RICE UNIVERSITY

Bench-Scale Studies of Natural Attenuation, Biostimulation, and Bioaugmentation for Remediation of Groundwater Contaminated with Benzene and Toluene in the Piceance Basin, CO

by

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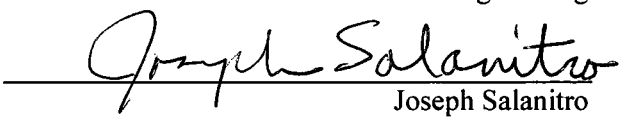

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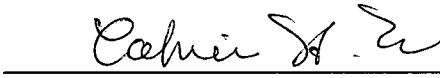

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ABSTRACT

The US EPA requires direct evidence of contaminant removal before bioremediation or natural attenuation can be used for site remediation. Microcosm studies are commonly used to provide this line of evidence; however, molecular biology tools may provide a better approach for biological forensic analysis of contaminated sites. DNA biomarker technology and laboratory scale microcosms were used to assess the feasibility of natural attenuation, biostimulation, and bioaugmentation for the remediation of a benzene and toluene (B/T) contaminated aquifer. Detection and subsequent increase of target aerobic catabolic and phylogenetic gene biomarkers corroborated aerobic B/T degradation observed in laboratory scale microcosms. Anaerobic biomarker and microcosm studies failed to produce evidence of anaerobic B/T biodegradation potential. Biostimulation (nitrate and sulfate addition) and bioaugmentation with a known anaerobic benzene degrading culture both failed to stimulate B/T removal. However, the addition of benzoate slightly stimulated the removal of benzene under anaerobic conditions. Collectively these results suggest this petroleum hydrocarbon aquifer is not strictly anaerobic and has the potential for natural attenuation

processes under aerobic conditions. This research demonstrated the value of using DNA biomarkers as a tool for biological and abiotic forensic site investigations.

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1: INTRODUCTION

Shell Exploration and Production Company implemented an in-situ conversion process (ICP) during 1998-2005 in an attempt to commercialize oil shale in the Piceance Basin located in the upper northwest portion of Colorado. ICP used subsurface heaters to pyrolyze kerogen into oil, gas, and water; however, water quality constituents of concern (COC) were also released during this process. Upon termination of ICP, inorganics (arsenic, boron, chloride, fluoride, iron, manganese, sulfate and sulfide) and organics (benzene and toluene) were above background concentrations and remain above regulatory levels in the upper and lower water bearing intervals.

Benzene and toluene are relatively soluble petroleum hydrocarbons and they have the greatest potential to migrate from the experimental site and impact environmental receptors. Benzene, in particular, is a cancer-causing agent and has the lowest MCL (0.5μg/L), therefore it is used as the proxy to assess reclamation efficacy.

The provisional subsurface reclamation plan by Shell Frontier Oil & Gas, Inc. targets monitored natural attenuation (MNA) as a possible remedial approach for benzene and toluene removal in combination with pump and treat. MNA has been approved by the Colorado Division of Reclamation, Mining and Safety (DRMS) for remediation and risk management of an adjacent Shell-leased site approximately two miles away from the MDP site. However, environmental, hydrological and microbial variation between sites is highly probable and further "proof of concept" will be required to gain DRMS approval of MNA for the MDP site. Currently the US EPA requires three lines of evidence for use of MNA including, 1) documented mass loss of contaminant, 2) geochemical evidence of

contaminant transformation and detoxification, and 3) direct evidence of biodegradation (28).

The objective of this study was to evaluate the potential for benzene and toluene (B/T) biodegradation and monitored natural attenuation as a remedial strategy for the MDP site. Laboratory microcosms and DNA-based biomarker technology were used to 1) assess anaerobic and aerobic B/T biodegradation rates, 2) quantify the presence of selected microorganisms capable of degrading B/T, 3) evaluate the effects of background total organic carbon on the biodegradation process, and 4) assess the potential to stimulate B/T biodegradation by adding terminal electron acceptors, auxiliary substrates, and through bioaugmentation.

2: LITERATURE REVIEW

2.1 Natural Attenuation and Bioremediation

Monitored natural attenuation (MNA) and enhanced natural attenuation (ENA) are widely used remediation technologies that take advantage of microorganisms to transform contaminants of concern to less hazardous components. The MNA or ENA microorganisms use a variety of enzymes, electron acceptors, and metabolic pathways to degrade COCs. MNA is the preferred remediation option since it is cost-effective, allows for treatment to occur *in situ* and minimizes the legal responsibility and dangers of alternatively transporting hazardous materials (38). ENA is similar to MNA but involves site manipulation in order to stimulate bioremediation through the addition of electron acceptors or donors, nutrients, or through the addition of adapted microorganisms (bioaugmentation). The major limitation of MNA is that it is not universally applicable. Factors that vary from site to site (Table 1) and may hinder the use of natural attenuation include, 1) types of microorganisms present, 2) their catabolic capabilities to degrade target pollutants and 3) environment abiotic factors such as pH, temperature, redox potential and available nutrients (61). Knowledge of these factors is necessary for each site to select and design a proper remedial approach that will lead to site closure (49).

Table 1: Summary of requirements for biodegradation to occur at a site (2)

1. Existence of organisms with degradation potential
2. Presence of specific degraders in contaminant zone
3. Accessibility of target pollutants to the microorganisms
4. Availability of necessary electron acceptors/donors
5. Availability of nutrients

6. Adequate pH and buffering capacity
7. Adequate temperature
8. Absence of toxic or inhibitory substances
9. Induction of degradative enzymes

2.2 Benzene and toluene biodegradation with various electron acceptors

MNA has been widely implemented in subsurface aquifer environments to remediate monoaromatic petroleum hydrocarbons such as, benzene, toluene, ethylbenzene, and xylenes (BTEX). BTEX components are highly soluble, hazardous components of gasoline and are mainly introduced into the environment through leaking underground storage tanks and petroleum industry processes.

To assess the potential efficacy of benzene and toluene ENA/MNA, critical site-specific parameters need to be understood. Electron acceptor occurrence within a hydrocarbon plume and surrounding groundwater is a crucial parameter for biodegradation processes. Under aerobic conditions, benzene and toluene are readily degraded using oxygen as the terminal electron acceptor for microbial respiration. Aerobic degradation pathways for aromatic hydrocarbons have been studied extensively (1, 33). In general, the aromatic compound goes through the convergent pathway to an intermediate metabolite and onto a central metabolite where it enters the divergent pathway(42). The central metabolite is transformed through either the *ortho*- (65) or *meta*-cleavage (22) pathways and then enters into the TCA cycle. Multiple pathways for aerobic toluene degradation have been elucidated from various microorganisms; many are of the *Pseudomonas* genus (Figure A1, Appendix A).

The initial ring attack on toluene is mediated by dioxygenase, monooxygenase, or methylhydroxylase enzymes. Dioxygenases, with an NADH cofactor, mediate the substitution of ring hydroxyls with two oxygen molecules, creating a *cis*-dihydrodiol. Alternatively, monooxygenases use two NADHs to incorporate one oxygen molecule into the ring, creating a cresol. A third option is mediated by a methylhydroxylase, which oxidizes the alkyl ring components to create a benzyl alcohol. The dihydrodiol, cresol, and benzyl alcohol are then transformed to a catechol, protocatechuate, or gentisic acid. These metabolites continue into the *ortho-* or *meta-* fission pathways (5). The ortho pathway involves enzymes that cleave the bond between the hydroxylated carbons, whereas the *meta* pathway cleaves adjacent to the two catechol hydroxyls. Benzene also undergoes oxidation to a catechol via benzene 1,2 -dioxygenase and then proceeds into the *meta-*cleavage pathway of catechol degradation (32)(Figure A2, Appendix A).

Oxygen, however, quickly becomes a limiting factor during aerobic B/T degradation in subsurface aquifers because little oxygen is initially present, is readily consumed during contaminant biodegradation, and the natural processes of restoring oxygen concentrations in groundwater are slow (1). Most subsurface environments become and remain anoxic after contamination (49). Under anaerobic conditions electron acceptors (EA) , alternative to oxygen, are used to oxidize hydrocarbons. Microorganisms will preferentially use the available EA from which they will gain the most energy. The availability of the electron acceptors tends to follow a zonation process within a stabilized BTEX plume. Oxygen tends to be available towards the fringe of the plume, followed by nitrate, iron (III), sulfate, and methanogenesis within the region of highest B/T concentration (Figure 1).

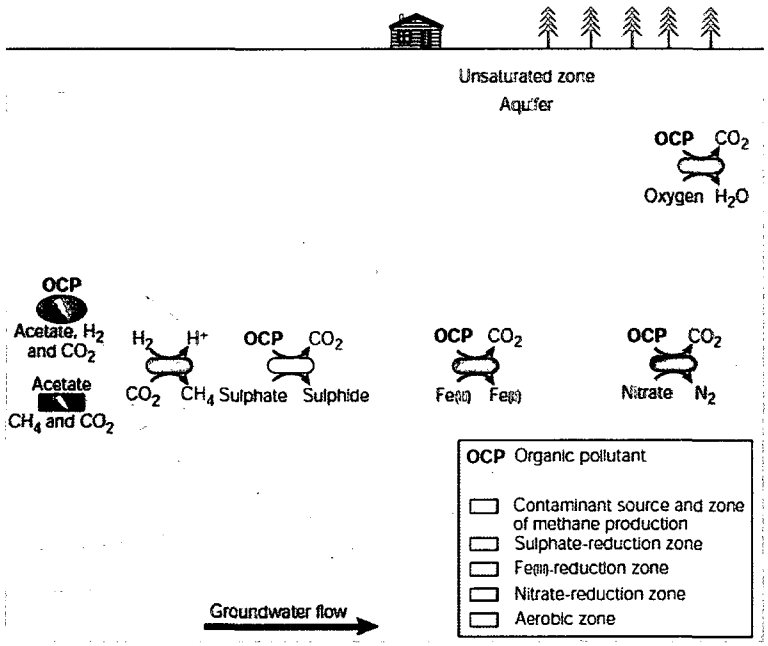

Figure 1. Zonation of electron acceptor availability adapted from (49)

Benzene was previously thought to be recalcitrant under anaerobic conditions because of environmental persistence and the high activation energy required for the initial enzymatic ring attack (45). However, laboratory and field scale studies have shown anaerobic benzene degradation linked to nitrate reduction (11, 16, 67), sulfate reduction (4, 15, 20, 21, 25, 41, 50, 60), iron (III) reduction (12, 39, 43, 51) and methanogenesis (14, 41). Benzene is the most recalcitrant of the BTEX constituents and the anaerobic pathway of degradation is essentially still unknown. Five potential pathways for the initial step in benzene biodegradation are proposed but two remain questionable. The initial step, via ring saturation, lacks the necessary literature support and fumarate addition may not be plausible because of the high activation energy required to remove hydrogen from the benzene ring (30). The three most feasible pathways include: hydroxylation followed by either carboxylation to an intermediate para-hydroxybenzoate or by ring reduction yielding

cyclohexanone; methylation of benzene to toluene followed by fumarate addition resulting in benzylsuccinate; and carboxylation resulting in benzoate (30). Intermediates produced from the three pathways converge on benzyl-CoA, which is a central intermediate for all BTEX components undergoing anaerobic degradation (13, 29, 30).

Of all the BTEX components, toluene is preferentially biodegraded under anoxic conditions by a variety of terminal electron acceptors, including nitrate (52), sulfate (9, 27, 56), ferric iron (39, 51), and methanogenesis (6, 26). The generally conserved pathway starts with a fumarate addition to the methyl group of toluene which is mediated by the enzyme benzylsuccinate synthase , resulting in benzylsuccinate formation (13)(Figure A3, Appendix A). Benzylsuccinate is then oxidized to E-phenylitaconate and eventually to benzyl-CoA which then undergoes ring reduction, cleavage, and oxidation to carbon dioxide (10, 30, 36).

2.3 Biodegradation by environmental and biological site enhancement

ENA involves manipulations of the environmental conditions (physical, chemical, and biological) to develop biodegradative capabilities. Amendments with terminal electron acceptors and/or donors, nutrients, and bioaugmentation with commercially available cultures have been successfully implemented in laboratory and field scale remediation efforts. Laboratory and field studies have shown biodegradation of B/T via biostimulation by the addition of terminal electron acceptors such as oxygen (34, 44), nitrate (18, 63), and sulfate (4, 40), resulting in the degradation of benzene and toluene to regulatory levels in aquifers that were previously not suitable for natural attenuation. Oxygen or air are commonly added through air sparging or the addition of oxygen release compounds or

peroxide. The addition of nitrate can serve as a key nutrient for enzyme or amino acid synthesis or as an electron acceptor for the degradation process. The optimal ratio of carbon: nitrogen: phosphorous (C:N:P) is approximately 30:5:1 for unrestricted microbial growth in aquifers (59).

