Effect of Fuel Ethanol on Subsurface Microorganisms and its Influence on Biodegradation of BTEX Compounds

by

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Abstract

Ethanol is used as fuel in neat form in some countries (Brazil and India) or blended with gasoline (Europe, Canada and the United States). The benefits of ethanol use include octane enhancement, a cleaner environment and a secure renewable energy supply. BTEX compounds (benzene, toluene, ethylbenzene, *m*-xylene, *p*-xylene and *o*-xylene) are aromatic hydrocarbons present in gasoline. The fate of these compounds in the environment is of great health concern due to their carcinogenic (benzene) and toxic properties, and due to their high solubility in water compared to the other gasoline hydrocarbons. Ethanol present in gasoline may affect BTEX degradation, in a event of a spill into the subsurface environment. To address the effects of ethanol on subsurface microorganisms, microbial activity and growth in the presence of ethanol (concentrations ranging 0 to 70% v/v) were assessed. Microcosms studies showed that ethanol at concentration ranging 0.5 to 3% (v/v) enhanced microbial activity and did not interfere in microbial growth at 10°C temperature, when another source of carbon was present (glucose). Ethanol at 0.5% concentration enhanced microbial activity over water soluble gasoline components and R2A medium combined. Both microbial activity and growth were not detected at ethanol concentrations equal and above 5%. Biodegradation study was conducted, in which subsurface material and ground water were exposed to BTEX and ethanol at 0.5 and 1.5% (v/v) concentration. The controls had BTEX alone and ethanol alone, sterile and nutrient-free. Total BTEX degradation was observed whenever ethanol was absent. Ethanol and BTEX were simultaneously degraded, however in microcosms containing 0.5% ethanol, BTEX degradation was slowed, compared to microcosms without ethanol. Competition for inorganic nutrients was the major problem in slowed BTEX degradation in the presence of ethanol. In microcosms where 1.5% ethanol was present, BTEX compounds and ethanol degradation were not observed.

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To
Ambrósio
Carolina
Carlson
Zelma

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Chapter 1

Introduction

The political instability of many nations that supply petroleum and the realization that petroleum is a finite source of energy has led some countries to invest in ethanol production to use as fuel. These countries, including Brazil and India, utilize ethanol from microbial fermentation as a principal energy source for vehicles and as a feed stock for industries. The use of ethanol as fuel was a result of an intense search for an alternative source of energy to power the transportation system and industries. In the late 1960s through the early 1970s Brazil invested in intense research for an alternative fuel and ethanol, produced from sugar cane, was chosen. In 1975 Brazil established the Programa Nacional do Álcool (National Alcohol Program), which promoted the use of ethanol as a fuel substitute for gasoline and increased ethanol production for industrial use [64]. Nowadays, filling stations provide pure alcohol or a blend of gasoline containing as much as 22% of ethanol [21], that are stored in two separated underground tanks [22].

Ethanol can be used as fuel in neat form or blended with gasoline. It is also used as a raw material in industrial and technological processes. Fuel ethanol is a high octane, water-free alcohol produced from the fermentation of sugar or converted starch. The benefits of ethanol use include octane enhancement, a cleaner environment and a secure renewable energy supply.

Ethanol is also used in Europe, Canada and the United States as an octane enhancer in lead-free gasoline replacing up to 10% of the petroleum [38]. In order to reduce the emissions of carbon monoxide and other pollutants to the atmosphere, the United States have introduced the addition of oxygenated compounds to gasoline. Ethanol, which is being produced from corn, is one of the most common oxygenates used to meet the

requirements for the U.S. EPA's [1] Reformulated Gasoline (RFG) and Oxygenated Fuel (Oxyfuel) Programs [29]. Other oxygenates currently used are methanol, MTBE (methyl tertiary butyl ether), ETBE (ethyl tertiary butyl ether) and TBA (tertiary butyl alcohol).

Benzene, toluene, ethylbenzene, *m*-xylene, *p*-xylene and *o*-xylene (BTEX) are aromatic hydrocarbons present in gasoline. The proportion of BTEX compounds in gasoline is low, consisting of only 2 to 8% by weight of gasoline. However, their high solubility in water, compared to the solubility of other gasoline components, has made them hazardous chemicals to ground water quality. The fate of these compounds in the environment is of great health concern due to their carcinogenic (benzene) and toxic properties [68]. Aromatic hydrocarbons are very stable molecules, formed by one or more benzene rings but they can be degraded by microorganisms that have evolved biochemical pathways, due to the natural presence of benzene structures in the environment [20].

1.1 Ethanol and BTEX Properties

Ethanol is a colorless, neutral, mobile liquid. This monohydric, short chain alcohol can be produced by fermentation of any raw material that contains carbohydrates [50]. There are three types of raw materials used in the manufacture of ethanol via fermentation. Sugars, from sugar cane, sugar beet, molasses and fruit, are converted to ethanol directly. Starches, from grains, potatoes and root crops, must first be hydrolyzed to fermentable sugars by action of enzymes from malt or moulds. Cellulose, from wood, agriculture wastes, and liquor from pulp mills must likewise be converted to simple sugars by the action of mineral acids [64].

The physical and chemical properties of ethanol and BTEX compounds are listed in Table 1.1. These properties are important to be taken into consideration when predicting the fate of these compounds in the environment. The Henry's law constant describes the air/water partitioning of a compound. It gives the ratio of the partial pressure of the compound in the gas phase to the concentration in the water that is at equilibrium with that partial pressure [77]. Henry's law constant (H) has a dimensionless value when it is divided by the product of the gas constant ($R = 8.2 \times 10^{-5}$ atm m^3/mol K) and the temperature (T = degrees K). H/RT gives the ratio between air and water concentration at equilibrium. A compound with a dimensionless Henry's law constant equal to or larger than 0.05 is considered to be very volatile from water [77]. Ethanol's solubility in water

[1] United States Environmental Protection Agency

is infinite and a dimensionless Henry's Law constant of approximately 0.0002 (Table 1.1) indicates that this alcohol is more likely to remain dissolved in water than to partition to the gas phase (vaporization).

The BTEX compounds exhibit dimensionless Henry's law constant of 0.2 to 0.3 (Table 1.1), indicating that these compounds are very volatile.

The octanol:water partition coefficient (K_{ow}) is the ratio of the solute concentration in an octanol phase to the solute concentration in the water phase of an octanol-water mixture [77]. Octanol was chosen because it mimics the lipids found in organisms and provides a simple way to assess if a specific compound would accumulate in biological tissue or not. Ethanol has a low K_{ow} (Table 1.1), meaning that it does not have a high bioconcentration effect if accumulated in biological tissue. In contrast, the BTEX have high K_{ow} values compared to ethanol and they do have a high bioconcentration effect, compared to ethanol, in biological tissue.

The Sorption Distribution Coefficient (K_d) indicates the tendency of a compound to sorb to solid surfaces when dissolved in water. When an organic compound is dissolved in ground water and that ground water is moving through a porous subsurface medium, the factor that determines the velocity of a compound relative to the ground water velocity is the soil/water partition coefficient [77]. K_d values are the product of the soil/water partition coefficient (K_{oc}) and the fraction of organic carbon in the soil (f_{oc}). The values of K_{oc} for BTEX compounds (Table 1.1) are not considered high, so there is almost no retardation on transportation of these compounds. Ethanol's K_{oc} value is far lower than the BTEX's, indicating that ethanol will move at the same velocity of ground water and will probably be the predominant compound in a leading edge of a plume from a ethanol-gasoline spill.

Table 1.1: Physical and chemical properties of BTEX and ethanol according to Zogorski *et al.* [77].

Property	Benzene	Toluene	Ethylbenzene	*m*-Xylene	*p*-Xylene	*o*-Xylene	Ethanol
Molecular weight (g/mole)	78.11	92.13	106.16	106.16	106.17	106.16	46.07
Boiling point, °C	80.1	110.6	136.25	139.3	137	144.4	78.2
Specific gravity at 20°C	0.88	0.86	0.87	0.88	0.86	0.88	0.79
Water solubility (mg/L)	1,780	534.8	161	146	156	175	∞
Vapor pressure at 20°C (mm Hg)	76	28.4	9.53	8.3	8.7	6.6	44
Henry's Law constant ($atm\ m^3\ g^{-1}\ mole^{-1}$)	5.43E-3	5.94E-3	8.44E-3	7.68E-3	7.68E-3	5.1E-3	6.17 - 5.13E-6
Dimensionless Henry's Law constant (H/RT)[a]	0.22	2.4E-1	3.4E-1	3.1E-1	3.1E-1	2.0E-1	2.52-2.09E-4
Log K_{oc}	1.50-2.16	1.56-2.25	1.98-3.04	2.04-3.15	2.05-3.08	1.68-1.83	1.21, 0.2
log K_{ow}	1.56-2.15	2.11-2.80	3.15	3.20	3.08-3.29	2.77-3.12	− 0.16

[a] T=25°C

1.2 Ethanol Toxicity

Alcohols have been used as disinfectants and preservatives for many years. Most microbial cells are killed at ethanol concentrations above 15%, with bacterial spores being considerably more resistant [38]. Most bacteria exhibit a dose-dependent inhibition of growth over the range from 1% to 10% ethanol, and a few grow at 10%. For example, growth of *Escherichia coli* is inhibited at ethanol concentrations above 6% (v/v), while *Zymomonas mobilis*, a bacterium that produces ethanol as an end product of its metabolism, can grow at concentrations up to 8% (v/v) [38]. Among eukaryotes, the genus *Saccharomyces* is the most tolerant to ethanol. These organisms are used for production of ethanol by fermentation. They are able to grow at concentrations of 8 -12% (v/v), to survive exposure up to 15% (v/v) and to produce saké, in which ethanol concentration reaches 20% (v/v) [38].

Temperature plays a role in the ethanol tolerance of some microorganisms. Elevated temperatures reduce ethanol tolerance in *Z. mobilis*, *Clostridium acetobutylicum* and *Cl. thermocellum* [38]. However, a thermophilic ethanol-producing bacterium was recently isolated from a hot spring in Iceland. *Thermoanaerobacter mathranii* sp. nov. is able to grow in the range of 50 - 75°C and at ethanol concentrations up to 4% (w/v) [43].

The basis of bacterial killing by ethanol appears to be disruption of the cellular permeability barrier. High concentrations of ethanol solubilize lipids and denature proteins leading to membrane destruction in prokaryotes [38]. Also, cell membranes of *E. coli* treated with ethanol appeared to be more fluid than membranes not treated with that alcohol [39]. Membrane leakage in the presence of ethanol also occurs in eukaryotes. *Saccharomyces* spp. are expected to have an unusual lipid composition, since they can tolerate exposure to ethanol concentrations as high as 15% [38]. *S. cerevisiae* was reported by Ingram and Buttke [38] to be able to adapt to ethanol during growth by altering its membrane fatty-acyl composition. *Tetrahymena pyriformis*, a unicellular ciliated free-living protozoan is also able to grow in the presence of ethanol, although it is not likely to find high concentrations of ethanol in its natural environment. This protozoan is able to adapt to ethanol by also changing its membrane lipid composition [38].

In a study on the response of fresh water green algae to ethanol as a solvent pollutant, it was found that growth of most species used in the experiment was not inhibited in media treated with ethanol. In fact, *Chlorella* and *Scenedesmus* doubled their growth rate when treated with ethanol concentrations ranging from 0.05 to 0.2% (v/v) [73]. Ethanol was not toxic to marine diatoms in a similar study of the response of these organisms to solvent

pollutants. *Nitzschia dissipata* and *Thalassiosira wessflogii* had their growth stimulated at ethanol concentrations of 0.2% [74].

1.3 Subsurface Microbial Ecology

Before discussing microorganisms in the subsurface environment, it is necessary to briefly describe the conventions established for discussion of the subsurface environment. For the point of view of microbiology the division of the subsurface environment is based on the hydrologic attributes, since water is the most important factor in microbial life. The environment is basically divided into two zones: the unsaturated zone and the saturated zone. The unsaturated zone has pore spaces that are filled with water or air and is usually aerobic. Air is easily exchanged with the atmosphere and water is always recharged with precipitation. This zone is subdivided into the soil zone, which contains plant roots and an intermediate zone, that contains sediments or rock that have not been exposed to extensive soil forming processes [20]. The water table delimits the saturated zone, where water is abundant, oxygen and nutrients are limited.

The soil zone, sub-division of the unsaturated zone, has the major variety of microorganisms and is the most biologically active subsurface environment. The food chain is mostly composed of heterotrophs and decomposers, which are represented by bacteria and fungi [20]. Microorganisms are found in the interstitial spaces filled with water or in its vicinity and they are predominantly aerobes. The unsaturated zone serves as a "filter" to chemicals that are released in the ground. Before they reach the water table, these compounds can be degraded by the microorganisms that inhabit this zone, avoiding ground water contamination [20].

Aerobic bacteria decompose most organic compounds into carbon dioxide, water, and mineral matter, such as sulfate, nitrate, and other inorganic compounds, and do not produce hydrogen sulfide or methane as reaction products. The soil aerobic bacteria are responsible for biodegradation of hazardous materials and the most commonly isolated organisms in areas of gasoline contamination are heterotrophic bacteria of the genera *Pseudomonas*, *Achromobacter*, *Arthrobacter*, *Micrococcus*, *Vibrio*, *Acinetobacter*, *Brevibacterium*, *Corynebacterium*, *Flavobacterium*, *Mycobacterium*, and *Nocardia* [63]. *Pseudomonas* species appear to be the most ubiquitous and have shown an ability to metabolize a large number of organic pollutants.

The role of fungi is more in detoxification than in biodegradation of contaminants.

However, there are two orders that have the ability to degrade hydrocarbons: the Mucorales and the Moniliales [63]. The genera most frequently isolated from hydrocarbon-contaminated soils are those producing abundant small conidia; e.g., *Penicillium* and *Verticillium* spp. Oil-degrading strains of *Beauveria bassiana*, *Mortieriella* spp., *Phoma* spp., *Scolecobasidium obovatum*, and *Tolypocladium inflatum* have also been isolated [63]. Fifty-six out of 500 yeasts studied were found to be able to degrade hydrocarbons and among them are the genera *Candida*, *Rhodosporidium*, *Rhodotorula*, *Saccharomyces*, *Sporobolomyces*, and *Trichosporon* that have been identified from soil samples [63].

The intermediate unsaturated zone has not been much studied in terms of identification and enumeration of bacteria. It seems that the number of microorganisms in this zone decreases with depth, although there are data that suggest the contrary. It is probable that bacteria found in deeper environments within the intermediate zone may be the result of transportation of them from the upper subsurface zones. There are no conclusive data to make any statement in this matter [20].

The water table is a region of intense movement of water. It is continuously subjected to rise and fall of water resulting from precipitation and discharge to surface water bodies, respectively [20]. Oxygen and nutrients are often renewed, depending on the frequency of water movement. Aerobic microorganisms will be abundant as long as this zone is being aerated. Deeper in the saturated zone the anaerobes are predominant. There are facultative anaerobes, microaerophiles and obligate anaerobes [9]. The anaerobes utilize other electron acceptors than oxygen and according to the predominance of a specific electron acceptor, the group of microorganisms capable of using that electron acceptor will prevail [20]. For example, if there are large amounts of nitrate in the environment, denitrifiers will be prevalent, since they are able to best metabolize carbon using nitrogen oxides as electron acceptors. The sulfate reducers, iron-reducers and methanogens would be inactivated by competition.

Photosynthetic bacteria are not found in the saturated zone since there is no penetration of light. Primary-production based in chemolithotrophy exists, but it is rare [20]. Heterotrophs are predominant and they are decomposers, represented mainly by bacteria, and secondary consumers represented by some protozoans that may feed on the decomposers. The abundance and diversity of bacteria in these environments depends on the sediment type. The greatest numbers and diversity are found in sandy sediments and clays have the lowest counts and diversity [20]. In general, these organisms that inhabit the subsurface are able to survive with scarce source of organic matter (oligotrophs), which is not constantly

replenished.

1.4 Fate of Fuel Components in Terrestrial Environments

Petroleum hydrocarbons and materials derived from them are largely consumed by industrialized societies. The annual worldwide production is about 800 billion gallons [20] and handling this huge amount of petroleum frequently results in spills and contamination of the environment, for instance, soil and ground water systems. Fuel tanks, either the aboveground or underground variety, are prone to corrosion, which often causes leaks. Data collected in Europe and in the United States have shown that a steel fuel tank will corrode and leak within 15 to 20 years after installation [57].

Once a spill happens, petroleum hydrocarbons are acted upon by a combination of physical, chemical and biological processes that tend to attenuate the contamination. If the spill reaches the ground water, the water-soluble components, such as the BTEX compounds, are flushed from the spill site creating a plume. These compounds are of great concern due to their relatively high solubility in water. Some of the physical processes acting to attenuate BTEX compounds in the subsurface include gravity, ground water flow, sorption, volatilization and hydrodynamic dispersion [20]. Among potential chemical processes, which may act on organic contaminants are hydrolysis, oxidation and polymeralization, although these processes do not play a major role in BTEX attenuation. And finally, the biological processes, in which native microorganisms metabolize partially or totally the contaminants. Among biological processes are fermentation, polymeralization and total degradation. It is important to notice that a compound may not be totally metabolized by one specific population of microorganisms. A whole community may be involved in mineralizing one compound, each kind of organism being responsible for one part of the total biochemical pathway, each working under its own requirements for performing the degradation.

There are some requirements for these biological processes to happen. These can be basic requirements common for the growth of all organisms, like suitable temperature, pH, necessary nutrients, electron acceptors and specific requirements, like the presence of enzymes involved in biochemical pathways necessary to metabolize a specific compound. The contaminant availability to the microorganisms is an important factor. Some contaminants may be insoluble in water or sorbed in soil particles, and hence not available to the cell. Once the contaminant is soluble in water, where the organisms are found, its

toxicity to the microorganism is another factor to be considered. The concentration of the contaminant should also be tolerable to the microorganisms since high concentrations may be toxic or very low concentrations may not be taken up by the cell.

Temperature influences microbial growth and consequently the rate of biochemical processes. If temperature is too elevated, it can denature proteins essential for growth or, if the temperature is too low, it can slow or even stop microbial activity. Each species of microorganism has an optimum temperature for growth and a temperature range for growth. Microorganisms known as psychrophiles are able to grow at a temperature range of 0-20°C [14]. Examples of such environments are the bottom waters of deep ocean basins [20] and some ground water systems. Mesophiles are microorganisms that live in a temperature range of 20-40°C. This temperature range represents many natural environments inhabited by the majority of microorganisms [20]. Microorganisms that are able to grow at temperatures above 45°C are termed thermophilic. These microorganisms can be isolated from very deep aquifer systems and petroleum reservoirs [20], so they could be significant in the study of petroleum formation and biodegradation at depth.

As a second factor to be considered, pH can vary in a wide range in the environment. There are microorganisms inhabiting acid to alkaline environments. These microorganisms must have proton-transporting mechanisms that enable them to maintain an internal pH of approximately 7.5, which is the optimum pH range for many enzymes to function properly. A product of a specific reaction can be released into the environment and alter the surrounding pH. For example, sulfide-oxidizing bacteria produce sulfuric acid as a by-product of some biochemical pathways altering the surrounding pH to a pH 3-4 range and are able to survive in it [20]. Or a simple presence of a contaminant can also alter the pH and be toxic to the cells, for example, aquifers contaminated by municipal waste leachates, with organic acids, can lower the pH to 3.0. As an opposite range, aquifers contaminated by sludge from cement manufacture, can reach pH values as high as 11 [20].

Some inorganic nutrients are vital for all living organisms. Among them are nitrogen and phosphorus. They are essential for the production of proteins, enzymes, DNA, RNA, and other important molecules for cell growth. Electron acceptors are important for the transference of energy during metabolic processes. For the obligate aerobes, oxygen is the only usable electron acceptor. Some microorganisms (facultative anaerobes) utilize oxygen as the primary electron acceptor, but another electron acceptor can be used if oxygen is unavailable. The microaerophilic bacteria require oxygen to grow, but cannot tolerate oxygen in high concentrations [20]. And finally there are the obligate anaerobes,

that cannot tolerate oxygen and use other electron acceptors, such as sulfate (sulfate-reducers), CO_2 (methanogens), nitrogen oxides (denitrifiers) and iron (iron-reducers). Some compounds are known to be only degraded under aerobic conditions, such as aliphatic hydrocarbons [7]. Some compounds can be degraded either under aerobic or anaerobic conditions, or the combination of both such as benzene, toluene and xylenes [46]. Barbaro *et al.* [10] tested for biodegradation of BTEX compounds under denitrifying conditions in both field and laboratory conditions. Toluene was the most biodegradable and benzene was the most recalcitrant compound under strictly-anaerobic, denitrifying conditions. Major *et al.* [46] reported BTX degradation under aerobic and denitrifying conditions. Nales *et al.* [54] tested benzene degradation in microcosms under a variety of electron acceptors, using subsurface material from six different sites and found that benzene was degraded under strictly anaerobic conditions. But the presence of high organic matter inhibited the biodegradation of benzene and also the presence of toluene, ethylbenzene and the xylene isomers inhibited benzene degradation when sulfate and nitrate were used as electron acceptors, but not under iron-reducing conditions. Corseuil *et al.* [23] reported ethanol degradation under denitrifying, iron-reduced, sulfidogenic and methanogenic conditions, in microcosms incubated at 25°C.

Remediation processes are often required on accidental gasoline spills. The focus of substance remediation is on the BTEX compounds, which are present in the gasoline, as they pose risks to human health and the environment. Gasoline spills containing fuel oxygenates have already occurred, since oxygenates (MTBE) have been added to gasoline since 1979 [77]. The monitoring of fuel oxygenates contamination is infrequent due to government regulations (many do not require monitoring of oxygenates in the environment) and to the difficulty in determining leak rates, leak durations and concentration of oxygenates in the gasoline stored in an underground storage tank [77]. Hubbard *et al.* [34] conducted an injection study to determine transport and fate of MTBE, methanol and BTEX in a sandy aquifer. The resulting contaminated plume exhibited high concentrations of MTBE in its leading edge, which indicates that MTBE showed a faster transport rate than benzene, toluene, ethylbenzene and xylene. Hubbard *et al.* [34] found no measurable effect of MTBE on BTEX degradation.

