Bioremediation of Trichloroethylene by Indigenous Microbes in Groundwater

by

Jennifer A. Humphries

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Jennifer A. Humphries

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Jennifer A. Humphries, Student — Date 4/29/03

Approvals:

Dr. Carl G. Johnston, Thesis Advisor — Date 4/29/03

Dr. Amy M. H. Ashe, Committee Member — Date 4/28/03

Dr. Jeffrey A. Smiley, Committee Member — Date 4/28/03

Peter J. Kasvinsky, Dean of Graduate Studies — Date 4/30/03

Abstract

Trichloroethylene (TCE) is a widespread contaminant of groundwater, which potentially can be degraded by indigenous microbes. Degradation can be augmented with the use of cometabolites, which stimulate bacteria to produce the enzymes essential to TCE degradation. Groundwater contaminated with TCE was received from a U.S Department of Energy site in Ohio to test for potential bioremediation of TCE by indigenous microbes. Groundwater treatments, augmented with various potential cometabolites including Compound C™ and phenol, were spiked with ^{14}C-TCE and the amount $^{14}CO_2$ produced was analyzed to determine rates of TCE degradation.
Culture conditions were manipulated by supplying nutrients and reducing equivalents and treatments were optimized to produce maximal TCE degradation. Under optimized conditions Compound C™ did not support increased TCE mineralization at this site. An optimized phenol treatment supported 14% mineralization within seven days.

Molecular techniques including RFLP and DNA sequencing of 16S rDNA were used to assess the microbial community structure of the three treatments, unamended groundwater, groundwater amended with phenol (Phenol Culture), and phenol culture after incubation with site concentrations of TCE (Phenol Culture + TCE), respectively. Phylogenetic trees were used to determine the evolutionary relationship between dominant species in each treatment and illustrate the shift in microbial community structure as a result of augmentation. The indigenous groundwater community was slightly more diverse than the augmented treatment communities. The clones extracted from the augmented communities related most closely with β-proteobacteria. One common RFLP pattern was observed to predominate in each of the three treatments. DNA sequencing revealed this predominant pattern to be closely related to *Variovorax* a β-proteobacteria. With the exception of this one common pattern, RFLPs were unique within the treatment groups. This study demonstrated that augmentation with phenol substantially increased TCE degradation as well as altered the community structure of indigenous bacteria in groundwater. The augmented phenol community was identified to support bacteria including *Variovorax, Pseudomonas,* and *Burkholderia,* which have previously been identified to be capable of degrading TCE.

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It is good to have an end to journey toward;
but it is the journey that matters, in the end.
-Ursula Le Quin

For my parents, my most faithful supporters…
…and for Erick my very best buddy.

Chapter 1: Introduction

I. Trichloroethylene

Trichloroethylene (TCE) is a chlorinated aliphatic hydrocarbon with the chemical formula $CHClCCl_2$. It was first manufactured in 1864, was commercially produced in the early 1900's, and achieved steadily increasing use in industry until recent years. According to the Environmental Protection Agency (EPA) over 124 million lbs. of TCE were produced in the US during 1974 and there continues to be over 4 million lbs. produced each year. (EPA, 2000). Due to overuse, improper disposal, and ignorance on the parts of the steel and dry cleaning industries, TCE is a widespread contaminant of groundwater and soils (Little et al., 1988; Ensley, 1991). Many instances of groundwater contamination have been documented (Ensley, 1991), which became of immediate concern to environmental safety as laboratory experiments began linking repeated high levels of exposure to cancer in rats and mice (ATSDR, 1997; Folsom et al.,1990; Alvarez-Cohen and McCarty, 1990). To determine the risks associated with chronic human exposure, the Agency for Toxic Substances and Disease Registry compiled medical data on over 4,000 residents who were exposed to TCE in Michigan and bordering states. Testing revealed no conclusive evidence that TCE caused an increase in cancer occurrence, however TCE exposure was linked to leukemia in women and children (ATSDR, 1997). Likewise, in 1979, a small community in Woburn, Massachusetts reported abnormally high levels of childhood leukemia after their drinking water was contaminated with industrial solvents (MA Dept. of Public Health, 1996). The direct cause and effect relationship associated with TCE exposure in humans is not fully understood and additional studies must be done to establish a direct causal relationship (ATSDR, 1997). Due to the carcinogenic properties and the largely unknown long-term

effects of TCE exposure, in 1989 the EPA set a maximum drinking water standard for trichloroethylene of five parts in one billion parts water (5ppb) (ATSDR, 1997).

II. Remediation

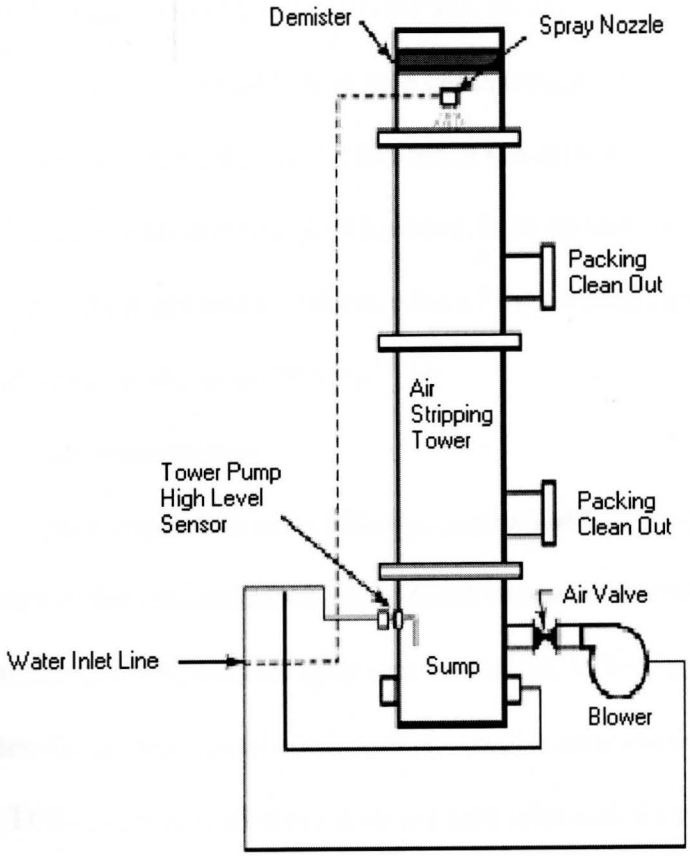
The properties of TCE, which make it useful as an industrial degreaser and solvent, are the same properties that make it difficult to remediate (Ensley, 1991). TCE degrades very slowly from natural environmental processes. Studies at a contaminated aquifer demonstrated TCE to exhibit a half-life of over 300 days (Wackett et al., 1989). Practical remediation techniques, including air stripping, activated carbon traps, UV light, ozone and peroxides, respectively have emerged to deal with this recalcitrant compound (Chang and Alverez-Cohen, 1997). Air stripping takes advantage of the volatile nature of TCE and serves to aerate the contaminated water to remove TCE. As the contaminated water is sprayed over an aeration column a fan blows air counter-current to the dispersed water droplets. By increasing the surface area of the contaminated water, the maximal amount of TCE is volatilized and can be subsequently trapped on an activated carbon filter, leaving the water free of contamination (Figure 1). Ultraviolet radiation is also effective at remediating TCE by breaking the chemical bonds through photodissociation (Wekhof, 1991). A chemical means of destroying TCE can be achieved though the use of both peroxide and ozone, which yield hydroxyl radicals (OH-) and rapidly degrade TCE (equation a and b, respectively.) (EPA, 2000).

$$3H_2O_2 + C_2HCl_3 \rightarrow 2CO_2 + 2H_2O + 3HCl \qquad \text{(Equation a)}$$

$$O_3 + H_2O + C_2HCl_3 \rightarrow 2CO_2 + 3HCl \qquad \text{(Equation b)}$$

Although effective, these different methods have many drawbacks, which limit the practical application of treatments in many sites. The effectiveness of photodissociation is

Figure 1: Diagram of a typical air stripping tower.
To remove TCE, contaminated groundwater is sprayed over an aeration column. The volatile nature of TCE allows it to be stripped from the groundwater and trapped on a carbon filter. Uncontaminated groundwater is then returned to the ground.



Source: Recovery Equipment Supply, Inc.

subject to the optimal wavelengths associated with each contaminant. Toxic breakdown products of TCE may occupy a different wavelength, resulting the accumulation of toxic intermediates (Wekhof, 1991). Both ozone and peroxide applications require a highly acidic system for maximal effectiveness, which may not be practical for in situ application. Also, ozone is highly reactive and unstable and requires on-site production. Air stripping is expensive to engineer and apply to a contaminated area (Chang and Alvarez-Cohen, 1997). Although air stripping is very efficient, it is a slow process and may take years to remediate large contaminant plumes. Furthermore, both air stripping and carbon trapping techniques do not cause the breakdown or destruction of the contaminant, but rather transfer the TCE from one medium to the next (Wekhof, 1991).

III. Bioremediation

Bioremediation is an effective method of TCE removal, which uses the indigenous bacteria of the contaminated site to break down the toxic TCE into daughter products. Bioremediation has advantages over traditional TCE remediation like air stripping because it causes the destruction of contaminant. Until a little over a decade ago, it was widely doubted that TCE could be biologically remediated (Fan and Scow, 1993; Kastner, 1991). Now, however, it has been shown that bioremediation of TCE is a successful and prominent solution to the remediation of severely contaminated sites (Nelson et al., 1986; Fan and Scow, 1993; Harker and Kim, 1990; Heald and Jenkins, 1994; Uchiyama et al., 1992). Upon the discovery of bacterial inhabitants in the contaminated groundwater and soils, significant work has been done in both laboratory studies and field studies to identify what enzymes are essential for metabolic degradation of the toxins (Sun and Wood, 1996; Nelson et al., 1987; Newman and Wackett, 1991; Wackett et al., 1989; Harker and Kim, 1990; Fries et al., 1997).

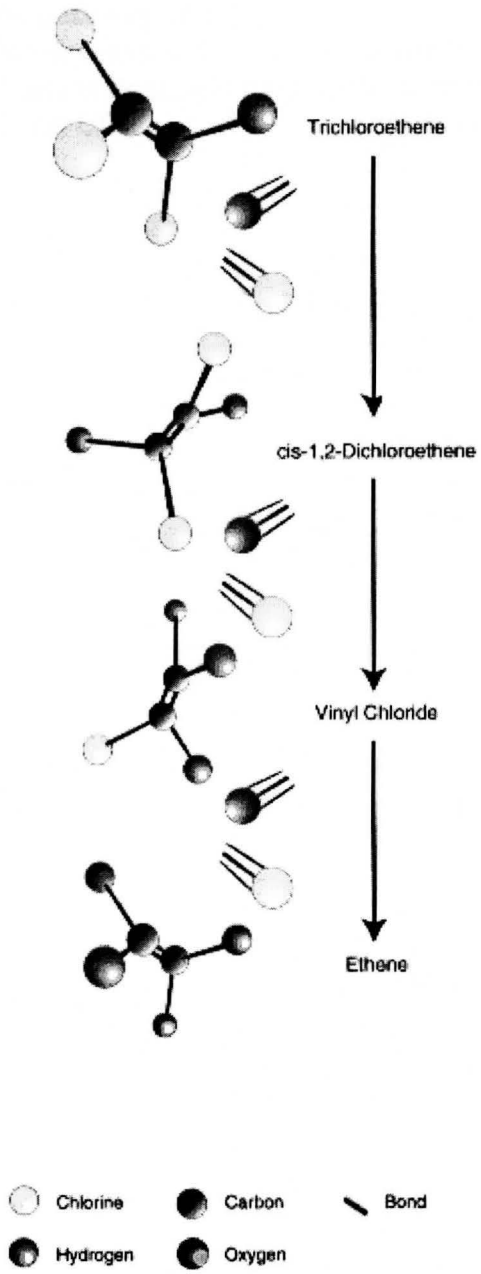
There are two pathways of degradation that differ primarily by the concentration of oxygen present in the subsurface, aerobic and anaerobic.

In an anaerobic system, where there is no oxygen present, the bacteria sequentially convert TCE to dichloroethylene to vinyl chloride (VC) to non-toxic ethene through reductive dehalogenation (Figure 2) (Folsom and Chapman, 1990; Kastner, 1991; Newman and Wackett, 1996). The solvents are used as electron acceptors and therefore require a surplus of electron donors (Nelson et al., 1986; Newman and Wackett, 1991). The partial dehalogenation of TCE is undesirable since it results in the formation of toxic intermediates including VC, a human carcinogen (Wackett et al., 1989; Harker and Kim, 1990; Phelps et al., 1990; Newman and Wackett, 1996). Additionally, this process occurs naturally at a low rate (Bradley et al., 1988), but can be enhanced by addition of hydrogen donors. Aerobic degradation, on the other hand, does not yield these undesirable byproducts (Fan and Scow, 1993; Enzien et al., 1994). Aerobic degradation is stimulated by the addition of a cometabolite, a compound that serves as a primary energy source for the bacteria. The bacterial enzymes used to metabolize the cometabolite fortuitously degrade the TCE to form an epoxide, which is further metabolized into non-toxic daughter products (Figure 3). As a cometabolic process, the bacteria do not receive any energy from the catabolism of TCE.

