Enhancement of BTEX biodegradation in subsurface environments.

Nataliya Kordonska
University of Windsor

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ENHANCEMENT OF BTEX BIODEGRADATION IN SUBSURFACE ENVIRONMENTS

by

Nataliya Kordonska

A Thesis
Submitted to the Faculty of Graduate Studies and Research through the Department of Civil and Environmental Engineering in Partial Fulfillment of Requirements for the Degree of Master of Applied Science at the University of Windsor

Windsor, Ontario, Canada

1997

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ABSTRACT

Massive consumption of petroleum and petroleum products in the world frequently leads to soil contamination by fuel components. The compounds of main concern are: benzene, toluene, ethylbenzene, and xylene, which are highly toxic. The bioremediation technology depends on the alteration of physical and chemical conditions in the subsurface environment to optimize microbial activity.

In this study, several techniques of soil remediation enhancement were investigated. BTEX- degraders were selected from the wastewater treatment sludge biota and used as inoculum later. The acclimation was performed on the granular activated carbon (GAC) columns supplied with aromatic hydrocarbons as the only source of carbon and energy. Enhancement of BTEX biodegradation in soil by addition of inoculum, nutrients, and oxygen was investigated in the batch experiments. Controls were run in parallel to account for abiotic losses. The study showed that all aromatic hydrocarbons were degraded under natural conditions by approximately 95% in a 45-day period in all experiments with BTEX concentration of 10 mg/L. The BTEX utilization rates were calculated and compared for different conditions. Supplemental nutrients improved the rate of biodegradation. Addition of hydrogen peroxide together with nutrients increased the utilization rates by 50%. Adding nutrients together with microorganisms increased the rates by more than 100% for TEX. Optimum conditions were created for biodegradation of all BTEX compounds when supplemental oxygen, nutrients and inoculum were added. The rate constants of utilization were increased more than twice. Addition of nitrate showed to be very effective in enhancing degradation rates of TEX. The degraders of these compounds were able to switch to the anaerobic pathway by using nitrate as electron acceptor when oxygen was depleted. Continuous soil reactor was used to compare the biodegradation abilities of soil indigenous microorganisms and preselected inoculum. It has been shown that addition of inoculum allows to start the onset of measurable biodegradation in a shorter time. After a certain time indigenous population develops the necessary catabolic abilities and grows to the certain density when the difference between indigenous and preselected biomass degradation abilities is no longer observed.
ACKNOWLEDGEMENTS

The author is greatly thankful to advisors Dr. N. Biswas and Dr. J.K. Bewtra for their guidance in development and completion of this work.

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LIST OF ABBREVIATIONS

BTEX = Benzene, Toluene, Ethylbenzene, Xylene
GAC = Granular Activated Carbon
GC = Gas Chromatography
MDL = Method Detection Limit
Chapter I

INTRODUCTION

1.1. Source and Scope of the Problem

The consumption of petroleum and petroleum products in the world is staggering. In 1989, the worldwide usage of petroleum was $2.76 \times 10^9$ US gallons per day, or $1 \times 10^{12}$ US gallons per year (Energy Information Administration, 1991 as discussed by Prince, 1992). Unfortunately frequently use, production and transportation of petroleum results in accidental discharges into the environment. These spills contaminate aquifers, soils and groundwater.

Massive releases, such as those following tanker accidents, get the most public attention, but in fact these probably represent only a small proportion of the total release into the environment (National Research Council, 1985 as discussed by Prince, 1992). While such spills have serious environmental impacts on their immediate surroundings, more people are likely to be affected by individual smaller spills such as those from leaking underground storage tanks (Prince, 1992).

There are several sources of petroleum contamination of soils and groundwater (La Rue, 1993):

- leaking underground storage tanks;
- petroleum pipelines breaks;
- spills of petroleum products;
- leaking aboveground tanks;
- leaks from petroleum refineries and bulk storage facilities;
• refinery residues;
• coal tar sites;
• chemical processing sites;
• wood treating sites; and
• production, transportation and refining of crude oil.

Petroleum contaminated sites need to be remediated because of the number of potential risks associated with this contamination. The most obvious hazard of hydrocarbons penetration to the subsurface environment is the possibility of contaminating groundwater. This can make groundwater unsuitable for consumption. One gallon of petroleum can render one million gallons of water unsafe for users. As discussed by Hills (1989), there are several potential risks associated with soil petroleum contamination. One of the reasons is the possibility of recontamination even after groundwater was remediated. This may happen if unsaturated soil has significant petroleum content. That is why not only does the water need to be treated if polluted with fuel, but the whole site may require a clean up. Also, significant concentrations of petroleum by-products can permeate PVC water lines and contaminate drinking water supplies.

Another potential risk is the possibility of explosion and fire if volatile components migrate and accumulate in the basements, sewer lines, or underground utility vaults.

Important constituents of petroleum and other hydrocarbon fuels are: benzene, toluene, ethylbenzene, xylene (BTEX) compounds. These substances are of main concern since they are fairly soluble in water and therefore frequently can penetrate groundwater. Also, BTEX compounds are considered to be toxic and carcinogenic (EPA, 1981 as discussed by Corseuil, 1992). Since a large number of people depend on groundwater as their supply of drinking water, the potential threat to public health is well understood.
1.2. Treatment Alternatives

Growing awareness of contamination consequences together with potential due-diligence prevention makes remediation of contaminated sites a fast-growing business. Many treatment alternatives have been proposed as capable of solving groundwater and soil contamination. Remediation strategies include (La Rue, 1993):

- chemical fixation and washing;
- enhanced biodegradation;
- soil venting (vapour extraction);
- thermal treatment (low and high temperatures);
- surface bioremediation (land - farming);
- solidification (stabilization);
- asphalt incorporation;
- soil leaching;
- solvent extraction;
- slurry phase bioremediation; and
- landfill disposal.

Excavation and shipment of contaminated soils to hazardous waste sites are increasingly being viewed as merely moving, not solving the problem (King et al. 1992). Enhanced bioremediation is one of the attractive options because it has the potential to completely destroy the contaminant compounds and its cost is lower than for physical - chemical treatment processes. A lot of research work has been performed in order to identify and understand the mechanism of biological transformation of BTEX in subsurface
environment as well as methods to optimize and enhance it. Enhanced biodegradation of these compounds using supplemental electron acceptor was shown under aerobic (Anid et al. 1993; Bianchi-Moscuera et al. 1994; Chiang et al. 1989; Corseuil and Weber, 1994), and anaerobic (Alvares and Vogel, 1991; Chaudhuri et al. 1995; Lovley et al. 1994; Haag et al. 1991) conditions. Positive effect of immobilization and adaptation was studied by Corseuil (1992), Li et al. (1994), Medina et al. (1995), Voice et al. (1992), and Weber and Corseuil (1994). Addition nutrients was investigated by Kao (1993), McCabe (1993), Wrenn et al. (1994), and Zhou and Crawford (1995). However, not many of these studies tried to combine these methods of biodegradation enhancement and to compare the effects of these methods on the biodegradation rate and extent. This research attempts to investigate different methods of biodegradation augmentation and make comparison of these methods.

1.3. Research Objectives

The specific objectives of this research were to:

- investigate the possibility of selecting the microorganisms that are able to degrade BTEX compounds while utilizing them as the source of carbon and energy for their metabolism;
- determine the effect of supplemental nutrients addition on the rate and extent of BTEX biodegradation in soil;
- determine the effect of supplemental source of electron acceptor on the rate and extent of biodegradation in soil;
- determine the effect of inoculation of soil with preselected microorganisms on
the rate and extent of biodegradation;

- determine the effect of combination of several methods of enhancement of the rate and extent of biodegradation;

- compare different BTEX biodegradation enhancement methods;

- determine the possibility and extent of anaerobic BTEX utilization using nitrate as a source of electron acceptor; and

- compare biodegradation rates and extent by indigenous and pre-selected microorganisms using different BTEX concentrations.

1.4. Scope of Work

The scope of this study included:

- adaptation of microorganisms capable of degrading the BTEX compounds using continuous granular activated carbon reactor and nutrient solution containing 10 mg/L total BTEX;

- conducting a series of batch tests by using soil and different biodegradation conditions with total initial BTEX concentration of 10 mg/L;

- to determine degradation of BTEX in soil under natural environment without addition of microorganisms, oxygen, and nutrients;

- to determine enhancement of biodegradation rate of BTEX using selected inoculum;

- to determine enhancement of biodegradation rate of BTEX using additional nutrients;

- to determine enhancement of biodegradation rate of BTEX using
supplemental electron acceptor source (H₂O₂);

- to determine enhancement of biodegradation rate of BTEX using a
  combination of selected inoculum and nutrients;

- to determine enhancement of biodegradation rate of BTEX using a
  combination of selected inoculum and supplemental electron acceptor;

- to determine enhancement of biodegradation rate of BTEX using
  supplemental nutrients and electron acceptor;

- biodegradation of BTEX in soil in concentrations of 20, 30, 50, 100,
  200 mg/L in batch tests enhanced by addition of inoculum, nutrients, and
  electron acceptor; and

- biodegradation of BTEX in soil continuous reactor by preselected and
  indigenous microorganisms with total BTEX concentrations of 10, 30, 40,
  70 mg/L.
Chapter II

LITERATURE REVIEW

2.1. Behaviour of Hydrocarbons in Soil

Development of the remediation technique depends largely on the understanding of the movement of leaking contaminant in the soil. Knowledge of contaminant behaviour and reasons behind it allows to develop strategies to induce or prohibit this behaviour in order to optimize the cleaning up process.

Released products can be retained in the soil for long periods of time, causing potential groundwater and lower atmosphere contamination. Research conducted by the Environmental Protection Agency (EPA) and discussed by Lyman, et al. (1992) has shown that a substance leaking from underground storage tanks will be present in and be transient between one or more of 13 identified locations in the subsurface environment. Each of these 13 loci represents a point and physical state of the leaked contaminant. Table 2.1.1 presents the list of these locations. Fate and transport processes for each of these locations are controlled by several physiochemical processes, as well as biodegradation and biosorption processes. Lyman et al. (1992) reports that general processes such as: mobilization, transformation and immobilization of hydrocarbons, can be predicted according to the partitioning and transformation processes between solid, liquid and gas phases, as well as bulk transport. Biodegradation and sorption on biomass play a considerable role in degradation of contaminants in soil. Dissolution, sorption, volatilization, bulk transport, and chemical oxidation are the abiotic processes governing the
<table>
<thead>
<tr>
<th>No</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Contaminant vapour as a component of soil gas in the unsaturated zone</td>
</tr>
<tr>
<td>2</td>
<td>Liquid contaminants adhering to &quot;water-dry&quot; soil particles in the unsaturated zone</td>
</tr>
<tr>
<td>3</td>
<td>Contaminants dissolved in the water film surrounding soil particles in the unsaturated zone</td>
</tr>
<tr>
<td>4</td>
<td>Contaminants sorbed to &quot;water-wet&quot; soil particles or rock surface (after migrating through water) in either the unsaturated or saturated zone</td>
</tr>
<tr>
<td>5</td>
<td>Liquid contaminants in the pore spaces between soil particles in the saturated zone</td>
</tr>
<tr>
<td>6</td>
<td>Liquid contaminants in the pore spaces between soil particles in the unsaturated zone</td>
</tr>
<tr>
<td>7</td>
<td>Liquid contaminants floating on the groundwater table</td>
</tr>
<tr>
<td>8</td>
<td>Contaminants dissolved in groundwater (i.e., water in the saturated zone)</td>
</tr>
<tr>
<td>9</td>
<td>Contaminants sorbed onto colloidal particles in water in either the unsaturated or saturated zone</td>
</tr>
<tr>
<td>10</td>
<td>Contaminants that have diffused into mineral grains or rocks in either the unsaturated or saturated zone</td>
</tr>
<tr>
<td>11</td>
<td>Contaminants sorbed onto or into soil microbiota in either the unsaturated or saturated zone</td>
</tr>
<tr>
<td>12</td>
<td>Contaminants dissolved in the mobile pore water of the unsaturated zone</td>
</tr>
<tr>
<td>13</td>
<td>Liquid contaminants in rock fractures in either the unsaturated or saturated zone</td>
</tr>
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*Source: Lyman et al. (1992)*
fate of contaminants in the subsurface environment (Lyman et al. 1992).