High concentrations of alcohol oxygenates can be found in water adjacent to gasoline spills because of their high solubility in water. In the case of an ethanol-amended gasoline leakage, it is more likely to find high concentrations of ethanol in the water than BTEX compounds. Ethanol has an infinite solubility in water and a very low value for the dimensionless Henry's law constant compared to the BTEX values (Table 1.1). Although

ethanol is expected to be more rapidly degraded than BTEX compounds, since ethanol has a simpler chemical composition, there are no data reporting the presence or absence of ethanol in ground water at spill sites [77]. Due to the natural occurrence of ethanol and other alcohols in the environment, as a result of production by biological or chemical processes [38], natural populations of microorganisms able to process ethanol will be widespread. Ethanol is a by-product of any sugar fermentation. For example, the glycolysis or the Embden-Meyerhof pathway, describes the fermentation of glucose, a primary sugar. Glucose is oxidized to pyruvate which is reduced to ethanol [45]. Ethanol can be incompletely oxidized to acetic acid by acetic acid bacteria (e.g., *Acetobacter*) [45]. Stevenson and Katznelson [71] reported that, with ethanol concentration up to 2%, oxidation of ethanol to acetate in soil occurred readily, with the rate of oxidation increasing as the concentration decreased. Acetate is further oxidized to carbon dioxide and water. What remains unknown is the concentration at which ethanol starts to be toxic to the soil/subsurface microorganisms. Alcohol production as a result of fermentation of carbohydrates by the indigenous communities of microorganisms is small compared to the amount found in a spill. In an event of a spill, there would be ethanol concentrations as high as 10% (in the case of North America) or up to 95% (in the case of Brazil and other countries that use pure ethanol as fuel). As ethanol penetrates the subsurface environment, ethanol is diluted either by the soil water in the unsaturated zone or by ground water if ethanol reaches the saturated zone.

Corseuil *et al.* [23] studied the biodegradation of ethanol and BTX (benzene, toluene and *o*-xylene) in microcosms under aerobic conditions. Ethanol was added at three concentrations: 20, 100 and 300 mg/L (0.0025, 0.01, 0.03% v/v, respectively) and microcosms were incubated at 20°C. BTX compounds were added at 20 mg/L. Ethanol at the concentration of 100 mg/L was completely degraded. Benzene, toluene and *o*-xylene were degraded after ethanol degradation was completed. Lag periods for benzene degradation were increased with the concentration of ethanol. Microcosms amended with 100 mg/L of ethanol had benzene degradation delayed if compared to microcosms amended with 20 mg/L of ethanol. At an ethanol concentration of 300 mg/L, the microcosms became anoxic while degrading ethanol. Benzene was then not degraded under this condition. Corseuil *et al.* [23] have demonstrated that ethanol was preferentially degraded over BTX, when ethanol concentration was at 20 and 100 mg/L. When ethanol was 300 mg/L, the degradation of ethanol exhausted the oxygen available in the microcosm, so benzene could not be degraded [23]. Ethanol present in a spill may delay BTX degradation.

There are very few data on the contamination of the subsurface environment with

ethanol. This problem is more prevalent in Brazil, since high amounts of ethanol are present in the gasoline and approximately 15% of the cars run on pure hydrated ethanol. Since Brazil has been using ethanol blended with gasoline for more than 20 years, it is likely that some of the storage tanks may be leaking [21]. In Brazil, ethanol is mixed with gasoline in a proportion of 22% to 78% (v/v) in the truck at the refinery. The mixture is then carried to the filling stations where the concentrations are measured again. The gasoline-ethanol mixture is then stored in underground tanks.

The expected fate of ethanol in the subsurface is loss by biodegradation processes or volatilization. Until ethanol levels become tolerable to the subsurface microorganisms, either by dilution or volatilization, it may kill or inhibit most of the population. If ethanol is blended with gasoline in the spillage, the BTEX compounds would pass through the soil without aerobic degradation, if the microorganisms are inactivated by ethanol. It is known that BTEX compounds are more easily metabolized under aerobic conditions [20]. Butler *et al.* [17] found that methanol partially inhibited microbial activity at 1-2% concentration and BTEX biodegradation was affected by methanol at 1.4% concentration, being completely prevented at 8.9%.

Another factor to be considered regarding the fate of BTEX compounds and ethanol is the effect of ethanol on the solubility of BTEX compounds. The BTEX compounds are more soluble in fuel oxygenates than in water [59], and that could increase the subsurface transport velocities of BTEX. Ethanol, as an oxygenate added to gasoline, reduces by dilution the proportion of BTEX in gasoline [59], so less BTEX would be available to dissolve in water in an eventual spill. However, the concentrations of fuel oxygenates are not high enough (in U.S.A. and Canada) to increase water solubility (co-solvent effect) or the transport of BTEX. Laboratory studies have shown that no co-solvent effect was observed for gasoline containing 15% MTBE, 10% ethanol, 10% TAME and 10% isopropyl alcohol [13, 60]. But in Brazil and India, the concentration of ethanol is high enough to cause co-solvent effects on BTEX compound's concentration [77].

1.5 Objectives

To study the effect of fuel ethanol on the subsurface microorganisms and its effec on BTEX biodegradation, the following were the objectives of this work:

1. To determine the effect of a wide range of ethanol concentrations on activity and growth of subsurface microorganisms;

2. To determine the effect of gasoline-ethanol mixtures on microbial activity;
3. To examine the biodegradation of ethanol in microcosms simulating a subsurface environment;
4. To examine the influence of ethanol on biodegradation of BTEX compounds in microcosms simulating a subsurface environment.

It is expected at the end of this work to have a better understanding of what happens to the subsurface microorganisms when a gasoline-ethanol mixture penetrates the subsurface environment.

To address some of the problems related to the influence of ethanol in the biodegradation of BTEX compounds, it was necessary to determine the ethanol concentrations over which subsurface microorganisms would survive and grow using ethanol as a nutrient (source of carbon and energy) and also, to determine the concentration at which ethanol starts to be inhibitory to subsurface microorganisms.

The first set of experiments measured the microbial activity at different concentrations of ethanol (expressed as the percentage of volume of ethanol per total liquid volume). Microbial activity was assessed by an enzyme assay that measures the electron transport system activity. The concentration of ethanol that stimulated the highest microbial activity was chosen to be tested with gasoline. Another set of experiments measured microbial activity in ethanol-gasoline mixtures.

The third set of experiments measured growth of subsurface microorganisms at various concentrations of ethanol under aerobic conditions. Growth rates were calculated for each ethanol concentration.

A final experiment determined the influence of ethanol on the biodegradation of BTEX compounds. Two concentrations of ethanol were chosen based on the results of the microbial activity and growth curve experiments.

Chapter 2

Materials and Methods

2.1 Field Site

Soil samples were collected in the unconfined, shallow sandy aquifer located at Canadian Forces Base Borden, Ontario. At the field site, the aquifer extends approximately 9 m beneath the bottom of an abandoned sand quarry down to a silty clay deposit. The composition consists of coarse to silty sand in thin, discontinuous beds [12]. These beds vary from 0.1 to 0.2 m in thickness, and from 1.5 to 3 m in length [72]. The water table is generally 1.0 - 1.5 m below ground surface. The flow of ground water is towards the northeast direction with a velocity of 9.0 cm/day [44]. The subsurface samples were collected from the saturated zone, over an interval extending about 1.5 m below the water table, in an uncontaminated site. Cores were obtained in ethanol-flamed aluminum core barrels using the method of Starr and Ingleton [70]. The cores were brought to the laboratory and kept at 4°C until used.

Uncontaminated ground water was collected from a shallow well at Canadian Forces Base, Borden, Ontario. Approximately 20 L of water were purged from the well before water was collected into a sterile glass carboy. The sample was brought to the laboratory and it was kept at 4°C incubator until used.

2.2 Measurement of Electron Transport System (ETS) Activity in Soil

This enzyme assay measures the electron transport system activity by measuring the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to iodonitrotetrazolium formazan (INT-formazan) [75]. INT was added to soil samples (see subsection 2.2.1), and after appropriate incubation, INT-formazan was extracted from subsamples of soil using methanol. The extraction consisted of removing approximately 1 g of soil from each vial and placing it in a 10 mL test tube. Each tube received 3 mL of methanol. The tubes were sealed with Parafilm (American National Can™) and mixed using a vortex mixer for one minute. The methanolic extract was poured through a paper filter (Whatman®, number 5) to remove soil particles. The absorbance of the filtrate was then measured in a spectrophotometer (Pharmacia LKB - Ultrospec Plus) at 480 nm against a methanol blank. The soil that remained in each tube was collected and pooled with that collected in the appropriate paper filter and dried overnight in a 100°C oven. The soil dry weight was measured the next day.

The absorbance readings of the filtrates were converted to micrograms (μg) of INT-formazan produced by using the equation for a INT-formazan standard curve (Figure A.1 in Appendix A). For the standard curve, a stock solution of 100 μg/mL INT-formazan (Sigma - I8377) in methanol was prepared. The stock solution was diluted to final concentrations of 50, 30, 20, 10, 5, 3, 2 μg/mL. The absorbance of the diluted solutions was then measured in a spectrophotometer (Pharmacia LKB - Ultrospec Plus) at 480 nm against a methanol blank. The absorbance readings were plotted against the INT-formazan concentrations.

2.2.1 ETS Activity of Subsurface Microorganisms Exposed to Ethanol

Microbial activity of subsurface material was measured by the electron transport system (ETS) assay described by Trevors *et al.* [75], as in section 2.2. The experiment was conducted in microcosms. Amy and Haldeman [6] define a microcosm as a closed container that holds samples of material collected in the environment, with or without the addition

amendments. These containers are incubated at controlled conditions and a disappearance of a reactant or a production of a substance is measured [6].

The microcosms used consisted of thirty-three 60-mL sterile vials, that received 15 g of subsurface material (wet weight) and 1.5 mL of an aqueous solution of INT (0.4% wt/v). The INT solution was sterilized by filtration through a 0.2 μm pore size cellulose membrane filter. One mL of modified Bushnell–Haas medium (MBH) [52] (a mineral salts solution) was added as nutrient supplementation. MBH medium consisted of K_2HPO_4 – 1.0 g; KH_2PO_4 – 1.0 g; NH_4NO_3 – 1.0 g; $MgSO_4 \cdot 7H_2O$ – 0.2 g; $CaCl_2 \cdot 2H_2O$ – 0.02 g; $FeCl_3$ – 0.005 g; distilled water – 1000 mL, adjusted to pH 7.0. Glucose was added at 1% (wt/v) of total MBH volume, as a carbon source. One set of triplicate vials did not receive nutrient supplementation. In these nutrient-free vials, instead of MBH-glucose medium, sterile distilled water was added.

Sterile controls were prepared by autoclaving subsurface material at 121°C for one hour for three consecutive days. The vials were sealed with Teflon®-faced silicon septa and aluminum crimp seals to avoid water loss during sterilization process. The sterile controls received 1.5 mL of INT solution, 1 mL of MBH-glucose solution and 3% (v/v) ethanol.

Microbial activity was measured in the presence of MBH medium and glucose and compared to similar microcosms that also received different concentrations of ethanol (95% (v/v) ethanol Omni Solv - CAS 64-17-5). The alcohol was added to distinct vials to a final concentration of 0.5, 1.5, 3, or 5% (v/v), per total liquid volume in each vial (3 mL). Sterile distilled water was used to adjust the concentration of ethanol by volume. Ethanol was added to a concentration of 3% (v/v) to the nutrient-free and sterile vials. Table A.2 in Appendix A shows the experiment's assembly schematically.

In order to test for the use of ethanol as the sole carbon source, microbial activity was measured in microcosms that did not receive glucose (Table A.2, in Appendix A). The only nutrient added was MBH medium (1 mL). The ethanol concentrations were the same as described above (0.5, 1.5, 3 and 5% v/v).

The microcosms were assembled in a sterile air flow cabinet and all equipment was

sterilized before use. The vials, which were prepared in triplicate, were sealed with Teflon®-faced silicon septa and aluminum crimp seal and incubated in the dark at 10°C.

The number of colony forming units (CFU) per gram of dry soil was also determined for each microcosm at the beginning and at the end of the experiment, using the plate count technique. For the initial colony count, 10 g of subsurface material were diluted in a initial bottle containing 90 mL of sterile saline solution. Serial dilutions were made from the initial bottle, plated onto R2A agar and the plates were incubated at 10°C. After six days of incubation, the colonies were counted. After the experiment was terminated, the remaining soil contained in each vial was transferred to bottles containing sterile saline solution. Each bottle was vigorously hand shaken for 5 minutes. To make serial dilutions, 1-mL was taken from the bottles and transferred to a series of 9-mL tubes and from them, the samples were plated onto R2A agar. The colonies were counted after incubation for six days at 10°C.

Another experiment was prepared exactly as described above, but using higher concentrations of ethanol (10, 20, and 45% v/v). The sterile and nutrient-free controls had 20% ethanol.

2.2.2 ETS Activity of Subsurface Microorganisms Exposed to Gasoline-Ethanol Mixtures

In a third set of microcosms, subsurface material was exposed to a mixture of gasoline-saturated water (as prepared and diluted 1:10) and 0.5% (v/v) ethanol. The gasoline was obtained from the American Petroleum Institute (API). The composition of the API gasoline is given in Table 2.1.

Gasoline-saturated water was prepared according to Brookman *et al.* [16]: in a sterile separatory funnel one part of API gasoline was mixed with 9 parts of sterile distilled water. The mixture was vigorously hand-shaken three times for 5 minutes and left overnight in a fume-hood. The next day, the water containing all the gasoline components that are water-soluble was collected and used for microcosm preparation.

In order to measure ETS activity of subsurface microorganisms exposed to gasoline-saturated water and ethanol, nine different microcosms conditions were prepared in

Table 2.1: Characteristics of API 91-01 gasoline [11]

Properties	Values
Molecular weight (g/mole)	94.3
Density (g/cm^3)	0.747
MTBE (vol %)	<0.10
Methanol (vol %)	<0.10
Hydrocarbon Classes (wt%)	
Paraffins	41.01
Naphthalenes	6.84
Aromatics	39.05
Olefins	11.74
Unknowns	1.26
Aromatic Hydrocarbons (wt% (mole fraction))	
Benzene	1.22 (0.0147)
Toluene	7.68 (0.0787)
Ethylbenzene	3.37 (0.0300)
m-Xylene	5.31 (0.0472)
p-Xylene	2.13 (0.0189)
o-Xylene	2.64 (0.0235)

triplicate. Fifteen g (wet weight) of subsurface material and 1.5 mL of an aqueous solution of INT (0.4% wt/v) were added to 60 mL sterile vials. The INT solution was sterilized as described in sub-section 2.2.1. R2A medium [61] (1 mL) was added as a nutrient supplementation, except for three vials that were left nutrient-free, in which 1 mL of distilled water was added instead to correct for the total liquid volume in the vial. R2A medium consisted of: yeast extract – 0.5 g; protease peptone #3 – 0.5 g; casamino acids – 0.5 g; dextrose – 0.5 g; soluble starch – 0.5 g; sodium pyruvate – 0.3 g; K_2HPO_4 – 0.3 g; $MgSO_4$ – 0.05 g; and distilled water – 1000 mL. The broth was autoclaved at 121°C for 15 minutes.

The amendments of gasoline-saturated water and ethanol were added to each microcosm as detailed in Table A.3. Gasoline microcosms contained 1-mL gasoline-saturated water, INT solution, R2A and no ethanol. Gasoline + ethanol microcosms contained 1-mL gasoline-saturated water, INT solution, R2A and 0.5% (v/v) ethanol. Gasoline (1/10) microcosm contained 1-mL of a 1:10 gasoline-saturated water dilution, INT solution, R2A and no ethanol. Gasoline (1/10) + ethanol contained 1-mL of a 1:10 gasoline-saturated water dilution, INT solution, R2A and 0.5% (v/v) ethanol. Ethanol microcosms contained 0.5% (v/v) ethanol, INT solution and R2A medium. There were two R2A-free microcosms: one with 1-mL gasoline-saturated water, INT solution and 0.5% (v/v) ethanol and another with 1-mL of a 1:10 gasoline-saturated water dilution, INT solution and 0.5% (v/v) ethanol. The sterile control, prepared using the same method described in sub-section 2.2.1, contained 1-mL gasoline-saturated water, INT solution, R2A and 0.5% (v/v) ethanol. And the R2A microcosms contained INT solution and R2A medium.

After the addition of all the amendments to the microcosms — which was done in a sterile air flow cabinet — the vials were sealed with Teflon®-faced silicone septa and aluminum crimp seals and incubated at 10°C without shaking. Sampling was performed once a week.

Microbial ETS activity measured in subsurface material amended with R2A medium was compared with: a) microcosms amended with R2A medium and gasoline-saturated water; b) microcosms amended with R2A medium, gasoline-saturated water and ethanol; c) microcosms amended with R2A medium and ethanol; d) sterile controls; and e) R2A-free microcosms, containing gasoline-saturated water and ethanol. The same comparisons were made using diluted gasoline-saturated water (1:10).

2.3 Growth Experiments

Growth experiments were carried out with cultivated subsurface microorganisms. Ten g of subsurface material were suspended in sterile distilled water (20 mL) and vigorously hand-shaken for 3 minutes. Three 1-mL aliquots of the suspension were drawn and used

to inoculate three 250-mL Erlenmeyer flasks, each containing 50 mL of MBH medium supplemented with 1% (wt/v of MBH medium) glucose. The flasks were incubated on a shaker operating at 100 rpm at 10°C for 6 days. After that period, the culture contained in the three flasks was centrifuged for 10 minutes at 12,000 g and the resultant cell pellet resuspended in MBH medium to an O.D. $_{650nm}$ of 0.15. This cell suspension was used in the growth experiments described in this section.

From a concentrated stock solution of MBH medium salts and 1% (wt/v) glucose, required volumes were removed and combined with ethanol and sterile water to provide a series of solutions with standard levels of MBH salts (sub-section 2.2.1) and a range of ethanol concentrations. Subsequently, 1 mL of the cell suspension was added to the flasks. Final ethanol ranged from 0.5 to 70% per volume of total liquid in each flask (50 mL). Control flasks had no ethanol. Each concentration of ethanol and the control was prepared in triplicate. Due to a limited number of flasks and space in the incubators, one set of flasks was prepared with ethanol concentrations ranging 5 to 70% (v/v) and incubated at three different temperatures (4, 10, and 25°C). After that experiment was complete, a second set was prepared with ethanol concentrations ranging 0.5 to 4% (v/v) and incubated at 10 and 25°C. In all incubations, the flasks were shaken at 100 rpm.

Sampling consisted of taking 1 mL of the culture from each flask and measuring its turbidity in a spectrophotometer at 650 nm. Growth rates of the exponential phase were calculated for all concentrations as described in Brock and Madigan [45] (equations B.1 and B.2 in Appendix B).

2.4 Biodegradation of Ethanol and BTEX compounds

2.4.1 Microcosm Preparation

Biodegradation of BTEX compounds (benzene, toluene, ethylbenzene, *m*-xylene, *p*-xylene and *o*-xylene) in the presence of ethanol was measured in glass bottle microcosms (average volume of 1200 mL), with o-ring tap stopcocks (J. Young Scientific Glassware Ltd.) and side arms equipped with Mininert™ valves (Figure 2.1). The Mininert™ valve maintains a

seal while sampling the gas head space of the microcosm.

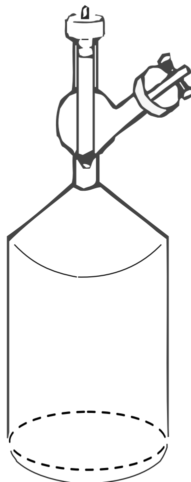

Figure 2.1: Schematic drawing of the bottle used for the microcosms [4]. The side arm was sealed with a Mininert™ valve and the main arm was sealed with O-ring tap stopcocks (J. Young Scientific Glassware Ltd.).

The microcosms were assembled within a sterile air flow cabinet. One hundred g of subsurface material (wet weight) and 100 mL of ground water were put into each microcosm. Control bottles received sterile subsurface material (autoclaved for 3 consecutive days for one hour) and 1.5 mL of 10% (wt/v) sodium azide solution. O-ring tap stopcocks and Mininert™ side arm caps were put in place and 5 mL of MBH medium was added to each microcosm (except for three bottles, which were left nutrient free) through the side arm.

A neat stock solution of the BTEX mixture was prepared by injecting quantities of each BTEX compound into a sterile flask in the proportion of 2 parts of both benzene and toluene for 1 part of ethylbenzene and each xylene isomer (2:2:1:1:1:1 BTEX). The BTEX mixture (3.5 μL per bottle) was added to the bottles followed by the addition of ethanol (1.5 mL per bottle for 1.5% (v/v) ethanol and 0.5 mL per bottle for 0.5% (v/v) ethanol). Table 2.2 details the volumes and concentrations of each compound in the neat stock and the final concentration in the microcosms. The sample concentration shown in Table 2.2 was calculated as follows:

$$concentration = \frac{injected\ volume \times density}{liquid\ volume} \tag{2.1}$$

Where:

injected volume = volume injected in the microcosm (from the neat stock solution)

liquid volume = volume of ground water in the microcosm (0.1 L)

Thus, a sample calculation for benzene concentration (Table 2.2):

$$\begin{aligned} benzene\ concentration &= \frac{0.8750 \times 10^{-6} L \times 0.8787\ g/10^{-3} L}{0.1 L} \\ &= 0.769 \times 10^{-2} g/L \\ &= 7.69\ mg/L \end{aligned} \tag{2.2}$$

Table 2.2: BTEX addition to the microcosms.

Compound	Neat Stock (μL)	3.5 μL of Neat Stock (μL)[a]	Density at 20-25°C	Sample Concentration[b] mg/L
Benzene	200.00	0.8750	0.8787	7.69
Toluene	200.00	0.8750	0.8669	7.59
Ethylbenzene	100.00	0.4375	0.8670	3.79
p-Xylene	100.00	0.4375	0.8611	3.77
m-Xylene	100.00	0.4375	0.8642	3.78
o-Xylene	100.00	0.4375	0.8802	3.83
Total BTEX	800.00	3.5	-	30.45

[a] Volume of each BTEX compound added to each microcosm.

[b] Concentration in the microcosm liquid.

According to Table 2.2 the total BTEX concentration added to each microcosm was 30.45 mg per 1000 mL. The microcosm components are detailed in Table 2.3.

Each microcosm condition was prepared in triplicate and the microcosms were incubated at 10°C in the dark. The microcosms containing BTEX and ethanol were compared to bottles containing ethanol only or BTEX only. Controls consisted of sterile and nutrient-free microcosms (Table 2.3).

