BIODEGRADATION OF PETROLEUM HYDROCARBONS BY HALOPHILIC AND HALOTOLERANT MICROORGANISMS

By

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ABBREVIATIONS AND SYMBOLS

1. BTEX ....benzene, toluene, ethylbenzene, and xylenes
2. bbl/d ....barrels per day
3. bp..base pairs
4. β ....beta
5. *cis*-DCE..*cis*-dichloroethylene
6. DGGE .... denaturing gradient gel electrophoresis
7. EDTA ....ethylenediaminetetraacetic acid
8. E&P.. exploration and production
9. GC ....gas chromatograph
10. LB ....Luria Bertani
11. μ .... micro
12. mm ....millimeter
13. mM..millimolar
14. MSM .... mineral salts medium
15. M .... molar
16. ng..nanogram
17. NTA .... nitrilotriacetic acid
18. nos. ....numbers
19. pmole..picomole

20. PCR .... polymerase chain reaction

21. RDP .... ribosomal database project

22. TCE .... trichloroethylene

23. UL .... universally labeled

24. VC .... vinyl chloride

25. v/v .... volume per volume

26. YE .... yeast extract

CHAPTER I

INTRODUCTION

Microbes have shown the ability to degrade a wide range of hydrocarbons, both those occurring naturally as well as environmental pollutants. Earlier studies have demonstrated biodegradation of a variety of pollutant compounds including phenol, benzoate, eicosane, 2, 4-dichlorophenoxyacetic acid (2, 4-D), polyaromatic hydrocarbons (PAHs), and certain components of crude oil under saline conditions. In addition, a few pure cultures of halophilic and halotolerant organisms have been isolated that degrade aromatic compounds. However, little is known about the degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) under saline environments. In addition, not much information exists in the literature on the isolation of pure cultures that degrade BTEX compounds. Therefore, evidence for the degradation of BTEX compounds under saline environment and isolation of relevant microbes that degrade BTEX as the sole carbon source is important for developing cost effective treatment technology for the cleanup of brine soil and produced water generated at oil exploration and production facilities.

Many naturally occurring hypersaline environments such as the Great Salt Plains are being increasingly threatened by environmental pollutant compounds. Most of these habitats are shown to harbor unique and ancient microbial life which could survive under earth's harsh early environments. The physiology and metabolic capabilities these organisms may have important implications on our fundamental understanding of the processes they catalyze. For example, halophiles living in naturally occurring

hypersaline habitats may employ novel strategies and pathways for the degradation of hydrocarbons thus these studies will provide insights into the microbiology, physiology, and metabolic pathways that might have existed once on this planet.

The ubiquity of aromatic rings in nature allows for the exposure of these compounds to microorganisms despite any lack of environmental contamination. This prior exposure to aromatics may allow for hypersaline organisms to carry the genes necessary for the degradation of pollutants if they are ever exposed to them. Therefore, it is important to survey naturally occurring hypersaline environments to try to understand the ability of native organisms in pristine hypersaline environments to degrade hydrocarbons, such as BTEX. These organisms could be used in bioremediation projects where the environment is co-contaminated with hydrocarbons since non-halophiles would not be able to survive.

The major objectives of this work were to find evidence for the biodegradation of BTEX compounds in natural and contaminated hypersaline habitats, enrich and isolate relevant organisms (bacteria or archaea), characterize the isolated strains with respect to their phylogenetic affiliation and physiological and metabolic potential for maximum degradation.

CHAPTER II

REVIEW OF LITERATURE

Background

The oil industry has been a frequent topic of discussion as of late, from the supply of crude oil down to the price consumers pay at the pump for a gallon of fuel. As long there is a supply and demand for oil, oil will continue to be produced. The United States ranks eleventh in the world with proven oil reserves of 21.9 billion barrels. Currently, there are over 500,000 producing oil wells in this country; however the vast majority only produces a few barrels of oil per day (Field 2005). In 2003, Oklahoma was the seventh most productive area in the United States with 179,000 bbl/d (including offshore reserves). This accounts for three percent of the crude oil production in the United States. In 2004, there were 83,750 producing wells and 159 rotary rigs in operation in Oklahoma (Hinton 2005).

In oil production and exploration operations spills are inevitable. Large volumes of crude oil spills occur during transportation, by leaking underground storage tanks and due to poor management. Contamination by crude oil results in the release toxic and carcinogenic compounds such as BTEX that are detrimental to human health and the environment. The high water solubility of BTEX poses special concern for rapid the contamination of subsurface and drinking water (Margesin and Schinner 2001).

Organisms living in diverse environments have the ability to degrade BTEX under aerobic (Deeb and Alvarez-Cohen 1999, Ridgeway *et al.* 1990, Gibson and Subramanian 1984) and anaerobic (Gieg *et al.* 1999, Chen and Taylor 1997a, Lovely *et al.* 1996)

conditions. This activity has also been observed under psychrophilic (Margesin and Schinner 2001, Braddock and McCarthy 1996, Bradley and Chapelle 1995) and thermophilic conditions (Chen and Taylor 1997 a and b, Chen and Taylor 1995). Limited evidence exists about BTEX degradation under hypersaline (Brusa *et al*. 2001), acidophilic, alkaliphilic and barophilic conditions (Margesin and Schinner 2001). Benzene, one of the BTEX compounds, is of particular concern. This priority pollutant, as listed by the United States Environmental Protection Agency, is highly stable, water soluble, and a known human carcinogen. Benzene and the other BTEX compounds constitute 1-2 % of crude oil.

Environments that have been exposed to contaminants for long periods of time select for microorganisms that can utilize these compounds for growth. Therefore, soils that have been contaminated by crude oil are likely to harbor organisms which are capable of breaking down these aromatics. Hayes *et al.* (1999) showed that sites with the highest level of contamination showed the most rapid degradation rates for the contaminants, while sites that were not contaminated with petroleum were unable to degrade added contaminants. The first evidences of BTEX degradation by microorganisms were noted in the early 1900's (Gibson and Subramanian 1984). Since that time, a wealth of knowledge has been accumulated about the organisms and conditions under which BTEX degradation occurs.

Aerobic BTEX Degradation

In nature, many compounds are transformed into common degradation intermediates before they are further degraded. This process of funneling compounds allows for common pathways in organisms that break down these substrates. For instance, many aromatic compounds are oxidized by the incorporation of oxygen into the ring. This can be accomplished via mono- or dioxygenases, which introduce one or two oxygen atoms, respectively, into the benzene ring. It is more common for organisms to employ dioxygenases to incorporate the entire oxygen molecule into benzene to form a *cis*-diol, which is then rapidly transformed (Bouwer and Zehnder 1993). Major breakdown intermediates of aromatic ring compounds are catechol or protocatechuate, (Harwood and Parales 1996). Catechol is the most common intermediate of aerobic BTEX degradation, and it may be substituted during the degradation of alkylated benzenes, as seen in Figure 1. Once catechol is formed, the ring can be cleaved and broken into fragments that can be further degraded (Dagley 1975). This oxidation can occur through *ortho*-fission (between the hydroxyl groups) or *meta*-fission (adjacent to one of the hydroxyl groups) (Cerniglia 1984). The o*rtho*-cleavage of catechol is catalyzed by catechol 1, 2-dioxygenase and generates *cis,cis*-muconic acid (Hayaishi *et al.* 1957) which can enter β–oxidation, with acetyl CoA and succinate CoA as products which can enter the citric acid cycle. *Meta*-cleavage of catechol is catalyzed by catechol 2,3-dioxygenase (C23O) and produces 2-hydroxymuconic semialdehyde (Bartilson and Shingler 1989), which is eventually broken into acetaldehyde and pyruvate. This enzyme can be chromosomal or encoded by a plasmid. Three plasmids that have been found that encode for C23O: the *xylE* gene

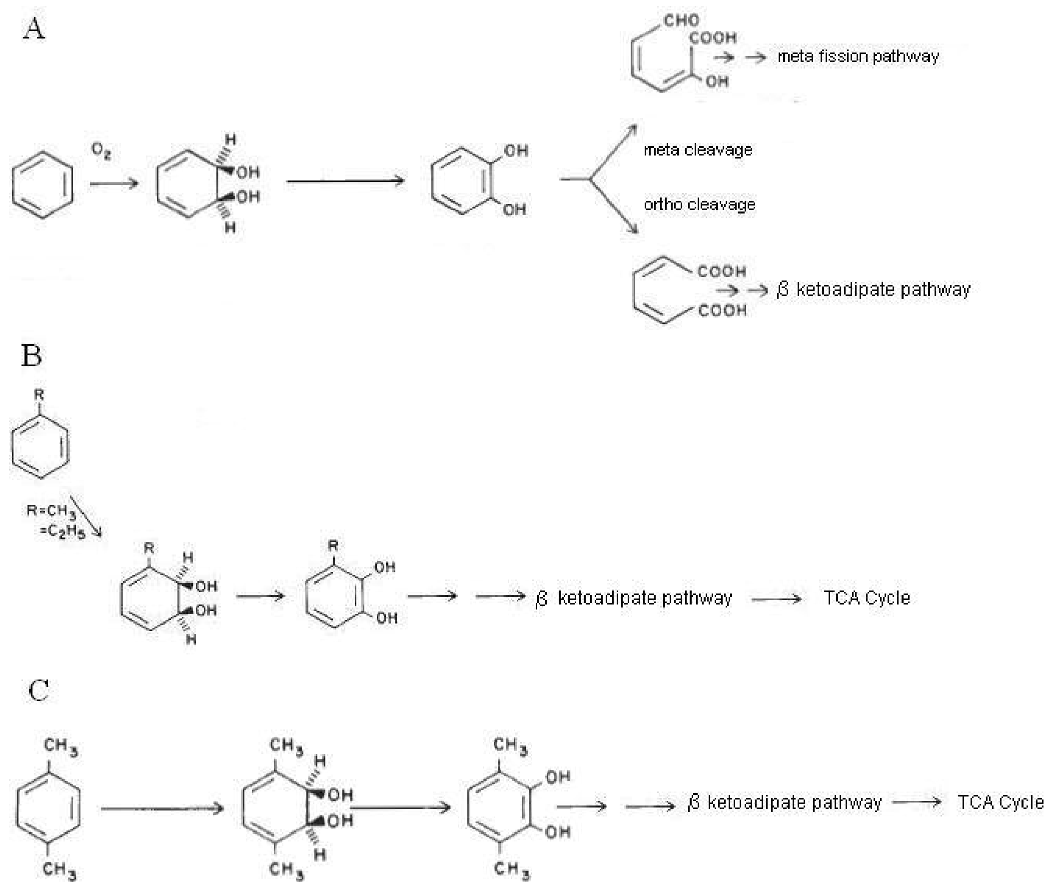

Figure 1. The aerobic degradation of BTEX compounds has common intermediates, such as catechol, which may be substituted in the case of alkylated benzenes. General examples for the oxidation of A) benzene, B) toluene and ethylbenzene, and C) *p*-xylene are given. (Modified from Gibson and Subramanian 1984)

of the IncP-9 Tol plasmid pWWO, the *nahH* gene of IncP-9 NAH7 plasmid, and the *dmpB* gene from plasmid pVI150 of *Pseudomonas* CF600. These three plasmids also show high degree of homology between nucleotide and amino acid sequences (Bartilson and Shingler, 1989). The C23O genes are widely distributed in nature (Brusa *et al.*2001).

While aerobic BTEX degradation has been shown by other species, such as *Moraxella* sp. (Högn and Jaenicke 1972), *Nocarida* sp., *Alcaligenes denitrificans*, *Micrococcs* sp., (Ridgeway *et al.* 1990), *Arthrobacter* sp. (Weber and Corseuil 1994), *Rhodococcus rhodochrous* (Deeb and Alvarez-Cohen 1999), and *Thermus* sp. (Chen and Taylor 1997b), much of the BTEX degradation activity has come from *Pseudomonas* sp. (Brusa *et al.* 2001, Yu *et al.* 2001, Reardon *et al.* 2000, Zamanian and Mason 1987, Gibson *et al.* 1970). Ridgeway *et al.* (1990) supported the dominance of *Pseudomonads* as degraders when they found that 86.9% of the bacterial species found in gasoline-contaminated aquifer were *Pseudomonas* sp.

Anaerobic BTEX degradation

Although anaerobic degradation proceeds at a slower rate than aerobic degradation, BTEX degradation under various anaerobic conditions plays a major role in subsurface BTEX containment and cleanup. Areas may be aerobic prior to contamination; however microbial respiration due to degradation of the contaminant rapidly depletes available oxygen. Once an environment becomes anaerobic, a succession of terminal electron acceptor processes occurs from the most energetically favorable to the least (Anderson and Lovley 1997). Likewise, BTEX degradation has also been found throughout this succession, which begins with brief periods of

manganese and nitrate reduction and then proceeds to iron (III) and then sulfate reduction, and teminates with methanogenic conditions (Anderson and Lovley 1997). Anaerobic degradation of toluene, ethylbenzene, and xylenes is well documented (Ball and Reinhard 1996, Morgan *et al.* 1993, Chen and Taylor 1997a), with toluene as the most extensively studied hydrocarbon under anaerobic conditions. Anaerobic BTEX degradation occurs either through ring reduction or oxidation with the oxygen atoms coming from water (Vogel and Grbi'c-Gali'c 1986). Many important oxidized intermediates that can be detected as BTEX compounds are biodegraded such as benzoic acid, phenol, and cresols (Caldwell and Suflita 2000, Grbi'c-Gali'c and Vogel 1987, Vogel and Grbi'c-Gali'c 1986).

The most energetically favorable of the anaerobic processes is manganese reduction. Manganese oxide is readily found in groundwater where its microbial reduction has recently been linked to the degradation of BTEX (Langenhoff *et al.* 1997). Villatoro-Monzon *et al.* (2003) have shown that sediments under Mn (IV)-reducing environments degrade BTEX at a faster rate than other alternative electron acceptors.

Nitrate is not generally abundant in groundwater unless there is nitrate contamination. It is highly soluble and can be easily added to groundwater to enhance the bioremediation of hydrocarbon contaminated materials (Anderson and Lovely 1997, Ball and Reinhard 1996). Early reports of the disappearance of benzene were not linked to reduction of nitrate, although other BTEX compounds were (Rabus and Widdel 1995, Evans *et al.* 1991, Dolfing et al 1990). Burland and Edwards (1999) reported that microbial benzene oxidation was coupled to the reduction of nitrate to N_2 in the absence of other electron acceptors. Several isolates of BTEX degrading organisms have been

obtained with nitrate as the terminal electron acceptor, such as *Thauera aromatica* T1 (Evans *et al.* 1991), *Azoarcus* sp. strain (Dolfing *et al.* 1990), *Dechloromonas* strain RCB and *Decholoromonas* strain JJ (Coates *et al.*2001).

Petroleum contaminated sediments with Fe (III)-reducing activity have proven to harbor the most efficient BTEX degraders, especially with the addition of chelating agents, such as NTA and EDTA, which solubilize Fe (III) (Kazumi *et al.* 1997, Lovley *et al.* 1996, Lovley and Woodward 1996). Studies with petroleum contaminated aquifers have demonstrated toluene as an electron donor in Fe (III) reducing conditions (Lovley and Lonergan 1990, Lovley *et al.* 1989). *Geobacter* sp. are the only know pure cultures that are able to oxidize toluene and benzoate under Fe (III)-reducing conditions (Coates *et al.* 1996).

Organisms from diverse sites have linked BTEX oxidation to sulfate reduction (Anderson and Lovely 2000, Chen and Taylor 1997a, Kazumi *et al.* 1997, Coates *et al.* 1996, Phelps *et al.* 1996, Lovley *et al.* 1995). Under sulfate-reducing conditions BTEX degradation has been enhanced upon the addition of sulfate when sulfate had already been depleted from petroleum-contaminated sediment (Anderson and Lovley 2000, Ball and Reinhard 1996).

Evidence of BTEX degradation under methanogenic conditions has been shown by several studies (Weiner and Lovley 1998, Kazumi *et al.* 1997, Grbi'c-Gali'c and Vogel 1987, Vogel and Grbi'c-Gali'c 1986). Although it was once believed that benzene could not be degraded under methanogenic conditions, Kazumi *et al.* (1997) conclusively demonstrated that greater than 80% of ^{14}C-benzene added to aquifer sediments was mineralized to $^{14}C\text{-}CH_4$ and $^{14}C\text{-}CO_2$.