In some cases, required nutrients for microbial growth and metabolism are minimal or not at all present. The addition of nutrients such as acetate and benzoate can stimulate growth by acting as a carbon source and result in higher densities of microorganisms, including specific degraders. However, if the added nutrients are central metabolites, this can inhibit further degradation of benzene and toluene by metabolic flux dilution, competitive inhibition, or catabolite repression (48).

Bioaugmentation can serve to remediate a site that is lacking the appropriate microorganisms needed to degrade petroleum hydrocarbons. While bioaugmentation of chlorinated solvents with commercially available cultures has been highly successful *in-situ*, there is little literature on field studies using cultures for anaerobic benzene and toluene degradation. Anaerobic benzene degradation has only recently been acknowledged and reported difficulties in culturing and isolating the responsible microorganisms may contribute to the lag in BTEX degrading culture commercialization. Laboratory and pilot scale studies have provided significant evidence in favor of bioaugmentation for BTEX degradation (20, 24, 31). Mixed cultures responsible for anaerobic BTEX degradation are currently being studied in an attempt to isolate the specific organisms and pathways responsible for anaerobic benzene degradation. A mixed culture of gasoline-contaminated subsurface sediment from Seal Beach, California was reported in 1992 as degrading benzene completely under strict anaerobic (methanogenic) conditions (27) and four

cultures were isolated from various petroleum hydrocarbon impacted sites and successfully enriched for benzene degradation under iron (III), nitrate, and sulfate-reducing conditions (53).

2.4 Bench scale studies to provide evidence of biodegradation potential

Feasibility studies are commonly used to determine bioremediation potential for a contaminated site. These studies typically involve the use of *in situ* microcosms and/or samples taken from the actual site to measure biological removal of the contaminant of concern. Biodegradation activity and rates of degradation under different environmental conditions can be acquired from laboratory microcosms (38, 49). However, it is difficult to reproduce the exact site conditions as they are heterogeneous and complex (62). While the degradation rates obtained in the laboratory can be used as input for models, they do not necessarily correlate directly to the degradation rates that will occur in the field. Moreover, feasibility studies cannot discern which microorganisms are present or the actual mechanisms of biodegradation. Additionally it may take months to years in order for the contaminant of concern to be degraded in microcosms. Alvarez et al 2006 reported an acclimation time of greater than two years for anaerobic benzene biodegradation in soil columns. New technologies are being developed based on nucleic acids to provide a more detailed assessment of the 'players' and activity within a complex microbial community involved in the degradation of contaminants (70).

2.5 Molecular Biotools

Over the last fifty years, the field of microbial molecular genetics has expanded tremendously and new technologies have been developed to investigate, characterize, and identify microbial communities and their catabolic processes. Revolutionary nucleic acid based technologies have provided the field of bioremediation culture-independent quantifiable approaches to better understand the diversity and dynamics of 'players' involved in contaminant degradation, their catabolic pathways responsible for biodegradation, and which enzymes (encoded for by genes) are implicated in contaminant transformation and how these genes are regulated. Table 2 summarizes the information that can be gained by targeting various genetic components.

Table 2 . Targets for molecular tools and the information can be acquired (adapted from (2))

Target	Information Gained	Question Answered
Ribosomal RNA (rRNA)	Taxonomic	What organisms are present
Genes for rRNA (DNA)	Taxonomic	What organisms are present
Catabolic genes (DNA)	Catabolic potential	What can be degraded
Messenger RNA (mRNA)	Gene activity	What genes are being expressed
Protein/other products	Enzymatic activity or phylogenetic identity	Who is active and what are they doing?

All living organisms possess DNA (usually chromosomal or independent plasmids) that contains information for vital processes, proteins, RNA, and has structural functions for genetic regulation. DNA is transcribed, via polymerases, into complimentary mRNA strands and ribosomes (active RNA/protein based machinery) then translate the mRNA into functional proteins/enzymes that can act in degradation processes. Ribosomes have a unique property in that their sequences are highly conserved yet contain small species-specific hypervariable regions. The hypervariable region in the small ribosomal subunit

(16S) can serve as a phylogenetic fingerprint, allowing for identification and quantification of specific microorganisms (57). Specific genera of microorganisms are linked to degradation of contaminants. *Dehalococcoides* spp. are known degraders of chlorinated solvents and are typically present at sites where natural attenuation of these solvents occur (35, 37). *Pseudomonas* spp. are linked to aerobic BTEX degradation. Recently, enrichment for anaerobic benzene degradation within a mixed culture provided a strong link between *Desulfobacterium* spp. and anaerobic benzene removal (19). The *Desulfobacterium* spp. open reading frame biomarker (*ORM2*), designed from an enrichment experiment, has not produced any false positives. This suggests anaerobic benzene degradation will likely occur at sites where this biomarker is detected.

Detection and quantification of catabolic genes (DNA) that encode for enzymes involved in degradation pathways can provide information on what contaminants can be degraded. Benzylsuccinate synthase (*bssA*) is an enzyme involved in the initial attack on toluene under anaerobic conditions (8, 47, 64). *bssA* was identified from the denitrifying bacterium *Thauera aromatica* and was subsequently sequenced for molecular targeting. This gene is commonly targeted to determine MNA potential for anaerobic environments as it is the only known biomarker for anaerobic toluene biodegradation. todC1 is a gene encoding for toluene dioxygenase which initiated toluene and xylenes degradation under aerobic conditions and is an indicator of aerobic BTEX degradation potential (46, 66, 68, 71). Typically there is a positive correlation between specific catabolic gene abundance and contaminant biodegradation potential (49). Therefore, detection and monitoring of catabolic genes can provide essential information on a site's bioremediation potential.

Real-time quantitative polymerase chain reaction (qPCR) uses probe/primers sets, designed from known gene sequences, to quantify/detect target genes that have been extracted from environmental samples. The target is amplified and the fluorescent signal is measured within the exponential phase which can then be used to back-calculate the initial quantity of target gene in the sample. This method is a culture free approach for assessing phenotypic potential, exploring microbial communities, and monitoring changes in catabolic and phylogenetic biomarkers. DNA biomarkers have been gaining popularity, as sequences of microorganisms become available and new catabolic or phylogenetic gene targets are correlated to degradation potential.

3: SITE CHARACTERIZATION

The Piceance Basin in Colorado is the largest single oil shale deposit of the Green River formations that span Wyoming, Colorado and Utah (Figure B1, Appendix B). Shell Oil Company experimented with an *in situ* heating conversion process (ICP) to enhance oil shale recovery. This process used in-ground thermal heaters to convert kerogen to recoverable products.

3.1 Hydrogeology

Water bearing intervals of this site are grouped into upper and lower aquifers separated by the R7 zone (Mahogany), a regional aquitard (Figure B4, Appendix B). The upper aquifer consists of a semi-confined/perched to dry A-groove (L7) while the lower consists of a seasonally unconfined B-groove (L6) and four fractured horizons (R6 and R5/L5 zones).The total water bearing interval thickness is 140 ft.

Table 3. Hydrostratigraphic Intervals at MDP

Hydrostratigraphic interval	**Depth (ft)**	**Thickness (ft)**	**Description**
L7 (A-groove)	86	15	Only seasonally saturated
L6 (B-groove)	201	20	Semi-confined, only seasonally saturated
R6 fracture zone (1)	310	10	Jointed and vuggy with partings
R6 fracture zone (2)	352	10	Jointed and vuggy with partings
L5 fracture zone	372	13	Jointed and vuggy with partings
L5/R5 fracture zone	410	40	Jointed and vuggy with partings

L3 and L4 are tight with negligible natural flushing rates. Packer permeability, pumping tests, and very long recovery times in the L3 zone confirm that there is no water bearing interval below the R5 zone. Chemistry and hydraulics of the L3/4 wells do not support

these zones for water use and were not monitored past the initial characterization. Groundwater flow for the test area is east-southeast with a short distance turning east-northeast towards the basin center and Piceance Creek. Pumping tests determined the fracture zones to be anisotropic and maximum transmissivity with an east-west major axis and rates from 4.9 to 10.6 ft^2/day (Figure B3, Appendix B).

Table 4. Summary of 1998 and 2000 Pumping Tests

Stratigraphic Interval tested	Transmissivity (ft^2/day)
L5 through L7	10.6
L6	5.6
Mean L5-R5	4.9

Recharge rates, based on hydraulic modeling and water balance, are thought to be between one-half to one inch per year. No immediate environmental receptors have been identified and MODFLOW projections predict a plume migration of a few hundred feet in 30 years (Figure B5, Appendix B).

3.2 Groundwater Chemistry

Groundwater in the fracture zones R5 through R6 is a sodium bicarbonate type with total dissolved solids (TDS) typically less than 1,300 mg/L. Lower intervals have elevated TDS levels at approximately 17,000-24,500 mg/L (85 percentile to maximum). Pyrolysis of oil shale in the MDP/MTE area generated hydrocarbons and/or released inorganic constituents into the ground water at concentrations higher than background levels. Production and perimeter producer wells removed the bulk of contaminated groundwater from the pyrolyzed area. Periodic measurements of M and PM wells (located

up and down gradient of the MDP/MTE research area) have shown inorganic and organic components that surpass regional water quality criteria. Six regulatory constituents (arsenic, fluoride, iron, manganese, sulfate, and sulfide) in L5 and above and two additional constituents (boron and chloride) in zones below L5 exceeded regulatory limits prior to pyrolysis.

Effectively, zones L3, R4, L4, and lower R5 were deemed irrelevant for monitoring progress, as these zones do not transmit adequate water to wells to be a flow pathway of concern. Produced groundwater from the MDP area exceeds quality criteria for 2,4 Dimethylphenol, benzene, toluene, boron, fluoride, manganese, and sulfide. The proposed reclamation plan focuses on aquifer L5 as it has the highest transmissivity however, L3, categorized as an aquiclude, was highlighted as harboring the highest concentrations of regulated analytes. Briefly, maximum concentrations of benzene for L5 and L3 measured at 21.5 μg/L and 984 μg/L, respectively, while toluene was present in concentrations of 1660 μg/L for L5 and 4980 μg/L for L3.

Table 5. Water sample well locations (Figure B2, Appendix B)

MDP M8L3
MDP M13L3
MDP M6L5
MDP M4B
MDP M10B
MDP M11B
MDP PM3
MDP M12R6
MDP M13L5
MDP PP08
MDP PP12

MIT M7L5
MIT M15L5
MIT M14B
MIT M13A
MIT M27B

4: MATERIALS AND METHODS

4.1 Microcosm construction and analysis

Water and shale samples were collected in five-gallon amber jugs and shipped on ice to Rice University. Upon arrival, the jugs were stored at 4°C until analysis could be completed. Baseline analysis prior to experimentation included total non-purgeable organic carbon and molecular analysis.

Aerobic microcosm construction consisted of 100 mL of site groundwater added to 240 mL glass amber bottles (Supelco), purged with air for one minute and sealed with a mininert cap (Supelco).

Anaerobic microcosms were constructed with 70 mL of site groundwater added to 125 mL serum bottles (Supelco). Ditholthrietol (0.0001% ; Sigma Aldrich) was added to enhance the reducing environment of the groundwater and Resazurin (0.001% ; Sigma Aldrich) was added as a colorimetric detector for the presence of oxygen. The samples were then purged with nitrogen until they became colorless and were capped with mininert valves (Supelco) and incubated in an anaerobic chamber (Coy) with a gaseous environment of 5% hydrogen, 5% carbon dioxide, and 90% nitrogen. Biostimulation experiments

utilized anaerobic microcosms constructed as in the previous description with the addition of sulfate or nitrate (100 mg; Fisher Scientific). Shale microcosms were constructed in 240 mL amber bottles using 20 g of ground shale (MDP CH6-44-432.1-432.4-F) and 80 g of shale groundwater (EC 401).

Abiotic controls were constructed using sterile water with trisodium phosphate (Fisher Scientific) to bring the pH up to 13 and were autoclaved. All microcosms were constructed in triplicate and spiked with toluene and benzene to a final concentration of 1 mg/L in the microcosm headspace. All microcosms were incubated in the dark at room temperature throughout the experiment.

Gas chromatography was used to monitor the concentration of toluene and benzene in the headspace of the microcosms during experimentation. 100 μL of headspace was taken up in a gas tight syringe and injected into an HP 5890 GC equipped with a packed column (6 ft x 1/8 in. OD) containing 60/80 Carbopack B/1% SP-1000 (Supelco) and a flame ionization detector (FID). The temperature program was 250°C for the inlet and detector and the oven at 200°C for 7 minutes.

Non-purgeable total organic carbon (TOC) in water samples was measured by a Shimadzu VCSH Total Organic Carbon Analyzer. Nitrate, sulfate, and acetate samples were prepared by filtering 3 mL through a 0.22 μM syringe filter into a 2.0 mL crimp top glass vial (Agilent Technologies). The samples were analyzed with a Dionex AS50 ion chromatograph equipped with an Ion-Pac ASII-HC 4x 250 mm column.