An extra 5 mL of MBH medium was added to one replicate (replicate 2) on day 18 of the experiment. The microcosms that received extra MBH were those containing 0.5% ethanol, 1.5% ethanol and ethanol alone.

Table 2.3: Detailed microcosm components

Microcosm	Ethanol (% v/v)	BTEX (μL)	MBH medium (mL)	Other
0.5% ethanol	0.5	3.5	5	-
1.5% ethanol	1.5	3.5	5	extra O_2 added
Ethanol-only	0.5	0	5	-
BTEX-only	0	3.5	5	-
Sterile	0.5	3.5	5	sodium azide
Nutrient-free	0.5	3.5	0	-

2.4.2 Analytical Procedures for BTEX Compounds

The BTEX compounds were measured by gas chromatography. The gas chromatograph was calibrated with an external standard method (Tables C.1 and C.2 in Appendix C). Prior to each sampling, a calibration check of the gas chromatograph was performed. Three μL samples of a 1:1:1:1:1:1 BTEX mixture were injected into two 1.038 L bottles (Table C.4 in Appendix C). The bottles were left undisturbed for one hour to achieve gas equilibrium. A 500 μL sample of BTEX was removed with a Hamilton Gastight® syringe from each bottle and injected in the GC. The BTEX concentrations were calculated according to Equation C.2, in Appendix C.

Microcosm sampling started at day one. Stopcock taps were opened and with a 1-mL gas

tight syringe, a 500 μL sample of head-space gas was removed through the mininert sidearm valve and quickly injected into the gas chromatograph. The gas chromatography equipment was a Shimadzu® GC 9A, with flame ionization detection. The sample was loaded on the column through a gas sampling valve (Valco Instruments) and a split injection port. The column used was a 0.32 mm I. D. x 60 m length, supelcowax 10 (Supelco) column with a 0.5 μm stationary phase of Carbowax 20. The chromatographic conditions were as follows: injection/detector temperature of 200°C; oven temperature of 105°C; helium carrier gas with a column flow rate of 5 mL/min and a helium make-up flow of 50 mL/min.

The BTEX compounds concentrations were measured by head-space analysis, then the values were converted to aqueous concentration according to equation C.5, in Appendix C. All results are reported as aqueous phase concentrations.

The method detection limits for the BTEX compounds are listed in Table C.3, in Appendix C.

2.4.3 Analytical Procedures for Ethanol

Ground water was periodically removed from each bottle and used to completely fill 2 mL glass vial which was sealed with a cap fitted with Teflon®-lined septum and stored at 4°C for measurement of ethanol concentration. These samples were preserved with sodium azide. A 2 μL aliquot of sample was removed for chromatographic analysis using a 10 μL syringe equipped with a Chaney adapter. The samples were analyzed on a Hewlett Packard® 5840A gas chromatograph equipped with a flame ionization detector. The column was 10 ft by 0.125 in inner diameter and packed with 3% SP1500 on Carbopack B (80/100 mesh). The analysis was run isothermally at 130°C, with a helium carrier gas at a flow rate of 20 mL/min. The detector temperature was 130°C and the injection temperature was 230°C. The gas chromatograph was calibrated in an external standard mode using several concentrations of ethanol in water. A linear regression equation for the standards was used to determine the aqueous ethanol concentration of the samples. The ethanol concentration was calculated in mg/L according to the standard curve equation showed in Figure C.1, in

Appendix C.

2.4.4 Oxygen Addition and Measurement of Dissolved Oxygen

The oxygen demand for total mineralization of ethanol at both concentrations (0.5 and 1.5%) and the oxygen demand for total mineralization of BTEX were calculated (Appendix C). The oxygen demand for 0.5% (v/v) ethanol was 823 mg. The oxygen demand for 1.5% (v/v) ethanol was 2468.8 mg. BTEX required 22.85 mg of oxygen to mineralize completely. The available oxygen in the head-space plus dissolved oxygen from the water in the each bottle totaled 285.5 mg. Hence, the bottles with the higher ethanol concentration (1.5%) were flushed with oxygen before the addition of ethanol and BTEX to avoid oxygen limitation. A 15-cm long cannula was inserted through the bottle side arm and pure oxygen was flushed through the microcosms at a flow rate of 800 mL/min for 10 minutes. The mininert side arm valve was left unscrewed to allow excess oxygen gas to escape while the o-ring tap stopcock remained closed. At the end of the flushing procedure, the mininert vials were closed off. Although the above situation does not resemble what happens in the environment, this procedure was necessary ensure adequate oxygen for complete mineralization of ethanol.

Occasionally, throughout the experiment, the dissolved oxygen in the microcosm fluid was measured. A 2 mL glass syringe with a sterile 22 gauge needle tip was inserted through the unlocked mininert valve and filled with 2 mL of ground water. The needle tip was removed from the syringe and the sample was quickly transferred to a 4 mL glass vial and dissolved oxygen was measured using a dissolved oxygen micro-probe (MI - 730 Oxygen Electrode and Meter, Microelectrodes Inc.). A 2% sodium sulfide solution was used as a zero standard, while air-sparged distilled water was used as an oxygen saturated water sample, to calibrate the micro-probe.

Chapter 3

Results

3.1 Microbial Activity of Subsurface Microorganisms

3.1.1 Microbial Activity with Ethanol

In order to determine the effect of ethanol on microbial activity of subsurface microorganisms, subsurface material collected from the Borden aquifer was exposed to ethanol concentrations ranging from 0 to 45% (v/v) in microcosms. Nutrient supplementation was provided as a mineral-salts medium (MBH) and glucose. Microbial activity was determined by measuring the production of INT-formazan from INT and the results obtained from microcosms amended with MBH medium and glucose were compared to results obtained from microcosms amended with glucose-MBH and ethanol at different concentrations. Figure 3.1 shows that microbial activity in microcosms amended with MBH medium and glucose only was lower than activity in microcosms that contained ethanol at concentrations of 0.5, 1.5 and 3%. Microbial activity was not detected at 5% ethanol.

INT-formazan production in microcosms amended with MBH medium and glucose only, after 35 days of incubation at 10°C was 15.8 μg/g dry weight (DW) soil. Comparing this production with microcosms that were amended with MBH medium, glucose and ethanol, the following results were observed: microcosms containing an ethanol concentration of 0.5%

(v/v), produced an average of 26.4 μg/g DW soil of INT-formazan, whereas microcosm with 1.5% and 3% ethanol both produced an average of 32.2 μg/g DW soil of INT-formazan. At 5% (v/v) ethanol, the average INT-formazan produced was 2.3 μg/g DW soil (Figure 3.1).

The nutrient-free (which represented endogenous microbial activity) and sterile microcosms had no detectable microbial activity (INT-formazan produced: 0.3 and 0.4 μg/g DW soil respectively) (Table 3.1).

Subsurface microorganisms exposed to higher concentrations of ethanol (ranging from 10 to 45% (v/v)) had no detectable microbial activity (Figure 3.2), with INT-formazan productions below 1 μg/g (Table A.4, in Appendix A).

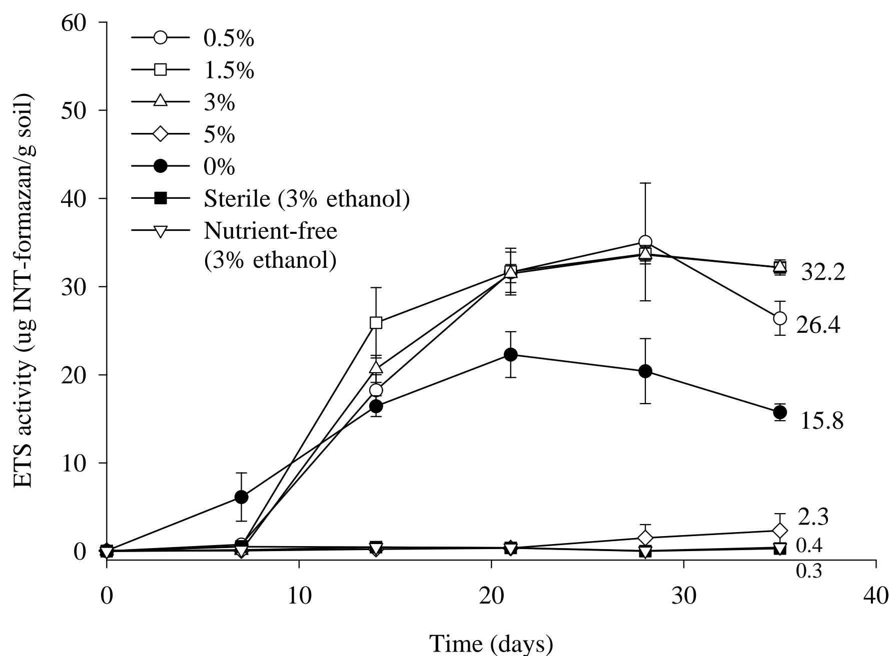

Figure 3.1: ETS activity of subsurface microorganisms exposed to ethanol concentrations ranging from 0 to 5% (v/v) in nutrient-amended (glucose + MBH) microcosms. The error bars represent the standard error of three replicates.

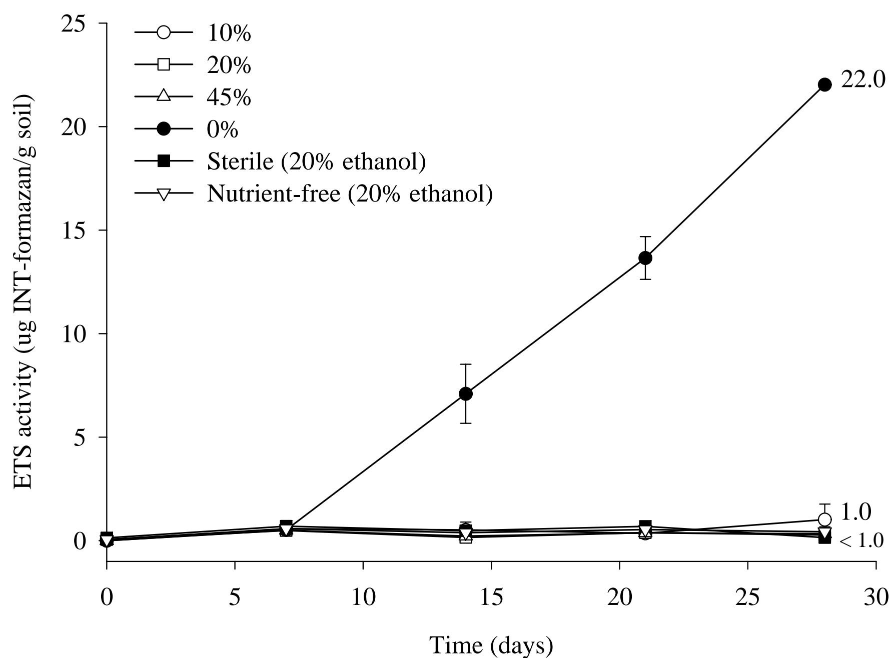

Figure 3.2: ETS activity of subsurface microorganisms exposed to ethanol concentrations ranging from 10 to 45% (v/v) in nutrient-amended (glucose + MBH) microcosms. The error bars represent the standard error of three replicates.

In order to determine if the subsurface microorganisms were able to use ethanol as the sole carbon source, subsurface material was exposed to different concentrations of ethanol and the nutrient supplementation consisted of a mineral-salts medium (MBH) only. The highest values of INT-formazan production were obtained at day 21, in the microcosms that contained 3% (v/v) ethanol (Table 3.1). Comparing microbial activity at specific ethanol concentrations with or without glucose, higher microbial activity was always observed when glucose was present (Table 3.1). A decrease in INT-formazan production was observed in all microcosms without glucose between days 21 and 35. No detectable microbial activity was observed for the 5% ethanol concentration (without glucose), nutrient-free and sterile

vials (Table 3.1).

Table 3.1: INT-formazan production in microcosms with and without glucose.[a]

Microcosm (ethanol %)	Glucose	INT-formazan (day 21) (μg/g DW soil)	INT-formazan (day 35) (μg/g DW soil)
0	present	22.3	15.8
0.5	present	31.6	26.4
0.5	absent	6.5	3.6
1.5	present	31.7	32.2
1.5	absent	9.1	5.9
3	present	31.5	32.2
3	absent	23.0	16.3
5	present	0.4	2.3
5	absent	0.2	0.2
nutrient-free[b]	-	0.4	0.4
sterile[b]	-	0.4	0.3

[a] All microcosms contained MBH medium, except for the nutrient-free control.

[b] The nutrient-free and sterile controls contained 3% (v/v) ethanol. An ethanol-free, glucose-free microcosm condition was not prepared.

3.1.2 Cell Counts

Cell counts were performed at the beginning and at the end of the microbial activity experiment as an attempt to relate microbial activity to a change in the number of cells. After appropriate dilutions, subsurface material was spread onto R2A medium agar and the plates were incubated at 10°C. The count at the beginning of the experiment (day 0) was 4.21 x 10^3 CFU/g dry weight soil. After 35 days, when the experiment was ended, the remaining subsurface material contained in the microcosms was plated onto R2A medium agar (after appropriate dilution) for cell counts. The number of cells counted in glucose-MBH amended, or MBH amended microcosms containing ethanol ranging from 0 to 5% are shown in Table 3.2. It was observed that when glucose was not added to the microcosms, the final cell count increased as ethanol concentration increased, reaching a maximum at 3% (v/v) ethanol. At 5% (v/v) ethanol, there was no growth detected. When glucose was present, the final cell count was approximately 100 times higher than the maximum observed in the absence of glucose. It is not possible to detect and effect of ethanol on the cells count comparing the presence or absence of ethanol at 3% concentration microcosms.

Table 3.2: Cell counts after 35 days in microcosms amended with ethanol, with or without glucose.[a]

Ethanol concentration (% v/v)	Presence of glucose	CFU/g dry weight soil
0	yes	2.1 x 10^7
0.5	yes	1.1 x 10^7
0.5	no	3.9 x 10^3
1.5	yes	1.7 x 10^7
1.5	no	3.5 x 10^4
3.0	yes	1.3 x 10^7
3.0	no	4.8 x 10^5
5.0	yes	4.5 x 10^3
5.0	no	8.0 x 10^2

[a] The cell count at day 0 was 4.21 x 10^3 CFU/g dry weight of soil. All microcosms contained MBH.

3.1.3 Microbial Activity with Gasoline and Ethanol

Microbial activity was also tested in microcosms containing gasoline-saturated water and ethanol. From the previous experiment, the concentration of 0.5% (v/v) ethanol was chosen to make gasoline-saturated water-ethanol mixtures because at this ethanol concentration, microbial activity reached one of the highest values (Figure 3.1).

The experiment set up followed the same procedures as the previous experiment of microbial activity, where subsurface material was exposed to ethanol. R2A medium was used as a nutrient supplementation to the microcosms instead of a combination of glucose and

MBH medium. Microbial activity of subsurface microorganisms measured in microcosms containing R2A medium produced 4.4 μg/g DW soil of INT-formazan after 22 days of incubation at 10°C (Figure 3.3). This result is compared to microcosms amended with R2A medium plus all the other combinations of gasoline-saturated water and ethanol. The microcosms amended with ethanol (0.5% v/v) and gasoline-saturated water-ethanol mixture produced double the amount of INT-formazan, compared to the R2A only microcosms (Figure 3.3). The "gasoline-saturated water" microcosms produced almost the same amount of INT-formazan as the R2A only microcosms. The sterile and nutrient-free vials showed no detectable activity (Figure 3.3).

Using the same result of INT-formazan production for R2A only microcosms (4.4 μg/g DW soil), the comparison was then made with gasoline-saturated water that was diluted 1:10. The diluted gasoline-saturated water-ethanol mixture had the highest microbial activity (Figure 3.4), followed by the vials containing ethanol. The controls, represented by sterile and nutrient-free microcosms had no detectable activity (Figure 3.4).

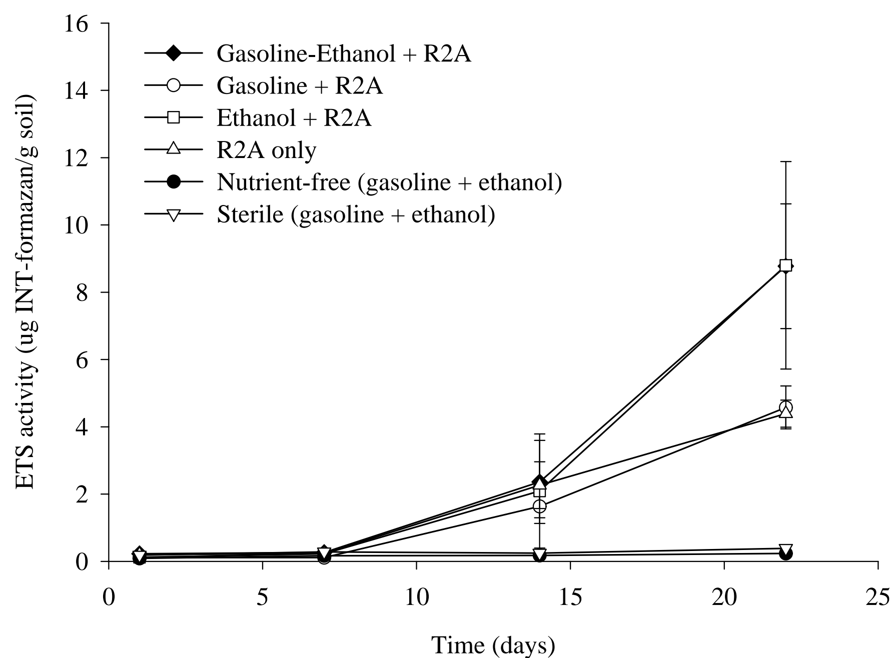

Figure 3.3: ETS activity of subsurface microorganisms exposed to gasoline-saturated water and 0.5% (v/v) ethanol in nutrient-amended (R2A) microcosms. The error bars represent the standard error of three replicates.

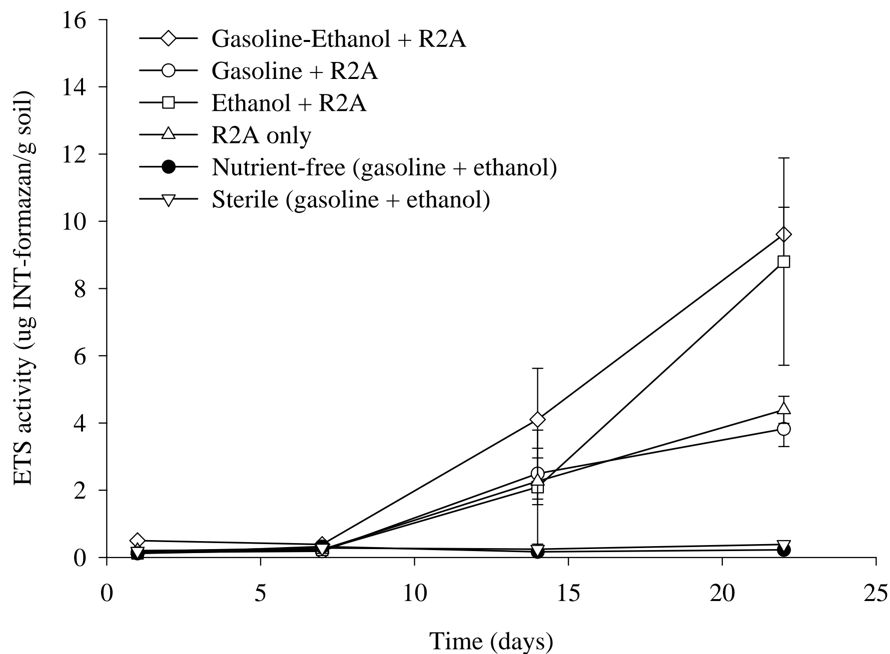

Figure 3.4: ETS activity of subsurface microorganisms exposed to diluted gasoline saturated water (1:10) and 0.5% (v/v) ethanol in nutrient-amended (R2A) microcosms. The error bars represent the standard error of three replicates.

3.1.4 Summary

Microbial activity achieved higher levels when subsurface material collected from the Borden aquifer was exposed to ethanol concentrations of 1.5 and 3% (v/v), compared to the control, which had no ethanol (Figure 3.1). Microbial activity was not detected at ethanol concentrations equal and over 5%.

When microbial activity was measured in microcosms that contained ethanol as the the sole given carbon source, the 3% ethanol microcosms exhibited the highest levels of INT-formazan production (Table 3.1).

Cell counts in MBH-amended microcosms exhibited an increase as ethanol concentration increased from 0.5 to 3% (Table 3.2). Comparing Tables 3.1 and 3.2, the increase in microbial activity levels corresponded to an increase in cell counts, as ethanol concentration increased, suggesting a direct relationship between microbial activity and cell counts, when ethanol was provided as a carbon source.

In microcosms exposed to gasoline-saturated water it was observed that whenever ethanol was present, either alone or blended with gasoline-saturated water, microbial activity achieved the highest levels compared to microcosms with gasoline-saturated water and R2A only (Figures 3.3 and 3.4).

3.2 Growth Experiments

These experiments examined the effect of ethanol on growth of subsurface microorganisms at three different incubation temperatures. Subsurface microorganisms were grown in a glucose-MBH medium at 10°C. Aliquots of the culture were added to several flasks containing glucose-MBH medium and ethanol concentrations ranging from 0 to 70% (v/v). The flasks were incubated at 4, 10 and 25°C. The growth curves show the effect of both ethanol and temperature on growth. Growth at 4°C was not detected in all ethanol concentrations tested (Figure 3.5). In the control, with 0% ethanol, the subsurface microorganisms were able to grow. There was a short lag phase after which the population started to grow. The exponential phase lasted 48 hours and the culture reached the stationary phase that lasted until the end of the experiment.

Growth at 10°C was observed at ethanol concentrations ranging from 0 to 4% (Figure 3.6). Growth on glucose-MBH medium showed the same curves for the control (ethanol 0%), ethanol 0.5% and ethanol 1.5%. However, an ethanol concentration effect was observed as the curves for 3 and 4% ethanol were below the control curve. The exponential phase for all concentrations lasted 48 hours. At higher concentrations of ethanol, growth was not observed in all ethanol concentrations (5 - 70%) tested (Figure 3.7).

