CHARACTERIZATION OF ANAEROBIC BENZENE-DEGRADING CULTURES

ENRICHMENT AND CHARACTERIZATION OF ANAEROBIC BENZENE-DEGRADING MICROBIAL CULTURES

By

SIOBHAN BURLAND, B.A.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Engineering

McMaster University

MASTER OF ENGINEERING (1998) McMaster University

(Civil Engineering) Hamilton, Ontario

TITLE: Enrichment and Characterization of Anaerobic Benzene-Degrading Microbial Cultures.

AUTHOR: Siobhan Burland, B.A.Sc. (University of Regina)

SUPERVISOR: Professor Elizabeth A. Edwards

NUMBER OF PAGES: xii, 157

ABSTRACT

Biodegradation of benzene, a common groundwater contaminant, occurs readily in the presence of oxygen; however, at contaminated sites, aerobic bacteria often deplete the available oxygen, resulting in anaerobic conditions. Field and laboratory studies have shown that the anaerobic biodegradation of other aromatic hydrocarbons such as toluene occurs readily, while anaerobic benzene biodegradation has only been documented in a handful of studies. Despite these difficulties, benzene biodegradation has been shown to occur under iron-reducing, sulphate-reducing and methanogenic conditions, but not under nitrate-reducing conditions.

The goal of this thesis research was to enrich and characterize the benzene-degrading microbial populations in microcosms and transfer cultures derived from soil from four different sites. Cultures were amended with potential exogenous electron acceptors (nitrate, sulphate, ferric iron) and the rates of biodegradation under different terminal electron accepting processes were determined. Sustained, anaerobic benzene biodegradation was obtained in transfer cultures containing less than 1% of the original soil inoculum. The rate of benzene degradation was variable, ranging from 1 μM/d to more than 75 μM/d. Growth of bacteria was linked to benzene degradation under sulphate-reducing and nitrate-reducing conditions. Growth was very slow, with doubling times of 9-30 days estimated by modelling benzene depletion curves to the

Monod kinetic equation. The rate of benzene degradation was influenced most by biomass concentration and much less by the terminal electron accepting process.

The ratio of moles of electron acceptor depleted to moles of benzene degraded was calculated and compared to the theoretically predicted ratios to confirm putative terminal electron acceptors. Anaerobic benzene degradation linked to iron reduction, sulphate reduction and methanogenesis was observed in enrichment cultures, corroborating results from previous studies. In addition, in some enrichment cultures, benzene degradation was linked to nitrate reduction. This is the first report demonstrating benzene degradation linked to nitrate reduction.

ACKNOWLEDGEMENTS

There are many people who contributed to the successful completion of this thesis. I would first like to thank my supervisor, Dr. Elizabeth Edwards, for allowing me to work with her for the past two years; your enthusiasm for your research is infectious. I would like to thank everybody at McMaster University, especially Anna and all the members of Phoenix Group Therapy. Thanks, Anna, for your technical expertise and invaluable assistance in the lab. I would especially like to thank Gail for making the move to Toronto easier, and for forwarding me my mail for a year.

At the University of Toronto, I would like to thank our new lab technician, Kirsten, for friendship, valuable technical assistance and taking over management of the lab – you're doing a great job! I also greatly appreciate the loan of equipment and all of the assistance I received from Professor Grant Allan's lab group in Chemical Engineering and Dr. Aled Edwards' group in the Best Institute.

I would like to thank Marit Nales for the excellent work that led to the success of my project, and for helping out when we first moved to Toronto.

The completion of this thesis was made possible by the financial support of the Natural Science and Engineering Research Council and McMaster University.

Finally, I would like to thank my friends, especially Monica and Steve for getting me out of Toronto on occasion, and Chris, for great phone conversations.

This thesis is dedicated my family: Mom and Dad, Pat and Elaine, Doug and Cindy, Shannon and Brock, Dave, and Joe. Thanks for being there.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

This thesis presents the results of experiments conducted with anaerobic benzene-degrading mixed microbial cultures derived from contaminated and uncontaminated soil. The microbial activity was transferred from microcosms to enrichment cultures under various anaerobic conditions: sulphate-reducing (Chapter 4), nitrate-reducing (Chapter 5), iron-reducing (Chapter 6) and methanogenic (Chapter 7). Benzene degradation linked to each of these terminal electron acceptors - sulphate, nitrate, ferric iron and carbon dioxide, respectively - was established. The enrichment procedure and associated benzene degradation rates and microbial growth rates are presented in Chapter 3. Sequential use of different electron acceptors by the same microbial community is discussed in Chapter 7.

1.1 Benzene as a Groundwater Contaminant

The extraction and use of petroleum products has resulted in concern over the quality of groundwater and soil at contaminated sites. Petroleum is needed for motive power, lubrication, fuel, dyes, industrial solvents, drugs and synthetics (Harwood and Gibson 1997; Smith 1990). Technological societies, such as Canada and the United States, consume vast amounts of petroleum every year. As of 1992, the United States consumed about 6 billion barrels of oil per year (Montgomery 1992). Due to the extensive production and use of petroleum in the industrialized countries, pollution of

groundwater is widespread in these areas. Pollution can be expected to be found wherever petroleum products have been stored, used or refined (Clark 1995).

Petroleum contains hundreds of different compounds. It occurs naturally in deposits under the earth's surface as the fossil remains of prehistoric forests and seabeds. It is a mixture of hydrocarbons, with some nitrogenous and sulfurous compounds. Petroleum hydrocarbons can be divided into four classes: saturates, aromatics, asphaltenes and resins. The saturates are normal and branched-chain alkanes and cycloparaffins; the aromatics include mono-, di- and polynuclear aromatic compounds with alkyl side chains and/or fused cycloparaffin rings; the asphaltenes include phenols, fatty acids, ketones, esters, and porphyrins; and the resins include pyridines, quinolines, carbazoles, sulfoxides, and amides (Ward et al. 1980; Leahy and Colwell 1990; Sugiura et al. 1997). The petroleum products that cause groundwater pollution are the hydrocarbons used as fuels (gasoline, diesel, jet fuels and heating oils) and as lubricants (oils, greases and cutting oils) (Clark 1995).

Gasoline is of particular concern because of the large number of gasoline storage tanks located across North America. Many of these tanks are buried underground and can leak undetected for many years. Additionally, many underground tanks have been abandoned, left buried, and are a continual source of groundwater contamination. In 1986, it was estimated that about 35% of the United States' 2 million underground storage tanks (UST) for gasoline were leaking. The gasoline from 40% of these leaking tanks had reached groundwater (Beller 1995; Borden et al. 1995; Reinhard et al. 1997).

Gasoline is a mixture of dozens of hydrocarbon compounds, most with molecular weight below 150 (Hadley and Armstrong 1991). Unleaded gasoline contains approximately 50% aliphatic and 50% aromatic compounds by weight; however, the water-soluble fraction is from 87 to 95% aromatic (Wilson et al. 1987; Ball and Reinhard 1996). The aromatics benzene, toluene, ethylbenzene and the three xylene isomers, collectively known as BTEX (Figure 1-1), are the most soluble components of gasoline, thus comprising over 50% by weight of the water-soluble fraction (Cozzarelli et al. 1990; Beller 1995; Vroblesky et al. 1996).

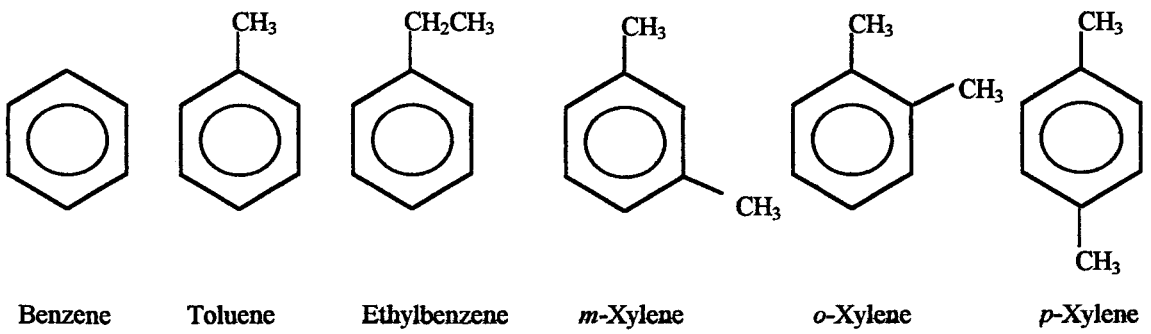


Figure 1-1 Structure of BTEX Compounds

Benzene, with a solubility of 1780 mg/l, is the most water-soluble of the BTEX compounds and typically represents about 2 - 5% of the total weight of gasoline (Hadley and Armstrong 1991). Toluene, with a solubility of 515 mg/l in water, comprises 5 to 7% of gasoline (Evans et al. 1991). Ethylbenzene and the xylenes are

less soluble than either benzene or toluene, at about 140 and 200 mg/l respectively. In comparison, the United States Environmental Protection Agency's drinking water standard for benzene is 0.005 mg/l, for toluene is 2 mg/l, for ethylbenzene is 0.7 mg/l and for the xylenes is 10 mg/l (Allen-King et al. 1994). Sites contaminated with gasoline can therefore readily exceed the drinking water limits for BTEX.

Benzene is of greatest concern because it is the most toxic of the BTEX compounds and is a known carcinogen. Toluene, although less toxic than benzene, is a depressant of the central nervous system (Sittig 1985). In addition, toluene is an enhancing agent in skin carcinogenesis even though it is not carcinogenic itself (Evans et al. 1991). Ethylbenzene and xylene can also affect the central nervous system, although they have not been linked to cancer.

Given that benzene is a common groundwater contaminant and a toxic and carcinogenic compound, benzene-contaminated sites need to be remediated. Bioremediation is an attractive alternative.

1.2 Bioremediation and Natural Attenuation

Bioremediation is an attractive option for remediation of contaminated sites because it results in the complete destruction of the contaminant: oxidation to non-harmful products such as carbon dioxide, methane and water. Bioremediation of gasoline-contaminated sites has traditionally focused on the aerobic degradation of benzene and the TEX compounds because they are known to degrade readily

aerobically. However, under natural conditions, the aerobic bacteria deplete oxygen in the contaminant plume resulting in anaerobic conditions (Zeyer et al. 1986; Grbic-Galic 1990). Therefore, for aerobic biodegradation to continue, additional oxygen must somehow be added to the groundwater. Most of the costs associated with aerobic bioremediation result from the difficulties associated with the introduction and transport of oxygen in the subsurface, as oxygen is very poorly soluble in water (Suflita and Sewell 1991; Lovley et al. 1994).

There has recently been increased interest in the processes of natural attenuation, especially intrinsic bioremediation, as an option for remediation of contaminated sites. Natural attenuation includes all natural processes that remove the source of contamination from the groundwater and soil: dilution, volatilization, sorption, chemical reactions, photodegradation as well as biodegradation. The biodegradation of a contaminant by the native microorganisms under native conditions is referred to as intrinsic bioremediation. If the native conditions are enhanced to stimulate the biodegradation of a contaminant by adding an electron acceptor, for example, the process is termed enhanced bioremediation. Bioaugmentation refers to the process of adding microorganisms capable of degrading the contaminant to the contaminated site. Intrinsic bioremediation is an attractive option for remediation because it is nonintrusive, completely destroys the contaminant by degrading it to metabolic end-products such as CO_2 and CH_4 (as opposed to processes such as volatilization which just move the contaminant to the air), and is usually less costly.

Enhanced bioremediation can also be a less intrusive and less costly alternative to traditional remediation.

Some field studies have confirmed that biodegradation of benzene occurs naturally or with the addition of electron acceptors under anaerobic conditions (Wilson et al. 1987; Hutchins et al. 1991; Davis et al. 1994; Borden et al. 1995). Although the presence of oxygen can never be completely ruled out in field experiments, there was strong evidence supporting the occurrence of anaerobic benzene biodegradation.

Bioremediation of any kind requires a very intensive site assessment and extensive monitoring to ensure that complete degradation of the contaminant is occurring. Monitoring of the contaminant, degradation intermediates and electron acceptors is necessary to characterize the biodegradation occurring at the site. However, the degradation pathways must be well known before intermediates can be used as biomarkers for contaminant degradation. Also, it must be known which electron acceptors can be coupled to the oxidation of the contaminant. Because of the anaerobic conditions usually prevalent at gasoline-contaminated sites, the elucidation of the anaerobic benzene degradation pathways under varying electron-accepting conditions is necessary if intrinsic bioremediation is to be used as a viable remediation strategy. At present, very little is known of the mechanisms of anaerobic benzene biodegradation. One step towards obtaining a pure culture of anaerobic benzene-degrading microorganisms to be used for studying mechanisms and pathways of benzene degradation is the enrichment of existing benzene-degrading mixed cultures.

1.3 Literature Review of Benzene Biodegradation

Hydrocarbon biodegradation is an oxidation-reduction reaction where the hydrocarbon is oxidized (electron donor) and the electron acceptor becomes reduced. Electron acceptors include oxygen, nitrate, iron oxides, sulphate, water, and carbon dioxide. These electron acceptors are present in many groundwaters (Evans et al. 1991). Microorganisms gain energy from the reaction; therefore preferential utilization of these electron acceptors is dictated by the amount of energy gained. Oxygen is most preferred, followed by iron oxides, nitrate, sulphate and carbon dioxide (Borden et al. 1995). However, because of the formation of microhabitats, denitrification, iron reduction, sulphate reduction and fermentation coupled to methanogenesis may occur simultaneously in the same soil (Grbic-Galic 1990; Cozzarelli et al. 1995; Lammey and Noseworthy 1997).

1.3.1 Aerobic Biodegradation of Benzene

Under aerobic conditions, organic matter, including most hydrocarbons, can be mineralized by a large variety of aerobic microorganisms using dioxygen (O_2) as the terminal electron acceptor (Bertrand et al. 1989; Borden et al. 1995). BTEX, including benzene, are rapidly degraded under aerobic conditions (Yadav and Reddy 1993; Reinhard et al. 1997). Because BTEX are fairly reduced compounds they are subject to oxidative transformations (Grbic-Galic et al. 1990).

Aerobic biodegradation is often limited by the low solubility of oxygen in water - less than 15 mg/l at 0°C at the surface - and the slow rate of reaeration to the subsurface groundwater. Therefore the rate of aerobic biodegradation in subsurface waters is controlled by the concentration of contamination, the rate of oxygen transfer into the water and the background oxygen content of the groundwater (Borden et al. 1995). The overall reaction for aerobic benzene mineralization can be simplified as shown below:

$$2\ C_6H_6 + 15\ O_2 \rightarrow 12\ CO_2 + 6\ H_2O \qquad \text{Equation 1.1}$$

The aerobic biodegradation of benzene has been well researched, and the pathways of degradation have been determined. Benzene reacts with molecular oxygen (O_2) in the presence of microbial oxygenases as catalysts. A dioxygenase enzyme adds two oxygen atoms to the benzene ring. This oxygenation results in an unstable ring, which subsequently undergoes ring cleavage (Major et al. 1988; Grbic-Galic 1990). As shown in Figure 1-2, there are two pathways for aerobic benzene biodegradation: the *ortho*-cleavage pathway and the *meta*-cleavage pathway. The two pathways are identical until ring cleavage; catechol is formed by oxidation in both pathways (Lapinskas 1989; Smith 1990).

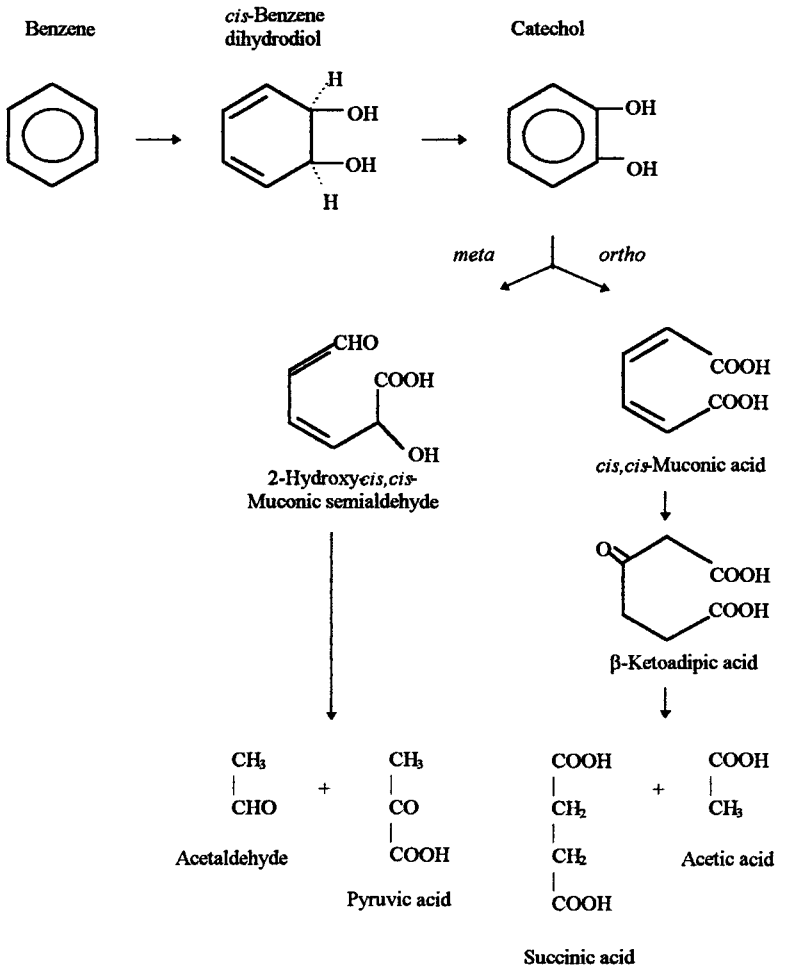


Figure 1-2 Aerobic Benzene Degradation (Lapinskas 1989)

Under natural conditions, the aerobic bacteria deplete oxygen in the contaminant plume. As a result, anaerobic conditions often predominate at contaminated sites.

1.3.2 Anaerobic Biodegradation of Benzene

The capability for anaerobic hydrocarbon degradation appears to be very widespread. Results from studies of contaminated sites in the United States and Canada have clearly demonstrated anaerobic biodegradation of BTEX compounds, including benzene. Although the ability of these microbes to degrade specific compounds under specific conditions is variable, there is some capability for anaerobic degradation of aromatic hydrocarbons in almost every environment (Chee-Sanford et al. 1996).

Toluene is degraded very readily under anaerobic conditions. Degradation generally begins quickly and occurs at a rapid rate. Consequently, anaerobic degradation of toluene has been intensively studied under many electron-accepting conditions. Pure cultures of anaerobic toluene-degraders have been attained, allowing elucidation of the metabolic pathways of anaerobic toluene degradation and identification of the enzymes involved. Benzene, a compound of similar structure, has been found to degrade anaerobically only very slowly if at all. It has proven very difficult to maintain the anaerobic benzene-degrading activity in microbial cultures; consequently no pure cultures of anaerobic benzene-degraders have yet been developed.

Anaerobic benzene degradation has been linked to ferric iron reduction, sulphate reduction and methanogenesis; however, benzene degradation linked to nitrate reduction has not been proven.

Ferric iron reduction. The rate of benzene degradation coupled to iron reduction is limited by the ability of the microorganisms to access the insoluble ferric iron. In fact, benzene has been verified to degrade under iron-reducing conditions only when Fe(III) chelators, such as nitrilotriacetic acid (NTA), disodium ethanol diglycine (EDG) or *N*-methyliminodiacetic acid (MIDA), are added to enhance the availability of Fe(III) for reduction (Coates et al. 1996; Lovley et al. 1996). Ferric sodium ethylenediaminetetraacetic acid (Fe(III)-EDTA) can also be a source of ferric iron for benzene degradation coupled to iron reduction (Lovley et al. 1996).

Sulphate Reduction. Degradation of benzene under sulphate-reducing conditions has been observed by several researchers (Edwards and Grbic-Galic 1992; Lovley et al. 1995; Kazumi et al. 1997; Reinhard et al. 1997).

Fermentation/Acetogenesis/Methanogenesis. Because of the competition between sulphate-reducers and methanogens, methanogenesis often does not begin until sulphate reduction ceases when the sulphate has been depleted (Grbic-Galic 1990). Under methanogenic conditions, degradation of benzene has been reported and a proposed pathway for the degradation to CO_2 and CH_4 was presented (Grbic-Galic and Vogel 1987). It was determined by studies with [^{18}O]water that, under anaerobic conditions, benzene was oxidized and the oxygen came from water. The first intermediate proposed in the anaerobic degradation of benzene was phenol, as shown in Figure 1-3.

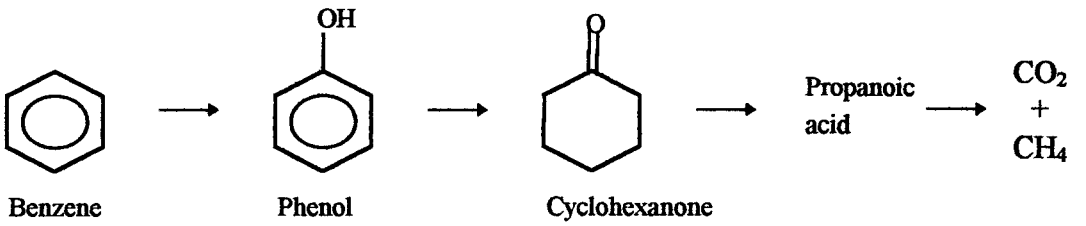


Figure 1-3 Anaerobic Benzene Degradation - Proposed Pathway (Grbic-Galic and Vogel 1987)

This pathway has not been confirmed and no proposed pathways have been published for benzene degradation under any other electron-accepting process.

Nitrate Reduction. Although the other BTEX compounds have been shown to degrade under denitrifying conditions, there is still some skepticism regarding benzene degradation linked to nitrate reduction (Borden et al. 1995). There have been many unsuccessful attempts to establish benzene degradation linked to nitrate reduction. A report summarizing field and laboratory studies indicated that of 34 laboratory studies, only 5 found that benzene degraded under nitrate-reducing conditions, but none of these studies could confirm the link between benzene degradation and nitrate reduction (Aronson and Howard 1997). Nitrate-reducing bacteria generally grow significantly faster than other types of anaerobic bacteria; therefore they are desirable for research studies. Additionally, because most nitrate-reducing bacteria are facultative aerobes, they can be grown quickly aerobically and then be switched to an anaerobic environment. Although benzene was found to degrade under denitrifying conditions in some cases (Major et al. 1988; Morgan et al. 1993; Nales et al. 1998), the link

between the two reactions could not be confirmed. In other cases, benzene was not degraded under denitrifying conditions (Hutchins 1991; Hutchins et al. 1991).

1.4 Research Goals

The goal of this research was to enrich and characterize anaerobic benzene biodegradation from microcosms derived from contaminated and uncontaminated soil:

- to establish growth conditions for sustainable biodegradation of benzene under anaerobic conditions;
- to determine rates of anaerobic benzene biodegradation; and
- to establish the terminal electron-accepting processes in enriched microcosms and transfer cultures derived from various sites.

2 BACKGROUND

2.1 Microbial Ecology of Subsurface Environments

One of the dominant environmental factors in subsurface environments is the availability of oxygen. Oxygen diffuses slowly from the surface and is quickly utilized by aerobic bacteria. The distribution of oxygen concentration from highly aerobic to anaerobic results in a distribution of microorganisms with varying affinity for oxygen. Obligate aerobes require oxygen, facultative aerobes can use oxygen but can also survive in the absence of it, microaerophiles require trace amounts of oxygen but do not grow well in the presence of high concentrations of oxygen, aerotolerant anaerobes do not require oxygen but will grow in its presence, and obligate anaerobes grow only in the absence of oxygen. Microniches develop in soil and sediments, so that even in a generally aerobic environment there will be some anaerobic areas. This means that in a given area, there may be several processes, aerobic and anaerobic, occurring simultaneously (Grbic-Galic 1990). When oxygen becomes depleted, bacteria capable of carrying out anaerobic processes will survive and flourish.

The amount of energy derived from the reduction of the electron acceptor determines which process dominates at a given time. After oxygen, the order of most energy-yielding to least energy-yielding electron-accepting processes is iron reduction, nitrate reduction, sulphate reduction and methanogenesis.

2.1.1 Iron-reducing bacteria

Ferric iron is one of the most common metals present in soils and rocks; thus, iron reduction is a very common process in anaerobic environments. The iron present in the sediments of aquifers can be in crystalline and amorphous forms. Amorphous and poorly crystalline Fe(III) hydroxides, Fe(III) oxyhydroxides and Fe(III) oxides are the most easily microbially reduced forms of iron (Borden et al. 1995). Iron-reducing bacteria reduce ferric iron to ferrous iron while oxidizing organic matter. Ferrous iron, the product of iron reduction, is more soluble in water than ferric iron; therefore the solubilization of iron from the soil into groundwater occurs as a result of iron reduction (Madigan et al. 1997). Because the solubility of ferric iron is very low, microbes must develop special mechanisms of obtaining it. Some studies have shown that most of the Fe(III) in sediments is not available for microbial reduction (Lovley and Phillips 1986). The extra effort thus required to access ferric iron may decrease the efficiency of the reaction. Regardless, iron-reducing bacteria can oxidize many compounds and are an important component of the subsurface microbial community.

2.1.2 Nitrate-reducing bacteria

Nitrate is present in many soils as the product of nitrogen oxidation by aerobic bacteria and electrical discharges (Sawyer et al. 1994). The most common source of groundwater nitrate, however, is fertilizer, as unused nitrate migrates down into the

groundwater (Viessman and Hammer 1993). Most nitrate-reducing bacteria are facultative aerobes; thus, in the presence of oxygen they use it as an electron acceptor and in the absence of oxygen they use nitrate as an electron acceptor. The enzymes required for nitrate reduction are usually repressed by oxygen, so that nitrate reduction occurs only under anaerobic conditions (Madigan et al. 1997). The first product of nitrate (NO_3^-) reduction is nitrite (NO_2^-). The enzyme that catalyzes this reaction is *nitrate reductase*. Subsequent reductions can follow one of two pathways: (1) reduction of nitrite to ammonia (NH_4^+), or (2) reduction of nitrite to nitric oxide (NO), nitrous oxide (N_2O) and nitrogen (N_2). Bacteria that follow the second pathway and reduce nitrate to nitrous oxide or nitrogen are called denitrifyers. Different enzymes are responsible for reduction past nitrite and in fact some bacteria can reduce nitrate only to nitrite, while others can reduce only nitrite (Madigan et al. 1997).

2.1.3 Sulphate-reducing bacteria

Sulphate is formed by oxidation of sulphides that are widely distributed in igneous and sedimentary rocks. Leaching of sulphate from the upper soil layers may also be significant, causing sulphate to be the principal anion of the underlying groundwater (Bouwer 1978). The sulphate-reducing bacteria are obligate anaerobes. Although they do not get as much energy from the reduction of sulphate as many other bacteria do from reducing other electron acceptors, they obtain enough energy to grow, although slowly. The first step is the reduction of sulphate (SO_4^{2-}) to sulphite

(SO_3^{2-}). Sulphite is readily reduced further, eventually to H_2S. There are many bacteria that can reduce sulphite but are unable to reduce sulphate (Madigan et al. 1997).

2.1.4 Fermentative/Acetogenic/Methanogenic bacteria

The catabolism of organic matter can occur by fermentation rather than aerobic or anaerobic respiration. The complete degradation of organic matter to CO_2 and CH_4 under these conditions is the result of the actions of at least three groups of bacteria: fermentative, syntrophic acetogenic and methanogenic. The bacteria previously discussed in this section – iron-reducing bacteria, nitrate-reducing bacteria and sulphate-reducing bacteria – have all been eubacteria. Methanogenic bacteria are evolutionarily distinct from eubacteria; they are Archaeabacteria. Methanogens are obligate anaerobes and have a very narrow substrate range, utilizing only very simple substrates such as hydrogen and acetate. There are two types of methanogenic bacteria: the hydrogen-consuming methanogenic bacteria utilize hydrogen produced by other types of bacteria to reduce CO_2 to CH_4 and the acetoclastic methanogens cleave acetate to CO_2 and CH_4. Therefore, if some other bacteria are present to convert a compound such as benzene to simple substrates, methanogenic bacteria can grow on these simple substrates. During fermentation, the substrate (benzene) acts as both electron donor and acceptor. The original substrate is the electron donor and one of the metabolites produced is the electron acceptor (Madigan et al. 1997). However,

during fermentation the substrate is not fully degraded to CO_2, resulting in a build-up of partially reduced compounds including alcohols and organic acids as well as hydrogen. Syntrophic acetogens convert the reduced intermediates to acetate and hydrogen (Beaty et al. 1986; Thiele et al. 1988; Thiele and Zeikus 1988). The reaction carried out by the acetogens is only energetically favourable under low concentrations of hydrogen; therefore, they rely on the methanogens to reduce the concentrations of hydrogen to low levels. The relationship between these three types of bacteria is known as inter-species hydrogen transfer. The fermentative bacteria produce hydrogen from the substrate, which the methanogens use as an energy source, thereby allowing the acetogens to reduce intermediates of fermentation to acetate and more hydrogen. The degradation of compounds such as benzene requires each member of the consortium to be active.

2.2 Theoretical Considerations

Chemical reactions are accompanied by changes in energy. The amount of energy released during a chemical reaction can be expressed as the total amount of energy released, enthalpy (H), or the energy released that is available to do useful work, free energy (G). The difference between H and G is the energy lost as heat during the reaction. The change in free energy during a reaction is expressed as $\Delta G^{\circ}{}'$, where the superscript ° denotes standard conditions of 25°C and all reactants and products at a concentration of 1 M, and the superscript ' denotes pH 7. The change in

free energy is determined as products minus reactants. A negative ΔG°' means that free energy is released and the reaction occurs spontaneously; a positive ΔG°' means that the reaction does not occur spontaneously (Madigan et al. 1997).