4.2 Molecular methods for determining biomarker concentrations

DNA was extracted from the provided groundwater samples using the MoBio™ UltraPure DNA water extraction kit (Carlsbad, CA, USA) according to the manufacturer's protocol with the exception of a bead beating device (Mini Beadbeater-8, Biospec) used for cell lysis. The extracted DNA was stored at -20°C prior to molecular analysis.

SYBR green and Taqman dye chemistries were used with corresponding primer or primer/probe sets (Table A1, Appendix A) respectively to determine the gene copy number of specific targets. Taqman PCR reactions contained 1x Taqman PCR Master Mix® (Applied Biosystems, Foster City, CA USA); 0.5 μM forward and reverse primers, 2.5 μM probe, 4 μL of extracted DNA and sterile water to make up a final reaction volume of 20 μL. For SYBR Green PCR reactions, the mixture consisted of 1x SYBR Green® (Applied Biosystems), 0.5 μM of forward and reverse primers, 4 μL of extracted DNA and sterile water to bring the reaction volume to 20 μL. qPCR reactions were performed using an ABI 7500 Sequence Detector (Applied Biosystems) with the following temperature program: 50°C for two min., 95°C for 10 min., and 40 cycles of 95°C for 15s and 60°C for 1 min. qPCR efficiency was determined by calculating the slope of the standard curve that was run during every reaction. A slope between -3.6 and -3.1 correlates to 90%-100% efficiency of the amplification reaction (www.appliedbiosystems.com). The equation used to calculate efficiency was:

$$\text{Efficiency} = -1+10^{(-1/\text{slope})}$$

Standard calibration curves were constructed for each biomarker target using a known quantity of extracted DNA (concentrations from 10^1 to 10^8 gene copies/μL) from

cultures of specific microorganisms known to harbor the targeted genes. Biomarker concentrations were estimated using the following equation:

Gene copy number/μL= (μg DNA μL^{-1}/bp genome) x ($9.1257x10^{14}$ bp μg DNA^{-1} x genes $genome^{-1}$)

Assumptions made for this equation include: the size of the microorganism's genome used for the standard curves is approximately $9.1257x10^{14}$ bp μg^{-1} of DNA (from http://www.genomesonline.org) and X gene copies per genome (http://rrndb.cme.msu.edu). While qPCR based assays provided targeted gene copy numbers, a one (gene copy) to one (bacterial cell) ratio cannot be assumed as microorganisms can harbor more than one gene copy per cell. Gene copy numbers were estimated using standard species harboring known quantities of target genes (above equation). However, the primer/probes target the gene of interest, which is present in variable quantities throughout a broad range of microorganisms.

Biodegradation of B/T under natural, biostimulated, and bioaugmented conditions as well as biomarker data was analyzed using Excel's Analysis of variance (ANOVA) two-factor and the alpha parameter was set to 0.05.

5: RESULTS AND DISCUSSION

5.1 Background total organic carbon and molecular analysis

Total organic carbon (TOC) and biomarker concentrations were measured in groundwater samples to serve as an initial baseline. The targeted biomarkers included *Total Bacteria* (16S rDNA), *Archaea* (typically methanogens), *Pseudomonas* spp., *todC1* (toluene dioxygenase), *bssA* (benzylsuccinate synthase), *ORM2* (phylogenetic target of anaerobic benzene degraders). The non-purgeable total organic carbon ranged from 21.35 – 30.77 mg/L; however, MDP M13L3 and MDP M8L3 measured 275.30 and 571.70 mg/L, respectively (Figure 2; Table C1, Appendix C). The elevated levels of TOC for the L3 zone are likely to exist as these samples are from a non-water baring heat-induced fracture zone.

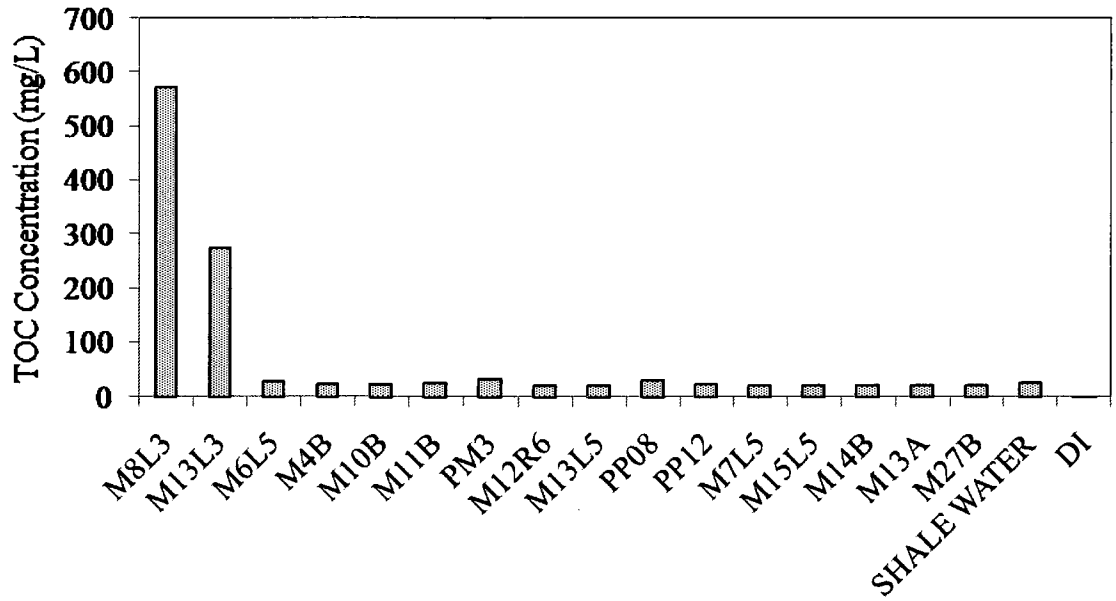

Figure 2. Total organic carbon measured in water samples from the MDP site

Elevated levels of TOC provide an environment of high nutrients that microorganisms can assimilate for growth, resulting in a greater concentration of microbes per volume of water. Baseline qPCR *Total Bacteria* gene concentrations measured $2.58 \pm 0.04 \times 10^8$ and $2.34 \pm 0.07 \times 10^7$ gene copies/mL for M13L3 and M8L3 and a range of 10^4 - 10^7 copies/mL for the additional samples (Figure 3). *todC1* (gene for the enzyme involved in the initial aerobic attack on toluene) was detected in all samples up to 7.87×10^4 copies/mL, suggesting that this site is capable of supporting aerobic microorganisms although it was believed to be anaerobic. Anaerobic toluene and benzene degradation biomarkers *bssA* and *ORM2* were not detected in water or shale samples (detection limit, 10 genes/mL) although Archaea were detected in half of the samples up to $3.93 \pm 1.3 \times 10^3$ copies/mL. The lack of anaerobic biomarkers, specifically linked to B/T degradation, indicate that anaerobic biodegradation of benzene and toluene at the MDP site is unlikely.

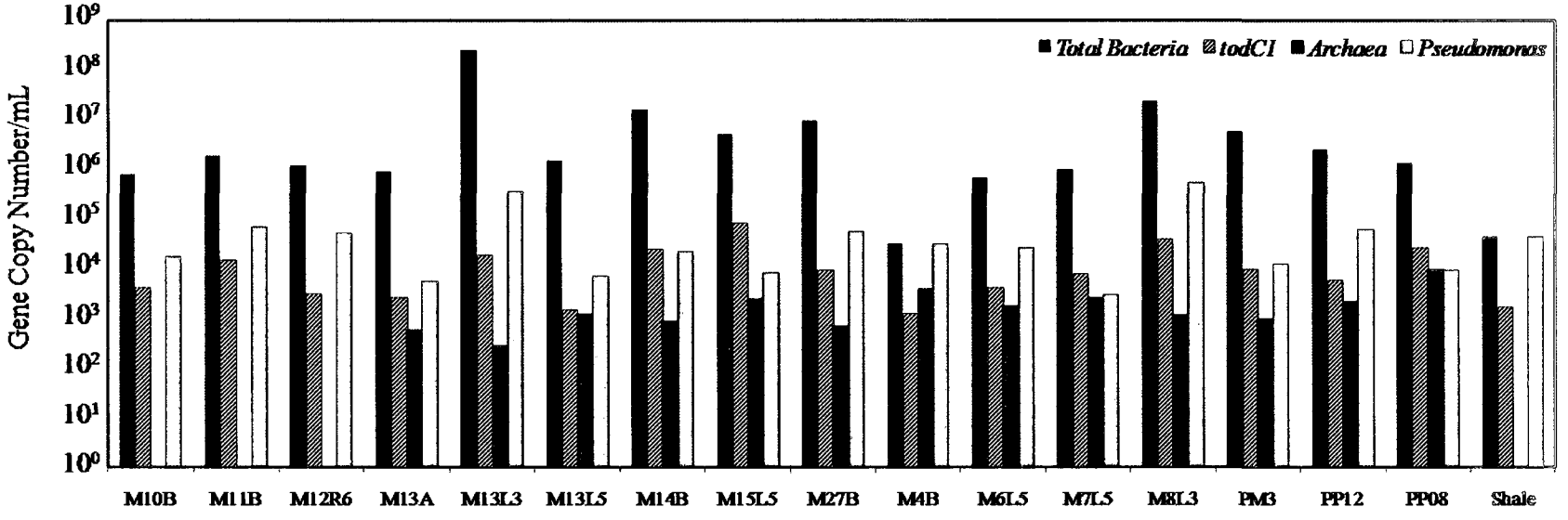

Figure 3. Baseline biomarker profile for MDP groundwater samples. Error bars represent ± 1 standard deviation from the mean of triplicate DNA extractions.

5.2 B/T degradation under anaerobic conditions

Unamended anaerobic microcosms did not demonstrate toluene or benzene biodegradation over a period of 420 days (Figure 4) and the biomarkers, *ORM2* and *bssA*, were not previously detected during baseline analysis. Loss of B/T was observed and attributed to volatilization; abiotic control microcosms demonstrated similar decreases in B/T. These findings do not indicate, unequivocally, that benzene and toluene will not be degraded anaerobically at the Mahogany site but clearly suggests that anaerobic degradation, if any will be severely retarded.

However, acclimation periods may extend past 2 years before anaerobic B/T biodegradation occurs (20) and *ORM2* and *bssA* biomarkers are not inclusive to all possible anaerobic B/T degraders (19).

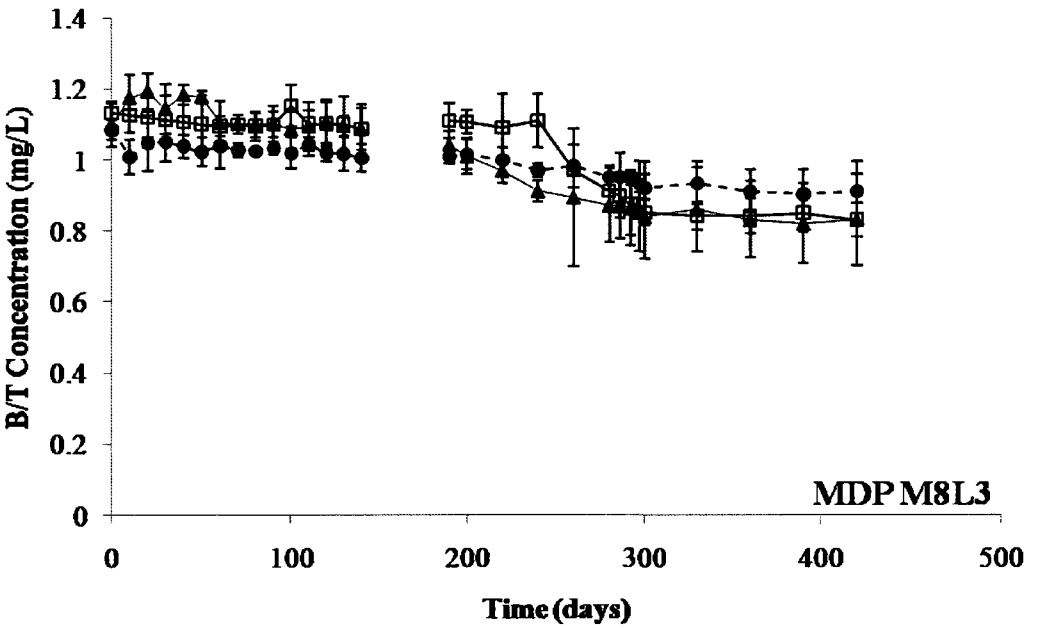

Figure 4. Symbols: ▲, Abiotic Control ; □, Toluene; •, Benzene. MDP M8L3 anaerobic microcosms demonstrate a lack of benzene and toluene biodegradation. The control samples provides evidence that the decrease in concentration is due to volatilization and not biological activity. (Break in data is due to a period of down equipment). All anaerobic microcosms retained B/T or had loss due to only volatilization. Error bars represent ± 1 standard deviation from the mean of triplicate microcosms.