Inhibitions to BTEX degradation

There are factors that limit BTEX degradation. For example, the microbial population in a given site may not have the capability to degrade all the BTEX compounds (Schreiber and Bahr 2002, Lee *et al*. 2002, Yu *et al*. 2001). The degradation of BTEX compounds can also be inhibited due to the presence of other petroleum compounds. Deeb and Alvarez-Cohen (1999) found that the presence of xylenes or ethylbenzene inhibited degradation rates of other aromatics such as benzene and toluene. High concentrations of contaminants can be toxic to microbial populations and therefore inhibit degradation (Alagappan and Cowan 2003).

Hypersaline Environments

Studies of the past three decades have revealed that an enormous diversity of microbial life exists in extreme environments such as extremes of temperature, pH, high salinity, or high pressure (Madigan and Marrs 1997). The organisms that live in these environments not only can tolerate such conditions, but also require them for their survival (Madigan and Oren 1999). These habitats represent the harsh and unique environments of an ancient earth, and organisms that survive in these areas have been shown to possess unique capabilities that could be exploited for innovative biotechnologies including degradation of environmental pollutants (Oesterhelt *et al.* 1998).

Halophilic and Halotolerant Organisms

Although osmotic stress can be fatal to many organisms, members of each of the three domains of life have adapted to high salinity (Madern *et al.* 2000, Martin *et al.* 1999, Oren 1999, Grant *et al.* 1998, Reed *et al.* 1984). The majority of the organisms present in saline sites belong to the bacterial and archaeal domains. Some genera of bacteria are found throughout saline environments. They include *Psuedomonas, Micrococcus, Bacillus, and Vibro* species (Trüper *et al.* 1991). In general, as salinity increases the diversity of microorganisms decreases (Benlloch *et al.* 2002, Rodriguez-Valera *et al.* 1985). However, in environments that approach NaCl saturation, dense microbial communities persist, perhaps because of limited predation and sometimes abundant nutrients. Archaea are most prevalent at the highest salt concentrations, while halotolerant bacteria tend to dominate the environments with moderate NaCl concentrations.

Halophilic archaea have been found in many hypersaline lakes, such as the Dead Sea (Israel), Great Salt Lake (United States), Solar Lake (Sinai) (Oren 2002), and Antarctic lakes (Vestfold Hills lake system) (Bowman *et al.* 2000). Hypersaline soda lakes such as Mono Lake (United States) and Lake Magadi (Kenya) have also been shown to harbor halophilic archaea (Oren 2002). Many of these organisms have high caroteniod content. Therefore dense populations often appear quite red (Oren 2002). Pure cultures of halophilic archaea have been isolated from many saltern crystallization ponds. *Halobacterium* species have been isolated from crystallizer ponds in Israel and in the United States (Oren and Litchfield 1999). *Haloarcula, Haloferax, Halorubrum,* and *Halobacterium* species are frequently recovered from saltern ponds in Spain (Benlloch *et*

al. 2001, Rodriguez-Valera *et al.* 1985). The Dead Sea has been a source of isolation of several archaeal species, such as *Haloferax volcanii and Halobaculum gomorrense* (Oren and Gurevich 1993a) as well as the algal species of *Dunaliella* (Oren 1999b). Alkaliphilic halophilic archaea such as *Natrialba magadii, Halorubrum vaculoatum, and Natronococcus occultus* were found in Lake Magadi in Kenya (Grant *et al.* 1999). Halophilic archaea have also been isolated from the brine inclusions in halite crystals (Mormile *et al.* 2003). A recent report shows that halophilic archaea are not restricted to only hypersaline conditions; they have also been detected in areas with lower salt concentrations such as sulfur-rich springs (Elshahed *et al.* 2004). Denitrifying halophilic archaea have been isolated and include *Haloarcula marismortui* and *Haloferax mediterranei* (Oren 2001, Mancinelli and Hochstein 1986).

Until recently, it was believed that only *Archaea* were the dominant members of hypersaline environments that contained saturated levels of NaCl. Culture independent methods were used with samples from crystallizer ponds and found that bacteria were indeed an important part of the microbiota inhabiting NaCl-saturated waters. The extremely halophilic bacteria *Salinibacter ruber* was isolated from these saltern crystallizer ponds (Antón *et al.* 2000). It is unusual in that this organism has many similarities with archaea including employing many of the same mechanisms for osmotic protection. There are few other bacteria that have been isolated that grow in saturated salts. *Halorhodospira halophila* (formerly *Ectothiorhodospira halophila*) is a halophilic phototrophic sulfur bacterium isolated from soda lakes that grows optimally near 25% total salts (Imhoff 1992). Also, the actinomycete *Actinopolyspora halophila* was isolated that can be grown in saturated salt (Johnson *et al.*1986).

Other halophilic bacteria include the sulfate reducing *Desulfovibro* sp., whose optimum NaCl concentrations are between 50-100 g/L and have been isolated from Solar Lake (Caumette *et al.* 1990, Krekeler *et al.* 1997). The Dead Sea is also home to many halophilic bacteria. The obligate anaerobic bacterium, *Selenihalanaerobacter shriftii* grows with selenate or nitrate as an electron acceptor (Switzer Blum *et al.* 2001). Methanogenic activity has been found in Dead Sea sediments by the methanogen, *Methanocalculus halotolerans* (Marvin DiPasquale *et al.* 1999). Various fermentative bacteria have been isolated from Dead Sea sediments, including *Halobacteroides halobius* (Oren *et al.* 1984) and *Sporohalobacter marismortui* (later renamed *Orenia marismortui*) (Oren *at al.* 1987). Also, *Nitrosococcus halophilus*, a halotolerant autotrophic bacterium can oxidize ammonium in concentrations up to 94 g/L was found in the Dead Sea (Koops *et al.* 1990). Halophilic bacteria have also been found in oil brines (Bhupathiraju *et al.* 1999, Huu *et al.* 1999, Tardy-Jacquenod *et al.* 1996, Adkins *et a.l* 1993) and salt marshes (Yoon *et al.* 2003). Halotolerant bacteria have been isolated from many saline environments. The aerobic alkaliphilic halotolerant methanotrophic bacterium, *Methylobacter alcaliphilus* was isolated from soda lakes (Khemelenina *et al.* 1997). The haloalkaliphiles *Bacillus arsenicoselanatis* and *Bacillus selenitrireducens* were isolated from Mono Lake and can respire arsenate and selenate respectively (Switzer Blum *et al.* 1998). Pelagic waters and hydrothermal vents of the Pacific Ocean have been shown to sustain *Halomonas* and *Marinobacter* species (Kaye and Baross 2000, Fernandez-Linares *et al.* 1996, Gauthier *et al.* 1992).

Osmoprotection

In order for an organism to survive in saline conditions, it must regulate the solute concentration in its cytoplasm with its environment. They do this in two ways: 1) by maintaining intracellular salt concentrations that are at least equivalent with their environment, or 2) by the production or accumulation of organic compatible solutes. The primary response in the osmoadaptation of bacteria and archaea involves maintaining a concentration of intracellular KCl similar to its environment. The increased cytoplasmic salt concentration not only balances the internal and external environments, but also results in greater intracellular stability (Zaccai *et al.* 1986, Welsh 2000). Although it is more energetically favorable to maintain high intracellular salt concentrations than to produce organic solutes, the presence of such high concentrations of intracellular salt requires special adaptations of proteins and enzymes (Oren and Gurevich 1993b, Rengpipat *et al.* 1988, Reed 1984). Accumulation (Csonka 1988) or *de novo* systhesis (Tempest *et al.* 1970) of intracellular glutamate additionally confers osmotic tolerance by countering the charges of salt cations. However, the upper limit of K^+ and its counter ion glutamate is ~ 400 mM in non-halophilic bacteria (Dinnbier *et al.* 1988). This limits osmotic protection through the accumulation of K^+ only up to ~ 0.5 M NaCl for these bacteria (Galinski 1995). Salt concentrations above this trigger a secondary response in bacteria.

The secondary response to osmotic stress for bacteria is the intracellular increase of osmoprotective compounds, either by their production or accumulation. These compounds do not interfere with cellular functions even at molar concentrations (Brown 1976) since they contain no net charge (Galinski 1995). Additionally, compatible solutes

stabilize proteins and enzyme activities. This can lead to protection against high temperature, freeze-thawing and drying in addition to salinity (Lippert and Galinski 1992, Welsh 2000). In times of starvation, organic compatible solutes may be utilized as carbon and energy sources (Wood 1988). Glycine betaine can be utilized for growth in a nitrogen- and carbon-free minimal media in the absence of osmotic pressures (Bernard *et al.* 1986). However, by increasing the osmotic pressure, the catabolism of glycine betaine is blocked to ensure it is preserved so it can provide osmoprotection (Oren 1999, Smith *et al.* 1988, Le Rudulier and Bernard 1986, Bernard *et al.* 1986). The intracellular concentrations of these solutes rise as the environmental salinity rises to confer protection from osmotic stress (Lai and Gunsalus 1992). The concentrations of non-ionic solutes often exceed that of what would be required by ionic solutes to balance environmental osmotic pressures (Measures 1975). Generally, halophilic bacteria will prefer uptake of compatible solutes to *de novo* synthesis and obtain these solutes from their environment when they are released from other organisms (Galinski and Trüper 1994). There are many compatible solutes, but the principle compatible solutes for bacterial osmoprotection are glycine betaine, carnitine, proline (Beumer *et al.* 1994), and ectoine (Ventosa *et al.* 1998, Galinski 1995). While glycine betaine (Galinski and Louis 1999, Reed *et al.* 1984) and ectoine are found most abundantly in nature, many prokaryotic cells contain cocktails of osmolytes rather than a single compound (Galinski 1995).

Hypo-osmotic shock can also be detrimental to cells. Rapid influxes of water (by rainfall, flooding, etc) can lead to an influx of water into the cell, due to the permeability of the cell membrane to water. However, bacteria have also adapted mechanisms for dealing with this stress by the efflux of water and solutes. Bacteria possess aquaporins

that facilitate the rapid movement of water in and out of the cell without dissipating the transmembrane potential (Engel *et al.* 2000, Calamita 2000). Bacterial cells also contain channels that release cytoplasmic solutes under hypo-osmotic conditions in order to maintain turgor pressure (Sukharev *et al.* 1994, Szabo *et al.* 1993, Zorratti and Petronelli 1988).

Biodegradation by Halotolerant and Halophilic Organisms

Bioremediation utilizes the metabolic activities of microorganisms to transform organic contaminants into non-hazardous forms. It is seen as a favorable method of cleanup technology because it seeks to accelerate the natural processes of contaminant degradation, is cost-effective, and does not destroy the surrounding areas. This technology can use several approaches. The addition of known degrading organisms to a site, known as bioaugmentation, can stimulate degradation; however the augmented organisms must compete with the indigenous microflora for nutrients. Another approach is to alter prevailing environmental conditions so that they are more suitable for microbial growth. Environmental conditions, such as pH value, nutrient availability, oxygen, concentration, composition, and bioavailability of the contaminants influence bioremediation activity (Margesin and Schinner 2001). An area that is polluted with organics may already have the organisms present to degrade the contamination; however essential nutrients, such as nitrogen and phosphorus may be limiting, especially in marine areas. Therefore, the addition of nutrients would allow growth and degradation by these indigenous organisms. Bragg *et al.* (1994) illustrated how the addition of nutrients to the Alaskan shoreline after the *Exxon Valdez* oil spill increased the degradation of crude oil

five-fold. The biodegradation of aliphatic and aromatic compounds in salt marshes has also been stimulated by the addition of nutrients, such as nitrogen and phosphorus (Jackson and Pardue 1999, Lin *et al.* 1999, Wright *et al.* 1997). Other environmental factors render bioremediation more difficult because the extreme conditions that prevail are not ideal for the majority of microbes that might otherwise be successful in bioremediation processes. These conditions include extremes in temperature and pH, high salinity, and high pressure. Therefore bioremediation activities in these areas must utilize organisms who display the needed metabolic activity under these extreme conditions (Oren *et al.*1992).

Industrial activities can contaminate saline and hypersaline environments and lead to environmental problems since biodegradation under these conditions is difficult. Some reports indicate increased salinity has negative impact on the biodegradation of petroleum hydrocarbons (Ward and Brock 1978, Mille *et al.* 1991, Rhykerd *et al.* 1995). It has been proposed that the salt contamination first be removed before further remediation takes place (Rhykerd *et al.* 1995). However, this type of activity is costly and damaging to the environment. The use of halotolerant and halophilic organisms in the cleanup of organic contaminants in saline environments would prevent costly remediation strategies that reduce or remove salt by dilution methods, reverse osmosis, ion exchange, or electrodialysis before biological treatment begins (Margesin and Schinner 2001). Bacteria are more promising candidates than archaea for bioremediation in areas co-contaminated with salt because they harbor a greater metabolic diversity. The intracellular salt concentration of bacteria is low and therefore the enzymes involved in degradation may be similar to those of non-halophilic organisms (Oren *et al.* 1992).

Organisms in saline environments have shown the ability to degrade numerous compounds including agar (Shieh and Jean 1998), atrazine (Shapir *et al.* 1998), eicosane (Fernandez-Linares *et al.* 1996, Betrand *et al.* 1990), 2, 4-dichlorophenoxyacetic acid (Maltseva *et al.* 1996), and organophosphorus compounds (DeFrank and Cheng 1991). Betrand *et al.* (1990) found that their halophilic archaebacterium, EH4, could degrade a number of odd and even carbon number saturated aliphatic and aromatic hydrocarbons. Bacteria have also shown the ability to degrade petroleum compounds at even the highest salinities (Kuznetsov *et al.* 1992, Kulichevskaya *et al.* 1992). Also, under anaerobic conditions, halophilic bacteria have shown the ability to reductively transform nitroaromatic compounds (Oren *et al.* 1991). Many halogenated compounds have been shown to undergo biodegradation under saline conditions. The halophilic *Methylomicrobium* sp. can oxidize TCE in 2-6% salt (Fuse 1998) and can be utilized for the bioremediation of contaminated sea water.

Generally, hydrocarbon-degrading bacteria make up < 1% of the bacterial population in pristine environments. Those which harbor this activity utilize hydrocarbons that are produced naturally by plants, algae and other living organisms. However, the population of degraders increases rapidly when an area is contaminated with hydrocarbons (Atlas 1981). Benzene rings are found throughout nature; therefore organisms must be present that can cleave these rings. For example, lignin is ubiquitous and without its degradation much carbon would be locked up in plants and not returned to the nutrient cycle after they die (Dagley 1975). However, organisms that possess the ability to degrade these rings, especially in saline environments have remained elusive. Bastos et al (2000) isolated two phenol degrading organisms from pristine Amazonian

forest soil. *Candida tropicalis* and *Alcaligenes faecalis* demonstrated the ability to degrade phenolic compounds in the presence of 15 % and 5.6 % salt, respectively. Likewise, *Halomonas* sp. has been found suitable for the biotreatment of moderately saline phenolic wastewater (Hinteregger and Streichsbier 1997). The halotolerant *Pseudomonas halodurans* is able to degrade benzoate and other aromatics by *ortho* ring cleavage (Rosenberg 1983). Indication of the degradation of more complex compounds has been found in brackish waters where the indigenous microflora has shown the ability to degrade PAHs (Abbondanzi *et al.* 2005, Plotnikova *et al.* 2001). *Halomonas* sp. have also demonstrated the ability to degrade PAHs (Melcher *et al.* 2002). A wide variety of aromatic compounds, including benzoic acid, hydroxybenzioc acid, phenylpropionic acid, and phenol could be degraded by isolates from industrial sites contaminated with low-molecular weight aromatics (Garcia *et al.* 2005). Halotolerant microorganisms from the water/brine interface of hypersaline anoxic basins in the Mediterranean Sea have demonstrated the ability to readily degrade toluene, xylenes and PAHs, while benzene was poorly degraded (Brusa *et al.* 2001). Therefore, halophilic and halotolerant organisms can be used in the bioremediation of halogenated, aliphatic, and aromatic hydrocarbons (Margesin and Schinner 2001, Oesterhelt*et al.*1998).

Oilfield brine soils have been shown to harbor organisms with various metabolic activities including organisms that carry out sulfate-reducing, fermentative, and methanogenic activities, as well as organisms that degrade petroleum compounds (Bhupathiraju *et al.* 1999, Huu *et al.* 1999, Ollivier *et al.* 1998, Tardy-Jacquenod et al 1996, Adkins *et al.* 1993). The halophic archaeon *Haloferax* sp. D1227 has shown the ability to degrade aromatic compounds, including benzoic acid, cinnamic acid, and 3-

phenylpropoinic acid as its sole carbon and energy sources (Emerson *et al.*1994).