In order to calculate the change in free energy as products minus reactants, the free energy of individual substances must be known. This is the free energy of formation, or the energy required for the formation of a given molecule from its constituent elements (Madigan et al. 1997). By convention, the free energy of formation of all elements is zero. The free energies of formation for compounds of interest to this thesis are presented in Appendix A.

The utilization of chemical energy in living organisms involves oxidation-reduction (redox) reactions (Madigan et al. 1997). An oxidation is defined as the removal of an electron from a substance; a reduction is the addition of an electron to a substance. Redox reactions involve electrons being donated by an electron donor and being accepted by an electron acceptor. The oxidation of benzene, an electron donor, can be expressed as follows:

$$1/30\ C_6H_6 + 3/5\ H_2O \rightarrow 1/5\ HCO_3^- + 6/5\ H^+ + e^- \qquad \text{Equation 2.1}$$

However, electrons cannot exist alone in solution (Madigan et al. 1997). Therefore Equation 2.1 does not represent a real reaction; it is a half reaction. For any oxidation to occur, a subsequent reduction must also occur. The oxidation of benzene could be

coupled to the reduction of many different substances, including oxygen. By convention, the half-reactions are written as oxidations:

$$1/2\ H_2O \rightarrow 1/4\ O_2 + H^+ + e^- \qquad \text{Equation 2.2}$$

The completed redox reaction for benzene oxidation coupled to oxygen reduction is therefore as follows:

$$1/30\ C_6H_6 + 1/4\ O_2 + 1/10\ H_2O \rightarrow 1/5\ HCO_3^- + 1/5\ H^+ \qquad \text{Equation 2.3}$$

In Equation 2.3, benzene is the electron donor and oxygen is the electron acceptor. Other electron acceptors can be coupled to benzene oxidation. Complete redox reactions for benzene oxidation coupled to iron reduction, nitrate reduction, sulphate reduction and carbon dioxide reduction (methanogenesis) can be constructed (Table 2-1).

Substances vary in their tendency to become oxidized or to become reduced; this tendency is designated as reduction potential (E_0') and is related to $\Delta G°$' by the following equation:

$$\Delta G^{\circ\prime} = -nF\ \Delta E_0' \qquad \text{Equation 2.4}$$

where n is the number of electrons transferred, F is the Faraday constant (96.48 kJ / V) and $\Delta E_0'$ is the E_0' of the electron-accepting couple minus the E_0' of the electron-donating couple (Madigan et al. 1997). O-R couples can be sorted by values of $\Delta G^{\circ\prime}$; the more negative the value, the more energy is released during the oxidation-reduction reaction (Table 2-1). It can be seen from the values of $\Delta G^{\circ\prime}$ in Table 2-1 that more energy is gained from benzene oxidation coupled to oxygen reduction, followed by iron reduction, nitrate reduction, sulphate reduction and methanogenesis. Therefore, aerobic bacteria will dominate as long as oxygen is available. Following depletion of oxygen, iron-reducing bacteria will flourish as long as ferric iron is available, and so on down the list of electron acceptors.

From Table 2-1 it can be seen that under iron-reducing conditions 30 moles of ferric iron are required to oxidize one mole of benzene; contrarily, 30 moles of ferrous iron are produced by the oxidation of each mole of benzene. Similarly, it can be seen that for nitrate-reducing conditions, 6 moles of nitrate are required (and 3 moles nitrogen are produced); for sulphate-reducing conditions, 3.75 moles of sulphate are required; and for methanogenic conditions, 3.75 moles of methane are produced. These calculations assume that all of the carbon in benzene is used for energy and results in the production of carbon dioxide and/or methane. However, the microorganisms carrying out this reaction use some of the carbon for cell synthesis.

Table 2-1 Energetic equations and standard free energy changes for benzene oxidation under aerobic, iron-reducing, nitrate-reducing, sulphate-reducing and methanogenic conditions

Electron Acceptor (ox/red)	Overall Energetic Equation	$\Delta G^{\circ\prime}$ (kJ/mol)[a]
O_2/H_2O	$C_6H_6 + 7.5\ O_2 + 3\ H_2O \rightarrow 6\ HCO_3^- + 6\ H^+$	-3180
Fe^{3+}/Fe^{2+}	$C_6H_6 + 18\ H_2O + 30\ Fe^{3+} \rightarrow 6\ HCO_3^- + 30\ Fe^{2+} + 36\ H^+$	-3070
NO_3^-/N_2	$C_6H_6 + 6\ NO_3^- \rightarrow 6\ HCO_3^- + 3\ N_2$	-2990
SO_4^{2-}/H_2S	$C_6H_6 + 3\ H_2O + 3.75\ SO_4^{2-} \rightarrow 6\ HCO_3^- + 1.875\ H_2S + 1.875\ HS^- + 35.625\ H^+$	-200
CO_2/CH_4	$C_6H_6 + 6.75\ H_2O \rightarrow 2.25\ HCO_3^- + 3.75\ CH_4 + 2.25\ H^+$	-116

[a] The data for calculating the standard free energy changes ($\Delta G^{\circ\prime}$) are from McCarty (1971), Thauer et al. (1977) and the CRC Handbook of Chemistry and Physics (1975).

The oxidation of benzene is defined as the removal of electrons to an electron acceptor. The fraction of these electrons that are utilized for cell synthesis is designated f_s. The remainder of electrons are utilized for energy, f_e, so that $f_s + f_e = 1$. A method to calculate f_s and f_e was proposed by McCarty (1975), and is described in Appendix A. Calculated values of f_s and f_e for benzene oxidation linked to each of the processes listed in Table 2-1 are presented in Table 2-2 along with the theoretical cell yield.

Table 2-2 Calculated f_e, f_s and theoretical yield for benzene oxidation coupled to various electron acceptors

O-R couple	f_e	f_s	Theoretical yield (g cells / g benzene)
O_2 / H_2O	0.41	0.59	3.46
Fe^{3+} / Fe^{2+}	0.43	0.57	3.39
NO_3^- / N_2	0.56	0.44	1.96
SO_4^{2-} / H_2S	0.93	0.07	0.22
CO_2 / CH_4	0.96	0.04	0.13

With values for f_s and f_e, the stoichiometric equation can be determined. The half reaction for cell synthesis is as follows:

$$1/24\ C_5H_7O_2N + 13/24\ H_2O \rightarrow 5/24\ HCO_3^- + 1/24\ NH_4^+ + H^+ + e^- \qquad \text{Equation 2.5}$$

If the half reaction for cell synthesis is designated R_c and those for the electron donor (benzene) and the electron acceptor are designated R_d and R_a, respectively, then the overall reaction, R, can be obtained as follows:

$$R = R_d - f_eR_a - f_sR_c \qquad \text{Equation 2.6}$$

The balanced stoichiometric equations for all of the electron acceptors discussed are listed in Table 2-3. These equations can be used to determine the amount of electron acceptor required to oxidize one mole of benzene, shown in bold in Table 2-3. This value can be used to evaluate if a particular electron-accepting process is coupled to benzene degradation by comparing it to measured values of benzene degraded and electron acceptor consumed.

Table 2-3 Stoichiometric equations for benzene oxidation coupled to various electron acceptors

Electron Acceptor (ox/red)	Stoichiometric Equation*
O_2/H_2O	$C_6H_6 + \mathbf{3.075}\ O_2 + 2.26\ H_2O + 0.74\ NH_4^+ \rightarrow 2.31\ HCO_3^- + 0.74\ C_5H_7O_2N + 6\ H^+$
Fe^{3+}/Fe^{2+}	$C_6H_6 + 8.74\ H_2O + \mathbf{12.9}\ Fe^{3+} + 0.71\ NH_4^+ \rightarrow 2.44\ HCO_3^- + 12.9\ Fe^{2+} + 0.71\ C_5H_7O_2N + 18.9\ H^+$
NO_3^-/N_2	$C_6H_6 + 0.77\ H_2O + \mathbf{3.36}\ NO_3^- + 0.55\ NH_4^+ \rightarrow 3.25\ HCO_3^- + 1.68\ N_2 + 0.55\ C_5H_7O_2N + 2.64\ H^+$
SO_4^{2-}/H_2S	$C_6H_6 + 2.91\ H_2O + \mathbf{3.49}\ SO_4^{2-} + 0.088\ NH_4^+ \rightarrow 5.56\ HCO_3^- + 1.74\ H_2S + 1.74\ HS^- + 0.088\ C_5H_7O_2N + 0.77\ H^+$
CO_2/CH_4	$C_6H_6 + 6.55\ H_2O + 0.05\ NH_4^+ \rightarrow 2.15\ HCO_3^- + \mathbf{3.6}\ CH_4 + 0.05\ C_5H_7O_2N + 2.4\ H^+$

*bolded numbers represent useful stoichiometric coefficients for determining the terminal electron accepting process

2.3 History of Cultures

Previous to this work, a survey of benzene-degrading activity form six sites in North America resulted in sustained benzene degradation in microcosms from four of the six sites. Enrichment and characterization of this benzene-degrading activity is the subject of this thesis.

The original study (Nales 1997) involved the construction of 18 microcosms containing soil and groundwater (or a defined medium) from each of the six sites. The 18 microcosms were prepared as duplicates of nine different treatments: groundwater alone; groundwater and TEX; groundwater and sulphate; medium alone; medium and sulphate; medium, sulphate and TEX; medium and nitrate; medium and ferric iron; and sterile controls. All of these microcosms were further amended with benzene. The microcosms were constructed in 250-ml glass bottles sealed with mininert caps and were stored in an anaerobic chamber. This section briefly describes each site and the results of the original study (Nales 1997).

Gas Station 1

Gas Station 1 was sampled June 1995. Soil and groundwater were collected from a decommissioned gas station in Toronto, Ontario. The gasoline tanks had been removed two years previous to sampling and bioremediation had been attempted. Benzene degradation linked to sulphate reduction was identified in microcosms from this site. In microcosms amended with nitrate, benzene degraded only in the presence

of nitrate and stopped when nitrate was depleted, suggesting but not proving that benzene degradation was linked to nitrate reduction.

Gas Station 2

Gas Station 2 was sampled June 1995. Soil and groundwater were collected from a decommissioned gas station in Toronto, Ontario. The gasoline tanks had been removed the day before sampling. There was no significant benzene degradation in any of the microcosms from this site (Nales 1997).

Oil Refinery

Oil Refinery was sampled July 1995. Soil and groundwater were obtained from an oil refinery site in Ponca City, Oklahoma. The site had been chronically exposed to hydrocarbons. Benzene degraded in all of the active (non-sterile) microcosms. Benzene degradation was linked to iron reduction; however, there was some evidence that sulphate-reduction may also have been involved.

Landfarm

Landfarm was sampled May 1995. Soil and groundwater were collected from a petroleum refinery land-farming site near Oakville, Ontario. The soil samples were collected from active land-treatment areas below the tilling depth. Benzene degradation linked to sulphate-reduction was identified in microcosms from this site. In the presence of nitrate, the sulphate was not depleted as the benzene was degraded,

suggesting that the nitrate-reducers out-competed the sulphate-reducers and that nitrate could be an electron acceptor coupled to benzene degradation.

Oil Refinery Terminal Site

The Oil Refinery Terminal Site was sampled August 1995. The soil was collected from below the water table at a terminal site in Columbia, South Carolina. The groundwater used with these microcosms was pristine water from the aquifer at CFB Borden in Ontario. The microcosms from this site produced very puzzling results (Nales 1997). In all of the microcosms, exactly three amendments of benzene were depleted; subsequently all benzene depletion ceased. One experiment was carried out to determine if there was a toxic compound associated with the sediment (Appendix B). However, there were no conclusive results and the microcosms from this site were not further studied.

Uncontaminated Swamp

The Uncontaminated Swamp material was collected from a pristine fresh water swamp that drains into a small lake near Perth, Ontario. Benzene degradation in the presence of sulphate and nitrate occurred after very long lag times.

These original microcosms were reamended with benzene when it became depleted. After several refeedings the microcosms were considered to be enriched for benzene-degrading microorganisms and are termed 'enriched microcosms'. After

sustained benzene-degrading activity was obtained in enriched microcosms, the activity was transferred to new bottles by removing some of the culture liquid and diluting with medium. These culture bottles are termed 'transfer cultures'. As part of the original study, transfer cultures were prepared from enriched microcosms from Gas Station 1 and Oil Refinery as shown in Appendix C. These transfer cultures and the enriched microcosms from Gas Station 1, Oil Refinery, Landfarm, and Uncontaminated Swamp were studied further, as described in this thesis.

3 GROWTH CONDITIONS AND CULTURE DEVELOPMENT

While the purpose of the previous study was to assess the potential for benzene-degrading activity by screening several sites, the focus of this study was to better understand the growth conditions required for sustaining anaerobic benzene degradation and to further characterize the microbial populations. One of the first tasks in this study was to develop enriched mixed cultures with steady degradation rates. Very few studies have been reported on anaerobic benzene degradation because the microorganisms responsible are difficult to sustain in culture. Benzene degrading activity may be observed in microcosms, but is lost on transfer or with time.

This chapter describes the enrichment and growth conditions that proved successful for long-term maintenance of anaerobic benzene-degrading microbial cultures. The procedures followed to obtain sustained anaerobic benzene-degrading activity in transfer cultures derived from enriched microcosms is described in Section 3.1. The rates of anaerobic benzene degradation were determined and the effect of culture dilution and increased concentration were evaluated (Section 3.2). An analysis of growth kinetics is presented in Section 3.3.

3.1 Culture Maintenance

3.1.1 Medium Composition

Transfer cultures were made with pre-reduced, defined mineral medium as described in Edwards et al.(1992). The following constituents were added to distilled, deionized water to make one litre: 10 ml of phosphate buffer (27.2 g KH_2PO_4 and 38.4 g K_2HPO_4 per litre), 10 ml of salt solution (53.5 g NH_4Cl, 7.0 g $CaCl_2 \cdot 6\ H_2O$, 2.0 g $FeCl_2 \cdot 4\ H_2O$ per litre), 2 ml of trace mineral solution (0.3 g H_3BO_3, 0.1 g $ZnCl_2$, 0.1 g $Na_2MoO_4 \cdot 2\ H_2O$, 0.75 g $NiCl_2 \cdot 6\ H_2O$, 1.0 g $MnCl_2 \cdot 4\ H_2O$, 0.1 g $CuCl_2 \cdot 2\ H_2O$, 1.5 g $CoCl_2 \cdot 6\ H_2O$, 0.02 g Na_2SeO_3, 0.1 g $Al_2(SO_4)_3 \cdot 18\ H_2O$, and 1 ml concentrated H_2SO_4 per litre), 2 ml of $MgCl_2 \cdot 6\ H_2O$ solution (48.8 g per litre), and 1 ml of redox indicator stock solution (1 g resazurin per litre). The mixture was autoclaved, purged with N_2-CO_2 (80%:20%) while cooling, and then transported into an anaerobic chamber. The remaining three constituents were added from sterile, anaerobic stocks: 10 ml of saturated bicarbonate solution (200 g of $NaHCO_3$ per litre), 10 ml of filter-sterilized vitamin stock solution (0.02 g of biotin, 0.02 g of folic acid, 0.1 g of pyridoxine hydrochloride, 0.05 g of riboflavin, 0.05 g of thiamine, 0.05 g of nicotinic acid, 0.05 g of pantothenic acid, 0.05 g of *p*-aminobenzoate [PABA], 0.05 g of cyanocobalamin, and 0.05 g of thioctic acid per litre), and 10 ml of an amorphous ferrous sulphide solution (39.2 g of $(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$ and 24.0 g of $Na_2S \cdot 9\ H_2O$, which has been washed three times with deionized water to remove free sulphide, per litre).

The redox indicator resazurin was included in the medium to indicate oxidation-reduction potentials above -0.042. The reduction of resazurin takes place in two stages: first, resazurin (blue) is reduced to resorufin (pink); second, resorufin (pink) is reduced to dihydroresorufin (colourless). The first reduction, from blue to pink, is non-reversible; the second reduction is reversible. Therefore, when the medium is first reduced, it changes colour from blue to colourless; if oxygen should subsequently enter the medium and raise the oxidation-reduction potential, the medium will change to a pink colour (Twigg 1945; Hungate 1969).

3.1.2 Storage of Enriched Microcosms and Transfer Cultures

All enriched microcosms and transfer cultures were stored in the dark in an anaerobic chamber (COY Laboratories Products Inc., Ann Arbor, Mich.), which was supplied with a gas mix of 10% H_2, 10% CO_2 and 80% N_2. All sampling and other procedures, except purging the headspace of culture vials, was performed in the anaerobic chamber. Purging of the headspace, although performed outside of the anaerobic chamber, did not introduce oxygen into the cultures as the resazurin indicator remained clear in all purged cultures. To minimize the time outside of the anaerobic chamber, cultures were removed one at a time and were replaced as soon as purging was complete.

3.1.3 Amendment of Cultures with Potential Electron Acceptors

To avoid adding oxygen with the sulphate, nitrate or ferric iron, stocks of each potential electron acceptor were prepared, purged with N_2/CO_2 gas mix and stored inside the anaerobic chamber. Sulphate was initially added as $FeSO_4$. The addition of Fe(II) is desirable in sulphate-reducing cultures because it reacts with the HS^- produced to precipitate as FeS, thus removing the toxic sulphide from solution. An anaerobic stock of $FeSO_4$ was prepared but did not remain reduced and was therefore not used. Subsequently, sulphate was added to enriched microcosms and transfer cultures from an anaerobic 500-mM stock of Na_2SO_4. The concentration of sulphate added to cultures was variable, but generally remained within the range of 2.5 - 10 mM. Nitrate was added to enriched microcosms and transfer cultures from an anaerobic 500-mM stock of $NaNO_3$. The concentration of nitrate added to transfer cultures was variable, but generally remained within the range of 2.5 - 10 mM. The form of ferric iron originally added to the microcosms was $Fe(OH)_3$. This form of iron was prepared as described in Nales (1997) and added to some cultures. However, the $Fe(OH)_3$ did not appear to stimulate benzene biodegradation; therefore, another source of ferric iron, ferric iron sodium ethylenediaminetetraacetic acid (Fe(III)-EDTA), was added as a powder (13% Fe^{3+}) to the cultures. In some experiments, the EDTA was added as a liquid from a 514 mM Fe^{3+} stock. The concentration of ferric iron added was variable, but generally remained within the range of 10 - 20 mM.

3.1.4 Enrichment Culture Development

Enriched microcosms were developed by repeatedly amending the original microcosms with benzene and sulphate or nitrate when they became depleted (Figure 3-1). Benzene was reamended from a neat anaerobic stock when the concentration fell below 1.0 mg/l. Typically, a liquid concentration of about 10 mg/l benzene was fed to the culture. Sulphate and nitrate were reamended when they could no longer be detected by ion chromatography, or when benzene biodegradation stopped and it was assumed that the sulphate or nitrate was depleted. In the latter case, samples were analyzed to confirm the depletion of the electron acceptor, but the cultures were reamended immediately.

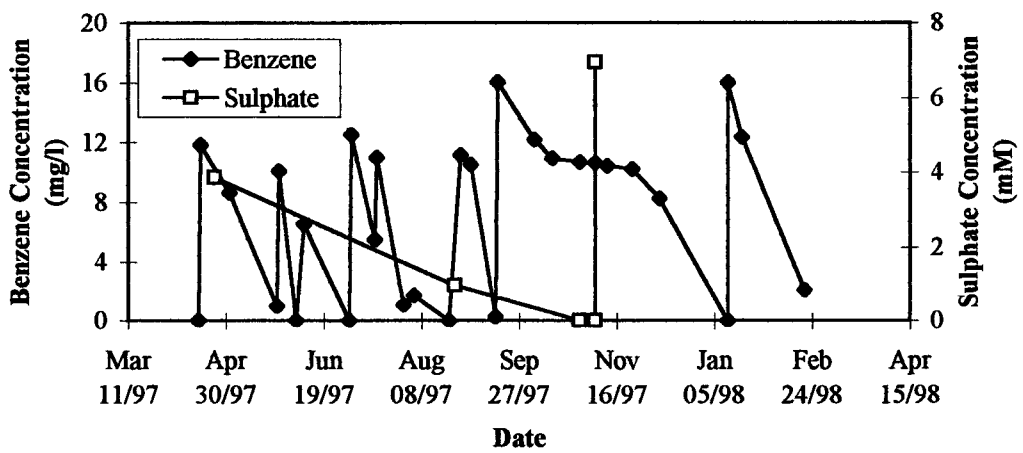


Figure 3-1 Enrichment process of a Gas Station 1 Microcosm

3.1.5 Transfer Culture Development

Transfer cultures were developed by transferring material from enriched microcosms into new bottles and sealing with mininert caps. Transfers were performed either by centrifuging the inoculum and resuspending in medium or by splitting the inoculum and diluting with the medium. The inoculum was either a settled, liquid only portion of the enriched microcosm or a well-mixed slurry sample. Some of the transferred cultures were further treated by purging the headspace of the new bottle with a nitrogen / carbon dioxide mix or with helium to remove any traces of hydrogen, which was present in the atmosphere of the anaerobic chamber. Transfer cultures were prepared in various dilutions, ranging from undiluted to 80% diluted. The summary of transfer culture development in Appendix C indicates the type of transfer performed for each transfer culture and the approximate percent of original culture remaining in the transfer source. Sustained anaerobic benzene degradation was maintained in transfer cultures with as little as 1% of the original culture. Transfer cultures were further enriched as previously described.

3.1.6 Analytical Procedures

<u>Benzene Analysis</u>

Benzene concentrations were monitored by removing a 300 µl sample of headspace from the enriched microcosms or transfer culture bottles with a 500 µl

Pressure-Lok® gas syringe (Precision Sampling Corp., Baton Rouge, Louisiana) and injecting the sample onto a gas chromatograph (Hewlett Packard 5890 Series II) equipped with a Supel-Q plot column (0.53 mm x 30 m, Supelco Co.) and a flame ionization detector. The injector temperature was 200°C, the oven temperature was 160°C and the detector temperature was 250°C. The carrier gas was helium at a flow rate of 11 ml/min. All sampling was performed in the anaerobic chamber.

Methane Analysis

Methane concentrations were measured as described for benzene above. One injection of a 300 µl sample was sufficient to analyze both benzene and methane. However, if methane concentrations exceeded 1%, the signal from the detector reached a maximum; therefore methane concentrations above this value were not accurately determined by this method. At higher concentrations, methane was measured more accurately in a separate injection of a 300 µl sample of culture headspace onto a gas chromatograph (Hewlett Packard 5700A) equipped with an Alltech carbosphere packed column and a flame ionization detector. The injector temperature was 200°C, the oven temperature was 200°C and the detector temperature was 200°C. The carrier gas was helium at a flow rate of 60 ml/min. All sampling was performed in the anaerobic chamber.

Sulphate / Nitrate / Nitrite Analysis

Sulphate, nitrate and nitrite concentrations were measured by removing a 0.5 ml liquid sample from the enriched microcosms or transfer culture bottles and injecting the supernatant of a centrifuged sample onto a Dionex ion chromatograph with an AS4A column. The eluent was a 1.8 mM sodium carbonate / 1.7 mM sodium bicarbonate solution at a flow rate of 2.0 ml/min. Samples were diluted by weight to below 0.5 mM before injection. Samples that contained EDTA could not be injected onto the ion chromatograph as the EDTA reacted with the column. These samples were taken as above, centrifuged and frozen in case a method of removing the EDTA was found.

Ferrous Iron and Total Iron Analysis

Ferrous iron analysis was performed as previously described (Lovley and Phillips 1986; Beller et al. 1992). A 0.2 ml slurry sample was removed from the transfer culture and extracted in 5 ml of 0.5 N HCl for 1 hour. After the extraction, 0.2 ml of the mixture was added to 5 ml of ferrozine (1 g per litre) in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer (pH 7). After shaking for 15 seconds, the A_{562} or A_{560} was determined. Before July, 1998, the A_{560} was determined on a Bausch & Lomb Spectronic 20; after July, 1998, the A_{560} was determined on a Milton Roy Company Spectronic 21. Standards were prepared by dissolving a known amount of ammonium iron(II) sulphate hexahydrate in 0.5 N HCl and preparing as above.

Total iron (ferrous iron plus hydroxylamine-extractable ferric iron) was determined as described by Lovley and Phillips (1987). The method is similar to that for determining ferrous iron described above except that the 0.2 ml sample was extracted in 5 ml of 0.25 M hydroxylamine hydrochloride in 0.25 M HCl. Following one hour extraction, 0.2 ml of this mixture was added to 5 ml of the ferrozine in HEPES and the A_{560} was determined. Ferric iron was then calculated by subtracting the measured Fe(II) from the measured total iron. The estimated detection limit for both ferrous iron and total iron measurements was 0.1 mM.

Protein Analysis

Protein was measured by the method of Bradford (1976) using a microassay kit (Biorad) and bovine serum albumin (BSA) as a standard. The cell pellet from 5 or 10 ml of culture was resuspended in 600 µl of 0.66 N NaOH for 12 hours at 35°C to solubilize protein. After centrifugation, the supernatant was removed, neutralized with 200 µl 2 N HCl, mixed with dye reagent (200 µl) and measured spectrophotometrically (Milton Roy Company Spectronic 21) at 595 nm. A blank was prepared with 800 µl ddH_2O and 200 µl dye reagent.

3.2 Benzene Degradation Rates

3.2.1 Factors Affecting Benzene Degradation Rate

The rate of benzene degradation in enriched microcosms and transfer cultures is an indication of the growth of the microorganisms in the culture bottle. As a culture becomes more enriched for benzene-degrading microorganisms, the rate of benzene degradation should increase. Figure 3-2 illustrates the increase in the rate of benzene degradation in Uncontaminated Swamp microcosm #5b. Note that an increase in the rate of sulphate utilization coincided with the increased rate of benzene degradation. This is strong evidence that sulphate was the electron acceptor in this microcosm.

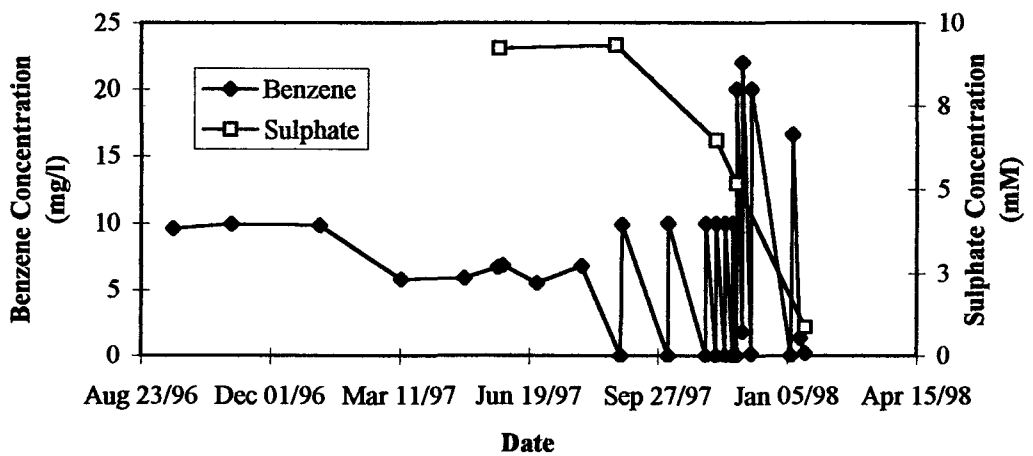


Figure 3-2 Enrichment of Uncontaminated Swamp Microcosm #5b

Some factors that might slow the rate of benzene degradation include dilution of the culture, build-up of a toxic compound and depletion of the electron acceptor. The effect of dilution is to decrease the concentration of microorganisms in the culture bottle. The rate of total benzene degradation will subsequently decrease. In the Gas Station 1 enriched microcosm shown in Figure 3-3, benzene degradation stopped when sulphate became depleted. Before addition of sulphate, the culture was diluted 40%; subsequent benzene degradation proceeded at a reduced rate for two feedings. The third feeding after dilution was degraded at approximately the same rate as before the dilution. If cultures were diluted too much, benzene biodegradation no longer occurred. For example, benzene biodegradation was not observed in 80%-diluted transfer cultures inoculated with liquid from a sulphate-reducing culture or from an iron-reducing culture. These data suggest that a critical mass of cells is required to sustain the activity on transfer.

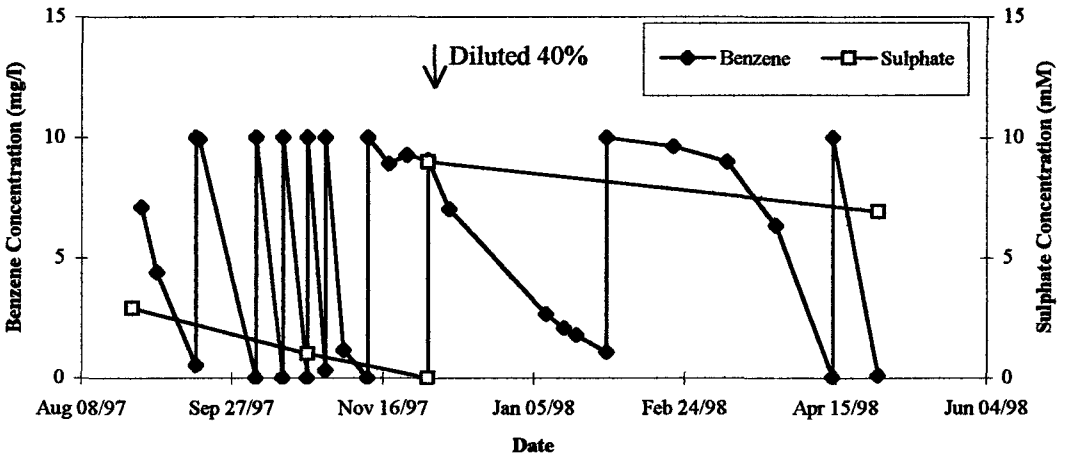


Figure 3-3 Effect of dilution on rate of benzene degradation

In a batch microcosm, by-products and waste are not continuously removed from the system. Thus a build-up of hydrogen sulphide in sulphate-reducing environments or a build-up of nitrite in nitrate-reducing environments can occur. These and other compounds are toxic to microorganisms and may result in a decrease in the rate of biodegradation. The rate of benzene degradation was also affected by the concentration of electron acceptor. When the electron acceptor for benzene degradation became limiting, benzene degradation slowed and eventually stopped until an acceptable electron acceptor was provided (Figure 3-3).