Total Bacteria response to acetate (a known biostimulant) amendment, TOC reduction, and methane generation were used as parameters to determine if the lack of biodegradation activity was a result of a non-viable culture. At day 210, *Total Bacteria* was quantified in sample MDP M8L3 followed by biostimulation with acetate (100 mg/L). After a four day incubation period, *Total Bacteria* concentrations increased by one order of magnitude ($7.08 \pm 0.01 \times 10^5$ to $5.25 \pm 0.03 \times 10^6$ copies/mL), indicating the culture was biologically viable (Figure 5). Additionally, the measured increase in concentration is in agreement with the theoretical cell yield from the anaerobic oxidation of acetate. Approximately 0.06 mg of cells are synthesized from 100 mg acetate and one *E. coli* cell weighs approximately 0.95 pg (54); therefore, an increase of 6.3×10^8 cells/microcosm (70mL) should be observed.

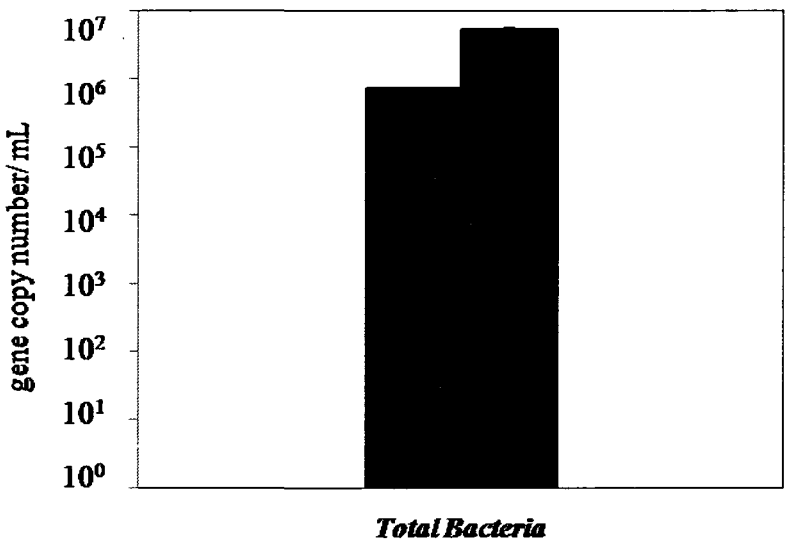

Figure 5. Acetate biostimulation of MDP M8L3 resulted in a one order of magnitude *Total Bacteria* increase. Error bars represent ± 1 standard deviation from the mean of triplicate DNA extractions.

TOC levels of MDP M13L3 and MDP M8L3 were significantly reduced over 14 months, 235.73 mg/L to 72.155 mg/L and 571.7 mg/L to 151.12 mg/L, respectively (Figure 8). MDP M6L5 (low initial TOC) demonstrated a reduction from 28.3 mg/L to 3.79 mg/L. Microbial activity could not account for the level of TOC removal however, precipitation in the microcosms could have contributed to TOC loss. Methane production and accumulation was monitored in microcosms M13A and M27B, providing further support for biological activity (Figure 11).

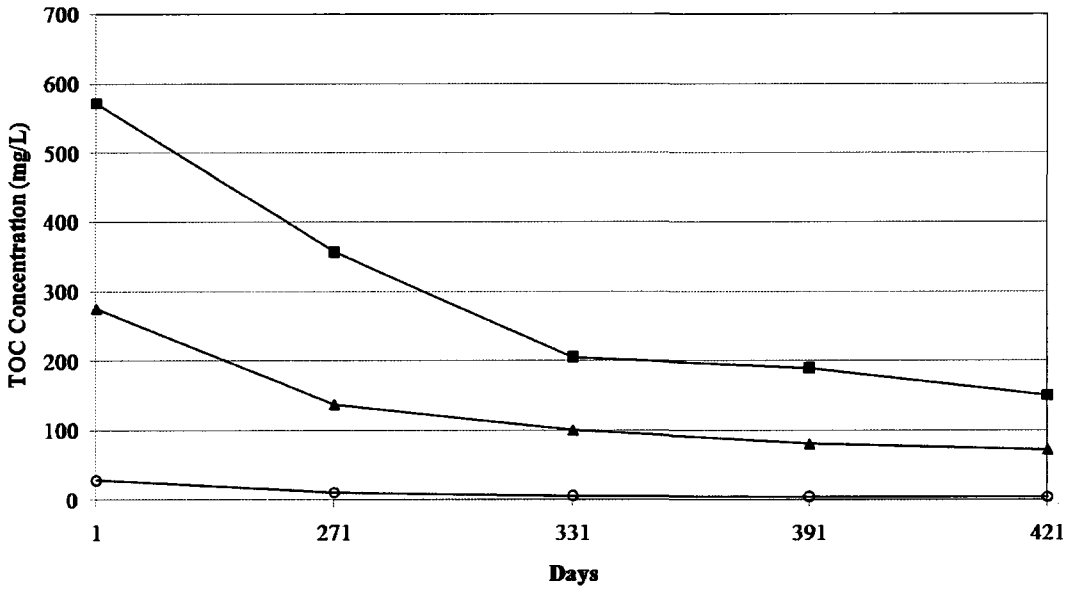

Figure 6. Symbols: ■, M13L3 ; ▲, M8L3; ○, M6L5. Reduction of TOC in anaerobic microcosms over 421 days.

5.3 Effects of biostimulation on B/T degradation

Air Treatment

Laboratory scale microcosms purged with air demonstrated complete ($p < 0.05$) aerobic biodegradation of benzene and toluene in all site samples (Figure 7). Biodegradation rates (k) for each sample were determined using first-order kinetics

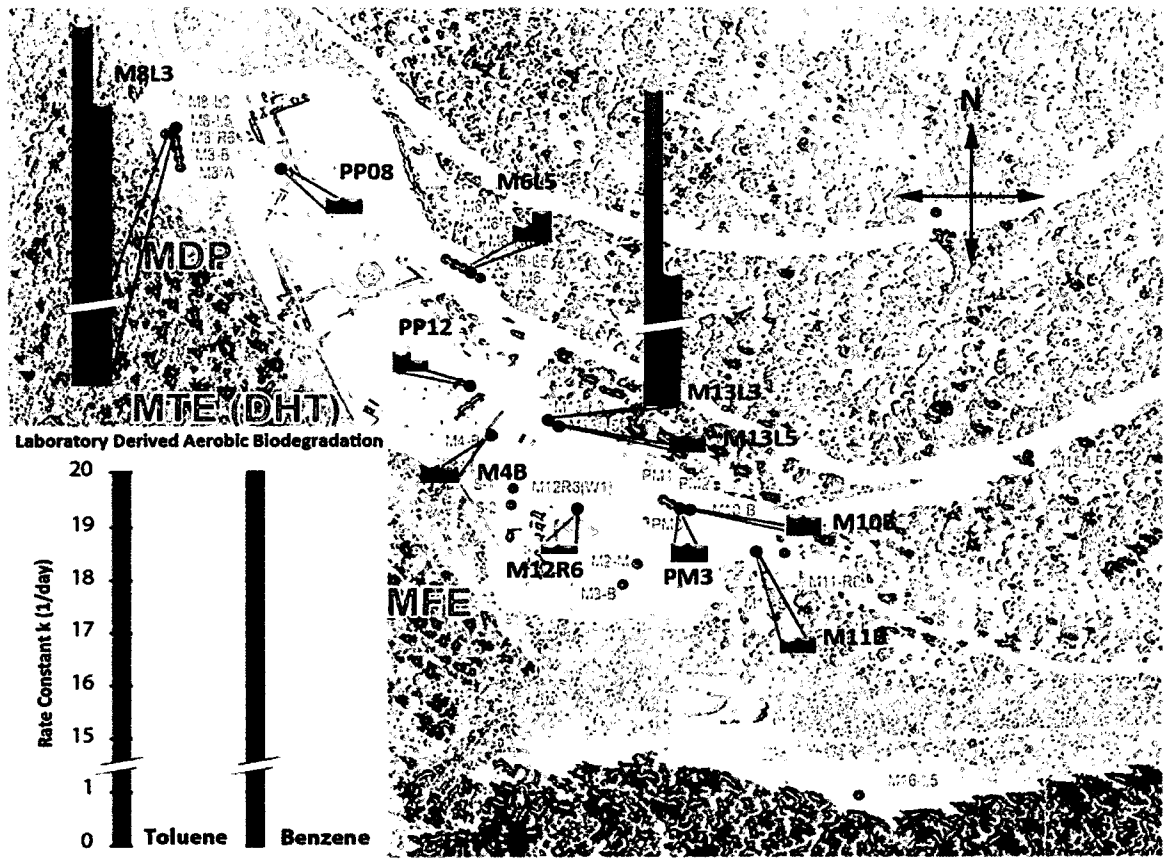

Figure 7. First order degradation rates determined by aerobic laboratory microcosms.

commonly used to describe degradation in aquifers (2)(Figure 8, Table C2, Appendix C). Microcosms MDP M8L3 and MDP M13L3 had B/T degradation rates of 18.33, 19.85 and 15.50, 18.98 (day^{-1}) respectively, a two order of magnitude greater *k* value in comparison to the other sites. The high *k* value for the L3 zone was corroborated by the presence of an abundant microbial population including bacteria that can degrade B/T (*Pseudomonas* spp. and *todC1*) and high organic carbon levels that support microbial growth. The aerobic biodegradation of benzene and toluene in the remaining groundwater samples was also substantiated by an increase in molecular biomarkers specific to genes involved in aromatic hydrocarbon degradation (Figure 9).

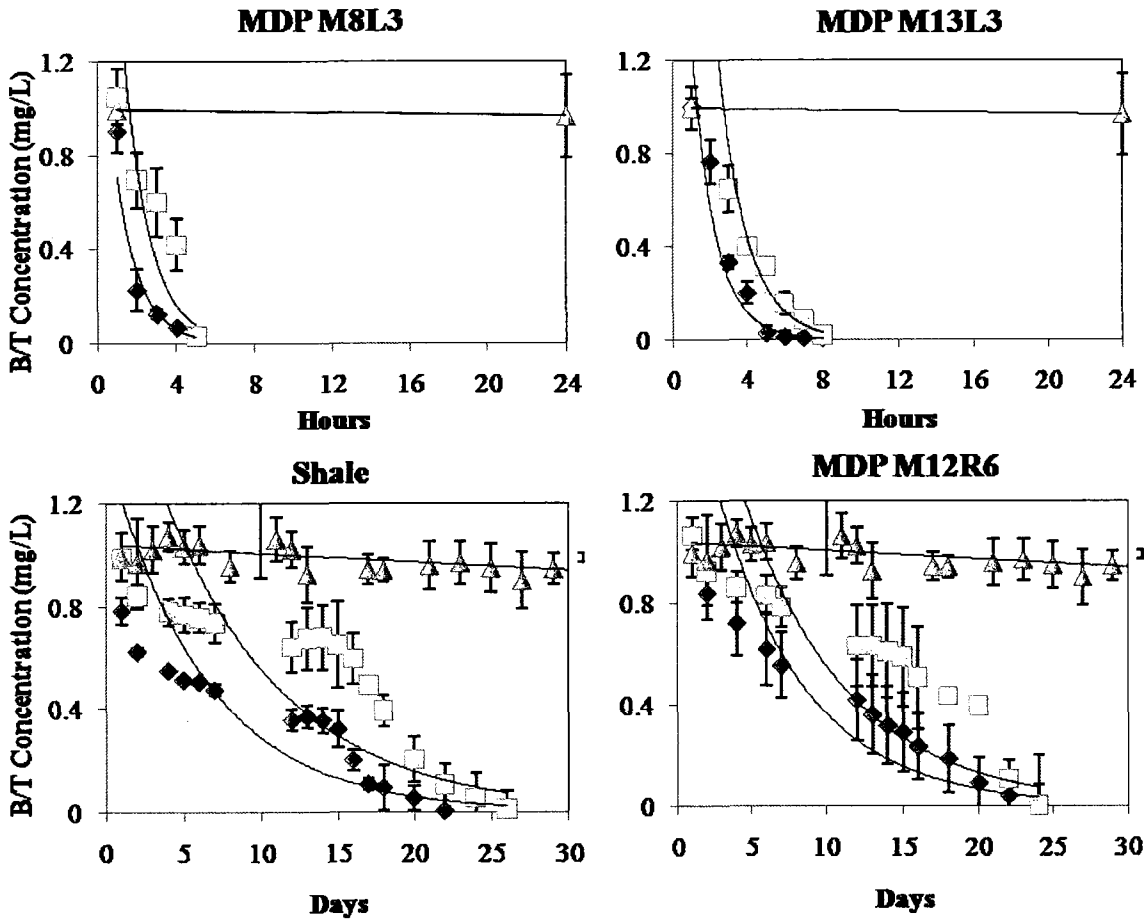

Figure 8. Symbols: ♦, Toluene ; ▫, Benzene; ▲, Abiotic Control. Aerobic degradation of B/T for high TOC (L3) and low TOC (Shale and R6). Error bars represent ± 1 standard deviation from the mean of triplicate microcosms. Solid lines represent first order exponential regression.