2.2. Background of Hydrocarbon Biodegradation

Biodegradation of naturally occurring organic compounds follows their synthesis. However, some man-made compounds may not be recognized by natural microbial population, and therefore can not be utilized via their metabolism (Rozgai, 1994). Those compounds found to be toxic to living organisms and released to the environment as a result of a human activity and in the amounts considerably exceeding the natural levels of the same or similar chemical are termed xenobiotics (Muller, 1992). Xenobiotics are refractory to microbial degradation or are degraded very slowly. As discussed by Rozgai (1994), physical and chemical characteristics of compounds as well as environmental factors may influence their biodegradability. Some xenobiotics may be transformed only when other compounds are present (Liou, 1995). Many compounds can be degraded only through a series of biotransformations performed by different microorganisms. The main natural degraders are microorganisms, mostly bacteria and some fungi. These organisms have rapid rates of multiplication and great metabolic potential. They are able to adapt to a new substrate and use xenobiotics as a source of carbon and energy. Selection of microorganisms, able to degrade certain chemicals and their successive adaptation to a naturally persistent compound, can be a solution to many environmental problems (Rozgai, 1994).

The biodegradation of oil and refined products is catalyzed by microorganisms, especially bacteria. The study of microbial oil degradation has been an important area of microbiology for at least 50 years (Prince, 1992). As discussed further by Prince (1992), non-photosynthetic microorganisms are dependent on energy-yielding oxidation reaction for their
source of energy. The amount of energy released depends on the source of carbon and type of compound used as an electron acceptor.

Bacteria degrading petroleum hydrocarbons use them as a source of carbon and energy. There are three pathways by which bacteria can utilize their metabolism: aerobic, anaerobic and fermentative respiration (Prince, 1992). Degradation of aromatic hydrocarbons is known to be performed via aerobic (using oxygen as the final electron acceptor) and anaerobic (using NO$_3^-$, SO$_4^{2-}$, CO$_3^{2-}$ as the final electron acceptor) pathways. As discussed by Corseuil (1992), bacteria are the most abundant microbial group in the soil environment, that is capable of utilizing aromatic hydrocarbons for growth. These microorganisms have a broad metabolic diversity and are considered to have an important role in the degradation of aromatic hydrocarbons released to the environment. The genera mostly isolated from contaminated sites are: *Pseudomonas*, *Arthobacter*, *Corynebacterium*, *Flavobacterium*, *Nocordia*, *Achromobacter*, *Micrococcus and Mycobacterium* (Corseuil, 1992).

Biodegradation of benzene, toluene and xylene under aerobic conditions has been extensively studied and the mechanism of it is described in literature (Gibbson and Subramanian, 1984; Muller, 1992,). Aerobic bacteria initiate the oxidation of aromatic hydrocarbons by incorporating two atoms of molecular oxygen into the aromatic ring to form a dihydrodiol with a cis-configuration. This dihydrodiol is then dehydrogenated to yield catechol. Further oxidation of cis-hydrodiols leads to the formation of catechol which is a substrate for enzymes that catalyze the fission of the aromatic nucleus via either the ortho- or the meta-pathways (Gibson and Subramanian, 1984). Cleavage between two hydroxy groups (ortho-) produces cis,cis-muconic acid. Meta-cleavage forms 2-hydroxymuconic semialdehyde (Figure 2.2.1). For toluene, ethylbenzene, and xylene two general ways of degradation are reported
(Figure 2.2.2). In one pathway the methyl group is oxidized stepwise to yield a carboxylic group. This group is then removed in the attack of dioxygenase, which introduces two hydroxy groups at the same time to form dihydrodiol, and then catechol. In the second pathway, the aromatic ring is attacked directly, and the degradation pathway is similar to the one for benzene (Muller, 1992).

Oil biodegradation is principally an aerobic process, but anaerobic degradation is also possible. Bertrand et al. (1989) reported that anaerobic hydrocarbon metabolism had been demonstrated under fermentative and denitrifying conditions. A number of researchers studied anaerobic degradation of BTEX, and mostly agreed that toluene, ethylbenzene and xylene were anaerobically biodegradable, but benzene remained recalcitrant (Hutchins, et al. 1991; Alvares and Vogel, 1991, Anid, et al. 1993).

Under aerobic conditions, aerobes or facultative anaerobes can utilize aromatic hydrocarbons as carbon and energy source. In this process cosubstrate of oxygenase enzymes is used for the initial attack of the aromatic ring and oxygen as the terminal electron acceptor for aerobic respiration (Su, 1994). However, some facultative or obligate anaerobes can degrade these hydrocarbons by using alternate electron acceptors, such as nitrate, iron, sulphates, or carbon dioxide for anaerobic respiration (Alvares and Vogel, 1991; Chaudhuri et al. 1995; Lovley et al. 1994; Haag et al.1991). Nitrate has been reported to be the most readily available electron acceptor for utilization under anaerobic or oxygen - limited conditions (Corseuil, 1992).

Biodegradation rate models for organic contaminants are usually based on Monod kinetics. In a batch reactor, substrate utilization rate is:
- \frac{dS}{dt} = \frac{kXS}{(Ks+S)},

where:

\( S \) = substrate concentration, mg/L;

\( t \) = time, days;

\( k \) = maximum specific substrate utilization rate,

\( \text{mg-substrate (mg-cells)}^{-1}\text{day}^{-1} \);

\( X \) = microbial concentration, mg/L; and

\( Ks \) = half-velocity coefficient, mg/L.

As discussed by Corseuil (1992), a wide range of Monod coefficients (k) for BTEX compounds have been reported in the literature. Values range from 0.004 to 4.7 for the maximum specific substrate utilization rate (k) and from 0.034mg/L to 17mg/L for the half-saturation coefficient (Ks). This can be explained by the difference in the types of microorganisms used and methods applied for estimation of these coefficients.

2.3. Factors Affecting Biodegradation

2.3.1. Role of Electron Acceptors

Electron acceptors are molecules such as \( O_2 \) and \( NO_3^- \) which transfer energy in the form of electrons during the metabolic process. As discussed by Corseuil (1992), lack of electron acceptors is one of the main limitations of natural hydrocarbon biodegradation. BTEX degradation is mostly an aerobic process, that is why insufficient amount of oxygen in subsurface environment is often the reason of aromatic hydrocarbons persistence in soil and groundwater. As reported by Corseuil (1992), a fairly large amount of oxygen is required to completely mineralize BTEX compounds. The oxidation reactions for benzene, toluene and
The reactions that occur without cell growth are:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reaction</th>
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<tbody>
<tr>
<td>benzene:</td>
<td>( \text{C}_6\text{H}_6 + 7.5 \text{O}_2 \rightarrow 6 \text{CO}_2 + 3 \text{H}_2\text{O} )</td>
</tr>
<tr>
<td>toluene:</td>
<td>( \text{C}_7\text{H}_8 + 9 \text{O}_2 \rightarrow 7 \text{CO}_2 + 4 \text{H}_2\text{O} )</td>
</tr>
<tr>
<td>ethylbenzene:</td>
<td>( \text{C}<em>8\text{H}</em>{10} + 10.5 \text{O}_2 \rightarrow 8 \text{CO}_2 + 5 \text{H}_2\text{O} )</td>
</tr>
<tr>
<td>xylene:</td>
<td>( \text{C}<em>8\text{H}</em>{10} + 10.5 \text{O}_2 \rightarrow 8 \text{CO}_2 + 5 \text{H}_2\text{O} )</td>
</tr>
</tbody>
</table>

Thus, for benzene, toluene, ethylbenzene and xylene, 3.08, 3.13, 3.5 and 3.5 mg of oxygen/mg of compound are required, respectively, if cell growth is not taken into consideration. That is why oxygen deficiency becomes one of the main limiting factors for in-situ biodegradation of hydrocarbons (Corseuil, 1992).

A number of research works confirmed the effect of oxygen concentration on the aerobic biodegradation of benzene, toluene and xylene. Chiang (1989) studied this process by sampling hydrocarbons and dissolved oxygen (DO) in a shallow aquifer beneath the field site at 42 monitoring wells. Material balance and nonlinear least-squares analyses were performed to evaluate the results. The results from 10 sampling periods over three years showed that the concentration of benzene in groundwater was significantly reduced over this time. The natural attenuation rate was calculated to be 0.95% per day. After oxygen had been depleted, the anaerobic conditions were established, and rate of BTX degradation decreased. In the research by Morgan et al. (1993), rate and extent of biodegradation of BTEX in groundwater were studied in samples from a contaminated site which contained total BTEX concentrations of up to 20 mg/L. Since elevation of incubation temperature, supply of organic nutrients, or addition of inorganic fertilizers did not significantly increase the rate or extent of biodegradation the
conclusion was made that the limiting factor of BTEX degradation was oxygen deficiency.

Several studies have proven the positive effect of supplemental oxygen supply on the BTEX biodegradation (Lodaya et al. 1991; Bianchini-Mosquera et al. 1994, Lovley et al. 1994). Stimulation of biodegradation with additional oxygen can be achieved using hydrogen peroxide. The physical properties of $\text{H}_2\text{O}_2$ allow it to be easily mixed with water in whatever concentrations desired. The reaction, producing oxygen is:

$$\text{H}_2\text{O}_2 \rightarrow \frac{1}{2} \text{O}_2 + \text{H}_2\text{O}$$

Lodaya et al. (1991) studied aerobic biodegradation of BTX using activated sludge immobilized in calcium alginate gel. Hydrogen peroxide was used as a source of dissolved oxygen. In this study, 60% of the benzene was degraded by immobilized biomass in batch tests after 24 hours using 100 mg/L as initial concentrations. The contact time of 17.4 hours was required to biologically reduce benzene concentrations from 600 mg/L to 1 mg/L during continuous operation. Although hydrogen peroxide can be toxic to microorganisms at high concentrations, Fiorenze (1992) has shown that subsurface microbes could adapt to increasing concentrations of it, if a carbon source was available. Bianchini-Mosquera et al. (1994) proposed to use a solid peroxide formulation as oxygen-releasing compound. This technique allowed to stimulate aerobic degradation, which resulted in decreasing benzene and toluene concentrations.

Several researches have shown stimulation of anoxic biodegradation using different electron acceptors. Lovley et al. (1994) has studied Fe (III) ligands as alternative potential oxidants. Insoluble Fe (III) oxides which are generally abundant in shallow aquifers can be
potential oxidants, but under natural conditions these are difficult for microorganisms to access. The above mentioned research reports that adding organic ligands binding to ferric ion considerably increased the bioavailability of electron acceptor. This resulted in enhancement of biodegradation rates of anaerobic hydrocarbon transformation. The rates reported were comparable to those under aerobic conditions. It has been found that even benzene, which is refractory in the absence of oxygen, can be rapidly degraded when Fe (III) oxides are available as electron acceptor. Another potential electron acceptor in anoxic conditions could be sulphate. Haag et al. (1991) reported that naturally present sulphate stimulated oxidation of toluene in anaerobic conditions.

