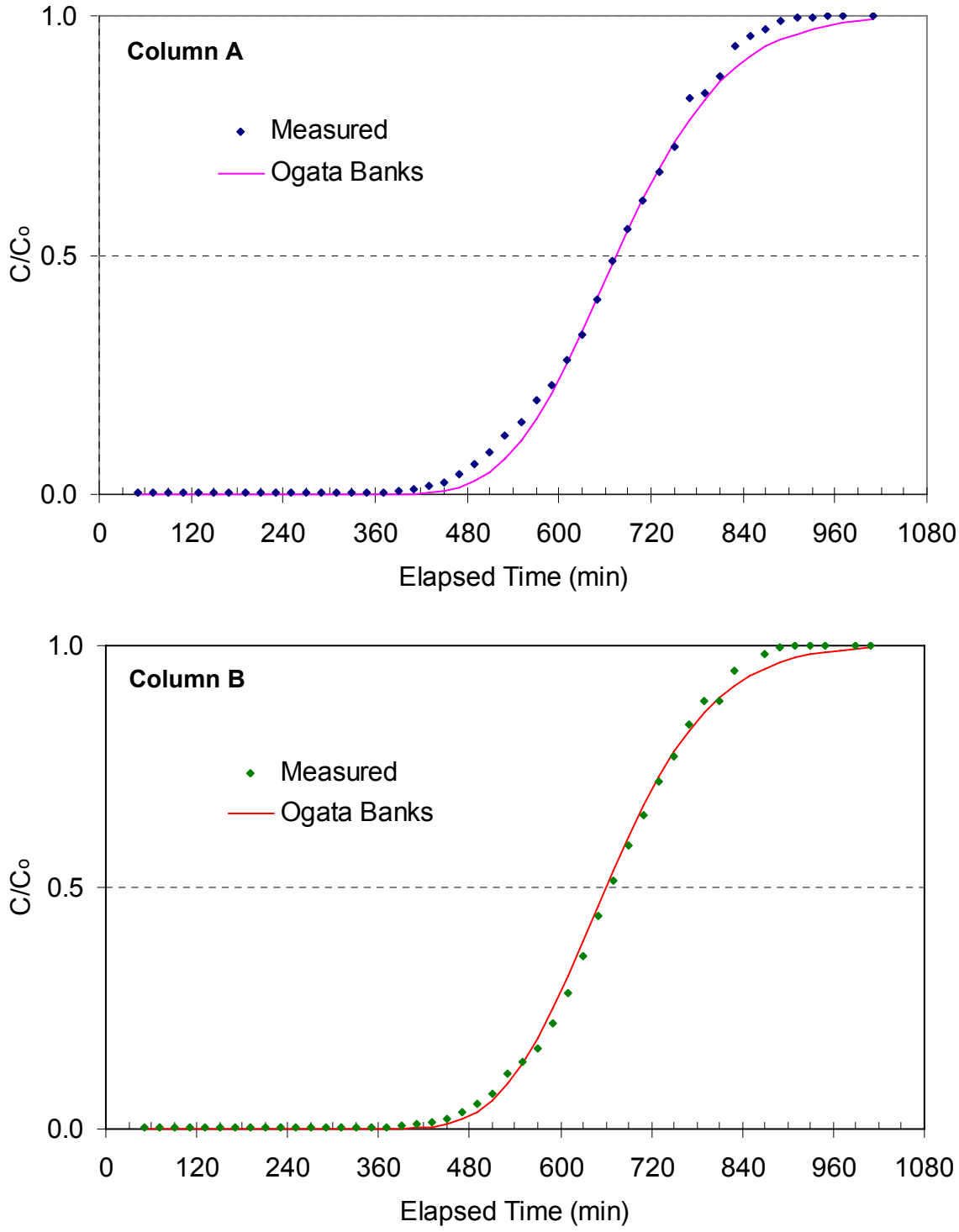


Figure H.2 Bromide breakthrough curves for Columns A and B.

Appendix I
MPN Test Results

Table I.1 Enumeration of aerobic BTEX-degrading bacteria using the three-tube Most Probable Number (MPN) test. Incubation period was 3 weeks at room temperature in the dark. Tubes were ranked positive if greater than 50% BTEX loss was observed. The BTEX analysis was performed by Marianne VanderGriendt on April 26, 2007.