When subsurface microorganisms exposed to ethanol were incubated at 25°C, growth in glucose-MBH medium showed no difference in the control (ethanol 0%) and ethanol 0.5% curves (Figure 3.8). There was a difference observed at 1.5% ethanol growth curve, where a slight decrease in growth rate was apparent. The cultures exposed to 3 and 4% ethanol were in a lag phase for the first 32 hours for 3% ethanol and 72 hours for 4% ethanol. Exponential growth for all ethanol concentrations lasted less time than the 10°C incubation, being 24 hours (except for ethanol 3%, which was 16). Growth was not observed for the 5% ethanol condition (Figure 3.9). In this experiment, the exponential growth for 0% ethanol lasted 48 hours, as observed in Figure 3.9. The measurement for this concentration stopped at day 15. Microbial biomass started to flocculate at day 4 in one of the replicates of the 0% ethanol concentration flasks and turbidity measurements were terminated day 15, when

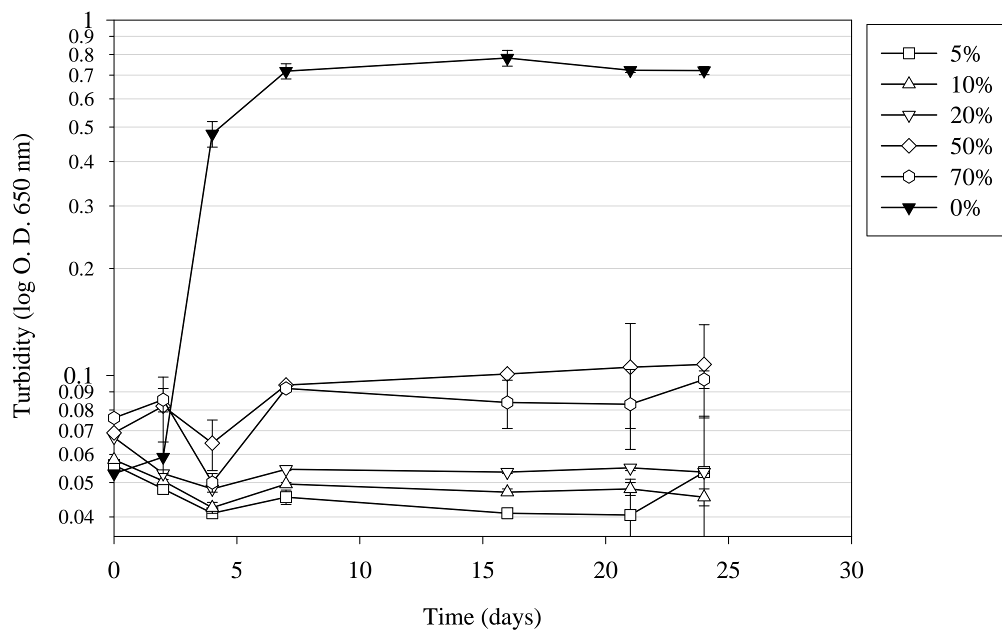

Figure 3.5: Growth curve of subsurface microorganisms exposed to ethanol concentrations ranging from 5 to 70% (v/v), incubated at 4°C in glucose-MBH medium. The error bars represent the standard error of the means of three replicates.

flocs were evident in all the 0% ethanol flasks. The reason for the flocculation is unknown.

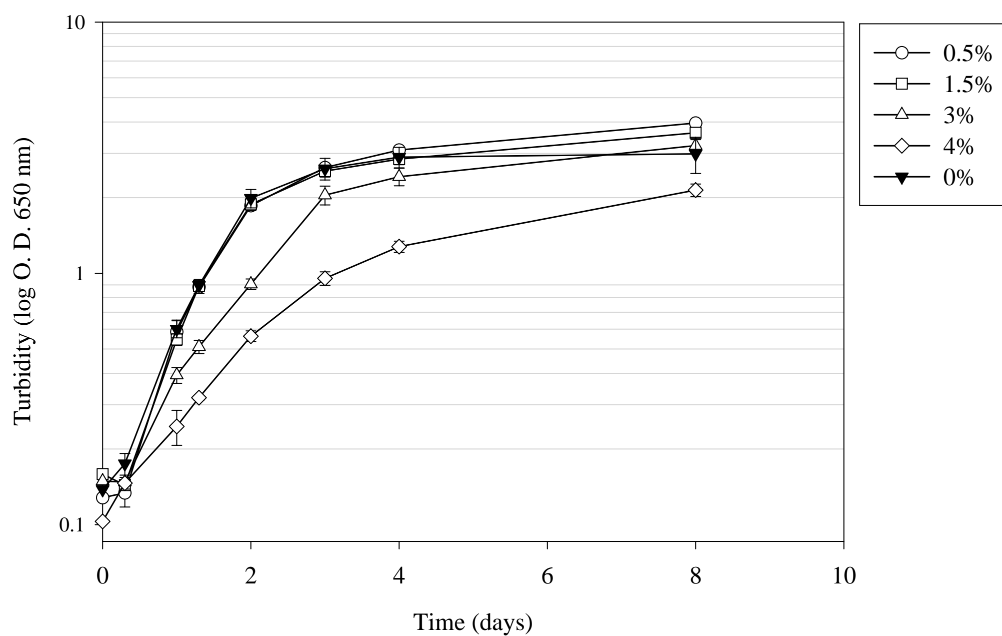

Figure 3.6: Growth curve of subsurface microorganisms exposed to ethanol concentrations ranging from 0 to 4% (v/v), incubated at 10˚C in glucose-MBH medium. The error bars represent the standard error of the means of three replicates.

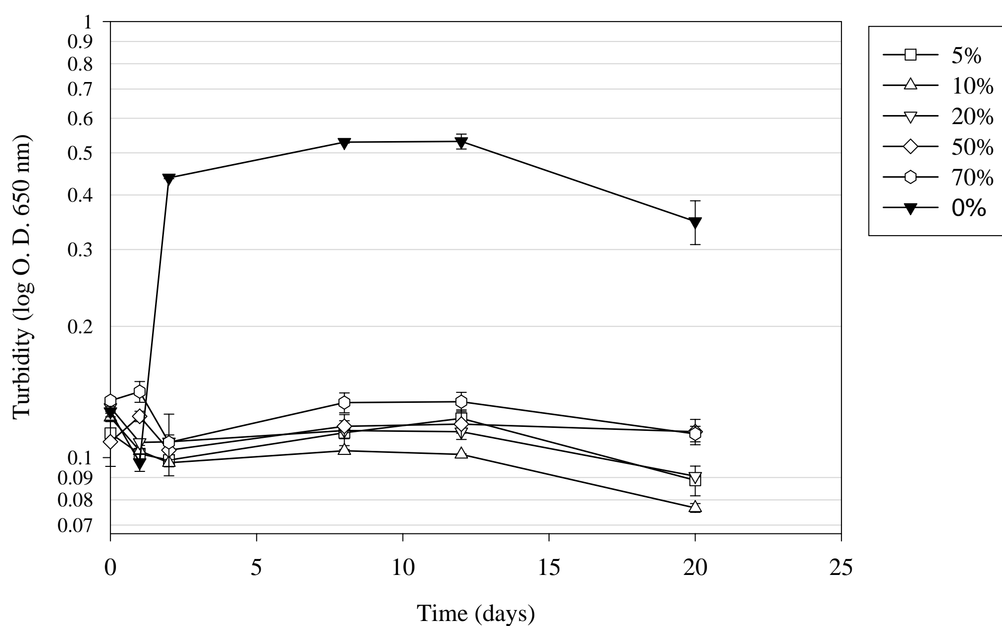

Figure 3.7: Growth curve of subsurface microorganisms exposed to ethanol concentrations ranging from 5 to 70% (v/v), incubated at 10°C in glucose-MBH medium. The error bars represent the standard error of the means of three replicates.

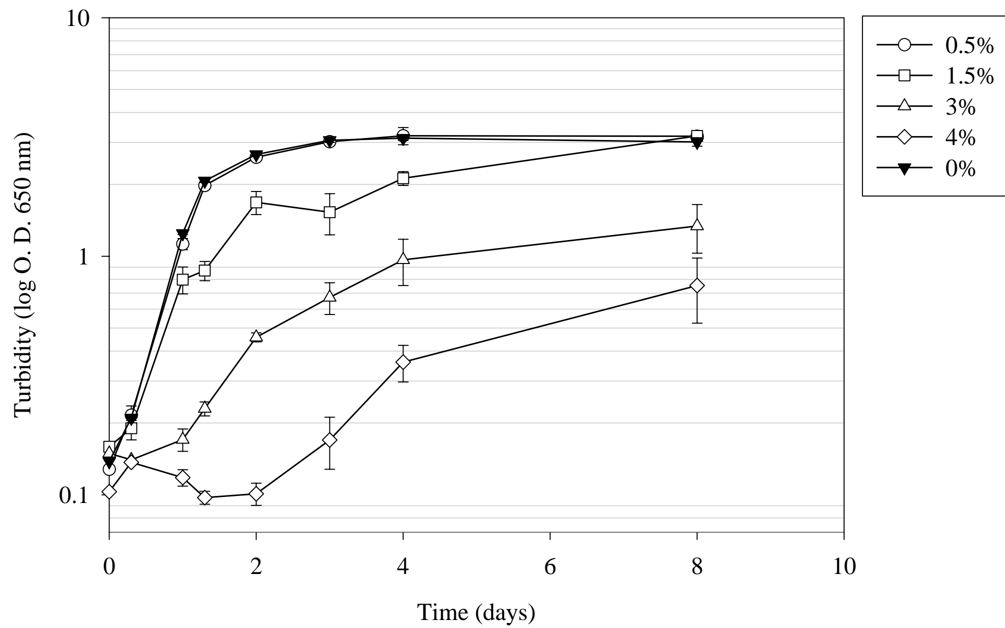

Figure 3.8: Growth curve of subsurface microorganisms exposed to ethanol concentrations ranging from 0 to 4% (v/v), incubated at 25°C. The error bars represent the standard error of the means of three replicates.

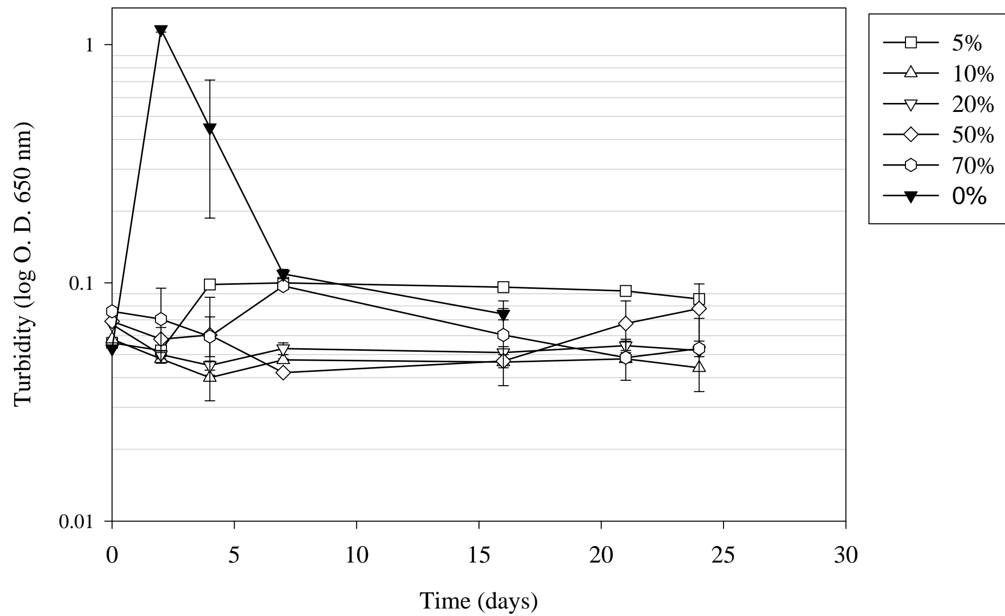

Figure 3.9: Growth curve of subsurface microorganisms exposed to ethanol concentrations ranging from 5 to 70% (v/v), incubated at 25°C. The error bars represent the standard error of the means of three replicates.

Using equations B.1 and B.2, in Appendix B, the number of generations (n) and generation time (g) for the 4, 10 and 25°C incubation were calculated and are listed in Table 3.3. At the 4°C incubation, the calculations were for the control only, since the other ethanol concentrations (5 to 70%) did not present an exponential phase in their growth curves.

Table 3.3: Growth rates at 4, 10 and 25°C

Temperature (°C)	ethanol concentration (%)	N_0[a]	N_t[b]	time[c](hours)	generation time (g)
4	0	0.06	0.48	48	16.14
10	0	0.14	1.99	48	12.55
10	0.5	0.13	1.86	48	12.51
10	1.5	0.16	1.88	48	13.56
10	3	0.15	2.05	72	19.17
10	4	0.10	0.96	72	22.56
25	0	0.14	2.07	24	6.19
25	0.5	0.13	1.98	24	6.11
25	1.5	0.16	0.87	24	9.85
25	3	0.23	0.46	16	16.26
25	4	0.17	0.36	24	22.14

[a] The values are the average O. D.$_{650}$ reading at the beginning of the exponential phase.

[b] The values are the average O. D.$_{650}$ reading at the end of the exponential phase.

[c] Elapsed time during which growth was measured (exponential phase).

According to Table 3.3 the values for the generation time (g) for the 10°C incubation temperature increased as ethanol concentration increased. Comparing the generation times for 0% ethanol and 0.5% ethanol, the difference was minimal. However, the generation time at 4% ethanol was almost two times higher than the control (0% ethanol).

For the 25°C incubation temperature, the exponential phase lasted the same for almost all concentrations (except for 3% ethanol). The differences in the generation times values were greater than at 10°C temperature. Again, there was not much difference between the

control and the 0.5% ethanol. But at 4% ethanol, the generation time was 3.6 times longer than for the control.

Growth of Borden's aquifer subsurface microorganisms in MBH-glucose solution was observed at 4, 10 and 25°C. There was no pronounced effect on microbial growth in MBH-glucose medium when ethanol was present at lower concentrations. However, there was some apparent restriction on growth, at as low as 1.5% ethanol when 25°C incubation was used, and growth was prevented at or above 5% ethanol at all three incubation temperatures.

3.3 Degradation of BTEX Compounds and Ethanol

3.3.1 BTEX Compounds Results

Biodegradation of two concentrations of ethanol and the effects of these two concentrations of ethanol in biodegradation of BTEX compounds was examined in microcosms simulating the subsurface environment. The microcosms contained subsurface material, ground water, and were amended with MBH medium as a nutrient supplementation, BTEX and ethanol at 0.5 and 1.5% concentration (Table 2.3). The microcosms were incubated at 10°C. BTEX compounds were sampled in the gas phase and concentration converted to mass values, using Equation C.8, in Appendix C.

Table 3.4 shows the percentage of BTEX mass lost from the various microcosm treatments over 64 days of incubation. The mean and standard error of the means for the 0.5% and 1.5% ethanol microcosms are derived from the measurements on two bottles that did not receive extra MBH medium. The mean and standard error for both the sterile and nutrient-free microcosms represent three bottles.

In order to compare BTEX and ethanol degradation, the mass values were converted to number of carbon moles and are displayed in Table 3.5. The moles of carbon were calculated from the BTEX mass shown in Table 3.4.

Table 3.4: Percentage of decrease in BTEX mass after 64 days of incubation at 10°C. BTEX mass values are from aqueous phase.

Compound	Microcosm	Initial mass (mg) mean (std. error)	Final mass (mg) mean(std. error)	% decrease
Benzene	0.5% ethanol	0.65 (±0.02)	0.37 (±0.01)	43.2
Benzene	1.5% ethanol	0.75 (±0.08)	0.59 (±0.00)	21.4
Benzene	BTEX only	0.75 (±0.05)	0.00	100
Benzene	sterile	0.76 (±0.01)	0.66 (±0.00)	13.5
Benzene	nutrient-free	0.81 (±0.04)	0.69 (±0.00)	14.8
Toluene	0.5% ethanol	0.60 (±0.02)	0.03(±0.01)	94.4
Toluene	1.5% ethanol	0.69 (±0.07)	0.51 (±0.00)	26.1
Toluene	BTEX only	0.68 (±0.04)	0.00	100
Toluene	sterile	0.6 (±0.02)	0.58 (±0.00)	14.0
Toluene	nutrient-free	0.76 (±0.04)	0.61 (±0.00)	20.4
Ethylbenzene	0.5% ethanol	0.34 (±0.02)	0.00	100
Ethylbenzene	1.5% ethanol	0.35 (±0.01)	0.16 (±0.02)	54.3
Ethylbenzene	BTEX only	0.38 (±0.01)	0.00	100
Ethylbenzene	sterile	0.37 (±0.01)	0.31 (±0.00)	18.0
Ethylbenzene	nutrient-free	0.45 (±0.02)	0.32 (±0.00)	28.3
m-Xylene	0.5% ethanol	0.30 (±0.02)	0.12 (±0.02)	60.4
m-Xylene	1.5% ethanol	0.33 (±0.02)	0.19 (±0.01)	42.7
m-Xylene	BTEX only	0.34 (±0.02)	0.00	100
m-Xylene	sterile	0.33 (±0.01)	0.26 (±0.00)	19.1

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Compound	Microcosm	Initial mass (mg) mean (std. error)	Final mass (mg) mean (std. error)	% decrease
m-Xylene	nutrient-free	0.38 (±0.02)	0.28 (±0.00)	26.3
p-Xylene	0.5% ethanol	0.35 (±0.02)	0.25 (±0.01)	30.3
p-Xylene	1.5% ethanol	0.37 (±0.02)	0.27 (±0.00)	28.1
p-Xylene	BTEX only	0.39 (±0.02)	0.00	100
p-Xylene	sterile	0.37 (±0.02)	0.31 (±0.00)	18.0
p-Xylene	nutrient-free	0.44 (±0.02)	0.33 (±0.00)	25.9
o-Xylene	0.5% ethanol	0.27 (±0.02)	0.21 (±0.00)	22.1
o-Xylene	1.5% ethanol	0.32 (±0.04)	0.21 (±0.00)	34.4
o-Xylene	BTEX only	0.32 (±0.01)	0.00	100
o-Xylene	sterile	0.29 (±0.01)	0.23 (±0.00)	20.7
o-Xylene	nutrient-free	0.35 (±0.02)	0.25 (±0.00)	28.6

Table 3.5: Decrease in number of carbon moles in BTEX degradation after 64 days of incubation at 10°C. BTEX mass values are from aqueous phase.

Compound	Microcosm	Number of moles of carbon (day 1)	Number of moles of carbon (day 64)	Number of moles lost
Benzene	0.5% ethanol	5.0×10^{-5}	2.8×10^{-5}	2.1×10^{-5}
Benzene	1.5% ethanol	5.8×10^{-5}	4.5×10^{-5}	1.2×10^{-5}
Benzene	BTEX only	5.8×10^{-5}	0.0	5.8×10^{-5}
Benzene	sterile	5.8×10^{-5}	5.1×10^{-5}	7.7×10^{-6}
Benzene	nutrient-free	6.2×10^{-5}	5.3×10^{-5}	9.2×10^{-6}
Toluene	0.5% ethanol	4.6×10^{-5}	2.3×10^{-6}	4.3×10^{-5}
Toluene	1.5% ethanol	5.2×10^{-5}	3.9×10^{-5}	1.4×10^{-5}
Toluene	BTEX only	5.2×10^{-5}	0.0	5.2×10^{-5}
Toluene	sterile	5.1×10^{-5}	4.4×10^{-5}	6.8×10^{-6}
Toluene	nutrient-free	5.8×10^{-5}	4.6×10^{-5}	1.1×10^{-5}
Ethylbenzene	0.5% ethanol	2.6×10^{-5}	0.0	2.6×10^{-5}
Ethylbenzene	1.5% ethanol	2.6×10^{-5}	1.2×10^{-5}	1.4×10^{-5}
Ethylbenzene	BTEX only	2.9×10^{-5}	0.0	2.9×10^{-5}
Ethylbenzene	sterile	2.8×10^{-5}	2.3×10^{-5}	4.5×10^{-6}
Ethylbenzene	nutrient-free	3.4×10^{-5}	2.4×10^{-5}	9.8×10^{-5}
m-Xylene	0.5% ethanol	2.3×10^{-5}	9.1×10^{-6}	1.4×10^{-5}
m-Xylene	1.5% ethanol	2.5×10^{-5}	1.4×10^{-5}	1.1×10^{-5}
m-Xylene	BTEX only	2.6×10^{-5}	0.0	2.6×10^{-5}
m-Xylene	sterile	2.5×10^{-5}	2.0×10^{-5}	5.3×10^{-6}

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Compound	Microcosm	Number of moles of carbon (day 1)	Number of moles of carbon (day 64)	Number of moles lost
m-Xylene	nutrient-free	2.9 x 10^{-5}	2.1 x 10^{-5}	7.5 x 10^{-6}
p-Xylene	0.5% ethanol	2.7 x 10^{-5}	1.9 x 10^{-5}	8.3 x 10^{-6}
p-Xylene	1.5% ethanol	2.8 x 10^{-5}	2.0 x 10^{-5}	7.5 x 10^{-6}
p-Xylene	BTEX only	2.9 x 10^{-5}	0.0	2.9 x 10^{-5}
p-Xylene	sterile	2.8 x 10^{-5}	2.3 x 10^{-5}	4.5 x 10^{-6}
p-Xylene	nutrient-free	3.3 x 10^{-5}	2.5 x 10^{-5}	8.3 x 10^{-6}
o-Xylene	0.5% ethanol	2.1 x 10^{-5}	1.6 x 10^{-5}	5.3 x 10^{-6}
o-Xylene	1.5% ethanol	2.4 x 10^{-5}	1.6 x 10^{-5}	8.3 x 10^{-6}
o-Xylene	BTEX only	2.4 x 10^{-5}	0.0	2.4 x 10^{-5}
o-Xylene	sterile	2.2 x 10^{-5}	1.7 x 10^{-5}	4.5 x 10^{-6}
o-Xylene	nutrient-free	2.6 x 10^{-5}	1.9 x 10^{-5}	7.5 x 10^{-6}

All BTEX compound masses decreased to zero by day five of the experiment in microcosms amended with BTEX “only”. Over 64 days, there was a decrease in BTEX mass in all the other microcosms (0.5% ethanol, 1.5% ethanol, sterile and nutrient-free). Comparing the percentages of loss in 0.5% ethanol and 1.5% ethanol microcosms, the higher mass losses were observed when ethanol concentration was 0.5%. However, only ethylbenzene was completely degraded in 0.5% ethanol microcosms (Table 3.4).