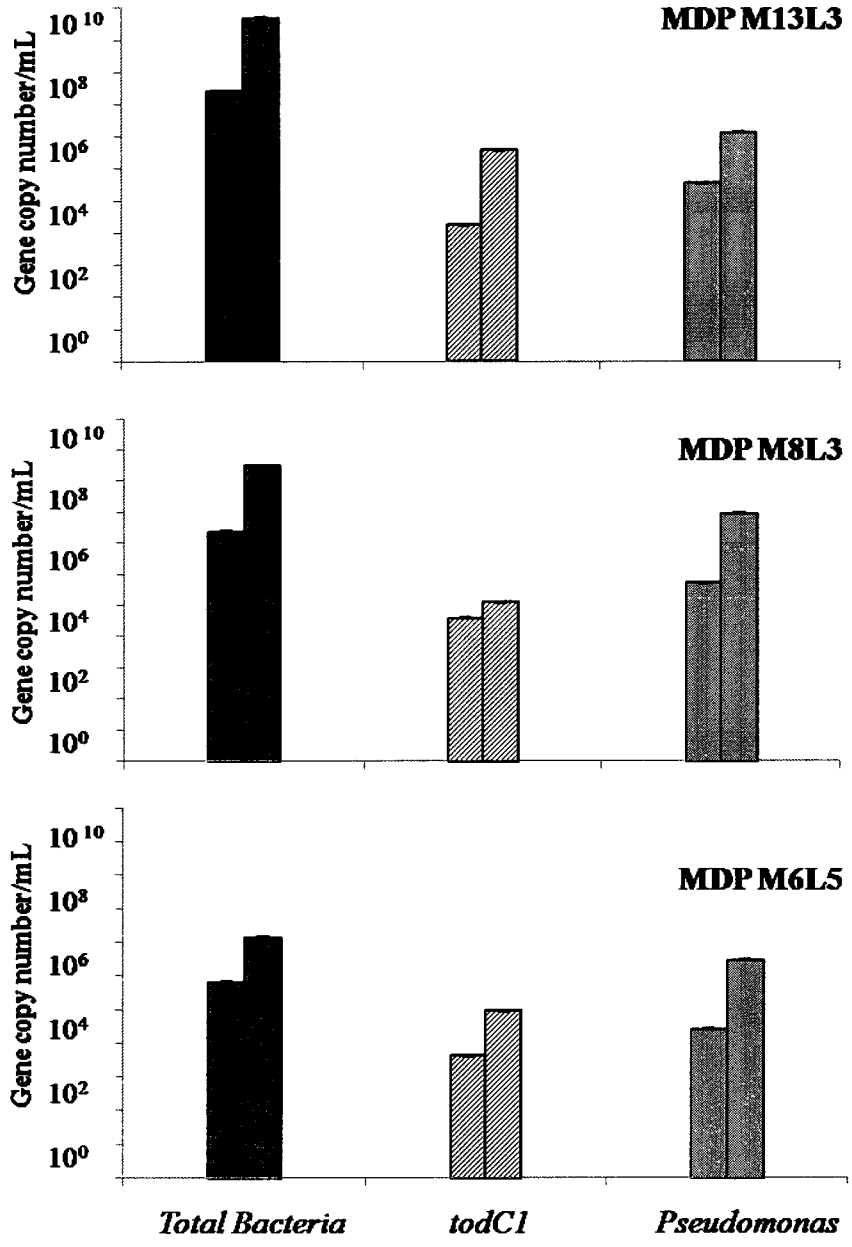

Figure 9. Biomarker concentrations before B/T spike and after degradation. Error bars represent ± 1 standard deviation from the mean of triplicate DNA extractions.

Nitrate and Sulfate Treatment

Anaerobic microcosms MDP M8L3, MDP M13L3, and MDP M6L5 were amended with nitrate (100 mg/L) and sulfate (100 mg/L) to stimulate the microbial oxidization of benzene and toluene. Samples with the highest levels of TOC were chosen because they had the highest number of microorganisms and are more likely to harbor a catabolically diverse community. However, large amounts of background organic carbon could hinder anaerobic B/T biodegradation by providing a preferential carbon source that may be more readily assimilated by the microbial community. Therefore, MDP M6L5, with low TOC (28.5 mg/L) was also selected for biostimulation with nitrate and sulfate.

Reduction of B/T with added electron acceptors was not observed. MDP M8L3 did show a statistically significant removal of nitrate compared to the control microcosm; however, the levels of benzene and toluene did not decrease over time (Figure 10). The decrease in nitrate could have been the result of a preferential carbon source within the background TOC that was microbially oxidized using nitrate as the electron acceptor. This possibility is supported by no removal of nitrate in the low TOC microcosm (MDP M6L5).

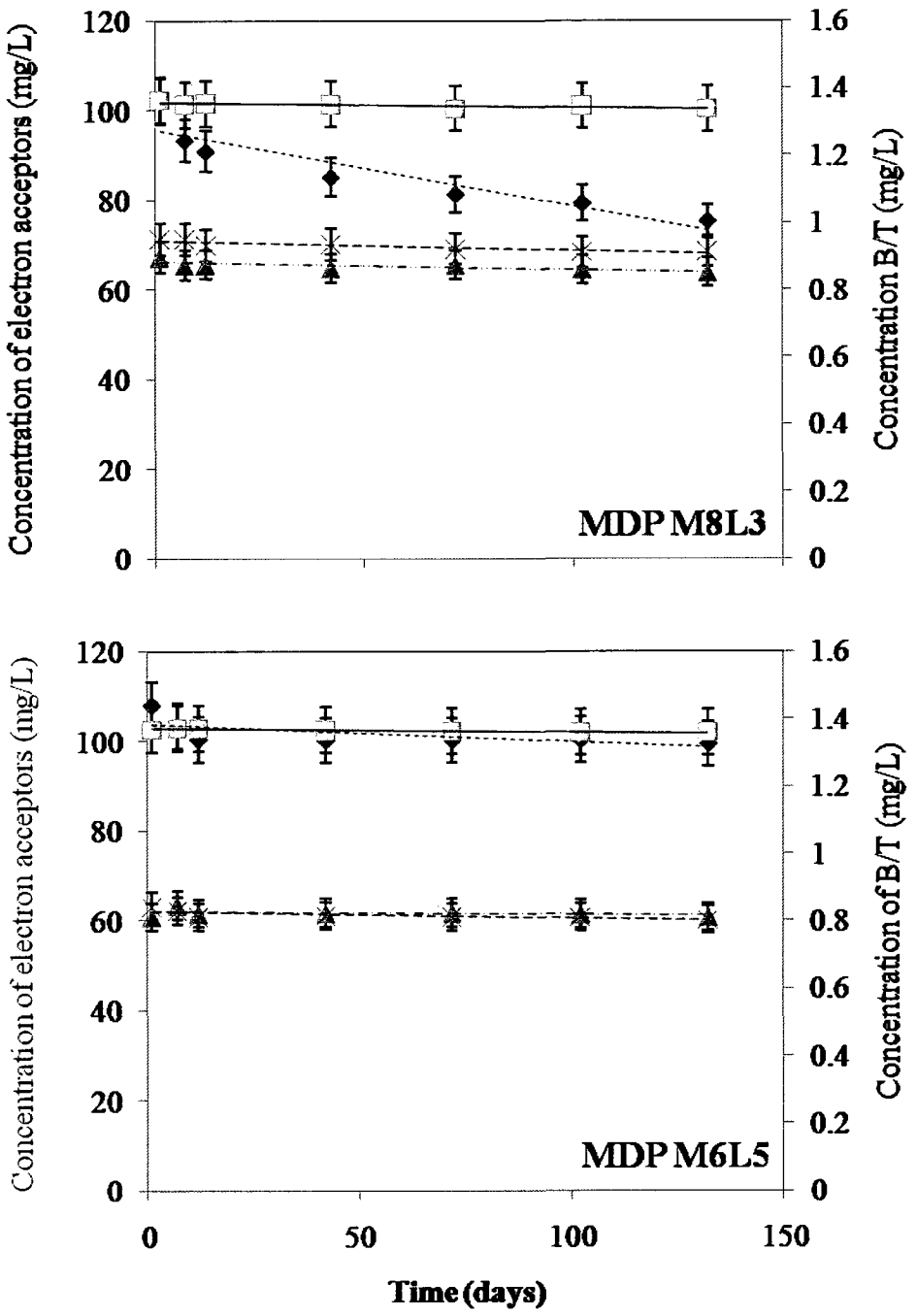

Figure 10. Symbols: ♦, Nitrate ; ■, Sulfate; ▲, Toluene; ×, Benzene. Consumption of nitrate occurred in high TOC (M8L3; $p < .05$) but not low TOC (M6L5) microcosms. Neither benzene nor

toluene was biodegraded in the presence of nitrate or sulfate. Error bars represent ± 1 standard deviation from the mean of triplicate microcosms. Lines represent the linear regressions.

Benzoate Treatment

Benzoate has been shown to stimulate hydrocarbon degradation in aquifer column studies (3). Benzoate is a common intermediate in many aromatic hydrocarbon biodegradation pathways and is thought to activate portions of the genes involved in hydrocarbon degradation (13).

Low TOC samples M13A and M27B were selected for stimulation to avoid any preferential use of substrates that may occur in the background TOC. Benzoate was biodegraded concomitantly with benzene removal. The levels of removal were minimal but statistically significant (p=.007) (Figure 11).

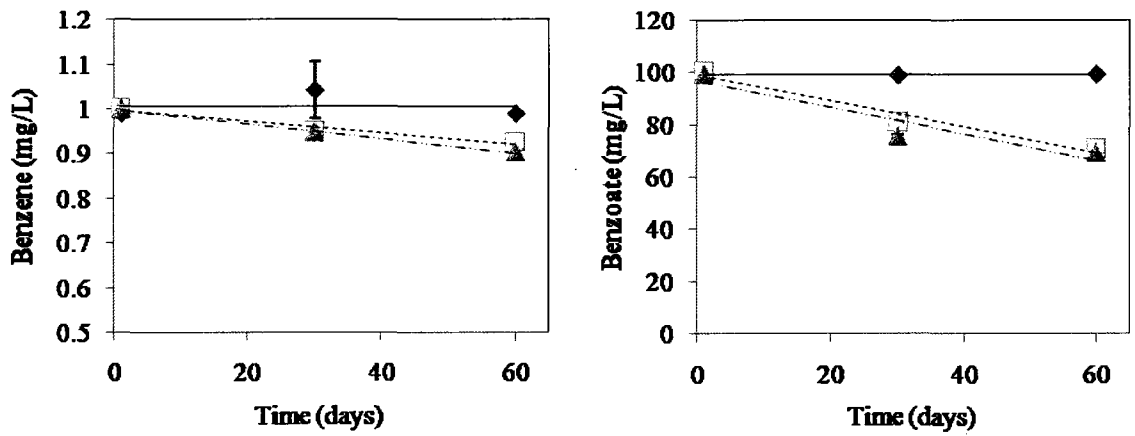

Figure 11. Symbols: ♦, Control ; ■, M27B; ▲, M13A. Lack of significant benzene degradation and removal of benzoate. Error bars represent ± 1 standard deviation from the mean of triplicate microcosms. Error bars smaller than the symbols not depicted. Lines represent linear regression trends.

5.4 Effects of bioaugmentation on B/T degradation

Bioaugmentation is the introduction of microorganisms with specific biodegrading capabilities to a contaminated aquifer or site (2).This technique has been shown to enhance or induce attenuation of specific contaminants. An anaerobic benzene degrading consortia

has been successfully used to bioaugment a contaminated aquifer column to catalyze benzene biodegradation (20). This microbial consortia (provided by Elizabeth Edwards, University of Toronto) was used to bioaugment M27B and M13A microcosms in an attempt to induce anaerobic benzene biodegradation.

The Elizabeth Edwards microcosm-based culture served as the positive control in which benzene was degraded. The M27B and M13A microcosms did not significantly degrade benzene when bioaugmented with 2 mL, 1 mL, or 0.5 mL of the same culture. Methane production was measured during the course of the experiment but did not accumulation to statistically significant levels compared with the negative control (non-bioaugmented microcosm) (Figure 12).

While the Edwards culture has been successfully used in the past for bioaugmentation (20), many factors, including nutritional requirements, environment, time, and concentration significantly affect its ability to thrive and degrade benzene anaerobically. These factors may contribute to the lack of biodegradation activity in the bioaugmented microcosms. Based on past laboratory experience with this culture, a ratio of 10% culture to 90% culture specific media is required for the microorganisms to survive and proliferate. Thus, it would be a challenge to grow up large quantities necessary for *in situ* use at the Mahogany site.