		Dilution						MPN	MPN (# bacteria	95% Confidence Limits	
Sample	Rep	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	code	per g soil)	Lower	Upper
Original	1	+	+	–	–	–					
Soil	2	+	+	+	–	–					
	3	+	+	–	–	–		3,1,0	4300	700	21000
Column A	1			+	+	+	–				
Bottom	2			+	+	+	–				
	3			+	+	–	–	3,2,0	930000	150000	3800000
Column A	1			+	+	–	–				
Middle	2			+	+	–	–				
	3			+	+	+	–	3,1,0	430000	70000	2100000
Column A	1			+	+	+	–				
Top	2			+	+	+	–				
	3			+	+	–	–	3,2,0	930000	150000	3800000
Column B	1		+	+	+	+	–				
Bottom	2		+	+	–	–	–				
	3		+	+	–	–	–	3,1,1	75000	14000	230000
Column B	1		+	+	+	–	–				
Middle	2		+	+	+	+	–				
	3		+	+	–	–	–	3,2,1	150000	30000	440000
Column B	1		+	+	+	+	+				
Top	2		+	+	+	–	–				
	3		+	+	–	–	–	3,2,2	210000	35000	470000
Colum C	1		+	+	+	+	–				
Bottom	2		+	+	+	+	–				
	3		+	+	+	+	+	3,3,1	4600000	710000	24000000
Column C	1		+	+	+	+	–				
Middle	2		+	+	+	+	–				
	3		+	+	+	+	–	3,3,0	2400000	360000	13000000
Column C	1		+	+	+	–	–				
Top	2		+	+	+	–	–				
	3		N/A	+	+	+	–	3,1,0	430000	70000	2100000

	Ranking		Ranking
Blank-1	–	BLANK #5	–
Blank-2	–	BLANK #6	–
Blank-3	–	BLANK #7	–
Blank-4	–	BLANK #8	–
Sterile Blank #1	–	Sterile Blank 5	–
Sterile Blank #2	–	Stirile Blank 6	–
Sterile Blank #3	–	Sterile Blank 7	–
Sterile Blank #4	–	Sterile Blank 8	–

Note: if BTEX concentration is less than half of the average blank value, then rank as positive.

Appendix J
Denaturing Gradient Gel Electrophoresis (DGGE) Results

microbialinsights

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DNA Analysis Report

Client: Erika Williams
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Phone: (519) 888-4567

Fax:

MI Identifier: 014EF **Date Rec:** 06/06/2007 **Report Date:** 06/22/2007

Client Project #: **Client Project Name:**

Purchase Order #:

Analysis Requested: DGGE, GenBank Accession Number (DGGE)

Comments:

All samples within this data package were analyzed under U.S. EPA Good Laboratory Practice Standards: Toxic Substances Control Act (40 CFR part 790). All samples were processed according to standard operating procedures. Test results submitted in this data package meet the quality assurance requirements established by Microbial Insights, Inc.

Reported By: Anita Biernacki

Reviewed By: Dora M Ogles

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DGGE

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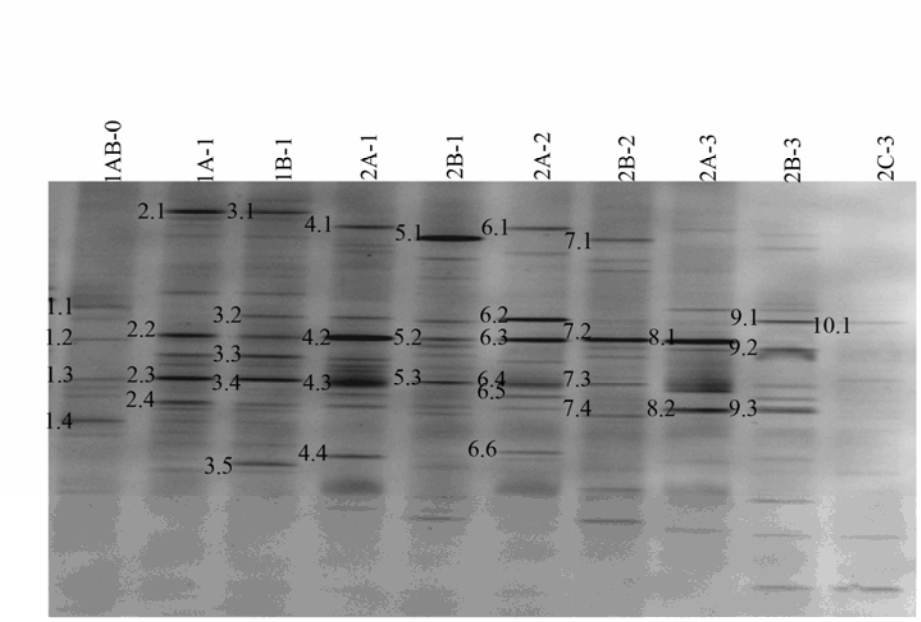

Figure 1. DGGE profile of amplified DNA from a portion of the 16S rRNA gene. Banding patterns and relative intensities of the recovered bands provide a means of comparing the communities. Bacteria must constitute at least 1-2% of the total bacterial community to form a visible band. Labeled bands were excised and sequenced. Results from sequencing can be found in the following table.