IV. Cometabolites

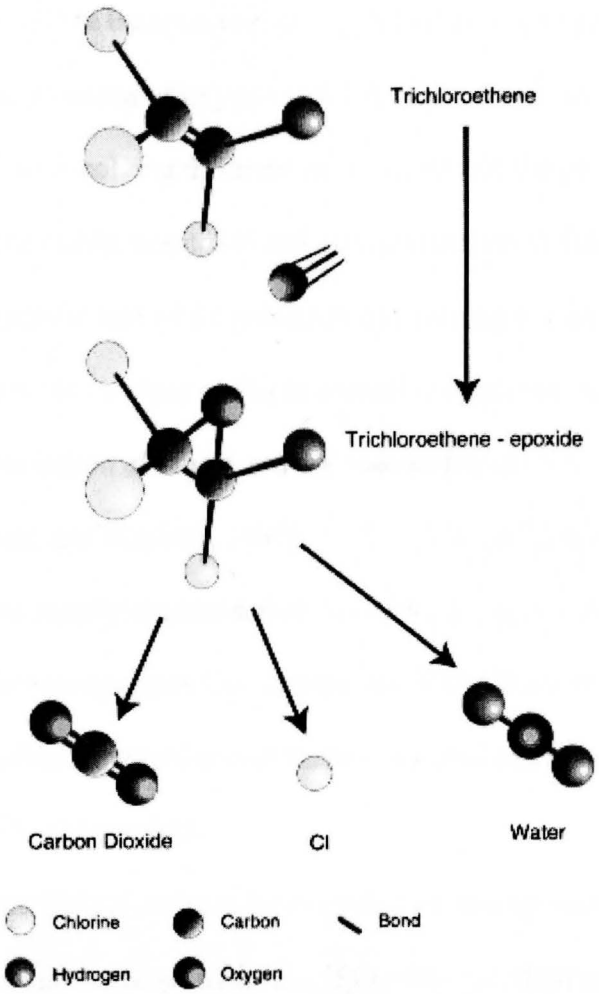
At a naval air station in 1986, indigenous microorganisms were observed to use an unknown component of the site water to aerobically metabolize TCE (Nelson et al., 1986). Since this discovery, significant studies have been preformed to determine the compounds which can serve as growth substrates for stimulating TCE degradation. Wide ranges of compounds have been identified, including methane (Newman and Wackett, 1991; Folsom and

Figure 2: Anaerobic degradation pathway of TCE.
Anaerobic degradation sequentially converts TCE to DCE to VC to ethene through reductive dehalogentaion.



Source: www.oceta.on.ca/

Figure 3: Aerobic degradation pathway of TCE
The first step in aerobic degradation converts TCE into an unstable TCE epoxide. The epoxide is rapidly broken down into organic acid intermediates, which are further metabolized to the final end product CO_2.



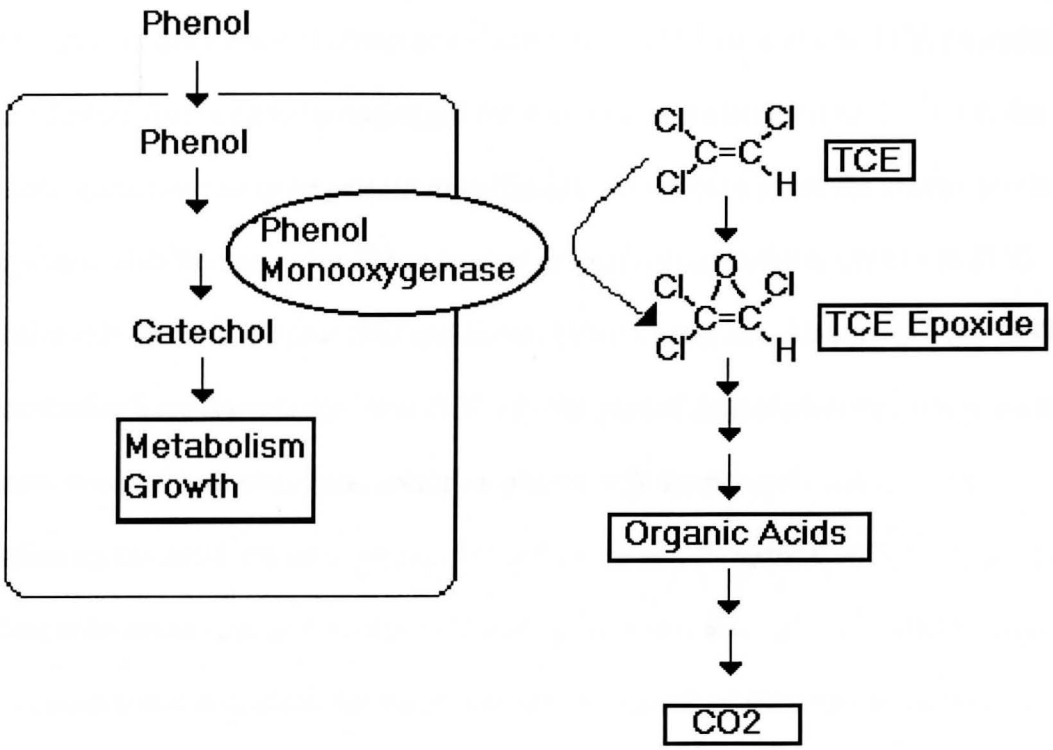
Source: www.oceta.on.ca/

Chapman, 1991; Little et al., 1988; Ensley, 1991), toluene (Mu and Scow, 1994; Newman and Wackett, 1996; Fan and Scow, 1992; Wackett and Gibson, 1988), and phenol (Folsom and Chapman, 1991; Harker and Kim, 1990). The phenol, toluene and methane growth substrates are metabolized using oxygenase enzymes specific to each compound. For example, to metabolize phenol the bacteria utilize the phenol monooxygenase (PMO) enzyme pathway and, in the presence of oxygen and NADH, convert phenol to catechol. Through subsequent reactions involving different enzymes within the pathway, catechol is eventually converted into the stable, non-toxic end product, carbon dioxide (CO_2) (Figure 4). The PMO pathway is nonspecific and while primarily functioning to convert phenol to carbon dioxide, can also serve to catalyze multiple oxidative reactions including hydroxylation of alkanes, oxidation of ethers and the epoxidation of TCE (Alvarez-Cohen and McCarty, 1990; Newman and Wackett, 1991). The TCE epoxide generated is a very unstable intermediate, and is rapidly degraded into two-carbon organic acid byproduct intermediates and one-carbon compounds like formate and CO_2 (Wackett and Householder, 1989). Each of these byproduct intermediates is further metabolized resulting in the final accumulation of a stable CO_2 end-product.

Like phenol, methane and toluene can be degraded by similar pathways: the methane monooxygenase and toluene dioxygenase pathway, respectively. Both pathways metabolize the growth substrates for energy, and like the PMO pathway, can convert TCE into the unstable TCE epoxide.

The ubiquity of TCE in the environment suggests that indigenous microbes are incapable of substantial aerobic TCE degradation without adequate stimulation by cometabolite growth substrates (Fan and Scow, 1993). It has been found through numerous

Figure 4: Phenol Monooxygenase (PMO) Pathway
The phenol monooxygenase enzyme converts phenol into catechol. Catechol is further metabolized and used for energy. The PMO enzyme is non-specific and can also catalyze the transformation of TCE into an unstable TCE epoxide. Subsequent breakdown of the epoxide will produce organic acid intermediates and finally the stable end product CO_2.


Phenol
Phenol
Phenol
Monooxygenase
Catechol
Metabolism
Growth
Cl
Cl
C=C
Cl
H
TCE
O
TCE Epoxide
Organic Acids
CO2

studies that by growing site-specific bacterial cultures in the presence of methane, toluene or phenol, scientists can augment the degradation of TCE in contaminated sites (Newman, and Wackett, 1991; Little et al., 1988; Folsom and Chapman, 1991). Although this technology has been extensively tested and proven to be effective in the laboratory, difficulties still remain for in situ application (Folsom and Chapman, 1991). Cometabolic TCE degradation has many factors that need to be optimized for maximal degradation to occur. First, the cometabolic substrate has to be present in sufficient quantities to allow for energy production and enzymatic viability, however if it is present in high concentrations relative to TCE, competitive inhibition will occur (Mu and Scow, 1994; Chang and Alvarez-Cohen, 1997). Previous studies have documented that TCE inhibits phenol degradation by 50% when both are present in equal concentrations; therefore phenol will theoretically inhibit TCE degradation by the same amount (Ensley, 1991; Folsom and Chapman, 1991). Since TCE is competing with phenol for active sites on the oxygenase enzyme, the cometabolite must be present in a ratio that will allow for the maximum expression of the enzyme, without inhibiting TCE from binding to enzyme active sites.

Second, oxygen levels must be sufficient to allow for oxygen to act as an electron acceptor and reducing equivalents in the form of NADH must be available to sustain the oxygenase-catalyzed reactions (Chang and Alvarez-Cohen, 1997). NADH is used during the conversion of TCE to TCE epoxide, and must be replenished or supplied exogenously to allow for subsequent reactions to occur. Formate, acetate, lactate and other sources of reducing equivalents have been shown to increase TCE degradation. Chang and Alvarez-Cohen concluded that addition of formate to degradative experiments significantly enhanced the ability of cells to degrade TCE. (Chang and Alvarez-Cohen, 1997; Alvarez-Cohen and

McCarty, 1990). Similar trends were seen for resting cell suspensions supplied with 20mM lactate (Sun et al., 1995).

Finally, TCE and its breakdown products are inherently toxic to bacterial cells and cause substantial intracellular destruction (Wackett and Householder, 1989; Alvarez-Cohen and McCarty, 1991) and catalytic inactivation (Ensley, 1991). The toxic metabolites produced during TCE degradation inhibit the PMO enzyme, leading to decreases in the amount of phenol oxidized, reducing power generated, biomass produced and fresh PMO enzyme present for subsequent degradation (Ensley, 1991). In addition to the unstable TCE epoxide, it has been determined that accumulating chloride ions are inhibitory to bacterial processes (Mars et al., 1995). Therefore, an improper ratio of contaminant to cometabolite and the depletion of nutrients, reducing equivalents and biomass are deleterious to cometabolic TCE degradation and must be optimized for efficient breakdown.

V. Compound C

Although methane, toluene and phenol are widely accepted cometabolites, they are regulated compounds and may exhibit deleterious effects on the environment in large quantities. Phenol has been demonstrated in laboratory and field applications to augment TCE degradation (Fries et al., 1997; Hopkins et al., 1993), however it is also recognized as an environmental toxin causing liver, kidney central nervous system and respiratory system damage in long-term exposure (ATSDR, 1989). Phenol addition to aquifers contaminated with TCE may be questioned since chlorination of groundwater containing phenol produces chlorinated phenols and leads to taste and odor problems (Hopkins et al., 1993). Likewise, methane is a widely accepted growth factor for TCE degradation, yet introduction of the explosive gas to field applications is obviously not favored. Seemingly, it is environmentally

counter-productive to introduce known toxins into an already polluted site in hopes of remediation. Experimental work is currently being done to develop new, effective compounds that will support faster, more effective and environmentally safe methods of TCE degradation.

Currently, in conjunction with BioRemedial Technologies Incorporated (BRT), I am testing the effectiveness of a proprietary cometabolite Compound C^{TM}, which preliminary studies have shown to be effective in removing TCE from contaminated soils and groundwater. Unlike phenol and methane, Compound C^{TM} is a non-toxic, food-grade material, which potentially can remove TCE from the environment without introducing additional chemical pollutants. Initial laboratory trials demonstrated that an indigenous culture amended with Compound C^{TM} resulted in 27% more TCE disappearance than a comparable culture amended with phenol (BRT unpublished report). Successful laboratory results led to the application of Compound C^{TM} in a field-scale study. A biological remediation system utilizing Compound C^{TM} was installed at a former RCRA drum storage pad, and an approximate area of 50,000ft^2 of contaminated soil and water was treated. Soil contaminated with 370ppm TCE and groundwater contaminated with 45ppm TCE were treated over a course of two years. Third party analysis of groundwater and soil demonstrated >99.6% removal of TCE from soil and >99.5% removal from groundwater. Although preliminary results show successful degradation of TCE in both the laboratory and the field, further research will be done to confirm preliminary data, assess the effectiveness of utilizing Compound C^{TM} in a novel site and optimize degradation with novel indigenous microbes.

VI. Community Structure

Successful remediation using cometabolic degradation depends on the presence, abundance and efficiency of suitable microbes possessing the appropriate enzymatic capabilities. As a contaminated site is augmented with cometabolites, the indigenous microbial community is manipulated and microbes capable of degrading the cometabolite are selected for. As a result of this manipulation, there are corresponding changes in the bacterial community structure over time. To characterize and identify the indigenous microbial population and determine the shift in the community structure following stimulation with cometabolic growth substrates, a variety of methods including biochemical analysis, Fatty Acid Methyl Ester (FAME) analysis and molecular identification are employed.