There was mass loss in the nutrient-free microcosms, suggesting some endogenous microbial activity. This microcosm condition was the closest simulation to the natural environment, compared to the others microcosm conditions, since nutrients were not added to it.

Some loss in BTEX mass was observed in the sterile microcosms. That loss was not expected since living microorganisms were not present to perform degradation. Abiotic

factors were probably involved in the mass loss in this microcosm condition.

Comparing the mass loss in the 0.5% ethanol microcosm to the mass loss in the sterile microcosm, it was necessary to determine if the mass loss in the former microcosm condition occurred in a significant level and if it occurred due to microbial degradation and not to the abiotic factors that caused mass loss in the sterile microcosm. A comparison between the 1.5% ethanol microcosm and the sterile microcosm was also required, for the same reasons.

Comparing the nutrient-free microcosm condition to the microcosms the received nutrients, there was mass loss in both conditions, but it was necessary to determine how significant was the addition of nutrients to biodegradation of BTEX compounds.

Finally, the comparison between the 0.5% and 1.5% ethanol microcosm conditions used in this experiment would determine the influence of ethanol concentration on BTEX mass loss.

In order to determine the significance of BTEX mass loss and to make a comparison among all microcosms conditions used, statistical analyses were applied and are described in subsection 3.3.3, on page 49.

3.3.2 Ethanol Results

Ethanol aqueous concentration was converted to mass of ethanol in the microcosm. In order to compare to BTEX compound degradation, the mass values were converted to number of carbon moles and are displayed in Table 3.6.

Table 3.6: Ethanol mass loss and carbon moles loss after 64 days of incubation at 10°C.

Measurement	0.5% Ethanol[a]	1.5% Ethanol[a]	Ethanol only[a] (0.5%)	Sterile[c]	Nutrient-free[c]
Initial mass (mg)	3.708	11.205	3.886	4.442	4.253
Final mass (mg)	2.763	10.389	2.770	4.557	4.300
% mass loss	25.4	7.3	28.7	0	0
Number of carbon moles (day 1)	0.016	0.049	0.017	0.019	0.018
Number of carbon moles (day 64)	0.012	0.045	0.012	0.019	0.019
Number of carbon moles lost	0.004	0.004	0.005	0.00	0.00

[a] Values are mean data from duplicate bottles.

[b] A single bottle of the original three replicates received extra MBH medium on day 18.

[c] Values are mean of three replicates.

There was not much ethanol mass loss observed in this experiment. Ethanol only and 0.5% ethanol microcosm conditions had similar values of mass degradation. Both had the same concentration of ethanol added with the difference being in the presence of BTEX in the 0.5% ethanol microcosm. The percentage of mass loss for the 1.5% ethanol microcosm condition, was low if compared to the microcosms with 0.5% ethanol. No ethanol loss was observed for the sterile and nutrient-free microcosms.

3.3.3 Statistical Analysis

To determine if the decrease in BTEX mass and ethanol concentration in the different sets of microcosms (0.5% ethanol, 1.5% ethanol, ethanol only, sterile and nutrient-free) was

significantly different from zero, statistical analysis was applied to the two bottles that did not receive extra MBH medium during the course of the experiment (0.5%, 1.5% ethanol, and ethanol "only" microcosms) and to the three sterile and three nutrient-free microcosms. The difference between day 3 and day 64 (for BTEX compounds) and between day 1 and day 64 (for ethanol) was calculated for each replicate (the subsamples were averaged) for all dependent variables (BTEX and ethanol) and the data were reduced to differences for all treatments (0.5% ethanol, 1.5% ethanol, ethanol "only", sterile and nutrient-free). The BTEX "only" case was not considered, since all compounds were completely degraded in 5 days.

Day 3 was chosen over day 1 for the analysis of BTEX degradation because day 1 measurement showed too much variability among the sub-samples (Tables C.6 and C.7). This variability was attributed to the lack of experience in sampling and injection of samples onto the gas chromatograph and to the short elapsed time after microcosm set up, which did not allow equilibration of the BTEX compounds between solid, aqueous and gaseous phases.

One way analysis of variance was conducted for each dependent variable to compare the treatments (microcosms conditions). The residuals were checked for the assumption of homogeneous variance and for outliers. A test of the null hypothesis of zero average difference between days 3 and 64 (for BTEX compounds) and days 1 and 64 (for ethanol) was conducted for each treatment (microcosm), using the Mean Square Error for the ANOVA as an estimate of the population variance. It is appropriate to conduct these tests regardless of the significance of the ANOVA F value since their validity does not depend on whether or not the treatments differ. The reason that the ANOVA had to be conducted first was to obtain the Mean Square Error value needed for the test statistics. Under the null hypothesis, the resulting statistics had a t-distribution with degrees of freedom corresponding to the Mean Square Error degrees of freedom. A one-sided alternative hypothesis was used for 0.5% ethanol, 1.5% ethanol, ethanol "only" and nutrient-free microcosms since a decrease in BTEX mass or ethanol concentration was the only result expected. A two-sided alternative hypothesis was selected for the sterile microcosms since the result expected

Table 3.7: p-values for BTEX compounds in the different treatments (microcosms).

Compound	0.5% Ethanol	1.5% Ethanol	Sterile	Nutrient-free
Benzene	3.7 x 10^{-6}	6.4 x 10^{-3}	1.52 x 10^{-3}	0.355
Toluene	1.6 x 10^{-7}	1.5 x 10^{-3}	1.44 x 10^{-3}	0.197
Ethylbenzene	0.010	6.38 x 10^{-6}	1.11 x 10^{-5}	1.84 x 10^{-5}
m-Xylene	1.2 x 10^{-5}	1.29 x 10^{-4}	5.54 x 10^{-4}	5.82 x 10^{-3}
p-Xylene	3.3 x 10^{-4}	6.24 x 10^{-4}	2.3 x 10^{-4}	5.48 x 10^{-3}
o-Xylene	1.2 x 10^{-3}	4.28 x 10^{-3}	7.08 x 10^{-4}	0.012

was neither decrease nor increase in BTEX mass or ethanol concentration. The p-value is highly significant if $p \leq 0.01$, is significant if $0.01 < p \leq 0.05$ and is marginally significant if $0.05 < p < 0.10$. The p-value is not statistically significant when $p \geq 0.10$.

According to Table 3.7, the p-values were, when considered to two decimal places, all highly statistically significant ($p \leq 0.01$), except for benzene and toluene in the nutrient-free microcosm.

If the null hypothesis of equality of the average differences for all treatments was rejected in the one way analysis of variance, multiple comparison procedures were then applied to compare the treatment averages. The analyses of variance are summarized in Appendix C, in Table C.8 for BTEX and Table C.15 for ethanol. The two-sided alternative hypothesis was used for all Tukey's multiple comparisons. BTEX mass loss was compared between the 0.5% and 1.5% ethanol microcosms, the 0.5% ethanol and sterile microcosms, the 0.5% ethanol and nutrient-free microcosms and the 1.5% ethanol and sterile microcosms. The p-values are displayed in Table 3.8.

Comparing the 0.5% and 1.5% ethanol microcosms, it was observed that the concentration of ethanol affected benzene, toluene and ethylbenzene degradation, since their mass values in the 0.5% ethanol microcosm were significantly lower than their mass values in the 1.5% ethanol microcosm. Ethanol concentration did not affect xylene isomers degradation.

The ethanol (0.5%) microcosm condition compared to the sterile microcosm condition

Table 3.8: Tukey's p-values for multiple comparisons of microcosms for BTEX compounds.

Comparison	Benzene	Toluene	Ethylbenzene	m-Xylene	p-Xylene	o-Xylene
0.5% Ethanol x 1.5% Ethanol	0.001	0.000	0.002	0.110	0.953	0.853
0.5% Ethanol x Sterile	0.001	0.000	0.004	0.011	0.997	0.990
0.5% Ethanol x Nutrient-free	0.000	0.000	0.020	0.002	0.107	0.339
1.5% Ethanol x Sterile	0.812	0.998	0.352	0.364	0.980	0.667

showed that mass loss was significant in the former condition for benzene, toluene, ethylbenzene and m-xylene.

The ethanol (0.5%) microcosms compared to the nutrient-free microcosms showed that benzene, toluene, ethylbenzene and m-xylene had significant mass losses in the 0.5% ethanol microcosm condition.

The differences between the 1.5% ethanol and sterile microcosms were not statistically significant for any of the dependent variables (Table 3.8).

Ethanol loss was highly significant in the ethanol "only", the 0.5% ethanol and 1.5% ethanol microcosms (Table 3.9). In sterile and nutrient-free microcosms, ethanol loss was not significant (Table 3.9). Tukey's multiple comparison was applied to compare ethanol loss among the different sets of microcosms.

Table 3.9: Summary of the p-values for ethanol in all microcosms separately and for Tukey's multiple comparison.

Microcosms	p-value
0.5% Ethanol	0.0011
1.5% Ethanol	0.0024
Ethanol only	7.86×10^{-5}
Sterile	0.44
Nutrient-free	0.394
Tukey's multiple comparisons for ethanol	**p-value**
0.5% Ethanol x 1.5% Ethanol	0.990
0.5% Ethanol x Ethanol only	0.407
0.5% Ethanol x Nutrient-free	0.037
0.5% Ethanol x Sterile	0.025
1.5% Ethanol x Sterile	0.045
Ethanol only x Sterile	0.003

The ethanol levels in the 0.5% ethanol, 1.5% ethanol and ethanol "only" microcosms were all significantly lower than those of the sterile microcosms (Table 3.9).

Ethanol (0.5%) mass loss was significantly lower than that of the nutrient-free microcosm. The addition of nutrients was important to ethanol degradation in this case.

The comparison between 0.5 and 1.5% ethanol microcosms showed that the concentration of ethanol was not a significant factor in ethanol degradation.

And the comparison between 0.5% ethanol and ethanol only microcosms showed that the presence of BTEX compounds did not affect ethanol degradation.

The complete statistical analyses are shown in Appendix C (Table C.17).

3.3.4 Effect of Nutrient Addition

Extra nutrients were added to one of the three bottles (replicate 2) on day 18 of the experiment as an attempt to enhance the biodegradation process in one of the replicates and compare to the ones that remained with the original amount of nutrients. MBH medium was added (5 mL) to replicate 2 of 0.5% ethanol, 1.5% ethanol and ethanol alone microcosm conditions.

Figures 3.10 to 3.15 show the effect of extra nutrients addition on the concentration of BTEX compounds in the 0.5% and 1.5% ethanol microcosms. In the 0.5% ethanol microcosm, benzene was completely consumed by day 38 (Figure 3.10), whereas in the 1.5% ethanol microcosm, despite the addition of extra MBH medium, benzene loss was not detected.

Toluene was completely degraded with the addition of extra nutrients to the 0.5% ethanol microcosm (Figure 3.11). Without extra nutrients toluene degradation was delayed, but it showed 94.4% of mass loss at the end of the experiment (Table 3.4). In the 1.5% ethanol microcosms, toluene concentrations remained stable, the addition of extra nutrients had no effect (Figure 3.11).

Ethylbenzene was depleted in the 0.5% ethanol microcosm before the addition of extra MBH medium (Figure 3.12). For the 1.5% microcosm, ethylbenzene concentration dropped until day 10, remaining stable after that time until the end of the experiment, even with the addition of extra nutrients.

Among the xylene isomers, only *p*-xylene was totally degraded with the addition of extra MBH medium to the 0.5% ethanol microcosm (Figure 3.13). The other two xylene isomers showed no concentration changes in the 0.5% ethanol microcosms (Figures 3.14 and 3.15). For the 1.5% ethanol microcosms, the addition of extra nutrients did not affect the concentration of any xylene isomer. Since only one bottle of each microcosm type received extra nutrients, the results could not be statistically tested for their significance.

The effect of nutrient addition was observed in the ethanol 0.5% and ethanol only microcosm conditions. Ethanol degradation was not very much affected by extra nutrient

addition (Figure 3.16).

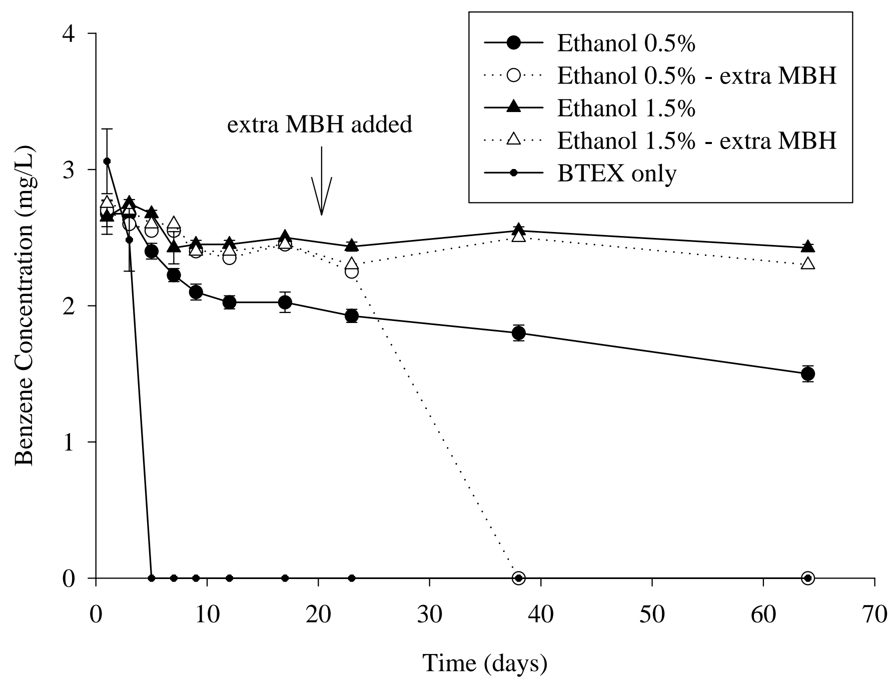

Figure 3.10: Effect of extra nutrients addition on benzene degradation in 0.5% and 1.5% ethanol microcosms. The error bars in microcosms that did not receive extra MBH medium represent the standard error of two replicates.

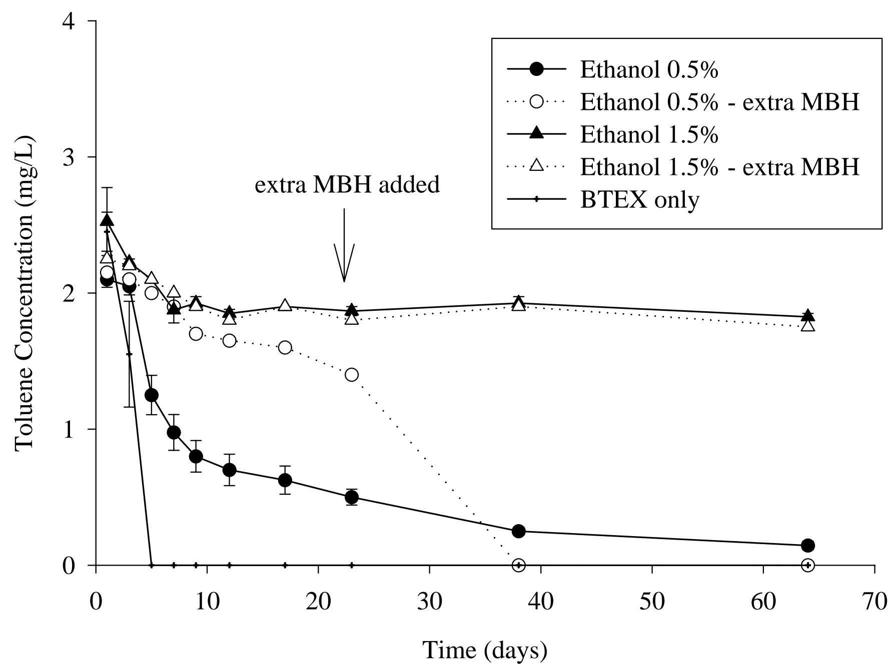

Figure 3.11: Effect of extra nutrients addition on toluene degradation in 0.5% and 1.5% ethanol microcosms. The error bars in microcosms that did not receive extra MBH medium represent the standard error of two replicates.

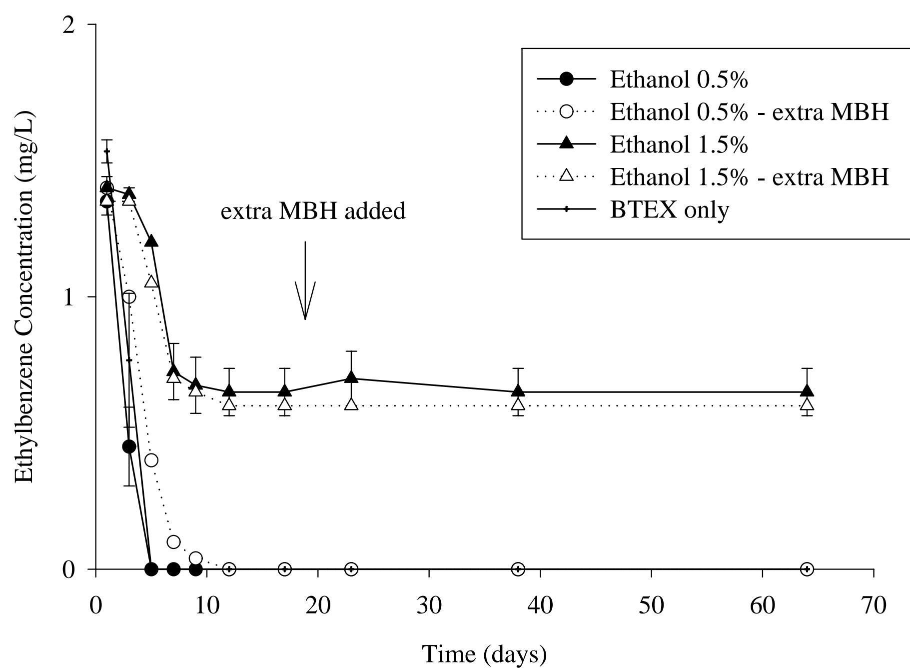

Figure 3.12: Effect of extra nutrients addition to ethylbenzene degradation in both 0.5% and 1.5% ethanol microcosms. The error bars in microcosms that did not receive extra MBH medium represent the standard error of two replicates.

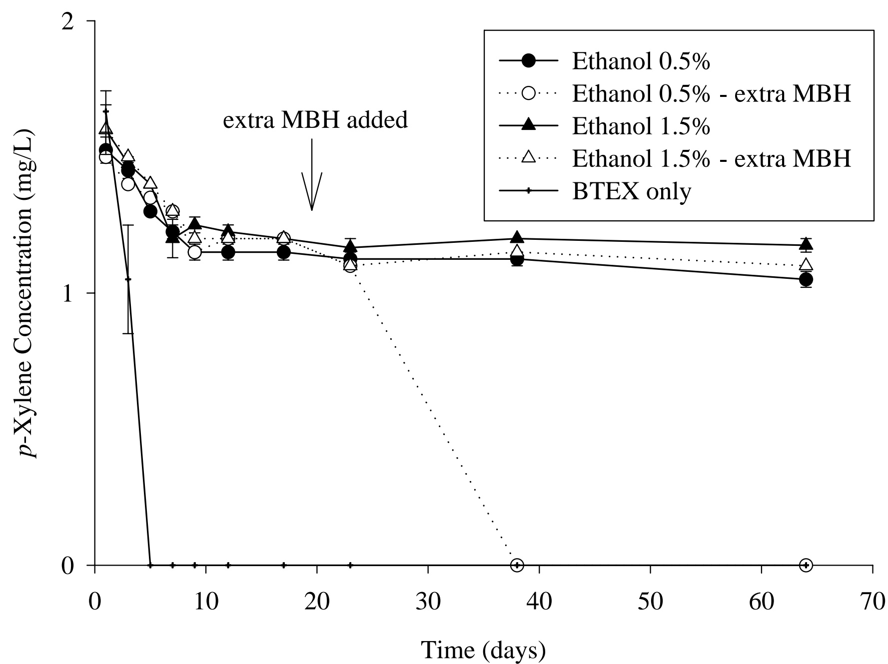

Figure 3.13: Effect of extra nutrients addition on *p*-xylene degradation in 0.5% and 1.5% ethanol microcosms. The error bars in microcosms that did not receive extra MBH medium represent the standard error of two replicates.

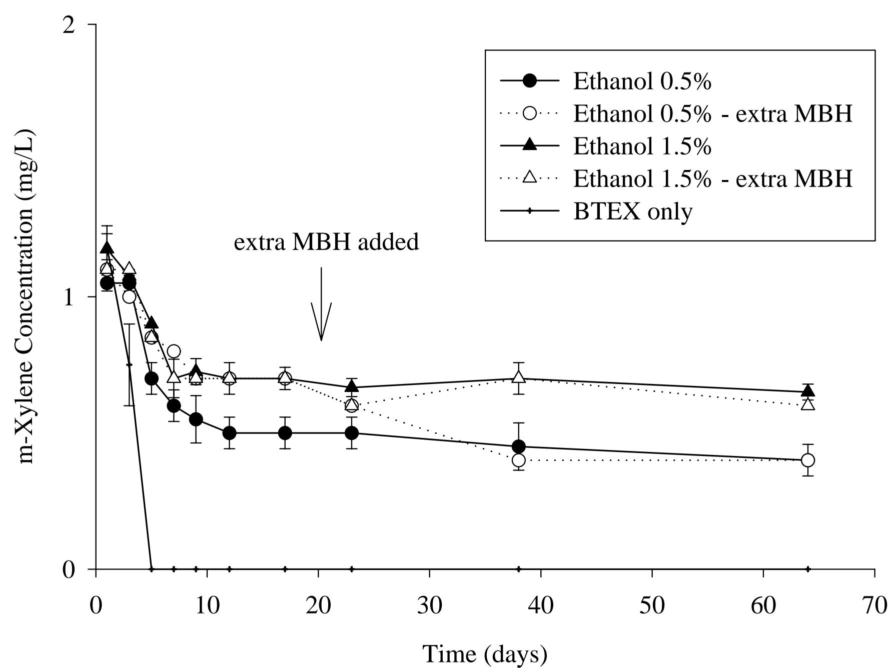

Figure 3.14: Effect of extra nutrients addition on m-xylene degradation in 0.5% and 1.5% ethanol microcosms. The error bars in microcosms that did not receive extra MBH medium represent the standard error of two replicates.