3.2.2 Experiment 3.1. Effect of Benzene Concentration and Culture Dilution

As described in the previous section, culture dilution resulted in a significant decrease in the rate of benzene biodegradation. In order to further study the effects of culture dilution and benzene concentration, the following experiment was designed with inoculum from a methanogenic Oil Refinery transfer culture.

Rationale. Enriched microcosms and transfer cultures were repeatedly amended with about 10 mg/l benzene. The ability to increase this concentration without harming the microorganisms was desirable for several reasons. First, it would decrease maintenance time by allowing cultures to be fed at less frequent intervals. Second, it would increase the microbial mass at a greater rate by supplying a larger source of carbon. Third, it would allow more accurate mass balances by increasing the amounts of both benzene and electron acceptor consumed.

Typically, transfer cultures were made by diluting an enriched culture about 50% with fresh medium. The ability to increase the dilution while sustaining benzene-degrading activity would allow transfer cultures to be made with less carryover of by-products from the source. Additionally, a pure culture could be obtained faster with higher dilutions.

Experimental design. The inoculum was added to 17-ml vials sealed with mininert caps. Three culture dilutions were tested - 100% (undiluted), 10% and 1% - and four benzene concentrations were tested - 10 mg/l, 25 mg/l, 50 mg/l and 100 mg/l.

Each condition was tested in duplicate; therefore there was a total of 24 vials. Benzene was measured as previously described in Section 3.1.6.

Results. The results of this experiment are shown in the following three graphs. In the 100% series, all concentrations of benzene were degraded at about the same rate (Figure 3-4). This indicates that, at least for Oil Refinery cultures, the concentration of benzene amended could be significantly increased without affecting the rate of benzene biodegradation.

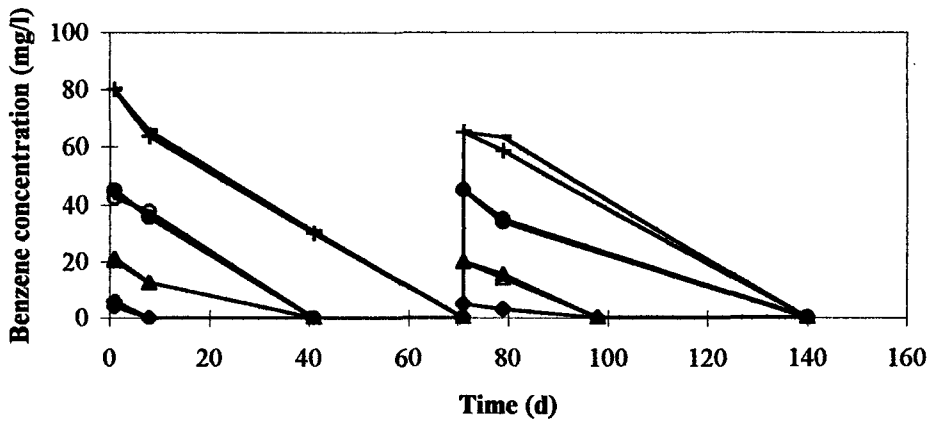


Figure 3-4 Experiment 3.1. Benzene concentration vs. time: Oil Refinery - 100% Inoculum

In the 10% series, the benzene was degraded in the vials containing 10, 25 and 50 mg/l benzene and in one of the two replicates containing 100 mg/l benzene. The degradation rate was slower than for the 100% series (Figure 3-5). In the 1% series, benzene degradation occurred only in one of the vials containing 10 mg/l benzene

(Figure 3-6). A very long incubation time may be required for benzene to be degraded in the 1% dilutions.

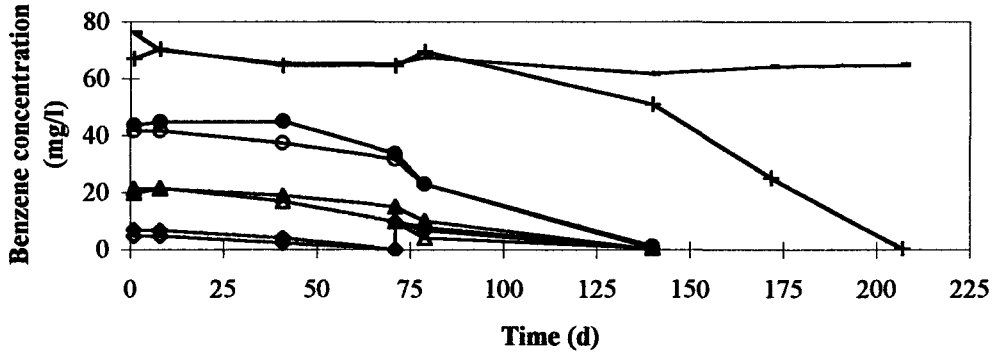


Figure 3-5 Experiment 3.1. Benzene concentration vs. time: Oil Refinery - 10% Inoculum

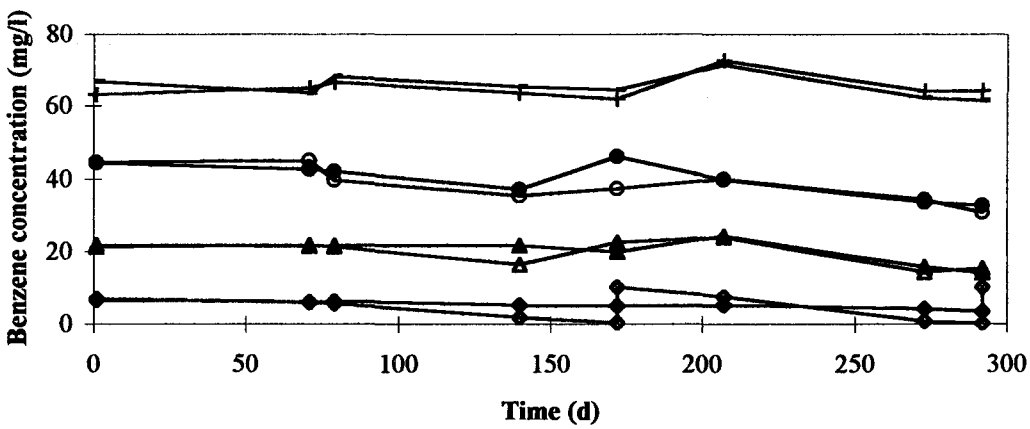


Figure 3-6 Experiment 3.1. Benzene concentration vs. time: Oil Refinery - 1% Inoculum

Conclusions. Transfer cultures could not be diluted substantially more than the typical 50% dilution without resulting in substantial loss of benzene-degrading ability. The concentration of benzene fed to enriched, undiluted cultures could be increased to at least 100 mg/l without decreasing the degradation rate. Cultures that have been significantly diluted, however, are affected by an increase in benzene concentration. The benzene concentration should only be increased if the biomass is well established. For a given cell concentration, this Oil Refinery transfer culture has a benzene degradation rate independent of benzene concentration - zero order kinetics at the concentrations of benzene tested. It was previously found that transfer cultures from Gas Station 1 also exhibited zero order kinetics (Nales 1997).

3.2.3 Summary of benzene biodegradation rates

A comparison of the rates of benzene biodegradation in enriched microcosms and transfer cultures revealed that the rate was not strongly dependent upon the type of electron acceptor nor the original inoculum source (Table 3-1 and Table 3-2), although sulphate-reducing cultures generally seemed to have the slowest rates of benzene degradation. The maximum rate of benzene degradation ranged from 1.3 to 75 μM/d with an average of about 15 μM/d. The rate was extremely variable within any given culture.

Table 3-1 Maximum benzene degradation rates

Site	Culture	Electron acceptor	Maximum rate (μM/d)	Average rate mean (SD)
Gas Station 1	6b(1)b	SO_4	17.9	
Gas Station 1	Combo1	SO_4	17.9	
Gas Station 1	5b(a)III	SO_4	17.9	
Gas Station 1	5b(a)II	SO_4	15.4	
Gas Station 1	5b	SO_4	15.4	
Gas Station 1	9b	SO_4	14.1	
Gas Station 1	5b(b)	SO_4	12.8	
Gas Station 1	3b	SO_4	12.4	
Gas Station 1	5b(a)1	SO_4	10.3	
Gas Station 1	6b(3)	SO_4	10.3	
Gas Station 1	2b	SO_4	10.1	
Gas Station 1	6b(1)a	SO_4	9.0	
Gas Station 1	10b	SO_4	7.7	
Gas Station 1	6b(2)a	SO_4	1.3	12.3 (4.7)
Oil Refinery	6b(2)	SO_4	16.7	
Oil Refinery	gen(ii)3	SO_4	12.8	
Oil Refinery	Combo2	SO_4	10.3	
Oil Refinery	gen(ii)1	SO_4	5.8	11.4 (4.6)
Uncontaminated swamp	5b	SO_4	57.7	
Gas Station 1	13b	NO_3	30.8	
Gas Station 1	14b	NO_3	16.7	23.7 (10)
Uncontaminated swamp	2b	NO_3	19.2	
Uncontaminated swamp	13b	NO_3	15.4	17.3 (2.7)
Gas Station 1	4b	CH_4	21.8	
Oil Refinery	11b	CH_4	33.3	
Oil Refinery	Combo1	CH_4	19.2	
Oil Refinery	9b	CH_4	14.1	
Oil Refinery	8b(1)	CH_4	9.6	
Oil Refinery	3b	CH_4	7.7	
Oil Refinery	6b(2)	CH_4	6.4	
Oil Refinery	6b(1)	CH_4	5.1	13.6 (10)

Rates of benzene biodegradation were also determined from data collected during experiments designed for establishing the terminal electron acceptor. For these experiments, the relative concentration of the inoculum could be approximated by the dilution of the culture prior to the experiment, and therefore the effect of cell concentration on degradation rate could be observed. In most cases, the first feeding of benzene after a culture was diluted was degraded slowly; subsequent feedings of benzene were degraded more rapidly. A summary of the rates of benzene degradation, electron-accepting condition and the reference to the chapter of this thesis in which the experiment is described is shown in Table 3-2. The experiments using undiluted nitrate-reducing inoculum (5.1 and 5.2) exhibited the highest rates of benzene degradation. The Oil Refinery iron-reducing and methanogenic cultures also had high rates of benzene degradation. The amount of culture dilution affected the rate of benzene degradation; those cultures diluted more than 50% or transferred with liquid only degraded benzene at the slowest rates (Gas Station 1 Experiments 5.6, 5.7 and 5.8 in Table 3-2). The rate of benzene biodegradation was dependent upon the cell concentration. The fact that dilution was the most significant rate-determining factor suggests that the growth rate of these anaerobic bacteria was very slow (see next section).

Table 3-2 Summary of rates of benzene degradation in experiments

Original Site	Experiment #	Electron Acceptor	Rate (μM/d)	Type of Transfer	Thesis section
Gas Station 1	4.1	SO_4	4.5	slurry; 50%	4.3
Oil Refinery	4.2	SO_4	3	slurry; 100%	4.3
Gas Station 1	5.1	NO_3	75	slurry; 100%	5.3.1
Gas Station 1	5.2	NO_3	20	slurry; 50%	5.3.2
Gas Station 1	5.4	NO_3	12	slurry; 50%	5.3.4
Gas Station 1	5.6	NO_3	7.5	liquid; 50%	5.3.6
Gas Station 1	5.7	NO_3	8.9	slurry; 25%	5.3.7
Gas Station 1	5.8	NO_3	7.5	liquid; 20%	5.3.8
Uncontaminated Swamp	5.7	NO_3	7.3	slurry; 100%	5.3.7
Gas Station 1	4.1	CH_4	1.3	slurry; 50%	4.3
Oil Refinery	3.1	CH_4	16	slurry; 100%	3.2.2
Oil Refinery	-	Fe(III)	15	slurry; 100%	7.3.1

3.3 Growth Kinetics

The growth rate of bacteria as a function of substrate concentration can be expressed as the Monod empirical equation (Monod 1949):

$$\mu = \mu_{max} S / (K_s + S) \qquad \text{Equation 3.1}$$

where μ is the specific growth rate (biomass formed per unit biomass per unit time), μ_{max} is the maximum specific growth rate occurring at high substrate concentration, S is limiting substrate concentration and K_s is the saturation coefficient or substrate

concentration at which specific growth rate is one half of μ_{max}. Equation 3.1 was modified to the following (McCarty 1971):

$$\mu = Y_m k_m S/(K_s + S) - b \qquad \text{Equation 3.2}$$

where Y_m is the maximum yield factor, mg cells / mg substrate, k_m is the maximum substrate utilization rate, d^{-1}, and b is the organism decay rate. When the value of S is large relative to K_s, the maximum growth rate occurs and, assuming the decay coefficient to be negligible for anaerobic conditions (McCarty 1971), the maximum growth rate can be defined as follows:

$$\mu_{max} = Y_m k_m \quad (b << Y_m k_m) \qquad \text{Equation 3.3}$$

The rate of growth of a microbial culture can be estimated by the following equation (Robinson and Tiedje 1983):

$$dX/dt = [\mu_{max} S / (K_s + S)]X \qquad \text{Equation 3.4}$$

where X is biomass concentration. The rate of change of substrate consumption by a bacterium growing in a batch culture can be described by the following equation (Robinson and Tiedje 1983):

$$dS/dt = -[\mu_{max}S / (K_s + S)] X/Y \qquad \text{Equation 3.5}$$

where Y is the yield coefficient. The biomass concentration at any time, t, can be determined by Equation 3.6:

$$X = Y (S_0 - S) + X_0 \qquad \text{Equation 3.6}$$

where S_0 and X_0 are the initial substrate concentration and initial biomass concentration, respectively. Equation 3.5 can then be rewritten as

$$dS/dt = -[\mu_{max}S/(K_s + S)][Y(S_0 - S) + X_0] / Y \qquad \text{Equation 3.7}$$

Integration of Equation 3.7 results in the following equation:

$$C_1 \ln\{[Y(S_0 - S) + X_0]/X_0\} - C_2 \ln \{S/S_0\} = \mu_{max} t \qquad \text{Equation 3.8}$$

where $C_1 = (K_sY + S_0Y + X_0)/(YS_0 + X_0)$ and $C_2 = K_sY / (YS_0 + X_0)$. If the substrate depletion curve (i.e. benzene concentration vs. time) is known, and the initial biomass concentration and yield are known or can be estimated then Equation 3.8 consists of

only two unknowns: μ_{max} and K_s. However, this equation must be solved by nonlinear regression analysis.

For a volatile substrate such as benzene, the calculations are complicated by the partitioning of the substrate into the liquid and gas phases. The liquid concentration is related to total mass in the bottle by the following equation:

$$C_l = M / (V_l + H V_g) \qquad \text{Equation 3.9}$$

where C_l is the liquid concentration, M is the total mass, V_l is the volume of the liquid phase, H is the dimensionless Henry's constant (H = 0.22 for benzene at 25°C) and V_g is the volume of the gas phase. Equation 3.5 can be rewritten for change in mass per unit time:

$$dM/dt = V_l k S X / (K_s + S) \qquad \text{Equation 3.10}$$

where S is the liquid concentration as calculated by Equation 3.9, M is mass of substrate consumed, and $k = \mu_{max} / Y$. By substituting $G = V_l + H V_g$ so that $S = M/G$, integration yields a form of the equation similar to Equation 3.8 as a function of substrate mass:

$$C_1 \ln\{(Y/V_l)(M_o-M) + X_o)/X_o\} - C_2 \ln\{M/M_o\} = \mu_{max} t \qquad \text{Equation 3.11}$$

where $C_1 = (K_sGY + M_oY + X_oV_l)/(YM_o + X_oV_l)$, $C_2 = (K_sGY) / (YM_o + X_oV_l)$, and M_o is the initial mass of substrate.

Nonlinear regression analysis was performed on a transfer culture that degraded benzene linked to sulphate reduction. The substrate depletion curve is shown in the figure as symbols; the model is shown as a line (Figure 3-7).

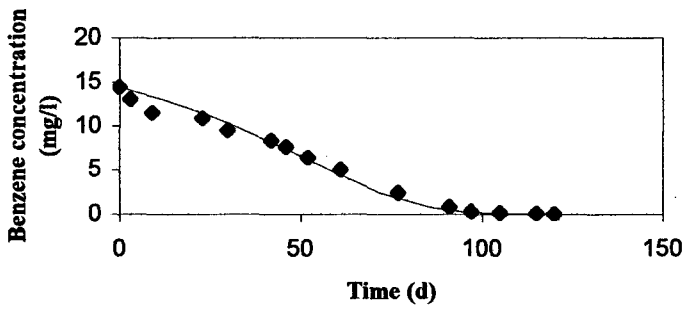


Figure 3-7 Results of non-linear regression analysis for a sulphate-reducing culture

The regression analysis for this culture resulted in values for μ_{max} and K_s of 0.023 d^{-1} and 3.888 mg/l, respectively. The details of the regression analysis are presented in Appendix D.

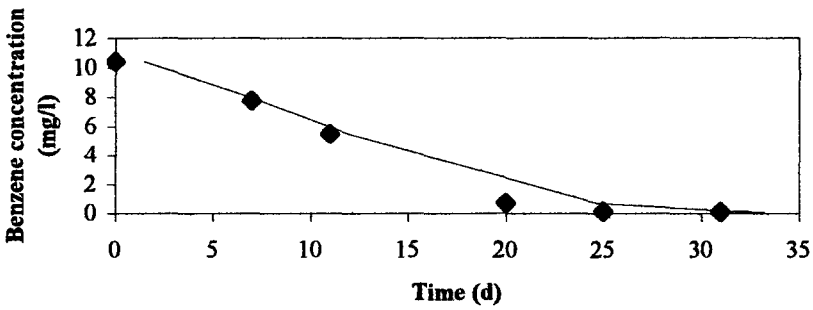


Figure 3-8 Results of a non-linear regression analysis for a nitrate-reducing culture

The results of a non-linear regression analysis of the benzene depletion curve for a nitrate-reducing culture are presented in Figure 3-8. The computed value for μ_{max} was 0.076 d^{-1} and for K_s was 4.961 mg/l. These values of μ_{max} and K_s compare well with values determined for anaerobic degradation of other compounds reported in the literature (Table 3-3). The doubling time can be calculated as $t_d = \ln(2)/\mu_{max}$ (Madigan et al. 1997).

Table 3-3 Comparison of K_s and μ_{max} with reported values

Electron donor	Electron acceptor	K_s (μM)	μ_{max} (d^{-1})	doubling time (d)	Reference
Toluene	CO_2	30	0.11	6	(Edwards and Grbic-Galic 1994)
Toluene	NO_3^-	92	-	-	(Alvarez et al. 1994)
Toluene	NO_3^-	-	6.2	0.11	(Dolfing et al. 1990)
o-Xylene	CO_2	20	0.07	10	(Edwards and Grbic-Galic 1994)
Glucose	O_2	-	29	0.02	(McCarty 1971)
Acetate	CO_2	-	0.27	61	(McCarty 1971)
Haloaromatics	CO_2	30 – 67	-	-	(Suflita et al. 1983)
Benzene	NO_3^-	64	0.076	9	This work
Benzene	SO_4^{2-}	40	0.023	30	This work

The doubling times shown in Table 3-3 indicate that the bacteria grow very slowly; in comparison, many aerobic bacteria have doubling times of hours or minutes. This is consistent with the results of slower growth after culture dilution described in the previous sections. Because the cultures do grow very slowly, culture transfers must be carefully planned to avoid diluting the culture too much to support degradation of benzene.

3.4 Conclusions

Sustained, anaerobic benzene degradation was obtained in cultures transferred into a defined, mineral medium. Successive transfer and enrichment resulted in viable transfer cultures with less than 1% of the original soil remaining. The rate of benzene biodegradation was variable, ranging from 1 μM/d to more than 75 μM/d. Generally, the sulphate-reducing cultures degraded benzene at the slowest rates and nitrate-reducing cultures degraded benzene at the fastest rates. The effect of culture dilution was to decrease the rate of benzene degradation temporarily. Undiluted cultures were unaffected by increases in benzene concentration up to 100 mg/l; however, dilution of the culture affected the ability of the culture to degrade benzene. This is because the bacteria grow only very slowly. Modelling of benzene depletion curves to the Monod kinetic equation resulted in estimates of K_s and μ_{max} of 3.9 mg/l and 0.023 d^{-1} for sulphate-reducers and 4.9 mg/l and 0.076 d^{-1} for nitrate-reducers. The resulting doubling times are 30 days and 9 days, respectively.

4 SULPHATE REDUCTION

4.1 Introduction

In microcosms prepared from Gas Station 1, Landfarm, Oil Refinery and Uncontaminated Swamp material, benzene degradation occurred in the presence of sulphate. Enriched microcosms in which benzene was degraded linked to sulphate reduction were developed from each type of material. Benzene-degrading activity was transferred to new culture bottles from Gas Station 1 and Oil Refinery microcosms.

The purpose of the experiments in this section was to confirm the link between benzene biodegradation and sulphate reduction. Two experiments were set up with sulphate-reducing inoculum: one from Gas Station 1 and one from Oil Refinery. Experiment 4.1 was designed to test the effect of sulphate on benzene degradation in material from a Gas Station 1 enriched microcosm. Experiment 4.2 was designed to test the effect of sulphate on benzene degradation in material from an Oil Refinery transfer culture.

4.2 Materials and Methods

4.2.1 Experimental Design

Experiment 4.1: Effect of sulphate on benzene degradation - Gas Station 1.

The first study was from Gas Station 1 enriched microcosm 9b. The inoculum (45 ml slurry) was split evenly between nine 40-ml vials (5 ml inoculum per vial). Each vial was then diluted 50% to 10 ml with medium. Three vials were amended with ca. 5 mM sulphate and 10 mg/l benzene, three vials were amended only with 10 mg/l benzene, and the three remaining vials were amended only with ca. 5 mM sulphate to establish background sulphate utilization. The concentrations of benzene and sulphate were measured over time.

Experiment 4.2: Effect of sulphate on benzene degradation - Oil Refinery.

The second experiment was from Oil Refinery transfer culture genII(1). The culture was split evenly into four 40-ml vials (16 ml per vial). No medium was added to dilute the culture. Two vials were amended with 5 mM sulphate; all four vials were amended with ca. 10 mg/l benzene. Benzene and sulphate were monitored. At the end of the experiment, protein content was determined.

4.2.2 Analytical Procedures

Benzene, sulphate and protein were measured as previously described in Section 3.1.6.

4.3 Results and Discussion

Experiment 4.1: Effect of sulphate on benzene degradation - Gas Station 1.

This experiment was designed to determine the dependence of benzene degradation on the presence of sulphate in an enriched microcosm from Gas Station 1. As can be seen from Figure 4-1, the benzene was degraded more readily in the cultures amended with sulphate. However, benzene degradation also occurred in the cultures without sulphate, with a corresponding methane production (see Chapter 7). The background sulphate utilization was determined and the ratio of sulphate reduced to benzene degraded was calculated (Table 4-1). There was more than enough sulphate depleted to account for the benzene degraded. The rate of benzene degradation doubled after the first feeding of benzene was degraded: the initial feeding was degraded in 100 days at a rate of 2.25 μM/d and the second feeding degraded in 50 days at a rate of 4.5 μM/d (Figure 4-1).

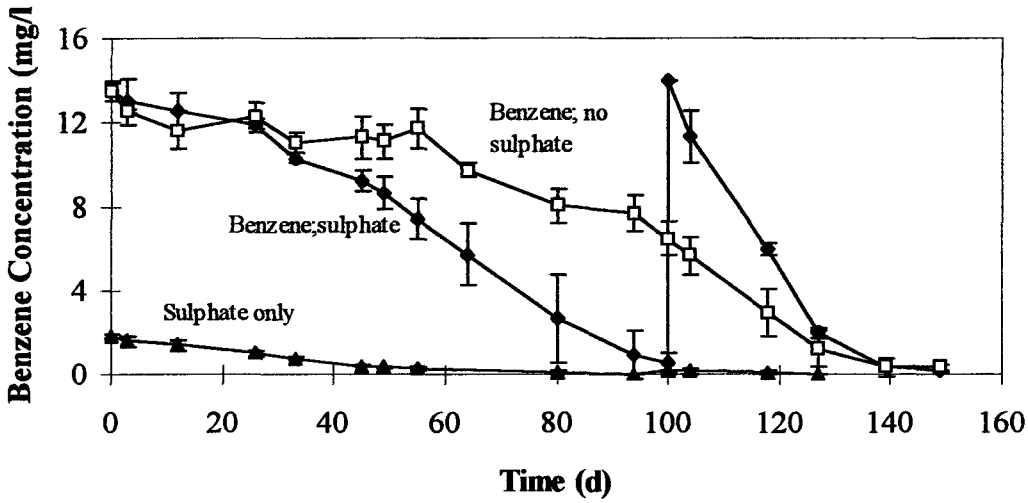


Figure 4-1 Experiment 4.1 Degradation of benzene in the presence and absence of sulphate

Table 4-1 Benzene Degraded and Sulphate Consumed in Experiment 4.1

Treatment	Benzene degraded (µmoles)	Sulphate utilized (µmoles)	Net sulphate utilized (µmoles)	Theoretical sulphate required (µmoles)	Ratio of sulphate utilized to sulphate required
Sulphate and benzene	2.26	49.1	12.4	8.5	1.46
Sulphate only	0.24	36.7	-	0.9	-

Experiment 4.2: Effect of sulphate on benzene degradation - Oil Refinery.

This experiment was set up from an Oil Refinery transfer culture to determine the effect of sulphate on this culture. In this case, sulphate was required for significant benzene degradation to occur, as shown in Figure 4-2. As in the experiment with the Gas Station 1 transfer culture, the rate of benzene degradation dramatically increased

after the first feeding of benzene was degraded. The initial rate of benzene degradation was 1.76 μM/d; the second feeding degraded in almost half the time, at a rate of 3.04 μM/d.

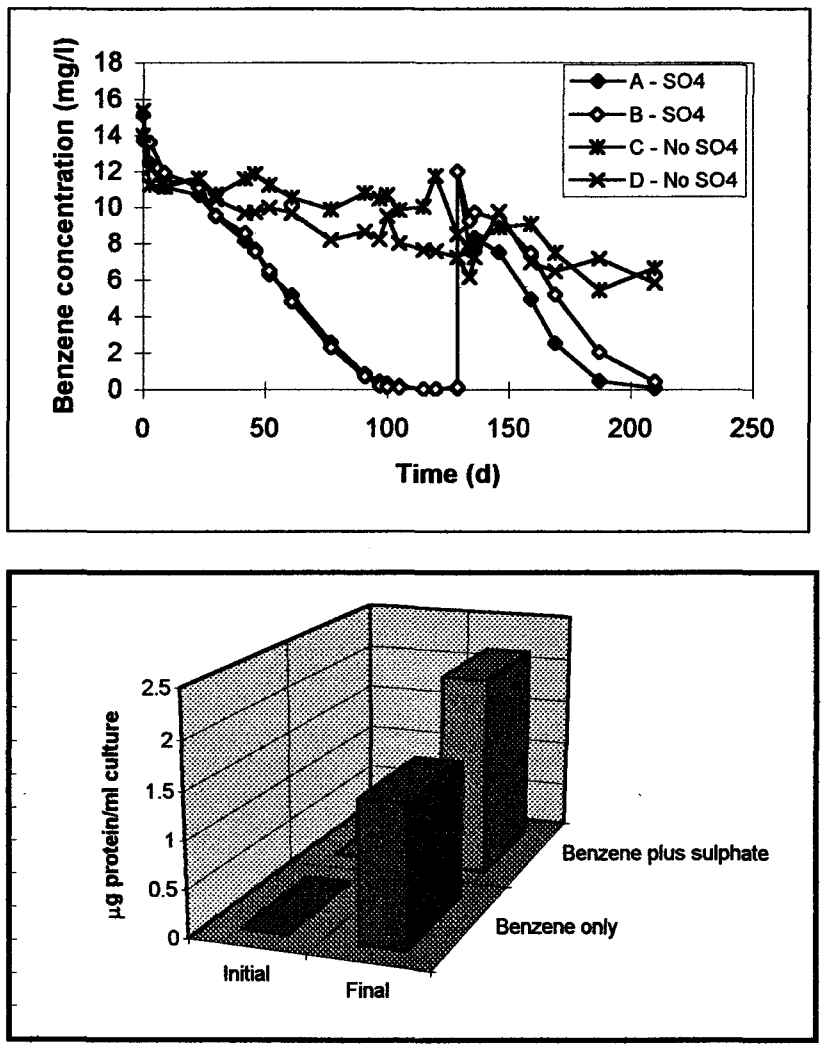


Figure 4-2 Benzene degradation and protein content in Experiment 4.2

Protein measurements were taken before and after the degradation of two feedings of benzene by the benzene-and-sulphate amended cultures. There was no detectable protein in the initial samples; results of analysis on Day 212 are summarized in Figure 4-2 B and Table 4-2. The calculation of a yield of 12.1 g cells / mol benzene was converted to units of electron equivalents to estimate the fraction of electrons from benzene used for cell synthesis, f_s. This gives a value of f_s equal to 6.5%. The method of McCarty (1971) predicts a value of f_s for benzene oxidation coupled to sulphate reduction of 0.08 or 8%, assuming a typical efficiency of electron transfer of 60%. The experimentally determined yield was close to this theoretical value.