Little work has been done on the metabolic pathways of halophiles. Fu and Oriel (1999) studied the biodegradation of aromatics by the archeon *Haloferax* sp. D1227. Such studies with *Archaea* are important since they provide an opportunity to compare similar aromatic degradation pathways in *Bacteria* and *Eukarya*. These studies with *Haloferax* sp. D1227 revealed that degradation of 3-phenylpropionic acid, cinnamic acid, and benzoic acid is regulated by the gentisate pathway catalyzed by gentisate 1, 2-dioxygenase (Fu and Oriel 1999). Likewise, Fairley *et al.* (2002) found that the halophilic archaeon *Haloarcula* sp. strain D1 also uses the gentisate pathway for the aerobic degradation of 4-hydroxybenzoic acid. It remains unknown at this time if other halophiles utilize similar or unique pathways in the degradation of petroleum compounds. Archaea balance osmotic pressures with the uptake of salts, therefore the enzymatic activity of the archeons *Haloferax* sp. D1227 and *Haloarcula* sp. strain D1 most likely differ from pathways of organisms from the other domains.

CHAPTER III

BIODEGRADATION OF BTEX COMPOUNDS IN BRINE SOIL FROM AN OIL PRODUCTION SITE

Background

Exploration and production activities generate large volumes of oily wastewater with a wide range of salinities. The oily wastewater or produced waters inhibit plant growth leading to erosion of topsoil and contamination of groundwater by both salt and hydrocarbons. Salinity also complicates bioremediation efforts of crude oil because it renders a harsh environment for the implementation of salt-sensitive microorganisms for bioremediation (Rhykerd *et al.* 1995). Therefore, the bioremediation of oilfield brine can only be accomplished by using indigenous bacteria capable of degrading petroleum compounds or through the addition of degradative halophilic or halotolerant organisms. Unfortunately, information on the degradation of petroleum compounds under saline conditions is limited. Only recently has limited degradation of BTEX compounds been shown under hypersaline aquatic conditions (Brusa *et al.* 2001). However, more work is needed to understand how well such degradative activity is distributed in geographically different saline environments which can be utilized in the efforts to clean up these areas. The main focus of this chapter is to evaluate the biodegradation of BTEX compounds in oil brine soil, obtain BTEX-degrading aerobic enrichment cultures, and optimize conditions for enhanced degradation rates.

Materials and Methods

Soil Samples

Five different soil samples were obtained from contaminated E&P sites in Seminole and Stephens Counties in Oklahoma. These samples contained varying levels of different petroleum contaminants and salt (Table 1). One of the samples (Sem 2) was of surface soil with relatively low total petroleum hydrocarbons (TPH). This sample was subsequently used in aerobic experiments and in the development of an enrichment culture with benzene as the sole carbon and energy source. The other soil samples were used to assay for anaerobic BTEX degradation.

Chemicals

^{14}C-UL-Benzene with a specific activity of 33.2 mCi/mol was purchased from Sigma Chemical Co., St. Louis, MO. The Carbon-14 cocktail used for trapping $^{14}CO_2$ was obtained from the R.J. Harvey Instrument Corp., Hillsdale, NJ. Benzene and xylenes (*o-m-p*-xylenes) were >99 % pure and purchased from Fisher Chemical Company (Fair Lawn, NJ). Toluene and ethylbenzene were >99 % pure and were obtained from Aldrich Chemical Company (Milwuakee, WI). All other chemicals were of reagent grade.

Microcosm Preparation

Microcosms were prepared with 160 ml capacity serum bottles filled with 10 g soil and 40 ml of mineral salts medium supplemented with 2.5 M NaCl (MSM-NaCl). The composition of MSM-NaCl (g/L) is: NaCl, 145; $MgCl_2$, 0.5; KH_2PO_4, 0.45; K_2HPO_4, 0.9; NH_4Cl, 0.3; KCl, 0.3. The bottles were closed with Teflon-coated rubber

TABLE 1
OIL BRINE SOIL ANALYSIS*

	EPA Method	Sem 1	Sem 2	Sem 3	Sem 4	Stephens
Sample depth (ft)		4.0	0-3	4.0	4.0	0-1.5
Benzene (mg/Kg)	8021 B	4.48	3.41	1.72	0.149	BDL**
Toluene (mg/Kg)	8021 B	9.72	10.4	7.48	0.715	0.241
Ethylbenzene (mg/Kg)	8021 B	30.2	7.95	14.1	14.1	BDL
TPH (mg/Kg)	8051 M	72744	6071	25198	120276	125580
Chloride (mg/Kg)	325.3	42200	14800	3740	220	1270

*Soil analyses were performed by the Beacon Environmental Assistance, Corp., Edmond, OK.
**Below detection limit

septa and aluminum caps. A gas-tight glass syringe was used to introduce 2 µl neat benzene, toluene, ethylbenzene, or xylenes (~25 µmole) into each serum bottle. Air in the headspace (110 ml) served as the source of oxygen. Bottles were incubated static in the dark at 30 °C. Biodegradation of BTEX was monitored by withdrawing 200 µl of headspace gas and injecting into a gas chromatograph (GC).

Development of Enrichment Culture

An aerobic enrichment culture was developed by adding 10 g soil (wet weight) from the Seminole County soil sample to duplicate 1-L bottles containing 500 ml of MSM-NaCl. The bottles were closed with a black rubber stopper with a hole in the middle that fit a cut 3-inch Hungate tube. The tubes were sealed with Teflon-coated septa and aluminum caps. A gas-tight glass syringe was used to introduce 22 µl neat benzene (~250 µmole) into each enrichment bottle. Air in the headspace served as the source of oxygen. Bottles were incubated static in the dark at room temperature. When the added benzene was depleted the bottles were opened to replenish the oxygen supply and respiked with benzene. This was repeated 6-7 times before a 10 % sediment-free mixed culture was transferred to freshly prepared MSM-NaCl medium. The bottles were repeatedly spiked with benzene, monitored for degradation, and transferred to fresh medium as before. This continued for the next 7-8 months, at which time highly enriched and stable cultures were developed that degraded 200 to 300 µmole of added benzene consistently in 2.5 weeks. The developed enrichment cultures were named Sem 2 enrichment cultures.

Biodegradation Studies with Sem 2 Enrichment

All biodegradation assays were carried out using 120 or 160 ml serum bottles filled with 45 ml of MSM-NaCl. Bottles were inoculated with 5 ml of Sem 2 enrichment culture. The bottles were spiked with 2 μl (~25 μmole/bottle) of undiluted benzene, toluene, ethylbenzene, or xylenes with a 10 μl gas-tight glass syringe to serve as the sole carbon and energy source. Triplicate active and duplicate autoclaved control bottles were prepared to study the biodegradation of individual BTEX compounds. Microcosms were incubated static in an inverted position in the dark at 30˚C. Biodegradation was monitored by injecting 200 μl of headspace gases into a GC.

Initial studies evaluated benzene degradation by the Sem 2 in the presence of low concentrations of growth promoting nutrients such as yeast-extract (YE), vitamins, or trace elements. Microcosms were prepared as above in 120 ml capacity serum bottles amended with 0.02 % YE, 1 μl of vitamin solution/ml, or 1 μl trace elements/ml (Löffler *et al.* 1996). To determine the effect of salt concentration on benzene degradation, triplicate active microcosms were prepared with 160 ml capacity serum bottles containing 45 ml of MSM amended with 0, 0.5, 1.0, 2.0, 2.5, 3.0, or 4.0 M NaCl. Duplicate autoclaved controls were also prepared similarly for each salt concentration tested.

GC Analysis

Biodegradation of BTEX compounds were assayed using a Hewlett Packard 6890 GC equipped with a flame ionization detector and a DB-1 capillary column (30 m x 0.320 mm x 1 μm; J&W Scientific, Inc., Folsom, CA). Helium served as both carrier and makeup gas at flow rates of 10 and 40 ml/min, respectively. The flow rates of hydrogen and air were set at 40 and 450 ml/min, respectively. The operating GC conditions were

the following: oven temperature, 70 ºC for 7 minutes; inlet temperature, 150 ºC; and detector temperature, 220 ºC. Approximately 200 µl of headspace gas was removed from microcosms using a gas-tight syringe and injected into the GC for quantification. The GC response for each compound tested was calibrated to give the total mass in that bottle. Assuming the headspace and aqueous phase concentrations were in equilibrium, the total mass present in the bottle was determined using standards prepared similarly. After equilibration (approximately 1 hour) at room temperature, the GC response for a range of mass (µmol/bottle) of each compound tested was plotted and the slopes were used to quantify the unknown. Benzene degradation in the enrichment bottles was accomplished as described above by using a calibration curve prepared with 1-L bottles containing 500 ml of MSM-NaCl. The GC detection limit for benzene using this method was < 1.0 µmole/bottle.

Mineralization of ^{14}C-Benzene Under Anaerobic and Aerobic Conditions.

In order to determine complete oxidation of benzene to CO_2 under anaerobic conditions, microcosms (120 ml serum bottles) were set up in an anaerobic glove box for each of the anaerobic soils obtained from Seminole and Stephens Counties, OK. Similarly, aerobic microcosms were setup using the Sem 2 enrichment culture to test its ability to mineralize added benzene to CO_2 under aerobic condition. Each bottle was spiked with 100 µL stock ^{14}C-benzene (specific activity of ^{14}C-benzene = 33.2 mCi/mmole). This amounted to 2.75E+04 dpm/bottle. Bottles were also spiked with 2 µl neat benzene. The bottles were closed with Teflon-coated septa and aluminum caps and incubated static at 30 ˚C in the dark. At the end of 4, 8, 12 weeks, triplicate active and

duplicate control bottles were sacrificed and stored at –20 °C until analyzed for radioactive CO_2.

Evidence of mineralization under anaerobic and aerobic conditions was determined by measuring the production of $^{14}CO_2$ from ^{14}C-benzene. Bottles were injected with 0.2 ml of 10 N HCl (pH < 2) followed by vigorous hand-shaking the contents for 5 minutes. It is anticipated that under strong acidic conditions, all aqueous $^{14}CO_2$ (^{14}C-HCO_3^-) is converted to gaseous $^{14}CO_2$. The bottles were then purged with N_2 (80-100 ml/min) for 40 min and the $^{14}CO_2$ was trapped in 5 glass vials connected in series. Each vial contained 10 ml of Harvey's Carbon-14 Cocktail and was sealed with Teflon-coated septum and an aluminum cap. In addition, a trap filled with activated carbon was placed between the flushing bottle and the first trapping vial to absorb volatile ^{14}C-benzene as well as to prevent contamination of the trapping vials with escaping liquids during flushing. Radioactivity in all 5 traps was measured using a Beckman LS 6000SC liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Microbial Community Analysis of Enrichment

Sem 2 enrichment grown on benzene and in the presence of 0 and 2.5 M NaCl were sent to Microbial Insights (Rockford, TN) for community analysis where samples were analyzed with the following protocols: Nucleic acid extraction was performed using a bead-beating method (Stephen *et al.* 1999). The DNA was purified by a glass-milk DNA purification protocol using a Gene Clean™ kit as described by the manufacturer (Qbiogene, Irvine, CA). PCR amplification of 16S rRNA gene fragments

was performed as described in Muyzer *et al.* (1993) with the following modifications: Thermocycling consisted of 35 cycles of denaturation for 45 seconds at 92 °C, annealing for 30 seconds at 55 °C, and extension for 45 seconds at 68 °C. The PCR mix contained 0.44 units of Clontech Advantage™ 2 polymerase (BD Biosciences, Mountain View, CA) and 12.5 pmole each primer in a total volume of 25 µl. Thermocycling was performed using a Robocycler™ PCR block (Stratagene, La Jolla, CA). Two primer sets were used in a nested PCR approach. The first primer set corresponded to *E. coli* bp positions 27 and 1492 of the 16S rRNA gene. The second set of primers targeted eubacterial 16S rDNA regions corresponding to *E. coli* positions 341-534, and the forward primer of this set contained a 40 bp GC-clamp. A portion (20%) of each PCR product was analyzed by agarose gel electrophoresis (1.5% agarose, 1x TAE buffer) and ethidium bromide fluorescence. The amount of DNA used for DGGE was standardized to 150 ng by comparison to molecular weight standards using Alpha Imager™ software (Alpha Innotech Corp., San Leandro, CA). DGGE was performed on a Bio-Rad (Hercules, CA) D-Code 16/16 cm gel system maintained at a constant temperature of 60°C in 6-L of 0.5x TAE buffer (20 mM Tris actate, 0.5 mM EDTA, pH 8.0). Denaturing gradients were formed at 30 – 65 % denaturant (with 100% denaturant defined as 7 M urea, 40% v/v formamide). A size gradient was imposed on the denaturing gradient by forming an 8 – 10 % acrylamide gradient (i.e., double gradient – DGGE) as described by Cremonesi et al (1997). Gels were electrophoresed at 35 V for 16 hours. Gels were stained with ethidium bromide (0.5 mg/L) and destained twice in 0.5 x TAE for 15 min. each. Gel images were captured using an Alpha Imager™ system. The central 1 mm portion of intensely fluorescing DGGE bands were excised using a

razor blade and soaked in 50 µl of purified water overnight. A portion (2 µl) was used as the template in a PCR reaction as described above. The products were purified using QiaQuick PCR purification kits (Qiagen, Valencia, CA). Purified DNA was sequenced with an ABI-Prism automatic sequencer model 377 with dye terminators. Sequence identifications were performed using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/Blast) and the "Sequence Match" facility of the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.html).

Results and Discussion

A highly enriched and stable aerobic enrichment was established with the Sem 2 soil that could utilize benzene as the sole carbon and energy source. After 7 to 8 months of continuous enrichment, the culture consistently degraded benzene within 18 days at room temperature at a rate of approximately 12 µmole/day. Figure 2 shows the repeated benzene degradation of the enrichment.

Mineralization of ^{14}C-Benzene to $^{14}CO_2$ by Oil brine Soil and the Sem 2 Enrichment

In order to show conclusively that benzene is mineralized to CO_2 under both aerobic and anaerobic conditions, universally labeled ^{14}C-benzene was added to microcosms consisting of soil and MSM-NaCl. As shown in Table 2, at the end of three months of incubation, about 5 to 10 % of the radiolabeled benzene was mineralized above that of the control bottles in all of the anaerobic soil samples obtained from

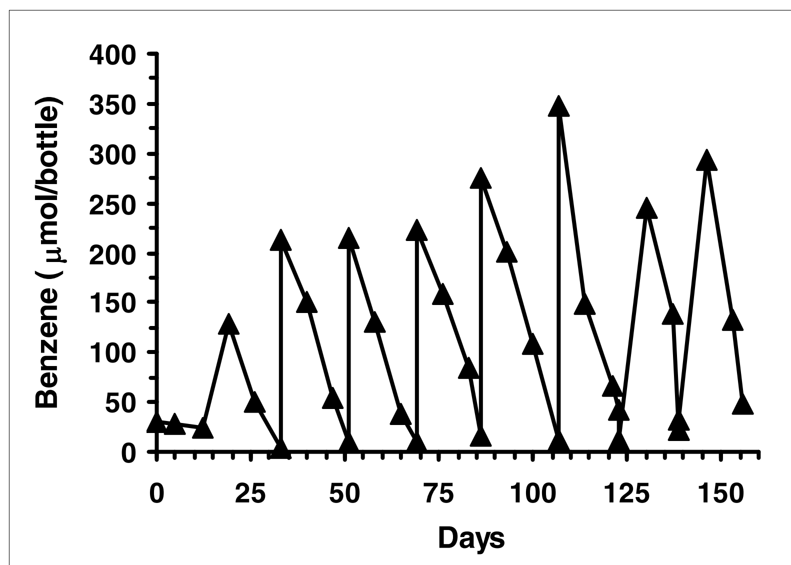

Figure 2. Repeated use of benzene (▲) as the sole carbon and energy source in the presence of 2.5 M NaCl by the Sem 2 enrichment culture. The enrichments were maintained in 1-L capacity bottles containing 500 ml of MSM-NaCl at room temperature. After an initial lag period, the enrichments degraded 200 to 300 µmole of added benzene/bottle consistently in 2.5 weeks. Results for only one bottle are shown; duplicate enrichments behaved similarly.