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Table 1. Sequence results from bands excised from Figure 1. Identifications are based on DNA sequences in the Ribosomal Database Project (RDP). Similarity indices above .900 are considered excellent, .700-.800 are good, and below .600 are considered to be unique sequences. Phylogenic affiliations are presented as phylum followed by family unless otherwise noted.

Band	Similar genus	Similarity Index	Donors	Acceptors	Description
1.3	*Hydrogenophaga spp.*	0.915	H2, organics	O2	Some Hydrogenophaga can degrade methyl-tert-butyl ether, and some can oxidize carbon monoxide.
2.1	*Acidobacterium*	1.00	H2	NO2, fumarate	Isolated in soils and deep sea vents
2.2	*Sphingomonas spp.*	1.00	many organics, recalcitrant compounds	O2	Aerobic phototrophic bacteria that do not possess the capability for anaerobic, photosynthetic growth. in which they utilized the contaminant as a carbon source demonstrating their degradative abilities. They also have the ability to grow and survive und
2.4	*Methylibium spp.*	0.890			
2.5	*Caulobacter spp.*	0.960	organics	O2	Can grow in distilled water
3.1	*Acidobacterium*	0.851	H2	NO2, fumarate	Isolated in soils and deep sea vents
3.2	*Acidovorax spp.*	0.781	H2, organic & amino acids	O2	Fixes N2. Isolated from soil and water with an optimal temperature of 28 degrees Celsius. Three recognized species.
3.3	*Aquabacterium spp.*	0.873			
3.4	*Methylibium spp.*	0.869			
4.1	*Erythrobacter spp.*	0.742	organics	O2	Members of this genus are aerobic phototrophic bacteria that do not possess the capability for anaerobic, photosynthetic growth.
4.2	*Erythrobacter spp.*	0.939	organics	O2	Members of this genus are aerobic phototrophic bacteria that do not possess the capability for anaerobic, photosynthetic growth.
4.2	*Sphingomonas spp.*	0.939	many organics, recalcitrant compounds	O2	Aerobic phototrophic bacteria that do not possess the capability for anaerobic, photosynthetic growth. in which they utilized the contaminant as a carbon source demonstrating their degradative abilities. They also have the ability to grow and survive und
4.4	*Bradyrhizobium spp.*	0.959	organics	O2	N2-fixing plant symbiote found in soil
5.1	*Chitinophaga spp.*	0.876	Organics incl. chitin	O2	Chemmoorganotrophic oxidative or fermentive.
5.2	*Sphingomonas spp.*	0.848	many organics, recalcitrant compounds	O2	Aerobic phototrophic bacteria that do not possess the capability for anaerobic, photosynthetic growth. in which they utilized the contaminant as a carbon source demonstrating their degradative abilities. They also have the ability to grow and survive und