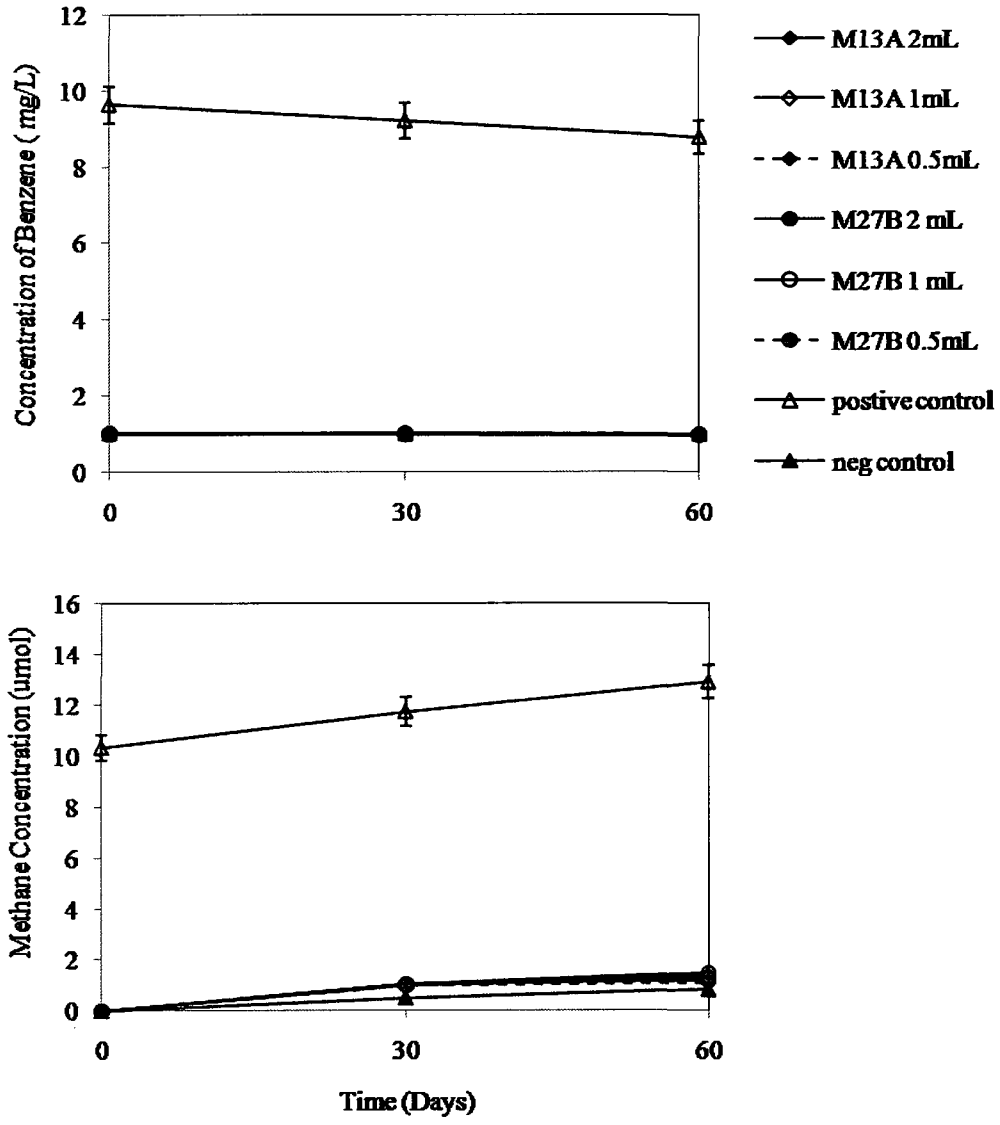

Figure 12. Benzene degradation occurred in the positive control but not in the experimental microcosms. Methane generation occurred but not in significant levels when compared to the negative control. Error bars represent ± 1 standard deviation from the mean of triplicate microcosms.

6: RECOMMENDATIONS AND CONCLUSIONS

6.1 Recommendations for the MDP site

This laboratory and molecular-based study has provided insight into the catabolic processes of the microorganisms present at the Mahogany site as well as the effects of the existing background TOC. Molecular assays detected significant quantities of phylogenetic (*Pseudomonas* spp.) and catabolic biomarkers (*todC1*) that support aerobic biodegradation potential at the Mahogany site even though the locations from which the samples were collected are presumed to be strictly anaerobic. Many aerobic microorganisms can survive on relatively low concentrations of dissolved oxygen and remain able to degrade hydrocarbons. For example, *Pseudomonas putida* F1, a common aerobic toluene degrader, was found to grow on toluene with as little as 0.1 mg L^{-1} dissolved oxygen present (17). Benzene and toluene biodegradation within the aerobic microcosms corroborated the molecular biomarker data and provided evidence that the areas of interest may not be completely anaerobic but possibly microaerophilic due to reoxygenation by natural processes or through the cone of depression created, as a result, of pumping.

Biomarkers indicative of anaerobic benzene and toluene biodegradation (*ORM2* and *bssA*) were not present above detection limits for any of the site samples nor did toluene or benzene degrade in anaerobic microcosms. Collectively these results suggest that anaerobic biodegradation may not occur at the Mahogany site however, other microorganisms, without the *ORM2* or *bssA* biomarkers, capable of degrading B/T anaerobically may exist at the site. The *ORM2* and *bssA* biomarkers are not inclusive to all

anaerobic B/T degraders because they are designed based on known catabolic and phylogenetic sequences.

Biostimulation with nitrate or sulfate was not successful in enhancing anaerobic biodegradation however, nitrate was significantly removed in high TOC samples which may indicate a lack of electron acceptors available for carbon removal. High TOC levels can inhibit the biodegradation of B/T because it may provide preferential carbon sources that are more easily assimilated by microorganisms. In order for benzene and toluene to biodegrade at sites MDP M13L3 and MDP M8L3 it may be essential to reduce the high levels of competing carbon sources. One way to achieve this would be to add nitrate and sulfate to meet the necessary electron acceptor requirements needed in an anaerobic environment for carbon utilization (1).

Benzoate was added to M13A and M27B (both with low TOC) to simulate anaerobic toluene or benzene biodegradation. Benzene was significantly degraded concomitantly with benzoate in both microcosms. Benzoate is a common intermediate in toluene and benzene biodegradation pathways and the addition of benzoate to stimulate B/T degradation has been a successful approach to reduce acclimation times (3). Microorganisms readily assimilate benzoate resulting in microbial proliferation, including specific degraders. Increasing population density of specific degraders can reduce acclimation periods and increase the rate at which benzene and toluene are removed.

Bioaugmentation of M13A and M27B was attempted with the Elizabeth Edwards consortia (19) to incorporate benzene degraders into the microbial population. Benzene loss and methane generation was not significant in bioaugmented microcosms, therefore a benzene degrading population failed to be established via bioaugmentation. This may have

been due to lack of required nutrients, electron acceptors, pH, or other factors that may negatively affect the viability of anaerobic benzene degrading microorganisms.

This study has provided multiple lines of evidence converging on the conclusion that the MDP site may not be completely anaerobic as previously thought. Oxygen at even the lowest concentrations is beneficial since natural attenuation of benzene and toluene is more likely to proceed in the presence of oxygen than in a completely anaerobic environment. Baseline detection and increase in the *todC1* and *Pseudomonas* spp. copy numbers confirmed the actual presence of microorganisms with the capability to aerobically degrade benzene and toluene. Air sparging or the addition of oxygen release compounds could be used to enhance B/T biodegradation at the MDP site.

Biomarkers indicative of anaerobic B/T degradation (*ORM2* and *bssA*) were not present above detection levels in the groundwater samples studied; however, these biomarkers do not detect all genes/organisms capable of anaerobic degradation of B/T. Benzoate was biodegraded under anaerobic conditions but failed to stimulate benzene removal. In light of the failure of biostimulation and bioaugmentation to enhance biodegradation of benzene and toluene in anaerobic microcosms, natural attenuation is unlikely to occur, or may require an extensive acclimation period at the test sites and may not be a practical reclamation approach.

If it is assumed that biodegradation of B/T will occur after a prolonged acclimation period, further (pilot-scale) studies should be conducted to assess the efficacy of enhancing B/T biodegradation by two potential approaches, 1) promotion of biodegradation of background organic carbon (which exerts a significant biochemical oxygen demand (BOD) and may be consumed preferentially over B/T) to enhance the potential for subsequent

aerobic B/T biotransformation. This could be accomplished by adding anaerobic electron acceptors such as nitrate or sulfate to lower the background BOD; and/or 2) biostimulation with benzoate to promote the fortuitous growth of anaerobic hydrocarbon degraders (i.e., enhanced acclimation through analogue enrichment) and thus enhance microbial utilization of B/T.

6.2 Future experiments for biomarker development

The use of DNA biomarkers provides insight into microbial community dynamics, the identity of specific degraders, and catabolic potential. Since microbial biomarkers are an emerging technology, critical assessments of the methods are necessary to determine limitations. Addressing the limitations will lead to a broader acceptance of the biomarkers and may result in a more complete picture of bioremediation on a molecular level.

While the stability of DNA contributes to the success of biomarker technology, it also creates a significant limitation because extracellular DNA and DNA in dead cells are both readily extracted with live cell DNA. Current biomarker qPCR protocols cannot discriminate between targets from viable or non-viable microorganisms, which results in an over estimation of relevant gene copy numbers. Differentiation between viable and non-viable DNA is critical for assessing biodegradation potential. RT-qPCR targeting of mRNA transcripts is an approach that can be used to measure gene activity of viable microorganisms. However, the decay rate of mRNA (half-life of approximately 30 seconds) makes it extremely difficult to use. Recent studies have provided an approach to circumvent the non-viable over estimation of gene copies by using ethidium monoazide or propidium monoazide (23, 55, 69). Ethidium monoazide (EMA) and propidium monoazide (PMA) are dyes that selectively pass through damaged cell membranes and intercalates into

the DNA via light activated cross linking (55). PCR amplification of the cross-linked DNA is strongly inhibited thereby reducing the detection of target genes from extracellular or dead cells. This technique has been successfully tested on environmental water samples (23) and activated sludge matrices(69). PMA and EMA can be utilized with the current DNA extraction technique to better quantify viable BTEX degraders and understand how BTEX concentrations affect the viability and recovery of the degraders. This altered approach could influence reclamation technology selection by providing a more accurate quantification of active B/T degraders. This critical information could then be used to determine if MNA is an appropriate approach for specific sites.

Additionally, DNA extraction efficiency/ recovery from environmental samples has yet to be standardized. Environmental samples are relatively complex in physical and biological nature, which can greatly affect DNA yield even when highly standardized extraction protocols are employed. One approach is the addition of competitor DNA or cells. Many studies have used Bacteriophage (λ) DNA as a recovery standard by spiking environmental matrices with a known concentration of Lambda phage DNA and measuring the amount recovered using qPCR. Lambda DNA is a 500bp fragment that is not amplified by *Total Bacteria* or other phylogenetic biomarkers during qPCR but only by the specific lambda DNA primer. Thus, it does not inadvertently increase *Total Bacteria* concentrations or other biomarker measurements when added to the environmental sample. However, DNA extraction protocols rely on physical beadbeating to disrupt cell walls. Therefore, adding 'naked' or unprotected DNA to an environmental sample prior to beadbeating presents an additional bias. This bias probably results from the shearing of Bacteriophage DNA during the beadbeating and, as a result, high variability in normalization rates

because the DNA fragments are too small or damaged for the primers to bind. Published studies that have used this technique (7, 19, 72), reported recovery percentages ranged from 23% to over 100% thereby indicating this approach is not ideal for calculating DNA extraction recovery.

A 2005 study (58) reported a new technique to better quantify recovery efficiency using green fluorescence protein (GFP) cloned into *Escherichia coli*. Known quantities of *E. coli* JM109 harboring a pEGFP-N1 plasmid were spiked into soil samples prior to DNA extraction. The pEGFP-N1 plasmids were quantified after extraction using SYBR green qPCR to calculate normalization factors. This technique provided the protection of a cell wall however, *E. coli* naturally occurs in the soil and could potentially increase measurements of *Total Bacteria*.

DNA biomarkers can be a powerful tool for quantifying and detecting specific contaminant degraders, monitoring attenuation progress, and providing insight into microbial communities of interest. However, issues such as extraction efficiency and viable, non-viable detection need to be addressed and standardized in order for this technology to move from the 'emerging' category to the 'commonly used' category for microbial forensic site investigation.