Nitrate is thermodynamically the best readily available electron acceptor after oxygen, which has been investigated and reported to be effective as an energy source for degradation of some aromatic hydrocarbons. Its use for subsurface remediation is attractive because it is less expensive and more water soluble than oxygen. Another source of supplemental oxygen in subsurface can be hydrogen peroxide, but it has been reported to be toxic at high concentrations. Besides, as discussed by Anid (1993), bacterial decomposition of hydrogen peroxide can prevent oxygen transportation to the contaminated areas. Anid (1993) tested the ability of indigenous microorganisms to degrade benzene, toluene, ethylbenzene, xylene in flow-through aquifer columns separately with hydrogen peroxide (110 mg/L) and nitrate (330 mg/L as NO₃⁻). Electron acceptors were added to air-saturated influent nutrient solution. Sterile columns were used as controls. BTEX compounds were removed more from the columns amended with supplemental source of electron acceptor than from sterile controls. This provided evidence for biodegradation. In the presence of H₂O₂, indigenous microorganisms degraded benzene and toluene (95%), meta- plus para- xylene (80%) and
ortho-xylene (70%). Removal of 90% of toluene and 25% of ortho-xylene was the result of a nitrate addition. Benzene, toluene, meta- and para-xylene concentrations were not significantly reduced after 42 days of operation under denitrifying conditions.

Alvares et al. (1991) studied addition of nitrate for enhancement of BTEX biodegradation. It has been reported in this research that addition of nitrate can aid in bioremediation of contaminated sites by increasing the electron acceptor pool. Addition of nitrate may reduce the total biochemical oxygen demand (BOD) of the system. This may result in less competition between benzene and other substrate for molecular oxygen, which would permit a greater extent of aerobic benzene biotransformation. As discussed further by these researchers, denitrifiers help to complete the biodegradation sequence initiated under oxic conditions by oxidizing metabolites of incomplete aerobic BTEX degradation. However, it has been shown in a number of studies that indigenous microorganisms are capable of degrading alkylbenzenes, but not benzene under denitrifying conditions (Anid et al. 1992; Hutchins, et al. 1991). In the research of Hutchins et al. (1991), laboratory tests were conducted to determine optimum conditions for BTEX biodegradation by indigenous microorganisms using nitrate as terminal electron acceptor. Toluene, ethylbenzene, meta- and para-xylene were degraded to below 5 μg/L when present as sole source of carbon and energy. Considering stoichiometric calculations, nitrate removal could account for 70 to 80% of the compounds transformation. Ortho-xylene was not transformed when present as a sole substrate, but was slowly degraded in the presence of other hydrocarbons. Benzene was not degraded in one year; regardless of whether it was available alone or with toluene, phenol, catechol used as cosubstrate. Gersberg et al. (1995) studied in-situ bioremediation of BTEX in oxygen-poor (approximately 1mg/L) fuel contaminated aquifer. In this experiment, extracted groundwater
was enriched with ammonium polyphosphate as a source of nutrients and KNO₃ as an electron acceptor. This water was piped to an infiltration gallery over the contaminated site. BTEX declined by 78% in water from the monitoring well which was the most contaminated initially and by nearly 99% in water from other extraction wells.

Although most researches report that benzene is no: degraded under anaerobic conditions, Chaudhuri and Wiessmann (1995) have shown that benzene can be degraded anaerobically via benzoic acid as a major intermediate in the benzene degradation pathway. The mixed culture was grown in mineral medium containing 1% glucose and then adapted on benzene as the carbon and energy source. In this research, degradation ability of microorganisms was improved stepwise by the number of enrichments and optimization of the culture medium.

2.3.2. Role of Nutrients

Historically, it has been discovered that biodegradation rates increased when fertilizers were added to soil. Several studies have proven that addition of phosphorus and nitrogen helps to enhance biodegradation (Kao, 1993; McCabe 1993; Wrenn, 1994; Zhou and Crawford, 1995). Very often, nutrient ratio C:N:P - 100:10:1, which is believed to be optimal (La Rue, 1993) for biological activity, is not found in natural ecosystems. In a study by Scherrer and Mille (1989), even though the soil was periodically oxygenated, aerobic microorganism activity in biodegradation of crude oil seemed to be limited. This was accounted for a nutrient deficiency, which resulted in slow mineralization of oil. This assumption was confirmed by stimulating biodegradation with a fertilizer. Zhou and Crawford (1995) also reported that gasoline hydrocarbons could be degraded faster after nutrients were added. As discussed by
McCabe (1993), nitrogen fertilizer has to be applied to the soil at the rate, which allows to maintain carbon: nitrogen ratio not lower than 30:1 for successful biodegradation of petrochemical waste.

Nitrogen source can be added in different forms, depending on the treatment method and the environment for remediation. In the study of Kao (1993), nutrient briquettes were developed to continuously supply nutrients and nitrate. In this case, nitrate has also been used as an electron acceptor. Results indicated that 85% of toluene and 71% of ethylbenzene were removed under anoxic condition. The proposed barrier system was recognized as a potentially useful remediation technology. Although all bacteria need nitrogen in some form to maintain cell growth, the choice of the proper nutrient supply is often influenced by abiotic factors. The effects of nitrogen source on crude oil biodegradation was studied by Wrenn et al. (1994). In this research, NH₄Cl and KNO₃ were investigated as sources of nitrogen for oil-degrading culture. Since sea salt medium used in this study was poorly buffered, addition of NH₄Cl decreased the pH of the culture, which resulted in complete seizure of biodegradation as measured by oxygen uptake. Adjustment of the culture pH allowed oil biodegradation to proceed normally. This severe decrease in pH of the medium and subsequent effect on biological activity was not observed in cultures supplied with KNO₃. In the research of Corseuil (1992), nitrate was used as a source of nitrogen in order to prevent competition for the electron acceptor between degraders of target compounds and nitrifiers, which grow if ammonia is used as nutrient source.
2.3.3. Role of Adaptation and Biomass Limitations

Inoculation of the subsurface with microorganisms with specific catabolic abilities is another method often employed to enhance the degradation of contaminants (Corseuil, 1992). Microorganisms capable of utilizing the target compounds can be selected by adaptation of microorganisms to the compound of interest with subsequent enrichment of cultures or by genetic manipulations. The success of inoculation technique depends on the concentration of compounds to be degraded, the presence of organic compounds used as cosubstrate, and the ability of the microorganisms to move through aquifer materials to areas containing the contaminants (Corseuil, 1992). Although several microorganisms capable of degrading toxic organic compounds have been found in natural environments, an acclimation or lag period is usually observed before mineralization can be detected. Acclimation period is defined as the time required for detection of mineralization after addition of a target compound (Corseuil, 1992). As further discussed by Corseuil (1992) there are three ways by which microbial acclimation can occur:

a) induction of enzymes;

b) genetic changes, and

c) increase in the number of microorganisms capable of degrading a compound of interest.

Several researches have observed that the time required for enzyme induction is usually in the range of minutes or hours (Richmond, 1968; Varma et al. 1976; Monticello et al. 1985; Robertson and Button, 1987 as discussed by Corseuil, 1992). Therefore, acclimation of microbial population to the specific xenobiotic, which usually takes several days of weeks, can not be explained solely by the enzyme induction. A number of research works confirmed that the time required for the biodegradation to be noticeable depends upon the number of
microorganisms with specific catabolic abilities. Biodegradation can be observed only after the substantial amount of substrate disappears, and this can be possible if a large population of bacteria participates in the utilization process. In the study of Wiggins et al. (1987), it has been found that microbial logarithmic growth phase began after one day, but substrate disappearance was only detectable after 8 days when degraders were present at $10^6$cells/mL. The lag period was not caused by enzyme induction, mutation, or toxins, but represented the time required for small active populations to multiply to levels where mineralization was measurable (Corseuil, 1992).

Weber and Corseuil (1994) studied the effect of inoculation of contaminated soils with enriched indigenous microbes on enhancement of bioremediation rates. The proposed method of enhancement was designed to increase the subsurface population of specific microorganisms. The technique was tested in laboratory soil columns using benzene, toluene and xylene as target compounds and sand as a soil medium. The technique involved small biologically active carbon reactors with low volume pumping scheme to acclimate and select microorganisms with specific catabolic activity. Then these microorganisms were sloughed from the reactor, grown on the nutrient medium, and reintroduced to the subsurface. This method was able to rapidly increase populations of such microbes to levels above $10^5$cells/g of dry solids in the previously uncontaminated aquifer sand studied. This has been shown to result in enhanced rates of in-situ degradation of the target hydrocarbons over a wide range of concentrations, from 25 to 9000 $\mu$g/L. The advantage of inoculation technique was also demonstrated by Corseuil and Weber (1994). The results of their experiments with indigenous and inoculated microorganisms illustrate the importance of inoculum size in enhancing the rate of biodegradation. At high benzene concentrations, an adapted population of $4 \times 10^6$
microorganisms/g of dry solids could completely utilize all electron acceptor available after only 10 hours. Indigenous organisms not enriched by inoculum could reach the same levels only after 10 days. Potential biomass limitations on rates of degradation of monoaromatic hydrocarbons by indigenous microbes in subsurface was investigated by Corseuil and Weber (1994). The rates of biodegradation of benzene, toluene and xylene by indigenous microorganisms in a natural aquifer sand were evaluated. Despite the fact that nutrients and electron acceptor conditions were highly favourable, the onset of measurable microbial oxidation of these readily degraded compounds was delayed in systems with small populations of biota. The delays varied inversely with the initial number of microbes capable of degrading a particular compound. Those delays suggested the need to accumulate some critical population of microorganisms. Thus, biodegradation can be stimulated by inoculation with the pre-selected microorganisms population of high density.

2.3.4. Role of Immobilization

Since microbes in nature tend to attach themselves to some surface rather than being free in the aqueous phase, it is important to take this fact into consideration while designing the biotreatment system. During the process of bioremediation, microorganisms are normally attached to the soil particles. It might be beneficial to use this idea for adaptation and growth of microorganisms with specific catabolic characteristics. A number of researchers have studied different types of supporting media for microbial attachment.

The advantages of using immobilized population with specific catabolic abilities over the suspended biomass have been shown in a number of works. Microorganisms that were attached to some media showed better degradation abilities. The rate and extent of organic
compounds utilization was higher. Wu (1994) used polyvinyl alcohol as entrapping agent for aerobic and anaerobic sludge. The comparison between the immobilized and free sludge was made. The results showed that volumetric loading for immobilized sludge was 1.3 to 2.1 times that of a free sludge. This proves that the immobilized sludge had a higher capacity of treating organics than a free sludge.

Activated carbon is one of the most frequently used media for growth and adaptation of microbes. Since activated carbon is used as a treatment method for monoaromatic hydrocarbon removal, its use for attachment of biomass and adsorption removal presents the area of scientific and practical interest. The surface of activated carbon allows hydrocarbons to be sorbed and desorbed relatively easily. It also helps to create favourable environment for growth and development of microorganisms. Cells attach themselves on the surface of carbon pores, forming biofilm and metabolizing organic compounds as they desorb from the pores (Corseuil, 1992). This lowers the compound concentration in the liquid phase and forces it's desorption from the carbon surface in order to reach equilibrium. Thus micropores are getting liberated from the hydrocarbons, sorbed on them, which is the process similar to regeneration of GAC. As discussed by Corseuil (1992), this activity, frequently called bioregeneration, can significantly increase the life time of GAC systems used for BTEX compounds adsorption.