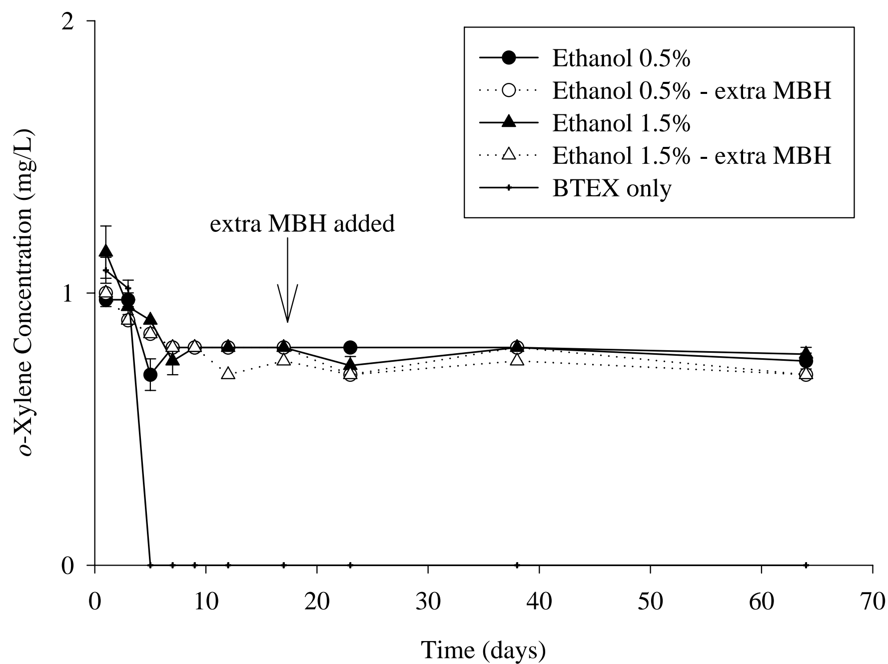

Figure 3.15: Effect of extra nutrients addition on *o*-xylene degradation in 0.5% and 1.5% ethanol microcosms. The error bars in microcosms that did not receive extra MBH medium represent the standard error of two replicates.

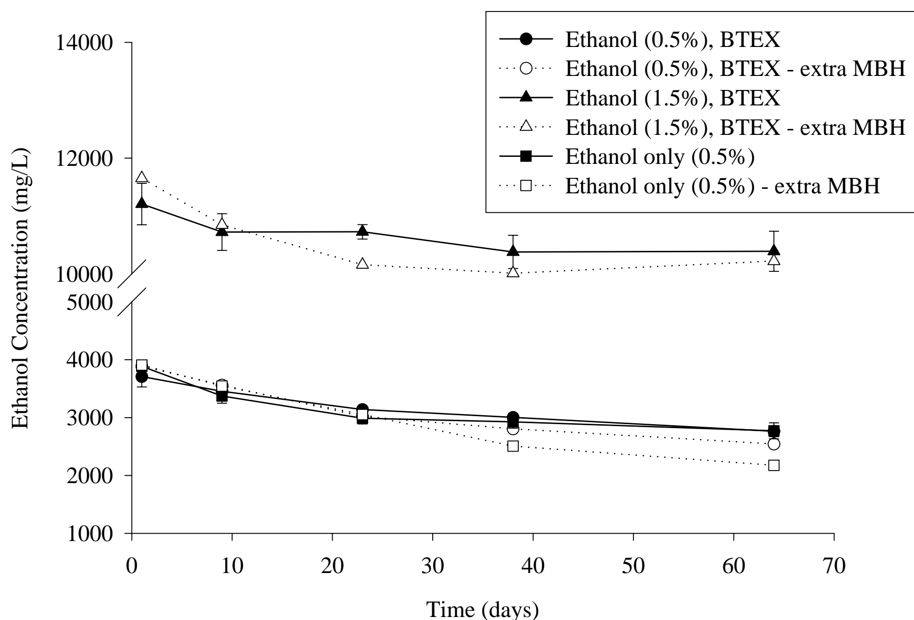

Figure 3.16: Ethanol concentrations in three different treatments, with and without extra MBH addition. The error bars in microcosms that did not receive extra MBH medium represent the standard error of two replicates.

3.3.5 Dissolved Oxygen

According to calculations of oxygen availability (shown in Appendix C, page 105) in the microcosms, oxygen would be exhausted before the total degradation of ethanol at 1.5% concentration if the microcosm headspace was air-filled. To prevent anaerobic conditions during the experiment, the 1.5% ethanol microcosms were therefore flushed with extra oxygen.

Dissolved oxygen (DO) was measured during the experiment until day 38. In the BTEX only microcosms dissolved oxygen was measured until day 23, when all the BTEX in those bottles were completely degraded. The ethanol only microcosm with extra MBH medium,

showed the greatest reduction in dissolved oxygen content (74%) (Figure 3.17). The 0.5% ethanol microcosms had 35% of the initial DO depleted and in the replicate where extra MBH was added, 48% of the initial DO was depleted. All the others microcosms showed no detectable change from the initial dissolved oxygen concentration (Figure 3.17). The percentages of DO lost from each microcosm is presented in Table C.18, in Appendix C.

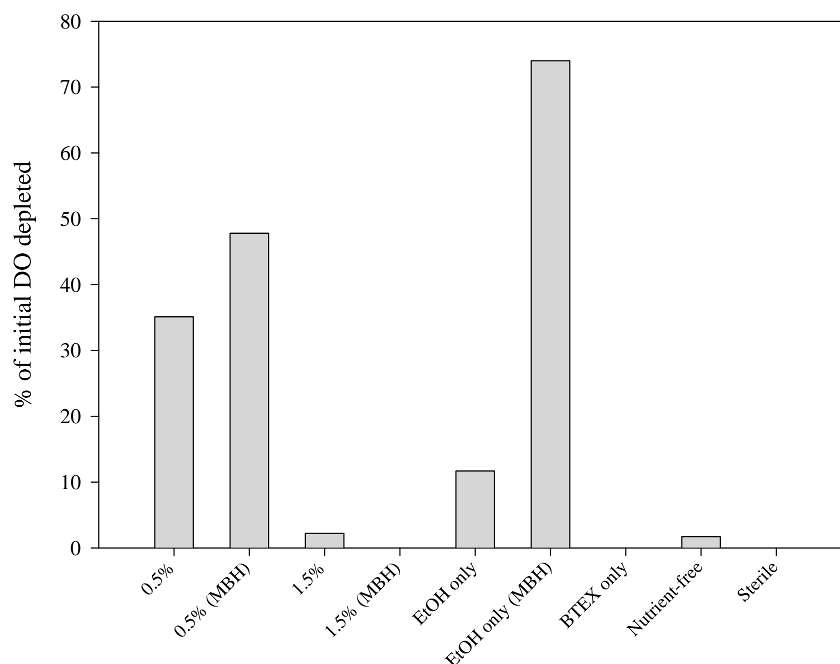

Figure 3.17: Dissolved oxygen depletion (%) on day 38. For BTEX only microcosm condition, on day 23.

3.3.6 Summary

BTEX compounds were completely degraded in the absence of ethanol (Table 3.4). In the 0.5% ethanol microcosm only ethylbenzene was totally degraded. Toluene and m-xylene had more than half of their mass degraded. The other compounds had less than 50% mass loss (Table 3.4).

In the 1.5% ethanol microcosm, BTEX degradation occurred to a lesser extent if compared to the 0.5% ethanol microcosm (Table 3.4).

This experiment showed the importance of nutrient supplementation in biodegradation of BTEX and ethanol performed by microorganisms of subsurface material collected from the Borden aquifer. When nutrients were added, it was observed that benzene, toluene and p-xylene concentrations decreased to zero in the 0.5% ethanol microcosm, compared to the 0.5% microcosms that did not receive extra nutrients (Figures 3.10, 3.11 and 3.13). Ethylbenzene concentration decreased to zero before the addition of extra nutrients (Figure 3.12). The concentrations of m-xylene and o-xylene remained stable regardless the addition of extra nutrients in the 0.5% ethanol microcosm condition (Figures 3.14 and 3.15).

In the 1.5% ethanol microcosm condition, nutrient addition did not cause any significant change in BTEX concentration (Figures 3.10 to 3.15).

Ethanol was not much degraded under any of the microcosm conditions tested (0.5% ethanol, 1.5% ethanol and ethanol only) (Table 3.6), except that extra nutrients addition increased degradation in the ethanol only microcosm condition (Figure 3.16).

The DO level decreased most in the ethanol only microcosm with extra MBH, followed by the 0.5% ethanol microcosm with extra MBH (Figure 3.17). Oxygen consumption was not observed in the BTEX only, 1.5% ethanol, sterile and nutrient-free microcosms.

Chapter 4

Discussion

4.1 Microbial Activity

4.1.1 Effect of ethanol concentration on microbial activity and growth

Microbial activity measurement was conducted in microcosms, simulating the subsurface environment. Microcosms have been used for a variety of purposes due to their convenience to control certain environmental features that would be difficult to do in a field study [6]. Nutrient-free microcosm had no detectable microbial activity in all experiments (Figures 3.1, 3.2, 3.3 and 3.4). Comparing the results of INT-formazan production with and without glucose, the microcosms amended with glucose had higher values than the microcosms without glucose (Table 3.1). With no nutrients provided, and at 10°C temperature, endogenous microbial activity in Borden subsurface material could not be detected by the INT-formazan assay which is relatively insensitive.

microbial activity on ethanol

Ethanol being utilized as carbon source, has a large demand for nutrients and electron acceptors [5]. Comparing INT-formazan production in Table 3.1 and cell counts in Table 3.2, an increase in the cell counts corresponded to an increase in the microbial activity. When

glucose was absent, the higher number in the cell counts was observed at an ethanol concentration of 3%, and that is when the higher level of INT-formazan production (microbial activity) was observed (Table 3.1).

Ethanol at 3% supplied enough carbon to support microbial activity and growth, as the amount of CFU/mg at this concentration was 100-fold more than the initial count (Table 3.2). The use of microbial quantification for soil biological activities has frequently been applied to interpret intensity of microbial metabolism in soil [15, 65], although Margesin *et al.* [47] concluded that the quantification of viable cells does not give information about the efficiency of the population because only a small portion of subsurface microorganisms can be isolated and cultivated in laboratory media. McCarthy and Murray [48] found that more oligotrophs were recovered from an environmental sample using MPN counts compared to CFU counts. It has to be taken into consideration that not all microorganisms are able to grow in agar and although R2A agar is used to enumerate bacteria from potable water [61], where usually oligotrophs are expected to be found, it may not contain all the substrates necessary for the culture of specific oligotrophs. The fact that fewer microorganisms were found in the 0.5 and 1.5% ethanol microcosms compared to the 3% ethanol microcosm could be associated with the less amount of carbon source in the former microcosms, compared to the latter microcosm and, in a lesser extent, to the medium onto which they were cultivated. Also, ethanol was not present in the medium, which may have prevented the growth of microorganisms that were using ethanol as a carbon source in the microcosms.

Microbial activity was observed at ethanol concentrations up to 3%, which indicates that ethanol was used as a carbon source. At 5% ethanol microbial activity was not observed, although cell counts demonstrated that the cells were not killed (Table 3.2), indicating that ethanol inhibited microbial activity at this concentration.

0 - 4% ethanol with glucose

Microbial activity in glucose-MBH containing microcosms was enhanced by ethanol concentrations ranging from 0.5 to 3% (Figure 3.1), compared to the control. Glucose is a readily available carbon source and is utilized by many microorganisms [47]. At low concentrations ethanol is a carbon and energy source and microorganisms that can use ethanol are very abundant in nature [5]. Due to more favorable thermodynamics, microbial growth in ethanol is faster compared to more complex compounds, like benzene ([36] cited by Alvarez and Hunt [5]). Microbial growth on glucose was not disturbed at 0.5 and 1.5% ethanol concentrations (Figure 3.6), compared to the control, without ethanol. Konopka *et al.* [42] reported that physiological studies in laboratory systems have shown that cells growing in batch cultures begin exponential growth immediately after nutrient addition. The ethanol concentrations of 0.5 and 1.5% were a carbon source in addition to glucose.

Cultures growing in the presence of 3 and 4% ethanol were affected by it since their curves were below the control curve (Figures 3.6 and 3.8). They exhibited, though, a sustained growth. Herrero and Gomez [32] investigating ethanol tolerance in *Clostridium thermocellum*, suggested that a culture growing under two carbon sources, need a time to adapt to a new carbon source, once the other in use was depleted. This work was conducted with a community of microorganisms, instead of one isolated organism, as Herrero and Gomez, there were probably organisms that were not able to adjust to the new situation and that selected the ones that were able to grow in ethanol. Two situations might have happened in the Borden cultures. The first one, as glucose was becoming scarce, the populations had to adapt to the next available carbon source, which was ethanol. The second situation could be that the populations were using glucose and ethanol simultaneously (Egli [28] reported simultaneous utilization of multiple substrates by microorganisms at carbon-limiting conditions) and acetate was being formed as a product of ethanol utilization. So, acetate could be the next available carbon source. Abbot [2] observed a lag phase in growth curves of *Acinetobacter calcoaceticus* growing on ethanol. Abbot suggested that, as *A. calcoaceticus* rapidly converts ethanol to acetate, the culture needed time to adapt to the new carbon source available, which was acetate.

more than 5% ethanol

Microbial activity was not detected at ethanol concentrations equal and above 5% (v/v) (Figure 3.1 and 3.2). Ethanol is known to cause cell damage and death in bacteria [18,19,27] yeasts [3, 58] and protozoan [55]. Hunt *et al.* [35], in a study on the effect of ethanol on aerobic biodegradation of BTX, reported lack of oxygen consumption in microcosms containing more than 4% (wt/wt) ethanol, showing that ethanol at this concentration was toxic to the microorganisms. In this experiment, although microbial activity was not detected at 5% ethanol, this concentration did not kill the cells, since colonies were able to grow onto R2A plates (Table 3.2). Rigomier *et al.* [62] reported that *Bacillus subtilis* cultures exposed to low concentrations of ethanol did not suffer permanent damage (death of cells) and the ethanol acted only on easily reversible molecular interactions in the plasma membrane of the cells. Microbial growth was inhibited at ethanol concentration above 5% (Figure 3.7).

4.1.2 Effect of ethanol and gasoline-saturated water mixture on microbial activity

The assessment of microbial activity in the presence of gasoline-saturated water was conducted as one experiment, although Figures 3.3 and 3.4 are shown separately, for clarity. Microbial activity in the R2A microcosm (control) was lower than ethanol and gasoline-saturated water-ethanol microcosms. Comparing the R2A microcosms to the gasoline-saturated water microcosms, they both showed similar levels of microbial activity (Figures 3.3 and 3.4). However whenever ethanol was present, either alone or mixed with gasoline saturated water, microbial activity reached higher values. Ethanol was an extra potential source of carbon, in addition to R2A medium, and at the concentration used for this experiment (0.5%) it appeared not to be toxic to microorganisms. The gasoline-saturated water microcosms provided water soluble gasoline components and R2A medium as carbon sources, but the microorganisms did not seem to use the extra carbon source (gasoline components) so easily, as with ethanol, since the values for INT-formazan

production for the microcosms were similar to the R2A microcosms. The use of gasoline-saturated water carbon might also have produced toxic substances that inhibited or killed susceptible microorganisms. In an experiment monitoring bioremediation of mineral-oil hydrocarbons by microbial activity, Margesin *et al.* [47] attributed the decrease in microbial activity to the reduced bioavailability of carbon sources and to accumulation of recalcitrant and/or toxic by-products. It was expected that the presence of gasoline-saturated water in the microcosm would result in a higher levels of microbial activity compared to the control, since dissolved gasoline components provided more source of carbons than R2A alone. The reason why the increase in microbial activity did not occur is unclear.

The combination R2A medium and ethanol promotes microbial activity and exceeds the activity in R2A only microcosms. At this concentration, ethanol is not inhibitory, but it served as a substrate in addition to R2A medium carbons.

4.1.3 Effect of temperature in ethanol tolerance

Several works have reported a link between ethanol tolerance and growth temperature [18, 24, 33, 37, 49, 66]. At 25°C the growth curves for the control and 0.5% ethanol were the same, indicating growth on glucose and no detectable influence of 0.5% ethanol (Figure 3.8). The generation times were very similar for both cultures (Table 3.3). A difference from the control curve was first observed at 1.5% ethanol concentration. At 10°C incubation, no difference was observed among the control, 0.5% and 1.5% curves until 3% ethanol (Figure 3.6). Comparing the two incubation temperatures, 10°C and 25°C, it was observed that when the temperature increased, ethanol tolerance decreased. Ingram and Buttke [38] reported reduction on ethanol tolerance in *Z. mobilis*, *Cl. acetobutylicum* and *Cl. thermocellum* when growth temperature was increased. One reason for the difference in the curve could be explained by the shift in temperature the cells were subjected to. The cells were cultured at 10°C and then transferred to flasks of ethanol-amended medium and incubated at 25°C. Temperature is known to affect the lipid composition of cell membranes [25, 40, 41, 56, 67]. Abbas and Card [1] found that when *Yersinia enterocolitica* cells were shifted from a lower to a higher temperature, the cells could not survive long

enough to make the adjustments necessary for growth at the higher temperature. In the present experiment the shift from 10°C to 25°C may have caused temperature stress-related changes in the cell membranes and decreased their tolerance to ethanol.

4.2 Biodegradation

There are a number of issues to be addressed on the results obtained in this experiment: i) the presence of ethanol at 0.5% slowed BTEX degradation compared to the microcosms without ethanol (BTEX only); ii) the lack of BTEX degradation in the 1.5% ethanol microcosm and the relation to oxygen consumption in this microcosm; iii) the effects of both nutrient addition and extra nutrient addition on both BTEX and ethanol degradation; and iv) interactions in 0.5% ethanol and BTEX containing microcosms.

4.2.1 Effect of 0.5% ethanol on BTEX degradation

BTEX compounds were completely degraded by day 5 of the experiment in the microcosms that did not contain ethanol. When ethanol was present, though, a different situation occurred. Ethanol slowed BTEX degradation at both ethanol concentrations. In studies on the influence of ethanol on BTX biodegradation, Corseuil *et al.* [23] suggest that ethanol is preferentially utilized as a carbon source and this increases the lag time before BTEX degradation begins. Hunt *et al.* [35] reported that ethanol was chosen to be degraded over benzene under aerobic conditions. It was observed in the present experiment that benzene, toluene, ethylbenzene and *p*-xylene were degraded at a significant level (Figures 3.10 to 3.13). The graphs show that 0.5% ethanol slowed BTEX degradation, since BTEX in the microcosm without ethanol were completely degraded at day 5 of the experiment (Figures 3.10 to 3.15). Comparing Figures 3.10 to 3.13 to Figure 3.16, both BTEX and ethanol were being degraded at the same time.

This might suggest a simultaneous ethanol/BTEX biodegradation, with no substrate preference. Egli [28] wrote an extensive review on multiple substrate utilization, that has been widely reported in the literature. It is possible that under carbon-limiting conditions,

which was Borden's subsurface material, the microbial population utilize more than one source of carbon at the same time. This hypothesis does not eliminate the effect of ethanol on BTEX degradation, since ethanol clearly slowed BTEX degradation, compared to BTEX only microcosm. There are some speculations that ethanol might be involved in repression of enzymes responsible for BTEX degradation, acting on the population of BTEX degraders, but there are only indirect evidences in the literature [5]. The competition for inorganic nutrients might have also played a role in the retardation of BTEX degradation in the presence of ethanol. It could be that there were distinct populations of BTEX degraders and ethanol degraders competing for inorganic nutrients like nitrogen and phosphorus, that are necessary for degradation of pollutants [69]. Since ethanol was present in a larger amount compared to BTEX compounds, the majority of the oxygen and inorganic nutrients present in the microcosms could have been used toward ethanol degradation.

Without the addition of extra nutrients, and in the 0.5% ethanol microcosm, only ethylbenzene was completely degraded (Figure 3.12 and Table 3.4). Xylene was resistant to biodegradation. Zhou and Crawford [76] also observed the same thing for ethylbenzene and *o*-xylene at 11°C.

The significant loss in all six BTEX compounds in the sterile microcosm was not expected (Table 3.4). This reduction can be attributed to leakage either during incubation or during sampling.

4.2.2 Effect of 1.5% ethanol on BTEX degradation

Figures 3.10 to 3.15 showed no apparent reduction in BTEX concentration in the 1.5% ethanol microcosm. Statistical analysis comparing the 1.5% ethanol microcosms to the sterile microcosms showed that BTEX mass loss in the 1.5% ethanol microcosms was not significant (Table 3.8), meaning that the factors that caused BTEX mass loss in the sterile microcosm (e.g., leakage), were also responsible for the mass loss in the 1.5% ethanol microcosm. Furthermore, the lack of change in the DO content of the 1.5% ethanol microcosm (Figure 3.17) indicates there was little or no microbial activity.

The idea that 1.5% ethanol was toxic to the cells, inhibiting microbial activity and consequently BTEX degradation, seems confusing since microbial activity at this concentration was detected (Figure 3.1). So, why was there microbial activity detected at 1.5% ethanol (Figure 3.1) and there was little biodegradation activity (Figures 3.10 to 3.15 and 3.16) and no oxygen consumption (Figure 3.17) detected at 1.5% ethanol biodegradation experiment microcosms?

First, although the concentration of ethanol was the same in the microbial activity and biodegradation experiments (1.5% v/v), the ratio of ethanol mass/1 gram of soil was different. While for the 1.5% ethanol in microbial activity experiment was 0.004 g ethanol/g soil, 1.5% ethanol in the biodegradation experiment was 0.012 g ethanol/g soil. There was three times more ethanol per gram of soil in the biodegradation experiment. That amount of ethanol might have been toxic to the cells and prevented them from performing normal cell functions or might have killed some populations of microorganisms. Also, the mass of ethanol added was 300 times more than the mass of BTEX compounds. When easily degradable substrates are present at high concentration, this may repress the expression of inducible enzymes responsible for degradation of pollutants [51]. However Alvarez and Hunt [5] noted that there is no direct evidence presented in the literature about the potential effects of ethanol on the expression of enzymes involved in BTEX degradation.