TABLE 2
MINERALIZATION OF BENZENE BY HALOPHILES UNDER AEROBIC AND ANAEROBIC CONDITIONS

	Sem 1	Sem 2	Sem 3	Sem 4	Stephens
Soil Type	Anaerobic	Aerobic	Anaerobic	Anaerobic	Anaerobic
Time incubated	> 12 weeks	4 weeks	12 weeks	12 weeks	12 weeks
Percent ^{14}C-benzene mineralized to $^{14}CO_2$*	5.93 ± 0.94	46.83 ± 14.09	10.16 ± 5.87	4.6 ± 0.12	<1%

*Percentage of ^{14}C-CO_2 recoveries are above the control values

Seminole County. However, no degradation was seen under the same growth conditions in microcosms prepared with soils from Stephens County. Although, the removal of benzene under anaerobic conditions seems poor, the rate and extent of degradation may be enhanced under more optimal growth conditions. Results also showed that roughly 46 % of the added benzene was mineralized under aerobic conditions by the Sem 2 enrichment culture above that of the controls.

BTEX Degradation by the Sem 2 Enrichment Culture

The Sem 2 enrichment showed the ability to degrade benzene, toluene, ethylbenzene, or xylenes as the sole carbon and energy source (Figure 3). This is not surprising since the enrichment was developed from a soil obtained from an oil production site where the microflora was exposed to crude oil (Hayes *et al.* 2001). Among the tested BTEX compounds, toluene degraded best. Approximately 20 µmole of toluene was completely degraded in less than one week, while benzene, ethylbenzene, and xylenes required 2 to 3 weeks for degradation. Autoclaved bottles showed no evidence of degradation. Although a few reports have documented the ability of halophilic or halotolerant organisms to degrade hydrocarbons such as phenol (Woolard and Irvine 1995), nitrophenols (Oren *et al.* 1992), benzoate (Emerson *et al.* 1994), pesticides (DeFrank and Chang 1991), herbicides (Maltseva *et al.* 1996), *n*-alkanes (Betrand *et al.* 1990), and PAHs (Plotnikova *et al.* 2001, Betrand *et al.* 1990), little evidence has been shown on the degradation of BTEX under saline conditions (Brusa *et a.l* 2001).

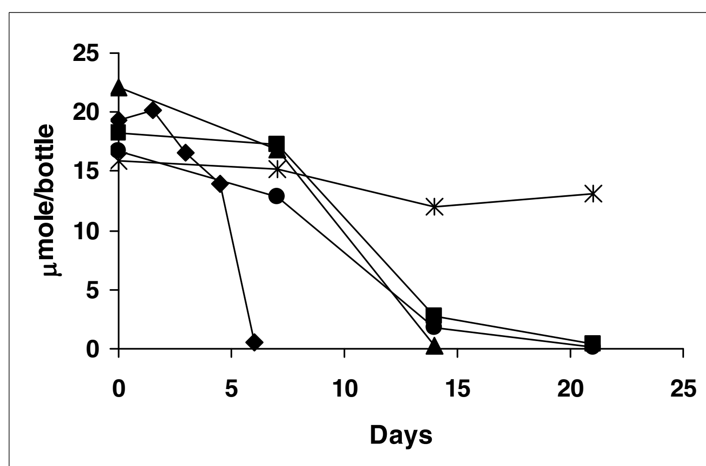

Figure 3. Biodegradation of benzene (▲), toluene (♦), ethylbenzene (■), and xylenes (●) in 160 ml capacity serum bottles containing 45 ml of MSM-NaCl and inoculated with 5 ml of Sem 2 enrichment culture. The microcosms were incubated at 30 °C. The data are means triplicate active microcosms and averages of duplicate autoclaved control bottles. Since all controls behaved similarly, only the control for xylenes (∗) is shown.

Optimization Studies

Biostimulation involves the addition of rate-limiting nutrients to accelerate the biodegradation process. Most of the hydrocarbon contaminated areas do not lack in carbon supply, but are limited in available nitrogen, phosphorus, and growth promoting trace nutrients. Therefore, these additions may stimulate the degradation of pollutants by the indigenous microflora (Jackson and Pardue 1999, Lin *et al.* 1999, Wright *et al.* 1997). The Sem 2 degraded benzene more quickly in the presence of low concentrations of YE, vitamins, or trace elements, compared to the culture devoid of added stimulants (Figure 4). It has been suggested that halophiles have more demanding nutritional requirements at high salt concentrations. Therefore, complex media may stimulate growth of halophilic bacteria at high salt concentrations (Ventosa *et al.* 1998). The halophilic archeon strain EH4 showed increased eicosane degradation in the presence of YE, peptone, and casamino acids (Betrand *et al.* 1990). Also, the addition of YE to growth media of halophilic organisms has been shown to increase the accumulation of the osmolyte glycine betaine (Wohlfarth *et al.* 1990).

Although the addition of osmolytes has been shown to reduce the lag time needed for the degradation pollutants under saline conditions (Shapir *et al.* 1998), benzene degradation by the Sem 2 enrichment was inhibited by the addition of osmolytes (Table 3). The exact reason for this inhibition is not known. It may be that these osmolytes were not compatible with organisms present in the enrichment culture. Alternatively, the added osmolytes might have served as the source of carbon for the organisms in the enrichment. Reports indicate that many halophilic bacteria are able to utilize compatible

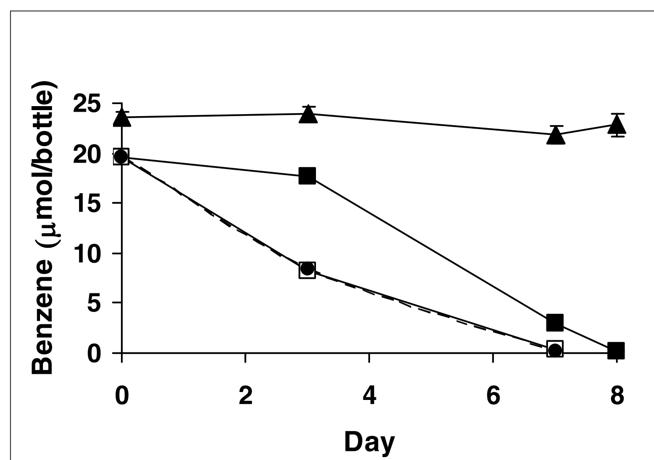

Figure 4. Biostimulation of benzene degradation by addition of YE, vitamin, or trace elements. Microcosms were established with 45 ml MSM-NaCl and were inoculated with 5 ml of Sem 2 enrichment. Biodegradation of benzene (20-25 µmol/bottle) was evaluated in the presence or absence of growth stimulants. Symbols: YE (■); vitamins (●); trace elements (-□-). Positive control bottles with no amendments completely degraded added benzene in 15 days (data not shown). The autoclaved microcosms were also amended with filter-sterilized 0.02 % YE, 1 µl of vitamin solution/ml, or 1 µl of trace elements solution/ml. The results are means of triplicate active microcosms and the averages of two autoclaved control bottles. Because all controls behaved similarly, only control data for YE (▲) are shown.

TABLE 3
BIODEGRADATION OF BENZENE IN THE PRESENCE OF KNOWN OSMOLYTES

Osmolyte	Concentration	% Degradation	Days
No osmolyte*		97.77 ± 0.87	12
Glycine	1 M	62.69 ± 0.22	61
Proline	1 M	42.30 ± 4.20	61
Betain	1 M	67.25 ± 2.38	61
KCl	1 M	56.41 ± 2.11	61

*Benzene degradation was monitored in the absence of an osmolyte.

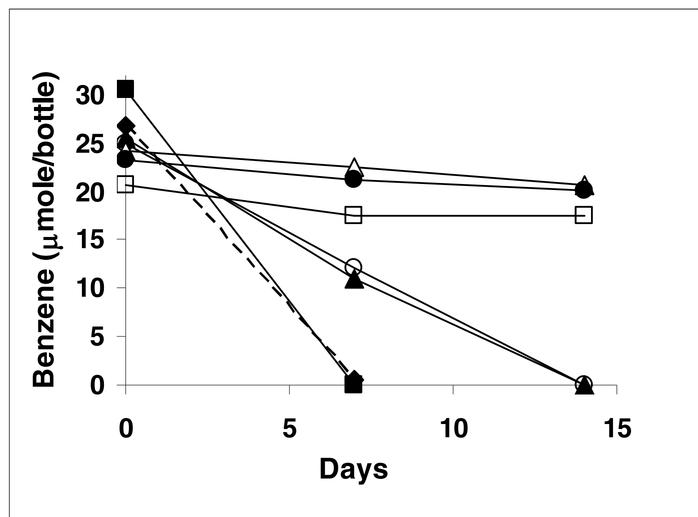

Figure 5. Biodegradation of benzene by Sem 2 enrichment culture in the presence of various NaCl concentrations. Microcosms were established in 160 ml capacity serum bottles containing 45 ml MSM and inoculated with 5 ml of Sem 2 culture. All microcosms were spiked with ~25 µmole benzene and amended with various concentrations of concentrations of NaCl. Symbols: 0 M (□); 0.5 M (-♦-); 1 M (■); 2 M (▲); 2.5 M (○); 3 M (Δ); or 4 M (●) NaCl. The results are means for triplicate active microcosms. Although results for 0, 3, and 4 M NaCl bottles are shown for only 2 weeks of incubation, no degradation occurred even after 4 weeks of incubation (data not shown).

solutes as osmoprotectants as well as for carbon sources (Fougère and Le Rudulier 1990, Smith *et al.*1988, Bernard *et al.* 1986, Le Rudulier and Bernard 1986).

The Sem 2 culture degraded 25 to 30 µmole of benzene in 7 to 14 days in bottles containing 0.5, 1, 2, or 2.5 M NaCl, as seen in Figure 5. No degradation occurred in bottles containing 0, 3, or 4 M NaCl, even after 4 weeks of incubation (data not shown). Our results show that degradation of benzene required the addition of at least 0.5 M NaCl to the growth medium since no degradation occurred in the absence of salt. This indicates that the enrichment harbored true benzene degrading halophiles. The ability of the culture to degrade benzene over a wide range of NaCl concentrations (0.5 to 2.5 M) suggests that this culture is well suited for field bioremediation applications because many produced waters or oilfield brines display a wide range of temporal or spatial salinity fluctuations. The reason for the lack of benzene degradation at 3 and 4 M NaCl is not known. Few studies have dealt with the effect of salinity on microbial degradation. Benlloch *et al.* (2002) and Rodriguez-Valera *et al.* (1985) observed that degradation activities decrease as salinity increases. In contrast, Fernandez-Linares *et al.* (1996) showed that increasing salt concentrations had no effect on eicosane degradation by a *Marinobacter* sp.

Microbial Community Analysis

The community structure of the Sem 2 culture grown in the presence and absence of added NaCl was characterized by profiling the 16S rDNA genes by using DGGE (Figure 6). Multiple bands were amplified, and sequences from each of these bands were amplified. Sequences from each of these bands aligned well (>99%) with *Marinobacter*

spp. sequences (GenBank accessions nos. AY136121, AF513448, and AF237685). From analysis, it appears that bands A and B were heteroduplexes of bands C and D (accession nos. AJ294359, AF212213, AY136121, AY129889, and AF546961). It may be that bands C and D represent two different yet closely related bacteria or that there are two ribosomal sequences for *Marinobacter* sp. Similar bands were missing in the DGGE obtained from the enrichment grown on benzene but no NaCl. These results are consistent with the degradation activity; benzene was not degraded in bottles that did not contain NaCl (Figure 5). This suggests that perhaps *Marinobacter* spp. were responsible for the degradation of benzene. *Marinobacter* spp. have been isolated from geographically different locations, including the French Mediterranean, from the mouth of a petroleum refinery outlet, from deep sea sediments in the western Pacific, and from oil wells off the coasts of Vietnam and California (Kaye *et al.* 2000, Gauthier *et al.* 1992). *Marinobacter hydrocarbonoclasticus,* a halotolerant bacterium, is able to degrade eicosane (Fernandez-Linares *et al.* 1996). BTEX degradation in saline environments been only recently been observed in marine environments (Brusa *et al.*2001).

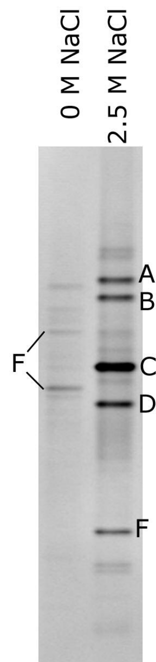

Figure 6. DGGE gel depicting the dominant members of the Sem 2 enrichment culture extracted from microcosms amended with 0 M and 2.5 M NaCl. Labeled bands were excised, sequenced, and compared to sequences in the database. Results indicate that the dominant members of the Sem 2 culture grown at 2.5 M NaCl have 97 to 100% sequence similarity to the members of the genus *Marinobacter* (bands A, B, C, and D). Similar bands were missing at 0 M NaCl. Bands labeled F failed to yield usable sequences due the lack of sufficient DNA. .

Conclusions

This study conclusively demonstrated the ability of halophiles to degrade benzene, toluene, ethylbenzene or xylenes as the sole carbon and energy source. A highly enriched aerobic consortium was developed from an oil-brine soil obtained from an E&P site in Seminole County, Oklahoma. Studies using ^{14}C-benzene showed that the enrichment was able to oxidize roughly 46 % of the added benzene to CO_2 in 4 weeks, thus suggesting mineralization ability of the organisms. Benzene degradation by this halophilic enrichment could be stimulated with the addition of low concentrations of YE, vitamins, or trace elements. Benzene degradation proceeded normally in the presence of NaCl ranging from 0.5 to 2.5 M, while at 3 M NaCl, degradation was inhibited. These results indicate that the enrichment can survive fluctuating salt concentrations commonly seen in the field. Community analysis using DGGE revealed that *Marinobacter* sp. were the dominant members of the consortium at 2.5 M NaCl, while there were no dominant members in the absence of NaCl. This corresponded well with the lack of benzene degradation in bottles lacking added salt. *Marinobacter* sp. have previously been shown to degrade petroleum hydrocarbons in saline environments.

CHAPTER IV

BIODEGRADATION OF BTEX COMPOUNDS IN A NATULLY HYPERSALINE ENVIRONMENT, THE GREAT SALT PLAINS, OKLAHOMA

Background

The Great Salt Plains National Wildlife Refuge is a naturally hypersaline environment located near Cherokee, Oklahoma. The surface of the salt flats is covered by a thin crust of salt deposited from the evaporation of Permian brine from underlying strata (Caton *et al.* 2004). Rainfall dissolves the salt crust and temporarily creates streams and ponds. Consequently, salt concentrations widely fluctuate from very low to saturation. Groundwater salinity ranges from 4 to 37 %, while surface salinity varies from 0.3 to 27 %, depending on prevailing weather conditions. The pH of the soil at the Salt Plains varies between 7.34 and 9.23 (Caton *et al.* 2004). The rapid changes in salt concentration, wide fluctuations in surface temperature (diel temperature change = 30 ˚C), and direct exposure to UV light makes this habitat an extreme environment. There is no known contamination, although nearby oil production activities may contaminate the shallow water table. Pristine environments contain natural genetic diversity that may be utilized for the degradation of problematic compounds (Bastos *et al.* 2000). Aromatic compounds are abundant in nature especially in the form of lignin and phenols (Bastos *et al.* 2000, Dagley 1975). Therefore, extremophiles who have not been exposed to oil contamination may still possess the ability to degrade BTEX compounds. Identifying and harnessing the unique capabilities of extremophiles inhabiting these areas may offer new solutions to longstanding challenges in environmental and waste cleanup.

Conversely, such habitats may be entirely devoid of microbes capable of degrading environmental pollutants. If this is the case, bacteria native to such habitats are likely to have been exposed to low-level, non-point sources of toxic contaminants from increased human activities. Therefore, it is important to understand how extremophiles modulate the fate of pollutants and quantify the degradative capacities to prevent the buildup of toxic levels of contaminants in these delicate and largely unexplored ecosystems.

The primary objective of this chapter is to assess the ability of indigenous bacteria at the Great Salt Plains to degrade BTEX compounds and to determine the impact of concentration of salt on degradation rate and bacterial community structure. This study is important because naturally hypersaline environments such as salt flats are ancient and may harbor unique organisms of considerable interest in terms of their evolution and as novel biocatalysts (Margesin and Schinner 2001, Hough and Danson 1999, Ventosa and Nieto 1995).