Table 4-2 Protein Concentration and Cell Yield for Oil Refinery Experiment 4.2

	Protein concentration (mg/l) mean (SD)	
	Benzene only (no benzene degraded)	Benzene and sulphate (benzene degraded)
T=0	0	0
T=212	1.47 (0.05)	2.14 (0.16)
Benzene degraded (μmol/vial)		5.64
Net protein formation (μg/vial)[a]		34.24
Yield (g cells / mol benzene)[b]		12.1
f_s (eq cells / eq benzene)[c]		0.065

[a]culture volume was 16 ml
[b]assuming 50% of the dry weight of a cell is protein
[c]assuming 6.27 g cells / eq cells and 30 eq benzene / mol benzene

Enriched microcosms and transfer cultures. The ratio of sulphate consumed per mole benzene degraded was calculated for many enriched microcosms and transfer cultures over the incubation period. The resulting mass balances are shown in Table 4-3. The theoretical ratio, including cell synthesis, is 3.49 moles sulphate per mole benzene. It can be seen from the table that the measured values are close to the theoretical value for benzene degradation linked to sulphate reduction.

Table 4-3 Summary of mass balances of sulphate-reducing cultures

Culture	Date	Ratio sulphate/benzene	n
Gas Station 1			
Enriched microcosms			
2b	1997	3.71	1
3b	1998	3.98	1
4b	1997	6.01 (0.16)	2
5b	1998	3.62 (1.26)	4
9b	1998	5.86 (1.58)	3
10b	1998	4.09 (0.47)	2
Transfer cultures			
Combo 1	1998	3.17 (1.22)	3
5b(a)I	1997	4.01 (1.57)	3
5b(a)II	1997	4.16 (1.28)	6
5b(a)III	1997	4.38 (1.56)	2
5b(b)	1997	3.23	1
6b(1)a	1997	3.13 (1.06)	4

6b(1)b	1997	3.49 (0.91)	4
6b(2)a	1997	11.04	1
6b(3)	1997	3.43 (0.69)	4
Oil Refinery			
Enriched microcosms			
9b	1997	4.12	1
10b	1997	2.97	1
Transfer cultures			
6b(2)	1997	4.45 (0.11)	3
GenII (1)	1997	3.81 (0.80)	3
GenII(3)	1997	4.66 (3.26)	4
Combo 2	1998	3.88 (0.18)	2
Uncontaminated Swamp			
Enriched microcosms			
5b	1997	3.58 (0.57)	2
Landfarm			
Enriched microcosms			
5b	1996	7.15	1
10b	1996	11.63	1

Most of the enriched microcosms and transfer cultures listed in Table 4-3 displayed a dependence on sulphate for benzene degradation. In the presence of sulphate, benzene degradation proceeded at the ratio of sulphate to benzene listed in the table; when sulphate became depleted, benzene degradation stopped. This behaviour is illustrated in Figure 4-3 for Gas Station 1 transfer 5b(b).

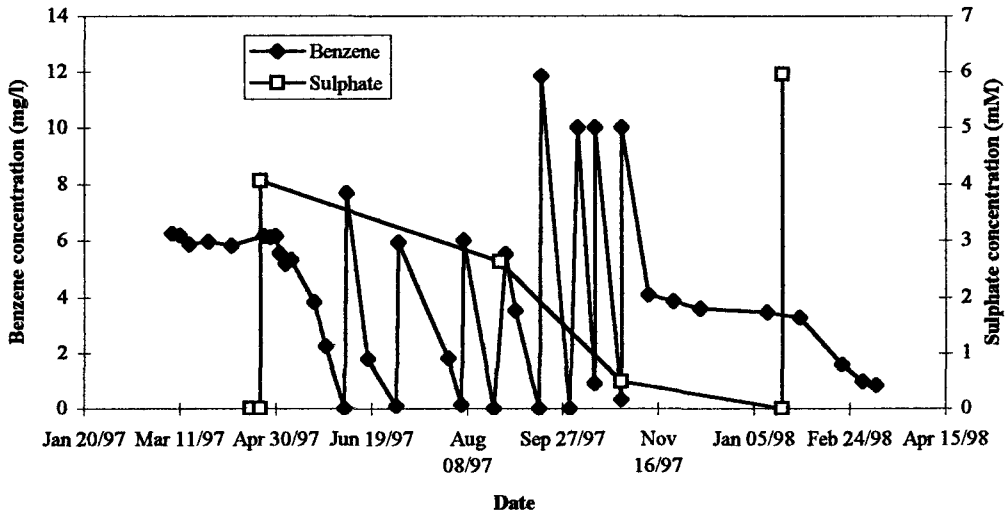


Figure 4-3 Benzene degradation is dependent upon the presence of sulphate

4.4 Conclusions

Anaerobic benzene biodegradation was linked to sulphate reduction in enriched microcosms and transfer cultures derived from three contaminated sites - Gas Station 1, Oil Refinery and Landfarm - and one uncontaminated site, Uncontaminated Swamp. Benzene degradation proceeded in the presence of sulphate, the measured sulphate demand was close to the theoretically-predicted demand, and cell growth was linked to benzene degradation. These conclusions corroborate other studies which have reported benzene biodegradation linked to sulphate reduction. In some transfer cultures, benzene continued to be degraded when sulphate became depleted; electrons

were diverted to methanogenesis in these cases. The observation that the terminal electron accepting process can switch prompted the studies reported in Chapter 7.

5 NITRATE REDUCTION

5.1 Introduction

The results presented in this chapter have been presented in a paper authored by S. M. Burland and E. A. Edwards accepted in Applied and Environmental Microbiology. This is the first confirmed report of benzene biodegradation linked to nitrate reduction. Although toluene is degraded readily under denitrifying conditions, benzene is usually recalcitrant. The results from the original study suggesting that benzene biodegradation was likely linked to nitrate reduction (Nales 1997) prompted the following in-depth study in order to confirm the link between the two processes.

In microcosms prepared from Gas Station 1, Landfarm, and Uncontaminated Swamp material, benzene degradation occurred in the presence of nitrate. Enriched microcosms, in which benzene was degraded linked to nitrate reduction, were developed from each type of material. Benzene-degrading activity was transferred to new culture bottles from Gas Station 1 and Uncontaminated Swamp microcosms. Refer to Appendix C for details of the development of transfer cultures.

The goal of this research was to link benzene biodegradation to nitrate reduction. Eight separate experiments with different objectives were set up with nitrate-reducing inoculum. Seven were carried out with inoculum from Gas Station 1 enriched microcosms or transfer cultures; one of these studies was duplicated with material from Uncontaminated Swamp. The other experiment was with an

Uncontaminated Swamp microcosm. Three experiments were designed to test the effect of nitrate on the degradation of benzene (Experiment 5.1, 5.2 and 5.3). The end products of benzene biodegradation (CO_2) and nitrate reduction (nitrogen) were monitored in Experiment 5.4 and 5.5, respectively. Other potential electron acceptors were tested in Experiment 5.6 (ferric iron) and 5.7 (sulphate). Experiment 5.8 was designed to link cell growth to benzene degradation under nitrate-reducing conditions. The sources of inoculum for these experiments are identified in Appendix C.

5.2 Materials and Methods

5.2.1 Experimental Design

Experiment 5.1: Effect of nitrate on benzene degradation - transfer culture. The inoculum (70 ml slurry) from a Gas Station 1 transfer culture was centrifuged and resuspended in 60 ml of medium. The resuspended inoculum was then split evenly between six 40-ml vials (10 ml per vial). Two vials were amended with ca. 5 mM nitrate and 10 mg/l benzene; three vials were amended only with 10 mg/l benzene; and the remaining vial was amended only with ca. 5 mM nitrate to estimate background nitrate utilization. The concentrations of benzene, methane, nitrate, nitrite, sulphate, ferrous iron and total iron were measured over time.

Experiment 5.2: Effect of nitrate on benzene degradation - enriched microcosm. The inoculum (45 ml slurry) from a Gas Station 1 enriched microcosm

was split evenly between nine 40-ml vials (5 ml per vial). Each vial was then diluted 50% by adding medium to a total volume of 10 ml per vial. Three vials were amended with ca. 5 mM nitrate and 10 mg/l benzene; three vials were amended with 10 mg/l benzene only; and three vials were amended with ca. 5 mM nitrate only to establish the background nitrate utilization. The concentrations of benzene, methane, nitrate, nitrite and sulphate were measured over time.

Experiment 5.3: Effect of nitrate on a groundwater microcosm. The original microcosm Uncontaminated Swamp #2b was constructed of groundwater and benzene, with no additional potential electron acceptors. It did not degrade benzene over the two year incubation. Nitrate (ca. 5 mM) was added to this microcosm. Nitrate and benzene were measured over time.

Experiment 5.4: [^{14}C]benzene experiment. This experiment was designed to establish biodegradation of benzene to carbon dioxide in the nitrate-reducing transfer cultures derived from Gas Station 1. The inoculum (60 ml slurry) was divided evenly between six 17-ml vials. Each vial was amended with ca. 20 mM nitrate. Two uninoculated controls (10 ml water poisoned with 0.1 ml HgCl) were also prepared in 17-ml vials. About 15 mg/l [^{14}C]benzene was added to each of the eight vials. [^{14}C]benzene, $^{14}CO_2$ and ^{14}C-labeled non-volatile compounds were measured before and after degradation of the benzene.

Experiment 5.5: End product of nitrate reduction. This experiment with nitrate-reducing inoculum was performed to determine if dinitrogen gas (N_2) was produced as benzene was degraded. The inoculum was that of Experiment 5.1,

pooled, redistributed into four 17-ml vials (12 ml per vial) and amended with ca. 10 mM nitrate. Two sets of controls were also prepared: two vials containing a sulphate-reducing culture and two vials containing uninoculated medium. All vials were purged with helium for twenty minutes to remove as much nitrogen from the headspace as possible (the atmosphere of the anaerobic chamber was about 80% nitrogen). Benzene (ca. 10 mg/l) was added to four of the vials: two of the nitrate-amended vials and the two vials containing a sulphate-reducing culture. Benzene and nitrogen were measured in all vials over time.

Experiment 5.6: Effect of iron-poor medium. This experiment was designed to eliminate the possibility that iron was acting as the electron acceptor for benzene degradation in the nitrate-amended transfer cultures. Two types of medium were used: the defined-mineral medium as described previously, and the same medium without the addition of FeS to reduce the medium. Furthermore, the regular medium used was allowed to settle so that very little FeS was added to the cultures. This was in an attempt to avoid adding iron to the system; however the medium without FeS was very pink and may have contained too much oxygen. The inoculum came from a Gas Station 1 transfer culture. The inoculum (40 ml of liquid only) was centrifuged in two 20-ml aliquots, washed and resuspended in 45 ml of the appropriate medium for an approximate 50% dilution. The resuspended inoculum was then evenly divided between eight 17-ml vials (four for each medium type; 10 ml per vial). Each vial was purged with N_2/CO_2 for 10 minutes to remove hydrogen from the headspace. All vials were amended with 10 mM nitrate. Two vials of each medium type were additionally

amended with ca. 10 mg/l benzene. Concentrations of benzene, methane, nitrate, nitrite, sulphate, ferrous iron and total iron were measured over time.

Experiment 5.7: Effect of molybdate. Many of the original microcosms had a significant concentration of sulphate (from the groundwater or subsequent amendment) and consequently many of the enriched microcosms and transfer cultures in which benzene was degraded in the presence of nitrate also contained sulphate. In order to eliminate sulphate as a possible electron acceptor in these cultures, the following molybdate inhibition experiments were conducted. Transfer cultures from each of Gas Station 1 and Uncontaminated Swamp were used as the inoculum for these experiments. The inoculum (40 ml slurry, containing nitrate and benzene) was split evenly between four 17-ml vials (10 ml per vial). Two of the vials were amended with 2 mM Na_2MoO_4 (0.2 ml of a 100 mM anaerobic stock) and the remaining two vials were amended with an equivalent volume of sterile, anaerobic water. The concentration of benzene was measured over time.

Experiment 5.8: Cell growth. A 30-ml liquid only portion of a Gas Station 1 enriched microcosm was diluted with 130 ml medium. This inoculum was then split evenly between four 40-ml vials (30 ml per vial). The remaining 40 ml was frozen for protein analysis. All four vials were amended with ca. 20 mM nitrate; two vials were amended with 10 mg/l benzene. Protein content was measured before and after benzene degradation.

5.2.2 Analytical Procedures

Benzene, methane, nitrate, nitrite, sulphate, ferrous iron, total iron and protein were measured as previously described in Section 3.1.6.

Nitrogen Analysis

Nitrogen was measured by injection of a headspace sample (100 µl) onto a gas chromatograph (Hewlett Packard 5890) equipped with a Molecular Sieve (Supelco) packed column and a thermal conductivity detector. The injector temperature was 200°C, the oven temperature was 50°C and the detector temperature was 200°C. The carrier gas and reference gas was helium at a flow rate of 30 ml/min.

[^{14}C]benzene Analysis

[^{14}C]benzene (Sigma) was diluted in neat benzene, and added to each vial to give a starting aqueous benzene concentration of about 15 mg/l and an initial activity of about 6000 dpm/ml. [^{14}C]benzene, $^{14}CO_2$, and ^{14}C-labeled non-volatile compounds were measured by scintillation counting of the radioactivity in the acid, base, and neutral fractions of an aqueous sample, following the method of Grbic-Galic and Vogel (1987). The ^{14}C activity was counted with a Liquid Scintillation Counter using UniverSol ES™ liquid scintillation cocktail (ICN Biomedicals, Inc. Costa Mesa, CA). Problems were encountered with the scintillation cocktail, similar to those reported in Nales (1997). Although various methods were experimented with, the best method,

described in Criddle (1989), resulted in clear mixtures of sample and scintillation cocktail. A 1-ml sample was added to each of three vials - one each for acid, base and neutral fraction analysis. The vial for the acid fraction contained 0.15 ml of 1 N HCl; the vial for the base fraction contained 0.15 ml of 1 N NaOH; and the vial for the neutral fraction contained 0.15 ml 1 N NaOH and 10 ml of scintillation cocktail. After addition of the sample directly into the liquid in the vials, the acid and base fractions were purged with CO_2/N_2 for 20 minutes. Following purging, 10 ml scintillation fluid was added to the acid and base fractions and then all three fractions were counted.

The acid fraction represents [^{14}C]non-volatiles; the base fraction represents [^{14}C]non-volatiles plus $^{14}CO_2$, which is soluble in basic solution; and the neutral fraction represents the total activity. Therefore, $^{14}CO_2$ was determined by the difference between the base and acid fractions and the [^{14}C]benzene (volatiles) was determined by the difference between the neutral and base fractions (Grbic-Galic and Vogel 1987).

5.3 Results and Discussion

5.3.1 Experiment 5.1: Effect of nitrate on benzene degradation - transfer culture

Before this study, benzene degradation linked to nitrate reduction had not been confirmed. To establish the dependence of the benzene-degrading microorganisms in the Gas Station 1 transfer cultures on nitrate, a culture was split into two different

treatments - one with nitrate and one without nitrate. The results are shown in Figure 5-1; benzene was degraded only in the culture vials amended with nitrate.

Potential electron acceptors were monitored over the duration of this experiment. Sulfate, nitrate, methane, ferrous iron and total iron were monitored and are summarized in Table 5-1. The theoretical amounts of methane and ferrous iron that must be produced and of nitrate and sulphate that must be consumed to account for the benzene that was degraded are presented in the last row of the table. From this data, only nitrate, with a ratio of 7.5 moles nitrate per mole benzene, was a possible electron acceptor. In fact, sulphate was produced and ferrous iron was depleted. This phenomenon occurred to the same degree in the background control as well as in the active cultures. It is possible that the nitrate was involved in the oxidation of sulphate to sulphide and ferrous iron to ferric iron. Approximately 12 μmoles nitrate would be required to oxidize the amount of sulfate and ferrous oxide that were oxidized in these culture vials. The background control utilized about 30 μmoles nitrate; the difference (19 μmoles) was likely depleted due to the hydrogen that formed a part of the headspace of these cultures.

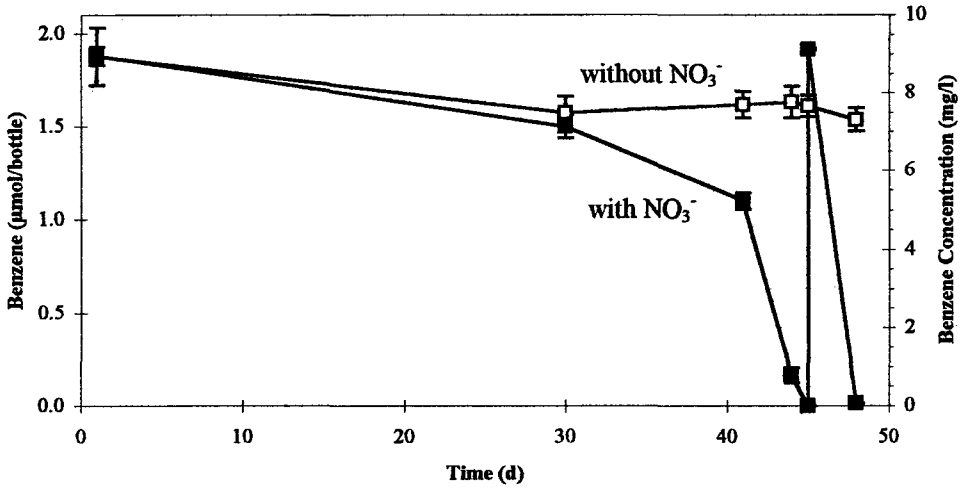


Figure 5-1 Benzene concentration versus time in Gas Station 1 transfer cultures in the presence and absence of nitrate (Experiment 5.1)

The rate of benzene degradation, once started, was very quick. After an approximate 30 day lag period, the benzene was degraded in 15 days at a rate of about 17 µM/d. The second feeding of benzene was degraded in 3 days at 75 µM/d. The third feeding of benzene was degraded somewhat slower in 9 days at a rate of 25 µM/day. By then, nitrate was limiting and nitrite was building up (Figure 5-2). Net nitrate consumption was determined by subtracting nitrate consumption in the background control (Table 5-1) from the nitrate consumed in the active culture vials. The background utilization of nitrate occurred before degradation of benzene began; thereafter the concentration of nitrate in the culture that was not amended with benzene remained constant. Initially, when benzene degradation was proceeding relatively slowly, the rate of nitrate reduction was also slow. At about Day 40, the

rates of benzene biodegradation and nitrate reduction increased significantly and simultaneously. During the period of rapid benzene degradation, Days 40 to 58, nitrate was converted nearly stoichiometrically to nitrite. When nitrate became depleted on Day 58, benzene degradation essentially ceased, and nitrite reduction began. These data suggest that nitrate was a much better electron acceptor for benzene oxidation than nitrite. Nitrite may have partially inhibited the microorganisms responsible for benzene degradation, resulting in a slower rate of benzene degradation. Similar results were reported for a pyridine-degrading denitrifying bacterium. Cell growth was linked only to degradation of nitrate to nitrite; further degradation of nitrite was not accompanied by pyridine degradation or cell growth (Rhee et al. 1997).

Table 5-1 Methane, sulphate, ferrous iron and nitrate concentrations in Experiment 5.1

	Methane	Sulphate	Ferrous Iron	Nitrate
Benzene/No Nitrate				
Day 0	0.29 ± 0.06[a]	0.4	78 ± 3.9	0.66
Day 44	1.7 ± 0.42	0.02 ± 0.03	82 ± 1.3	0.02 ± 0.04
Day 92	8.8 ± 2.1	0.00 ± 0.00	87 ± 8.1	0.00 ± 0.00
No Benzene/Nitrate				
Day 0	0.27	0.3	78 ± 3.9	81
Day 44	0.27	1.3	39	55
Day 92	0.34	1.8	24	51
Benzene/Nitrate				
Day 0	0.31 ± 0.05	0.3	78 ± 3.9	81
Day 44	0.23 ± 0.01	1.2 ± 0.35	10 ± 14	27 ± 8.0
Day 92	0.31 ± 0.04	1.4 ± 0.23	15 ± 3.9	0.00 ± 0.00
Theoretical Requirements[b]	+26	-26	+204	-41

[a]Results are mean ± SD (except for single measurements)

[b]Based on benzene actually degraded (6.8 μmoles/bottle) and the stoichiometries presented in Chapter 2

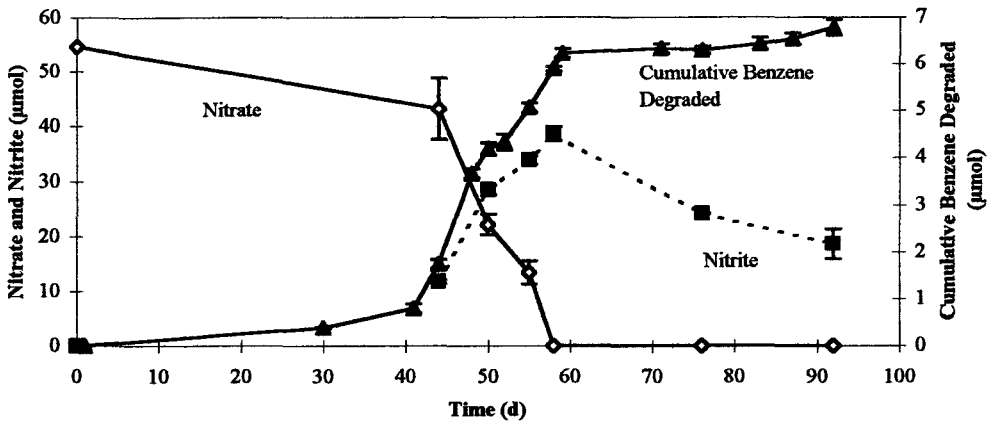


Figure 5-2 Nitrate consumed, nitrite produced, and cumulative benzene degraded in Gas Station 1 culture vials (Experiment 5.1)

5.3.2 Experiment 5.2: Effect of nitrate on benzene degradation - enriched microcosm

This experiment was performed as a confirmation of Experiment 5.1, to confirm the dependence of benzene degradation on the presence of nitrate. The inoculum from Gas Station 1 enriched microcosm #13b was split into nine vials, three each of three treatments: benzene and nitrate, benzene only, and nitrate only. As in Experiment 5.1, the benzene was significantly degraded only in the presence of nitrate (Figure 5-3). There was significant nitrate use in the background controls. The nitrate was depleted in all six nitrate-amended vials; however, as expected, the second amendment of nitrate was depleted faster in the benzene-amended cultures than in the background controls. The greater use of nitrate in the background controls in this

experiment than in Experiment 5.1 was likely due to the greater volume of headspace (30 ml) and thus a greater volume of hydrogen in the culture vials of this experiment. The ratio of net nitrate utilized to benzene degraded in this experiment was estimated to be 5.2. Even accounting for the nitrate use in the background controls, there was still enough nitrate utilized in the benzene-and-nitrate-amended vials to account for all of the benzene that was degraded.

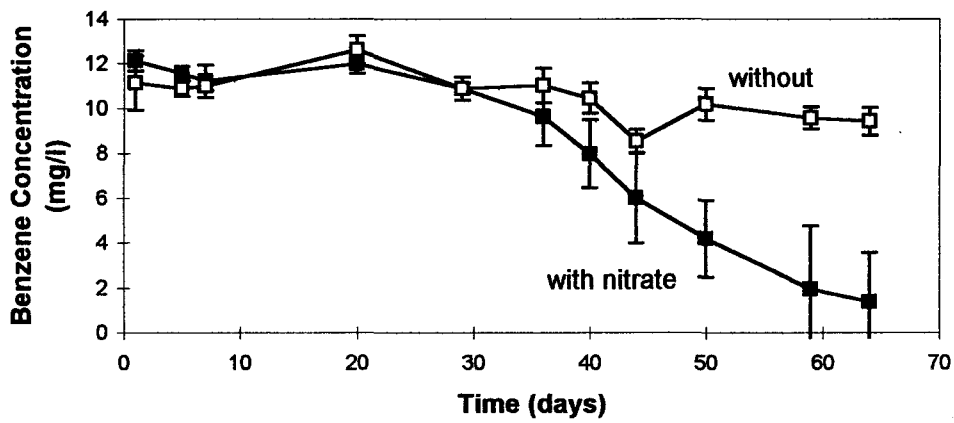


Figure 5-3 Benzene concentration vs. time in the presence and absence of nitrate (Experiment 5.2)

The rate of benzene degradation of the initial feeding of benzene was 3.5 μM/d. Subsequent feedings degraded much faster at about 20 μM/d until nitrate became limiting. The initial slower degradation was expected because the culture was significantly diluted (50%) before the experiment.

The vials amended with only benzene (no nitrate) exhibited much greater methane production than any of the nitrate-amended treatment vials. However, since the benzene was not degraded, this methane production was likely due to the hydrogen in the headspace. Contrary to the results from sulphate reduction Experiment 4.1, benzene degradation did not occur under methanogenic conditions; when the nitrate became depleted, benzene biodegradation stopped and resumed only when additional nitrate was added to the culture.

5.3.3 Experiment 5.3: Effect of nitrate on a groundwater microcosm

During the original microcosm study, benzene was degraded in the presence of nitrate in microcosms from Uncontaminated Swamp amended with nitrate and medium. However, microcosm #14b was sacrificed for analysis, leaving 13b as the only representative of benzene degradation linked to nitrate reduction from this site. In an attempt to increase the volume of nitrate-reducing culture from this site, and to test the effect of adding nitrate to a microcosm previously unexposed to nitrate, nitrate was added to a groundwater-only microcosm, #2b. In this microcosm, representing *in situ* conditions, benzene had not degraded during the two-year incubation. Upon addition of nitrate (Figure 5-4) benzene degradation began immediately and continued at a rapid rate. Subsequent reamendments of benzene and nitrate have resulted in an enriched microcosm for benzene degradation linked to nitrate reduction.

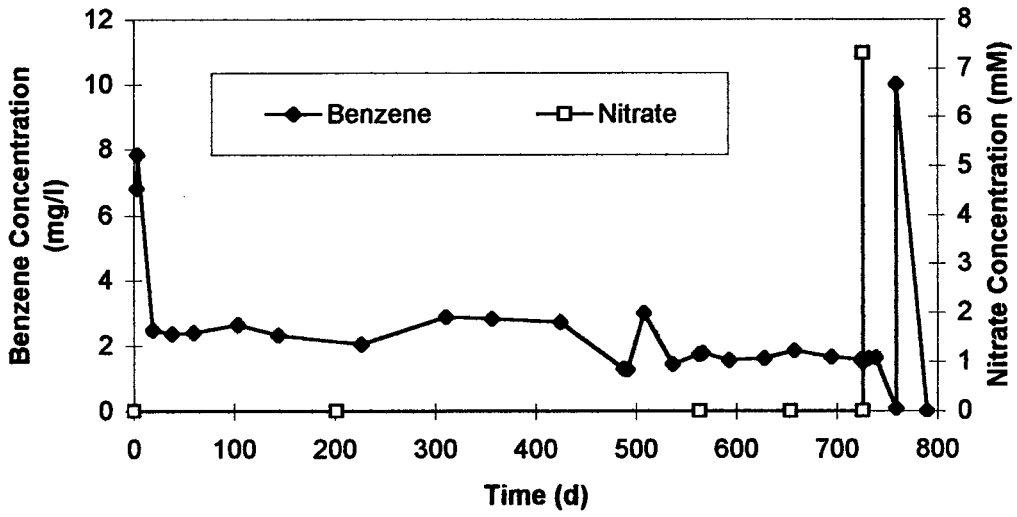


Figure 5-4 Addition of nitrate to Uncontaminated Swamp Microcosm #2b

5.3.4 Experiment 5.4: [^{14}C]benzene experiment

To confirm that benzene was being mineralized to CO_2, a set of enrichment cultures was set up and amended with [^{14}C]benzene. About 94% of the initial [^{14}C]benzene was recovered as $^{14}CO_2$ and the remaining 6% was found in the nonvolatile fraction, presumably as biomass (Figure 5-5). This portion to biomass was in agreement with the fraction to cell synthesis of 5% determined from the protein measurements of Experiment 5.8.