The beneficial aspects of combined biological/adsorption systems were reported by Weber et al. (1970) as discussed by Corseuil (1992). Researches observed that adsorption columns continued to effectively remove organic compounds beyond the adsorption capacity exhaustion. This removal has been shown to be a result of growth of microorganisms on the surface of activated carbon particles and the subsequent biodegradation performed by these microbes. Medina et al. (1995) compared the efficiency of granular activated carbon (GAC) as
a support medium with the compost biofilter. It has been shown that initial adsorption provided high percentages of removal, until it was saturated. After that the treatment efficiency had dropped dramatically. Later, biological activity increased to the point where effective treatment was possible. The average and maximum elimination capacities were higher than those of compost biofilter.

As discussed in Corseuil (1992) the bioregeneration process consists of four phases:

- Phase one: adsorption is the dominant process, biomass is growing;
- Phase two: short period of rapid microbial growth, and availability of substrate;
- Phase three: a longer period of decrease in biodegradation rates because it is controlled by diffusion of substrate, and transport phenomena in GAC;
- Phase four: a period of low rate bioregeneration, approaching the steady state value

A good illustration of above-mentioned four-stage process and advantages of biological activated carbon reactor (both biological and adsorption processes) over abiotic fluidized bed reactor (only adsorption, no biological processes) and biological fluidized bed reactor (only biological process, no adsorption) were reported by Voice et al., (1992). These three systems were fed groundwater contaminated with BTX. During start-up, although the same amount of inoculum was added to the two biological systems, the time required for the onset of effective biodegradation was 200 hours for the reactor using GAC as a carrier for biomass, and 500 hours for the system using non-activated carbon. During this period, before a fully functional biofilm had developed, the substrate was removed primarily by adsorption. After the biofilm was established, biodegradation took over and was dominating the system performance. Significantly less BTX was found in the effluent during this period from the reactor with
combined removal mechanisms. The development of a biofilm was more rapid for this reactor. During step increase in organic loading, the combination of biological and adsorptive removal capacity resulted in enhanced BTX removal and more stable operation. Combined system was found to remove 82, 74 and 64 % of benzene, toluene, xylene compared to 65, 56 and 46% respectively in the biological system. The abiotic system was getting saturated and adsorption capacity was getting exhausted.

There is a wide-spread impression that biological systems are not sufficiently stable to consistently meet the stringent regulation requirements for the removal of contaminants. The research by Xing and Hickey (1994) addressed the problem of responses of immobilized system to periods of interruption in oxygen and nutrient supply. The following results were found out in their research: greater than 99% BTX removal was achieved at an organic loading rate of 6.7 kg COD /day and an empty hydraulic retention time of 3 minutes; interruption of nutrient supply for 24 hours did not significantly impact system performance; and a nutrient pulse feeding mode of 30 min on / 30 min off was successfully used without affecting removal efficiency. This means that granular activated carbon was proven to be an excellent immobilizing agent for selection and adaptation of microorganisms used to degrade BTEX compounds.

2.3.5. Mixed Populations and Cometabolism

Natural polluted environments are quite different from those in the laboratory. In contrast to the pure culture and single substrate systems in the laboratory, the field environment is extremely varied and complex. A single microbial species can never be responsible for the degradation and destruction of pollutants in the field (King et al. 1992). It has been shown that
mixed culture has the better ability to degrade the BTEX mixture than a pure culture alone, especially when it has undergone the acclimatization period (Malakin, 1994).

As discussed by King et al. (1992), most natural environments are loaded with microbes. Every polluted site contains the variety of microbial species, many of which have become acclimated to existing contaminants. It is common for these mixed populations to act in concert in metabolizing and degrading the organics available. Valenti et al. (1994) reports that although the mechanism of microbial hydrocarbon utilization has been successfully modelled in the laboratory, in nature the extent of hydrocarbon degradation is higher. This degradation is performed by mixed cultures, which are more versatile in nature than in the laboratory. This natural versatility and complexity of food chains makes it possible for microbiota to interact during the catabolic processes. One of the interaction is termed cometabolism. Cometabolism is the biotransformation of a non-growth (or secondary) substrate in the obligate presence of a growth (or primary) substrate (Liou, 1995). Different substrate interactions and cometabolic combinations have been reported in literature. Alvares and Vogel (1991) indicate that substrate interactions of BTEX compounds during biodegradation could be both beneficial (cometabolism) and detrimental (inhibition). Beneficial interactions include enhanced degradation of benzene and para-xylene by the presence of toluene. Detrimental substrate interactions include retardation in benzene and toluene degradation by the presence of para-xylene. In the study of Chang et al. (1993), paired substrates (BT, TX, or BX) revealed competitive inhibition, while para-xylene was reported to be cometabolically transformed during toluene utilization. The positive effects of cometabolism on xenobiotic wastes biodegradation were reported also by Liou (1995). From the above-mentioned it would be natural to make a conclusion that cometabolism of bacteria consortium
adapted to metabolizing the specific substrate/group of substrates can be beneficial for biodegradation of target compounds.
Figure 2.2.1 - Mechanism of Benzene Biodegradation (Müller, 1992)
Figure 2.2.2. The Initial Steps in the Two Pathways for the Bacterial Degradation of Toluene (Muller, 199
Chapter III

MATERIALS AND EXPERIMENTAL METHODS

3.1. Introduction

A three-phase experimental program was implemented in this study. Selection of microorganisms adapted to BTEX as the only source of carbon and energy was the purpose of Phase I. The study of the extent of BTEX degradation enhancement using different stimulation methods was done in Phase II. Transformation of monoaromatic compounds in soil in the presence of indigenous and adapted microorganisms was studied in Phase III.

In Phase I, adaptation of microorganisms to BTEX was performed by using granular activated carbon column reactor, and substantial density of microbial population was achieved. In Phase II, different ways of biodegradation enhancement were studied and compared in batch experiments. In Phase III, soil columns were used to study the rate and extent of biotransformation using inoculation technique in a continuous flow reactor.

Due to sorption ability and volatility of monoaromatic hydrocarbons, controls had to be prepared and analyzed to account for abiotic BTEX transformation and losses.

3.2. Chemicals

3.2.1. Organic Compounds

The monoaromatic hydrocarbons, benzene, toluene, ethylbenzene, and meta-xylene were used in this study because they are often found in soil and groundwater contaminated with gasoline spills. Since all four BTEX compounds are present in gasoline and oil
simultaneously, all experiments were conducted using a mixture of them. Although these compounds have the highest water solubility of all gasoline chemicals, stability and homogeneity of the aqueous solution in the presence of nutrient salts, even at low BTEX concentration, was difficult to achieve. During the first 6 weeks of study, water-based solutions were employed, and even though mixing for 24 hours was used before feed, the analyses reflected hydrocarbon's separation in aqueous solutions. It was decided to use methanol as a basis for solution of monoaromatic compounds in order to avoid the necessity to mix solutions for long periods of time, and to prevent potential problems with dissolving higher BTEX concentrations in water. All BTEX compounds were mixed with methanol easily at all concentrations studied, and methanol-based solutions were mixed with water-based nutrient solutions. Benzene was purchased from Fisher Scientific (Fair Lawn, NJ). Toluene, ethylbenzene, meta-xylene, and methyl alcohol were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Some of the physical and chemical properties of these compounds are listed in Table 3.2.1

Table 3.2.1 - Physical and Chemical Properties of Monoaromatic Hydrocarbons

<table>
<thead>
<tr>
<th>Property</th>
<th>Benzene</th>
<th>Toluene</th>
<th>Ethylbenzene</th>
<th>m-Xylene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiling Point, °C</td>
<td>80.1</td>
<td>110.6</td>
<td>136.2</td>
<td>139.1</td>
</tr>
<tr>
<td>Melting Point, °C</td>
<td>5.5</td>
<td>-95.0</td>
<td>-94.97</td>
<td>-47.87</td>
</tr>
<tr>
<td>Specific Gravity</td>
<td>0.8765</td>
<td>0.8669</td>
<td>0.8670</td>
<td>0.8642</td>
</tr>
<tr>
<td>Molecular Mass</td>
<td>78.11</td>
<td>92.13</td>
<td>106.17</td>
<td>106.17</td>
</tr>
</tbody>
</table>

Source: Corseuil, 1992, Handbook of Chemistry and Physics, 1972

3.2.2. Feed Solution

Nutrient salts and BTEX compounds were used to provide adequate chemical
conditions for growth of microorganisms. Medium containing major macro- and micro-
elements was prepared to supply sufficient amount of nutrients required for development
of healthy microbial population. Nitrogen and phosphorus, as well as other trace elements
were added in excess to meet biosynthesis needs. Nitrate was used as a nitrogen source
instead of ammonia to limit the growth of nitrifiers, which can increase the oxygen
demand, and thus, limit the availability of an electron acceptor for the degradation of
target compounds (Corseuil, 1992). Another reason for choosing nitrate was that it could
be used as an alternative source of electron acceptor after free dissolved oxygen in water
had been depleted by aerobic metabolism. The composition of nutrient medium used is
presented in Table 3.2.2. The concentrations listed were used in the experiments with the
total concentration of BTEX of 10 mg/L. They were increased slightly when higher
concentration of hydrocarbons were employed. In soil experiments only nitrogen,
phosphorus and potassium were added as supplemental source of nutrients.

Table 3.2.2- Composition of Nutrient Solution

<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄·7H₂O</td>
<td>200</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>100</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.05</td>
</tr>
<tr>
<td>NaMoO₄·2H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>43.6</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>22.9</td>
</tr>
<tr>
<td>KNO₃</td>
<td>50</td>
</tr>
</tbody>
</table>

Source: Corseuil, 1992
The following procedure was used to prepare feed solution used in the experiments. Distilled-deionized water was aerated for 15 min prior to mixing, which was sufficient to reach the oxygen saturation (9.2 mg/L) as measured by Biological Oxygen Monitor. Nutrient salts were weighed and dissolved in water. Care was taken to assure complete dissolution of salts for which heating and mixing of the solution was employed. A mixture of equal mass of each of BTEX compounds was prepared gravimetrically from pure chemicals as needed and dissolved in 10 mL of methanol. Then oxygenated water, salts and BTEX were mixed together and brought to a 1 L volume. The flask with the feed solution was closed and sealed tightly to avoid volatilization of BTEX hydrocarbons.

3.3. Activated Carbon

Granular activated carbon (GAC) was selected as a medium for biomass immobilization because of the reports in literature of studies where GAC was successfully used for adsorption of volatile organic compounds as well as support for the growth of microorganisms (Corseuil, 1992; Voice et al. 1992). GAC (0.42 - 0.50mm retained sieve size) was washed with distilled-deionized water to remove fines, then dried at 100°C and placed into reactors.

3.4. Soil Preparation

The soil material used in this study was top-soil collected from a flower-bed at the University of Windsor. Soil was dried at room temperature (approximately 20°C) and sieved, using mesh 0.42 - 1.68mm retained to achieve uniform size particles.
The amount of liquid required to achieve soil moisture saturation was determined experimentally. The results obtained showed that 2 mL of water was enough to saturate 5 g of soil under conditions studied.