Biochemical analysis requires the growth, culturing and isolation of bacterial colonies to characterize microbes. Colonies can be initially differentiated by morphological analysis (color, texture, shape, etc.), and can be further characterized using a variety of biochemical tests designed to detect the presence or absence of characteristic enzymes. In addition to biochemical tests, FAME analysis is routinely used to identify and characterize microbes, providing information regarding genus and species (Heyrman et al., 1999; Descheemaeker, 1995). FAME was used to distinguish between and determine the genus of three bacteria involved in beer spoiling (Beverly et al., 1997). Likewise, the phospholipid fatty acids were analyzed to determine profiles of microorganisms residing in trichloroethylene degrading bioreactors (Phelps et al., 1990). Fatty acids are present in the phospholipid layer of the cellular membranes in all microorganisms, but vary in composition and abundance between species. By growing the bacteria in controlled environments, the fatty acid profile can be

generated and used to rapidly identify genus and species of large numbers of bacterial isolates.

Finally, the most widely used and accepted method for the characterization of microbes involves molecular methods, which include but are not limited to DNA extraction, Polymerase Chain Reaction (PCR), restriction enzyme digestion, cloning and DNA sequencing. Nucleic acid characterization of bacteria has become increasingly prominent over the last decade (Madsen, 1999). The genetic blueprint of all microorganisms is contained in deoxyribonucleic acid (DNA), which codes for three types of ribonucleic acid (RNA): messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA), respectively. The genes that code for the 16S subunit of rRNA (rDNA) are highly conserved throughout evolution and are frequently analyzed to determine phylogeny (Madsen, 1999). Molecular techniques of extracting community DNA, PCR amplification, cloning and DNA sequencing are highly favored over conventional methods of microbial identification because they do not encourage the introduction of as many sampling artifacts. For example, identification of a bacterial community using biochemical analysis relies on the culturability of the individual microorganisms. Less than 10% of total microorganisms present in a sample are culturable, and therefore culturing results in an under representation of the community (Dunbar et al., 1997). On the other hand, molecular techniques can be applied with minimal sampling and handling artifacts. RFLP was used to determine microbial the succession in a field site before and after augmentation with phenol (Fries et al., 1997). Likewise, RFLP, DNA sequencing and other molecular techniques were used by both Bakermans et al. and Reysenback et al., to determine the shift in microbial community structure of naphthalene-degrading microbes from a contaminated coal-tar-waste-site, and the

phylogenetic relationship of microbes in a mid-Atlantic ridge hydrothermal vent, respectively. (Bakermans and Madsen, 2002; Reysenback et al., 2000).

Additional techniques employed to analyze the 16S subunit of rDNA include both denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE). Instead of determining an RFLP pattern for individual cloned sequences, these techniques are used to identify a community 16S rDNA pattern. These techniques are therefore useful in determining the shifts in community structure over time. Although this procedure does not allow for direct analysis of predominant species by DNA sequencing, bands of interest can be excised, purified and sequenced to determine genus and species. Further identification and verification of microbes may be achieved through the combination of molecular methods and FAME (Dierksen, 2002; Hanan et al., 2001; Fries et al., 1997).

In this study, groundwater contaminated with TCE was received from a Department of Energy site in Ohio and similar molecular techniques were applied to analyze the microbial community and assess changes in community structure as a result of augmentation with a cometabolite in the laboratory. The microbial diversity of the indigenous population was monitored in the laboratory, before and after augmentation with site-specific concentrations of both phenol and TCE.

Chapter 2: Method Development and Biodegradation Assays

Specific Aims:

The goal of this research was to optimize the degradation of TCE by microbes amended with different cometabolites. Preliminary laboratory experiments and field studies demonstrated that growth of indigenous bacteria on experimental cometabolite Compound C™ substantially increased TCE degradation above that of experimental controls. Degradative assays using radiolabeled ^{14}C-TCE were developed to quantitate both the amount of TCE that was mineralized to CO_2, and the amount of organic acid intermediates produced. The rate of TCE degradation supported by potential cometabolites was determined and optimized to maximize TCE mineralization. Process optimization was attained by modifying several factors including the ratio of cometabolite to contaminant, the age of culture used in degradative assays, and the abundance of nutrients and reducing equivalents.

Materials and Methods

Sample Collection:

Site water was received from a TCE contaminated Department of Energy site on 10/2/01 and on 2/6/02, and was kept at 4°C.

Culture development:

In a preliminary experiment, four cultures amended with Compound C™, Compound C-723™, toluene, and dextrose, respectively were started from site water received on 10/2/01 to test the effectiveness of different cometabolites on TCE degradation, as described previously (Humphries et al., 2002).

Cultures used in all subsequent experiments received 125ml sterile basal salts media (BSM) (Appendix A) and 125ml site water. Cometabolic carbon sources were added at 100ppm three times a week. Cultures were maintained under semi-continuous growth conditions by performing serial transfers with fresh media three times a week.

^{14}C-TCE Mineralization Assay:

This assay was developed to determine rates of mineralization of TCE by monitoring the accumulation of $^{14}CO_2$ from ^{14}C-TCE. Similar methods of monitoring ^{14}C-TCE mineralization have been documented (Nelson et al., 1986, Uchiyama et al., 1992; Fogel et al., 1986; Humphries et al., 2002). For this study, incubations were performed in 10ml serum vials (Wheaton, NJ), which were capped with grey TFE-faced butyl rubber septa (Wheaton, NJ) and sealed with aluminum crimp caps (Fisher, NH). A 9 x 30mm glass shell vial (Daigger, IL) filled with a 2M KOH soaked filter paper wick was placed in each incubation vial. To each incubation vial, 0.8ml of culture and 0.2ml of BSM were added. All supplies were autoclave sterilized prior to addition of culture. Incubation vials were sealed and spiked with ~100,000CPM or 10-15µl ^{14}C-TCE (Sigma, MI) (Appendix B) through the septa to give a final concentration of 0.6ppm TCE in the liquid phase (based on site concentrations). Vials were incubated in the dark at room temperature throughout the incubation. At designated time points, vials were sampled by spiking 100µl 1M HCl through the septa. Acidification of vials forced all $^{14}CO_2$ produced to bind to the basic wick. After allowing one hour for equilibration, vials were opened and the wicks were removed and placed in scintillation vials containing 10ml scintillation fluid for analysis (Packard, 19900CA Tricarb Liquid Scintillation Analyzer).

To validate the mineralization protocol and assess the absorption of $^{14}CO_2$ and ^{14}C-TCE to the wick, two treatments, a $^{14}CO_2$ treatment and ^{14}C-TCE treatment, were set up in duplicate as follows. Each treatment received 1ml sterile water and a shell vial with a basic wick. Vials were sealed and spiked accordingly, where the first treatment designed to assess the binding efficiency of $^{14}CO_2$ was spiked with 220cpm ^{14}C-$NaHCO_3$, and the second treatment designed to assess binding of ^{14}C-TCE to the wick was spiked with 100,000cpm ^{14}C-TCE. Vials were allowed to equilibrate for one hour and were sampled as described above.

Mass Balance:

An additional protocol was employed during the sampling of the incubation vials to quantitate mass balance of the system. Prior to removing the wick, 0.5ml headspace was removed using a gas-tight 1ml glass syringe (Supelco, PA), and was placed into an airtight scintillation vial for counting. The total headspace of the incubation vial was multiplied by the counts in 0.5ml to determine total ^{14}C in the headspace. Upon removal of the headspace, the wick was removed and prepared for counting as described previously. Finally, to account for ^{14}C in the aqueous layer and adhered to the glassware, the incubation vial was filled with 10ml scintillation fluid and counted. The sum of the total headspace counts, the wick counts and the aqueous and glassware counts is equal to the total ^{14}C-TCE added minus ^{14}C-TCE lost from the system due to volatilization.

To validate the current experimental design and determine the total recoverable ^{14}C in the system, a small-scale experiment was set up in triplicate as follows. Sterile vials received 1ml of sterile deionized water, were sealed and spiked with 100,000ppm ^{14}C-TCE as

described previously. Vials were incubated at room temperature and sampled over a range of time points to determine ^{14}C-TCE loss from the system.

Organic Acid Extraction:

To account for organic acid intermediate production as a result of incomplete TCE degradation, the unreacted ^{14}C-TCE was separated from ^{14}C-organic acids. This procedure is set up the same as described above, but differs in the sampling protocol. Like the aforementioned procedure, at the appropriate time points, HCl was added through the septa to drive off the $^{14}CO_2$. The KOH soaked wick was removed and counted. The vial was resealed and 100µl of 2M KOH was injected through the septa, making the aqueous solution basic thereby causing the ^{14}C-organic acids produced as intermediates to remain in the aqueous layer. To extract the ^{14}C-TCE remaining in the aqueous layer, 1ml of hexane was added through the septa, and the vials were sonicated (VWR Scientific Aquasonic 750HT) for five minutes. The hexane and aqueous layers were transferred by pipette to a 5ml glass test tube. After allowing for separation of phases, the hexane layer was removed by pipette and placed in a scintillation vial for counting. The aqueous layer was returned into the original vial, and re-extracted with another 1 ml of hexane. This process was repeated three times. After the final hexane extraction was removed for counting, the aqueous layer was returned to the original vial, which was prepared for counting by filling with 10ml scintillation fluid.

The ability of hexane to act as a solvent in TCE extraction was verified as follows. Triplicate sterile vials received 1ml sterile water, were sealed and spiked with 100,000cpm ^{14}C-TCE. Vials were sampled by the aforementioned protocol to determine the amount of TCE remaining in the aqueous layer after triplicate hexane extractions.

A subsequent experiment was designed to confirm that any ^{14}C-organic acids produced as intermediates in ^{14}C-TCE degradation would remain in the aqueous layer and not be extracted during the hexane extractions. A set of duplicate vials containing 1ml sterile water was spiked with 9500cpm ^{14}C-Uracil-5-Carboxylic acid and extracted with hexane following the method described previously. The amount of organic acids extracted was determined by scintillation counting of the ^{14}C recovered in the hexane layers.

Most Probable Number (MPN):

The MPN method was set up in a sterile 50ml Erlenmeyer flask by adding 25ml sterile BSM and a variable concentration of carbon source (cometabolite). A 2mM solution of iodonitrotetrazolium (INT) was made by dissolving 30mg INT into 250µl dimethyl formamide (DMF) (Mosher et al., 2002). The INT was added to the BSM and was hand swirled. Next, 900µl of the solution was pipetted into sterile 1.5ml Eppendorf tubes. 100µl of test culture was added to the Eppendorf tubes in triplicate to produce 10^{-1} dilution tubes. The tubes were vortexed (Scientific Instruments Vortex Genie 2, model G560) for two seconds. Serial dilutions were made for the remaining MPN tubes to a final dilution of 10^{-6}. Dilution tubes were incubated at 30°C until no further color change to pink was observed, approximately two weeks.

A small-scale experiment examining the four treatments, Compound C-723 ™ (100ppm C), C-723 ™ (1000ppm C), 100ppm Glucose and a BSM, were set up as described above to test the effectiveness of MPN as a means for determining bacterial concentrations. Triplicate dilutions were made in each treatment from 10^{-1} to 10^{-6}. Color change was monitored over two weeks.

Gas Chromatograph Analysis of TCE Disappearance:

EPA method 551.1 (Appendix C) was modified and optimized for use on a Trace™ Gas Chromatograph, Model 2000, equipped with an electron capture detector (ECD) and a flame ionization detector (FID), in tandem. The TCE was eluted after 4.5 minutes using a glass capillary column [30m x 0.25mmID, 0.25um film thickness (Model EC-1; Alltech EconoCap, IL]. Helium was used as a carrier gas. The oven temperature held at 45°C for 4.50 minutes and increased at a rate of 100°C/minute to a final temperature of 150°C and held at that temperature for 2 minutes. The ECD was maintained at 200°C and had a base temperature of 250°C.

Trial experiments were designed to determine sensitivity and reproducibility of both the ECD and FID in detecting TCE disappearance using a 10ppm TCE standard gas (Supelco, PA). Controlled incubations were designed by adding 30ml sterile water to 60ml sterile serum vials. Vials were sealed with teflon-faced septa and aluminum crimp caps. Vials were spiked with 1-10ppm TCE, were inverted, incubated and sampled over the course of the incubation by injecting 250µl of headspace over the column. The area under the TCE peak was integrated (Xcaliber software package) and compared to a standard curve generated using the 10ppm TCE standard gas, and the total concentration of TCE was determined. The feasibility of using the GC as a method of TCE quantitation was determined by assessing the reproducibility of results, the sensitivity of the detectors and the degree of TCE loss from the system from repeated septa puncture.

Optimization of Cometabolic TCE degradation

Round 1 – Preliminary Assessment of Compound C™

Two different formulations of Compound C ™, C-723 ™ and C-Original ™, respectively, were experimentally tested to determine the level of TCE degradation supported. Methods, experimental design and treatment set-up are described previously (Humphries et al., 2002).