A second reason might have been the high amount of oxygen pumped into the 1.5% ethanol microcosms. In experiments on the effect of oxygen on gasoline biodegradation by soil microorganisms, Zhou and Crawford [76] reported that higher oxygen concentration did not necessarily result in higher rates of aerobic degradation of gasoline. There was an optimal oxygen concentration of 10% oxygen in the air in the microcosm. High concentrations of oxygen can be toxic to some microorganisms. For example, it is known that oxygen inhibits the activity of nitrogenase, the enzyme responsible for nitrogen fixation, and this process is more effective under low oxygen concentrations [45]. It could be that the amount of oxygen in the 1.5% microcosm affected enzymes responsible for BTEX biodegradation.

4.2.3 Nutrient addition

Figures 3.10, 3.11 and 3.13 show the clear impact of extra nutrient addition on benzene, toluene and *p*-xylene degradation in the 0.5% ethanol microcosm. Their concentrations were reduced to zero between days 23 and 38 of the experiment (Table C.11). The absence of inorganic nutrients was one of the limiting factors of toluene biodegradation reported by Davis and Madsen [26]. They found that the soil contained high numbers of degrading microorganisms but toluene degradation was not occurring [26]. Inorganic nutrients (e.g. nitrogen and phosphorus) equilibrate the C/N/P ratio and stimulate biodegradation in gasoline-contaminated sites [26, 31, 76]. The addition of extra nutrients to one of the replicates accelerated degradation, suggesting that ethanol and BTEX simultaneous degradation exhausted the inorganic nutrients provided initially. The DO measurement showed that dissolved oxygen levels decreased at the addition of extra nutrients, which also indicated renewed microbial activity.

Table 3.4 shows that in the nutrient-free microcosm condition degradation did not occur. The statistical analyses confirm it, since there was no significant degradation of BTEX and ethanol in the nutrient-free microcosms (Tables 3.8 and 3.9). These support the fact that the indigenous nutrient concentration at Borden site was not enough to support degradation of the supplied levels of BTEX and ethanol, so inorganic nutrient supplementation was required.

Figures 3.10 to 3.15 show that for the 1.5% ethanol microcosms, the addition of extra nutrients did not promote BTEX degradation, in contrast to the results for 0.5% ethanol microcosms.

4.2.4 Ethanol degradation

Comparing the amount of ethanol carbon moles in the 0.5% microcosms (Table 3.6) to the amount of carbon moles of total BTEX (Table 3.5), ethanol was in a larger amount. The number of ethanol carbon moles lost in the 0.5% ethanol microcosms is higher than the amount of BTEX carbon moles lost in the same microcosm. Ethanol degradation was

consuming a large part of inorganic nutrients and oxygen, due to its larger concentration, compared to the BTEX concentration.

Of all the microcosms, the highest ethanol loss occurred when BTEX compounds were absent (Table 3.6). Corseuil *et al.* [23] stated that in all ethanol concentrations they tested, ethanol was degraded before BTX. This was not observed in this experiment, as some BTEX compounds were totally degraded before ethanol was reduced to zero. It has to be taken into account that the ethanol concentrations used in this experiment were higher than those in Corseuil's experiments. Ethanol statistics (Table 3.9) show that in the 0.5% ethanol microcosms, ethanol loss was significant compared to the sterile (control). Also, the addition of nutrients was important for ethanol degradation, as the comparison between 0.5% ethanol microcosms and nutrient-free microcosms shows that ethanol in the former microcosms had significant loss. The dissolved oxygen consumption data confirms it, since oxygen demand increased when extra nutrients were added (Figure 3.17 and Table C.18). The concentration of ethanol in a ground water contaminated plume is expected to be higher than the BTEX concentration, since ethanol concentration in gasohol is higher than BTEX compounds. The aqueous ethanol will have a high demand for oxygen compared to the soluble components of gasoline [5].

4.2.5 Summary

Biodegradation of BTEX and ethanol occurred simultaneously, but BTEX degradation was delayed when 0.5% ethanol was present in the microcosms. The large amount of ethanol, compared to the concentration of BTEX, required more oxygen and inorganic nutrients for its degradation, leaving little for BTEX degradation. Consequently more ethanol carbon moles was being utilized than BTEX carbon moles (Tables 3.5 and 3.6). Extra MBH addition was critical to enhance BTEX and ethanol degradation, evidence that inorganic nutrients were the limiting factor for degradation in this experiment. Ethanol at 1.5% concentration prevented BTEX and ethanol degradation, compared to 0.5% ethanol (Figures 3.10 to 3.15 and 3.16).

4.3 Implications

Relating to what could happen to a gasoline-ethanol mixture spill in environments such as the Borden aquifer, with the low levels of inorganic nutrients, it can be expected that ethanol and BTEX will take a long time to be degraded. The concentration of ethanol should be taken in account, since 1.5% ethanol inhibited both BTEX and ethanol degradation (Figures 3.10 to 3.15 and 3.16).

If a gasoline-ethanol spill occurs in an environment where inorganic nutrients are abundant, total degradation of ethanol and BTEX compounds would happen more quickly if compared to low nutrient environments. Still, there are some other factors to be considered, such as ethanol concentration, aquifer temperature and oxygen availability. Microbial activity could be inhibited by ethanol and its presence affects BTEX degradation. Competition for inorganic nutrients and oxygen among ethanol and BTEX degraders will affect these compounds' degradation rates. Temperature plays a role in microbial tolerance to ethanol. High temperatures reduces microbial tolerance to ethanol. High concentrations of ethanol require high amounts of oxygen for degradation, so thatoxygen depletion would likely occur in an environment such as an aquifer, where oxygen is not readly replenished.

Further studies on population of ethanol and BTEX degraders would help determine if there are different populations using each carbon source or if there is one population that "switches" from one carbon source to another, according to its availability.

An experiment on biodegradation of BTEX with ethanol would be necessary to determine if there would be a depletion of oxygen when all ethanol is degraded. Enough nutrients must be added at the beginning of the experiment to ensure there will not be limitation of nutrients.

Chapter 5

Conclusions

In soil, water microcosms, ethanol enhanced microbial activity at 3% concentration. Ethanol at concentration ranging 0.5 to 3% (v/v) enhanced microbial activity when combined with a second carbon source (glucose).

Ethanol at 0.5% concentration enhanced microbial activity over water soluble gasoline components and R2A medium combined.

Microbial growth in liquid culture (in glucose-MBH medium) was not affected by 0.5% and 1.5% ethanol and was partially inhibited at 3% and 4% ethanol at 10°C. At 25°C, 1.5%, 3% and 4% ethanol were partially inhibitory. Thus, an increase in incubation temperature (from 10 to 25°C) decreased ethanol tolerance in cultivated subsurface microorganisms.

Both microbial activity and growth were not detected at ethanol concentrations equal and above 5%.

Ethanol slowed BTEX degradation at 0.5% concentration and prevented BTEX degradation at 1.5%.

Mineral nutrients were essential for BTEX and ethanol degradation.

Appendix A

ETS Activity

A.1 INT-formazan Standard Curve

A standard curve was prepared before the experiment sampling, to calculate the INT-formazan production in the microcosms. For the standard curve, a stock solution of 100 μg/mL INT-formazan in methanol was prepared. The stock solution was then diluted to final concentrations of 50, 30, 20, 10, 5, 3, 2 μg/mL. The dilutions were then measured in a spectrophotometer at 480 nm against a methanol blank. The O.D. readings were plotted against the INT-formazan concentrations.

Raw data for the standard curve (Figure A.1) are listed in Table A.1:

Table A.1: Raw data for INT-formazan standard curve

Concentration of INT-formazan (μg/mL)	O. D.$_{480}$ nm
0	0
2	0.072
3	0.099
5	0.161
10	0.364
20	0.657
30	1.216
50	1.912

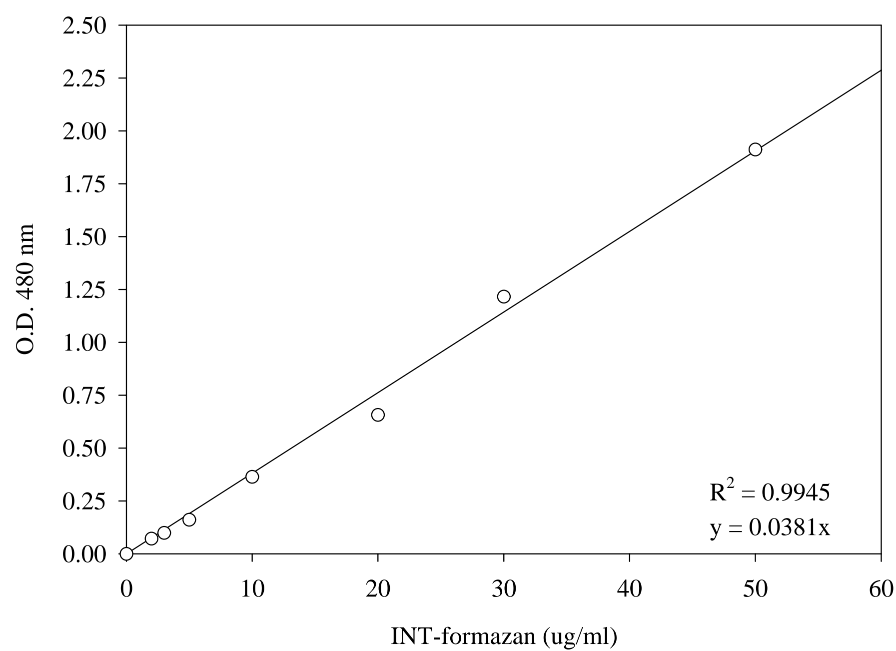

Figure A.1: INT-formazan standard curve

A.2 Detailed Microcosm Assembly

Table A.2 shows the detailed microcosm assembly of the ETS experiment in the presence of ethanol. The microcosms that did not receive MBH medium received 1 mL of sterile distilled water. Each microcosm received 15 g of subsurface material.

Table A.2: Microcosm assembly for ETS experiment using ethanol.

Microcosm[a]	INT solution (mL)	MBH + glucose (mL)	MBH only (mL)	Ethanol % (v/v) per vial
Ethanol 0.5%	1.5	1	0	0.5
Ethanol 1.5%	1.5	1	0	1.5
Ethanol 3%	1.5	1	0	3
Ethanol 5%	1.5	1	0	5
Ethanol 0.5% (no glucose)	1.5	0	1	0.5
Ethanol 1.5% (no glucose)	1.5	0	1	1.5
Ethanol 3% (no glucose)	1.5	0	1	3
Ethanol 5% (no glucose)	1.5	0	1	5
Nutrient-free	1.5	0	0	3
Sterile	1.5	1	0	3
No ethanol	1.5	1	0	0

[a] Microcosms were prepared in triplicate.

Table A.3 shows the detailed microcosm assembly of the ETS experiment in the presence of gasoline-saturated water and ethanol mixtures. The microcosms that did not receive MBH medium received 1 mL of sterile distilled water.

Table A.3: Microcosm assembly for ETS experiment using gasoline saturated water-ethanol mixtures.

Microcosm[a]	INT solution (mL)	R2A medium (mL)	Gasoline-saturated water (mL)	Ethanol % (v/v) per vial
Gasoline	1.5	1	1[b]	0
Gasoline + ethanol	1.5	1	1[b]	0.5
Gasoline (1/10)	1.5	1	1[c]	0
Gasoline (1/10) + ethanol	1.5	1	1[c]	0.5
Nutrient-free	1.5	0	1[b]	0.5
Nutrient-free	1.5	0	1[c]	0.5
Ethanol	1.5	1	0	0.5
Sterile	1.5	1	1[b]	0.5
R2A	1.5	1	0	0

[a] Microcosms were prepared in triplicate.

[b] Gasoline-saturated water as prepared.

[c] Gasoline-saturated water diluted 1:10.

A.3 INT-formazan Production

Table A.4 shows INT-formazan production at the end of the experiment (day 28), for microcosms exposed to ethanol concentrations ranging 10–45% (v/v). The nutrient-free and sterile microcosms had 20% (v/v) ethanol each.

Table A.5 shows INT-formazan production in microcosms amended with gasoline-

Table A.4: INT-formazan production in microcosms exposed to ethanol only with concentrations ranging 10–45% (v/v). Values are mean of three replicates.

Microcosm (ethanol %)	INT-formazan (μg/g DW soil)
0	22.0
10	1.0
20	0.3
45	0.5
nutrient-free	0.4
sterile	0.1

Table A.5: INT-formazan production in microcosms exposed to gasoline-saturated water and ethanol (0.5% v/v). Values are mean of three replicates.

Microcosm	INT-formazan (μg/g DW soil)
Gasoline 1:10 + ethanol	9.6
Gasoline 1:10	3.8
Gasoline + ethanol	8.8
Gasoline	4.6
Ethanol	8.8
R2A	4.4
Nutrient-free (gasoline 1:10 + ethanol)	0.2
Nutrient-free (gasoline + ethanol)	0.2
Sterile (gasoline + ethanol)	0.4

saturated water (as prepared or diluted 1:10) and/or 0.5% ethanol. The INT-formazan values are from the last day of the experiment (day 22).

Appendix B

Growth Curve

B.1 Growth Rate Calculation

In order to calculate the growth rate constant for the exponential phase, it is necessary to know the number of generations n that have occurred during t, the period of exponential growth. The generation time, which is the time required for the cell population to double, is calculated as t/n. These calculations were used according to Madigan *et al.* (2000) using the following equations [45]:

B.1.1 Number of Generations (n)

$$n = \frac{logN_t - logN_0}{0.301} \tag{B.1}$$

Where:

n = number of generations

$\mathbf{N}_t$ = O. D. reading (absorbance) at the end of exponential growth

$\mathbf{N}_0$ = O. D. reading (absorbance) at the beginning of exponential growth

0.301 = $\log_2$ = doubling time has occurred when $N_t/N_0 = 2$

B.1.2 Generation Time (g)

$$g = \frac{t}{n} \tag{B.2}$$

Where:

g = generation time

n = number of generations

t = elapsed time during which growth is measured

Appendix C

Biodegradation Experiments

C.1 BTEX Head-Space Analysis

The gas chromatography (GC) was calibrated according to Nales [53]. Using a one point calibration, 25 μL of a neat stock was injected into three 1.038 L bottles and left undisturbed for one hour in order to equilibrate. Table C.1 shows the high end standard calibration. Three high end standards were run on the GC and response factor (R_f) values were calculated. The R_f value is obtained by injecting a known amount of a compound onto the GC and dividing it by the area of the resulting peak, according to the equation below [53]:

$$R_f = \frac{concentration}{area} \tag{C.1}$$

Where:

concentration = theoretical concentration of the compound injected onto the GC

area = area of the resulting peak

Table C.1: Standard GC calibration (high end) [a]

Compound	Neat Stock (mL)	25 μL of Neat Stock (μL)	Density at 20-25°C	Sample Concentration[b] μg/L
Benzene	1.75	5.8333	0.8787	4935.28
Toluene	1.75	5.8333	0.8669	4862.42
Ethylbenzene	1.00	3.3333	0.8670	2778.85
p-Xylene	1.00	3.3333	0.8611	2759.94
m-Xylene	1.00	3.3333	0.8642	2769.87
o-Xylene	1.00	3.3333	0.8802	2821.15

[a] Oven temperature = 105°C; injection temperature = 200°C; Supelcowax 10 capillary column, with FID detector.

[b] See equation C.2 on page 84 for sample calculation. These values are gaseous concentrations.

In order to calculate the theoretical concentration of each BTEX compound in the 1.038 L bottle, the following equation was used (equation C.2).

$$concentration = \frac{injected\ volume \times density}{bottle\ volume} \tag{C.2}$$

Where:

injected volume = volume injected in the bottle (from the neat stock solution)

bottle volume = 1.038 L

Sample calculation for benzene concentration (Table C.1):

$$\begin{aligned} benzene\ concentration &= \frac{5.83\ \mu L \times 0.8787}{1.038L} \\ &= 4.93\ \mu g/L \\ &= 4.93\ mg/L \times 1000 \\ &= 4935.28\ \mu g/L \end{aligned} \tag{C.3}$$

A low end standard was also run on the GC for a linear calibration. A sample of 6μL of a neat stock was injected into a 1.038 L bottle and left undisturbed for one hour in order to equilibrate. Table C.2 corresponds to the low end standard calibration. Three low end standards were run on the GC and response factor (R_f) values were calculated.

Table C.2: Standard GC calibration (low end) [a]

Compound	Neat Stock (mL)	6 μL of Neat Stock (μL)	Density at 20-25°C	Sample Concentration[b] μg/L
Benzene	1.25	1.00	0.8787	844.86
Toluene	1.25	1.00	0.8669	833.56
Ethylbenzene	1.25	1.00	0.8670	833.65
p-Xylene	1.25	1.00	0.8611	827.98
m-Xylene	1.25	1.00	0.8642	830.96
o-Xylene	1.25	1.00	0.8802	846.35

[a] Oven temperature = 105°C; injection temperature = 200°C; Supelcowax 10 capillary column, with FID detector.

[b] Equation C.2 shows sample calculation. These numbers are gaseous concentration.

The method detection limit for gas-phase benzene concentration was 2 μg/L [54]. Using equation C.7, the method detection limit was calculated for aqueous concentration.

Table C.3: Method detection limits (MDL) for the BTEX compounds in the aqueous phase

Compound	MDL for gas-phase[a] concentration (μg/L)	MDL for aqueous phase[b] concentration (μg/L)
Benzene	2.0	14.5
Toluene	2.0	14.5
Ethylbenzene	4.0	29.1
p-Xylene	5.0	36.3
m-Xylene	6.0	43.6
o-Xylene	6.0	43.6

[a] According to Nales *et al.* [54]

[b] See sample calculation on page 86 (equation C.4).

$$\begin{aligned} \frac{C_g}{C_{aq}} &= 0.1376 \\ \frac{2.0}{C_{aq}} &= 0.1376 \\ C_{aq} &= 14.5\ \mu g/L \end{aligned} \tag{C.4}$$

The GC calibration was checked on every sampling day. Samples of 3 μL of a neat 1:1:1:1:1:1 BTEX mixture were placed in two 1.038 L bottles. The bottles were left undisturbed for one hour to allow gas equilibration. A 500 μL sample was removed with a Hamilton Gastight® syringe from each bottle and injected in the GC to check the calibration. Table C.4 shows the calculated concentration (theoretical) of each BTEX compound in the bottle. The BTEX concentrations values measured by the GC were always approximately the same values displayed on Table C.4.

Table C.4: Theoretical BTEX concentrations for the check of the standard calibration with 3 μL of stock solution [a]

Compound	Neat Stock (mL)	3 μL of Neat Stock (μL)	Density at 20-25°C	Sample Concentration[b] μg/L
Benzene	1.25	0.5	0.8787	422.43
Toluene	1.25	0.5	0.8669	416.78
Ethylbenzene	1.25	0.5	0.8670	416.83
p-Xylene	1.25	0.5	0.8611	413.99
m-Xylene	1.25	0.5	0.8642	415.48
o-Xylene	1.25	0.5	0.8802	423.17

[a] Oven temperature = 105°C; injection temperature = 200°C; Supelcowax 10 capillary column, with FID detector.

[b] See equation C.2 on page 84 for sample calculation. These values are gaseous concentrations.

C.2 BTEX Aqueous Concentrations

The headspace BTEX concentrations were converted to aqueous concentrations by using Henry's law constants. Concentrations were calculated using the equation C.5, according to Granger [30]:

$$\frac{C_{aq}}{C_g} = \frac{R \times T}{H} \tag{C.5}$$

Where:

$\mathbf{C}_{aq}$ = equilibrium concentration in the aqueous phase (μg/L)

$\mathbf{C}_g$ = head-space gas concentration (μg/L)

R = ideal gas constant = 8.207×10^{-5} atm.m^3/mol.K

T = absolute temperature (K)

H = Henry's law constant (atm.m^3/mol)

The Henry's law constant varies according to temperature. The incubation temperature for the biodegradation experiments was 10°C. The Henry's Law constants for the BTEX compounds were calculated by Granger [30] according to a temperature regression equation:

$$H = e^{A - \frac{B}{T}} \tag{C.6}$$

Where:

H = Henry's Law constant (atm.m^3/mol)

T = absolute temperature (K)

A = regression coefficient (dimensionless)

Table C.5: Coefficients of Ashworth *et al.* [8] and Henry's law constants

Compound	Coefficient A	Coefficient B	Henry's Law Constant at 10°C (atm.m^3/mol)
Benzene	5.534	3194	0.003195
Toluene	5.133	3024	0.0039
Ethylbenzene	11.92	4994	0.003289
p-Xylene	5.541	3220	0.002935
m-Xylene	6.28	3337	0.004065
o-Xylene	6.931	3520	0.004084

B = regression coefficient (K)

The coefficients used in the equation C.6 and the Henry's law constants for the BTEX compounds at 10°C are listed in Table C.5.

Using the equation C.5, a sample calculation for benzene ratio (gaseous concentration : aqueous concentration) is shown in equation C.7.

$$\begin{aligned}
\frac{C_{aq}}{C_g} &= \frac{R \times T}{H} \\
&= \frac{8.207 \times 10^{-5} atm.m^3/mol.K \times 283K}{0.003195\ atm.m^3/mol} \\
&= 7.2694 \\
\frac{C_g}{C_{aq}} &= \frac{1}{7.2694} \\
\frac{C_g}{C_{aq}} &= 0.1376
\end{aligned} \tag{C.7}$$

C.3 Biodegradation of BTEX Compounds

C.3.1 BTEX Mass Loss

Table 3.4 shows the percentage of BTEX mass loss (in aqueous phase) at the end of the experiment (day 64). Equation C.8 was used in order to calculate the mass of BTEX compounds.

$$\begin{aligned} Compound\ Mass &= Mass\ water + Mass\ air \\ Compound\ Mass &= C_{aq} \times V_{aq} + C_g \times V_g \end{aligned} \tag{C.8}$$

Where:

$\mathbf{C}_{aq}$ = equilibrium concentration in the aqueous phase (mg/L)

$\mathbf{V}_{aq}$ = liquid volume in the microcosm (0.1 L)

$\mathbf{C}_g$ = head-space gas concentration (mg/L)

$\mathbf{V}_g$ = head-space gas volume in the microcosm (1.050 L)

Sample calculation for benzene mass. The value calculated is posted in Table 3.4 under "Initial mass" column.

$$\begin{aligned} Benzene\ Mass &= C_{aq} \times V_{aq} + C_g \times V_g \\ &= 2.7 \times 0.1 + 0.1376 \times C_{aq} \times 1.050 \\ &= 0.27 \times 0.1376 \times 2.7 \times 1.050 \\ &= 0.65\ mg \end{aligned} \tag{C.9}$$

Where:

$\mathbf{C}_g$ = 0.1376 x C_{aq} (Equation C.7, shows sample calculation of C_g:C_{aq} ratio for benzene.)