3.5. Analytical Techniques

Analysis of the BTEX compounds present in water and in soil was done using gas chromatography preceded by head-space analysis. HP 5890 Gas Chromatograph (GC) was equipped with a Methyl Silicone Gum column (HP-1 Instrument Test, 5m X 0.53 mm X 2.65 μm film thickness), and a flame ionization detector (FID). Nitrogen was used as a carrier gas, with the flow rate of 1 mL/min. Head-space analyzer model 19395A (Hewlett Packard, Avondale, Pa) was used to reach the equilibrium in the test vials and to inject the sample into the column. In the experiments of Anid et al. (1992), vials were equilibrated at 35°C before head-space analysis. In these experiments 45 min at 45°C was used for equilibration since lower concentrations of BTEX compounds were employed. According to Corseuil (1992), BTX compounds can be separated isothermally at 75°C. In this study, the temperature of 30°C was enough to separate all target compounds isothermally. The signal from the detector was integrated using Hewlett Packard 3393A integrator. The injector temperature was 250°C, and the detector temperature was 250°C. The GC oven temperature was 30°C held for 5 minutes which showed to be enough to separate all BTEX compounds.

The integrated peaks and area counts were translated to BTEX levels using standard calibration curves. Standard BTEX solutions were prepared gravimetrically in methanol from pure compounds as needed. Calibration was performed by plotting an 8
point standard calibration curve from 0.01 to 50 mg/L for each aromatic compound studied. The calibration curve was plotted as a concentration-versus-area counts plot using a best fit line. Standard calibration curves for BTEX in soil and in water are shown in Appendix A.

Method detection limits (MDL) were determined for each BTEX compound according to Method No. 1030E in Standard Methods (APHA, 1992). A set of seven solutions for each hydrocarbon at a level of approximately 0.01 mg/L for water and 0.1 mg/L for soil were prepared and analyzed as all other samples. Standard deviation of the seven replicate measurements was calculated for each compound. Methanol blanks free from hydrocarbons were analyzed as well. The MDL for each hydrocarbon was calculated using the formula:

\[
\text{MDL} = t (s) + b
\]

where \( t = 3.143 \) (t-value for 6 degrees of freedom and 99% confidence level);

\( s = \) standard deviation of seven replicate analyses; and

\( b = \) mean value of hydrocarbon-free methanol blanks.

The MDLs for each aromatic hydrocarbon are listed in Table 3.5.1.

Dissolved oxygen was measured using an oxygen monitor (YSI model 5700 Biological Oxygen Monitor, Yellow Spring, OH).

Standard plate counts were used to quantify the microbial population detached from the carbon and soil columns. Trypton Soy Agar (Weber and Corseuil, 1994) of standard composition was used as a media for growth. Standard dilutions in a potassium
phosphate buffer solution (Corseuil, 1992) were made. Plates were cultured at 35°C, and colonies were counted after 48 hours of incubation according to Method No 9215C in *Standard Methods* (APHA, 1992).

**Table 3.5.1 - BTEX method detection limits (MDLs)**

<table>
<thead>
<tr>
<th>Hydrocarbon Name</th>
<th>Method Detection Limit in Water, mg/L</th>
<th>Method Detection Limit in Soil, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>0.012</td>
<td>0.16</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.04</td>
<td>0.13</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>m-Xylene</td>
<td>0.01</td>
<td>0.13</td>
</tr>
</tbody>
</table>

### 3.6. Activated Carbon Reactors

The GAC reactors consisted of 1.8-cm ID glass columns (60 mL empty bed volume), packed with activated carbon (10/40 sieve size) to achieve a 50 mL volume. After being filled with activated carbon, both reactors were rinsed with distilled-deionized water to remove air and fines from the pores. The BTEX and nutrient solution prepared and mixed earlier were fed to the system using syringe pump model A-35696 (Harvard Apparatus, South Natick, MA). Two 60 mL - syringes were used with the pump. The system was operated in an up-flow mode. The flow rate was 1.25 mL/h, which provided uninterrupted operation time of 48 hours, after which feeding solution was refilled. The retention time in the packed bed reactor was 60 hours. The details of the column set up are illustrated in Figure 3.6.1.
Figure 3.6.1 GAC Column Reactor
3.7. Selection of the BTEX-Degraders

Activated sludge from the Little River Pollution Control Plant (Windsor, ON) was used as a source of microorganisms. Catabolic diversity of microorganisms used in the secondary treatment of waste water, enables their use for selection of specific compound-degraders.

Two identical columns with GAC were employed in this part of the study. One column (Reactor I), was used as a bioreactor, and the other (Reactor II) as a control to account for abiotic losses. Both columns were packed with GAC up to 60 mL bed volume. Reactor I was seeded with microorganisms. The inoculum solution was prepared as follows: 30 mL of nutrient solution mixed with 30 mL of activated sludge and fed to the system at the rate of 1mL/min. The system was left for 12 hours in order for microorganisms to attach to carbon particles under stagnant conditions. Control reactor was rinsed with sodium azide solutions to prevent any microbial activity and seeded with the solution containing 30 mL of nutrients and 30 mL of previously autoclaved activated sludge at the rate of 1mL/min. It was then rinsed with sodium azide solution again. Microbial counts of the effluent from the control reactor after 12 weeks of operation showed that the biological activity in it was completely suppressed. Samples of the influent were taken prior to start of the new feed (every 48 hours). Sampling of the effluent was performed every 24 hours. This phase of the experimental program was conducted for 14 weeks.

3.8. Batch Studies

Soil samples (5 g) were incubated at the room temperature (20°C) in 20 mL vials
sealed with teflon-coated rubber septum and aluminum caps. Depending on the experimental conditions, 3 mL of liquid with different composition was added to each of the vials. This amount of liquid was enough to reach soil saturation with moisture, as determined experimentally.

Controls were prepared by autoclaving soil and adding the same chemicals, except microorganisms. Distilled water was used to maintain the same level of soil saturation. Control vials were kept in the refrigerator at 4°C to prevent any biological activity.

Solutions of benzene, toluene, ethylbenzene, and meta-xylene (BTEX) in methanol were prepared in the following concentrations: 10, 20, 30, 50, 100, and 200 mg/L of total BTEX, with approximately one fourth of this concentration for each of the BTEX components.

In experiments where nutrients effect was studied, the macronutrients in the following concentrations were added: K₂HPO₄ - 43.6 mg/L, KH₂PO₄ - 22.9 mg/L, KNO₃ - 50 mg/L (Corseuil, 1992). In vials where addition of oxygen was investigated, 0.002% hydrogen peroxide was added to the BTEX solution. In experiments where addition of pre-selected microorganisms was studied, 1 mL of effluent of the GAC column employed for Phase I, was used as inoculum. Standard plate counts showed that the density of microorganisms was 10¹⁰ cells/mL of effluent. One milliliter of suspension was added per 5 g of soil. Since GAC column was fed with BTEX as a sole source of carbon and energy for several months, all microorganisms in the effluent were considered to be the selected BTEX-degraders. An adequate number of vials was prepared for each experiment to be analyzed daily for a 45-day period. Controls were prepared for each of the concentration studied.
3.9. Experiments with Soil Columns as Continuous Flow Reactors

The same reactor, as for the Phase I (Figure 3.6.1), was used for these experiments. Columns were packed with soil up to 50 mL of bed volume. The adapted microorganisms obtained from the GAC column in Phase I were used to seed one of the reactors were. Potassium phosphate buffer solution was used at a rate of 1 mL/min to wash out microorganisms from the GAC column. Plate counts of the effluent indicated a cell density of about $1 \times 10^{10}$ viable cells/mL. This suspension was used to inoculate one of the two columns. After seeding at the rate of 1 mL/min, the column was left for 12 hours to allow microorganisms to attach to soil particles. Degradation in the presence of indigenous microbes was studied in the other column, in which no inoculum was added. After 12 hours, the continuous operation of the system was started.

Several concentrations of total BTEX were investigated: 10, 30, 40, and 70 mg/L. The flow rate in all the experiments was 2.5 mL/h with the retention time of 26 hours as determined experimentally. Breakthrough of BTEX compounds was achieved after 7 to 9 days. Every time after the concentration of BTEX compounds in the effluent stabilized at a certain level, higher initial concentration of monoaromatic compounds was used in the feed solution.

3.10. Biodegradation Rate Constants Determination

The biodegradation rate coefficient, $k$, was determined from the degradation curves of BTEX compounds. First order removal kinetics was assumed for transformation
of all aromatic hydrocarbons (Neufeld et al. 1994). The removal of hydrocarbons can be modelled as:

$$\frac{C}{C_0} = e^{-k(t-t_0)}$$

where 

- $C =$ target compound concentration after incubation, mg/L;
- $C_0 =$ initial target compound concentration, mg/L;
- $k =$ removal constant based on compound disappearance, day$^{-1}$;
- $t =$ time of incubation, day; and
- $t_0 =$ time of measurable onset of biodegradation.

Since sorption effects did not play important role in the hydrocarbon removal at total BTEX concentration of 10mg/L, the change in concentration described by this equation can be credited to biodegradation.

Plotting $\ln(C/C_0)$ versus $(t-t_0)$, the $k$ coefficient can be obtained as a slope of the line: $\ln \left( \frac{C}{C_0} \right) = -k(t-t_0)$. 

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Chapter IV
RESULTS AND DISCUSSION

4.1. General

Biodegradation of BTEX was studied in three phases as discussed earlier. The following results were obtained.

4.2. Phase One: Selection of BTEX-degraders

The objective of phase one was to select microorganisms that are able to utilize BTEX as the source of carbon and energy for their metabolism. Granular activated carbon was selected as a media for immobilization and growth of biomass because of the several reports in the literature on the successful use of GAC for growth and adaptation of microorganisms with specific catabolic activity. The activated sludge from the wastewater treatment plant was chosen as the source of inoculum because of the catabolic diversity of microbial population represented in the sludge. The GAC columns were fed with BTEX-nutrient solution continuously for a period of 16 weeks. Mixture of all BTEX compounds was used to prepare a solution. Care was taken to account for abiotic BTEX removal by operating the control column under the same conditions except for being sterilized. During the first 5 weeks, sorption process was dominating and no BTEX was detected in the effluent from either columns.

Breakthrough of all BTEX compounds occurred in the following order: benzene (37 days), toluene (40 days), ethylbenzene and xylene (45 days). These results showed that activated carbon had a considerable potential for sorption of BTEX compounds. The order of breakthrough for different compounds agrees with the data reported by other
researches (Corseuil, 1992; Voice et al. 1992). Overall, approximately 2.7 mg of benzene, 3 mg of toluene and 3.3 mg of ethylbenzene and xylene were sorbed on 40 g of carbon before the breakthrough occurred.

During the first 6 weeks of experiments, the BTEX-nutrient solution was prepared in water and mixed for 12 hours to achieve homogeneity. Despite this, the analyses showed erroneous results for influent BTEX concentration. Based on these results, it was assumed that during the delivery time BTEX compounds were coming out of the solution and accumulating on the surface, thus making the system unstable. The reason for this might be low solubility of these compounds in water, which became even lower when salts were present. Most salts have negative effect on the solubility of aromatic hydrocarbons. Also, the time required to deliver the feed to the system was long enough for the solution to separate in layers, and thus, loose homogeneity. This instability of influent concentration was reflected in the analysis of the effluent concentration. However, almost in all cases, the concentrations of BTEX in the effluent from biologically active column were lower than those from the sterile column. Figure 4.2.1 illustrates the data obtained during this phase of experiments. During the first 9 weeks (60 days), the effluent concentration of all BTEX compounds had high and low peaks, which reflects the instability of the influent concentration. After 6 weeks, it was decided to employ methanol as additional solvent for BTEX solution as discussed in Chapter III. Homogeneity of BTEX distribution was achieved easily without mixing and was maintained during the delivery time. Biodegradation of hydrocarbon compounds could not be observed before the breakthrough occurred.