Round 2 – Optimization and Screening of Different Compound C ™ Variations

By adding nutrients and increasing miscibility with water, the two proprietary formulations of Compound C ™ were modified. Variations in existing formulas produced a total of seven experimental cometabolites, Compound C -Original ™, C-723 ™, C-62330 ™, C-T ™, C-C ™, C-155 ™ and C-156 ™, respectively. Cultures were prepared by amending 125ml site water and 125ml BSM with 100ppm of their respective cometabolites. Each culture was maintained in semi-continuous growth by tri-weekly serial transfer and supported between $3x10^6$ and $1x10^7$ CFU/ml. Each formulation was experimentally tested to determine the most effective growth substrates for TCE degradation. Incubation vials containing 0.8ml culture and 0.2ml BSM were spiked with ^{14}C-TCE, incubated at room temperature and sampled at different time points throughout the incubation as described above.

Round 3 – Quantitation of Organic Acid Intermediates Produced

Analysis of three cometabolite formulations supporting high levels of TCE mineralization, Compound C-723 ™, C-155 ™ and C-156 ™ respectively, was performed to account for the amount of ^{14}C-organic acids produced as intermediates of ^{14}C-TCE degradation. Five treatments, including the three cometabolite varieties, phenol positive control and groundwater negative control were set up in triplicate as follows. Sterile incubation vials received 1ml of the respective culture, and a basic wick. Vials were spiked

with ^{14}C-TCE, incubated at room temperature, and sampled at different time points following the organic acid extraction protocol described earlier.

Round 4 – Optimization of Culture Age

The level of TCE degradation supported by cultures two and a half hours, four hours and twelve hours after serial transfer with fresh media was experimentally monitored to determine the optimal growth period for maximal TCE degradation. Two cultures, groundwater amended with phenol and groundwater amended with C-156 ™, were maintained in semi-continuous growth and supported >10^7CFU/ml. At time 0, serial transfers were performed and cultures were fed 100ppm of their respective cometabolite. Treatment groups were set up as follows: 1- phenol culture two and a half hours after serial transfer; 2-phenol culture four hours after serial transfer; 3- phenol culture twelve hours after serial transfer; 4- C-156 culture four hours after serial transfer; 5- C-156 culture twelve hours after serial transfer; 6- unamended groundwater. Corresponding killed controls were performed for all treatments. To set up the experiment, 1ml of culture from each respective treatment was placed in sterile incubation vials. Vials were provided with a basic wick, spiked with ^{14}C-TCE, and incubated as described above. At the appropriate time points, vials were sacrificed and sampled following the organic acid extraction protocol. The level of ^{14}C-TCE degradation supported by each treatment was determined by analyzing the amount of $^{14}CO_2$ bound to the wick and the amount of ^{14}C-organic acids present in the aqueous layer.

Round 5 – Optimization of Cometabolite to Contaminant Ratio

In order to control the ratios of cometabolite to TCE, cultures were centrifuged and residual cometabolite was washed from the cells prior to use in incubation studies following similar methods from the literature (Folsom et al., 1990; Newman and Wackett, 1996).

Using data collected from the optimal culture growth studies, cultures were sub-cultured into fresh media and fed cometabolite four hours prior to the set-up of the study. Culture was harvested in a refrigerated centrifuge (Sorvall RC5B Plus) at 8,000rpm. The pellet was both washed and resuspended in the same volume of buffered water to remove residual cometabolite. Cometabolite was then added in a known ratio to TCE. Each 10ml vial received 1.8ml of harvested culture (approximately 10^7 CFU's) and 0.2ml of 1:10 diluted BSM. The vials received a glass shell vial filled with KOH-soaked wick, and were spiked with ^{14}C-TCE as described above.

Five treatments were designed to test the effect of different ratios of TCE to phenol on TCE degradation. Treatments included an unwashed phenol culture, a 1:1 TCE to phenol treatment, a 1:10 TCE to phenol treatment, a 1:20 TCE to phenol treatment and a 1:50 TCE to phenol treatment. In a parallel but independently performed experiment four additional treatments were set up to test the effect of different ratios of TCE to C-156 ™ on TCE degradation. Treatments included an unwashed C-156 culture, a 1:1 TCE to C-156 treatment, a 1:5 TCE to C-156 treatment and a 1:15 TCE to C-156 treatment.

Round 6 – Assessment of Additional Experimental Cometabolites

Additional compounds were tested for use as cometabolites for TCE degradation based on similarity in structure to common cometabolites. Groundwater was amended with 100ppm of sodium benzoate, 100ppm of anisole, 100ppm of limonene and 0.3ppm phenol plus 1ppm toluene (based on MCL's), respectively three times per week and were maintained in semi-continuous growth through tri-weekly serial transfer with fresh media. Incubations were set up and compared to a phenol positive control as described above, using 0.8ml of the respective culture and 0.2ml of BSM. Vials were incubated at room temperature and

sampled over a range of time points to determine their respective abilities to support TCE degradation.

Round 7 – Optimization with Nutrient Supplements and Amendments:

Further optimization was performed on four different cometabolic treatments by supplying nutrients and reducing equivalents to the incubations during the experimental set-up. Groundwater was amended with 100ppm phenol; 0.3ppm phenol plus 1ppm toluene, 100ppm C-156 ™ and 100ppm C-723 ™, respectively. Cultures were maintained in semi-continuous growth, and were serially diluted 4 hour prior to the set up of the incubations. Incubation vials were set up for each treatment as follows. 0.8ml culture and 0.2ml BSM and a basic wick were added to sterile vials. Prior to spiking with ^{14}C-TCE, either lactate, formate, acetate, YEP or nicotinamide, were added through the septa to final concentrations similar to those suggested in literature of 33mM, 10mM, 5mM, 2ppm and 0.1mM, respectively (Folsom and Chapman, 1991; Alvarez-Cohen and McCarty, 1990; Sun and Wood, 1995; Henry and Grbic-Galic, 1991). Vials were incubated and sampled over a range of time points to measure the increase in TCE degradation due to the various culture amendments.

Assay Validation and Optimization Results and Discussion:

^{14}C-TCE Mineralization:

The treatment vials spiked with 220cpm ^{14}C-$NaHCO_3$ reported an average of 256.8cpm on the wick. The treatment vials spiked with 100,000cpm ^{14}C-TCE reported an average of 538.8cpm on the wick. These results show that ~100% of any $^{14}CO_2$ that is produced as a result of ^{14}C-TCE mineralization was captured and counted on the wick with current methods. Additionally, background levels of 0.5% of ^{14}C-TCE added to the

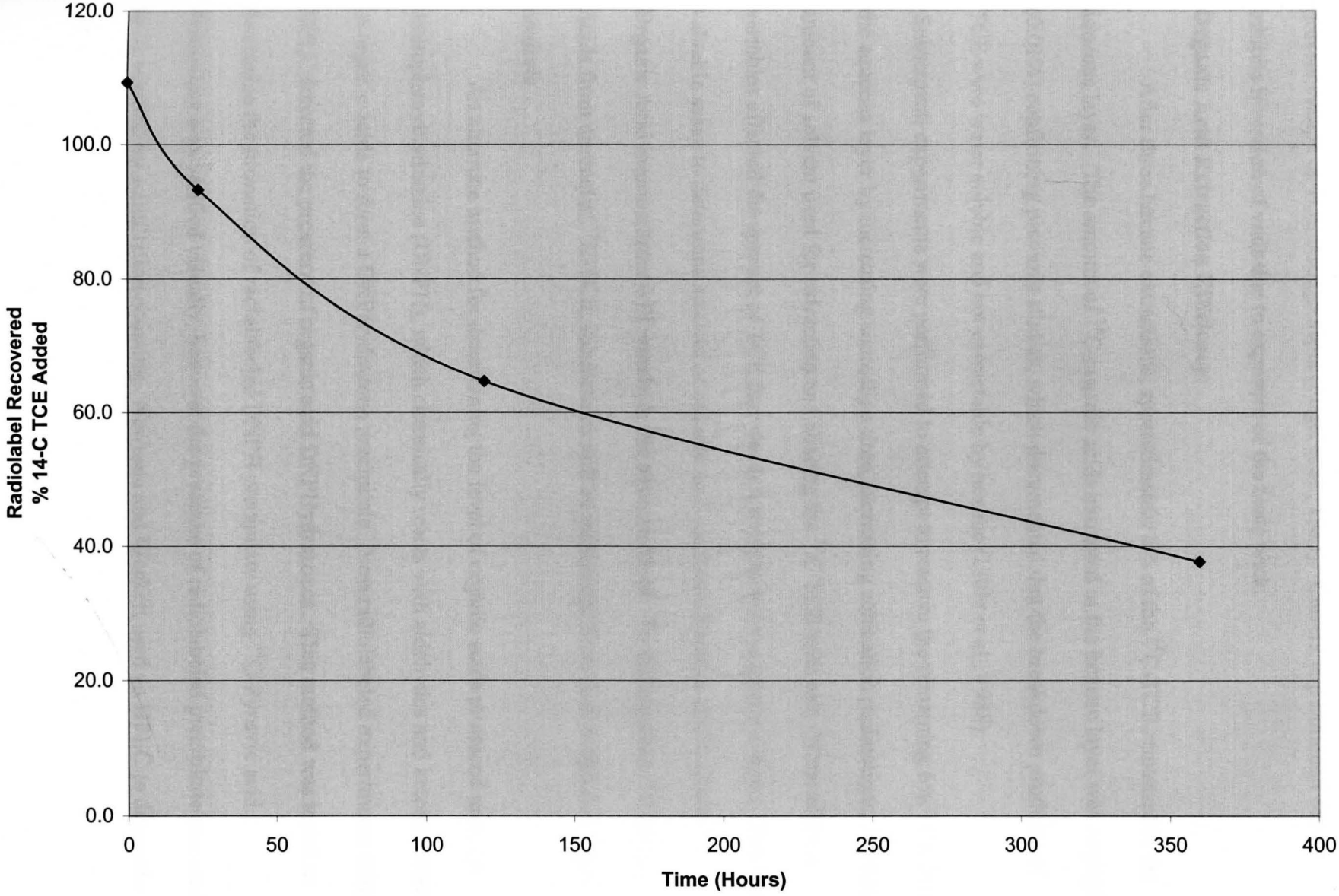
incubation vials will adhere to the wick. To differentiate between $^{14}CO_2$ produced and ^{14}C-TCE background counts, treatment vials were compared to killed controls.

This assay effectively quantitates TCE mineralization by monitoring the accumulation of the end product of TCE degradation, $^{14}CO_2$. $^{14}CO_2$ produced during ^{14}C-TCE mineralization is accurately captured on the basic wick at 100% efficiency. This assay does not account for the production of intermediate breakdown products associated with TCE degradation. Only one-third of breakdown products will go to CO_2, where the remaining two-thirds will go to metabolic byproducts and biomass (Cox et al. 1994). During attack by oxygenases, TCE is sequentially degraded from TCE epoxide, to organic acid intermediates like formate and glyoxylate, and finally to non-toxic CO_2. Therefore, accounting for the production of radiolabeled byproduct intermediates would better represent the rate of TCE degradation occurring. To quantify organic acid intermediate formation, the aforementioned procedure was modified to account for the presence of radiolabeled byproducts.

Mass Balance:

By accounting for TCE adsorption to the glassware, partitioning into the aqueous and headspace phases and binding to the wick, recovery at time zero is 100%. This yield decreases as incubation time increases (Figure 5). TCE is most likely lost from the system due to volatilization and escape through the pierced septa. Nelson et al., attributed the volatile nature of TCE to explain a 53% loss of ^{14}C-TCE after a 2-day incubation (Nelson et al., 1986). Although teflon septa with crimp caps have been employed to maintain air-tight seals in similar systems (Heald and Jenkins, 1994; Uchiyama et al., 1992; Alvarez-Cohen and McCarty, 1990), frequent leakage occurs after a single puncture with syringe (Fan and Scow, 1993). A possible solution to retain lost TCE from the system requires inverting the vial to

Figure 5: Validation of mass balance assay to determine the percentage of ^{14}C-TCE lost from the system due to volatilization.
Mass balance was experimentally monitored to determine the percentage of ^{14}C-TCE lost from a sterile, sealed system over the course of 360 hours.


120.0
100.0
80.0
60.0
40.0
20.0
0.0
Radiolabel Recovered
% 14-C TCE Added
0
50
100
150
200
250
300
350
400
Time (Hours)

prevent escape of TCE through septa (Fogel et al., 1985). Current experimental design inhibits inversion of vials due to exposure of the basic wick.

Organic Acid Extraction Efficiency:

After three hexane extractions, approximately 6% of the ^{14}C-TCE remained in the aqueous layer. The amount of ^{14}C-organic acids extracted in the hexane layer was negligible (0.01%) confirming previous studies, which documented that the breakdown products of TCE were water soluble and not extractable by hexane (Little et al., 1988).

Subsequent experiments were performed to attempt to remove the remaining 6% TCE from the aqueous layer by increasing sonication time, increasing sonication replications, increasing amount of solvent used for extraction and chasing the ^{14}C-TCE with salt. None of these variables affected the amount of TCE that was left unextracted the aqueous layer. This is a valuable assay to determine amount of organic acid produced because 100% of the ^{14}C-Organic Acid intermediates will remain in the aqueous layer. To differentiate ^{14}C-Organic Acids from unreacted ^{14}C-TCE, incubations will be subtracted from their respective killed controls.