C.3.2 Statistical Analysis for BTEX Mass Loss

Table C.6 and Table C.7 compare the raw data for BTEX measurements in days 1 and day 3, respectively. The numbers were converted to mass and shows the variability between the sub-samples in day 1 and in day 3. The measurements taken in day 3 were chosen over day 1 for the statistical analysis.

Table C.6: BTEX mass (mg) measured on day 1.

Sample	Benzene	Toluene	Ethylbenzene	*m*-Xylene	*p*-Xylene	*o*-Xylene
Ethanol 0.5%						
A1	0.65	0.60	0.30	0.29	0.38	0.28
A1	0.59	0.54	0.35	0.27	0.41	0.26
A2	0.66	0.63	0.36	0.32	0.28	0.29
A2	0.65	0.62	0.36	0.32	0.38	0.29
A3	0.68	0.61	0.34	0.31	0.40	0.28
A3	0.68	0.60	0.36	0.30	0.40	0.27
Ethanol 1.5%						
B1	0.97	0.88	0.36	0.39	0.41	0.42
B1	0.75	0.71	0.35	0.35	0.39	0.34
B2	0.67	0.59	0.33	0.29	0.34	0.26
B2	0.66	0.60	0.34	0.30	0.35	0.26
B3	0.63	0.63	0.33	0.32	0.37	0.28
B3	0.65	0.61	0.34	0.31	0.36	0.28
BTEX only						
D1	0.73	0.68	0.40	0.35	0.41	0.33
D1	0.71	0.66	0.40	0.34	0.41	0.32

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Sample	Benzene	Toluene	Ethylbenzene	m-Xylene	p-Xylene	o-Xylene
D2	0.62	0.58	0.35	0.31	0.36	0.30
D2	0.66	0.60	0.35	0.30	0.35	0.28
D3	0.84	0.69	0.40	0.31	0.34	0.31
D3	0.93	0.85	0.40	0.40	0.45	0.38
Sterile						
E1	0.70	0.64	0.38	0.33	0.38	0.30
E1	0.75	0.68	0.39	0.35	0.41	0.31
E2	0.77	0.60	0.30	0.26	0.28	0.24
E2	0.81	0.71	0.38	0.33	0.38	0.29
E3	0.76	0.70	0.39	0.35	0.40	0.31
E3	0.76	0.69	0.39	0.35	0.40	0.30
Nutrient-free						
F1	0.98	0.86	0.36	0.39	0.45	0.36
F1	0.86	0.91	0.46	0.48	0.54	0.44
F2	0.81	0.75	0.43	0.39	0.44	0.35
F2	0.74	0.68	0.45	0.35	0.40	0.31
F3	0.79	0.73	0.54	0.38	0.44	0.35
F3	0.69	0.63	0.43	0.32	0.37	0.29

Table C.7: BTEX mass (mg) measured on day 3.

Sample	Benzene	Toluene	Ethylbenzene	m-Xylene	p-Xylene	o-Xylene
Ethanol 0.5%						
A1	0.64	0.53	0.05	0.29	0.33	0.27

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Sample	Benzene	Toluene	Ethylbenzene	*m*-Xylene	*p*-Xylene	*o*-Xylene
A1	0.63	0.52	0.05	0.27	0.32	0.27
A2	0.66	0.59	0.17	0.31	0.35	0.28
A2	0.68	0.61	0.17	0.31	0.35	0.28
A3	0.63	0.57	0.25	0.29	0.33	0.26
A3	0.64	0.58	0.25	0.29	0.33	0.25
Ethanol 1.5%						
B1	0.68	0.62	0.35	0.31	0.36	0.27
B1	0.67	0.61	0.34	0.31	0.35	0.26
B2	0.67	0.61	0.33	0.30	0.35	0.26
B2	0.67	0.61	0.34	0.31	0.35	0.27
B3	0.66	0.60	0.32	0.30	0.34	0.26
B3	0.68	0.62	0.34	0.31	0.35	0.27
BTEX only						
D1	0.46	0.10	0.00	0.09	0.10	0.29
D1	0.44	0.09	0.00	0.09	0.11	0.28
D2	0.69	0.58	0.28	0.26	0.30	0.29
D2	0.56	0.48	0.24	0.23	0.26	0.25
D3	0.76	0.66	0.31	0.31	0.35	0.32
D3	0.74	0.65	0.31	0.32	0.36	0.32
Sterile						
E1	0.76	0.68	0.39	0.35	0.40	0.30
E1	0.74	0.67	0.39	0.35	0.39	0.30
E2	0.77	0.70	0.38	0.35	0.40	0.30

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Sample	Benzene	Toluene	Ethylbenzene	m-Xylene	p-Xylene	o-Xylene
E2	0.75	0.68	0.40	0.35	0.39	0.30
E3	0.75	0.67	0.39	0.34	0.39	0.29
E3	0.73	0.66	0.38	0.33	0.38	0.28
Nutrient-free						
F1	0.68	0.65	0.38	0.34	0.38	0.30
F1	0.71	0.65	0.38	0.35	0.39	0.31
F2	0.56	0.52	0.30	0.27	0.31	0.24
F2	0.71	0.64	0.36	0.32	0.37	0.28
F3	0.76	0.69	0.38	0.35	0.39	0.30
F3	0.66	0.62	0.35	0.31	0.35	0.27

The summary of ANOVA that were carried out to test the null hypothesis of no differences amongst microcosms for each BTEX compounds is displayed in Table C.8.

Table C.8: Summary of ANOVA for each BTEX compound.

BTEX compound	Mean Square Error	F-value	df	p-value
Benzene	8.306×10^{-4}	41.91	3, 6	< 0.0005
Toluene	9.278×10^{-4}	129.21	3, 6	< 0.0005
Ethylbenzene	2.431×10^{-3}	18.36	3, 6	0.002
m-Xylene	4.611×10^{-4}	16.11	3, 6	0.003
p-Xylene	3.778×10^{-4}	3.96	3, 6	0.071
o-Xylene	3.528×10^{-4}	2.19	3, 6	0.189

The complete results for the Post-Hoc test are displayed in Table C.9.

Table C.9: Test results for the hypothesis of zero decrease in BTEX mass from day 3 to day 64.

Variable	Mean	Standard Error	df	t-value	p-value
Microcosm: 0.5% Ethanol					
Benzene	0.285	0.20	6	14.25	3.7 x 10^{-6}
Toluene	0.535	0.22	6	24.32	1.6 x 10^{-7}
Ethylbenzene	0.110	0.035	6	3.14	0.010
m-Xylene	0.175	0.015	6	11.66	1.2 x 10^{-5}
p-Xylene	0.090	0.014	6	6.43	3.3 x 10^{-4}
o-Xylene	0.065	0.013	6	5.0	1.2 x 10^{-3}
Microcosm: 1.5% Ethanol					
Benzene	0.070	0.20	6	3.5	6.4 x 10^{-3}
Toluene	0.105	0.22	6	4.77	1.5 x 10^{-3}
Ethylbenzene	0.455	0.035	6	13.0	6.38 x 10^{-6}
m-Xylene	0.115	0.015	6	7.66	1.29 x 10^{-4}
p-Xylene	0.080	0.14	6	5.71	6.24 x 10^{-4}
o-Xylene	0.050	0.013	6	3.84	4.28 x 10^{-3}
Microcosm: Sterile					
Benzene	0.093	0.17	6	5.49	1.52 x 10^{-3}
Toluene	0.100	0.018	6	5.55	1.44 x 10^{-3}
Ethylbenzene	0.373	0.028	6	13.32	1.11 x 10^{-5}
m-Xylene	0.080	0.012	6	6.66	5.54 x 10^{-4}
p-Xylene	0.086	0.011	6	7.82	2.3 x 10^{-4}
o-Xylene	0.070	0.011	6	6.36	7.08 x 10^{-4}
Microcosm: Nutrient-free					
Benzene	-0.0066	0.017	6	-0.39	0.355

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Variable	Mean	Standard Error	df	t	p-value
Toluene	0.016	0.018	6	0.92	0.197
Ethylbenzene	0.303	0.028	6	10.82	1.84 x 10^{-5}
m-Xylene	0.043	0.012	6	3.58	5.82 x 10^{-3}
p-Xylene	0.040	0.011	6	3.63	5.48 x 10^{-3}
o-Xylene	0.033	0.011	6	3.0	0.012

In order to compare the mean differences between the treatments (microcosms), Tukey's multiple comparison was applied. Table C.10 shows Tukey's statistical analysis results for BTEX mass loss.

Table C.10: Tukey's multiple comparisons of BTEX mass loss between microcosms.

Variable	Mean Difference	Std. Error	p-value
Microcosms: 0.5% Ethanol x 1.5% Ethanol			
Benzene	0.2150	0.028	0.001
Toluene	0.4300	0.0304	0.000
Ethylbenzene	-0.3450	0.049	0.002
m-Xylene	0.06	0.021	0.110
p-Xylene	0.010	0.019	0.953
o-Xylene	0.015	0.019	0.853
Microcosms: 0.5% Ethanol x Sterile			
Benzene	0.1917	0.026	0.001
Toluene	0.4350	0.028	0.000
Ethylbenzene	-0.2633	0.045	0.004
m-Xylene	0.095	0.019	0.011
p-Xylene	0.0033	0.018	0.997

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Variable	Mean Difference	Std. Error	p-value
o-Xylene	-0.005	0.017	0.990
Microcosms: 0.5% Ethanol x Nutrient-free			
Benzene	0.2917	0.026	0.000
Toluene	0.5183	0.028	0.000
Ethylbenzene	-0.1933	0.045	0.020
m-Xylene	0.1317	0.019	0.002
p-Xylene	0.050	0.018	0.107
o-Xylene	0.032	0.017	0.339
Microcosms: 1.5% Ethanol x Sterile			
Benzene	-0.023	0.026	0.812
Toluene	0.005	0.028	0.998
Ethylbenzene	0.082	0.045	0.352
m-Xylene	0.035	0.019	0.364
p-Xylene	-0.0066	0.018	0.980
o-Xylene	-0.020	0.017	0.667

Table C.11: Aqueous concentration of BTEX compounds and ethanol in each replicate of the 0.5% ethanol-amended microcosms.

Sample	Ethanol mg/L	Benzene mg/L	Toluene mg/L	Ethylbenzene mg/L	*m*-Xylene mg/L	*p*-Xylene mg/L	*o*-Xylene mg/L
replicate 1							
day 23	3,079.5	1.8	0.4	0	0.4	1.1	0.8
day 38	2,975.6	1.7	0.2	0	0.3	1.1	0.8
replicate 2[a]							
day 23	3,017.7	2.2	1.4	0	0.6	1.1	0.7
day 38	2,804.9	0	0	0	0.4	0	0.7
replicate 3							
day 23	3,200.7	2.0	0.6	0	0.6	1.2	0.8
day 38	3,032.5	1.9	0.3	0	0.6	1.2	0.8

[a]Five mL of MBH medium were added to the replicate 2 bottle on day 18.

C.4 Effect of Extra Nutrient Addition

The addition of extra MBH medium (5 mL) on day 18 of the experiment had an effect on BTEX and ethanol degradation. Tables C.11 and C.12 show a comparison in aqueous concentration between bottles that received extra MBH medium during the experiment and those that did not.

Table C.12: Aqueous concentration of BTEX compounds and ethanol in each replicate of the 1.5% ethanol-amended microcosms.

Sample	Ethanol mg/L	Benzene mg/L	Toluene mg/L	Ethylbenzene mg/L	*m*-Xylene mg/L	*p*-Xylene mg/L	*o*-Xylene mg/L
replicate 1							
day 23	10,600.5	2.4	1.8	0.5	0.6	1.1	0.7
day 38	10,090.8	2.5	1.9	0.5	0.6	1.2	0.8
replicate 2[a]							
day 23	10,115.2	2.3	1.8	0.6	0.6	1.1	0.7
day 38	10,009.2	2.5	1.9	0.6	0.7	1.2	0.8
replicate 3							
day 23	10,850.3	2.5	1.9	0.8	0.7	1.2	0.7
day 38	10,664.8	2.6	2.0	0.8	0.8	1.2	0.8

[a] Five mL of MBH medium were added to the replicate 2 bottle on day 18.

C.5 Aqueous Ethanol Analysis

Ethanol was measured in the aqueous phase. In order to calculate the concentration of ethanol in the sample, a standard curve was run before sampling. Figure C.1 shows the standard curve for aqueous ethanol.

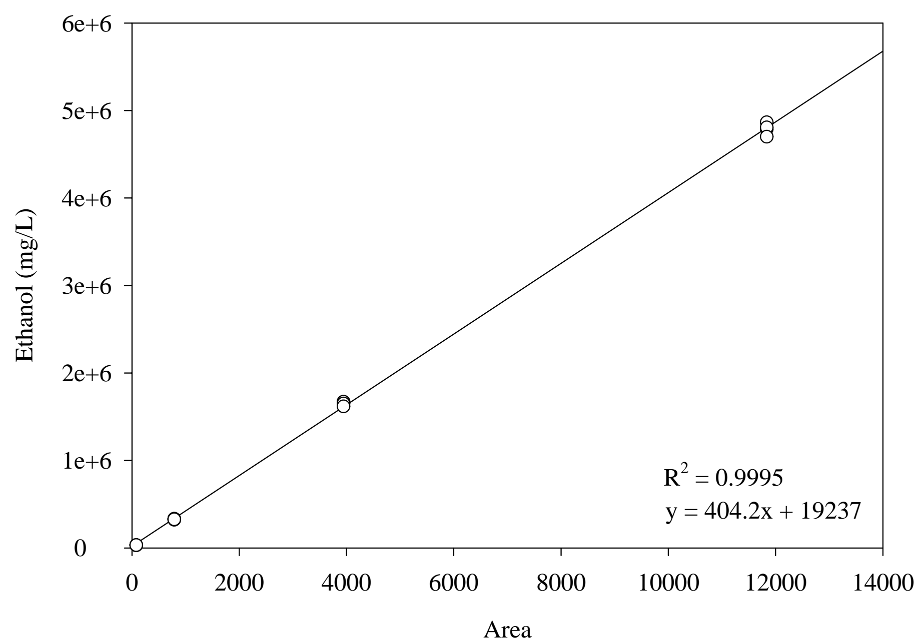


Figure C.1: Standard curve for aqueous ethanol measurements

Raw data for the standard curve (Figure C.1) is listed in the Table C.13 below:

Table C.13: Raw data for aqueous ethanol standard curve

Ethanol Concentration (% v/v)	Ethanol Concentration (mg/mL)	Area
1.5	11,835	4788000
1.5	11,835	4866000
1.5	11,835	4807000
1.5	11,835	4701000
0.5	3,945	1671000
0.5	3,945	1672000
0.5	3,945	1652000
0.5	3,945	1619000
0.1	789	334300
0.1	789	323600
0.1	789	330400
0.1	789	328000
0.001	78.9	31750
0.001	78.9	32690
0.001	78.9	33730
0.001	78.9	33960

Statistical analysis for the aqueous ethanol standard curve (Figure C.1) is listed in Table C.14:

Table C.14: Regression analysis

Regression with computed y-intercept	
Constant	19237.268
Standard Error of Y Est	41794.116
R Squared	0.9995
No. of Observations	16
Degrees of Freedom	14
X Coefficient	404.204
Standard Error Coefficient	2.240
Equation for Regression through zero	Area = 404.2 x + 19237.27

C.5.1 Statistical Analysis for Ethanol Loss

The summary of ANOVA's that was carried out to test the null hypothesis of no differences amongst microcosms for ethanol is displayed in Table C.15.

Table C.15: Summary of ANOVA for ethanol.

Compound	Mean Square Error	F-value	df	p-value
Ethanol	81046.79	14.24	4, 7	0.002

Results for the Post-Hoc test for ethanol are displayed in Table C.16.

Table C.16: Test results for the hypothesis of zero decrease in ethanol concentration from day 1 to day 64.

Variable	Mean	Standard Error	df	t-value	p-value
0.5% Ethanol	944.8	201.3	7	4.69	0.0011
1.5% Ethanol	816.6	201.3	7	4.06	0.0024
Ethanol only	1478.5	201.3	7	7.34	7.86×10^{-5}
Sterile	-134.6	164.4	7	-0.82	0.44
Nutrient-free	-46.7	164.4	7	-0.28	0.394

In order to compare the mean differences between the treatments (microcosms), the Tukey's multiple comparison was applied. Table C.17 shows the results for the Tukey's statistical analysis.

Table C.17: Tukey's multiple comparisons for ethanol.

Microcosms	Mean Difference	Std. Error	p-value
0.5% Ethanol x 1.5% Ethanol	128.2	284.7	0.990
0.5% Ethanol x Sterile	1079.4	259.9	0.025
0.5% Ethanol x Ethanol only	-533.6	284.7	0.407
0.5% Ethanol x Nutrient-free	991.5	259.9	0.037
1.5% Ethanol x Sterile	951.2	259.9	0.045
Ethanol only x Sterile	1613.1	259.9	0.003

C.6 Dissolved Oxygen Data

C.6.1 Oxygen Demand Calculations

Total oxygen available in each microcosm was calculated following the equation C.10 below.

For 0.5% ethanol microcosms

With a 1000 ml oxygen head-space in the microcosm (1000 ml x 0.2 = 200 ml of oxygen).

$$PV = nRT, \quad (Standard\ Gas\ Law) \tag{C.10}$$

then,

$$\begin{aligned} n &= \frac{PV}{RT} \\ n &= \frac{1\ atm \times 0.2\ L}{0.0821\ l\ atm.\ mole^{-1}\ K^{-1} \times 283\ K} \\ n &= 0.008923\ mole \\ mass &= 0.008923\ mole \times 32\ g/mole \\ mass &= 285.5\ mg \end{aligned} \tag{C.11}$$

There is 285.5 mg of oxygen in the microcosm head-space.

Dissolved oxygen from the water in the microcosm would contribute another 0.7 mg of oxygen per 1000 mL. There was 100 mL of ground water in the bottle. Hence, dissolved oxygen from the water would contribute 0.07 mg.

Total oxygen available in the microcosm: 275.5 + 0.07 = 285.6 mg.

The reaction of ethanol mineralization shows how many moles of oxygen is required to completely mineralize 1 mole of ethanol.

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

One mole of ethanol requires 3 moles of oxygen to mineralize completely.

1 mole of ethanol = 46 g

requires

3 moles of oxygen = 96 g

Hence, 1 mg of ethanol requires approximately 2.086 mg of oxygen to degrade to carbon dioxide and water.

0.5% ethanol in microcosm (0.5 mL × 0.789 density) corresponds to 0.3945 g or 395 mg therefore it requires:

395 × 2.086 = 823 mg of oxygen to completely degrade all ethanol.

There is only 285.6 mg of oxygen in the microcosm.

For 1.5% ethanol microcosms

With a 1000 mL pure oxygen head-space in the microcosm

$$PV = nRT, \quad (Standard\ Gas\ Law)$$

then,

$$\begin{aligned} n &= \frac{PV}{RT} \\ n &= \frac{1\ atm \times 1.0\ L}{0.0821\ l\ atm.\ mole^{-1}\ K^{-1} \times 283\ K} \\ n &= 0.04461 mole \end{aligned} \tag{C.12}$$

$$mass = 0.04461 \times 32\ g/mole$$

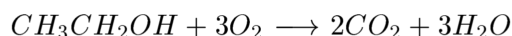
$$mass = 1427.7\ mg$$

There is 1427.7 mg of oxygen in the microcosm head-space.

Dissolved oxygen from the water in the microcosm would contribute another 0.7 mg of oxygen per 1000 mL. There was 100 mL of ground water in the bottle. Hence, dissolved oxygen from the water would contribute 0.07 mg.

Total oxygen available in the microcosm: 1427.7 + 0.07 = 1427.8 mg.

The reaction of ethanol mineralization shows how many moles of oxygen is required to completely mineralize 1 mole of ethanol.



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

One mole of ethanol requires 3 moles of oxygen to mineralize completely.

1 mole of ethanol = 46 g

requires

3 moles of oxygen = 96 g

Hence, 1 mg of ethanol requires approximately 2.086 mg of oxygen to degrade to carbon dioxide and water.

1.5% ethanol in microcosm (1.5 mL × 0.789 density) corresponds to 1.1835 g or 1183.5 mg

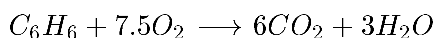
therefore it requires:

1183.5 × 2.086 = 2468.78 mg of oxygen to completely degrade all ethanol.

There is only 1427.8 mg of oxygen in the microcosm.

For BTEX compounds

The reaction of benzene mineralization shows how many moles of oxygen is required to completely mineralize 1 mole of benzene.



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

One mole of benzene (used as a representative for BTEX only microcosm) requires 7.5 moles of oxygen

1 mg of BTEX requires approximately 3 mg of oxygen for complete degradation

Total BTEX addition to the microcosm was 3.04 mg (Table 2.2, in Material and Methods Section).

Table C.18: Percentage of dissolved oxygen consumed.

Microcosm	Initial concentration mg/L	Final concentration mg/L	% decrease
0.5% Ethanol[a]	7.8	5.1	35.1
0.5% Ethanol (extra MBH)[b]	7.8	3.7	47.8
1.5% Ethanol[a]	45.4	44.4	2.2
1.5% Ethanol (extra MBH)[b]	40.5	43.7	-
Ethanol only[a]	8.3	7.3	11.7
Ethanol only (extra MBH)[b]	8.1	2.1	74.0
BTEX only[c]	10.3	10.9	-
Nutrient-free[c]	10.2	10.0	1.7
Sterile[c]	10.5	11.4	-

[a] Values are mean data from two replicates.

[b] Values are results from one replicate. A single bottle of the original three replicates received extra MBH medium.

[c] Values are mean of three replicates.

therefore

BTEX would require 22.85 mg of oxygen to degrade completely.

C.6.2 Dissolved Oxygen Measurement

Dissolved oxygen was measured during the experiment. The measures were done up to day 38. BTEX only microcosms had its D.O. measured until day 23, when all BTEX compounds were degraded.

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