Materials and Methods

Soil Samples and Chemicals

Two soil samples, A and B were obtained from the surface of the salt flats at the Great Salt Plains Wildlife Refuge, OK. The coordinates (obtained using GIS) are N 36° 42.435' W 98° 15.620' and N 36° 42.485' W 98° 15.700' for samples A and B respectively. ^{14}C-UL-naphthalene with a specific activity of 31.3 mCi/mole was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were described in Chapter 3.

Microcosm Setup

Initially, microcosms were prepared with soils A and B in 160 ml serum bottles containing 10 g soil and 40 ml MSM-NaCl (described in Chapter 3). Bottles were closed with Teflon-coated septa and aluminum caps. Each bottle was spiked with approximately 25 µmole of benzene and incubated static in the dark at 30 ˚C. Triplicate microcosms were prepared for each soil. Benzene degradation was monitored using a GC as described in Chapter 3. After five weeks of lag time, all three bottles from soil A and one of the three bottles from soil B degraded the added benzene in seven days.

Development of Enrichment Culture

A stable and highly enriched aerobic microbial consortium that degraded benzene as the sole carbon source was developed for each soil sample (soil A and B) by transferring the entire contents of a serum bottle (approximately 50 ml of slurry) to 1-L capacity bottles containing 450 ml MSM-NaCl. Bottles were closed with black rubber septa with a hole in the middle that fit a cut 3-in. Hungate tube. The tubes were sealed with Teflon-coated septa and aluminum caps. Enrichment cultures were developed by repeatedly spiking the bottles with benzene and transferring 10 % of the sediment-fee culture to fresh MSM-NaCl medium as described in Chapter 3.

Biodegradation Studies with the Salt Plains Enrichment

All studies involving the Salt Plains enrichment culture were carried out in 120 ml or 160 ml serum bottles as described before (Chapter 3). Microcosms were spiked with 25 to 30 µmoles of benzene, toluene, ethylbenzene, or xylenes as sole carbon and energy

sources. Biodegradation of BTEX compounds was monitored with a GC as described in Chapter 3. The ability of the enrichment culture to mineralize ^{14}C-benzene and ^{14}C-naphthalene was also evaluated. Bottles were spiked with 100 µl of ^{14}C-benzene (5.38E+4 dpm/bottle) or ^{14}C-naphthalene (7.01E+5 dpm/bottle) from an aqueous stock. After 4 weeks of incubation, bottles were sacrificed and analyzed for the production of $^{14}CO_2$ as described in Chapter 3.

Biodegradation of benzene by the Salt Plains enrichment culture was assessed under various electron accepting conditions, including fermentative (no externally added electron acceptor), nitrate reducing (150 µmole $NaNO_3$/bottle), and iron reducing (750 µmole $FeCl_3$/bottle). Since $FeCl_3$ is highly insoluble in aqueous phase, we also setup microcosms with $FeCl_3$ (750 µmol/bottle) and NTA (4 mM). NTA is an iron-chelating agent that increases the solubility of $FeCl_3$. All bottles were purged with N_2 (80-100 ml/min) for 15 minutes to remove dissolved oxygen prior to the addition of 25 µmole of benzene and an alternative electron acceptor. Bottles were closed with Teflon-coated septa and aluminum caps.

The Salt Plains enrichment culture was studied for its ability to degrade benzene under various growth conditions. Microcosms were amended with low concentrations of growth promoting nutrients to study if the rate of benzene degradation could be enhanced. These include peptone (0.02 %), casamino acids (0.02 %), YE (0.02 %), vitamins (1 µg/ml) or trace elements (1 µg/ml). The composition of vitamins and trace elements can found elsewhere (Löffler *et al.* 1996). The effect of salinity on benzene degradation was determined in microcosms using MSM supplemented with 0, 0.5, 1.0, 2.0, 2.5, 3.0, or 4.0 M NaCl. Also, degradation of benzene was evaluated in the presence

of different types of salts such as KCl, $MgCl_2$, or $CaCl_2$ at 2.5 M each. Duplicate autoclaved controls were prepared for each salt concentration and salt type. Biodegradation of benzene at different temperatures was evaluated by incubating bottles at different temperatures ranging from 5 to 60 °C. Microcosms were brought to room temperature prior to GC analysis.

Analytical Techniques

Biodegradation of individual BTEX compounds was monitored by analyzing headspace gases using the GC as described in Chapter 3. A separate set of standard plots were prepared using 160 ml serum bottles for 2.5 M $MgCl_2$, 2.5 M KCl, or 2.5 M $CaCl_2$ since partitioning of benzene in headspace was different for each of the salt types tested.

Microbial community analysis was performed by Microbial Insights, Inc. (Rockford, TN). Genomic DNA from the Salt Plains enrichment grown on benzene and in the presence of different concentrations of NaCl (0 to 4 M) was extracted using the bead-beating method (Stephen *et al.* 1999). Details of PCR amplification of 16S rDNA and community analysis using DGGE are described in Chapter 3. Sequence identifications were performed using the "Sequence Match" facility of the Ribosomal Database Project II (http://rdp.cme.msu.edu/html/).

Results and Discussion

The results of this study demonstrate that organisms living in hypersaline soils can readily degrade simple aromatic compounds such as benzene and toluene. Although biodegradation of BTEX has been extensively studied by non-saline aerobic and

anaerobic bacteria, little is known about the degradative ability of organisms native to natural hypersaline environments that have no history of contamination. However, the abundance of aromatics such as phenols (Bastos *et al.* 2000) and lignins (Dagley 1975) in the natural environment may sustain microbial populations that can degrade aromatic compounds in pristine environments.

Highly enriched microbial consortia capable of using benzene as the sole carbon and energy source in the presence of 2.5 M NaCl were developed. The enrichments consistently degraded added benzene (~250 µmole/bottle) within one week with no apparent lag time, as seen in Figure 7. Microcosm studies were initiated with MSM-NaCl and inoculated with 5 ml of the enrichment culture (10 % inoculum size) to study the degradation of individual BTEX compounds. The culture completely degraded added benzene or toluene under aerobic conditions. No degradation of ethylbenzene or xylenes occurred even after incubating for more than 50 days (Table 4). This was also true for the enrichment developed from Soil A. This lack of degradation activity towards ethylbenzene and xylenes is interesting because these compounds are readily degraded by non-saline aerobic bacteria of various genera (Alvarez and Hunt 2002). We also assessed the ability of the enrichment to degrade benzene and toluene under alternative electron accepting conditions. Results shown in Table 5 illustrate that no degradation of benzene or toluene occurred under denitrifying, iron-reducing, and fermentative/methanogenic conditions even after incubating for more than four weeks. The lack of degradation activity by the enrichment under denitrifying conditions could not be explained since nitrate is prevalent at the Salt Plains (Caton *et al.* 2004). Moderately halophilic bacteria have been shown to grow under both aerobic and denitrifying conditions (Vreeland *et al.*

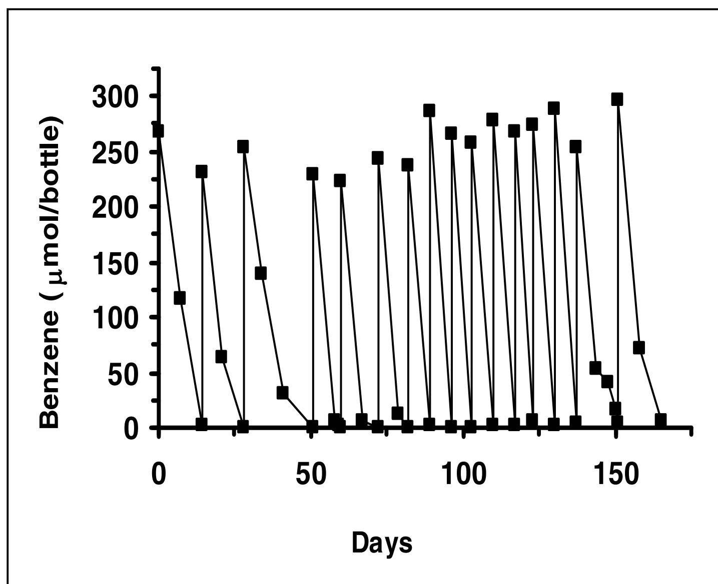

Figure 7. Repeated degradation of added benzene by the Salt Plains enrichment culture. Duplicate enrichments behaved similarly therefore only degradation for one enrichment is shown.

TABLE 4
DEGRADATION OF AROMATIC COMPOUNDS
BY THE SALT PLAINS ENRICHMENT

Compound	Degradation*
Benzene	+
Toluene	+
Ethylbenzene	—
o-, *m*-, *p*-xylenes	—
Naphthalene	—

* “+” denotes degradation, “—” denotes no degradation by the Salt Plains enrichment

TABLE 5
BENZENE AND TOLUENE DEGRADATION BY THE SALT PLAINS ENRICHMENT WITH VARIOUS ELECTRON ACCEPTORS

Electron Acceptor	Benzene or Toluene Degradation**
Oxygen	+
Fermentation	—
Nitrate	—
Iron (III)	—
Iron (III) + NTA*	—

*Nitrilotriacetic acid, ** "+" denotes degradation, "—" denotes no degradation by the Salt Plains enrichment

1980). It is probable that the growth conditions used during the enrichment process did not select for denitrifying activity. Autoclaved control bottles did not degrade BTEX compounds indicating that microorganisms are responsible for the observed degradation under hypersaline conditions. Studies also evaluated the ability of the enrichment to mineralize ^{14}C-benzene or ^{14}C-naphthalene to $^{14}CO_2$. Roughly 33% of the initially added radiolabeled benzene was converted to $^{14}CO_2$ in 4 weeks, while little (<1 % above control) of radiolabeled naphthalene was converted to $^{14}CO_2$ within the same time period (data not shown). Perhaps longer incubations were needed for greater mineralization of added benzene or naphthalene. The ability of the cultures to degrade benzene and toluene under hypersaline conditions is significant since these compounds are highly soluble and can easily find their way to the salt flats from non-point sources, human activities, and/or spills originating from oil production sites, which are widespread in Oklahoma.

Optimization Studies

Although a previously established halophilic enrichment culture from an oil brine soil showed increased benzene degradation in the presence of low concentrations of YE, vitamins, and trace elements (Figure 4 in Chapter 3), similar amendments to the Salt Plains enrichment culture neither helped nor hindered benzene degradation (Figure 8), Ventosa et al (1998) have proposed that complex media may stimulate growth of halophilic bacteria due to more demanding nutritional requirements at high salt concentrations. In our studies, we did not see such stimulation with the Salt Plains enrichment culture. The exact reason is not known.

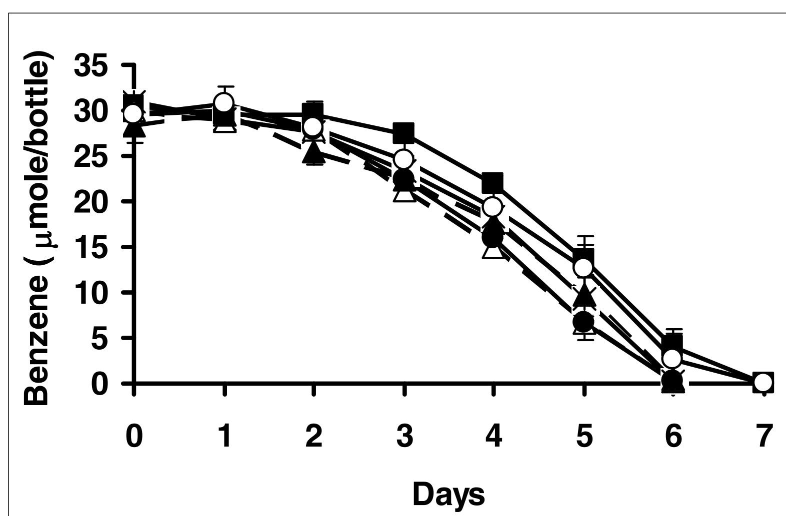

Figure 8. In an effort to stimulate benzene degradation by the Salt Plains enrichment culture, low amounts of peptone (■), casamino acids (-Δ-), YE (●), vitamins (-▲-) or trace elements (○) were added to microcosms containing MSM-NaCl. Control bottles with no addition (×) degraded benzene similarly.

Biodegradation of benzene was evaluated at various salt concentrations ranging from 0 to 4 M. As seen in Figure 9, benzene was rapidly degraded within 7 days in microcosms containing moderate levels of salt including 1, 2, and 2.5 M NaCl, while in microcosms containing 0, 0.5, and 3 M NaCl degradation occurred at reduced rates requiring up to 2 weeks for complete degradation. At the highest salt concentration tested (4 M NaCl), the growth rate decreased (Le Rudulier and Bernard 1986) and longer lag times were noted for degradation (Hinteregger and Streichsbier 1997). This could be due to several reasons including the lack of sufficient number of bacteria that can tolerate high salinity, and/or reduced availability of dissolved benzene due to high partitioning of benzene into the headspace. Tests have shown that more than 68 % of the added benzene was partitioned to the headspace at 4 M NaCl, compared to only 41 % at 1 M NaCl (data not shown). The ability of the enrichment to degrade benzene at higher salt concentrations (3 M and 4 M) is interesting because the enrichment was developed and maintained in MSM containing 2.5 M NaCl. Few studies have dealt with the effect of salinity on microbial degradation of hydrocarbons. These studies have found that, in general, biodegradation of hydrocarbons is negatively affected at elevated NaCl concentrations (Diaz *et al.* 2002, Betrand *et al.* 1990). In contrast, Fernandez-Linares *et a.l* (1996) showed no significant effect on eicosane degradation when the salinity was increased from 0.2 to 2.5 M NaCl.

Studies evaluated the degradation of benzene in the presence of different monovalent and divalent salts including NaCl, KCl, $MgCl_2$ or $CaCl_2$ each added at 2.5 M, as seen in Figure 10. Benzene was completely degraded within 10 days only in microcosms supplemented with NaCl and no degradation was seen in the presence of

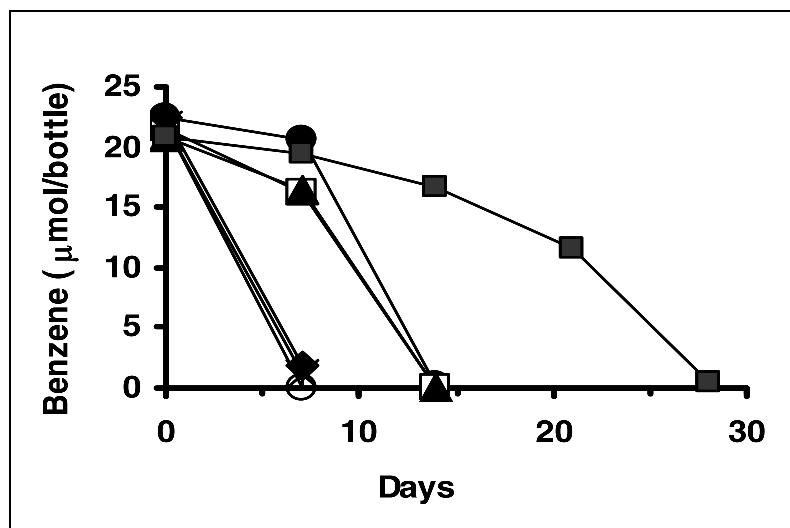

Figure 9. Biodegradation of benzene by the enrichment at different NaCl concentrations. Microcosms were amended with benzene (~22 µmole/bottle) and varied concentrations of NaCl: 0 M (●); 0.5 M (□); 1.0 M (◆); 2.0 M (○); 2.5 M (✳); 3.0 M (▲); 4.0 M NaCl (■). The Salt Plains enrichment could degrade benzene in MSM with 0 to 4 M NaCl added. The results are averages of triplicate active microcosms. Autoclaved controls established for each salt concentration tested showed no degradation of benzene (data not shown).