An alternate method for determining the level of organic acids produced uses 2,4-dinitophenylhydrazine (DNPH), which chemically reacts with aldehydes and ketones present in organic acids to form a DNPHydrazone precipitate. Non-radiolabeled experiments using a HPLC detected the presence of organic acid DNPHydrazones. This method was tested to determine the formation of radiolabeled DNPH precipitate using ^{14}C-Pyruvic acid. Precipitate was verified visually, however the presence of radiolabeled precipitate was unable to be verified by scintillation counting. Newman and Wackett used an HPLC to detect the

occurrence of ^{14}C-organic acid fractions (Newman and Wackett, 1996). Facilities were unavailable for further testing.

MPN:

Since the carbon sources of the Compound C ™ cometabolites are colloidal, it is impossible to detect visual bacterial growth without the aid of an indicating dye. Idonitrotetrazolium violet (INT) indicates metabolic activity by turning from clear to pink when it is reduced. Rapid color change throughout the dilutions was observed in the glucose positive control treatment after a 48-hour incubation indicating positive growth (Figure 6). The BSM treatments, which served as a negative control, remained clear throughout the incubation, indicating negative growth. The 10^{-1} dilution of both the 1000ppm and the 100ppm C-723 ™ treatment turned pink after 48 hours. Further incubation was required for complete color change for both of these treatments. After incubating for one week, all treatments demonstrated total color change. Further incubation served to increase the intensity of the color produced, but did not cause color change in higher dilutions. The 100ppm C-723 ™ treatment supported growth in the 10^{-3} dilution, whereas the 1000ppm C-723 ™ treatment supported growth in the 10^{-4} dilution. This is most likely due to amount of carbon.

This method is advantageous because it provides a means of gathering data regarding cometabolite-degrading bacteria that cannot be gathered by traditional plate counts. Previous studies have addressed the use of the GC to detect loss of toluene from a similar MPN system to indicate the presence of contaminant degraders (Mu and Scow, 1994). This colorimetric assay is favored due to the rapid two-week determination of bacterial concentrations as opposed to the 28-day incubation required for GC analysis. Our method was not used to

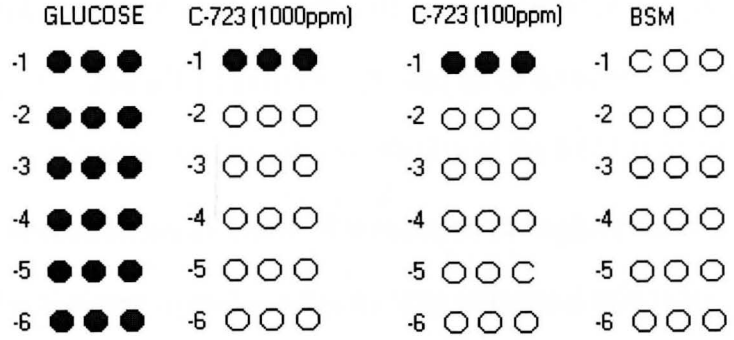
Figure 6 – Use of the most probable number assay to determine the success of INT as an indicator of microbial growth.

Legend:

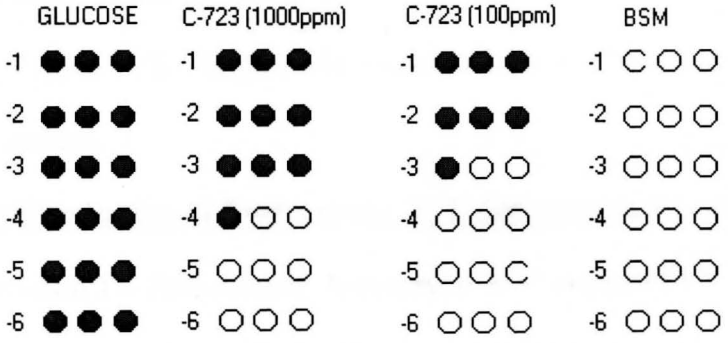
● = Positive Growth

○ = Negative Growth

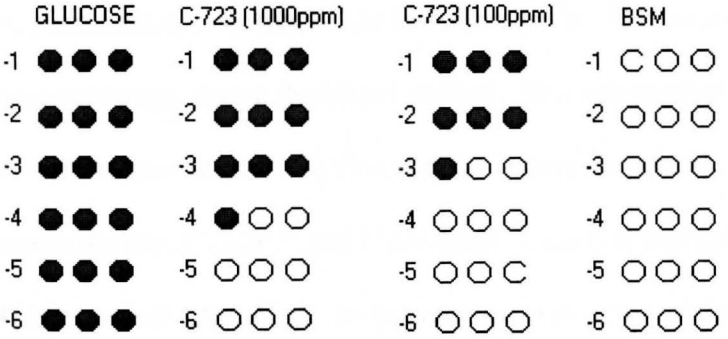
48-Hour Incubation



One-Week Incubation



Two week Incubation:



quantitate bacterial numbers in the further incubation studies. Focus was instead placed on heterotrophic CFU's to determine total culturable bacterial numbers.

Gas Chromatograph Analysis of TCE Disappearance:

The use of both the FID and ECD detectors were incompatible with the current experimental design. The sensitivity of the ECD allowed for detection of TCE at site concentrations of 1ppm. It was found through repeated sampling that the ECD did not allow for accurate reproducibility by way of manual injections. An autosampler was determined to be impractical for current applications due to incubation size restrictions. Being less sensitive than the ECD, the FID achieved more reliable and reproducible results by way of manual injection, however it was not sensitive enough to accurately detect the disappearance of 1ppm TCE. Higher site concentrations of TCE would be necessary for application of this method.

Optimization of Cometabolic TCE degradation Results and Discussion:

Round 1 – Preliminary Assessment of Compound C™

As shown in Figure 7, the Compound C™ treatment showed a 4.6% mineralization of TCE over that of the killed controls. Likewise, the Compound 723 ™ treatment exhibited a 3.8% mineralization over that of the killed controls. The toluene treatment showed no increase in mineralization above the killed control. The unamended treatment supported a 0.2% mineralization above its killed control (Humphries et al., 2002). It is apparent that cometabolites C-723™ and C-Original™ support increased mineralization above that of the killed control. Although the percent mineralization obtained from this study is lower than originally expected (based on preliminary BRT experiments), this data supports the fact that compounds C-723™ and C-Original™ may be valuable cometabolites and warranted further

Figure 7: Preliminary assessment of the levels of TCE degradation supported by two proprietary cometabolites, Compound C™ and Compound C-723™, compared to a toluene positive control.

Legend:

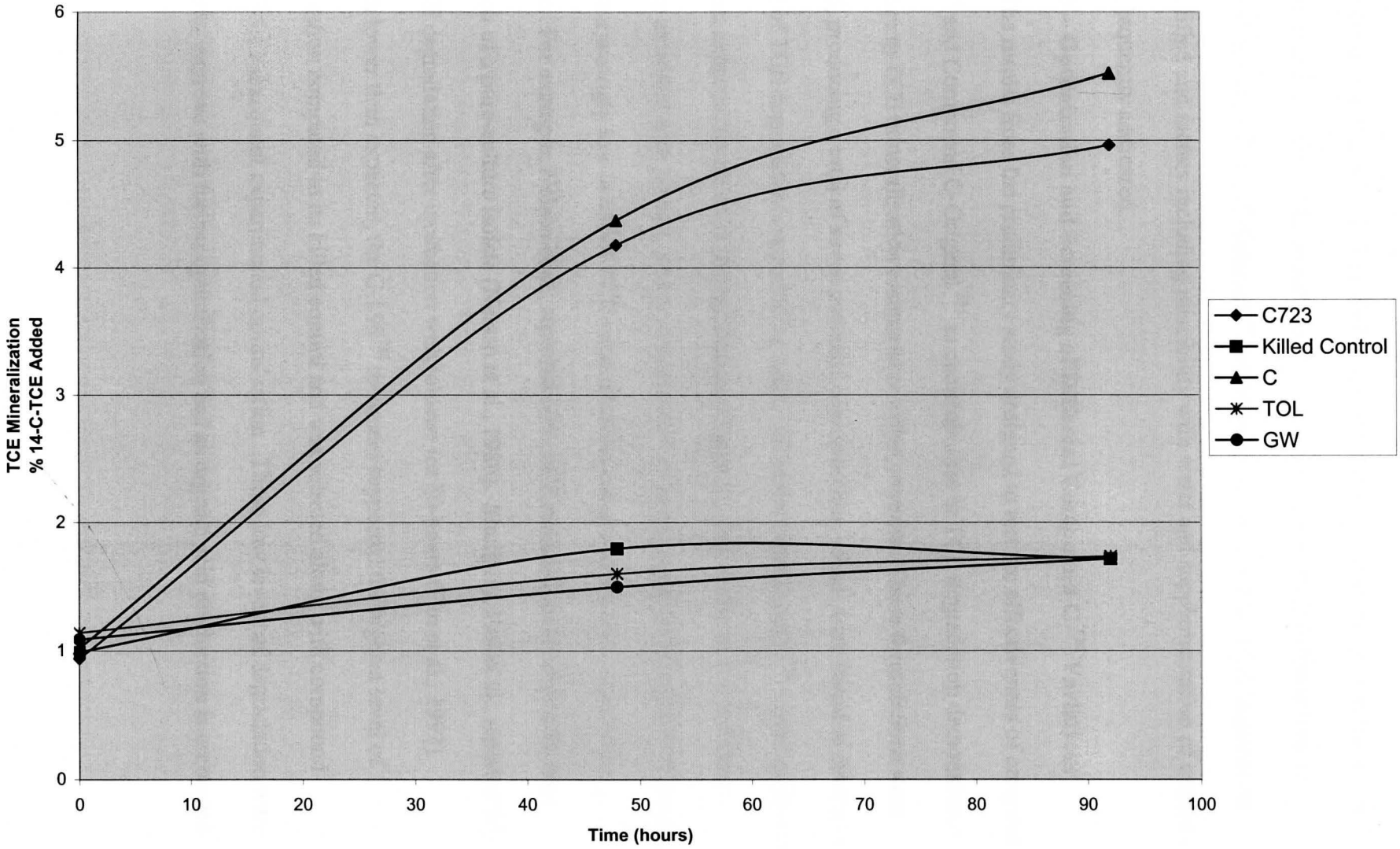
C-723 – represents the percentage of ^{14}C-TCE added to the Compound C-723 treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

Killed Control – represents the percentage of ^{14}C-TCE added to the Compound C-723 killed control treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

C - represents the percentage of ^{14}C-TCE added to the Compound C treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

Tol - represents the percentage of ^{14}C-TCE added to the Toluene treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

GW- represents the percentage of ^{14}C-TCE added to the unamended groundwater treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.


TCE Mineralization
% 14-C-TCE Added
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Time (hours)
C723
Killed Control
C
TOL
GW

study. Increased levels of TCE degradation and mineralization may be anticipated as the treatments and cometabolite formulations are optimized. The current formulations of cometabolites C-723™ and C-Original™, which support low levels of TCE degradation, were modified and factors including miscibility with water and supplementation of nutrients were subsequently addressed.

Round 2 – Optimization and Screening of Different Compound C ™ Variations

The results from the preliminary study designed to test the effectiveness of compound C-723 ™ and Compound C-Original ™ as cometabolites in TCE degradation demonstrated an increase in TCE mineralization compared to killed controls. These formulations were modified, producing a total of seven potential cometabolites, which were tested to determine the level of TCE degradation supported by each. All seven Compound C™ varieties showed lower than expected levels of TCE degradation (Figure 8). Likewise, the groundwater treatment amended with phenol, which was intended to be the experimental positive control, showed surprisingly low levels of TCE mineralization compared to those reported in literature. For example, Nelson et al., reported 60% TCE mineralization after a 48-hour incubation of a pure-culture isolate (Nelson et al., 1986). Similarly, Fan et al., reported 60-75% TCE degradation after incubation with toluene for 80-hours (Fan et al., 1992). Although lower than expected, the C-156™ treatment supported the highest level of mineralization compared to its killed control and was selected along with compound C-155 ™ for subsequent experimental optimization. These low levels of degradation were expected to improve with further optimization and as organic acid production is accounted for.

Figure 8: Extent of TCE mineralization supported by seven experimental variations of the proprietary cometabolite Compound C™ and compared to a phenol positive control.