According to the other researchers, acclimation and accumulation of the sufficient biomass usually happens during the first 10 days of feeding the microorganisms with BTEX as the only source of carbon and energy (Corseuil, 1992). Apparently some biomass was adapted during the first 6 weeks of experiment, but the biodegradation could not be measured, since no BTEX was detected in the effluent. After the sorption capacity
of carbon was achieved, the presence of BTEX compounds was detected in the effluent. The concentration of aromatic hydrocarbons in the reactor with biological activity was lower than that in the sterile column, and continued to decrease steadily. This served as an evidence for biodegradation, since all other conditions were maintained the same in the two reactors.

After 70 days of operation, the concentration of BTEX compounds stabilized at less than 0.1 mg/L of each hydrocarbon in the effluent from the biologically active carbon system. At the same time, in the effluent from the column without biological activity, the BTEX level stabilized at approximately 1.2 - 1.4 mg/L of each compound. Some BTEX was still sorbed on the activated carbon surface, but this could account only for 30-40% of the total reduction in aromatic hydrocarbons concentration. The total reduction in hydrocarbon concentration reached 80-90% in the biologically active column. Since all other conditions were the same, the difference can be credited to biodegradation. After 10 weeks, the density of biomass grew to a level where microorganisms were able to degrade almost all of benzene, toluene, ethylbenzene and xylene supplied to the columns. Steady-state conditions were achieved. In general, for all BTEX compounds, rates and extend of biodegradation were approximately the same.

Sorption effect was more significant for toluene than for benzene, and even more for ethylbenzene and xylene.

The effluents from both columns were seeded on nutrient agar, and plate counts were performed. They showed that biomass density in the effluent from the biologically active column was $10^{10}$ cells/mL, while the control reactor remained sterile, and no microorganisms were detected in the effluent. This can be interpreted as an indirect proof of successful biodegradation. The BTEX compounds were the only source of carbon and energy supplied for 10 weeks, and since sufficient biomass grew, and maintained its viability, the conclusion was made that those microorganisms were acclimatized to utilizing aromatic compounds for their metabolism. Thus the main objective of this phase
of the experiment was achieved. The biodegradation of all BTEX compounds was proven to be possible by adaptation of consortium of microorganisms to aromatic hydrocarbon compounds as the only source of carbon and energy.
Figure 4.2.1. - BTEX Breakthrough on GAC Column, 10 mg/L total BTEX
4.3. Phase Two: Study of the Efficiency of Enhancement Methods

Biodegradation of mixture of benzene, toluene, ethylbenzene, and meta-xylene was studied in batch experiments. Different methods of enhancement of BTEX compounds were investigated and compared. Addition of supplemental electron acceptor (in the form of hydrogen peroxide H_2O_2 and nitrate NO_3), nutrients, and preselected microorganisms capable of degrading BTEX were studied independently as well as in combination. In order to account for abiotic BTEX transformation, controls containing autoclaved soil, nutrients, electron acceptor and no microorganisms were run in parallel. Instead of suspension with microorganisms, distilled water was added to autoclaved soil to maintain the same level of soil saturation. Depending on the factor variable studied, microorganisms, nutrients, and hydrogen peroxide were added along with aromatic hydrocarbons to the soil. Nitrate as KNO_3 was used primarily as a source of nitrogen, and was present in the nutrients added. Controls were prepared for each BTEX concentration studied. The number of control vials was sufficient for analyses under all conditions. The amounts of hydrocarbons sorbed on soil particles were considerably higher when higher initial concentrations of BTEX were used. For example, in case of xylene, for example, at initial concentration of 54 mg/L, approximately 33% was irreversibly sorbed on soil. This agrees with the theoretical knowledge of the nature of sorption following the Freundlich Isotherm, according to which the amount of compound sorbed is proportional to the concentration of it in the solution.

Results of biodegradation under different conditions are shown in Figures 4.3.1 to 4.3.7 and the rate constant, k, values are tabulated in Table 4.3.1. The values of R^2 ranged between 0.91 and 0.99, where R is the coefficient of correlation. Confidence limits of 95% were determined for regression estimate, k, using Student’s t-test, and are presented in Table 4.3.1. The time by which BTEX compounds were degraded by 90% in each case is tabulated in Table 4.3.2.
Results of biodegradation under different conditions showed that benzene, toluene, ethylbenzene and meta-xylene were degraded by approximately 95% in a 45-day period in all experiments with total BTEX concentration of 10 mg/L. Soil environment to which no nutrients, microorganisms, and supplemental electron acceptor source were added was considered natural. Natural soil environment was altered only by addition of water up to the soil saturation level as discussed in Chapter III. Under these conditions, BTEX concentration reached the level of 0.1 mg/L after 35-40 days, but did not drop any further during next 5-10 days (Figure 4.3.1). The onset of measurable biodegradation was observed after 5-6 days depending on the compound. The concentration of BTEX decreased at a fast rate until Day 9-13. Then the degradation slowed down. The resultant concentrations ranged from 0.2 to 0.5 mg/L. The natural biodegradation rates, $k$, for all BTEX compounds was estimated as approximately 0.20-0.23 day$^{-1}$ under these conditions.

Addition of nutrients enhanced biodegradation rates as compared to those under natural conditions. Significant reduction in benzene concentration was achieved on Day 4 as opposed to Day 6 without supplemental nutrients. However, after 9-11 days, oxygen was depleted and when anaerobic utilization was not possible, as in case of benzene, the rate of degradation slowed down considerably. The degradation rate for benzene had increased from 0.20 to 0.24 day$^{-1}$ due to nutrients addition. At the same time, degradation rates for toluene increased from 0.23 to 0.31 day$^{-1}$; from 0.21 to 0.31 day$^{-1}$ for ethylbenzene, and from 0.22 to 0.30 day$^{-1}$ for m-xylene. Figure 4.3.2 demonstrates the enhancement of biodegradation by supplemental nutrients. In case of toluene, ethylbenzene an xylene, addition of KNO$_3$ as a source of nitrogen exhibited the possibility of anaerobic pathway by switching to nitrate as electron acceptor since in all cases when nitrate was added, those compounds were degraded faster than benzene, which is known to be recalcitrant under anaerobic conditions. While benzene was still detected after 45 days of incubation, toluene, ethylbenzene and xylene were not detected after 35-37 days.
Addition of microorganisms considerably increased the degradation rates compared to natural conditions. This also reduced the lag-phase, which was observed to be 5-6 days under normal conditions without addition of supplemental microorganisms. Measurable onset of biodegradation in case of inoculum addition was observed soon after the start of the experiment. Indigenous microorganisms in soil under natural conditions needed an acclimation time to produce necessary enzyme, and develop the population large enough to make biodegradation measurable. Inoculation of the subsurface with microorganisms which already possess necessary enzyme production activity and are capable of utilizing the BTEX compounds, can considerably shorten the lag-phase. Also, increased number of microorganisms, $10^{10}$/mL in the suspension added, enhanced the biodegradation rates and reduced the time required for BTEX removal from 40 days in case of natural environment to 17-20 days (Figure 4.3.3). The biodegradation rate constant for benzene increased from 0.20 day$^{-1}$ under natural conditions to 0.32 day$^{-1}$ when inoculum was added. The degradation rate for toluene, ethylbenzene, and xylene constants also increased on average by 0.10-0.15 day$^{-1}$. There was no significant difference in enhancement of degradation for different aromatic hydrocarbons studied. This can be explained by the fact that adaptation of microorganisms was performed using the mixture of BTEX compounds, and, most probably degraders of all compounds were selected and subsequently grown to certain density. It is also possible that cometabolism of microbial population enhanced biodegradation, and all hydrocarbons were successfully utilized for microbial metabolism. As it has been shown by other researchers (Corseuil, 1992; Weber and Corseuil, 1993), that the main factor influencing the rates of biodegradation is the actual number of microorganisms with specific catabolic abilities. Since the number of microbes able to degrade all BTEX compounds was probably the same or close to it for different hydrocarbons, the rate and extent of utilization of each of them were almost the same under the conditions studied. Overall, addition of the pre-selected microorganisms increased the rate and degree of biodegradation. Addition of large bacteria population
with necessary catabolic abilities made the onset of degradation possible without a lag-phase, and the whole degradation process was more effective.

Addition of microorganisms together with electron acceptor source shortened complete biotransformation of all BTEX compounds as compared to natural conditions (Figure 4.3.4). Biodegradation constants were almost the same in case of addition of microorganisms alone and when combined with oxygen. Supplemental electron acceptor addition appeared to be not very effective in improving the microorganisms metabolic activity. It may be possible, that at this level of concentration of BTEX (10 mg/L), electron acceptor was not the limiting factor of biodegradation of monoaromatic hydrocarbons. Another possible explanation could be the technique used to inoculate soil and add hydrogen peroxide at the same time. The most obvious reason why addition of supplemental oxygen did not speed up the biodegradation process, was probably the lack of necessary nutrients. Microbiological activity is at its best when the balance between carbon, nitrogen, phosphorus and oxygen is maintained. In this case BTEX as a source of carbon was supplied in concentration, which considerably exceeded the natural level of hydrocarbons. At the same time, nutrients level was that of natural environment. That is probably why the biodegradation rate and extent did not increase significantly.

Addition of nutrients and electron acceptor increased biodegradation rates, and BTEX was not detected in the samples after 17-20 days (Figure 4.3.5). The biodegradation rate constants for benzene increased from 0.20 to 0.29 day\(^{-1}\) as compared to natural conditions. Significant increase in rate constants for toluene, ethylbenzene, and xylene was also observed: 0.23 to 0.39 day\(^{-1}\) for toluene; 0.21 to 0.35 day\(^{-1}\) for ethylbenzene; and 0.22 to 0.37 day\(^{-1}\) for m-xylene compared to degradation under natural conditions. This confirms that regardless of whether electron acceptor is in deficit or not, there should be a balance of macro- and micro- nutrients in order for microbial population to maintain healthy activity. At the same time, it proves that nutrients can work as additional source of electron acceptor in case of toluene, ethylbenzene, and xylene.
Benzene, although in low concentrations, was still detected even after 20 days of experiment, while toluene and xylene were not found after 13-14 days, and ethylbenzene after 17 days. Overall, addition of supplemental nutrients and electron acceptor increased the biodegradation rate and extent considerably. In this study, addition of supplemental oxygen and nutrients decreased time needed for BTEX biotransformation from 40 days in case of natural environment to 17-20 days.

Addition of microorganisms and nutrients allowed not only to start the degradation in a shorter time, but also complete it in 20 days for benzene (without switching to nitrate as an electron acceptor after oxygen depletion) and 13 days for toluene, ethylbenzene, xylene (Figure 4.3.6). Addition of microorganisms helped to minimize the lag-phase, while addition of nutrients created proper element balance between carbon, nitrogen, and phosphorus necessary for energy production and biosynthesis of microbial cells. Addition of nitrate was playing a dual role: as a source of nitrogen and as electron acceptor. That was the reason why toluene, ethylbenzene, and xylene were degraded faster than benzene. Biodegradation rate constants in this case were increased: from 0.20 to 0.33 day\(^{-1}\) for benzene; 0.23 to 0.53 day\(^{-1}\) for toluene; 0.21 to 0.55 day\(^{-1}\) for ethylbenzene, and 0.22 to 0.52 day\(^{-1}\) for xylene, as compared to rates obtained under natural conditions.