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APPENDIX A: BENZENE AND TOLUENE DEGRADATION PATHWAYS

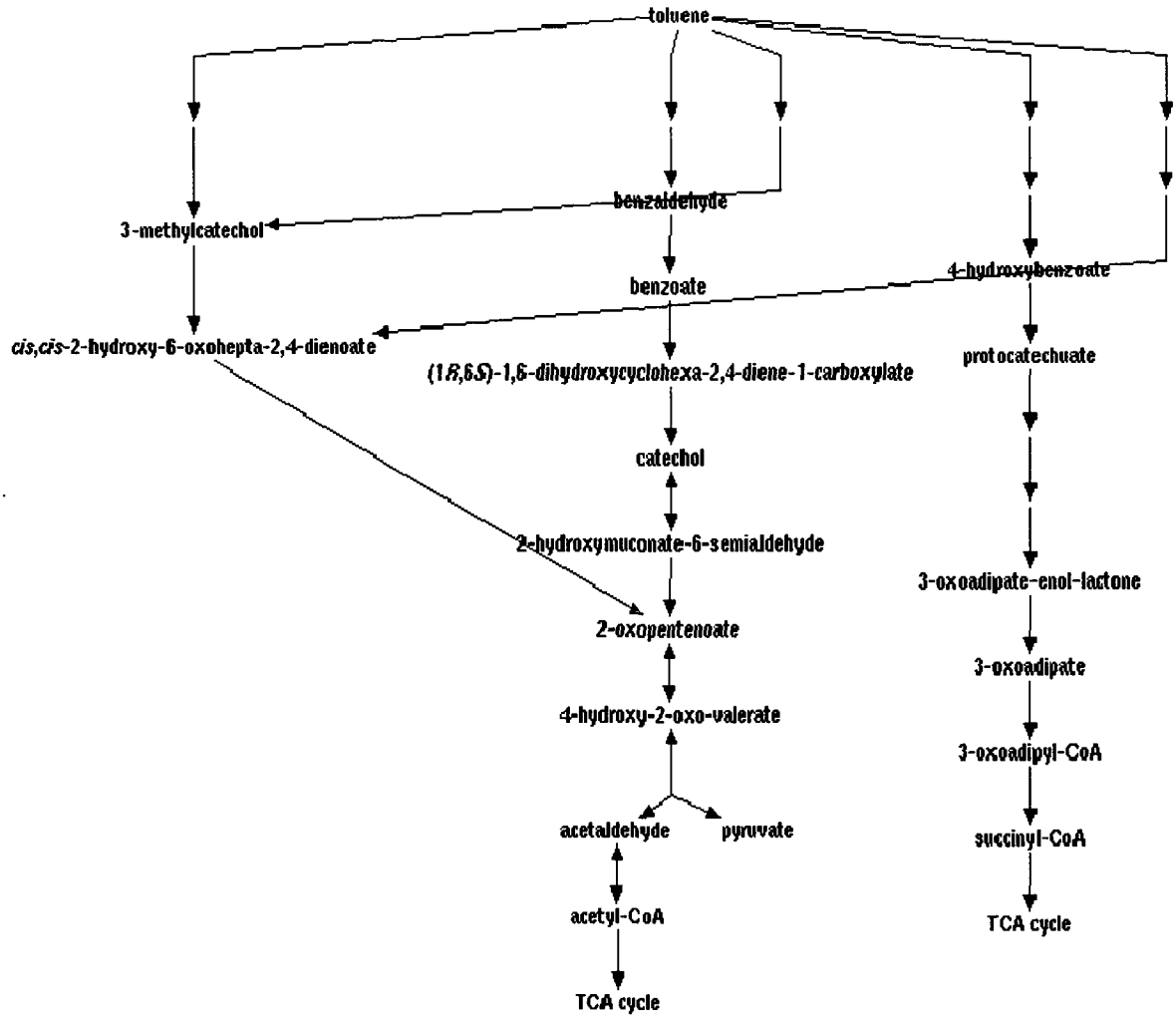

Figure A1. Aerobic toluene degradation(http://metacyc.org/)

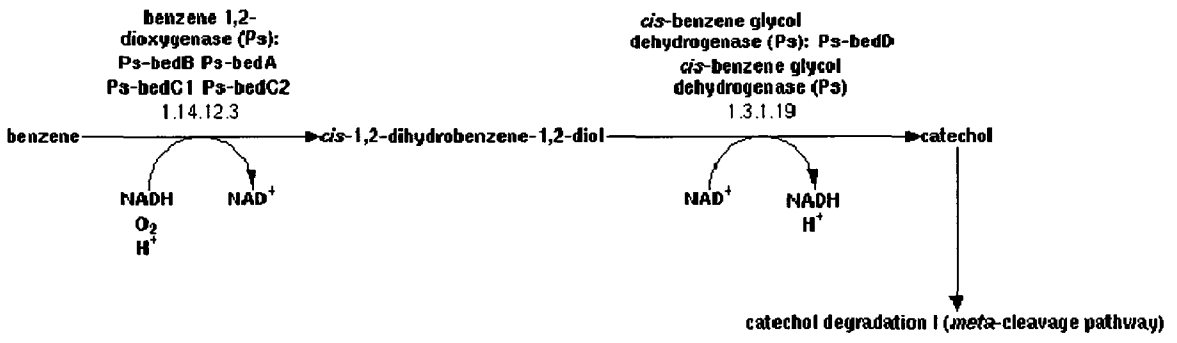

Figure A2. Aerobic Benzene Degradation (http://metacyc.org/)

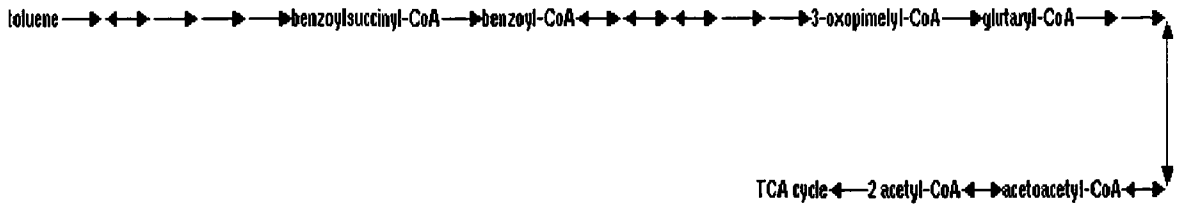

Figure A3. Anaerobic toluene degradation (http://metacyc.org/)

Table A1. Phylogenetic and Catabolic Biomarkers Used to Detect and Quantify Specific Degraders

Target	Primer/Probe	Sequence	Reference
Total Bacteria (16S rDNA)	BACT1369-F	5'-CGGTGAATACGTTCYCGG-3'	Suzuki et al. 2000
universal primers to detect total bacteria	PROK1492-R	5'-GGWTACCTTGTTACGACTT-3'	
	TM1389F	FAM-5'-CTTGTACACACCGCCCGTC-3'-BHQ-1	
Archaea	ARCH1-1369-F	5'-CGGTGAATACGTCCCTGC-3'	Suzuki et al. 2000
universal primers to detect Archaea including methanogens	ARCH2-1369-R	5'-CGGTGAATATGCCCCTGC-3'	
	PROK1541-R	5'-AAGGAGGTGATCCTGCCGCA-3'	
	probe	FAM-5'-CTTGTACACACCGCCCGTC-3'-BHQ-1	
Pseudomonas spp.(16S rDNA)	Psu-F	5'-ACTGCATCCAAAACTGGCAA-3'	Duteau et al. 1998
catabolically versatile genus commonly involved with BTEX biodegradation	Psu-R	5'-TCTCTGCATGTCAAGGCCT-3'	
	Probe	SYBR Green	
Toluene dioxygenase (TOD)	TOD-F	5'-ACCGATGA(A/G)GA(C/T)CTGTACC-3'	Baldwin et al. 2003
enzyme that initiates aerobic degradation of BTEX	TOD-R	5'-CTTCGGTC(A/C)AGTAGCTGGTG-3'	
	Probe	SYBR Green	
Benzylsuccinate synthase (bssA) enzyme involved in anaerobic toluene degradation	bssA-F	5'-ACGACGGYGGCATTTCTC-3'	Beller et al. 2002
	bssA-R	5'-GCATGATSGGYACCGACA-3'	
	Probe	FAM-5'-CTTCTGGTTCTTCTGCACCTTGGACACC-3'-TAMRA	
Desulfobacterium sp. (ORM2)	ORM2-F	5'-CCACGAAAGTCGATTATACCAGAA-3'	Da Silva et al. 2007
genus linked to anaerobic benzene degradation	ORM2-R	5'-AACCATACCTTAGGCGCTTATCTC-3'	
		FAM-5'-CGCTGGTTTAACCCCCGATTTATCTCG-3'-TAMRA	

This table was adapted from (Alvarez & Illman, 2006. Bioremediation and Natural Attenuation)