Legend:

C-723 – represents the percentage of ^{14}C-TCE added to the Compound C-723 treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

C-C – represents the percentage of ^{14}C-TCE added to the Compound C-C killed control treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

C-155 - represents the percentage of ^{14}C-TCE added to the Compound C-155 treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

C-156 - represents the percentage of ^{14}C-TCE added to the Compound C-156 treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

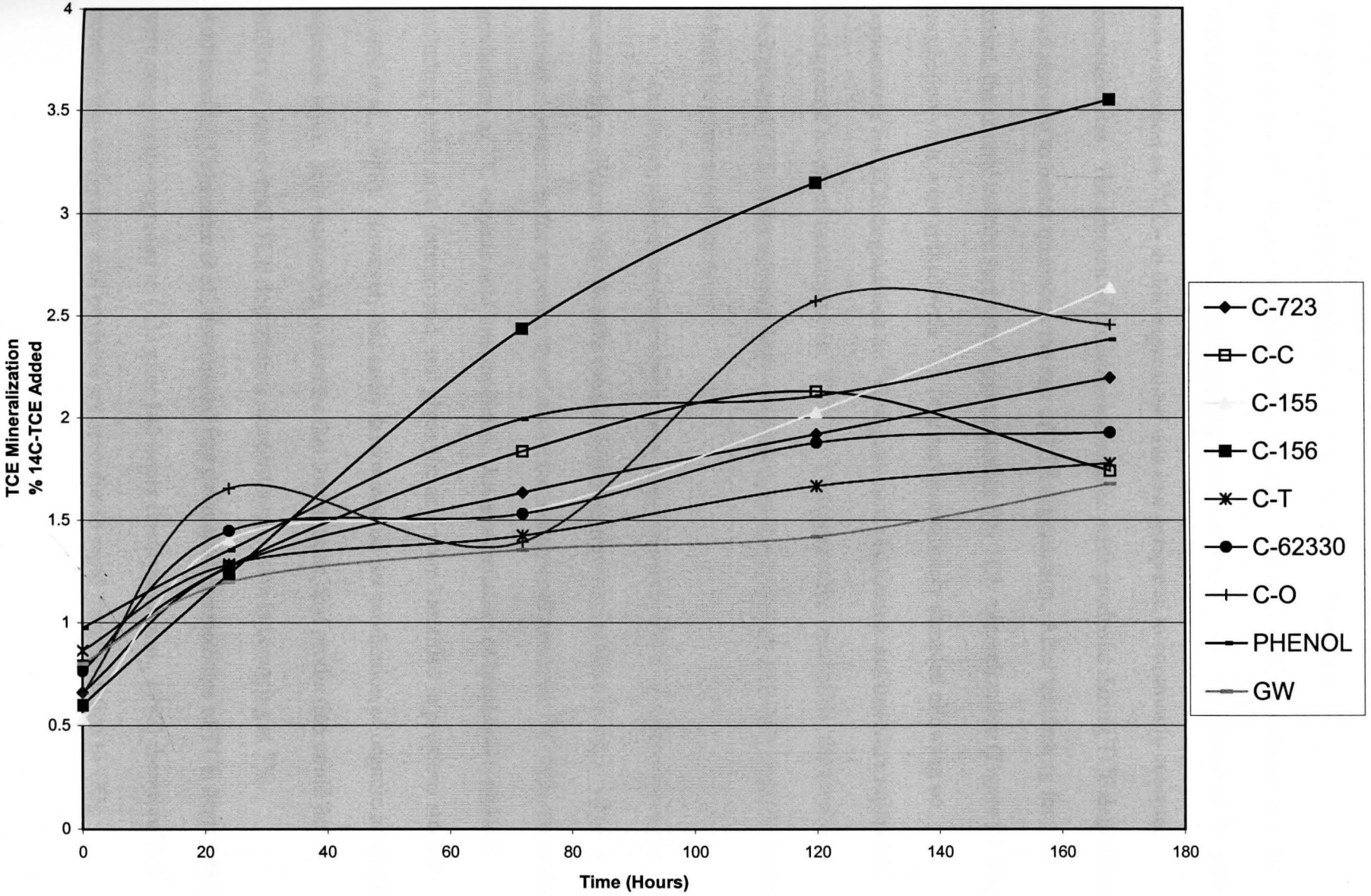
C-T- represents the percentage of ^{14}C-TCE added to the Compound C-T that was mineralized and trapped on the basic wick as $^{14}CO_2$.

C-62330 – represents the percentage of ^{14}C-TCE added to the Compound C-62330 killed control treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

C-O - represents the percentage of ^{14}C-TCE added to the Compound C-O treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

Phenol - represents the percentage of ^{14}C-TCE added to the Phenol positive control treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

GW- represents the percentage of ^{14}C-TCE added to the unamended groundwater treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.


TCE Mineralization
% 14C-TCE Added
Time (Hours)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
20
40
60
80
100
120
140
160
180
C-723
C-C
C-155
C-156
C-T
C-62330
C-O
PHENOL
GW

Round 3 – Quantitation of Organic Acid Intermediates Produced

By accounting for organic acid production, the total amount of TCE degradation is more realistically assessed. Consistent with the results from round 1 and 2, the level of ^{14}C-TCE mineralization as $^{14}CO_2$ in this experiment was low compared to commonly accepted cometabolites. The amount of radiolabeled organic acids produced during TCE degradation was assessed to better quantitate the rate of TCE degradation. After incubating for 160 hours, the phenol culture supported approximately 7% TCE mineralization (Figure 9). Day 0 incubation vials were spiked with ^{14}C-TCE and immediately sampled allowing no opportunity for TCE degradation or ^{14}C-organic acid production and therefore represent background levels of unextractable ^{14}C-TCE. Throughout the incubation, the level of background TCE in the aqueous layer decreased, except in the groundwater negative control where they remained constant.

On day 0, after extracting with hexane, approximately 5% TCE remained in the aqueous layer (Figure 10). Results were expected to show an increase in the level of radiolabel present in the aqueous layer, above the background levels of ^{14}C-TCE, signifying production of ^{14}C-organic acid intermediates. An accumulation of breakdown product including acetic acid, formic acid, and glyoxylic acid were identified in previous studies (Little et al., 1988). However, this assay determined no net production of organic acids in the aqueous layer. It is reasonable to assume that low organic acid production could be an artifact of low overall TCE degradation since mineralization is occurring at 7%. Additionally, Uchiyama et al., determined that the major intermediates of TCE degradation were completely degraded to CO2 after 140 hours (Uchiyama et al., 1992) therefore, it is possible that no organic acid products are seen due to rapid transformation to CO_2.

Figure 9: Extent of TCE mineralization supported by three Compound C™ varieties and phenol. Each treatment was subsequently sampled to determine the percentage of ^{14}C-organic acids produced (Data shown in Figure 10).

Legend:

C-723 CO2 – represents the percentage of ^{14}C-TCE added to the Compound C-723 treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

C-156 CO2 – represents the percentage of ^{14}C-TCE added to the Compound C-156 killed control treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

C-155 CO2 – represents the percentage of ^{14}C-TCE added to the Compound C-155 treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

Phenol CO2 – represents the percentage of ^{14}C-TCE added to the Phenol killed control treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

GW CO2 – represents the percentage of ^{14}C-TCE added to the unamended groundwater treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

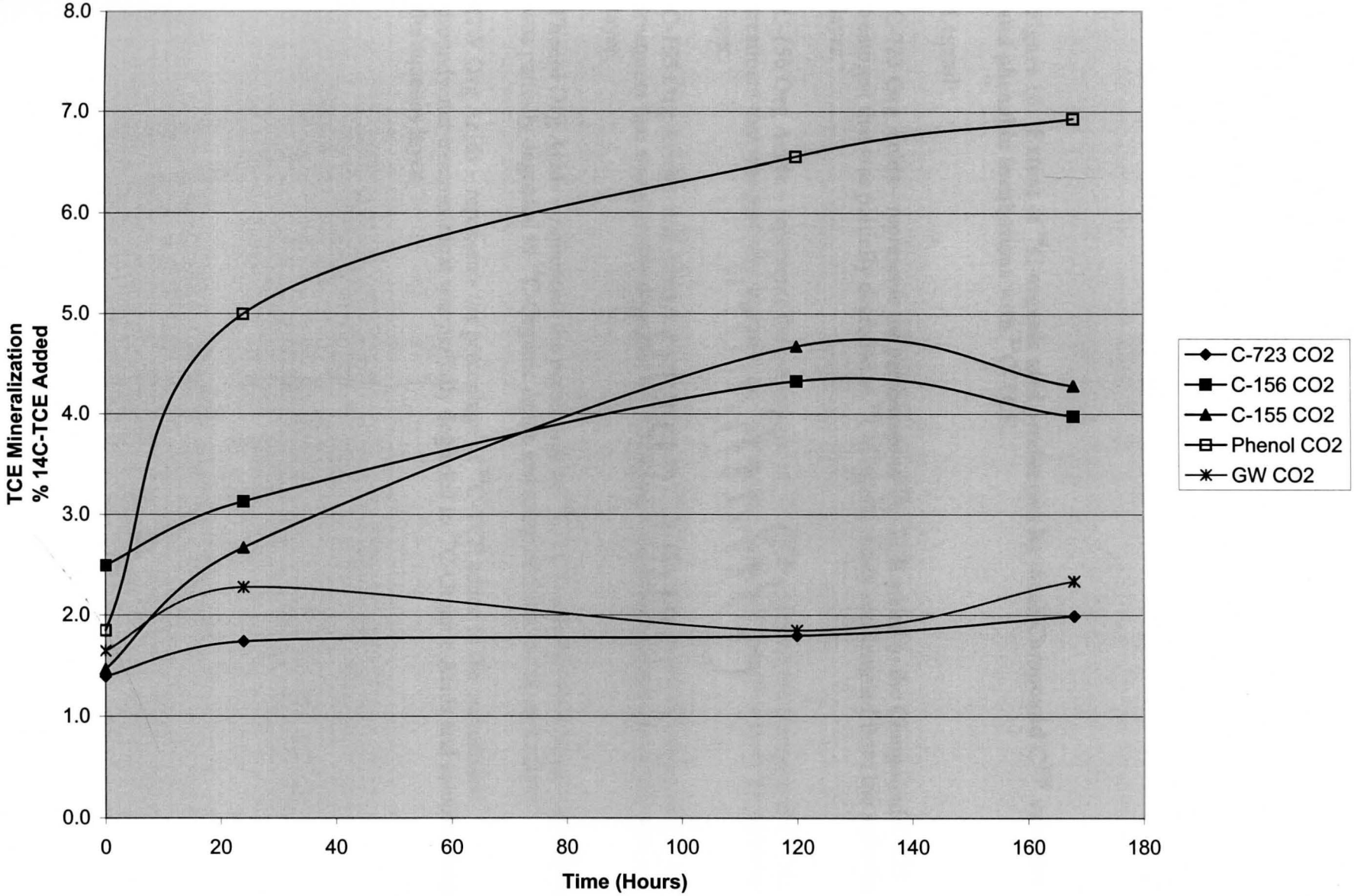

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
TCE Mineralization
% 14C-TCE Added
0
20
40
60
80
100
120
140
160
180
Time (Hours)
C-723 CO2
C-156 CO2
C-155 CO2
Phenol CO2
GW CO2

Figure 10: Extent of ^{14}C-organic acid production by three Compound C^{TM} varieties and phenol in incubations with ^{14}C-TCE.

Legend:

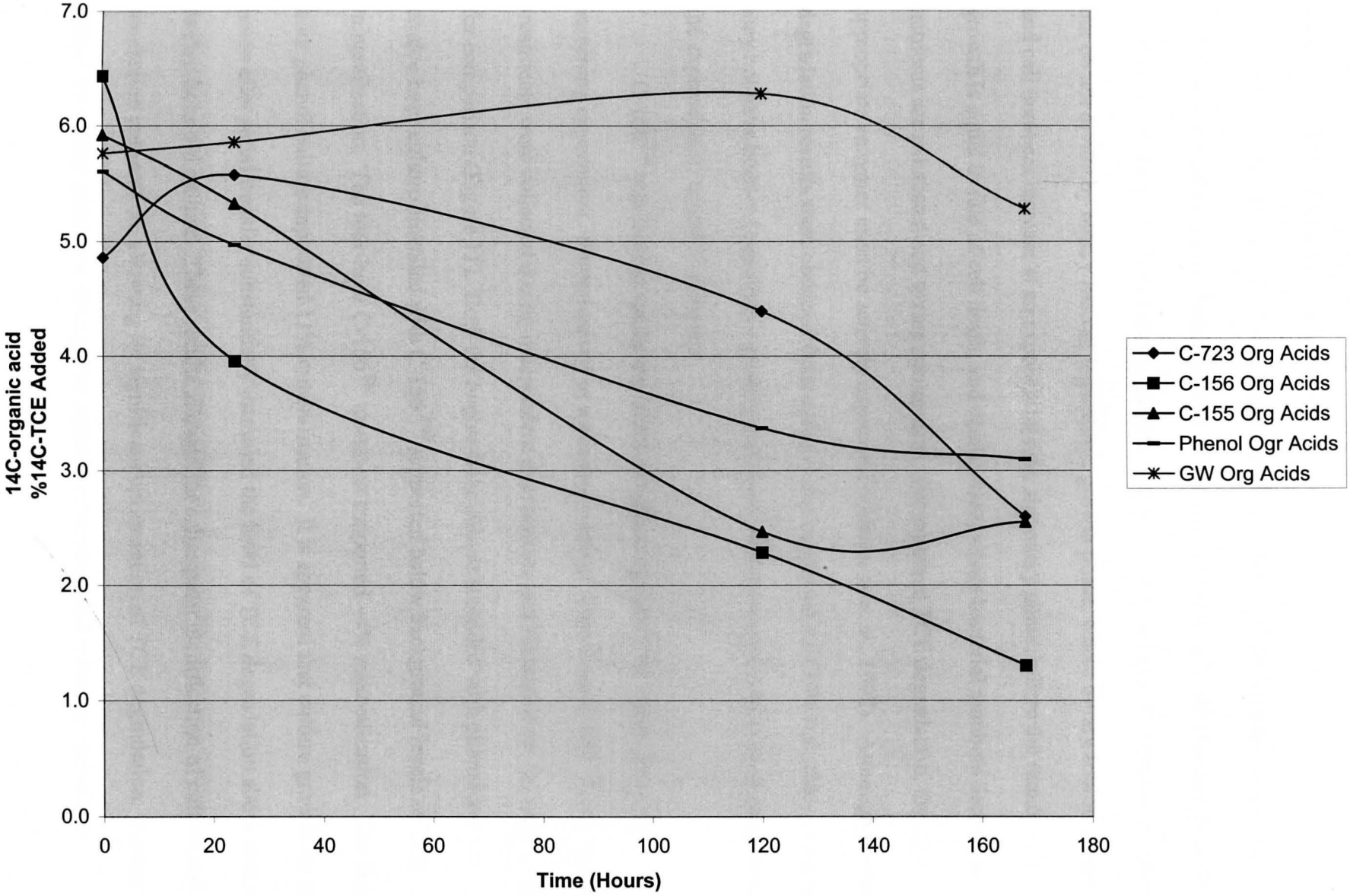
C-723 Org Acids – represents the percentage of ^{14}C-TCE added to the Compound C-723 treatment that was partially degraded to ^{14}C-Organic Acids and sampled from the aqueous layer.