The optimum conditions for biodegradation of all BTEX compounds were created when supplemental electron acceptor, nutrients and microorganisms were added. In this case, complete biotransformation was achieved after 8-9 days. Figure 4.3.7 illustrates the biodegradation under optimum conditions when all microbial requirements for efficient metabolism were maintained. This allowed microorganisms to utilize all BTEX supplied at a high rate. Oxygen was used to start the biodegradation sequence for all compounds, and nitrate was used as an electron acceptor at subsequent stages of degradation. Competition for oxygen in water was reduced because degraders of toluene, ethylbenzene, and xylene were able to switch to nitrate. Electron acceptor supplied was enough to complete the degradation of benzene at almost the same rate as other BTEX compounds.
Biodegradation rates in this case reached the following maximum values as compared to those obtained under natural conditions: 0.37 day\(^{-1}\) versus 0.20 day\(^{-1}\) for benzene; 0.57 day\(^{-1}\) versus 0.23 day\(^{-1}\) for toluene; 0.55 day\(^{-1}\) versus 0.21 day\(^{-1}\) for ethylbenzene; and 0.55 day\(^{-1}\) versus 0.22 day\(^{-1}\) for m-xylene.

Further experiments were conducted with BTEX concentrations of 20, 30, 50, 100, and 200 mg/L after adding microorganisms, nutrients, and hydrogen peroxide. The results are plotted in Figures 4.3.8 to 4.3.12. BTEX in total concentration of 20 mg/L was degraded under favourable conditions in 13-15 days. There was no significant difference observed in degradation rates of different BTEX compounds (Figure 4.3.8). This might be because at this concentration additional electron acceptor supplied, along with natural oxygen dissolved in water was enough to start the degradation sequence for all compounds. The amount of oxygen was sufficient to complete it for benzene, and nitrate served as additional electron acceptor for degradation of toluene, ethylbenzene, and xylene. Thus, addition of nitrate helped in reducing the competition for oxygen.

Approximately 7-8 mg/L of each aromatic compound was degraded during a 45 day-period in the experiment with total BTEX concentration of 30 mg/L. Benzene degradation was to a less extent, probably due to the lack of electron acceptor after a certain time. Toluene, ethylbenzene and xylene were degraded to 0.2 mg/L from 7-8 mg/L (Figure 4.3.9). At this level of BTEX concentration, additional electron acceptor provided was not sufficient to complete biodegradation of benzene. Degraders of other aromatic compounds were able to use nitrate as terminal electron acceptor for their metabolism. That is why those compounds were degraded more than benzene.

The same tendency was observed when the starting total concentration of monoaromatic compounds was increased to 50 mg/L. On the average, benzene was reduced from 14 mg/L to 5 mg/L, and toluene, ethylbenzene and xylene were degraded from 13-14 mg/L to 2-3 mg/L (Figure 4.3.10). This difference was due to the ability of toluene-, ethylbenzene-, and xylene- degraders to switch to nitrate as an electron acceptor.
after oxygen had been depleted. At this concentration, addition of supplemental oxygen in the form of H$_2$O$_2$ was probably not enough and this lack of oxygen became a limiting factor in biodegradation.

In the experiment with total BTEX concentration of 100 mg/L, benzene was degraded from 28 mg/L to 10 mg/L during a 45-day period, while toluene concentration was reduced by 25mg/L, and ethylbenzene and xylene concentrations were reduced by 26 mg/L (Figure 4.3.11). Sorption was also higher at this level of concentration. The difference in the removal efficiency can be explained by additional degradation of toluene, ethylbenzene, and xylene under anaerobic conditions, after depletion of oxygen.

Similar behaviour was observed in the experiment with total BTEX concentration of 200 mg/L, as shown in Figure 4.3.12. While 34 mg/L of benzene was degraded during a 45-day period, 50 mg/L of toluene and ethylbenzene were biotransformed at the same time.

In this study, biodegradation refers to the reduction in BTEX concentration as a result of microbiological activity. No attempts were made to investigate the extent of oxidation. No analyses were performed to measure the products of complete oxidation or metabolic byproducts. Theoretically the extent of BTEX degradation observed required higher concentration of oxygen than was provided (3mg/L of O$_2$ per 1mg/L of BTEX) Thus, the biotransformation was, most probably, not carried out to complete mineralization. The GC analysis also showed accumulation of some intermediate products of microbial metabolism. However, since the BTEX in the concentration up to 20 mg/L was not detected, oxygen present was enough to initiate the degradation sequence.

As results have shown, after available oxygen was depleted, some microorganisms switched to nitrate as alternative electron acceptor. Nitrifiers could degrade toluene, ethylbenzene, and m-xylene, but not benzene. This was confirmed by several experiments using different degradation conditions and BTEX concentrations. In all cases, where nitrate was present, toluene, ethylbenzene, and m-xylene were degraded to a higher degree
than benzene. This could be explained by the fact, that benzene-degraders that were strictly aerobes, could not use alternative electron acceptor for their metabolism. Those microorganisms seized their activity after oxygen had been depleted. The ability of hydrocarbon degraders to utilize nitrate as alternative electron acceptor can be explained by the specific conditions under which these microorganisms were selected. The consortium of microorganisms was cultivated in the reactor where oxygen was provided only by saturation of water used to prepare a feed solution. In this case, oxygen was probably all utilized by the time solution reached the top end of the column. The majority of microbes in the effluent used as inoculum was most likely washed out from the top of the column. Microbes near the top of the column would have to be facultative aerobes, and possess the ability to use alternative electron acceptor to maintain their metabolic activity. Nitrate is thermodynamically the most readily available electron acceptor after oxygen, and it was supplied in abundance, being used as a source of nitrogen. That is why most probably the selected consortium contained facultative anaerobes, able to use nitrate as a terminal electron acceptor. In most cases, the denitrification process was much slower than aerobic respiration. The rate of BTEX transformation was slowing down after 8-9 days, probably due to oxygen depletion, and even in case of toluene, ethylbenzene, and xylene where further degradation was possible, the rates were considerably lower.

Combination of microorganisms, nutrients, and electron acceptor addition has been shown to be the optimum alteration of condition for successful biodegradation of all monoaromatic compounds. BTEX compounds in total concentration of 10 - 20mg/L were degraded after 10 - 15 days respectively. Almost the same effect on the rate of toluene, ethylbenzene, and m-xylene utilization was achieved by addition of microorganisms and nutrients only. This shows that oxygen and nitrate in nutrients were sufficient for TEx-degraders to initiate the biodegradation sequence.

Top-soil used for the experiments was rich in organic content, and irreversible sorption of monoaromatic compounds played significant role with the increase in BTEX
concentration. Sorption could be neglected in experiments, where BTEX concentration was low (10 mg/L - 20 mg/L), but for higher concentration it reached substantial values. In all cases, benzene, toluene, ethylbenzene, and m-xylene were transformed to a higher extent in vials containing microbes, than in sterile controls. But since at high concentration, substantial amounts of monoaromatic compounds, especially ethylbenzene and xylene were sorbed, the actual biodegradation was difficult to assess.

Based on this study, conclusions about the rates and exact pathway of biodegradation process can not be made with a high degree of certainty. Special conditions (small ecosystem) were created in each particular vial, which might possibly be considerably different in another. Different particle size and soil composition, as well as different density and composition of indigenous and inoculated microorganism population, could alter the process of BTEX biotransformation. However, the general tendencies and extent of biodegradation were observed and measured. Working with soil requires use of statistical analysis for verification of any data obtained, and more replicates should have been prepared in order to perform it.

Overall, the biodegradation of the BTEX in the subsurface environment can be achieved by indigenous microorganisms. Different methods can be employed to optimize this process. The best conditions for BTEX microbial utilization were created when the subsurface environment was amended with addition of preselected microorganisms (to start the degradation process), supplemental oxygen (to be used as terminal electron acceptor for aerobic respiration), and nutrients. It is possible to use nitrate both, as a source of nitrogen for cellular growth, and oxygen for anaerobic respiration. Nitrate can be successfully used as a source of oxygen for degradation of toluene, ethylbenzene, and xylene. However, biodegradation of benzene seems to be either impossible or very slow under the denitrifying conditions.
Table 4.3.1 - Biodegradation Rate Constants with Total BTEX Concentration of 10 mg/L

<table>
<thead>
<tr>
<th>Hydrocarbon</th>
<th>Rate Constants, day(^{-1}) with 95% confidence interval, and R(^2) Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Natural Environment</td>
</tr>
<tr>
<td>Benzene</td>
<td>0.20 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>(0.95)</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.23 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>(0.97)</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>(0.97)</td>
</tr>
<tr>
<td>m-Xylene</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>(0.98)</td>
</tr>
</tbody>
</table>
Table 4.3.2 - Time of 90% BTEX Biodegradation with Total Initial Concentration of 10 mg/L

<table>
<thead>
<tr>
<th>Hydrocarbon</th>
<th>Natural Environment</th>
<th>Addition of Nutrients</th>
<th>Addition of Nutrients and Oxygen</th>
<th>Addition of Inoculum</th>
<th>Addition of Inoculum and Nutrients</th>
<th>Addition of Inoculum and Oxygen</th>
<th>Addition of Inoculum, Nutrients, and Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>23</td>
<td>23</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Toluene</td>
<td>20</td>
<td>15</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>20</td>
<td>17</td>
<td>11</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>m-Xylene</td>
<td>20</td>
<td>17</td>
<td>9</td>
<td>11</td>
<td>6</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 4.3.1 - BTEX biodegradation, natural environment
Figure 4.3.3 - BTEX biodegradation enhancement by microorganisms addition
Figure 4.3.4 - BTEX biodegradation enhanced by microorganisms alone and with oxygen addition
Figure 4.3.5 - BTEX biodegradation enhancement by addition of nutrients and oxygen
Figure 4.3.6 - BTEX biodegradation enhancement by addition of microorganisms and nutrients
Figure 4.3.7 - BTEX biodegradation enhancement by addition of microorganisms, nutrients, and oxygen
Figure 4.3.8 - BTEX Biodegradation, 20 mg/L, microorganisms, nutrients, and oxygen added
Figure 4.3.9 - BTEX biodegradation, 30mg/L, microorganisms, nutrients, and oxygen added
Figure 4.3.10 - BTEX Biodegradation, 50 mg/L, microorganisms, nutrients, oxygen added
Figure 4.3.11 - BTEX biodegradation, 100 mg/L, microorganisms, nutrients, and oxygen added
Figure 4.3.12 - BTEX biodegradation, 200 mg/L, microorganisms, nutrients, and oxygen added.
4.4. Phase Three: Biodegradation of BTEX by Inoculated and Indigenous Microorganisms in Soil