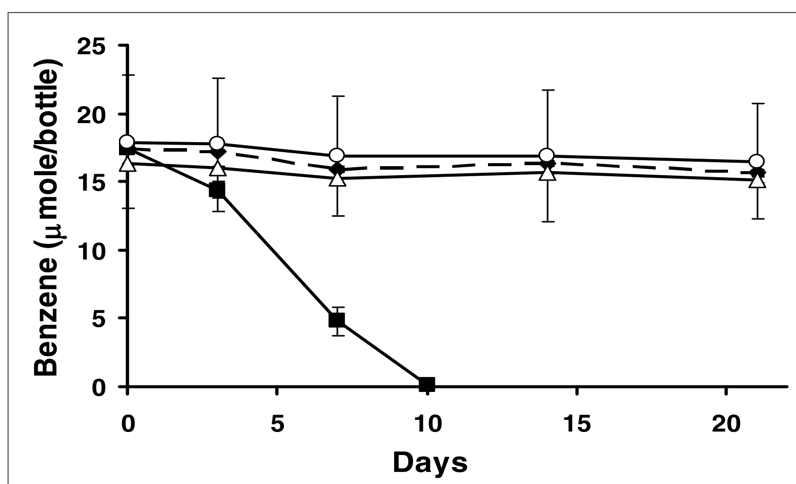

Figure 10. Benzene degradation by the Salt Plains enrichment culture in the presence of 2.5 M NaCl (■), KCl (-◆-), $MgCl_2$ (○), or $CaCl_2$ (Δ). Benzene degradation was only observed in the presence of 2.5 M NaCl. The results are averages of triplicate microcosms. Control bottles for each salt showed no degradation (data not shown).

KCl, $MgCl_2$ or $CaCl_2$ even after incubating for 4 weeks. These results are interesting considering the presence of other salts such as $CaSO_4$ available at the Salt Plains. This lack of activity is unlike that of organisms isolated from the Dead Sea which can tolerate relatively higher concentrations of Mg^{2+} and Ca^{2+}, perhaps due to their higher concentrations in the Dead Sea (Oren *et al.* 1984). Therefore, it is possible that the addition of 2.5 M KCl, $MgCl_2$, or $CaCl_2$ proved to be toxic to benzene-degrading populations. Also, since the culture was enriched in medium with only NaCl, organisms which could survive in the presence of other salts were not selected. For example, no degradation of benzene occurred in the presence of $CaCl_2$, despite the presence of high levels of $CaSO_4$ at the salt flats. Salt requirements and tolerance are highly variable among different species and may vary greatly according to the growth temperature and the nutrient availability (Kushner 1993).

Biodegradation of benzene was evaluated at different temperatures ranging from 5 °C to 60 °C. Although the enrichment was developed and maintained at 30 °C, results showed that benzene was degraded at maximum rates of 6.44 and 5.96 µmol/bottle/day at 37 °C to 45 °C, respectively (Figure 11). Also, slow degradation occurred at 50 °C (0.77 µmol/bottle/day). The capacity of the enrichment to degrade benzene at high temperatures could be attributed to the site conditions. In summer, surface temperatures at the salt flats often reach 45 °C to 50 °C with a daily diel temperature range of 30 ºC.

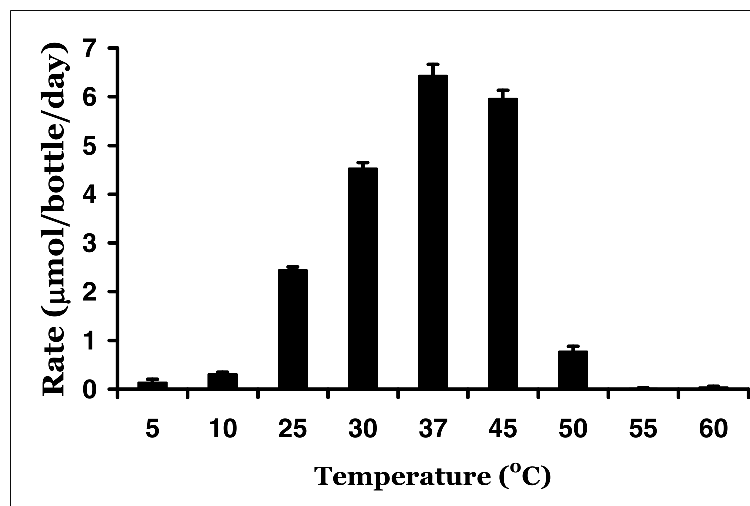

Figure 11. Effect of temperature on rate of benzene degradation by the Salt Plains enrichment culture. Although the enrichment culture was maintained at 30 °C, maximum degradation rates were seen at 37 °C and 45 °C. Slow degradation was seen at 50 °C. Bars are averages of triplicate bottles plus standard deviations.

Microbial Community Analysis

This study also evaluated the impact of salt on the bacterial diversity of the enrichment grown on benzene at various salt concentrations (0 to 4 M NaCl). DGGE analysis showed the presence of different phylotypes dominating at different NaCl concentrations (Figure12). In the absence of added NaCl, only one prominent band was seen whose sequence places it in the family Comamonadaceae, with a best match (Sequence Match S_ab score of 1.0) to a strain of *Acidovorax delafieldii* and *Pseudomonas sp.* This lack of diversity at 0 M NaCl correlates well with the relatively slow benzene degradation observed (Figure 9). Band B was dominant in the enrichment grown at 1.0 and 2.5 M NaCl. The sequence places it in the Bacillaceae family, with closest match to a strain of *Halobacillus salinus*, (Sequence Match S_abscore of 0.842). Band C was detected in the enrichment grown at broad salt concentrations ranging from 1 to 4 M NaCl thus suggesting that this organism can tolerate varying degrees of NaCl concentration. The band C sequence matched closely (Sequence Match S_ab score of 0.92) to the type strain of *Bacillus simplex*, which is a well-characterized metal-adsorbing bacterium. Band D was prominent only at higher salt concentrations including 3 and 4 M NaCl. The sequence for band D yielded no close matches to characterized bacteria (Sequence Match S_ab score of 0.529). Repeated DGGE analysis of the enrichment grown with 3 M NaCl yielded similar profiles and sequence matches, possibly indicating a novel organism. Band E was seen at the highest salt concentrations (2.5 to 4.0 M) tested. Sequence analysis again resulted in only a poor match (similarity score of 0.719) to Cytophagales from inland waters of remote Hawaiian islands, more specifically the

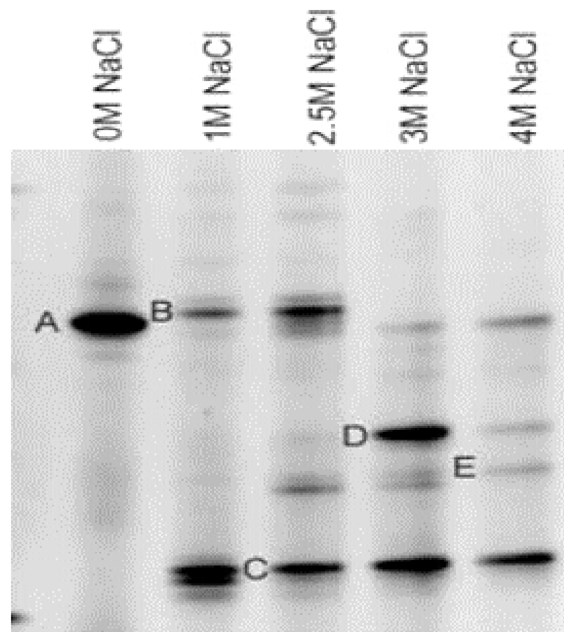

Figure 12. Comparison of DGGE profiles of PCR-amplified bacterial 16S rRNA gene fragments derived from the Salt Plains enrichment culture grown on benzene in the presence of different concentrations of NaCl. The figure shows the DGGE separation pattern of PCR fragments. The prominent bands were excised, reamplified and sequenced. The sequences were compared with those in the RDP database using the Sequence Match similarity function and the putatively identified organisms are indicated by the letters to the left of bands. The Genbank access number for the letters are: A, AF078767, AF015487; B, AB021195, AF500003; C, AJ439078; D, AF507866, AF235111 ; E, AF513957.

hypersaline Lake Laysan and a brackish water pond. These results clearly suggest that the bacteria yielding bands D and E needed higher salt concentrations to become dominant. The DGGE analysis provided a sensitive and efficient method for measuring diversity and changes in community structure due to changes in growth conditions, which will lead to a better understanding of microbial dynamics. However, it should be noted that these profiles obtained from the enriched culture do not necessarily represent the community structure at the salt flats. Further, these results only show bacterial and not archaeal responses to salt concentrations.

Conclusions

A highly enriched aerobic consortium was developed from a hypersaline soil with no known history or source of contamination. The enrichment rapidly degraded benzene and toluene, but not ethylbenzene and xylenes. The enrichment mineralized 33 % of the added ^{14}C-benzene to ^{14}C-CO_2 in 4 weeks. Despite the presence of nitrate at the salt flats, the enrichment did not degrade benzene or toluene under denitrifying or other alternative electron accepting conditions. Benzene degradation occurred over a wide rang of NaCl concentrations ranging from 0 to 4 M NaCl. These observations suggest that the enrichment is comprised of mainly halotolerant and halophilic microorganisms that can withstand varying level of salt. Such results can be attributed to the site conditions where microorganisms are exposed to rapid fluctuations in salinities both temporally and spatially. Analysis of microbial community structure of the enrichment exposed to varying concentrations of salt revealed that a great diversity of organisms was present in

the enrichment and different phylotypes dominated at different salt concentrations. Results also showed that degradation of benzene occurred at relatively higher temperatures such as 45 °C and 50 °C. This can be attributed the site conditions where the surface temperature often exceed 45 °C in the summer. Overall, these results show that extreme environments with no known history or source of contamination have the potential to rapidly degrade toxic pollutants. However, the exact mechanism, pathways, and organisms involved in the degradation are not known from this study. The ability of the enrichment to degrade benzene over a wide range of salt concentrations and the corresponding changes in the microbial community structure reflects natural attenuation potential of the culture and its ability to adapt to the fluctuating salt concentrations, which are often encountered in the field.

CHAPTER V

ISOLATION AND CHARACTERIZATION OF A HALOPHILIC BENZENE-DEGRADING BACTERIUM

Background

While there are many isolates of aerobic and anaerobic BTEX degrading organisms, few have been isolated from hypersaline environments. Halophiles with biodegradative potential can be used in the bioremediation of saline environments contaminated with organic pollutants (Margesin and Schinner 2001, Bastos *et al.* 2000). Isolation of halophilic and halotolerant organisms and characterization of their phylogenetic affiliation and metabolic capabilities are important for elucidating degradation pathways and developing bioremediation technologies (Garcia *et al.* 2004).

Two highly enriched microbial consortia, the Sem 2 and Salt Plains enrichments, were developed from an oil production facility and the Great Salt Plains, respectively (Chapters 2 and 3). These consortia were able to rapidly degrade BTEX compounds under aerobic conditions thus giving hope for the isolation of pure cultures that can degrade aromatic hydrocarbons under saline conditions. This chapter deals with the isolation and characterization of a novel halophilic bacterium isolated from the Sem 2 enrichment that assimilated benzene or toluene as the sole carbon and energy source. Here physiological, phylogenetic, and metabolic capability of the isolated microorganism is reported. Optimization studies on benzene degradation were carried out using the isolate. Such optimization studies are important for natural attenuation and *in-situ* bioremediation of hydrocarbon-impacted brine soil and produced water at E&P facilities. Attempts to isolate microorganisms from the Salt Plains failed.

Materials and Methods

Chemicals Used

VC, *cis*-DCE, and TCE were 98 % pure and obtained from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were described in Chapter 3.

Isolation Procedures

A benzene-degrading halophilic bacterium was isolated from the Sem 2 enrichment. The isolation was accomplished by performing a 10-fold serial dilution of the enrichment and streaking 0.1 ml of diluted aliquots onto agar plates prepared with MSM containing 1 M NaCl and 0.1x LB. The plates were incubated at 30 ˚C. Colonies first appeared after 10 days. After three weeks, colonies appeared smooth, round, beige and measured 1.5 - 2 mm in diameter. Single colonies were aseptically transferred to 120 ml serum bottles containing 50 ml of sterile MSM-NaCl, 0.01 % YE, and 25 μmoles of benzene. Headspace samples were withdrawn periodically and monitored for degradation using the GC. Please refer to Chapter 3 for microcosm set-up and analysis of hydrocarbons. The bottles that showed benzene degradation were further plated and single colonies were picked and monitored for benzene degradation as before. Purity of the culture was confirmed by routine microscopic observations and by culturing the isolated strain on LB plates for three consecutive times.

Phylogenetic Analysis

Phylogenetic analysis of the isolate was performed by comparative 16S rRNA gene sequence analysis. A 10 ml sample of the pure culture was centrifuged at 12,000 rpm for 15 minutes. The supernatant was decanted and the cells were washed twice with 5 ml of 10 mM potassium phosphate buffer (pH 7). The cells were resuspended in 0.5 ml MSM-NaCl. The cells were subjected to a freeze/thaw cycle (-20 °C overnight and 65 °C for 10 minutes) to aid in cell lysis. Genomic DNA was extracted from the pure culture using UltraClean™ Soil DNA Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Three primers were used in the amplification of 16S rDNA. These include: Bact 27f (5'-AGAGTTTGATC(A/C)TGGCTCAG-3'), Bact 1492r (5'-TACGG(C/T)TACCTTGTTACGACTT-3'), and Bact 1098r (5'-AAGGGTTGCGCTCGTTGCG-3') (Chang et al 2000). Theoretically, amplification with Bact 27f -1492r should yield 1505 bp and amplification with Bact 27f -1098r should yield 1108 bp from the 16S rDNA. Amplifications with these two primer sets were used to obtain the nearly full-length sequence (1453 bp) of the 16S rDNA of the isolate. For sequence, see Appendix.

PCR amplification was performed in a total volume of 50 µl in a Bio-Rad My Cycler (Hercules, CA). Each PCR mixture contained 25 ng of template DNA, 0.6 µM of each primer, 1.75 mM $MgCl_2$, 200 µM of dNTPs, 1.25 U of *Taq* polymerase in Buffer A (M1865, Promega Chemicals, Madison, WI). Amplification of 16S rDNA using both primer sets consisted of an initial denaturation of the genomic DNA at 94 ºC for 3 minutes, followed by 30 cycles of denaturation at 94 ºC for 1 minute, annealing at 53 ºC for 1 minute, and extension at for 2 minutes, and a final extension at 72 ºC for 8 minutes.

PCR products were checked for expected size on 1% agarose gels. The PCR product was purified with shrimp alkaline phosphatase and exonuclease I (DaSilva *et al.* 2003) and was directly sequenced using an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA).

The 16S rDNA sequence of the isolate was compared to sequences of other microorganisms obtained from GenBank (http://www.nbci.nlm.nih.gov). Sequences were aligned using the integrated Clustal-W (MEGA 3.0). The phylogenetic tree was constructed using neighbor-joining algorithm and *p*-distance estimation method implemented in MEGA, version 3.0 (Kumar *et al.* 2004). The confidence for individual branches of the resulting tree was estimated by performing 1000 bootstrap replicates. *Novosphingobium pentaromativorans* was used as the outgroup.

Experimental Setup for Batch Culture Studies

In order to provide a consistent source of inoculum for all experiments, a stock culture (500 ml) of the isolated microorganism was prepared in 1-L bottle. The bottle was amended with 250 μmoles of benzene twice as the sole carbon source and when all the added benzene was degraded, the culture was used as an inoculum. All studies involving the pure cultures were carried out in 120 ml capacity serum bottles as described before (Chapter 2).

The ability of the isolate to degrade BTEX compounds was assessed in bottles containing MSM-NaCl and inoculated with 5 ml of the stock inoculum and spiked with 2 μl (~25 μmole/bottle) of undiluted benzene, toluene, ethylbenzene, or xylenes as the sole carbon and energy source. Triplicate active and duplicate autoclaved control bottles were

prepared to study biodegradation. Bottles were incubated static at 30 °C in the dark. Headspace samples were withdrawn periodically and analyzed for the target compound as before (Chapter 2).

The isolate's ability to degrade benzene at different initial concentrations was studied in bottles containing MSM-NaCl and amended with 6, 12, 25, 35, or 50 µmole/bottle of benzene. The bottles were inoculated with 5 ml of the stock culture. Similarly, the isolate's ability to degrade benzene in the presence of different concentrations of NaCl ranging from 0 to 4 M was assessed. Bottles were also setup with MSM-NaCl and different concentrations of YE, including 0, 0.01, 0.02, 0.03, 0.04, or 0.05 % to determine the stimulatory effects of YE on benzene degradation.

Experiments with Chloroethenes

The ability of the isolate to degrade chlorinated ethenes such as VC, *cis*-DCE or TCE was assessed. Microcosms were prepared in bottles containing MSM-NaCl as described in Chapter 3. Microcosms were amended with VC, *cis* -DCE or TCE in the presence or absence of benzene. Biodegradation of the chlorinated ethenes and benzene were monitored with a GC.