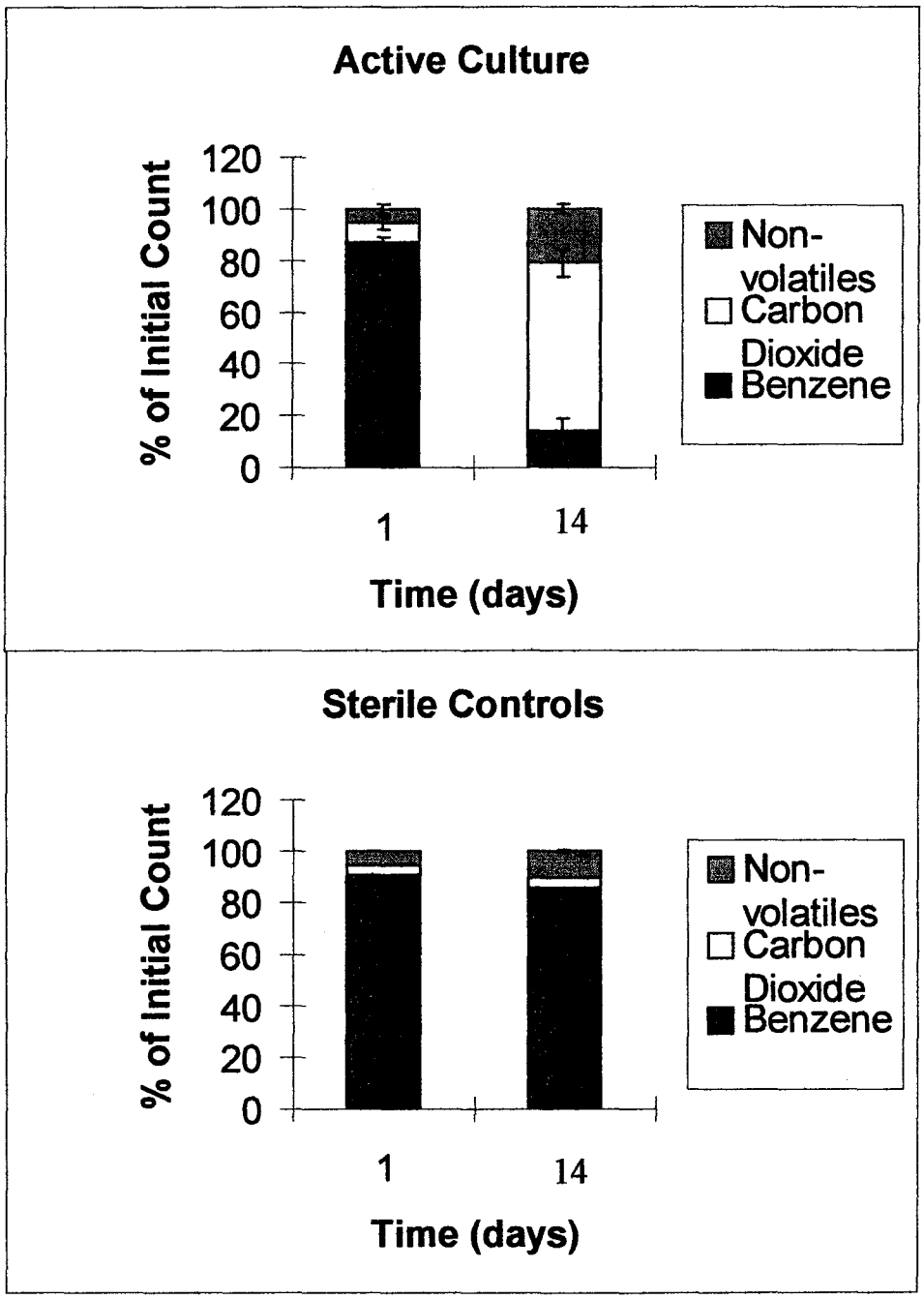


Figure 5-5 Results of [^{14}C]benzene Experiment 5.4

The rate of benzene biodegradation was about 12 μM/d before the experiment, when the cultures were amended with benzene. The rate was essentially the same

when the cultures were amended with [^{14}C]benzene. There was no lag period associated with the degradation of benzene because these cultures were not diluted. The results of this experiment confirmed that biodegradation did occur in the nitrate-reducing transfer cultures: benzene was mineralized to carbon dioxide.

5.3.5 Experiment 5.5: End product of nitrate reduction

The first product of nitrate (NO_3^-) reduction is nitrite (NO_2^-). Further reduction of nitrite can proceed directly to ammonia (NH_3) or to nitrogen gas (N_2) via nitric oxide (NO) and nitrous oxide (N_2O); the latter pathway is referred to as denitrification. Nitrogen production was monitored in culture vials amended with nitrate and benzene, as well as in control cultures, to confirm denitrification in these culture vials. Nitrogen production was significant only in the culture vials that were amended with nitrate-reducing cultures and benzene (Table 5-2). Therefore, it is likely that denitrification was occurring in these cultures.

Table 5-2 Nitrogen production in Experiment 5.5

Treatment	Benzene degraded (μmol)	Nitrate consumed (μmol)	Initial N_2 concn (%)	Final N_2 concn (%)	N_2 produced (μmol)
Benzene; nitrate	1.6	16.0	5.2 (1.2)	11.8 (0.6)	13.7 (3.7)
Nitrate only	0	1.3	5.2 (1.2)	6.5 (0.7)	2.6 (4.0)
Not inoculated	0	0	5.2 (1.2)	5.4	0.4 (2.5)

Two other observations support the conclusion that denitrification occurred in these cultures: gas bubbles, presumably nitrogen gas, developed in active cultures, and the redox dye resazurin changed colour from clear to pink during active nitrate reduction. The intermediates of denitrification - nitric oxide and nitrous oxide - oxidize resazurin and change the colour to pink (Suflita et al. 1997). When nitric oxide and nitrous oxide are further reduced to nitrogen, the resazurin changes back to clear.

5.3.6 Experiment 5.6: Effect of iron-poor medium

The results from Experiment 5.1 indicated that ferrous iron in the medium was oxidized to ferric iron in the presence of nitrate. In order to eliminate this newly formed ferric iron as the electron acceptor coupled to benzene degradation in the nitrate-amended enrichment cultures, an experiment was designed in an iron-poor medium. Benzene degradation was rapid in the cultures amended with nitrate and benzene in the iron-poor medium; however, the FeS-free medium did not support

sustained benzene degradation (Figure 5-6). Benzene degradation did occur in one of the replicates in the FeS-free medium; however, upon reamendment, the benzene was not degraded. The cultures inoculated into the FeS-free medium were very pink (the colour of oxidized resazurin); there may have been too much oxygen in the medium to support denitrification.

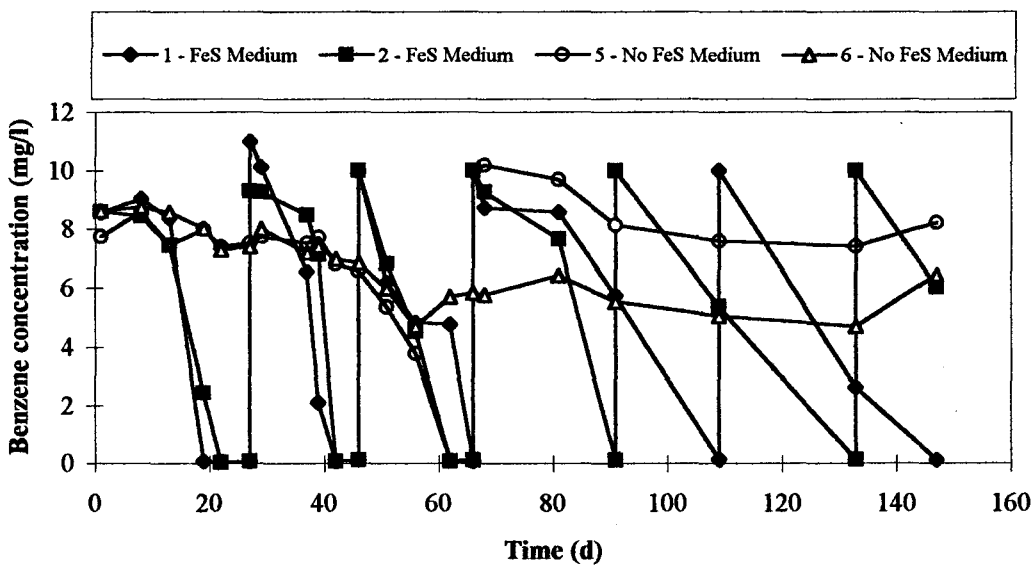


Figure 5-6 Benzene concentration vs. time in benzene-amended culture vials from Experiment 5.6

The four cultures prepared in the iron-poor FeS medium were monitored to determine the likely electron acceptor corresponding to the benzene degradation (Table 5-3). It can be seen from the table that there was not enough methane

produced, or enough sulphate or ferric iron consumed, to account for the 3.38 μmoles benzene that was degraded. There was, however, enough net nitrate consumed (about 36 μmoles) to account for the benzene degraded. The calculated ratio of nitrate consumed to benzene degraded was 10.7.

Table 5-3 Methane, sulphate, ferric iron and nitrate concentrations in benzene-degrading cultures (μmoles per vial) from Experiment 5.6

		Methane	Sulphate	Ferric iron[b]	Nitrate
Nitrate; no benzene	Day 0	0.002 (0.00)[a]	0.71 (0.10)	<10[c]	113.1 (0.9)
	Day 66	0.012 (0.002)	0.84 (0.00)	<10	89.5 (0.5)
	Change	+0.010	+0.13	<10	-23.6
Nitrate; benzene	Day 0	0.004 (0.001)	0.71 (0.10)	<10	113.1 (0.9)
	Day 66	0.013 (0.002)	0.91 (0.21)	<10	53.9 (0.03)
	Change	+0.011	+0.21	<10	-59.2
Theoretical production or consumption[d]		+13	-13	-101	-20

[a]data are presented as the mean (SD) of triplicates.
[b]Fe(III) measured as the difference between total iron and Fe(II)
[c]below the detection limit of 0.1 mM or 10 μmoles per 10 ml vial. Both Fe(II) and total iron measurements were below the detection limit
[d]based on benzene actually degraded (3.38 μmoles) and the stoichiometries presented in Chapter 2

The initial rate of benzene biodegradation was 5.6 μM/d; this rate increased somewhat to about 7.5 μM/d for subsequent feedings of benzene. This rate was consistent with the rate of benzene biodegradation in cultures of similar dilution, such as Experiment 5.8.

5.3.7 Experiment 5.7: Effect of molybdate

Results from Experiments 5.1 and 5.6 indicated that sulphate could be produced, presumably from sulphide present in the medium, by the addition of nitrate. In order to eliminate the possibility that sulphate was acting as the electron acceptor for the degradation of benzene, molybdate, a specific inhibitor of sulphate reduction (Taylor and Oremland 1979), was added to benzene-degrading cultures. Inoculum from two sites - Gas Station 1 and Uncontaminated Swamp - was split into four vials each and treated with nitrate and either molybdate and benzene or benzene alone. In both experiments, the benzene was degraded at the same rate, regardless of the presence of molybdate (Figure 5-7). Therefore, sulphate reduction was not the terminal electron accepting process for benzene degradation in these transfer cultures.

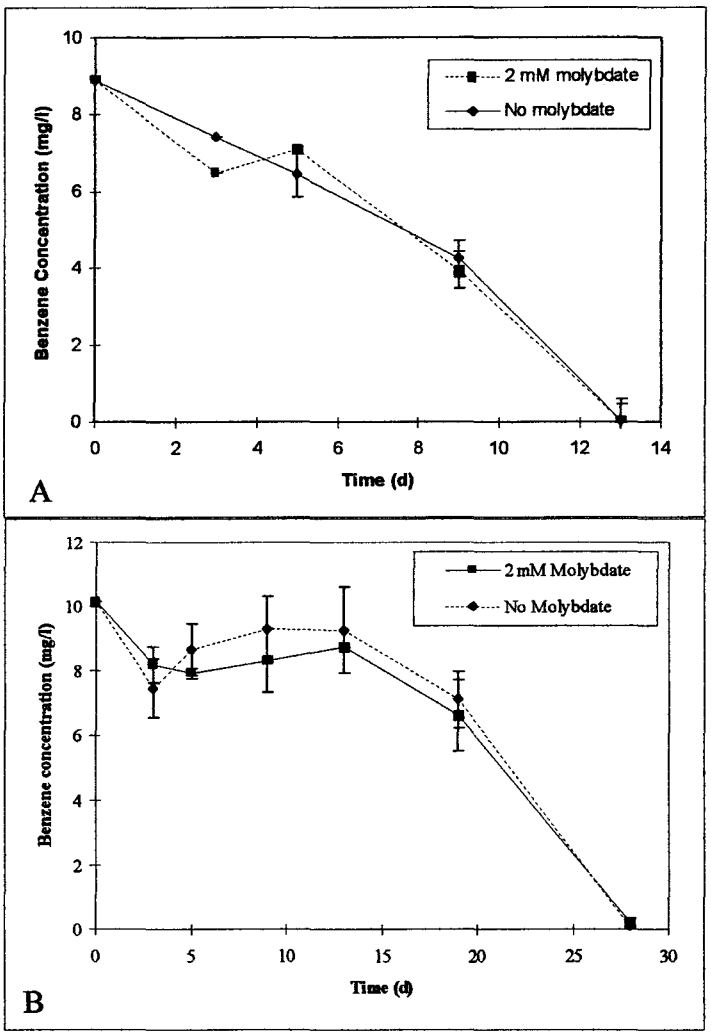


Figure 5-7 Benzene degradation in the presence and absence of molybdate for (A) Gas Station 1 and (B) Uncontaminated Swamp

5.3.8 Experiment 5.8: Cell growth

To determine if benzene was being used as a carbon source for cell synthesis, the growth of a transfer culture was linked to benzene biodegradation. Increase of protein in microbial cultures was used as an indicator of cell growth. Protein was assumed to comprise about 50% of the cell's dry weight. Experiment 5.8 was set up to verify cell growth; four vials were inoculated with a benzene-degrading culture and protein was measured before and after degradation of 13.5 μmoles benzene in the benzene-amended cultures. The increase of protein was greater in the vials in which benzene was degraded than in the vials without benzene (Figure 5-8).

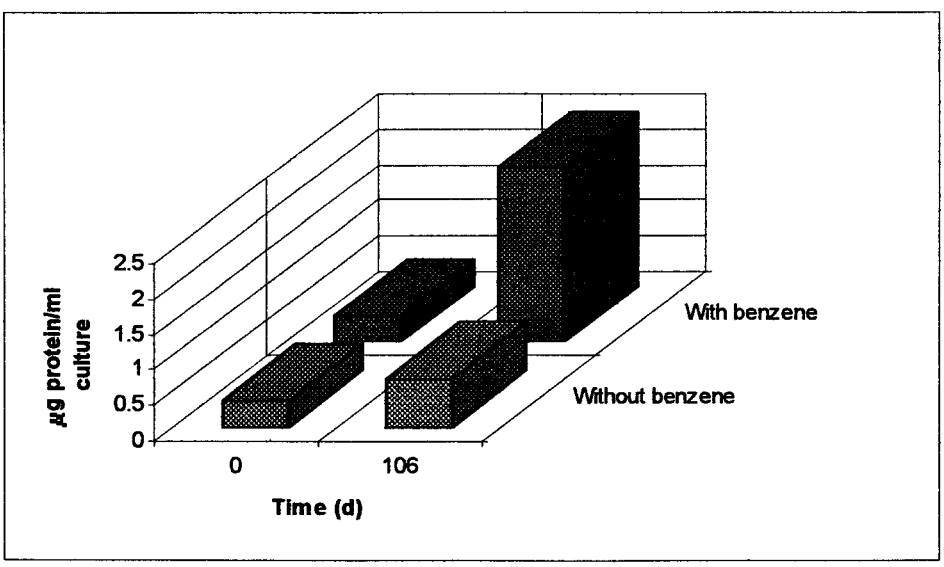


Figure 5-8 Protein concentration in Experiment 5.8

The calculation of a yield of 8.8 g cells / mol benzene was converted to units of electron equivalents to estimate the fraction of electrons from benzene used for cell synthesis, f_s. This gives a value of f_s equal to 5% (Table 5-4), which is in agreement with the 6% calculated in the [^{14}C]benzene experiment. The method of McCarty (1971) predicts a value of f_s for benzene oxidation coupled to nitrate reduction to nitrogen gas of 0.45 or 45%, assuming a typical efficiency of electron transfer of 60%. An f_s of 0.35 is predicted for benzene oxidation coupled to nitrate reduction to nitrite (McCarty 1971). The experimentally determined yield was much less than either of these theoretical values. This may indicate that the actual efficiency of electron transfer in these cells was far lower than the average of 60%, perhaps due to sub-optimal growth conditions, the presence of inhibiting substances such as nitrite, or an inefficient pathway for benzene metabolism. In this case, the assumption that microbial decay rate is negligible may be erroneous (see Section 3.3).

Table 5-4 Protein concentration and cell yield in Experiment 5.8

	Protein concentration (mg/l) mean (SD)	
	Background control (nitrate; no benzene)	Active treatment (nitrate and benzene)
T=0	0.44 (0.07)	0.44 (0.07)
T=106 days	0.84 (0.36)	2.82 (0.25)
Benzene degraded (μmol/vial)		13.5
Net protein formation (μg/vial)[a]		59.4
Yield (g cells / mol benzene)[b]		8.80
f_s (eq cells / eq benzene)[c]		0.047

[a]background subtracted; culture volume was 30 ml
[b]assuming 50% of the dry weight of a cell is protein
[c]assuming 6.27 g cells / eq cells and 30 eq benzene / mol benzene

The rate of benzene biodegradation was slow initially (3.7 μM/d) but doubled for the second and third feedings, 6.5 and 7.5 μM/d respectively. This rate was slower than the rates observed in Experiment 5.1 and 5.2 because the culture was diluted much more for this experiment. However, the low initial rate of benzene biodegradation was due entirely to an initial lag period of about 20 days. If the rate is determined after degradation of benzene degradation began at day 20, the initial rate was 7.2 μM/d, the same as subsequent rates. This is illustrated in Figure 5-9; the three areas of benzene degradation - Day 20 to 40, Day 65 to 85, and Day 90 to 110 - have approximately the same slope on a plot of cumulative benzene degraded versus time.

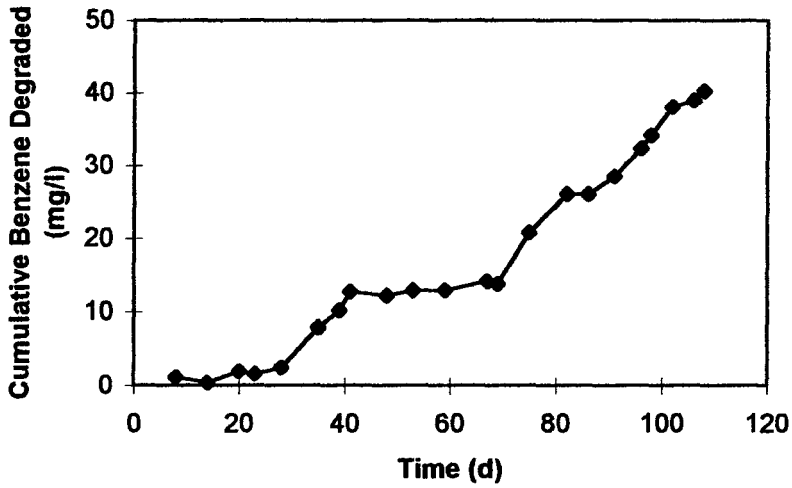


Figure 5-9 Cumulative benzene degradation in benzene-amended culture vials in Experiment 5.8

5.3.9 Enriched microcosms and transfer cultures

The ratio of moles nitrate consumed per mole benzene degraded was calculated frequently in enriched microcosms and transfer cultures (Table 5-5). The ratio has tended to stabilize at about 10 moles nitrate consumed per mole benzene degraded. Those cultures with higher ratios are less enriched than those with lower ratios. As the microcosms became enriched, the rate of benzene degradation increased and the ratio of moles nitrate consumed per mole benzene degraded approached 10 (Table 5-6).

Table 5-5 Summary of mass balances in nitrate-reducing enriched microcosms and transfer cultures

Culture	Date	Ratio nitrate/benzene	n
Gas Station 1			
Enriched microcosms			
13b	1998	12.11 (0.57)	5
14b	1998	13.30 (4.36)	2
Transfer cultures			
13NO3	1998	11.81 (4.16)	2
14(1)	1998	36.72	1
14(2)	1998	14.00	1
Uncontaminated Swamp			
Enriched microcosms			
2b	1998	10.45	1
13b	1998	17.74 (3.22)	2
Transfer culture			
13NO3	1998	10.50 (0.59)	2
Landfarm			
Enriched microcosm			
14b	1996	28.28	1

Table 5-6 Benzene biodegradation in nitrate-amended microcosms and transfer cultures

	Rate of benzene degradation (μM/d)	Nitrate / benzene (mol / mol)
Original microcosms (1995)	3.2 (0.14)[a]	49.6 (15.8)
Enriched microcosms (1995-1997)	7.6 (0.10)	12.6 (3.3)
1st generation transfers (1997)	11.0 (2.5)	11.1 (2.5)
Subsequent transfers (1997-1998)	18.7 (13.0)	10.1 (1.7)

[a]values are mean (SD)

Although the theoretical ratio of nitrate required per mole benzene is 3.36, the experimental ratio tended to stabilize at about 10. Because enriched sulphate-reducing cultures tended to a measured ratio of sulphate utilized per mole of benzene degraded very close to the theoretical ratio (Chapter 4), and because the result from Experiment 5.1 suggested that benzene degradation in the nitrate-reducing cultures might be coupled only to nitrate reduction to nitrite, the energetic equation for benzene degradation coupled to nitrate reduction to nitrite only was determined:

$$C_6H_6 + 15\ NO_3^- + 3H_2O \rightarrow 6\ HCO_3^- + 15\ NO_2^- + 6\ H^+ \qquad \text{Equation 5.1}$$

The standard free energy change for the reaction in Equation 5.1 is –2000 kJ/mol, yielding somewhat less energy than nitrate reduction to nitrogen but significantly more

than is yielded by either sulphate reduction or methanogenesis (refer to Table 2-1). The stoichiometric equation including cell formation is as follows:

$$C_6H_6 + 2.56\ H_2O + 9.75\ NO_3^- + 0.44\ NH_4^+ \rightarrow 3.81\ HCO_3^- + 9.75\ NO_2^- + 0.44\ C_5H_7O_2N + 6\ H^+ \qquad \text{Equation 5.2}$$

Therefore, the theoretical ratio of nitrate required to benzene degraded for nitrate reduction to nitrite is about 10. However, Equation 5.2 assumes an f_s of 0.35, whereas the measured f_s in the nitrate-reducing cultures was actually about 0.06. Using an f_s of 0.06, about 14 moles nitrate are required to degrade one mole of benzene. The reality may be somewhere in-between, with most of the cells' energy derived during the first stage of nitrate reduction, and a smaller amount during subsequent stages.

The role of nitrite in benzene biodegradation in these cultures remains unclear; nitrite is produced as nitrate is reduced but subsequent nitrite reduction does not appear to be linked to benzene biodegradation. However, the nitrite does become completely reduced to nitrogen and does not build up in cultures which are routinely amended with nitrate.

5.4 Conclusions

This study has linked benzene mineralization to nitrate reduction via nitrite to nitrogen gas. This conclusion is supported by the following experimental results: (1) benzene was completely mineralized to CO_2; (2) benzene biodegradation was

concurrent with nitrate reduction and the ratio of nitrate consumed to benzene degraded was constant; (3) other electron acceptors (sulphate, iron(III), and carbon dioxide) were not involved in the degradation; (4) nitrate was first reduced stoichiometrically to nitrite as benzene biodegradation proceeded (with subsequent reduction of nitrite); and (5) degradation of benzene and nitrate was accompanied by cell growth. This is the first confirmed report of benzene biodegradation linked to nitrate reduction.

Benzene biodegradation was linked to nitrate reduction in enriched microcosms and transfer cultures from two different sites – Gas Station 1 and Uncontaminated Swamp. The microcosms from Landfarm also degraded benzene in the presence of nitrate. One possible reason why this study has been so successful in obtaining benzene-degrading cultures is that the cultures were monitored for a very long time, even if benzene did not degrade. The long initial lag times before onset of benzene biodegradation may explain why so many studies report benzene as recalcitrant. Most of the studies reported in the literature were of only a few months duration or even less. The initial lag time before benzene degradation in the Uncontaminated Swamp and Landfarm microcosms was close to one year.

6 FERRIC IRON REDUCTION

6.1 Introduction

In the original microcosms prepared from Oil Refinery material benzene was degraded regardless of the treatment. Measurement of ferrous iron production in these microcosms indicated that iron-reduction was likely the terminal electron accepting process for benzene degradation (Nales 1997). The source of ferric iron in the original microcosms was presumably some mineral in the soil, but was not identified. However, enrichment cultures that degrade benzene in the presence of iron-chelated ethylenediaminetetraacetic acid (Fe(III)-EDTA) were developed. It appears that when the source of soil-derived ferric iron became depleted, other electron acceptors were subsequently used to drive benzene degradation. Chapters 4 and 7 describe sulphate-reduction and methanogenesis in Oil Refinery enriched microcosms and transfer cultures that were originally shown to be iron-reducing. The ability of these cultures to switch electron acceptors is described in detail in Chapter 7.

The goal of this research was to link benzene biodegradation to iron reduction in enriched microcosms and transfer cultures derived from the Oil Refinery site. Two experiments were set up with inoculum from iron-reducing Oil Refinery cultures to determine the effect of iron on benzene degradation. First, Experiment 6.1 was designed to test the effect of adding a source of ferric iron, $Fe(OH)_3$, to a transfer culture which was degrading benzene only very slowly. Second, Experiment 6.2 was

designed to test the effects of adding various sources of potential electron acceptors, including some of the original soil and groundwater, on benzene degradation.

6.2 Materials and Methods

6.2.1 Experimental Design

Experiment 6.1: Effect of $Fe(OH)_3$. Approximately 20 mM Fe(III) was added as $Fe(OH)_3$, from a 400 mM stock, to Oil Refinery transfer culture 6b(1)4, which was only very slowly degrading benzene.

Experiment 6.2: Effect of various sources of electron acceptors. The source of inoculum was from several Oil Refinery cultures pooled together, as shown in Appendix C. All transfer cultures were pooled, to a total volume of approximately 350 ml, into two centrifuge tubes, centrifuged at 4000 rpm for 30 minutes and resuspended in medium supernatant (medium in which the FeS had settled). This mixture was then re-centrifuged and resuspended in only 10 ml of sterile, anaerobic ddH_2O to concentrate the inoculum. Twelve vials were prepared as outlined in Table 6-1 (2 replicates of each treatment). The groundwater and sediment from the original site, which had been stored at 4°C since sampling in 1995, were added as indicated. Inoculated vials were amended with 1 ml of the concentrated inoculum. Two additional vials (treatment 7 in Table 6-1) were subsequently prepared with leftover inoculum from the centrifuge tubes rinsed with medium; the inoculum of these two

vials was therefore not identical to that of the other vials. Benzene concentrations and Fe(II) production were monitored over time.

Table 6-1 Set up of Experiment 6.2

Treatment	Inoc.[a] (ml)	Sed[b] (ml)	SO_4^{2-} (mM)	$Fe(OH)^3$ (mM)	EDTA (mM)	GW[c] (ml)	Med[d] (ml)
1-Medium only	1	-	-	-	-	-	29
2-Groundwater only	1	-	-	-	-	29	-
3-Sediment & medium	1	5	-	-	-	-	up to 29
4-Medium & sulphate	1	-	5	-	-	-	28.7
5-Medium & $Fe(OH)^3$	1	-	-	16	-	-	27.8
6-Sediment & medium (not inoculated)	-	5	-	-	-	-	up to 29
7-Medium & EDTA	rinse	-	-	-	16	-	30

[a]inoculum from pooled Oil Refinery transfer cultures
[b]sediment from the original Oil Refinery site
[c]groundwater from the original Oil Refinery site
[d]defined mineral medium

6.2.2 Analytical Procedures

Benzene, methane, nitrate, sulphate, ferrous iron and total iron were measured as previously described in Section 3.1.6.

6.3 Results and Discussion

6.3.1 Experiment 6.1: Effect of $Fe(OH)_3$

The rate of benzene degradation in enrichment culture Oil Refinery 6b(1)4 had slowed considerably. It was hypothesized that the ferric iron present in the soil had become limiting. In an attempt to speed up the benzene degradation, 20 mM Fe(III) was added to this culture as $Fe(OH)_3$. As can be seen from Figure 6-1, the addition of this potential electron acceptor did not increase the rate of benzene degradation.

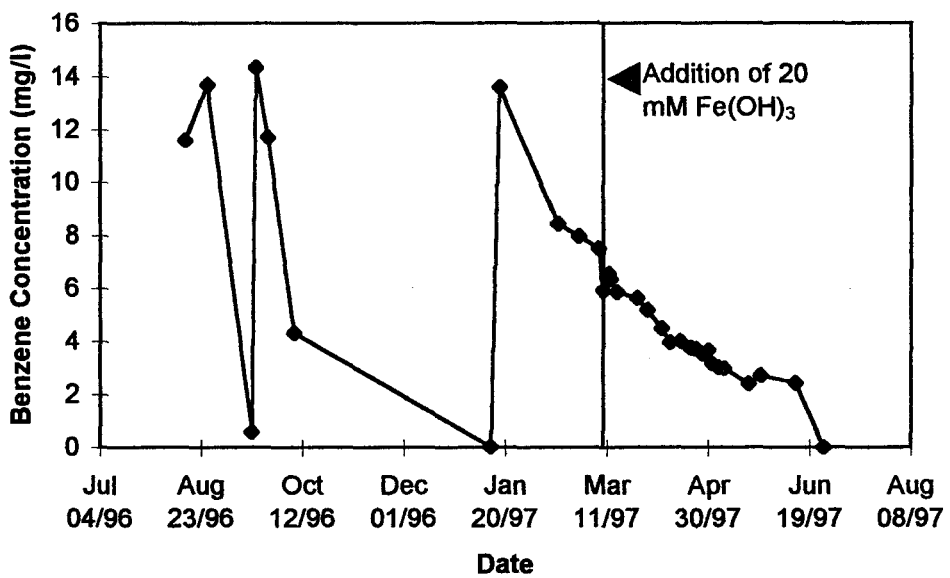


Figure 6-1 Addition of $Fe(OH)_3$ to an Oil Refinery Transfer Culture - Experiment 6.1

Some forms of ferric iron are more accessible to microorganisms than others (Lovley and Phillips 1986). It has been difficult to find an appropriate form of ferric iron to add to these transfer cultures when the source in the original soil becomes depleted.