C-156 Org Acids – represents the percentage of ^{14}C-TCE added to the Compound C-156 treatment that was partially degraded to ^{14}C-Organic Acids and sampled from the aqueous layer.

C-155 Org Acids – represents the percentage of ^{14}C-TCE added to the Compound C-155 treatment that was partially degraded to ^{14}C-Organic Acids and sampled from the aqueous layer.

Phenol Org Acids – represents the percentage of ^{14}C-TCE added to the Phenol treatment that was partially degraded to ^{14}C-Organic Acids and sampled from the aqueous layer.

GW Org Acids – represents the percentage of ^{14}C-TCE added to the unamended groundwater treatment that was partially degraded to ^{14}C-Organic Acids and sampled from the aqueous layer.


7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
14C-organic acid
%14C-TCE Added
0
20
40
60
80
100
120
140
160
180
Time (Hours)
C-723 Org Acids
C-156 Org Acids
C-155 Org Acids
Phenol Ogr Acids
GW Org Acids

Round 4 – Optimization of Culture Age

Serial transfer removes accumulated biomass, inhibitors and cellular toxins, and therefore approximates logarithmic growth of bacteria. According to the standard bacterial growth curve, there are four phases of growth: lag phase, where bacteria increase in cell size, but do not divide by binary fission; logarithmic growth phase, where binary fission occurs and cell numbers increase at an exponential rate; stationary phase, where the rate of cell growth is equal to rate of cell death; and death phase, where bacterial numbers decline as nutrients are exhausted and toxins accumulate. For maximal TCE degradation, the appropriate enzymes must be actively expressed (Uchiyama et al., 1992). Although previous degradation results were obtained from cultures that supported $>10^6$CFU/ml, these cultures may not have been in logarithmic growth, and therefore they may not have been supporting the expression of essential enzymes.

C-156^{TM} was used to assess the effect of culture age based of results from the screening experiment. Phenol served as a positive control. Data from C-156 ^{TM} and Phenol treatments were collected during independent experiments and illustrated on the same graph for comparison (Figure 11). Both the twelve-hour culture amended with phenol and the twelve-hour culture amended with C-156 ^{TM} supported below background levels of TCE mineralization. The four-hour C-156^{TM} treatment supported ~4% mineralization. The four-hour phenol culture supported 11% mineralization. It is apparent that culture growth for four hours after serial transfer substantially increased the level of TCE degradation above that of a twelve-hour old culture. These results allowed for subsequent modification of culture and incubation preparation, allowing for significant optimization of TCE degradation. Further optimization was attempted through the addition of nutrient and reducing equivalents.

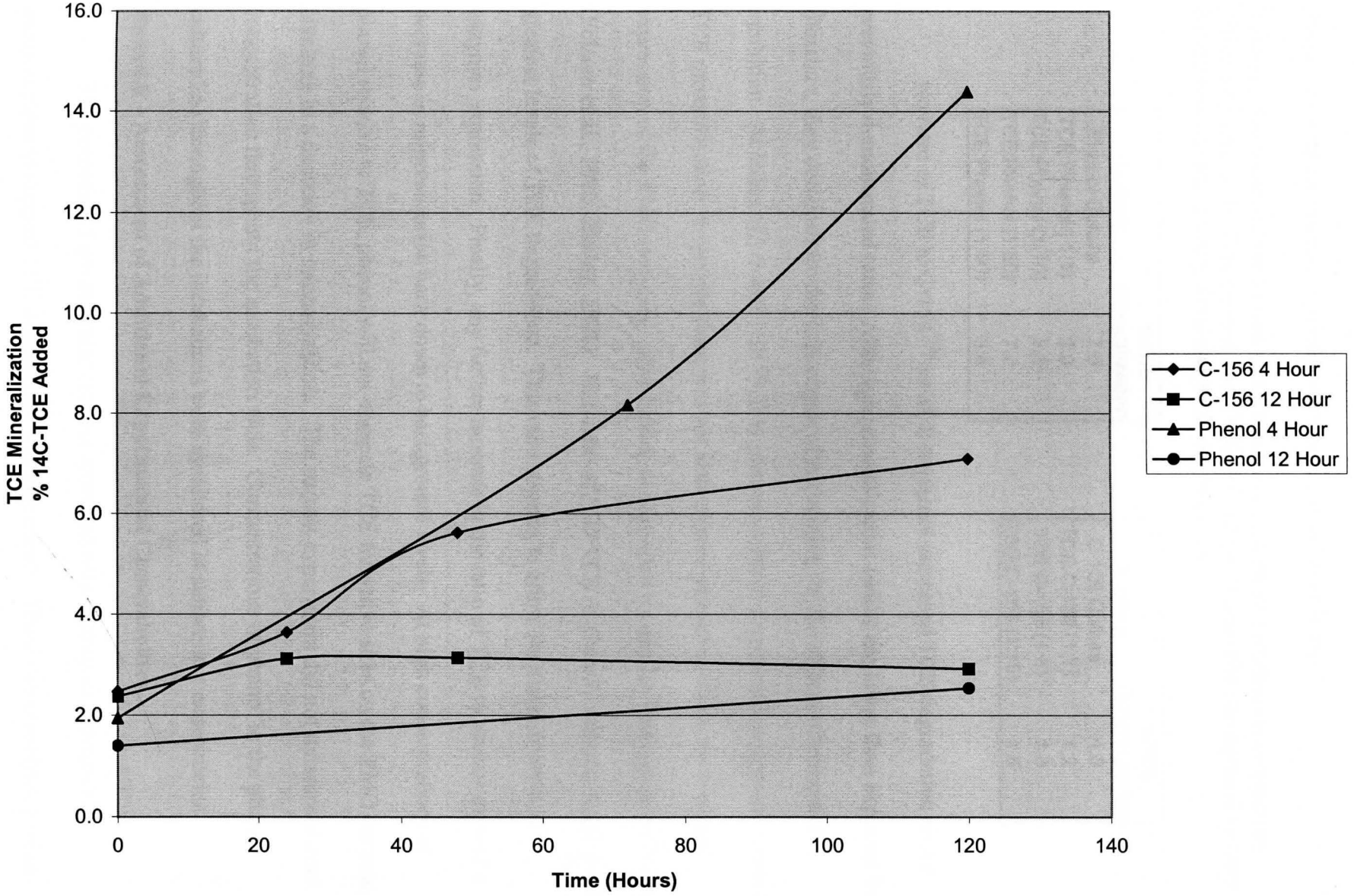
Figure 11 – Extent of TCE mineralization supported by treatments subjected to varied culture growth times to determine the optimal culture conditions.

C-156 4 Hour – represents the percentage of ^{14}C-TCE added to a 4-hour old Compound C-156 treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

C-156 12 Hour – represents the percentage of ^{14}C-TCE added to a 12-hour old Compound C-156 treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

Phenol 4 Hour – represents the percentage of ^{14}C-TCE added to a 4-hour old Phenol treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

Phenol 12 Hour – represents the percentage of ^{14}C-TCE added to a 4-hour old Phenol treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.


16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
TCE Mineralization
% 14C-TCE Added
0
20
40
60
80
100
120
140
Time (Hours)
C-156 4 Hour
C-156 12 Hour
Phenol 4 Hour
Phenol 12 Hour

Round 5 – Optimization of Cometabolite to Contaminant Ratio

Table 1: Summary of the extent of TCE mineralization by treatments containing different ratios of TCE to phenol and TCE to C-156 to determine the optimal culture conditions for stimulating maximal TCE degradation.

Culture	%TCE Mineralization
Phenol Culture	7.6
TCE:Phenol (1:1)	2.0
TCE:Phenol (1:10)	2.3
TCE:Phenol (1:20)	7.2
TCE:Phenol (1:50)	3.4

Culture	%TCE Mineralization
C-156 Culture	4.8
TCE:C-156 (1:1)	1.2
TCE:C-156 (1:5)	1.8
TCE:C-156 (1:15)	1.6

No ratio of TCE to C-156 ^{TM} or TCE to phenol increased TCE degradation above previously demonstrated rates. Although mineralization results are lower than expected from literature, they demonstrate the importance of accounting for the effects of competitive inhibition. At ratios of 1:1 and 1:10 TCE to phenol, cultures supported background levels of TCE mineralization. It appears there was too little phenol present to allow for maximal expression of the PMO enzymes. This finding is supported by similar findings in literature (Folsom et al., 1990; Ensley, 1991). At a ratio of 1:20 TCE to phenol, cultures supported the greatest levels of TCE degradation. This ratio appears to allow for a maximum level of enzymes expression. Finally, any further increase in the ratio of TCE to phenol showed a decrease in mineralization back down to background levels. At high concentrations of phenol relative to TCE, phenol will out-compete TCE for active sites on the PMO enzyme, resulting in a decrease in mineralization. The current experiment did not measure phenol concentration throughout the incubation vials. Concentrations of phenol in the phenol culture and throughout the incubations were monitored in subsequent experiments.

Round 6 – Assessment of Additional Experimental Cometabolites

The potential of groundwater cultures amended with various experimental cometabolites to support TCE degradation was monitored. These cometabolites, sodium

benzoate, limonene, anisole, and phenol plus toluene, respectively, were chosen for analysis due to structural similarity with aromatic compounds that have been proven to stimulate TCE degradation (Nelson et al., 1987) (Figure 12).

Table 2: Summary of the level of TCE mineralization supported by groundwater treatments augmented with experimental cometabolites after a 120-hour incubation with ^{14}C-TCE.

Culture	% TCE Mineralization
100ppm Anisole	1.4
100ppm Limonene	2
100ppm Sodium Benzoate	2.8
0.3ppmPhenol+1ppmToluene	10
100ppm Phenol	16.8

Although sodium benzoate, limonene and anisole all have similarities in structure to the aromatic ring seen in phenol and toluene, they did not support levels of TCE mineralization above background levels. The culture amended with a combination of 0.3ppm phenol and 1ppm toluene supported increased levels of mineralization comparable to that of the phenol positive control. The application of a cometabolic combination of phenol and toluene for in-situ TCE remediation is advantageous due to the low drinking water standards employed by the EPA for both phenol and toluene. If the TCE concentration is relatively high, phenol may not be able to be added to the appropriate levels without exceeding the maximal government regulated limits; however if a combination of phenol and toluene can be used to stimulate TCE degradation, each could be added in smaller concentrations.

Round 7 – Optimization with Nutrient Supplements and Amendments:

Using previously documented amendments from literature as a reference point, amendments including formate (Chang and Alvarez-Cohen, 1997; Alvarez-Cohen and McCarty, 1990), lactate (Sun and Wood, 1995; Folsom and Chapman, 1991) nicotinamide, acetate and

Figure 12: Structure of experimental cometabolites used in mineralization studies to determine effectiveness in stimulating TCE degradation by indigenous microbes.