Analytical Techniques

Biodegradation of BTEX compounds was monitored with a GC as described in Chapter 3. Production of $^{14}CO_2$ from ^{14}C-benzene was measured using a scintillation counter as described in Chapter 3. Analysis of VC, *cis*-DCE, and TCE was accomplished using a GC as described by Singh *et al.* (2004). Total protein was estimated by

withdrawing 0.5 ml of liquid culture (grown in the absence of YE) using a sterile 1 ml syringe. Cells were mixed with 0.5 ml of 2 N NaOH and heated at 90 °C for 10 min. The samples were analyzed for total protein as described by Lowry *et al.* (1951). The standards were prepared using bovine serum albumin treated with 1 N NaOH and heated at 90 °C for 10 min. The resulting plot was used to estimate the concentration of total cell protein.

Scanning Electron microscopy

Colonies grown on MSM-LB plates were scraped with a sterile spatula and cells were fixed with 2% cacodylate buffered glutaraldehyde solution at room temperature for 2 hours. Cells were placed onto a poly-L-lysine coated cover slip and 2 % cacodylate buffered glutaldehyde was added and allowed to stand for 15 minutes. The cover slip was washed with 0.1 M cacodylate buffer. The samples were dehydrated through a series of ethanol-water washes (50 %, 70 %, 90 %, 95 %, and three 100 % ethanol). The cells were dried using a critical point drying apparatus and the specimens were coated with gold/palladium (60-40) using a Balzer MED 010 sputter coater. Cells were observed in a JEOL JXM 6400 Scanning Electron microscope with an Evex Analytical Imaging Package.

Results and Discussion

An halophilic bacterium was isolated from the Sem 2 enrichment using benzene as the sole carbon and energy source. Phylogenetic analysis of the nearly full length 16S rDNA revealed that the isolate had > 95 % sequence similarity with *Arhodomonas aquaeolei*—a gram negative rod that was isolated from an oil brine in Payne County, OK

(Adkins *et al.* 1993). *A. aquaeolei* is a halophile that can utilize a number of different organics as a carbon source. The isolate is tentatively referred to as *Ahrodomonas* sp. strain Seminole. The isolate is a Gram negative, rod-shaped (0.5 x 2-3 µm in length), oxidase positive, and catalase negative bacterium (Figure 13).

Figure 14 is a phylogenetic tree showing the relationship of *Arhodomonas* sp. strain Seminole to other halotolerant/halophilic organisms. The strain Seminole closely clustered with *A. aquaeolei* and *Ralstonia pickettii*. Although, the closely related *A. aquaeolei* has been isolated from an oil-brine environment, its ability to degrade hydrocarbons is not known. On the other hand, *Ralstonia pickettii* is not a halophilic organism that degrades aromatic compounds (Kukor and Olsen 1996, Kukor and Olsen 1991). Phylogenetically *Marinobacter* spp. formed a separate cluster. Many members of the *Marinobacter* are shown to degrade aliphatics and PAHs, but no degradation of BTEX compounds have been reported. In addition, most members of *Marinobacter* have been isolated from marine environments. Phylogenetically, *Halomonas* spp. form a distinct group. *Halomonas* have been isolated from a variety of habitats with a wide range of salinities. Many *Halomonas* spp., including the recently isolated *Halomonas organivorans,* have been implicated in the degradation of aromatic compounds at high salt concentrations (Garcia *et al.* 2004). The moderately halophilic bacterium *Novosphingobium pentaromativorans* was isolated from a saline habitat, has been shown to degrade aromatic compounds (Sohn *et al.* 2004) and was used as an out group on the phylogenetic tree. Overall, this analysis indicates that biodegradation potential appears to be widely distributed among phylogenetically different halophiles and halotolerant microorganisms.

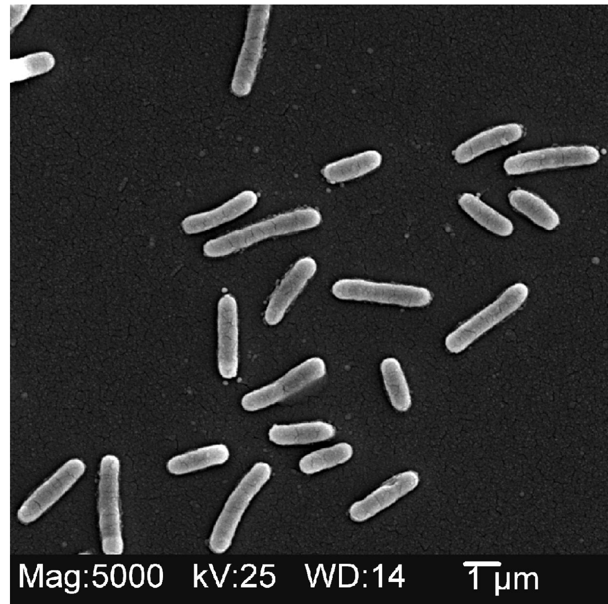

Figure 13. Scanning electron microscopy of the pure culture obtained from the Sem 2 enrichment. Cells were grown on agar plates prepared with MSM amended with 1 M NaCl and 0.1x LB.

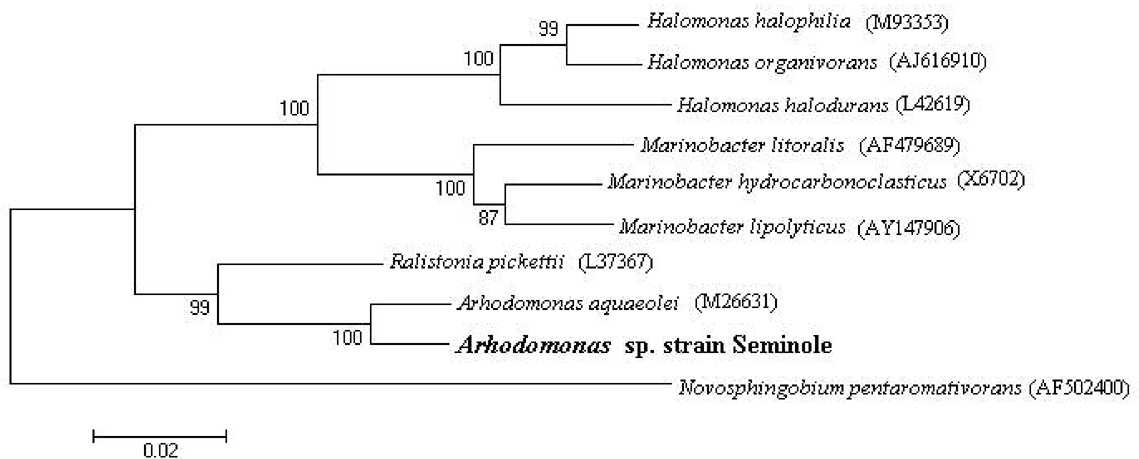

Figure 14. Phylogenetic tree based on 16S rDNA sequences showing the relationship of *Arhodomonas* sp. Stain Seminole to other halophilic/halotolerant organisms. Accession numbers of the sequences used in this study are shown in parentheses after the strain designation. Numbers at nodes are estimated confidence levels in percentages and were determined by bootstrap analysis with 1000 replicates. Bar denotes 2 % sequence divergence.

BTEX Degradation

Unlike the Sem 2 enrichment which was able to degrade each of the BTEX compounds (Chapter 3), *Arhodomonas* sp. strain Seminole could only degrade benzene and toluene. The isolate did not degrade ethylbenzene or xylenes (Table 6). Therefore, the degradation of each of these compounds in the original enrichment culture must have been due to other salt-tolerant organisms. Analysis with radiolabeled compounds showed that 40-60% of the added ^{14}C-benzene was converted to $^{14}CO_2$ in 3 weeks, suggesting complete mineralization potential of the isolate (data not shown). As shown in Figure 15, degradation of benzene by the isolate was associated with a corresponding increase in cell protein indicating that the isolate was able to use benzene as the sole source of carbon and energy. Biodegradation of benzene was also tested in the presence of nitrate as an alternative electron acceptor. No degradation of benzene occurred in the presence of nitrate thus suggesting that the isolate is a strict aerobe.

Results in Figure 16 show that the *Arhodomonas* sp. is capable of degrading benzene at different concentrations ranging from 6 to 50 µmole/bottle with an apparent lag of 3 days. Although the exact reason for the observed lag time is not known, it is reasonable to assume that 3 days may be required for the induction of degradation enzymes. Corseuil and Weber (1994) found that a sufficient number of cells must be present to immediately initiate biodegradation of added benzene by non-saline organisms. Consequently, a larger inoculum size may be necessary to decrease the lag time before degradation begins.

TABLE 6
AROMATIC COMPOUNDS DEGRADED BY
ARHODOMONAS SP. STRAIN SEMINOLE

Compound	Degradation*
Benzene	+
Toluene	+
Ethylbenzene	—
Xylenes	—

* "+" denotes degradation by *Arhodomonas* sp. strain Seminole, "—" denotes no degradation

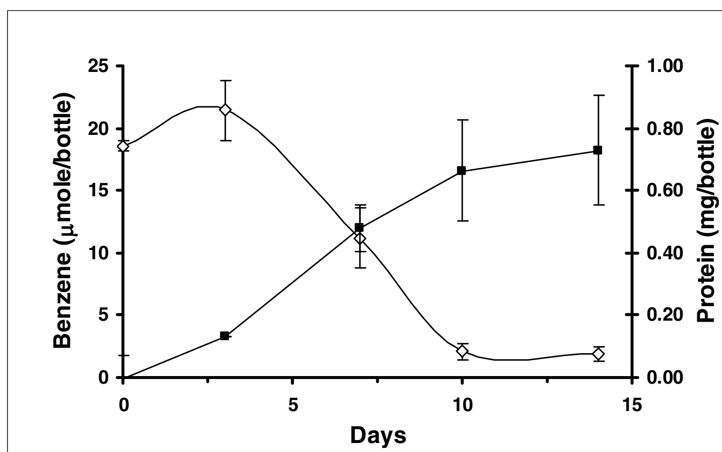

Figure 15. Biodegradation of benzene coupled to growth. As benzene (◇) is degraded, total protein (■) is accumulated.

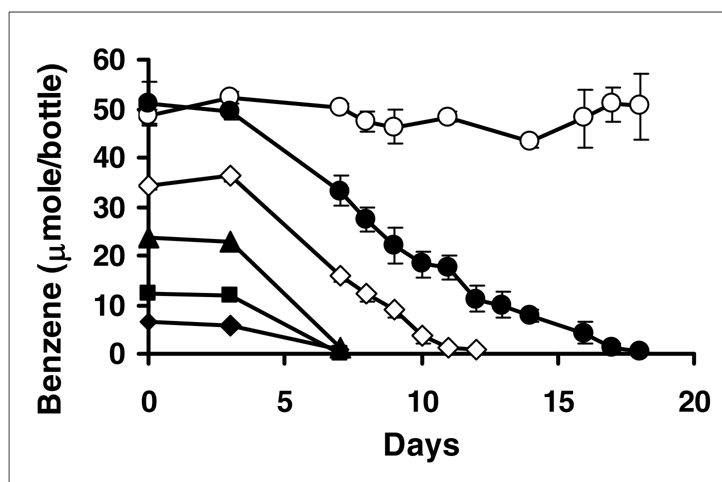

Figure 16. Biodegradation of various concentrations of benzene by *Arhodomonas* sp. strain Seminole. Symbols: 6 μmoles (◆); 12 μmoles (■); 25 μmoles (▲); 35 μmoles (◇); 50 μmoles (●). Autoclaved control bottles with 50 μmoles of benzene (○).

Optimization Studies

Several studies were performed with *Arhodomonas* sp. Seminole to evaluate benzene degradation under various growth conditions. Results showed that benzene was not degraded in MSM devoid of added NaCl suggesting that the isolate is a true halophile that requires salt for benzene degradation (Figure 17). This is not surprising since the *Arhodomonas* sp. was isolated from the Sem 2 enrichment. However, the isolate has a greater minimum requirement for salt than the enrichment since benzene was not degraded in the presence of 0.5 M NaCl (see Figure 5 in Chapter 3 for comparison). Maximum benzene degradation occurred at 2 M NaCl, with 1 M and 2.5 M behaving similarly. Degradation proceeded slowly at 3 M NaCl resulting in only 60 % degradation of the added benzene after 60 days. No degradation occurred at 4 M NaCl. A recently isolated hydrocarbon degrading *Halomonas organivorans* is shown to grow in salinities ranging from 0.25 to 5 M NaCl and also shows optimal growth around 2 M NaCl (Garcia *et al.* 2004).

Previous studies with the Sem 2 enrichment found that benzene degradation could be stimulated with the addition of low concentrations of YE (Figure 4 in Chapter 3). However, studies with the pure culture showed no such stimulation. Instead, degradation of benzene was inhibited in bottles containing >0.03 % YE (Figure 18). Estimation of cell protein in bottles amended with different concentrations of YE did not result in increased cell protein. This indicates that YE was not used as a source of carbon (data not shown).

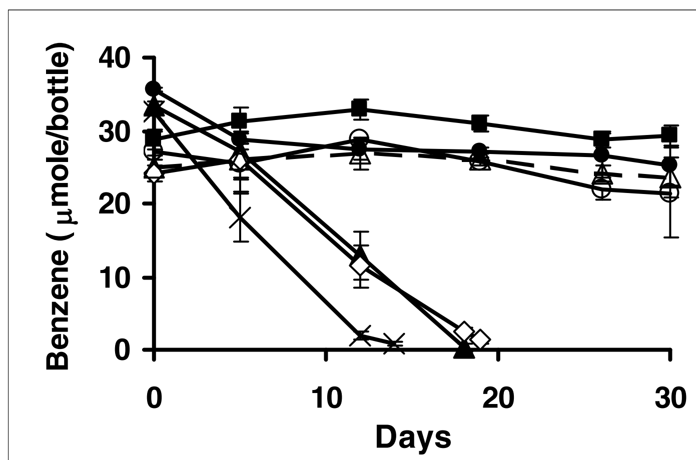

Figure 17. Effect of salt concentration on benzene biodegradation by *Arhodomonas* sp. strain Seminole. Symbols: 0 M (●); 0.5 M (■); 1 M (▲); 2 M (×); 2.5 M (◇); 3 M (○); 4 M (Δ) NaCl. No benzene degradation occurred at 0, 0.5, or 4 M NaCl. Maximum benzene degradation occurred in the presence of 2 M NaCl. Bottles amended with 3 M NaCl showed > 60% degradation of benzene after 60 days (data not shown).

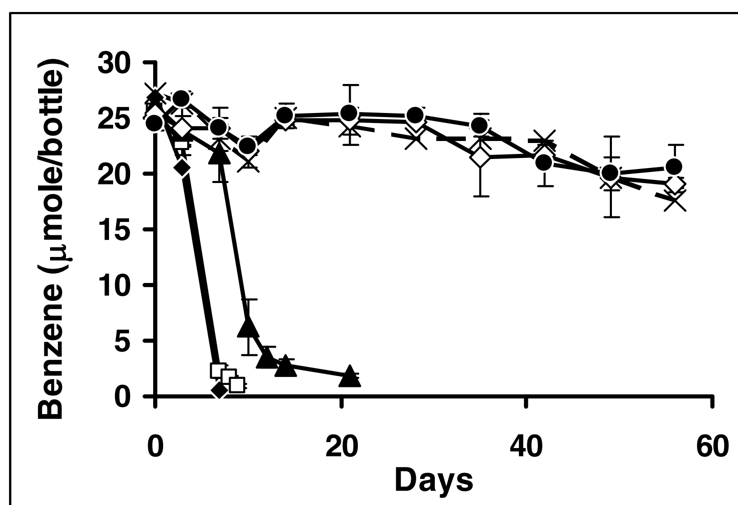

Figure 18. Benzene degradation in the presence of various concentrations of YE. Symbols: 0 % (♦); 0.01 % (□); 0.02 % (▲); 0.03 % (×); 0.04 % (◇); 0.05 % (●). There was no difference in benzene degradation in bottles with no YE and bottles containing 0.01% YE. Addition of 0.02% YE slowed benzene degradation and no degradation occurred in the presence of >0.03% YE.