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Band	Similar genus	Similarity Index	Donors	Acceptors	Description
5.3	*Sphingomonas spp.*	0.931	many organics, recalcitrant compounds	O2	Aerobic phototrophic bacteria that do not possess the capability for anaerobic, photosynthetic growth. in which they utilized the contaminant as a carbon source demonstrating their degradative abilities. They also have the ability to grow and survive und
6.1	*Methylibium spp.*	0.777			
6.2	*Acidovorax spp.*	0.904	H2, organic & amino acids	O2	Fixes N2. Isolated from soil and water with an optimal temperature of 28 degrees Celsius. Three recognized species.
6.3	*Sphingomonas spp.*	0.821	many organics, recalcitrant compounds	O2	Aerobic phototrophic bacteria that do not possess the capability for anaerobic, photosynthetic growth. in which they utilized the contaminant as a carbon source demonstrating their degradative abilities. They also have the ability to grow and survive und
6.4	*Hydrogenophaga spp.*	0.791	H2, organics	O2	Some Hydrogenophaga can degrade methyl-tert-butyl ether, and some can oxidize carbon monoxide.
6.5	*Sphingomonas spp.*	1.00	many organics, recalcitrant compounds	O2	Aerobic phototrophic bacteria that do not possess the capability for anaerobic, photosynthetic growth. in which they utilized the contaminant as a carbon source demonstrating their degradative abilities. They also have the ability to grow and survive und
7.1	*Chitinophaga spp.*	0.870	Organics incl. chitin	O2	Chemmoorganotrophic oxidative or fermentive.
7.2	*Zoogloea spp.*	0.870	organics	O2, Mn, metals	Found in rivers, streams, sludge. Typical activated sludge bacterium. Matrix slightly water soluble, stable at pH 3-10 and temperatures -15 to 90 degrees C.
7.3	*Methylibium spp.*	0.888			
7.4	*Nitrospira spp.*	0.764	NH4+	O2	microaerophilic marine bacteria
8.1	*Sphingomonas spp.*	0.899	many organics, recalcitrant compounds	O2	Aerobic phototrophic bacteria that do not possess the capability for anaerobic, photosynthetic growth. in which they utilized the contaminant as a carbon source demonstrating their degradative abilities. They also have the ability to grow and survive und
8.2	*Caulobacteraceae (family)*	0.701			
9.1	*Acidovorax spp.*	0.871	H2, organic & amino acids	O2	Fixes N2. Isolated from soil and water with an optimal temperature of 28 degrees Celsius. Three recognized species.
9.2	*Sphingomonas spp.*	0.737	many organics, recalcitrant compounds	O2	Aerobic phototrophic bacteria that do not possess the capability for anaerobic, photosynthetic growth. in which they utilized the contaminant as a carbon source demonstrating their degradative abilities. They also have the ability to grow and survive und

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Band	Similar genus	Similarity Index	Donors	Acceptors	Description
9.3	*Caulobacter spp.*	0.819	organics	O2	Can grow in distilled water
10.1	*Acidovorax spp.*	0.821	H2, organic & amino acids	O2	Fixes N2. Isolated from soil and water with an optimal temperature of 28 degrees Celsius. Three recognized species.

Excised bands not included in this table did not produce phylogenetic matches.

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GBAN

Client: **Univ. of Waterloo - Waterloo ON**
Project:

MI Project Number: 014EF
Date Received: 06/06/2007

Identifications are based on DNA sequences in the Ribosomal Database Project (RDP). Similarity indices above .900 are considered excellent, .700-.800 are good, and below .600 are considered to be unique sequences. Phylogenic affiliations are presented as phylum followed by family unless otherwise noted.

Band	Similar genus	Similarity Index	GenBank Accession Number
1.3	*Hydrogenophaga spp.*	0.915	AY569978, AF019073
2.1	*Acidobacterium*	1.00	Z95709
2.2	*Sphingomonas spp.*	1.00	Y10677
2.4	*Methylibium spp.*	0.890	AF176594
2.5	*Caulobacter spp.*	0.960	AJ007805
3.1	*Acidobacterium*	0.851	Z95709
3.2	*Acidovorax spp.*	0.781	AJ420324
3.3	*Aquabacterium spp.*	0.873	AM696981
3.4	*Methylibium spp.*	0.869	AF176594
4.1	*Erythrobacter spp.*	0.742	DQ396311
4.2	*Erythrobacter spp.*	0.939	Y16267
4.2	*Sphingomonas spp.*	0.939	Y15514
4.4	*Bradyrhizobium spp.*	0.959	AY649437
5.1	*Chitinophaga spp.*	0.876	AJ581603
5.2	*Sphingomonas spp.*	0.848	AY212693
5.3	*Sphingomonas spp.*	0.931	Y10677
6.1	*Methylibium spp.*	0.777	AF176594
6.2	*Acidovorax spp.*	0.904	AJ420324
6.3	*Sphingomonas spp.*	0.821	AY328595
6.4	*Hydrogenophaga spp.*	0.791	AY569978
6.5	*Sphingomonas spp.*	1.00	AB033945
7.1	*Chitinophaga spp.*	0.870	AJ581603
7.2	*Zoogloea spp.*	0.870	AJ505853
7.3	*Methylibium spp.*	0.888	AF176594
7.4	*Nitrospira spp.*	0.764	AF035813
8.1	*Sphingomonas spp.*	0.899	AY245435
8.2	*Caulobacteraceae (family)*	0.701	DQ532288
9.1	*Acidovorax spp.*	0.871	AJ420324
9.2	*Sphingomonas spp.*	0.737	L22759, AB099636
9.3	*Caulobacter spp.*	0.819	AY512823
10.1	*Acidovorax spp.*	0.821	AJ420324

Excised bands not included in this report did not produce phylogenetic matches.