APPENDIX B: MDP SITE MAPS

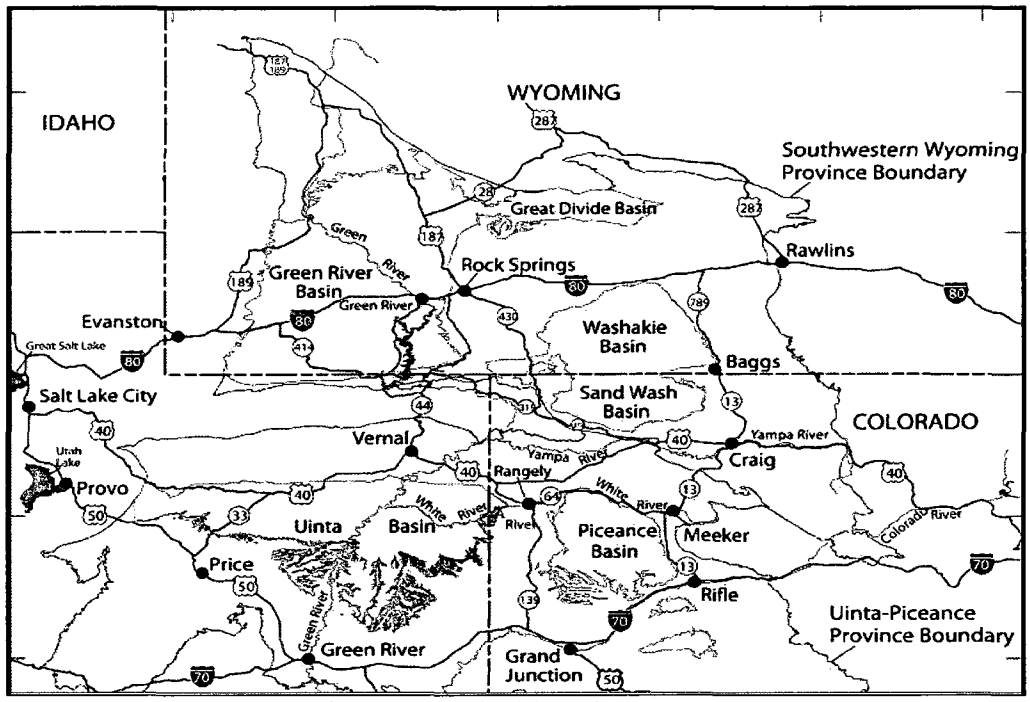

Figure B1. Piceance Basin geographical location

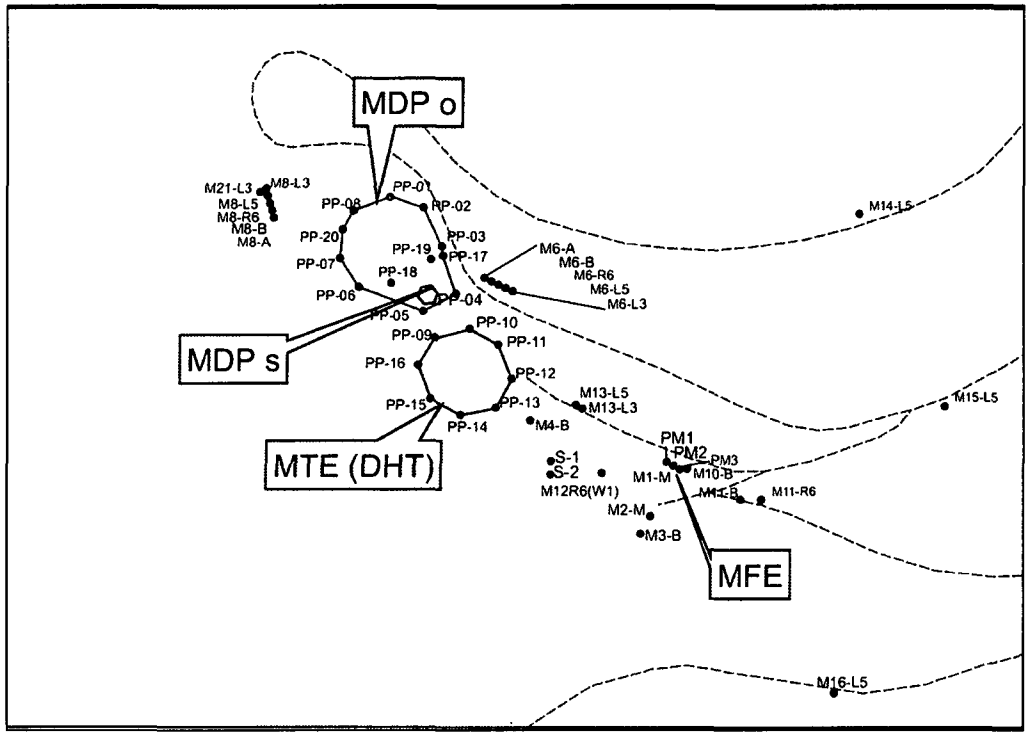

Figure B2. Perimeter pumping and monitoring well locations

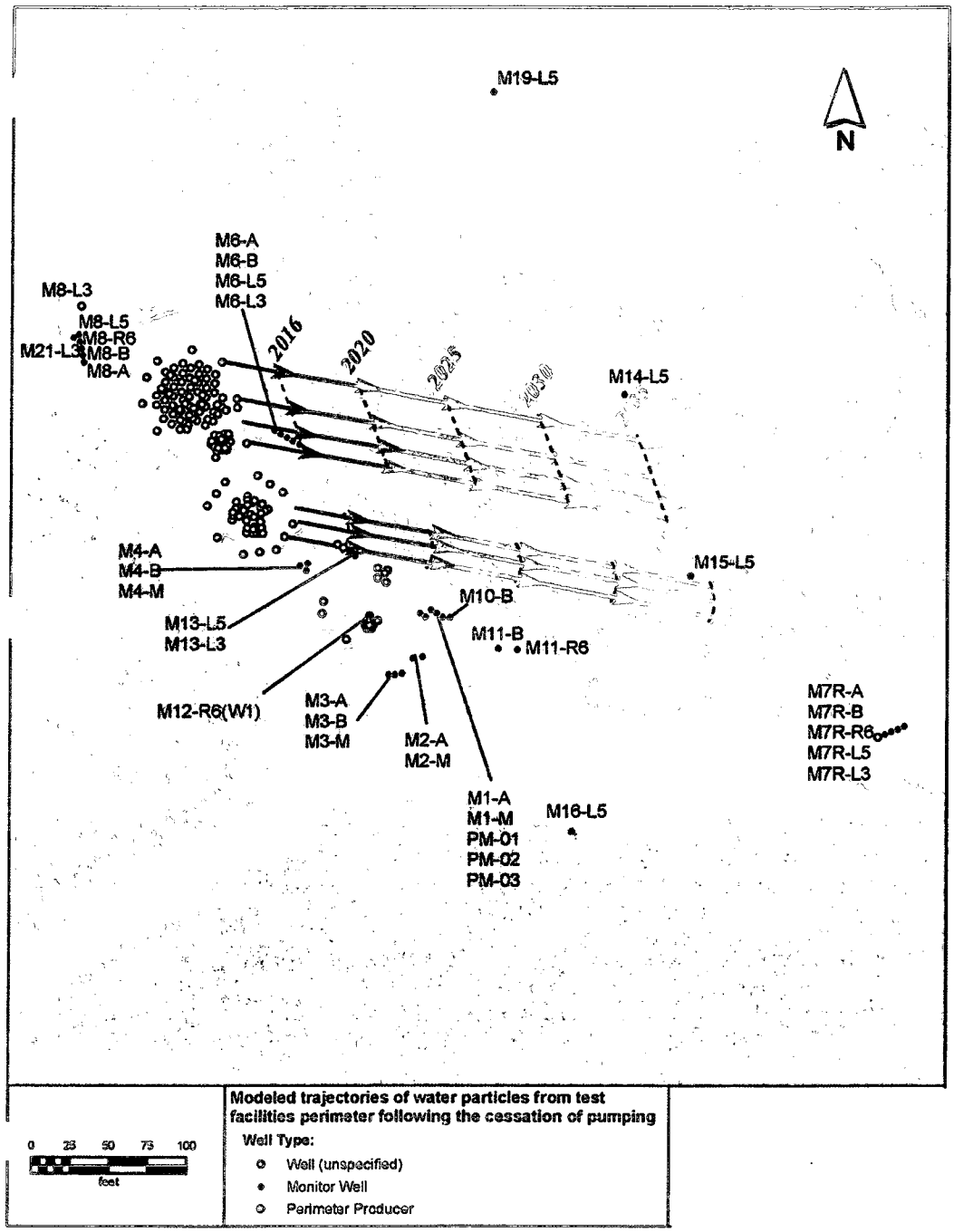

Figure B3. MODFLOW model of groundwater travel time

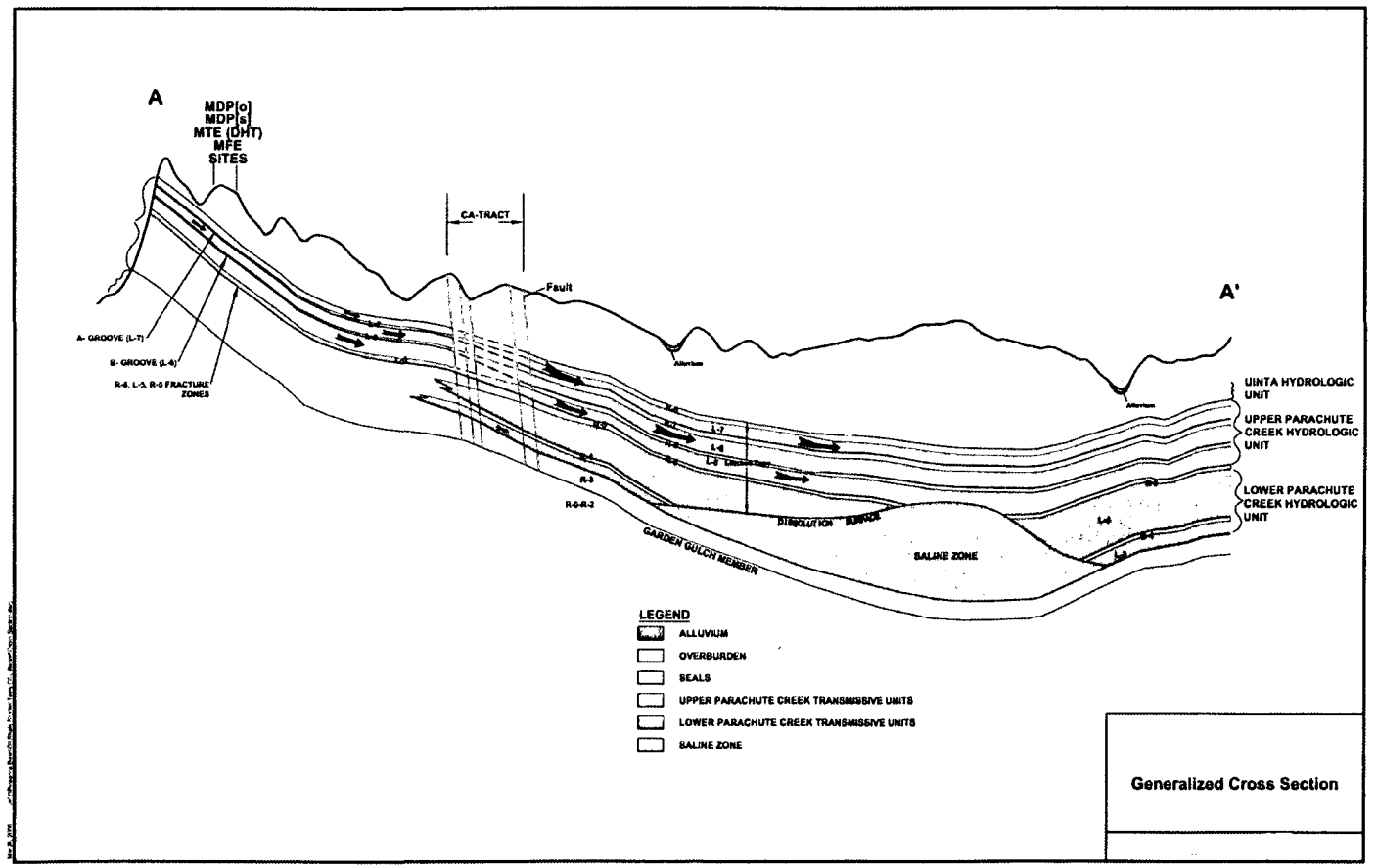

Figure B4. Cross section of the MDP location

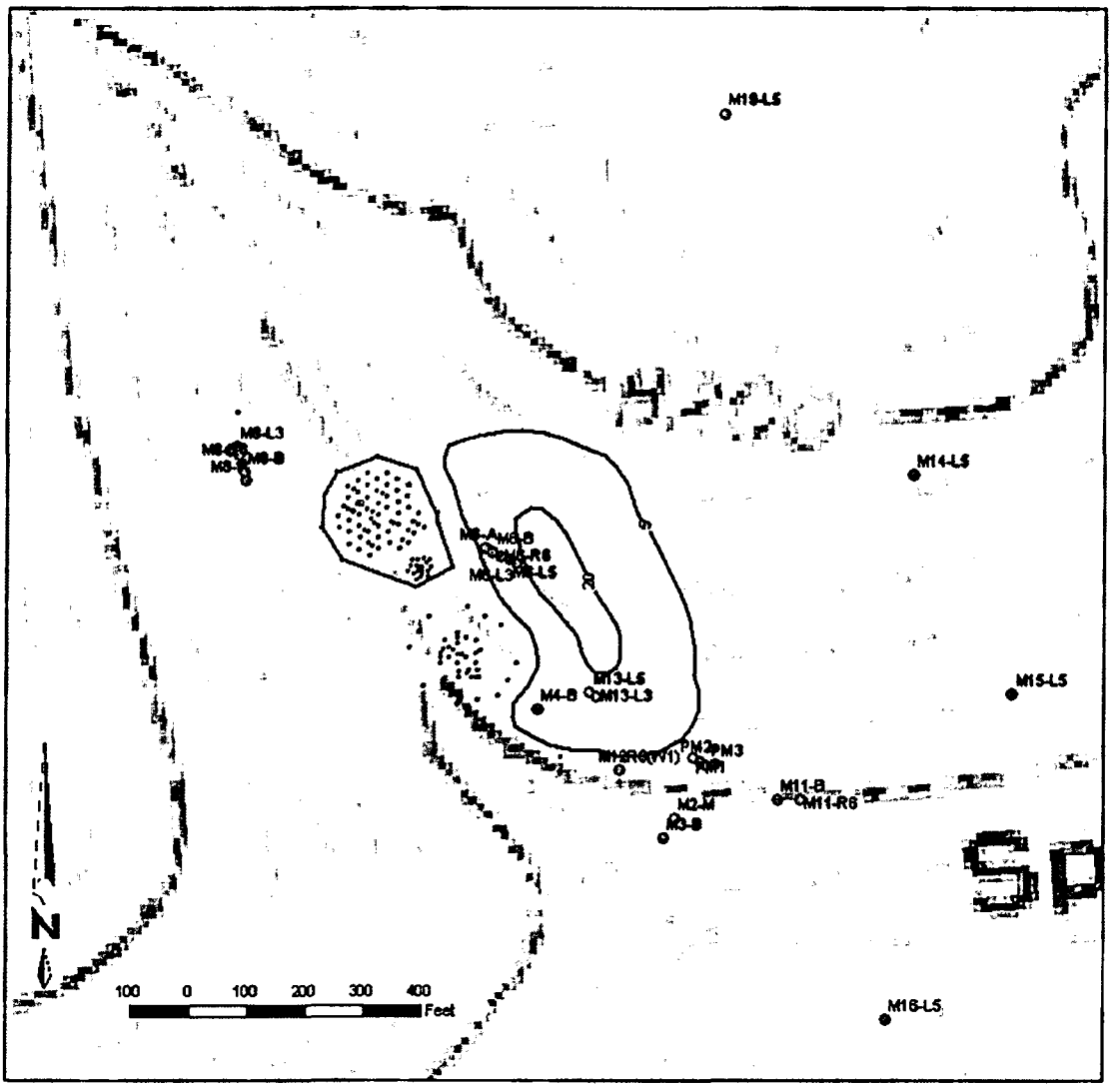

Figure B5. Predicted benzene plume migration after 30 years. (Originating at red cluster)

APPENDIX C: SUPPLEMENTAL DATA

Table C1. Non-purgeable organic carbon measured in groundwater samples

Sample Location	*NPOC (mg/L)*
MDP M8L3	571.7
MDP M13L3	275.3
MDP M6L5	28.3
MDP M4B	22.99
MDP M10B	24.28
MDP M11B	26.17
MDP PM3	33.65
MDP M12R6	22.41
MDP M13L5	22.27
MDP PP08	30.72
MDP PP12	23.84
MIT M7L5	21.83
MIT M15L5	22.75
MIT M14B	22.82
MIT M13A	21.83
MIT M27B	21.35
Shale water	27.26
DI water	0.181

Table C2. Aerobic first order degradation rate constants (k)(day^{-1}) for benzene and toluene

Sample location	*toluene*	*benzene*
MDP M8L3	19.85	18.33
MDP M13L3	18.98	15.50
MDP M6L5	0.33	0.56
MDP M4B	0.34	0.28
MDP M10B	0.38	0.33
MDP M11B	0.27	0.27
MDP PM3	0.35	0.29
MDP M12R6	0.17	0.14
MDP M13L5	0.34	0.30
MDP PP-8	0.36	0.19
MDP PP-12	0.30	0.26
MIT M7L5	0.22	0.20
MIT M15L5	0.85	0.83
MIT M14B	1.01	0.54
MIT M13A	0.54	0.29
MIT M27B	0.39	0.49
MIT Shale	0.16	0.12