Degradation of Chlorinated Compounds

Industrial sites are often contaminated with a mixture of compounds. It is not uncommon for chlorinated compounds and hydrocarbons to be co-contaminants. Just as aromatic hydrocarbons can use monooxygenases for degradation; these enzymes are also used in the degradation of chlorinated alkenes. Oxygenases have broad substrate specificity, oxidizing halogenated aliphatics, aromatics, and cyclic compounds. Bouwer and Zehnder (1993) reported that chlorinated solvents, such as TCE can be aerobically co-metabolized with oxygenases. Studies were carried out to evaluate the impact of chloroethenes such as VC, *cis*-DCE, or TCE on benzene degradation. In addition, the ability of *Arhodomonas* sp. strain Seminole to degrade the chloroethenes in the presence and absence of benzene was also evaluated. As shown in Figure 19, benzene degradation was not affected in the presence of 20-25 μmoles of VC. However, the addition of higher chlorinated ethenes such as *cis*-DCE or TCE resulted in a reduced rate of benzene degradation. Although the reason for this inhibition is not known, high concentrations of chloroethenes have been shown to be toxic to microorganisms (Yu and Semprini 2004). Results also showed that the *Arhodomonas* sp. was able to degrade *cis*-DCE in bottles amended with benzene (Figure 20). No *cis*-DCE was degraded in bottles devoid of added benzene (data not shown). These results suggest that the isolate is capable of degrading *cis*-DCE co-metabolically in the presence of benzene as the carbon source. However, VC and TCE were not degraded in the presence or in the absence of benzene. Such co-metabolic degradation of chloroethenes has been seen in many non-halophilic bacteria that degrade aromatic hydrocarbons (Vogel *et al.*1987, Bouwer and Zehnder 1993).

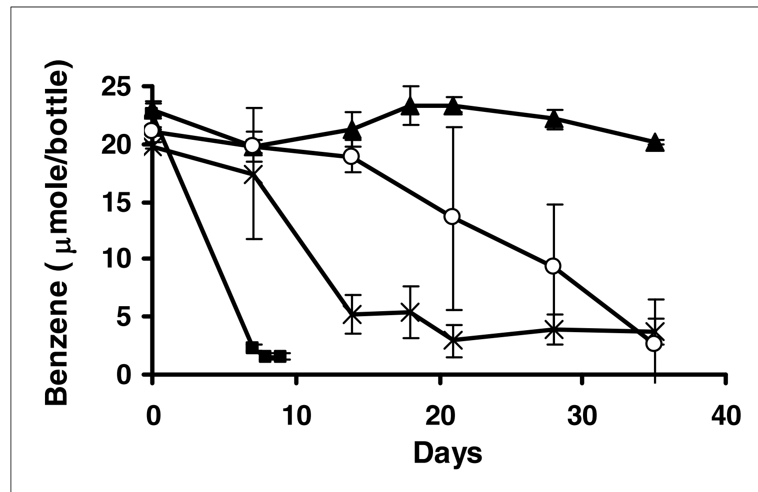

Figure 19. Degradation of benzene by the isolate in the presence of VC (■); *cis*-DCE (×); TCE (○). Degradation rate of benzene was unchanged in the presence of VC, while benzene was degraded at reduced rates in the presence of *cis*-DCE or TCE. Since all controls behaved similarly, only the control bottles with benzene and *cis*-DCE (▲) is shown.

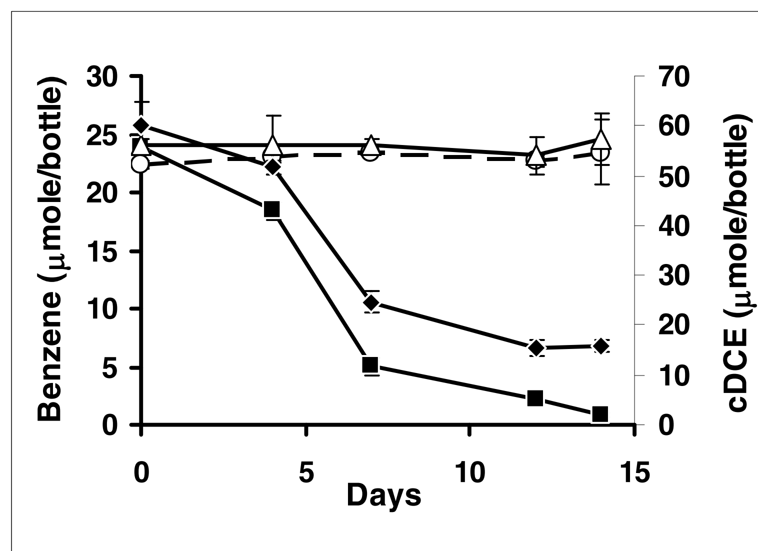

Figure 20. Biodegradation of *cis*-DCE in the presence of benzene (♦); biodegradation of benzene in the presence of *cis*-DCE (■). Autoclaved control showed no degradation of benzene (○) or *cis*-DCE (Δ).

Halophilic organisms have been shown to have the ability to degrade chlorinated compounds such as 2, 4-dichlorophenoxyacetic acid (2, 4-D) (Maltseva *et al.*1996).

Conclusions

Although limited BTEX degradation by cultures of *Pseudomonas* sp. isolated from anoxic brine from a marine environment has been reported (Brusa *et al.* 2001), this is the first report of a halophile isolated from an oil brine soil that is able to completely oxidize benzene to CO_2. Additionally, the isolate could degrade toluene but not ethylbenzene and xylenes. The isolate also degraded *cis*-DCE co-metabolically in the presence of benzene as the sole carbon source. Phylogenetic analysis showed >95 % sequence similarity with *Arhodomonas aquaeolei* which was isolated from an oil brine environment. The isolate degraded benzene optimally in the presence of 2 M NaCl, while no degradation occurred in the absence of added NaCl.

CHAPTER VI

OVERALL CONCLUSIONS

Hypersaline areas may be found across the earth. They may occur naturally or as a result of anthropogenic activities. Organisms that live in such areas are able to tolerate the harsh conditions found at these sites or may even require them for survival. Halotolerant and halophilic microorganisms have the potential to degrade aliphatic, aromatic, and chlorinated compounds and may therefore be useful in bioremediation technologies to clean up contaminated hypersaline areas. Little information exists on the biodegradation of BTEX compounds under these conditions. Hypersaline soils obtained from oil production facilities as well as from naturally occurring uncontaminated saline environments in Oklahoma were used to assess the ability of indigenous microorganisms to degrade BTEX compounds.

Oil brine contaminated soils from oil production sites were used to assess the ability of microorganisms to degrade BTEX compounds under hypersaline conditions. Aerobic and anaerobic soils showed the ability to completely mineralize ^{14}C-benzene to $^{14}CO_2$. An aerobic halophilic enrichment was developed that could degrade each of the BTEX compounds separately. The enrichment converted 46 % of added ^{14}C-benzene to $^{14}CO_2$ in 4 weeks. Benzene degradation was stimulated by the addition of low concentrations of YE, trace elements, and vitamins. Microbial community analysis using DGGE showed *Marinobacter* spp. as the dominate members of the enrichment.

Naturally hypersaline soils from the Salt Plains National Wildlife Refuge were used to assess the potential of organisms in a pristine environment to degrade BTEX. An

aerobic halotolerant enrichment was developed that could degrade benzene in up to 4 M NaCl. This enrichment could degrade benzene and toluene but not ethylbenzene and xylenes. The enrichment was able to completely mineralize 33 % of the added ^{14}C-benzene to $^{14}CO_2$ in 4 weeks. Efforts to stimulate benzene degradation with the addition of low concentrations of YE, trace elements, peptone, casamino acids, and vitamins proved unsuccessful. Although the enrichment was maintained at 30 °C, maximum rates of benzene degradation were observed at 37 and 45 °C. This can be attributed to high surface temperatures at the Salt Plains during the summer months. Microbial community analysis with DGGE showed the bacterial diversity was impacted when the enrichment was exposed to various salt concentrations.

An aerobic halophilic bacterium was isolated from the enrichment developed from oil brine contaminated soil. Phylogenetic analysis with the nearly full length sequence of 16S rDNA indicated the isolate contained > 95 % sequence similarity to *Arhodomonas aquaeolei*, an halophilic bacterium isolated from oil brine that has shown the ability to utilize a number of organics as a carbon source, although it is not known if it can degrade BTEX. The isolate is tentatively referred to as *Arhodomonas* sp. strain Seminole. The isolate is halophilic and could degrade benzene in the presence of 1 to 3 M NaCl. Radiolabled assays indicated 40 to 60 % of added ^{14}C-benzene to $^{14}CO_2$ in 3 weeks. This bacterium could also degrade toluene but not ethylbenzene or xylenes. Additionally, the isolate showed the ability to co-metabolically degrade *cis*-DCE in the presence of benzene.

More studies are needed to assess the ability of *Arhodomonas* sp. strain Seminole to degrade BTEX compounds under optimal conditions such as temperature, pH, and in

the presence of osmolytes. Using the isolated strain, the mechanisms and pathways for benzene biodegradation under saline conditions should be elucidated. Such studies are important for understanding if the genes and enzymes involved in degradation of aromatic compounds in halophilic bacteria are similar to those found in non-saline degraders. Also, using the isolated pure culture, phylogenetic probes can be developed to detect the presence and distribution of similar phylotypes in different hypersaline habitats (Crocetti *et al.* 2000). Furthermore, phylogenic probes can be used to monitor the persistence of this bacterium in the environment if it is found suitable for projects.

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APPENDIX A

SEQUENCE OF ISOLATE FROM SEM 2 ENRICHMENT

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   1 ACGCTGGCGG CATGCCTAAC ACATGCAAGT CGAGCGGCAG CAGCTCCTTC
0051 GGGAGGCTGG CGAGCGGCGG ACGGGTGAGT AACGCGTGGG AATCTGCCCT
0101 TCGGTGGGGG ATAGCCCGGG GAAACTCGGA TTAATACCGC ATACGCCCTG
0151 CGGGGCAAAG TGGCCCTCTG TTTCATGGTC ACGCCGAAGG ATGAGCTCGC
0201 AGTCCGATTA GCTAGTTGGT GAGGTAATGG CTCACCGAGG CGACGATCGG
0251 TAGCTGGTCT TAGCGGACGA TCAGCCACAC CGGGACTGAG ACACGGCCCG
0301 GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGGACAATG GGCGAAAGCC
0351 TGATCCAGCA ATGCCGCGTG GGTGAAGAAG GCTTGCGGGT TGTAAAGCCC
0401 TTTCAGCCGG GAGGAAAAGC GTTCGGTTAA TACCCGGACG TCTTGACGTT
0451 ACCGGCAGAA GAAGCACCGG CTAACTCCGT GCCAGCAGCC GCGGTAATAC
0501 GGAGGGTGCA AGCGTTAATC GGAATTACTG GGCGTAAAGC GCGCGTAGGC
0551 GGTCGGATAA GTCGGGTGTG AAAGCCCCGG GCTCAACCTG GGAACAGCAT
0601 TCGATACTGT TCGGCTAGAG TCTGGCAGAG GGAGGTGGAA TTTCCGGTGT
0651 AGCGGTGAAA TGCGTAGATA TCGGAAGGAA CACCAGTGGC GAAGGCGACC
0701 TCCTGGGCCA AGACTGACGC TGAGGTGCGA AAGCGTGGGG AGCAAACAGG
0751 ATTAGATACC CTGGTAGTCC ACGCCGTAAA CGATGAGAAC TAGCCGTTGG
0801 CCTCATTTAA GAGGTTCGTG GCGCAGCTAA CGCGATAAGT TCTCCGCCTG
0851 GGGAGTACGG CCGCAAGGTT AAAACTCAAA GGAATTGACG GGGGCCCGCA
0901 CAAGCGGTGG AGCATGTGGT TTAATTCGAT GCAACGCGAA GAACCTTACC
0951 TGCCCTTGAC ATCCTGGGAA CTTGGCAGAG ATGCCTTGGT GCCTTCGGGA
1001 GCCCAGTGAC AGGTGCTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT
1051 TGGGTTAAGT CCCGCAACGA GCGCAACCCT TGTCCCTGGT TGCCAGCGGT
1101 TCGGCCGGGA ACTCCAGGGA GACTGCCGGT GACAAACCGG AGGAAGGTGG
1151 GGATGACGTC AAGTCATCAT GGCCCTCATG GGCAGGGCTA CACACGTGCT
1201 ACAATGGCTG GTACAACCGG TTGCCAACCC GCGAGGGGGC GCTAATCCGA
1251 TAAAGCCAGT CCCAGTCCGG ATTGGAGTCT GCAACTCGAC TCCATGAAGT
1301 CGGAATCGCT AGTAATCGCG GATCAGCATT GCCGCGGTGA ATACGTTCCC
1351 GGGCCTTGTA CACACCGCCC GTCACACCAT GGGAGTCGGC TGCACCAGAA
1401 GTCGGTAGTC TAACTTCGGG AGGACGCCGC CCACGGTGTG GTCGAGACNG
1451 GGG
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VITA

Carla Annette Nicholson

Candidate for the Degree of

Master of Science

Thesis: BIODEGRADATION OF PETROLEUM HYDROCARBONS BY HALOPHILIC AND HALOTOLERANT MICROORGANISMS

Major Field: Microbiology and Molecular Genetics

Biographical:

Personal Data: Born on September 28, 1978 in Wynne, Arkansas to Bill and Charlene Nicholson.

Education: Graduated from Cross County High School in Cherry Valley, Arkansas 1997; Received Bachelors of Science in Biology from Southern Arkansas University in Magnolia, Arkansas May 2001, completed the requirements for Masters of Science Degree in Microbiology, Cell and Molecular Biology at Oklahoma State University in Stillwater, Oklahoma in December 2005.

Experience: Teaching Assistant, School of Arts and Sciences, Oklahoma State University, August 2001 through May 2002 and August 2004 through May 2005; Graduate Research Assistant, Department of Microbiology and Molecular Genetics, July 2002 through June 2004.

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Name: Carla Annette Nicholson Date of Degree: December, 2005

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: BIODEGRADATION OF PETROLEUM HYDROCARBONS BY HALOPHILIC AND HALOTOLERANT MICROORGANISMS

Pages in Study: 92 Candidate for the Degree of Master of Science

Major Field: Microbiology and Molecular Genetics

Scope and Method of Study: Hypersaline environments have demonstrated a diversity of life. While halophilic and halotolerant organisms have shown the ability to degrade contaminants such as pesticides and aliphatic and aromatic hydrocarbons, little is known of the capacity of salt-tolerant organisms to degrade benzene, toluene, ethylbenzene, and xylenes (BTEX). These compounds are of concern because they are stable and highly water soluble. Benzene is a United States Environmental Protection Agency priority pollutant because it is a known carcinogen. Hypersaline soil samples were obtained from oil production sites in Seminole County, OK and the naturally occurring hypersaline soils of the Salt Plains National Wildlife Refuge in Oklahoma. These samples were used to assess the ability of native organisms to degrade BTEX.

Findings and Conclusions: Evidence of BTEX degrading organisms was found in the hypersaline soils tested. Highly enriched cultures were developed which consistently degraded added benzene as the sole carbon and energy source. The aerobic enrichment from an oil production site showed the ability to degrade each of the added BTEX compounds separately. This halophilic culture also showed the ability to mineralize 46 % of the added ^{14}C-benzene to $^{14}CO_2$ in 4 weeks. A halotolerant aerobic enrichment was developed with pristine soil from salt flats in the Salt Plains National Wildlife Refuge. This enrichment degraded benzene and toluene but not ethylbenzene and xylenes. This halotolerant enrichment could degrade benzene in up to 4 M NaCl. In the presence of 2.5 M NaCl, 33 % of added ^{14}C-benzene was mineralized to $^{14}CO_2$ in 4 weeks. An aerobic halophilic bacterium was isolated from the Sem 2 enrichment developed from an oil brine soil obtained from Seminole County that could degrade benzene and toluene but not ethylbenzene or xylenes. The isolate degraded benzene in the presence of 1 to 3 M NaCl with maximum degradation at 2 M NaCl. Studies using radiolabeled benzene showed that the isolate could mineralize 40 to 60 % of added ^{14}C-benzene to $^{14}CO_2$ in 3 weeks. The 16S rDNA of the isolate showed > 95 % sequence similarity to *Arhodomonas aquaeolei* and is therefore tentatively referred to as *Arhodomonas* sp. strain Seminole.

ADVISER'S APPROVAL Babu Z. Fathepure