6.3.2 Experiment 6.2: Effect of various sources of electron acceptors

Because of the unsuccessful results of Experiment 6.1, an experiment was designed to test the effects of various electron acceptors on the benzene-degrading activity of transfer cultures from the Oil Refinery site. In addition to ferric iron as both $Fe(OH)_3$ and Fe(III)-EDTA, original soil and groundwater were added as a source of the original electron acceptor, presumably iron. Sulphate was also tested as a possible electron acceptor. The results, shown in Figure 6-2, confirm that the original soil and groundwater supplied enough electron acceptor to degrade the benzene. In addition, benzene degradation proceeded much faster in the vials amended with Fe(III)-EDTA than in any other vials. It must be restated, however, that the inoculum was different for this treatment than for all of the others in this experiment, and may have contained a greater initial concentration of microorganisms (see above).

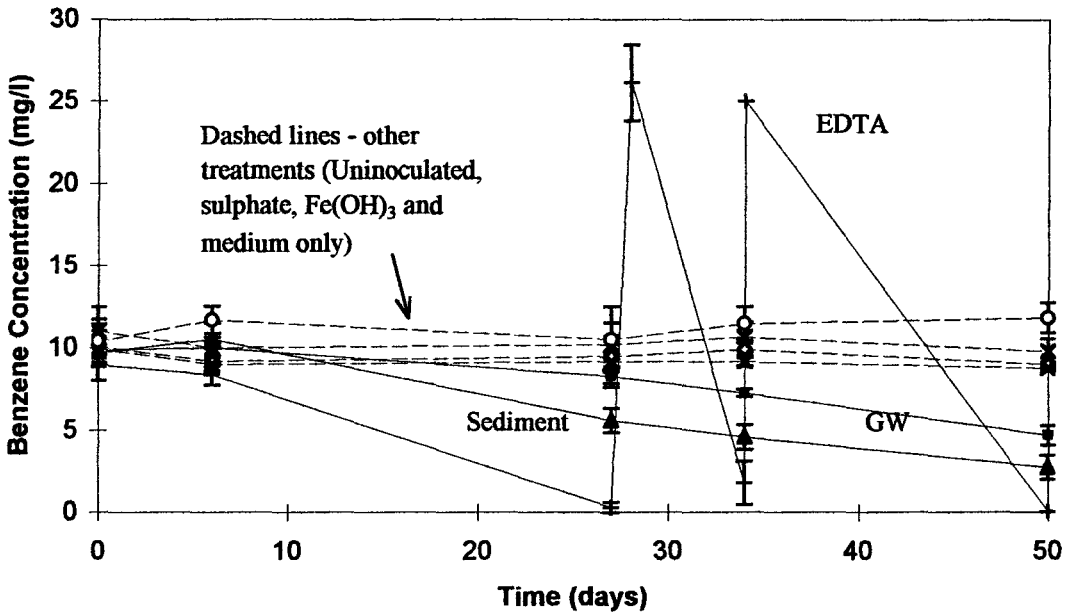


Figure 6-2 Degradation of benzene in Oil Refinery Transfer Cultures - Experiment 6.2

6.3.3 Mass Balances

Because the natural source of ferric iron in the enriched microcosms was depleted before this thesis research was started, benzene degradation linked to iron reduction was observed only with addition of Fe(III)-EDTA. Experiment 6.2 resulted in benzene degradation in the two vials amended with Fe(III)-EDTA, but the ratio of ferrous iron produced to benzene degraded was well below the theoretical; 8.33 and 9.47 for the two vials. A subsequent transfer culture, (1)EDTA, produced 27.44 mol ferrous iron per mol benzene degraded, which is close to the predicted value of 30.

Transfers from this transfer culture, prepared for Experiment 7.1, produced 21.37 and 22.76 mol ferrous iron per mole of benzene degraded. Refer to Chapter 7 for a detailed description of this work.

6.4 Conclusions

Addition of some original soil or groundwater from the Oil Refinery site stimulated benzene degradation, presumably because of the presence of an electron acceptor. The addition of Fe(III)-EDTA also stimulated benzene degradation in some Oil Refinery transfer cultures. A ratio of ferrous iron produced to benzene degraded of about 22-28 was determined. This value is close to the theoretical value of about 30 moles ferrous iron per mole benzene. A non-chelated source of microbially-available Fe(III), such as $Fe(OH)_3$ or other iron oxides, was not found. As reported in Lovely (1986) it is difficult to find a source of iron which can be used as an electron acceptor for contaminant oxidation.

7 METHANOGENESIS AND SEQUENTIAL ELECTRON ACCEPTOR UTILIZATION

7.1 Introduction

Benzene degradation linked to methanogenesis was not found during the original study of material from the six sites (Nales 1997). However, at least one culture derived from Gas Station 1 soil and several derived from Oil Refinery soil have since produced significant amounts of methane as benzene biodegradation proceeds. When the available ferric iron in Oil Refinery microcosms and transfer cultures became depleted, benzene continued to be degraded, presumably linked to methanogenesis or sulphate reduction. This chapter describes experiments designed to confirm that the terminal electron acceptor for benzene degradation changed in these cultures.

7.2 Materials and Methods

7.2.1 Amendment of Experimental Cultures with Potential Electron Acceptors

The source of ferric iron was ferric iron sodium ethylenediaminetetraacetic acid (EDTA). It was added as a liquid from a 514-mM anaerobic stock for Experiment 7.1 and as a powder (13% Fe^{3+}) in all other instances. Sulphate was added as sodium

sulphate from a 500-mM anaerobic stock. Nitrate was added as sodium nitrate from a 500-mM anaerobic stock.

7.2.2 Amendment of Experimental Cultures with Specific Inhibitors

Specific inhibitors of sulphate reduction and/or methanogenesis were added to some of the experimental cultures in Experiment 7.2. Molybdate is an inhibitor of sulphate reduction (Taylor and Oremland 1979). The first step in sulphate reduction is the activation of sulphate to adenosine 5'-phosphosulphate (APS) catalyzed by the enzyme adenosine 5'-triphasphate (ATP) sulfurylase:

$$SO_4^{2-} + ATP = APS + PP \qquad \text{Equation 7.1}$$

Other group VI oxyanions can be substrates for this reaction; but with molybdate, chromate or tungstate, the ATP is converted into adenosine 5'-monophosphate (AMP) and pyrophosphate (PP) via a putative unstable AMP-anion anhydride (Taylor and Oremland 1979). Molybdate and chromate are the most effective oxyanions in destroying ATP. However, chromate is a generally toxic compound, whereas molybdate is usually a nutrient. Therefore, molybdate is lethal to sulphate-reducing bacteria but does not detrimentally affect other bacteria (Taylor and Oremland 1979). The molybdate oxyanion, MoO_4^2, was added from a 100-mM anaerobic stock of sodium molybdate to a concentration in the vials of 2 mM.

Bromoethanesulfonic acid (BESA), is an inhibitor of methanogenesis (Madigan et al. 1997). The final step in methane formation involves 2-mercaptoethanesulfonic acid (coenzyme M) as the carrier of the methyl group that is reduced to methane:

$$CH_3\text{-S-CoM} + 2\ H \rightarrow HS\text{-CoM} + CH_4 \qquad \text{Equation 7.2}$$

BESA is a potent inhibitory analog of coenzyme M, causing 50% inhibition of methyl reductase activity at a concentration of 10^{-6} M and inhibiting the growth of methanogenic bacteria (Madigan et al. 1997). BESA was added from a 100 mM anaerobic stock to a concentration in the vials of 2 mM.

7.2.3 Experimental Design

Two observations of the change in terminal electron accepting process for benzene biodegradation in transfer cultures derived from the Oil Refinery soil led to the design of the following two experiments.

Experiment 7.1. This experiment was designed to determine if an Oil Refinery transfer culture had the ability to degrade benzene under iron-reducing, sulphate-reducing and methanogenic conditions. This culture was originally iron-reducing, then had switched to methanogenesis. The culture was allowed to settle before 300 ml was poured into a sterile, anaerobic beaker. Fifteen milliliters of well-shaken inoculum was distributed into each of 18 40-ml vials. The remaining 10 ml was saved for protein

analysis. The vials were diluted 50% to 30 ml with medium supernatant (medium in which the FeS was allowed to settle). Nine of the vials (three of each treatment) were amended with ca. 10 mg/l benzene and the other nine (three of each treatment) were background controls with no benzene. The three treatments were Fe(III)EDTA (17 mM added as a liquid), sulphate (10 mM), and nothing (for methanogenesis). Each vial was purged with N_2/CO_2 for 10 minutes. Benzene, methane, sulphate (in vials without EDTA) and ferrous iron were measured over time.

Experiment 7.2. This experiment was designed to determine if the transfer cultures derived from Oil Refinery material were able to switch electron acceptors as benzene degradation continued. Three transfer cultures derived from Oil Refinery Combo 1 were used as three separate inocula in this experiment: Inoculum #1 Iron-reducing; Inoculum #2 Sulphate-reducing and Inoculum #3 Methanogenic. Each inoculum was split into several 17-ml vials. Duplicate vials were amended with electron acceptors and specific inhibitors as shown in Table 7-1.

Table 7-1 Experiment 7.2 Set up: Electron Acceptor Use in Oil Refinery Transfer Cultures

Treatment	EDTA (mM)	BESA (mM)	Molybdate (mM)	SO_4^{2-} (mM)	NO_3^- (mM)
Inoculum #1 Iron-reducing					
1 – None	0	0	0	0	0
2 – EDTA	17.5	0	0	0	0
3 – EDTA & BESA	17.5	2	0	0	0
4 – EDTA & Molybdate	17.5	0	2	0	0
5 – EDTA & BESA & Molybdate	17.5	2	2	0	0
6 – Nitrate	0	0	0	0	5
Inoculum #2 Sulphate-reducing					
7 – None	0	0	0	0	0
8 – Sulphate	0	0	0	5	0
9 – Sulphate & Molybdate	0	0	2	5	0
10 – Sulphate & BESA	0	2	0	5	0
11 – Sulphate & BESA & Molybdate	0	2	2	5	0
12 – Nitrate	0	0	0	0	5
Inoculum #3 Methanogenic					
13 – None (4 replicates)	0	0	0	0	0
14 – BESA	0	2	0	0	0
15 – EDTA & Sulphate	17.5	0	0	5	0
16 – Nitrate	0	0	0	0	5

Medium was added to make each culture 10 ml (50% dilution) and each vial was amended with 0.1 µl benzene from a neat, anaerobic stock.

In addition to the 34 experimental cultures, control vials for BESA and molybdate were prepared from a toluene-degrading methanogenic (Edwards and

Grbic-Galic 1994) and a benzene-degrading sulphate-reducing culture, respectively. The addition of molybdate to a sulphate-reducing benzene-degrading culture inhibited benzene degradation. The addition of BESA to a methanogenic toluene-degrading culture slowed the degradation of toluene but did not stop it; the addition of more BESA (an additional 2 mM) slowed the degradation of toluene further but still did not stop it.

7.2.4 Analytical Procedures

Benzene, methane, sulphate, nitrate and ferrous iron were measured as previously described in Section 3.1.6.

7.3 Results and Discussion

7.3.1 Initial Observations of a Changing Terminal Electron Acceptor

The first indication of a changed terminal electron accepting process was the production of methane in a Gas Station 1 enriched microcosm (previously sulphate-reducing) and in Oil Refinery transfer cultures (previously iron-reducing). Generally, in transfer cultures from all sites, methane was rapidly produced when added electron acceptors such as sulphate, nitrate and Fe(III)EDTA became depleted. Benzene biodegradation continued under methanogenic conditions in some transfer cultures but was inhibited in others.

Gas Station 1

The first indication of methanogenesis linked to benzene degradation in the Gas Station 1 material was the continuation of benzene biodegradation in microcosm 4b even when sulphate was depleted (Figure 7-3). Additionally, the concentration of methane in the headspace of the microcosm, was much greater than the methane produced in other Gas Station 1 microcosms in which benzene degradation occurred in the presence of sulphate.

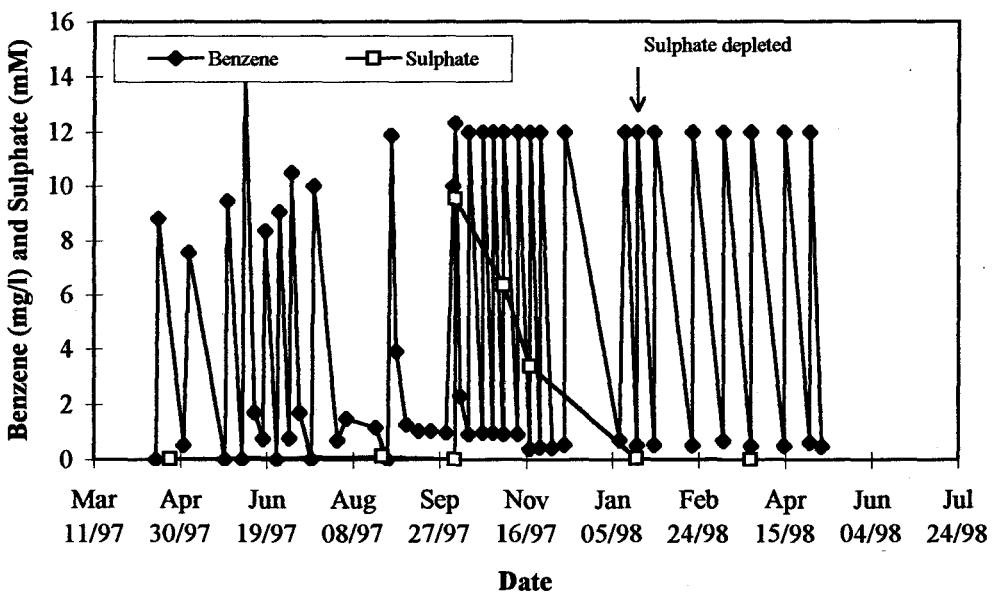


Figure 7-1 Benzene biodegradation and sulphate utilization in microcosm Gas Station 1 #4b

Experiment 4.1 was set up with inoculum from Gas Station 1 microcosm 9b to establish that benzene degradation was dependent upon sulphate (see Chapter 4).

Although benzene was degraded more rapidly in the presence of sulphate (refer to Figure 4-1), benzene degradation also occurred in the culture vials without sulphate. There was significantly more methane produced in the vials not amended with sulphate than in the sulphate-amended vials.

Oil Refinery

During the original study (Nales 1997), there was no significant methane production in the Oil Refinery microcosms or transfer cultures; it was determined that ferric iron was the likely electron acceptor. However, the source of ferric iron in the original soil was not determined. Sustained benzene biodegradation may have depleted the original ferric iron in the soil, allowing other bacteria to flourish. In many of the Oil Refinery microcosms and transfer cultures benzene degradation now appears to be linked to sulphate reduction or methanogenesis (Figure 7-2).

The data shown in Figure 7-2 are for the transfer culture described as Inoculum #3 in Experiment 7.2. There was no sulphate or nitrate present in the culture, eliminating sulphate and nitrate reduction as potential electron-accepting processes coupled to benzene degradation. During the one-year incubation, almost 240 μmoles of benzene were degraded by this enrichment culture, corresponding to at least 90 mM Fe(II) production (assuming a ratio of 30 mol Fe(II) produced for each mol benzene degraded) if iron reduction was the terminal electron acceptor. Less than 14 mM Fe(II) was produced in this culture bottle, eliminating iron reduction as the terminal electron acceptor. However, there was sufficient methane produced (440000

ppm in the headspace) to account for the benzene degraded: the experimentally measured ratio of 3.04 mol CH_4 produced per mol benzene degraded is close to the theoretical ratio of 3.75 mol CH_4 per mol benzene, confirming methanogenesis as the terminal electron accepting process.

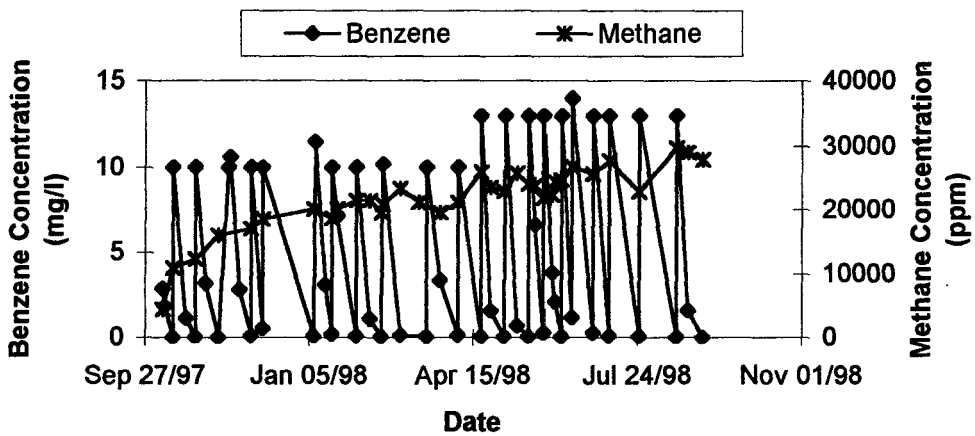


Figure 7-2 Methane production and benzene degradation in an Oil Refinery transfer culture

In two different instances the apparent switch of electron acceptor coupled to benzene biodegradation was observed in an Oil Refinery transfer culture. First, benzene degradation under sulphate-reducing conditions was occurring in transfer culture 6b(2) at a constant ratio of about 4.4 moles sulphate utilized per mole benzene degraded. When the sulphate became depleted, there was a decrease in the rate of benzene degradation; however, methane production and the resumption of benzene degradation coincided (Figure 7-3). A ratio of methane produced per mole benzene

degraded could not be calculated because the methane concentrations were significantly underestimated after about 10000 ppm.

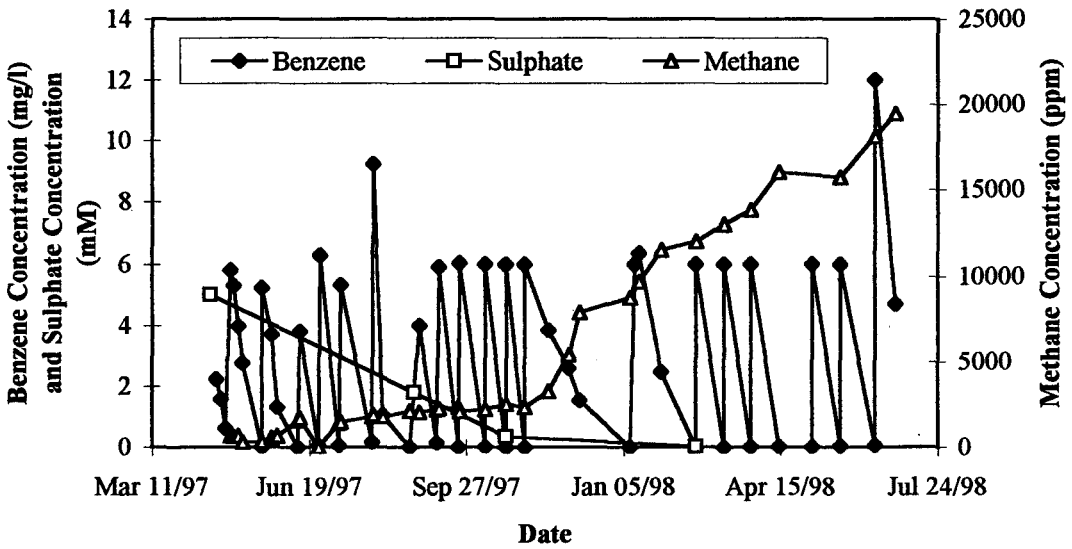


Figure 7-3 Sulphate depletion and methane production in an Oil Refinery transfer culture

Second, the three transfer cultures from Combo 1 exhibited intermittent methane production (Figure 7-4). In the presence of Fe(III)-EDTA or sulphate there was insignificant methane production. However, when the source of ferric iron or sulphate became depleted, methane was produced. Benzene degradation continued at the same rate in all cultures.

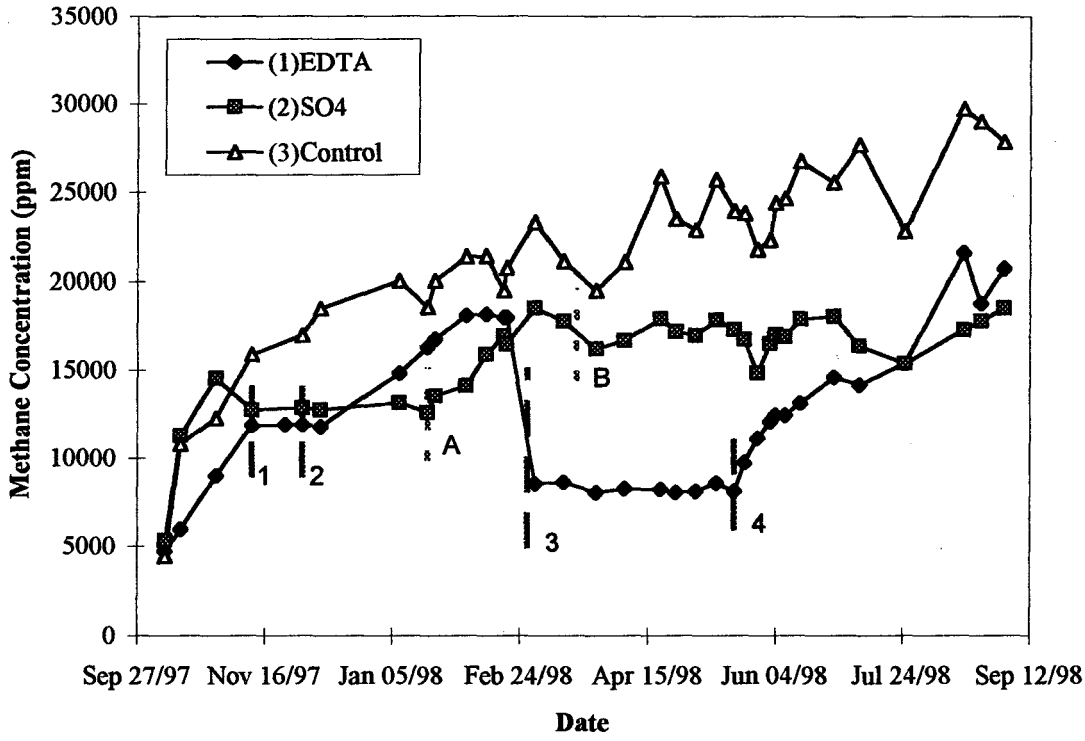


Figure 7-4 Methane production in Oil Refinery transfer cultures

The source of inoculum for these transfer cultures contained no sulphate, nitrate or added ferric iron (such as EDTA). Methane production in the source culture was very significant, although accurate measurements were not made. Initially, when the transfers were made, methane was produced in all three cultures (Figure 7-4), however, methane production essentially stopped in the Fe(III)-EDTA and sulphate amended cultures after a few weeks.

The Fe(III)EDTA-amended culture may have been methanogenic up to point 1 (Figure 7-4). The flat portion of the curve from 1 to 2 represents reduction of the 8 mM ferric iron at a ratio of 30 moles ferrous iron produced per mole benzene degraded. At point 2, when the source of ferric iron would theoretically be depleted, methane production resumed. On February 24, 1998 a transfer was made from this culture bottle; therefore the bottle was opened and methane was removed from the headspace. However, when 16 mM Fe(III)-EDTA was again added to the culture at point 3, methane was not produced. For a ratio of 30 moles ferrous iron produced per mole benzene degraded, the source of ferric iron would be depleted at point 4; subsequently, methane production resumed. Measurement of Fe(II) at two points between 3 and 4 confirmed a ratio of 27.44 moles ferrous iron produced per mole benzene degraded.

It appears that the sulphate-amended culture continued benzene degradation under methanogenic conditions for a few weeks after the transfer, before switching to sulphate reduction. Sulphate was measured in the culture at various times and the ratio of sulphate consumed per mole of benzene degraded was calculated. Assuming the first amendment of benzene was degraded methanogenically, the initial 2.5 mM sulphate was consumed at a ratio of 3.35 moles sulphate per mole benzene degraded; the sulphate was depleted at point A (Figure 7-4). After point A, the methane concentration increased until additional sulphate was added to the culture at point B, at which time, methane production stopped. Fortunately, this culture was not opened before the actual methane concentration in the culture bottle was determined: 132000

ppm. The total sulphate added to the culture over the one-year incubation (680 μmoles) accounts for 181 μmoles of the 237 μmoles benzene degraded. The degradation of the remaining 56 μmoles benzene would result in the production of 210 μmoles methane if degraded by methanogenesis; 207 μmoles methane was produced in this culture bottle. Therefore, between sulphate reduction and methanogenesis, the benzene degradation was entirely accounted for. The culture was able to degrade benzene under both conditions and switched back and forth.

In order to confirm the capability of these Oil Refinery transfer cultures to degrade benzene under various electron-accepting conditions, two experiments were carried out. The next two sections describe these two experiments and the preliminary results.

7.3.2 Experiment 7.1

The results from Experiment 7.1 confirmed that transfer cultures derived from Oil Refinery Combo 1 could degrade benzene linked to iron reduction, sulphate reduction and methanogenesis (Figure 7-5). Although the replicates did not respond identically, there was significant benzene degradation in eight of the nine active vials.

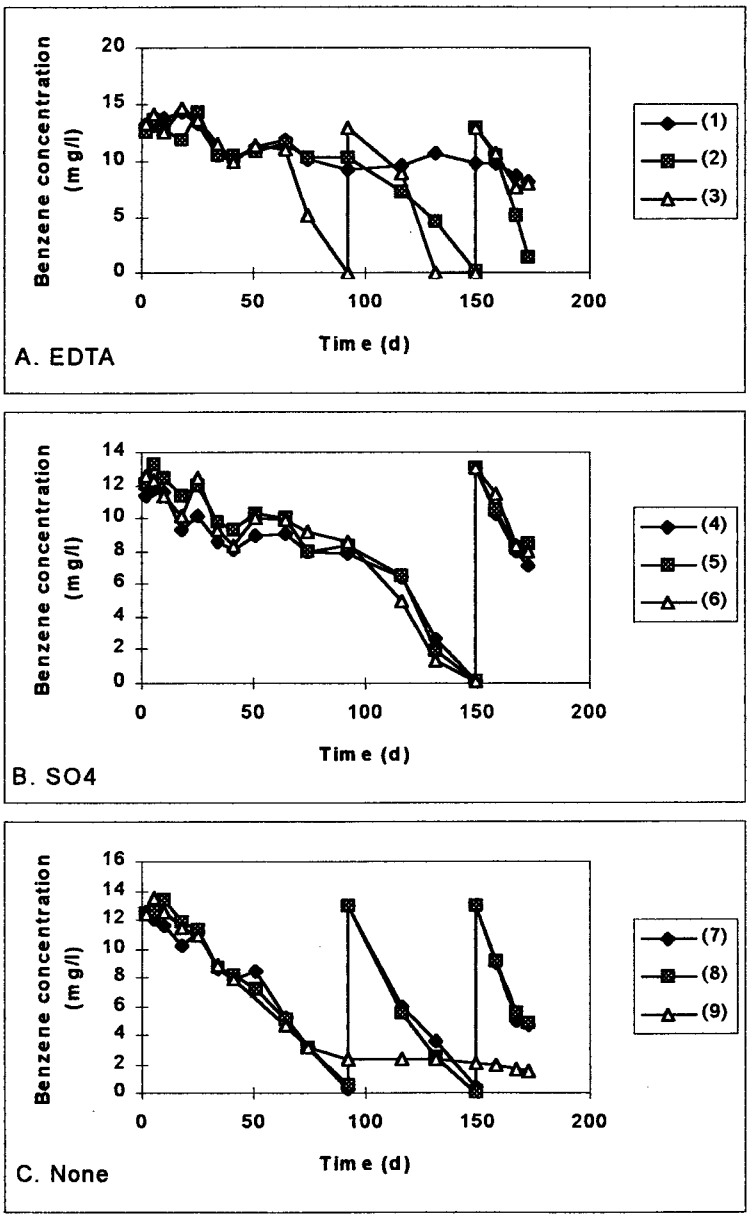


Figure 7-5 Benzene concentration vs. time in benzene-amended vials of Experiment 7.1. A. Ferric iron amended. B. Sulphate amended. C. No amendment (carbon dioxide present).

The three vials amended with EDTA are shown in Part A of Figure 7-5; benzene degradation occurred in two of the vials. Benzene was degraded in all three of the vials amended with sulphate (Part B). Several feedings of benzene were degraded in two of the vials with no additional amendment (Part C); however, in one of the vials benzene was degraded only to 2 mg/l and then was not degraded further.

Ferrous iron, sulphate and methane were monitored in these nine vials as well as in the nine background controls with no benzene. The ratios of ferrous iron produced, sulphate utilized and methane produced to benzene degraded were calculated for each active vial and compared to the theoretical ratios in Table 2-1. Background ferrous iron production and methane production, calculated as the average of the three background controls for each treatment, were subtracted before the ratio was calculated (Table 7-2). There was insignificant background sulphate utilization.

Table 7-2 Evaluation of potential electron accepting conditions in Experiment 7.1

Vial	Benzene degraded (μmol)	Ratio Fe(II) produced / benzene degraded	Ratio sulphate utilized / benzene degraded	Ratio CH_4 produced / benzene degraded	Electron-accepting process[a]
1	1.69	0	0	0	benzene not degraded
2	9.03	21.37	0	0	iron reduction
3	10.72	22.76	0	0	iron reduction
4	6.21	0.15	11.47	0	sulphate reduction
5	6.21	0.47	12.80	0	sulphate reduction
6	6.21	0	12.26	0	sulphate reduction
7	11.85	0.14	0	4.38	methanogenesis
8	11.85	0.14	0	4.59	methanogenesis
9	3.84	0	0	4.85	methanogenesis

[a]by comparison with Table 2-1

These data show that this particular inoculum harboured sulphate-reducing, iron-reducing and methanogenic bacteria, and that each of these processes can drive benzene oxidation.