In this experiment, soil columns were used to study the degradation of BTEX by indigenous and preselected microorganisms. One of the soil columns was seeded with suspension of microorganisms washed from the activated carbon reactor. The suspension contained about $10^{10}$ cells/mL of viable microorganisms. Several concentration in the range from 10 mg/L total BTEX to 70 mg/L total BTEX were investigated in this experiment. The experiment was started with the lowest concentration and a flow rate of 2.5 mL/h. Concentration was increased after the steady-state was achieved, and BTEX removal was maintained at the same level. The results of the effluent from two columns were compared. During the first 20 days, the reactor was fed with solution with total BTEX concentration of 10 mg/L (Figure 4.4.1). During this period, the reactor seeded with preselected microorganisms showed higher degradation potential. The breakthrough from the both column appeared almost at the same time. However, the difference in the effluent concentration from the column with preselected and indigenous microbes was significant. Concentration of benzene in effluent from the column with adapted biomass was 0.005 mg/L as opposed to 0.590 mg/L in effluent from the column with indigenous soil microorganisms. Effluent concentration from the column without inoculum reached almost the influent level on Day 11, because of the saturation of the sorption capacity. By this time, however, acclimation and accumulation of BTEX-degraders in this column was taking over, and the effluent concentration started to decrease slowly. By Day 17, concentrations of effluent from both reactors were almost the same. This meant that ability to utilize BTEX of biomass on the column with only indigenous microbes almost reached
the degradation ability level of preselected biomass. Steady-state removal was achieved. Overall removal efficiency for 10 mg/L total BTEX reached approximately 84% for benzene, 98% for toluene, 96% for ethylbenzene, and 99% for m-xylene. Biomass was more active in utilizing toluene, m-xylene and ethylbenzene than benzene. The lower benzene degradation was due to the limited oxygen availability. Water used to prepare nutrient solution was aerated before being fed to the reactor, but obviously, the oxygen in the water was not enough to utilize all benzene supplied. As discussed before, toluene, ethylbenzene, and m-xylene degraders could switch to nitrate as a terminal electron acceptor. Figure 4.4.1 shows the biodegradation of BTEX compounds on the soil in continuous reactors using preselected and indigenous microorganisms. The influent concentration reflects the average value. It was rather difficult to maintain the level of BTEX in the feed solution for a period of 20 days, since the new solution had to be prepared after several days. The peaks in influent BTEX concentrations were reflected in the concentration levels of effluent. The peaks in the effluent from the columns with adapted and indigenous microorganisms were recorded at the same time, which left no doubts that the reason behind it was the instability of the influent concentration. Overall, during this phase of the experiment adapted microorganisms showed much better BTEX utilization abilities compared to the indigenous microorganisms in soil.

After steady-state removal of 10 mg/L was achieved, the concentration was raised to 30 mg/L, and the same experiments were performed. At this time both reactors had acclimated biomass, and there was no significant difference in the removal efficiency of preselected and indigenous biomass. Analyses showed that the concentration observed at 24 hours after the beginning of the experiment did not change during the next 36 hours,
and was maintained at the level of 1.36 mg/L for inoculated column, and 1.45 mg/L for indigenous microbes column for benzene; 0.08 mg/L and 0.11 mg/L for toluene; 0.045 mg/L and 0.052 mg/L for ethylbenzene; and 0.02 mg/L and 0.06 mg/L for m-xylene respectively. These results show that biomass in the column without additional microbes had caught up with the inoculated column microorganisms. Both columns were able to remove approximately 8 mg/L of toluene, ethylbenzene, and xylene using nitrate as electron acceptor. Oxygen dissolved in water was enough only to start the degradation sequence, and oxidize approximately 6.7 mg/L of benzene. The removal efficiency for TEX compounds reached 96-99%, while the removal efficiency for benzene was 82% in average. Table 4.4.1 illustrates the removal efficiency of soil columns with adapted and indigenous microorganisms for 30 mg/L total BTEX feed solution.

Similar results were obtained in the experiment with 40 mg/L BTEX. Benzene concentration was steadily removed from 11.01 mg/L to the average of 1.79 mg/L for inoculated, and 2.55 mg/L for indigenous microorganisms, which represents 83% and 77% correspondingly. Much higher removal efficiency was achieved for toluene: 96%, 95% for ethylbenzene, and xylene. Again, by this time, the difference in removal efficiency for columns with indigenous and preselected microorganisms was insignificant - approximately 1% for all compounds (Table 4.4.2).

In the experiments with 70 mg/L total BTEX, the benzene was removed by 83% in the column with indigenous microorganisms, and 86% in the column with preselected microorganisms. At the same time, the level of removal of toluene, ethylbenzene, and xylene was maintained at 96-98% (Table 4.4.3).

In general, inoculation of the subsurface environment with preselected
microorganisms can be a useful factor in increasing the times and rates of biodegradation of BTEX compounds. Differences in the time required for the onset of measurable biodegradation in unaugmented and inoculated soil columns were found to be significant. At the same time, after a certain time, the indigenous population caught up with the preselected population when adequate conditions were provided. This shows that indigenous microorganisms in soil were able to degrade BTEX compound under favourable conditions, but sufficient biomass of degraders had to be accumulated before the biodegradation rates became equal to those for inoculated column. The use of inoculation was beneficial during the initial phase of bioremediation process to shorten the lag-phase before the onset of measurable biodegradation; however oxygen and nutrients were required in the later part of biodegradation.
Figure 4.4.1 - BTEX breakthrough on soil columns with indigenous and adapted microorganisms, 10 mg/L.
<table>
<thead>
<tr>
<th>Aromatic Compound</th>
<th>Average Influent Concentration (mg/L)</th>
<th>Steady-State Effluent Concentration, Indigenous Microorganisms (mg/L)</th>
<th>Percent Removal in the Soil Column</th>
<th>Percent Removal in the Soil Column, Adapted Microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>8.14</td>
<td>1.45</td>
<td>82%</td>
<td>98%</td>
</tr>
<tr>
<td>Toluene</td>
<td>6.47</td>
<td>0.11</td>
<td>98%</td>
<td>99%</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>8.25</td>
<td>0.05</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>m-Xylene</td>
<td>7.05</td>
<td>0.06</td>
<td>99%</td>
<td>99%</td>
</tr>
</tbody>
</table>

Table 4.4.1 - BTEX removal in the soil columns with indigenous and adapted microorganisms, 30 mg/L total BTEX
Table 4.4.2 - BTEX removal in the soil columns with indigenous and adapted microorganisms, 40 mg/L total BTEX

<table>
<thead>
<tr>
<th>Aromatic Compound</th>
<th>Average Influent Concentration mg/L</th>
<th>Steady-State Effluent Concentration, Indigenous Microorganisms mg/L</th>
<th>Percent Removal in the Soil Column</th>
<th>Steady State Effluent Concentration, Adapted Microorganisms mg/L</th>
<th>Percent Removal in the Soil Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>11.01</td>
<td>2.45</td>
<td>77%</td>
<td>1.79</td>
<td>83%</td>
</tr>
<tr>
<td>Toluene</td>
<td>8.54</td>
<td>0.29</td>
<td>96%</td>
<td>0.16</td>
<td>98%</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>10.13</td>
<td>0.60</td>
<td>94%</td>
<td>0.52</td>
<td>95%</td>
</tr>
<tr>
<td>m-Xylene</td>
<td>8.27</td>
<td>0.36</td>
<td>95%</td>
<td>0.35</td>
<td>96%</td>
</tr>
<tr>
<td>Aromatic Compound</td>
<td>Average Influent Concentration mg/L</td>
<td>Steady-State Effluent Concentration, Indigenous Microorganisms mg/L</td>
<td>Percent Removal in the Soil Column</td>
<td>Steady State Effluent Concentration, Adapted Microorganisms mg/L</td>
<td>Percent Removal in the Soil Column</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------------------------</td>
<td>---------------------------------------------------------------------</td>
<td>-----------------------------------</td>
<td>-----------------------------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Benzene</td>
<td>18.39</td>
<td>3.08</td>
<td>83%</td>
<td>2.46</td>
<td>86%</td>
</tr>
<tr>
<td>Toluene</td>
<td>17.38</td>
<td>0.30</td>
<td>98%</td>
<td>0.18</td>
<td>98%</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>18.95</td>
<td>0.82</td>
<td>96%</td>
<td>0.64</td>
<td>96%</td>
</tr>
<tr>
<td>m-Xylene</td>
<td>16.33</td>
<td>0.62</td>
<td>96%</td>
<td>0.45</td>
<td>97%</td>
</tr>
</tbody>
</table>
Chapter V

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

The following conclusions are drawn from this research:

- BTEX compounds can be successfully degraded by microorganisms able to utilize them as the only source of carbon and energy;
- GAC continuous reactors can be used for selection and growth of BTEX-degraders as well as efficient contaminant removal systems;
- BTEX-degraders can be grown up to sufficient density over a period of time if favourable conditions are created;
- Biodegradation of BTEX can be enhanced by addition of nutrients to provide the necessary balance between carbon, nitrogen, and phosphorus;
- Biodegradation of BTEX can be enhanced by addition of supplemental source of electron acceptor: hydrogen peroxide and/or nitrate;
- Nitrate can be used as electron acceptor by degraders of toluene, ethylbenzene, and m-xylene, but not benzene;
- Nitrate play a dual role in enhancement of the biodegradation: as a source of nitrogen for biosynthesis, and source of electron acceptor for microbial metabolisms;
- Addition of preselected microorganisms at certain density allows the onset of measurable biodegradation in shorter time, and complete it at higher
rates;
- Addition of preselected microorganisms is effective in increasing the biodegradation rates at the initial phases of remediation, but later the natural selection takes over and indigenous microbes acquire the ability to degrade target compounds at similar rates;
- BTEX compounds in total concentrations of up to 100 mg/L can be degraded under conditions when supplemental nutrients, oxygen, and microorganisms are supplied;
- Sorption of BTEX on GAC and soil is more significant for m-xylene and ethylbenzene, than for benzene and toluene;
- Sorption of BTEX is playing more significant role as concentration of aromatic hydrocarbons increases.

5.2. Recommendations

In this study, no attempt was made to estimate the degree of mineralization of aromatic hydrocarbons. The presence of intermediate byproducts as a result of microbial activity in biotransformation of BTEX should be investigated since these products may be toxic.

If the analysis shows that by-products are formed and accumulate in the amounts which may pose a risk, the study should be undertaken to identify the possibilities to improve the biodegradation process, so that no hazardous compounds are formed.

In order to determine the real pathway for biodegradation process in soil, the statistical approach to the analysis should be undertaken, and significant amount of
replicates should be analyzed.

For higher concentrations of BTEX, the continuous supply of nutrients and oxygen must be investigated, since biodegradation will exhaust the amounts provided.
REFERENCES


Chaudhuri, B.K., Wiesmann, U., 1995, Enhanced Anaerobic Degradation of Benzene by Enrichment of Mixed Microbial Culture and Optimization of the Culture Medium,


Kao, C.M., 1993, Bioremediation of BTEX Contaminated Aquifers Using Biologically Active Barriers (Benzene, Toluene, Ethylbenzene, Xylene), Ph.D Dissertation, North Carolina State University.


Appendix - Standard Calibration Curves
Benzene Water Standard Calibration Curve,

\[ y = 61908.75x + 2815, \quad R^2 = 0.9995 \]
Toluene Water Standard Calibration Curve,

\[ y = 68414.24x + 1229.1, \quad R^2 = 0.9999 \]
Ethylbenzene Water Standard Calibration Curve,

\[ y = 67320.71x + 1139.2, \quad R^2 = 0.9999 \]
m-Xylene Water Standard Calibration Curve,
y = 71964.26x + 1908.57, R^2 = 0.9997
Benzene Soil Standard Calibration Curve,

\[ y = 31409.72x - 1811.1, \quad R^2 = 0.9999 \]
Toluene Soil Standard Calibration Curve,
\[ y = 18531.08x + 286.72, \quad R^2 = 0.991 \]
Ethylbenzene Soil Standard Calibration Curve,

\[ y = 13971x + 138.38, \quad R^2 = 0.9889 \]
m-Xylene Soil Standard Calibration Curve,
\[ y = 14114.61x + 1405.09, \quad R^2 = 0.9908 \]
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