7.3.3 Experiment 7.2

The results from Experiment 7.2 confirmed that the electron-accepting process switched from sulphate reduction to methanogenesis and back to sulphate reduction. Neither iron reduction nor nitrate reduction was observed in this experiment.

Inoculum #1 Originally Iron-reducing. None of the cultures amended with Fe(III)EDTA significantly degraded benzene. These cultures should have been active with Fe(III)EDTA. The reason this addition of Fe(III)EDTA inhibited this culture, which had previously degraded benzene in the presence of Fe(III)EDTA, is unknown. Benzene was degraded only in the unamended (methanogenic) vials. The average ratio of moles methane produced per mole benzene degraded in these vials was 2.45.

Inoculum #2 Originally Sulphate-reducing. In the vials amended only with sulphate, three feedings of benzene were degraded with no methane production, presumably via sulphate reduction. In the unamended treatments, benzene was degraded at the same rate, with 2.66 moles methane produced per mole of benzene degraded. The addition of sulphate and molybdate partially inhibited benzene degradation; the benzene that did degrade was accompanied by methane production at a ratio of 3.00 moles methane per mole benzene, indicating that the sulphate-reducing bacteria were inhibited by the molybdate and the methanogens were inhibited by the sulphate. This result is in agreement with results from other experiments where the presence of sulphate inhibited methanogens. The addition of sulphate and BESA completely inhibited methane production; three feedings of benzene were degraded, presumably by sulphate reduction. The addition of sulphate, molybdate and BESA completely inhibited benzene degradation and methane production, indicating that benzene biodegradation in these transfer cultures is likely linked to either sulphate reduction or methanogenesis.

Inoculum #3 Originally Methanogenic. Benzene degradation occurred in the four replicates with no added electron acceptor or specific inhibitor (methanogenic conditions). Three feedings of benzene were degraded in each vial at an average ratio of 1.74 moles methane produced per mole benzene degraded. The addition of BESA considerably slowed both benzene degradation and methane production; the partial degradation of one feeding of benzene resulted in 2.48 moles methane produced per mol of benzene degraded.

7.4 Conclusions

Although during the original microcosm study benzene degradation linked to methanogenesis was not observed, some of the enriched microcosms and transfer cultures from the Gas Station 1 and Oil Refinery sites switched to methanogenesis when alternate electron acceptors, such as sulphate or ferric iron, became depleted. This result supports findings reported in the literature linking benzene biodegradation to methanogenesis, and that methanogenesis often does not begin until other electron acceptors have become depleted (Rogers 1987; Grbic-Galic 1990).

The experiments with molybdate and BESA confirmed that sulphate reduction and methanogenesis were significant processes in these transfer cultures. Transfer cultures derived from Oil Refinery material were able to switch the terminal electron-accepting process coupled to benzene biodegradation. In one experiment, the electron accepting process switched from methanogenic to iron-reducing and sulphate-

reducing. In another experiment the electron-accepting process switched from methanogenic to sulphate-reducing and from sulphate-reducing to methanogenic (when sulphate was depleted). These results are supported by the theoretical energy calculations which predict that iron-reducers should out-compete sulphate-reducers which should out-compete methanogens. The ability of these enriched transfer cultures to degrade benzene linked to more than one electron acceptor indicates that the cultures remain very mixed; much more enrichment is necessary to obtain a pure benzene-degrading culture.

Perhaps these data suggest that the benzene-degrading microorganism is a fermentative bacterium that produces extracellular metabolites (hydrogen and acetate, for example) that can be used by iron-reducing, sulphate-reducing or methanogenic bacteria, depending upon electron acceptor availability. The fact that the rates of benzene biodegradation don't change appreciably between electron acceptors suggests that the rate is controlled by the fermentation step and not by the terminal electron accepting process.

8 CONCLUSIONS AND RECOMMENDATIONS

Sustained, anaerobic, benzene biodegradation was obtained in enriched microcosms and transfer cultures derived from four different sites. The benzene-degrading activity was successfully transferred from soil and groundwater microcosms into a defined, mineral medium with little or no soil from the original microcosms. Viable transfer cultures with less than 1% of the original microcosm were developed.

The rate of benzene biodegradation was variable, ranging from 1 μM/d to more than 75 μM/d, with an average of about 15 μM/d. Benzene degradation linked to nitrate reduction degraded at faster rates in some experiments; benzene degradation linked to sulphate reduction generally had the slowest degradation rates. The rate of benzene biodegradation linked to methanogenesis in Oil Refinery transfer cultures did not depend on the concentration of benzene for concentrations ranging from 1 to 100 mg/l. The zero-order kinetics determined for this culture are in agreement with the results from a similar experiment conducted by Nales (1997) with a Gas Station 1 sulphate-reducing culture.

Benzene depletion curves from a sulphate-reducing culture and a nitrate-reducing culture were fit to the Monod kinetic equation by nonlinear regression analysis. The results confirmed the slow growth of the bacteria in these cultures. The calculated doubling time for the sulphate-reducing culture was 30 days and for the nitrate-reducing culture was 9 days.

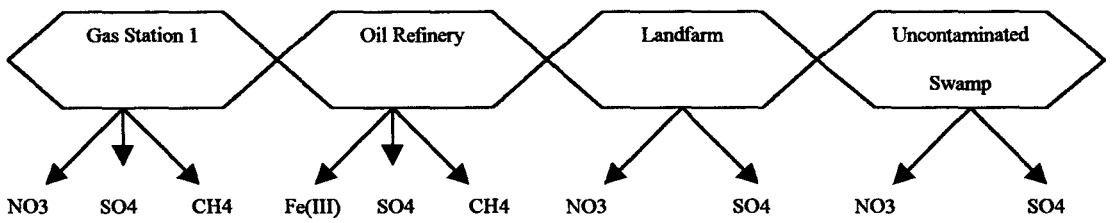


Figure 8-1 Terminal electron accepting processes for benzene biodegradation in enriched cultures derived from each site

Anaerobic benzene biodegradation was linked to sulphate reduction, nitrate reduction, iron reduction and methanogenesis (Figure 8-1). Sulphate reduction linked to benzene degradation was confirmed by the following results: (1) experimentally-determined ratios of sulphate depleted per mole benzene degraded agreed with the theoretical ratios; and (2) degradation of benzene and sulphate was accompanied by cell growth.

Benzene degradation was linked to nitrate-reduction. This important conclusion was supported by the following experimental results: (1) benzene was completely mineralized to CO_2; (2) benzene biodegradation was concurrent with nitrate reduction and the ratio of nitrate consumed to benzene degraded was constant; (3) other electron acceptors – sulphate, ferric iron and carbon dioxide – were not involved in the degradation; (4) nitrate was first reduced stoichiometrically to nitrite as benzene biodegradation proceeded, with subsequent reduction of nitrite to nitrogen; and (5)

degradation of benzene was accompanied by cell growth. Benzene-degrading nitrate-reducing cultures were enriched from two of the four sites – Gas Station 1 and Uncontaminated Swamp. In two microcosms from the Landfarm site, benzene was degraded only in the presence of nitrate, although the link was not established in these cultures. This is the first study linking benzene degradation to nitrate reduction.

Transfer cultures derived from Oil Refinery material showed the ability to switch the terminal electron-accepting process linked to benzene degradation. The terminal electron acceptor was putatively ferric iron in the original microcosms; transfer cultures linked to sulphate reduction, iron reduction and methanogenesis were developed. A reliable source of ferric iron to replenish iron-depleted cultures with was not found; Fe(III)-EDTA was used by some cultures as a source of ferric iron, but not by others. The ability of Oil Refinery transfer cultures to switch electron acceptors while degrading benzene was confirmed by (1) the same inoculum degrading benzene under iron-reducing, sulphate-reducing and methanogenic conditions; (2) the production of methane only when iron or sulphate became depleted; and (3) inhibition experiments with molybdate and BESA. A summary of changes in the terminal electron accepting process for benzene degradation observed in any enriched microcosms and transfer cultures is presented in Table 8-1.

Table 8-1 Summary of observed changes in terminal electron accepting process for benzene biodegradation

Starting Electron acceptor	Switched to iron reduction?	Switched to nitrate reduction?	Switched to sulphate reduction?	Switched to methanogenesis ?
Fe(III)	-	no	yes	yes
NO_3^-	n.d.*	-	no	no
SO_4^{2-}	n.d.	no	-	yes
CO_2	yes	no	yes	-

*not determined

This research was successful in developing and maintaining anaerobic benzene-degrading microbial cultures. The initial characterization of these cultures has raised many questions regarding the mechanisms of anaerobic benzene degradation. Future work that would help to further characterize these cultures includes (1) investigating the role of nitrite in the nitrate-reducing cultures; (2) determining the effect of oxygen on the nitrate-reducing cultures – can they aerobically degrade benzene?; (3) finding a reliable source of ferric iron to add to iron-reducing cultures; and (4) purifying cultures of each type of electron acceptor with the aim of identifying metabolic pathways.

9 REFERENCES

Allen-King, R. M., J. F. Barker, R. W. Gillham and B. K. Jensen (1994). "Substrate-limited and nutrient-limited toluene biotransformation in sandy soil." Environmental Toxicology and Chemistry **13**(5): 693-705.

Alvarez, P. J. J., P. J. Anid and T. M. Vogel (1994). "Kinetics of toluene degradation by denitrifying aquifer microorganisms." Journal of Environmental Engineering-Asce **120**(5): 1327-1336.

Aronson, D. and P. H. Howard (1997). Anaerobic biodegradation of organic chemicals in groundwater: a summary of field and laboratory studies. North Syracuse, NY, Environmental Science Center.

Ball, H. A. and M. Reinhard (1996). "Monoaromatic hydrocarbon transformation under anaerobic conditions at Seal Beach, California - laboratory studies." Environmental Toxicology and Chemistry **15**(2): 114-122.

Beaty, P. S., M. J. McInerney and N. Q. Wofford (1986). Energetics of H2-Producing Syntrophic Bacteria. Biotechnological Advances in Processing Municipal Wastes for Fuels and Chemicals. A. A. Antonopoulos, Argonne National Laboratory.

Beller, H. R. (1995). "Byproducts of anaerobic alkylbenzene metabolism useful as indicators of *in situ* bioremediation." Environmental Science and Technology **29**(11): 2864-2870.

Beller, H. R., D. Grbic-Galic and M. Reinhard (1992). "Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron on the process." Applied and Environmental Microbiology **58**: 786-793.

Bertrand, J. C., P. Caumette, G. Mille, M. Gilewicz and M. Denis (1989). "Anaerobic biodegradation of hydrocarbons." Science Progress **73**(291): 333-350.

Borden, R. C., C. A. Gomez and M. T. Becker (1995). "Geochemical indicators of intrinsic bioremediation." Ground Water **33**(2): 180-189.

Bouwer, H. (1978). Groundwater Hydrology. New York, McGraw Hill Inc.

Bradford, M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal. Biochem. **72**: 248-254.

Chee-Sanford, J. C., J. W. Frost, M. R. Fries, J. Z. Zhou and J. M. Tiedje (1996). "Evidence for acetyl coenzyme A and cinnamoyl coenzyme A in the anaerobic toluene mineralization pathway in *Azoarcus tolulyticus* Tol-4." Applied and Environmental Microbiology **62**(3): 964-973.

Clark, L. (1995). "Hydrocarbon pollution control and remediation of groundwater: a brief review." Quarterly Journal of Engineering Geology **28**(S2): S93-S100.

Coates, J. D., R. T. Anderson, J. C. Woodward, E. J. P. Phillips and D. R. Lovley (1996). "Anaerobic hydrocarbon degradation in petroleum-contaminated harbor sediments under sulfate-reducing and artificially imposed iron-reducing conditions." Environmental Science and Technology **30**(9): 2784-2789.

Cozzarelli, I. M., R. P. Eganhouse and M. J. Baedecker (1990). "Transformation of monoaromatic hydrocarbons to organic acids in anoxic groundwater environment." Environ. Geol. Water Sci. **16**(2): 135-141.

Cozzarelli, I. M., J. S. Herman and M. J. Baedecker (1995). "Fate of microbial metabolites of hydrocarbons in a coastal plain aquifer: the role of electron accepters." Environmental Science & Technology **29**(2): 458-469.

Criddle, C. (1989). Reductive Dehalogenation in Microbial and Electrolytic Model Systems. Department of Civil Engineering, Stanford University: 162.

Davis, J. W., N. J. Klier and C. L. Carpenter (1994). "Natural biological attenuation of benzene in ground water beneath a manufacturing facility." Ground Water **32**(2): 215-226.

Dolfing, J., J. Zeyer, P. Binder-Eicher and R. P. Schwarzenbach (1990). "Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen." Arch Microbiol **154**: 336-341.

Edwards, E. A. and D. Grbic-Galic (1992). "Complete mineralization of benzene by aquifer microorganisms under strictly anaerobic conditions." Applied and Environmental Microbiology **58**(8): 2663-2666.

Edwards, E. A. and D. Grbic-Galic (1994). "Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium." Applied and Environmental Microbiology **60**(1): 313-322.

Edwards, E. A., L. E. Wills, M. Reinhard and D. Grbic-Galic (1992). "Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions." Applied and Environmental Microbiology **58**(3): 794-800.

Evans, P. J., D. T. Mang, K. S. Kim and L. Y. Young (1991). "Anaerobic degradation of toluene by a denitrifying bacterium." Applied and Environmental Microbiology **57**(4): 1139-1145.

Grbic-Galic, D. (1990). Anaerobic microbial transformation of nonoxygenated aromatic and alicyclic compounds in soil, subsurface, and freshwater sediment. Soil Biochemistry. J. M. B. a. G. Stotzky.

Grbic-Galic, D., N. Churchman-Eisel and I. Mrakovic (1990). "Microbial transformation of styrene by anaerobic consortia." Journal of Applied Bacteriology **69**(2): 247-260.

Grbic-Galic, D. and T. M. Vogel (1987). "Transformation of toluene and benzene by mixed methanogenic cultures." Applied and Environmental Microbiology **53**(2): 254-260.

Hadley, P. W. and R. Armstrong (1991). "Where's the benzene - examining California groundwater quality surveys." Ground Water **29**(1): 35-40.

Harwood, C. S. and J. Gibson (1997). "Shedding light on anaerobic benzene ring degradation: a process unique to prokaryotes?" Journal of Bacteriology **179**(2): 301-309.

Hungate, R. E. (1969). A Roll Tube Method for Cultivation of Strict Anaerobes. Methods in Microbiology. J. R. Norris and D. W. Ribbons. New York, Academic Press Inc.: 117-132.

Hutchins, S. R. (1991). "Biodegradation of monoaromatic hydrocarbons by aquifer microorganisms using oxygen, nitrate, or nitrous oxide as the terminal electron acceptor." Applied and Environmental Microbiology **57**(8): 2403-2407.

Hutchins, S. R., W. C. Downs, et al. (1991). Nitrate for biorestoration of an aquifer contaminated with jet fuel. Ada, OK, United States Environmental Protection Agency.

Hutchins, S. R., G. W. Sewell, D. A. Kovacs and G. A. Smith (1991). "Biodegradation of aromatic-hydrocarbons by aquifer microorganisms under denitrifying conditions." Environmental Science & Technology **25**(1): 68-76.

Kazumi, J., M. E. Caldwell, J. M. Suflita, D. R. Lovely and L. Y. Young (1997). "Anaerobic degradation of benzene in diverse anoxic environments." Environmental Science and Technology **31**(3): 813-818.

Lammey, J. P. and D. F. Noseworthy (1997). Innovative hydrocarbon reduction techniques for cold climates. 6th Symposium on Groundwater and Soil Remediation, Montreal, Quebec.

Lapinskas, J. (1989). "Bacterial degradation of hydrocarbon contamination in soil and groundwater." Chemistry & Industry(23): 784-789.

Leahy, J. G. and R. R. Colwell (1990). "Microbial degradation of hydrocarbons in the environment." Microbiological Reviews **54**(3): 305-315.

Lovley, D. R., J. D. Coates, J. C. Woodward and E. J. P. Phillips (1995). "Benzene oxidation coupled to sulfate reduction." Applied and Environmental Microbiology **61**(3): 953-958.

Lovley, D. R. and E. J. P. Phillips (1986). "Availability of ferric iron for microbial reduction in bottom sediments of the freshwater tidal Potomac River." Appl. Environ. Microbiol. **52**(4): 751-757.

Lovley, D. R. and E. J. P. Phillips (1987). "Rapid assay for microbially reducible ferric iron in aquatic sediments." Applied and Environmental Microbiology **53**(7): 1536-1540.

Lovley, D. R., J. C. Woodward and F. H. Chapelle (1994). "Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe(III) ligands." Nature **370**(6485): 128-131.

Lovley, D. R., J. C. Woodward and F. H. Chapelle (1996). "Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms." Applied and Environmental Microbiology **62**(1): 288-291.

Madigan, M. T., J. M. Martinko and J. Parker (1997). Brock Biology of Microorganisms, Prentice-Hall.

Major, D. W., C. I. Mayfield and J. F. Barker (1988). "Biotransformation of benzene by denitrification in aquifer sand." Ground Water **26**(1): 8-14.

McCarty, P. L. (1971). Energetics and bacterial growth. Organic Compounds in Aquatic Environments. S. D. Faust and J. V. Hunter, Marcel Dekker. **7**: 157-172.

McCarty, P. L. (1975). "Stoichiometry of bacterial reactions." Progress in Water Technology 7: 157-172.

Monod, J. (1949). "The growth of bacterial cultures." Annu. Rev. Microbiol. **3**: 371-394.

Montgomery, C. W. (1992). Environmental Geology, Wm. C. Brown Publishers.

Morgan, P., S. T. Lewis and R. J. Watkinson (1993). "Biodegradation of benzene, toluene, ethylbenzene and xylenes in gas-condensate-contaminated groundwater." Environmental Pollution **82**(2): 181-190.

Nales, M. (1997). Evaluation of Anaerobic Benzene Biodegradaiton for *In Situ* Passive Bioremediation. Biology. Waterloo, University of Waterloo: 151.

Nales, M., B. Butler and E. Edwards (1998). "Anaerobic benzene biodegradation: a microcosm survey." Biormediation Journal **2**(2): 125-144.

Reinhard, M., S. Shang, P. K. Kitanidis, E. Orwin, G. D. Hopkins and C. A. Lebron (1997). "*In situ* BTEX biotransformation under enhanced nitrate- and sulfate-reducing conditions." Environmental Science and Technology **31**(1): 28-36.

Rhee, S.-K., G. M. Lee, J.-H. Yoon, Y.-H. Park, H.-S. Bae and S.-T. Lee (1997). "Anaerobic and aerobic degradation of pyridine by a newly isolated denitrifying bacterium." Applied and Environmental Microbiology **63**: 2578-2585.

Robinson, J. A. and J. M. Tiedje (1983). "Nonlinear estimation of Monod growth kinetic parameters from a single substrate depletion curve." Applied and Environmental Microbiology **45**(5): 1453-1458.

Rogers, J. E. (1987). Anaerobic transformation processes: a review of the microbiological literature. Athens, Georgia, U.S.EPA.

Sawyer, C. N., P. L. McCarty and G. F. Parkin (1994). Chemistry for Environmental Engineering. New York, McGraw-Hill Inc.

Sittig, M. (1985). Handbook of Toxic and Hazardous Chemicals and Carcinogens. Park Ridge, NJ, Noyes Publications.

Smith, M. R. (1990). "The biodegradation of aromatic hydrocarbons by bacteria." Biodegradation **1**: 191-206.

Suflita, J. M., K. L. Londry and G. A. Ulrich (1997). Determination of anaerobic biodegradation activity. Manual of Environmental Microbiology. C. J. Hurst. Washington D.C., ASM Press: 790-801.

Suflita, J. M., J. A. Robinson and J. M. Tiedje (1983). "Kinetics of microbial dehalogenation of haloaromatic substrates in methanogenic environments." Applied and Environmental Microbiology **45**(5): 1466-1473.

Suflita, J. M. and G. W. Sewell (1991). Anaerobic biotransformation of contaminants in the subsurface, EPA.

Sugiura, K., M. Ishihara, T. Shimauchi and S. Harayama (1997). "Physicochemical properties and biodegradability of crude oil." Environmental Science and Technology **31**(1): 45-51.

Taylor, B. F. and R. S. Oremland (1979). "Depletion of adenosine triphosphate in *Desulfovibrio* by oxyanions of group VI elements." Current Microbiology **3**: 101-103.

Thauer, R. K., K. Jungermann and K. Decker (1977). "Energy conservation in chemotrophic anaerobic bacteria." Bacteriological Reviews **41**: 100-180.

Thiele, J. H., M. Chartrain and J. G. Zeikus (1988). "Control of interspecies electron flow during anaerobic digestion: role of floc formation in syntrophic methanogenesis." Appl. Environ. Microbiol. **54**(1): 10-19.

Thiele, J. H. and J. G. Zeikus (1988). "Control of interspecies electron flow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs." Appl. Environ. Microbiol. **54**(1): 20-29.

Twigg, R. S. (1945). "Oxidation-Reduction Aspects of Resazurin." Nature: 401-402.

Viessman, W. and M. J. Hammer (1993). Water Supply and Pollution Control. New York, Harper Collins College Publishers.

Vroblesky, D. A., P. M. Bradley and F. H. Chapelle (1996). "Influence of electron donor on the minimum sulfate concentration required for sulfate reduction in a petroleum hydrocarbon-contaminated aquifer." Environmental Science and Technology **30**(4): 1377-1381.

Ward, D. M., R. M. Atlas, P. D. Boehm and J. A. Calder (1980). Microbial biodegradation and chemical evolution of oil from the Amoco spill. AMBIO.

Weast, R. C. (1975). Handbook of Chemistry and Physics, CRC Press.

Wilson, B. H., B. Bledsoe and D. Kampbell (1987). Chapter 8: Biological processes occurring at an aviation gasoline spill site. Chemical Quality of Water and the Hydrologic Cycle. R. C. A. a. D. M. Mcknight. Chelsea, Michigan, Lewis Pulishers Inc.: 125-136.

Yadav, J. S. and C. A. Reddy (1993). "Degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*." Applied and Environmental Microbiology **59**(3): 756-762.

Zeyer, J., E. P. Kuhn and R. P. Schwarzenbach (1986). "Rapid microbial mineralization of toluene and 1,3- dimethylbenzene in the absence of molecular oxygen." Applied and Environmental Microbiology **52**(4): 944-947.

Appendix A

Calculation of Theoretical Yield

The details of the theoretical calculation of f_e and f_s, the fraction of electron donor used for energy and cell synthesis, respectively, are provided in this appendix.

In order to calculate the free energies of reactions, the free energies of formation of the compounds in the reactions must be known. The free energies of formation for compounds necessary to calculate the free energies of reactions used in this thesis are provided in Table A-1.

Table A-1 Free energies of formation for various compounds*

	State	ΔGf°
Benzene	l	30.989
CO_2	g	-94.26
H_2O	l	-56.69
H^+	l	-9.67
HCO_3	l	-140.31
CH_4	g	-12.14
Fe^{+2}	aq	-20.3
Fe^{+3}	aq	-2.52
NO_3^-	aq	-26.41
NO_2^-	aq	-7.91
SO_4^{-2}	aq	-177.34
HS^-	aq	3.01
H_2S	aq	-6.54

* The free-energy values are in kcal/mol
* (McCarty 1971; Weast 1975; Thauer et al. 1977).

Calculation of f_e and f_s:

The fraction of electron donor used for energy (f_e) and the fraction of electron donor used for cell synthesis (f_s) can be estimated by the method of McCarty (1971). The electron equivalent of the total substrate is assigned the value of A+1, so that the substrate converted for energy is A and the substrate converted for cell synthesis is 1. The substrate converted for energy per electron equivalent of cells synthesized is therefore A/1, or A:

$$A = - (\Delta G_p / k^m + \Delta G_c + \Delta G_n / k) / (k\Delta G_r)$$

ΔG_p, half reaction for electron donor – half reaction for pyruvate

ΔGp is the free energy of conversion of one electron equivalent of cell carbon source (benzene) to intermediate (pyruvate). Pyruvate is used by convention as the intermediate energy level as it represents about the same energy content on an electron equivalent basis as "active acetate", which is the usual level of entrance into the tricarboxylic acid cycle for synthesis of proteins and fats (McCarty 1971). The half reaction and free energy of reaction for the electron donor, benzene is

$$1/30\ C_6H_6 + 3/5\ H_2O \rightarrow 1/5\ HCO_3^- + 6/5\ H^+ + e^-$$

$$\Delta G^\circ = -6.470 \text{ kcal / mol } e^-$$

The half reaction and free energy of reaction for pyruvate is

$$1/10\ CH_3COCOO^- + 2/5\ H_2O \rightarrow 1/5\ CO_2 + 1/10\ HCO_3^- + H^+ + e^-$$

$$\Delta G^\circ = -8.545\ \text{kcal / mole } e^-$$

ΔG_c, free energy of conversion of one electron equivalent of intermediate to one electron equivalent of cells

The value for ΔG_c was estimated from the yield of 10.5 grams of bacterial dry weight per mole of ATP and from the free energy released by hydrolysis of ATP under physiological conditions of –12.5 kcal (McCarty 1971). This resulted in a value of 7.5 kcal per electron equivalent of dells. However, if average energy transfer efficiency, k, is included in the reaction, ΔGc would be modified to 4.5 / k, which gives a value of 7.5 for a k equal to 60%.

ΔG_n, free energy per electron equivalent of cells for reduction of nitrogen source to ammonia

The energy requirement for nitrogen reduction per electron equivalent of cells formed is based upon the need for 1/20 mole of nitrogen per electron equivalent of cells formed. The value of ΔG_n is dependent upon the nitrogen source. For ammonia as the nitrogen source, the reduction equation is

$$1/20\ NH_4^+ = 1/20\ NH_4^+$$

$$\Delta G_n = 0.00$$

For nitrate as the nitrogen source, the reduction equation is

$$1/20\ NO_3^- + 1/10\ H^+ + 1/20\ H_2O = 1/20\ NH_4^+ + 1/10\ O_2$$

$$\Delta G_n = 4.17 \text{ kcal / cell equiv.}$$

<u>ΔG_r, half reaction for electron donor – half reaction for electron acceptor</u>

The free energy per electron equivalent of substrate converted for energy is calculated as the $\Delta G°$ for electron donor (benzene) less the $\Delta G°$ for the electron acceptor. The half reactions and $\Delta G°$ for the four most common electron acceptors are as follows:

Sulphate: $1/16\ H_2S + 1/16\ HS^- + ½\ H_2O \rightarrow 1/8\ SO_4^{2-} + 19/16\ H^+ + e^-$

$\Delta G° = -5.085$ kcal / mole e^-

Nitrate: $1/10\ N_2 + 3/5\ H_2O \rightarrow 1/5\ NO_3^- + 6/5\ H^+ + e^-$

$\Delta G° = +17.128$ kcal / mole e^-

Ferric iron: $Fe^{2+} \rightarrow Fe^{3+} + e^-$

$\Delta G° = +17.780$ kcal / mole e^-

Bicarbonate: $1/8\ CH_4 + 3.8\ H_2O \rightarrow 1/8\ HCO_3^- + 9/8\ H^+ + e^-$

$\Delta G° = -5.763$ kcal / mole e^-

k, the efficiency of energy transfer

Transfer efficiencies range from 12 to 100 percent (McCarty 1971). However, the efficiencies for anaerobic heterotrophic growth generally ranged from 40 to 70 percent and an average of about 60 percent is used (McCarty 1971).

m, constant

The constant, m is equal to +1 when ΔG_p is positive, and –1 when ΔG_p is negative. If the substrate is at a lower energy level than pyruvate ($\Delta G_p>0$), energy is required to raise the substrate to the energy level of pyruvate; therefore more energy is required ($\Delta G_p/k$). If the substrate is at a higher energy level than pyruvate ($\Delta G_p<0$), the cell obtains energy from the conversion of substrate to pyruvate, although some energy is lost in the transfer ($\Delta G_p k$).

The fractions f_e and f_s can now be calculated:

$$f_e = A / (A+1) \qquad f_s = 1 - f_e$$

The theoretical cell yield can be determined:

$$Y_{max} = c / dA$$

where, c = 6.27 g cells per electron mole of carbon synthesized (if synthesized from ammonia)

c = 4.49 g cells per electron mole of carbon synthesized (if synthesized from nitrate)

d = equals the electrons actually transferred from a donor molecule divided by the electron equivalents per molecule (usually 1.0 except in certain fermentations)

Appendix B

Oil Refinery Terminal Site Experiment

This appendix describes an experiment designed to investigate the unusual results from the Oil Refinery Terminal Site microcosms observed during the original study (Nales et al. 1998). All of the microcosms derived from this site degraded exactly three feeding of benzene and then stopped.

In order to determine if the sediment of Oil Refinery Terminal Site microcosms contained some compound toxic to the microorganisms, the following experiment was carried out. A methanogenic toluene-degrading culture (Edwards and Grbic-Galic 1994) was used as inoculum for three different treatments: a positive control in methanogenic medium, filtered liquid from Oil Refinery Terminal Site microcosm #7b, and a slurry of soil and water from this same microcosm. Each treatment was prepared in duplicate in 17-ml vials and all vials were amended with toluene.

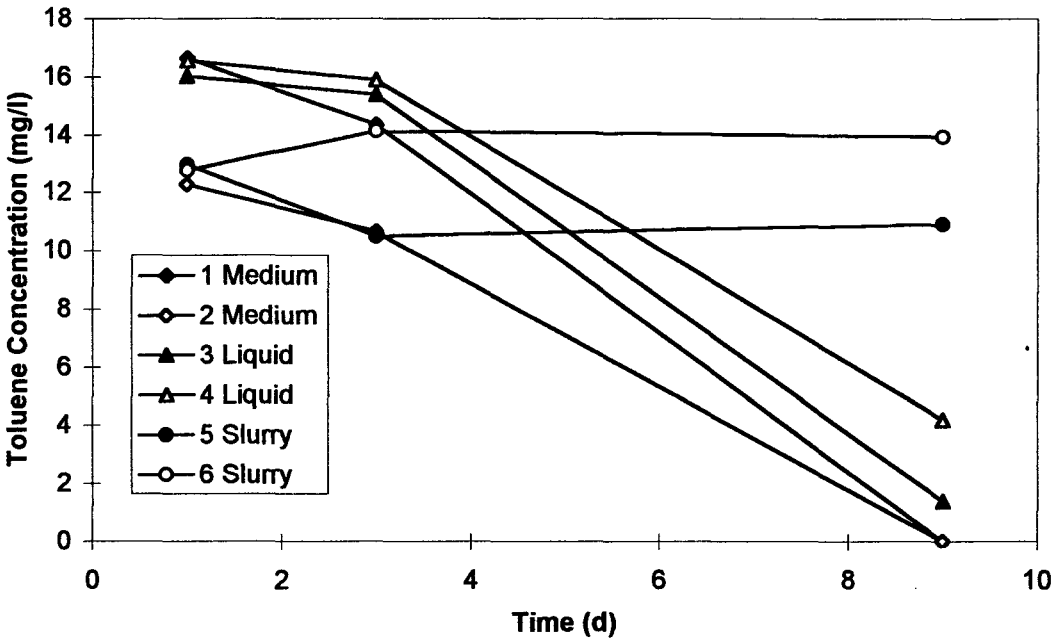


Figure A-1 Oil Refinery Terminal Site Experiment

As can be seen from Figure A-1, it appears that the sediment had an effect on the toluene-degrading ability of these bacteria. The toluene was degraded in both the medium-only and liquid-only treatments, but was not significantly degraded in the vials with slurry from an Oil Refinery Terminal Site microcosm. It appears as though some component of the soil from this site inhibits microbial activity, which may explain the strange results observed. It is possible that the degradation produced a build-up of some toxic compound, which then affected the bacteria; however, these results are not conclusive.

Appendix C

Development of Transfer Cultures

This appendix provides details of the enrichment and transfer processes of microbial cultures derived from four different sites. The results from the original study (Nales et al. 1998) are summarized in the following four tables, one for each of the sites that have been studied further. Each table lists the 18 microcosms and whether or not benzene was degraded. The last two columns identify which microcosms were studied during this thesis research and from which microcosms transfer cultures were developed. Following the tables are several figures which outline the process of transfer culture development from these microcosms. The transfer processes illustrated in Figures C-1, C-2 and C-3 were completed by Marit Nales during the original study; the remaining Figures illustrate transfers which were completed for this thesis.

Table C-1 Summary: Gas Station 1

	Treatment	Benzene degraded?	Degradation sustained?	Studied further?	Transfers?
1b	GW	yes	no	no	
2b	GW	no	-	yes*	no
3b	GW & BTEX	no	-	yes	no
4b	GW & BTEX	no	-	yes	no
5b	GW & SO4	yes	yes	yes	yes
6b	GW & SO4	yes	yes	yes	yes
7b	Medium	no	-	no	
8b	Medium	yes	no	no	
9b	Med & SO4	yes	yes	yes	no
10b	Med & SO4	yes	yes	yes	no
11b	Med & SO4 & BTEX	no	-	no	
12b	Med & SO4 & BTEX	no	-	no	
13b	Med & NO3	yes	yes	yes	yes
14b	Med & NO3	yes	yes	yes	yes
15b	Med & Fe(III)	no	-	no	
16b	Med & Fe(III)	no	-	no	
17b	Sterile control	no	-	no	
18b	Sterile control	no	-	no	

* degradation was stimulated by the addition of sulphate

Table C-2 Summary: Oil Refinery

	Treatment	Benzene degraded?	Degradation sustained?	Studied further?	Transfers?
1b	GW	yes	yes	no	
2b	GW	yes	yes	yes	yes
3b	GW & BTEX	yes	yes	yes	no
4b	GW & BTEX	yes	yes	no	
5b	GW & SO4	yes	yes	no	
6b	GW & SO4	yes	yes	yes	yes
7b	Medium	yes	yes	yes	yes
8b	Medium	yes	yes	yes	yes
9b	Med & SO4	yes	yes	yes	no
10b	Med & SO4	yes	yes	yes	no
11b	Med & SO4 & BTEX	yes	yes	yes	no
12b	Med & SO4 & BTEX	yes	yes	no	
13b	Med & NO3	yes	yes	yes	no
14b	Med & NO3	yes	yes	yes	no
15b	Med & Fe(III)	yes	yes	yes	yes
16b	Med & Fe(III)	yes	yes	yes	yes
17b	Sterile control	no	-	no	
18b	Sterile control	no	-	no	

Table C-3 Summary: Landfarm

	Treatment	Benzene degraded?	Degradation sustained?	Studied further?	Transfers?
1b	GW	yes	yes	no	
2b	GW	yes	yes	no	
3b	GW & BTEX	no	-	no	
4b	GW & BTEX	no	-	no	
5b	GW & SO4	yes	yes	yes	no
6b	GW & SO4	yes	yes	no	
7b	Medium	yes	?	no	
8b	Medium	yes	?	yes*	no
9b	Med & SO4	yes	?	no	
10b	Med & SO4	yes	yes	yes	no
11b	Med & SO4 & BTEX	no	-	no	
12b	Med & SO4 & BTEX	no	-	no	
13b	Med & NO3	yes	yes	yes	no
14b	Med & NO3	yes	yes	yes	no
15b	Med & Fe(III)	?	?	no	
16b	Med & Fe(III)	?	?	no	
17b	Sterile control	no	-	no	
18b	Sterile control	no	-	no	

* degradation was sustained only with addition of sulphate

Table C-4 Summary: Uncontaminated Swamp

	Treatment	Benzene degraded?	Degradation sustained?	Studied further?	Transfers?
1b	GW	no	-	no	
2b	GW	no	-	yes*	no
3b	GW & BTEX	no	-	no	
4b	GW & BTEX	no	-	no	
5b	GW & SO4	yes	yes	yes	no
6b	GW & SO4	no	-	no	
7b	Medium	no	-	no	
8b	Medium	no	-	no	
9b	Med & SO4	no	-	no	
10b	Med & SO4	no	-	no	
11b	Med & SO4 & BTEX	no	-	no	
12b	Med & SO4 & BTEX	no	-	no	
13b	Med & NO3	yes	yes	yes	yes
14b	Med & NO3	yes	yes	no	
15b	Med & Fe(III)	no	-	no	
16b	Med & Fe(III)	no	-	no	
17b	Sterile control	no	-	no	
18b	Sterile control	no	-	no	

* degradation was stimulated by addition of nitrate

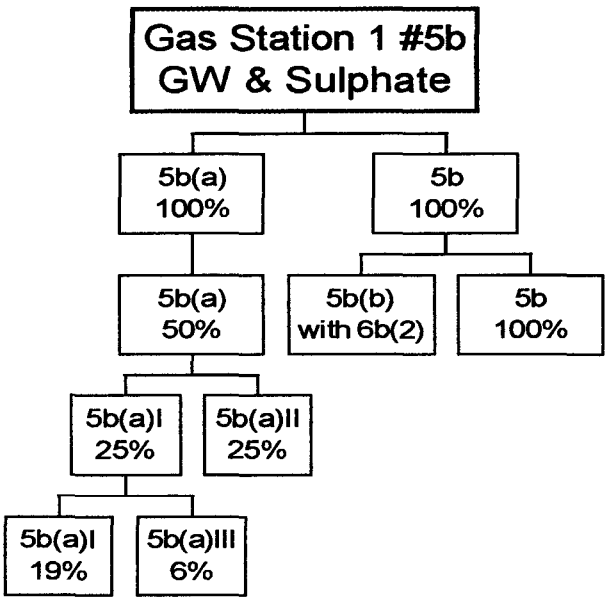


Figure C-1 Development of Transfer Cultures from Gas Station 1 Microcosm #5b

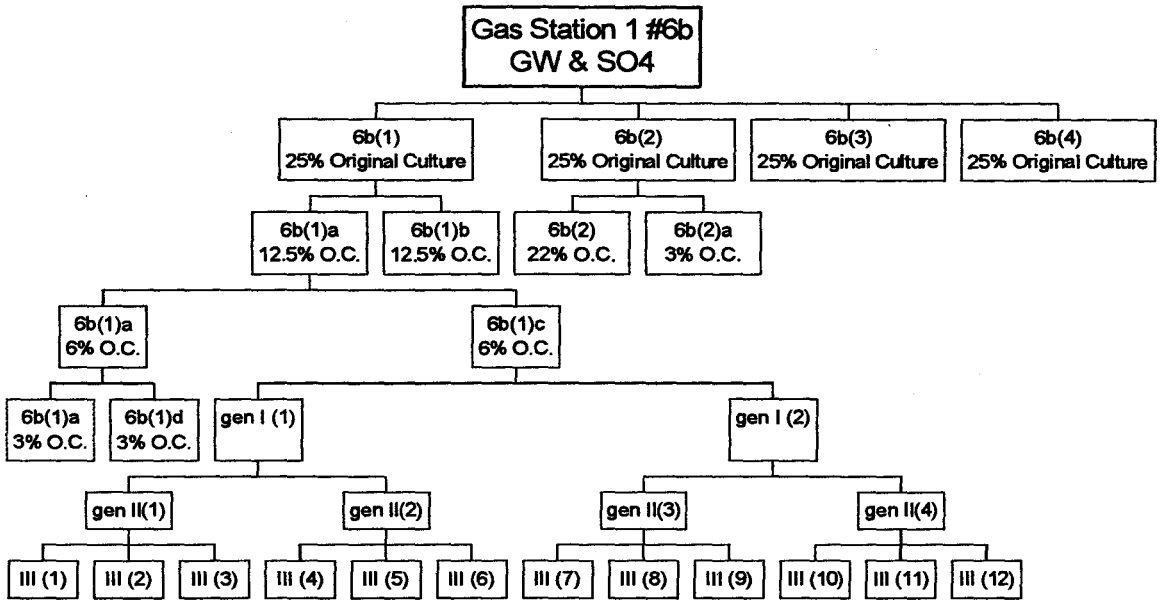
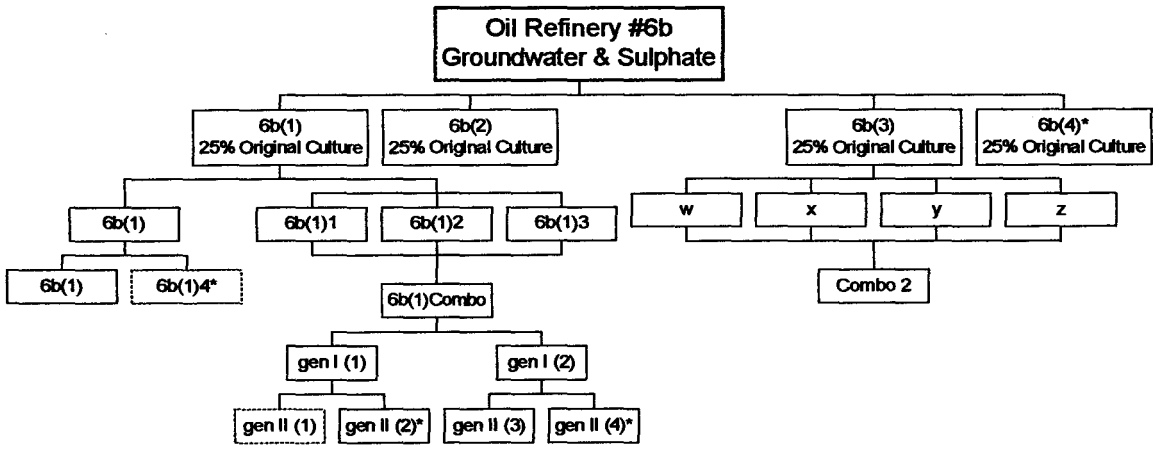
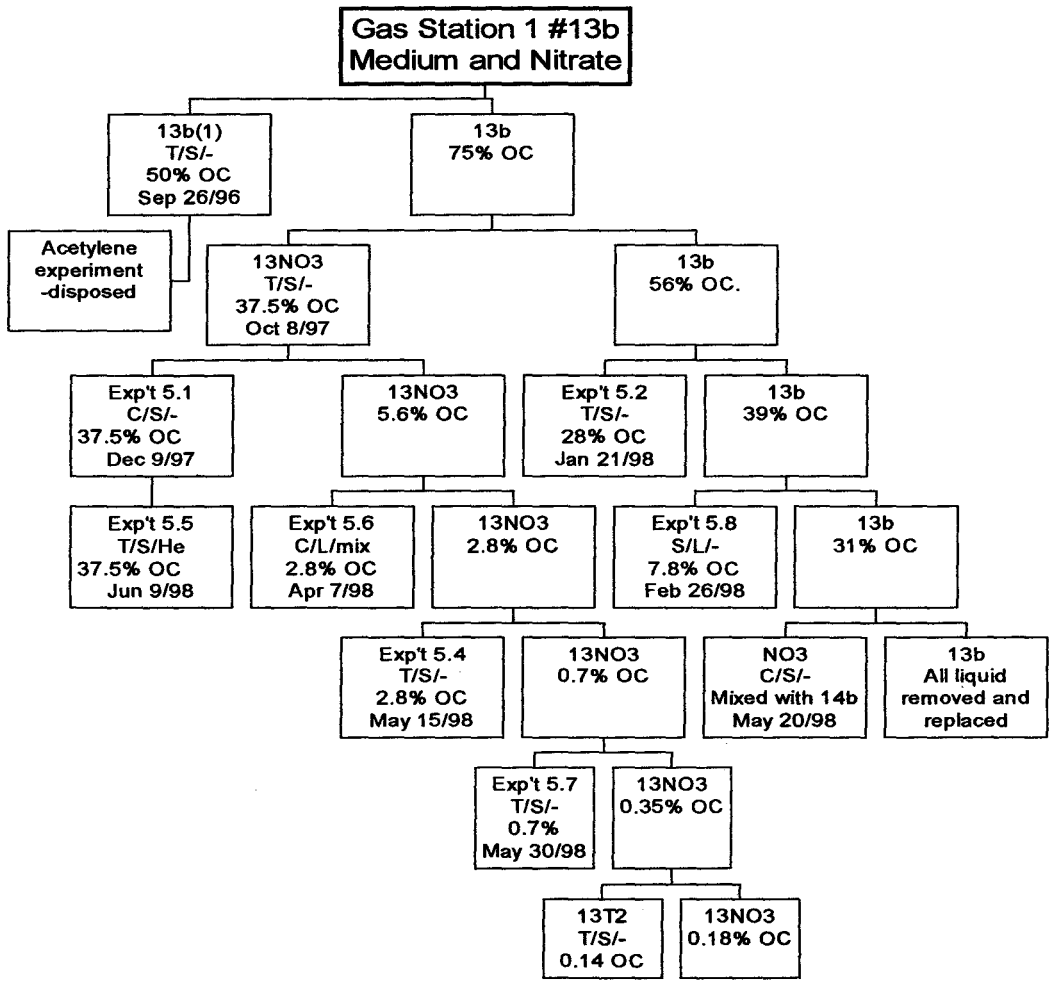


Figure C-2 Development of Transfer Cultures from Gas Station 1 Microcosm #6b



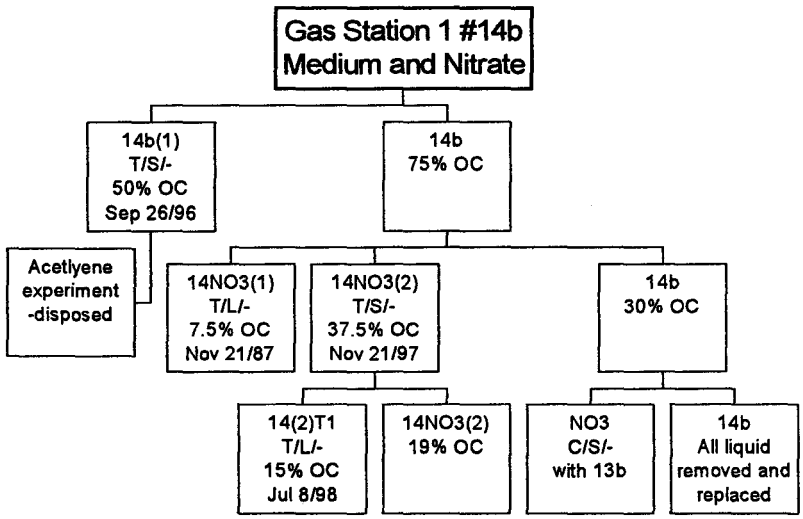
* Pooled, with Transfer 9b+10b, to make Experiment 6.2

Figure C-3 Development of Transfer Cultures from Oil Refinery Microcosm #6b



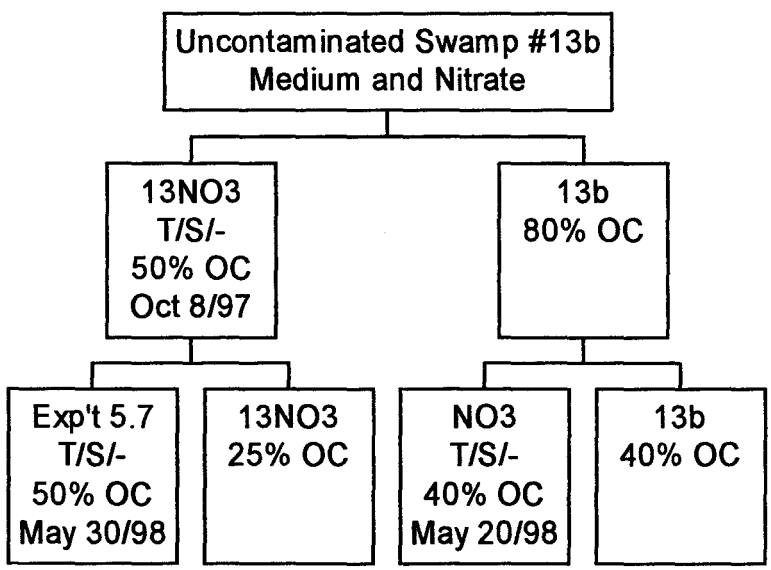
(T-split transfer; C-centrifuged/S-slurry; L-liquid/purged)

Figure C-4 Development of transfer cultures from Gas Station 1 Microcosm #13b



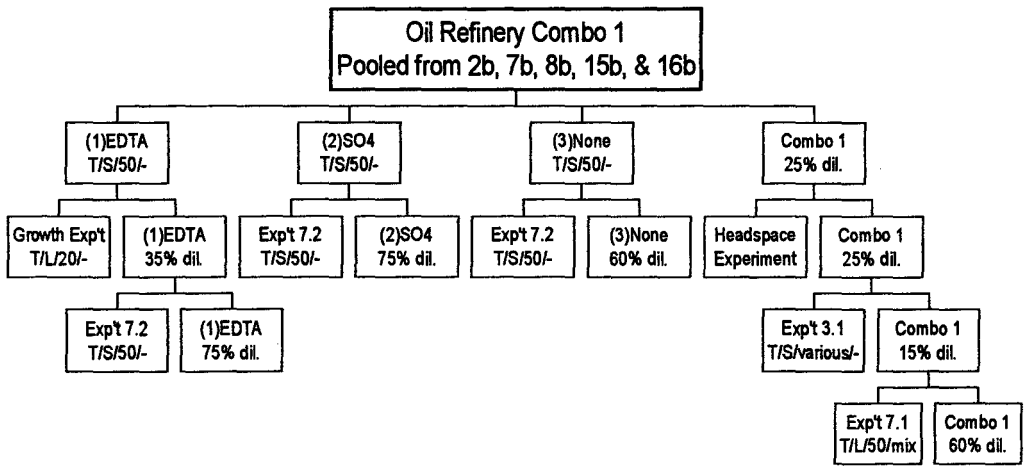
(T-split transfer; C-centrifuged/S-slurry; L-liquid/purged)

Figure C-5 Development of transfer cultures from Gas Station 1 Microcosm #14b



(T-split transfer; C-centrifuged/S-slurry; L-liquid/purged)

Figure C-6 Development of transfer cultures from Uncontaminated Swamp Microcosm #13b



(T-split transfer; C-centrifuged/S-slurry; L-liquid/%diluted/purged)

Figure C-7 Development of transfer cultures from Oil Refinery Combo 1

Appendix D - Mathcad Models

Nonlinear regression analysis - nitrate-reducing culture

$Vl := .03$ volume of liquid, l

$Vg := 0.01$ volume of gas, l

$Hcc := 0.22$ Henry's constant for benzene at 25 C

$Xo := 0.88$ initial concentration of cells, mg/l

$G := 0.0322$ G = Vl + Hcc*Vg

$Y := 0.113$ cell yield, mg cells / mg benzene

$Co := 13$ initial benzene concentration, mg/l

$Mo := Co \cdot G$ Mo = 0.419

$A := Y \cdot Mo + Xo \cdot Vl$ A = 0.074

$B := G \cdot \frac{Y}{A}$ B = 0.049

$D := \frac{Y}{Vl \cdot Xo}$ D = 4.28

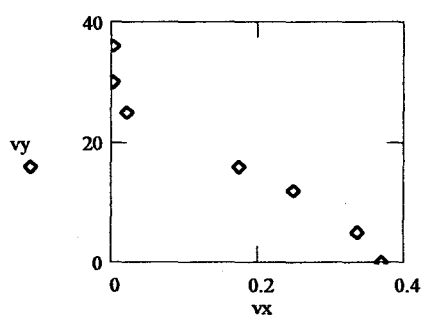
$E := D \cdot Mo$ E = 1.792

The data to be used is t(time) and M(mass):

$$vy := \begin{bmatrix} 0 \\ 5 \\ 12 \\ 16 \\ 25 \\ 30 \\ 36 \end{bmatrix} \quad vx := \begin{bmatrix} 0.369 \\ 0.336 \\ 0.249 \\ 0.176 \\ 0.022 \\ 0.004 \\ 0.002 \end{bmatrix} \quad vg := \begin{bmatrix} .5 \\ .03 \end{bmatrix}$$

$$F(z,u) := \begin{bmatrix} \frac{(u_0 \cdot B + 1)}{u_1} \cdot \ln(E - D \cdot z + 1) - \frac{u_0 \cdot B}{u_1} \cdot \ln\left(\frac{z}{Mo}\right) \\ B \cdot \frac{\left(\ln(E - D \cdot z + 1) - \ln\left(\frac{z}{Mo}\right)\right)}{u_1} \\ \frac{-\ln(E - D \cdot z + 1) \cdot (u_0 \cdot B + 1) + u_0 \cdot B \cdot \ln\left(\frac{z}{Mo}\right)}{(u_1)^2} \end{bmatrix}$$

Scatter plot of benzene mass vs time:

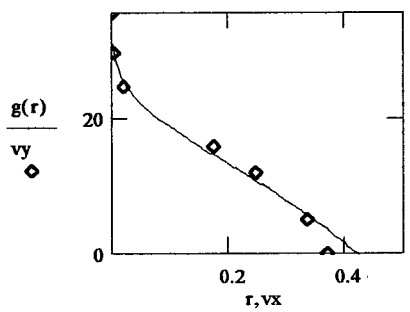


$p := genfit(vx, vy, vg, F)$

$i := 0..6$

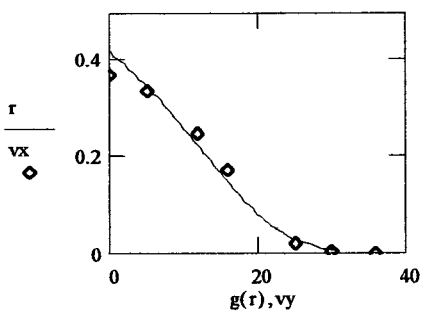
$r := 0.005, .01 .. 4.5$

$g(r) := F(r,p)_0$ $\quad p = \begin{bmatrix} 4.961 \\ 0.076 \end{bmatrix}$ $\quad K := p_0$ $\quad U := p_1$



$K = 4.961$

$U = 0.076$

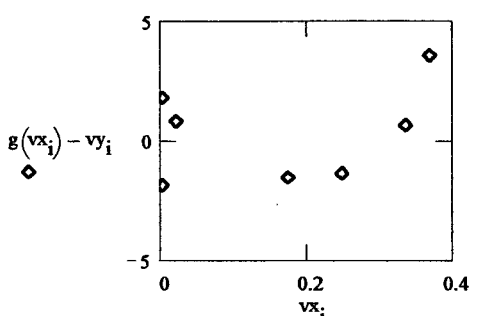


$scale := \max\left(\left|\overrightarrow{g(vx) - vy}\right|\right) \cdot 1.1$

$g\left(vx_1\right) = 5.695$ $\quad vy_1 = 5$

$scale = 3.935$

Residual Plot



$sum := \sum_i \left| g\left(vx_i\right) - vy_i \right|$

$sum = 11.657$

Nonlinear regression analysis - sulphate-reducing culture

$Vl := .03$ liquid volume, l

$Vg := 0.01$ gas volume, l

$Hcc := 0.22$ Henry's constant for benzene at 25C

$Xo := 1$ initial cell concentration, mg/l

$G := 0.0322$ $G := Vl + Hcc \cdot Vg$ $G = 0.032$

$Y := 0.156$ yield, mg cells / mg benzene

$Co := 14.5$ initial benzene concentration, mg/l

$Mo := Co \cdot G$ $Mo = 0.467$

$A := Y \cdot Mo + Xo \cdot Vl$ $A = 0.103$

$B := G \cdot \frac{Y}{A}$ $B = 0.049$

$D := \frac{Y}{Vl \cdot Xo}$ $D = 5.2$

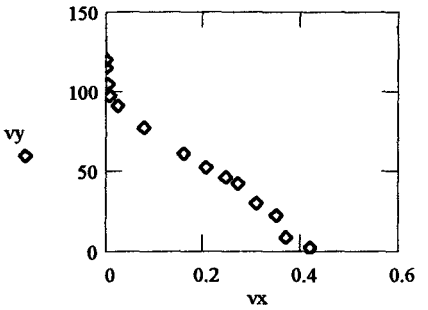
$E := D \cdot Mo$ $E = 2.428$

The data to be used is t(time) and M(mass):

$$vy := \begin{bmatrix} 3 \\ 9 \\ 23 \\ 30 \\ 42 \\ 46 \\ 52 \\ 61 \\ 77 \\ 91 \\ 97 \\ 105 \\ 115 \\ 120 \end{bmatrix} \qquad vx := \begin{bmatrix} .4199 \\ .3706 \\ .3498 \\ .3062 \\ .2694 \\ .2449 \\ .2056 \\ .1608 \\ .0780 \\ .0250 \\ .0099 \\ .0046 \\ .0012 \\ .0003 \end{bmatrix} \qquad vg := \begin{bmatrix} .05 \\ .005 \end{bmatrix}$$

$$F(z,u) := \begin{bmatrix} \dfrac{\left(u_0 \cdot B + 1\right)}{u_1} \cdot \ln(E - D \cdot z + 1) - \dfrac{u_0 \cdot B}{u_1} \cdot \ln\left(\dfrac{z}{Mo}\right) \\ B \cdot \dfrac{\left(\ln(E - D \cdot z + 1) - \ln\left(\dfrac{z}{Mo}\right)\right)}{u_1} \\ \dfrac{-\ln(E - D \cdot z + 1) \cdot \left(u_0 \cdot B + 1\right) + u_0 \cdot B \cdot \ln\left(\dfrac{z}{Mo}\right)}{\left(u_1\right)^2} \end{bmatrix}$$

Scatter plot of benzene mass vs time:

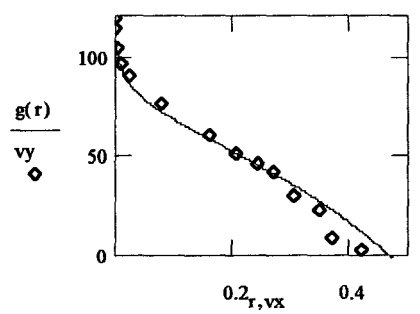


$p := genfit(vx, vy, vg, F)$

$i := 0 .. 13$

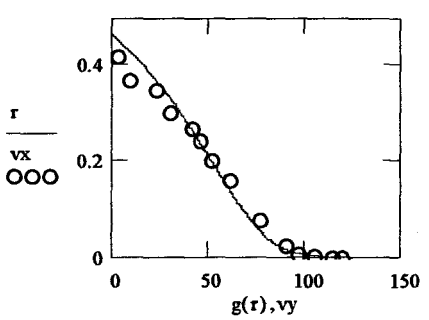
$r := 0.0005, .001 .. 4.5$

$g(r) := F(r, p)_0$ $\quad p = \begin{bmatrix} 3.888 \\ 0.023 \end{bmatrix}$ $\quad K := p_0$ $\quad U := p_1$



K = 3.888

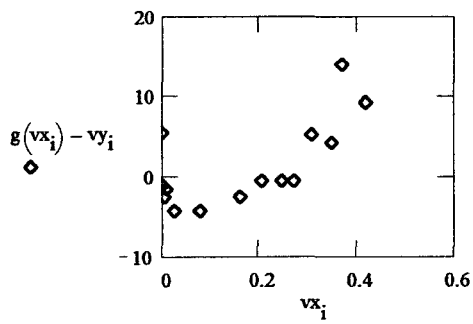
U = 0.023



$$scale := \max\left(\left|\overrightarrow{g(vx) - vy}\right|\right) \cdot 1.1$$

$g(vx_1) = 23.111$ $vy_1 = 9$ scale = 15.523

Residual Plot



$$sum := \sum_i \left(\left| g(vx_i) - vy_i \right|\right)^2$$ sum = 415.244