Legend:

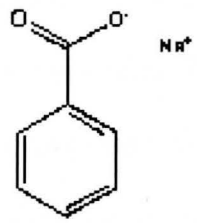
A. Sodium Benzoate

B. Anisole

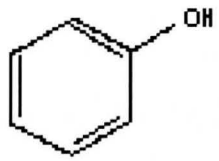
C. Limonene

D. Phenol

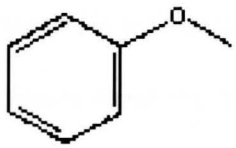
E. Toluene



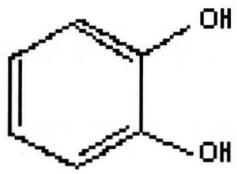
A. SODIUM BENZOATE



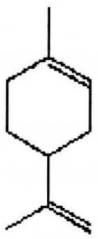
D. PHENOL



B. ANISOLE



E. TOLUENE



C. LIMONENE

vitamin supplements like YEP (Fogel et al., 1986) were experimentally tested to determine their potential effect on TCE degradation rates. Compounds such as formate serve to increase the reducing power available to augment the reducing power requirements for the different oxygenase enzymes (Chang and Alvarez-Cohen, 1997). As the enzymes degrade TCE, NADH is utilized and must be replenished for subsequent degradation. Formate, lactate and acetate are all tricarboxycilic acid intermediates, which can be metabolized to produce NADH. Following the same logic, cultures were augmented with YEP, which primarily served to act as a nutrient supplement for the indigenous culture. Because little is known about the community structure or nutrient demands of our TCE degrading bacterial cultures, it was reasoned that the addition of YEP may provide the culture with a limiting cofactor and serve to increase degradation levels. To prevent YEP from being used as a carbon source for bacterial growth, it was added in very low concentrations (1:50) relative to total organic carbon added.

Table 3 - Summary of the percent ^{14}C-TCE mineralization supported by cultures supplemented with nutrients and amendments after a 120-hour incubation.

Culture	Unamended	33mM Lactate	10mM Formate	5mM Acetate	2ppm YEP	0.1mM Nicotinamide
Phenol	16.8	13	8	10	8.7	14
C-723	1.6	1.5	N/A	1.9	N/A	2.3
Ph + Tol	10	5.7	N/A	4.5	N/A	12.9
C-156	3.8	N/A	N/A	N/A	3.6	N/A

Results indicate that none of the amendments increased TCE degradation above that observed in the unamended cultures. In the case of phenol, results seemed to decrease below the results obtained in the unamended control, after addition of the amendments. There may be some inhibition occurring due to the presence of the added amendment, however, the discrepancy is likely due to the experimental groups being set up and tested in different

experiments. A small amount of variability has been observed between cultures tested in different experiments due differences in enzyme activity and culture maintenance. Regardless of this variability, it is apparent that none of the amendments increased TCE degradation above that of the positive control. Similarly, previous studies documented that cultures supplied with amendments including glucose, lactose and acetate did not increase TCE degradation (Nelson et al., 1986). Conversely, studies detailed an increase in TCE degradation by cultures amended with formate to a saturating level of 20mM suggesting the reducing equivalent limitations were overcome at this concentration (Chang and Alvarez-Cohen, 1997). Therefore, the benefit of supplementing indigenous cultures with amendments is largely site-specific and dictated primarily by the limitation of reducing power in each individual system. The cultures from the current site did not appear to be limited by the availability of reducing power.

Chapter 3– Community Structure Analysis

Specific Aims:

The goal of this study was to analyze the effect of phenol addition on microbial community structure. Molecular techniques including RFLP and DNA sequencing of 16S rDNA were used to assess the microbial community structure of the three treatments, unamended groundwater, groundwater amended with phenol (Phenol Culture), and phenol culture after incubation with site concentrations of TCE (Phenol Culture + TCE), respectively. Phylogenetic trees were constructed to compare the shift in dominant species between each treatment. The radiolabeled assay described in Chapter 1 was used to draw a correlation between levels of TCE mineralization and resultant changes in the indigenous microbial community structure after augmenting with phenol and incubating with site concentrations of TCE.

Chapter 3 Materials and Methods:

Treatment Preparation:

Using site water received from a contaminated DOE site on 10/4/02, two treatment groups were set up as follows: 1- site water amended with mineral nutrients and fed 100ppm phenol 3x/week; 2- unamended site water. The phenol culture was maintained in semi-continuous growth by serial transfer as described previously.

^{14}C-TCE Assay:

To correlate community structure with TCE mineralization, a subset of treatment vials were set up as follows. As described previously in Chapter 1, 0.8ml of the respective treatment, 0.2ml of BSM and a basic wick were added to sterile incubation vials. The vials were sealed and spiked with ^{14}C-TCE to a final concentration of 0.6 ppm (reflecting site

concentrations). Autoclaved killed controls were preformed for each treatment. Vials were incubated in the dark at room temperature and sampled by sacrificing individual vials at different time points over seven days. As vials were sampled, mass balance was calculated as described previously. The level of ^{14}C-TCE mineralization was determined by analyzing the accumulation of $^{14}CO_2$ on the basic wick.

In addition to the complete set of incubation vials spiked with ^{14}C-TCE, a duplicate set of vials were set up for each treatment and their respective killed controls which were spiked with non-radioactive TCE at a concentration of 0.6ppm. These vials were sacrificed over the course of seven days and used to determine colony forming units (CFUs), and phenol concentration as described below.

Phenol Concentration:

A sub-set of non-radioactive vials were sacrificed on days 0, 2, 5, and 7. Phenol concentration in each treatment and corresponding killed controls were analyzed in triplicate using colorimetric test kits according to manufacturer's instructions (Chemetrics, VA). Vials were sacrificed at each time point to avoid loss of phenol from volatilization.

Plate Counts:

On days 0, 2, 5, and 7, treatment vials spiked with non-radioactive TCE were sacrificed and used for determination of CFUs. Both the culture and the sacrificed biotic treatment vials were serially diluted and plated on Plate Count Agar (PCA) to a dilution of 10^{-7}, while abiotic treatments were plated to a dilution of 10^{-1}. Plates were incubated at room temperature for a minimum of 48 hours before counting.

DNA Extraction:

On day 0, 6L of the unamended groundwater treatment and 9ml of the amended phenol treatment (phenol culture) were filtered with 0.22um Durapore membrane filters (Millipore, MA) and 60ml luer-lock syringes. Additionally, on day 7, 9ml of phenol culture was filtered from the sub-set of non-radioactive vials, representing the phenol culture + TCE treatment. Each treatment was aseptically filtered and filters were sealed in Ziploc baggies and placed at –80°C.

Bulk DNA was extracted from the filters following the procedure described previously (Hohnstock-Ashe et al., 2001). The procedure is summarized below. Filters from each treatment were aseptically crushed while frozen and placed in sterile 40ml centrifuge tubes. Next, 5ml of lysis buffer#1 (Appendix D), preheated to 85°C, was added to each centrifuge tube. The vials were vortexed and were placed in a boiling water bath for five minutes. After re-vortexing, the supernatant was removed, added to a fresh sterile tube, and placed on ice. The filter pieces were extracted three times with 5ml of lysis buffer #2 (Appendix D) by vortexing for 30 seconds. The four liquid extracts were combined and placed on ice for 15 minutes. 15ml of pH-equilibrated molecular biology grade phenol (Sigma, St. Louis, MO) was added, mixed vigorously, and placed on ice for 5 minutes. The mixture was centrifuged at 10,000xg (Sorvall RC5B Plus) at 4°C for 15 minutes, upon which time the aqueous layer was removed and placed in a separate tube. The aqueous layer was re-extracted with an equal volume of phenol-chloroform-isoamyl alcohol (125:25:1) and centrifuged at 10,000Xg for 15 minutes. The aqueous layer was removed and extracted with an equal volume of chloroform-isoamyl alcohol (25:1) and centrifuged again at 10,000Xg for 15 minutes. The aqueous layer was removed. The DNA in the aqueous layer was

precipitated overnight at –20°C with 0.5 volume of 7.5M ammonium acetate, 2 volumes of absolute ethanol and 2µl of 20mg/ml glycogen (Invitrogen, CA). Following the precipitation, the DNA was centrifuged at 12,000Xg at 4°C for 30 minutes and the supernatant was removed. The pellet was washed with 5ml 70% ethanol and centrifuged again for five minutes. The supernatant was removed and the tube was allowed to dry inverted in a sterile hood. The pellet was resuspended in 2.1ml of buffer #3 (Appendix D) at room temperature for 30min and then mixed by gently pipetting up and down. The DNA was reprecipitated in three eppendorf tubes overnight at –20°C by adding 1/10 volume of 3M NaOAC, 2 volumes of absolute ethanol and 1.5µl of 20mg/ml glycogen. Following the precipitation, the DNA was centrifuged at 4°C in a tabletop microcentrifuge (Sorvall, D-37520) for 30 minutes. The supernatant was removed and the pellet was washed with 70% ethanol at –20°C. After centrifuging for 5min, the supernatant was removed and the inverted tube was allowed to dry for 15min in a sterile hood. The DNA was resuspended by adding Tris buffer (Appendix D), incubated at room temperature for 30 minutes and gently pipetted up and down.

To verify successful extraction of DNA from each treatment, extraction product was PCR amplified (as described below) and quantitated by gel electrophoresis. The extracted DNA product was diluted by a factor of 1:1, 1:10, 1:100 and 1:1000, respectively to optimize the PCR amplification reaction. The optimal concentration of DNA was determined and used for subsequent reactions.

Polymerase Chain Reaction:

Table 4: Reagents for Polymerase Chain Reaction (PCR)

Reagent	Amount per RXN (µl)
H_20	**29.4**
10X PCR Buffer (Gibco)	**5**
50 mM $MgCl_2$ (Gibco)	**3**
10uM Primer 27F (IDT)	**0.2**
10uM Primer 1492R (IDT)	**0.2**
10mM dNTPs (Roche)	**2**
Taq Pol, 5U/µl (Gibco)	**0.2**

Each PCR reaction tube received 40µl of reaction mixture and 10µl of sample. The 27F (5'-AGA GTT TGA TCC TGG CTC AG- 3' [IDT, IA]) and 1492R (5' –GGT TAC CTT GTT ACG ACT T- 3' [IDT, IA]) primers are short pieces of DNA, which will recognize and bind to the DNA template (Reysenbach et al 2000). These primers are universal for eubacteria and have been shown to be successful in amplifying the 16S region of rDNA (Bakermans and Madsen, 2002; Reysenbach et al., 2000).

An MJ Research PTC-200 thermal cycler was used with the following program:

5 min @ 94°C – initial denaturation
30 sec @ 94°C – denaturation
1 min @ 55°C – annealing **35X**
1min @ 72°C – elongation
5 min @ 72°C – final elongation

Gel Electrophoresis:

The PCR product was run on a 1% agarose gel (High Resolution, Sigma, MO) at ~84V for 35min. The expected PCR product was ~1500bp. The gel was stained in ~1% ethidium bromide solution for 20 minutes. Using a UV light, the DNA bands were

visualized and compared to a molecular weight marker (Biomarker Low, Invitrogen, CA) to determine size of the PCR product.

Cloning:

Fresh 16S rDNA PCR product was ligated into plasmid pCR2.1 (TA cloning kit, Invitrogen, CA) following the manufacturer's instructions. The ligation reaction was set up as follows:

Ligation Reaction:

3µl	Fresh PCR product
1µl	10x Ligation Buffer
2µl	pCR2.1 vector (25ng/µl)
3µl	Sterile water
1µl	T4 DNA Ligase (4.0 Weiss units)
10µl	Final volume

The ligation reaction was transformed into INVaF one-shot competent *E. coli* cells following manufacturer's instructions. 25-100µl of the transformation product was spread on appropriately labeled LB+ampicillin plates and incubated inverted at 37°C for 24 hours. (Appendix E). Prior to plating of transformation product, the plates were spread with 40µl of 40mg/ml X-gal to allow for blue/white screening. Blue/white screening determines the presence of PCR insert within the transformed cells. The plasmid vector contains an intact Lac Z gene, which encodes for β -galactosidase. The enzyme β -galactosidase can metabolize X-gal, and cause a colormetric change from clear to blue. Successful insertion of the PCR product within the plasmid vector will disrupt expression of the lacZ gene and colonies will be white. For blue/white screening to be effective in determining presence of insert, ampicillin is used to inhibit growth of extraneous colonies. The pCR2.1 vector is resistant to ampicillin and allows for successful selection and maintenance of *E.coli* cells.

For each treatment, 80-100 white colonies were selected and isolated on fresh LB plates. Insert was verified for each clone by PCR amplification using the 27f and 1492r primers.

Restriction Digestion:

Each amplified clone was digested using the restriction enzymes HaeIII and CfoI (Roche Molecular Biochemicals, Boeringer Mannheim, Germany) as described in Table 5.

Table 5: Reagents for restriction digestion reaction

Reagent	Volume/reaction (µl)
H2O	9.6
10X Buffer	3
R.E. Hae III	0.2
R.E. Hha I	0.2
PCR Product	17

Restriction digestion reactions were incubated at 37°C for 2-4 hours. The reaction was halted with the addition of 6 µl 6X gel loading solution (Type I, Sigma-Aldrich, MO) and was stored at 4°C. Restriction digests were run on 3% agarose gel and RFLP patterns were compared to a 100bp ladder (Biomarker low, Gibco BRI). RFLP clone libraries were constructed for each treatment (groundwater, phenol culture and phenol+TCE). Statistical analyses, including the Shannon-Weaver diversity index (equation c) and % clone coverage (equation d), were determined. These measure the diversity of the community and the percent of the community sampled, respectively. (Bakermans and Madsen, 2002; Dunbar et al., 1999; Nubel et al., 1999; Ravenschlag et al., 1999).

Equation c: Clone Coverage

(C=[1-(S/N]*100%, where S is the number of species and N is the number of individuals)

Equation d: Shannon-Weaver diversity index

(H' = C/N NlogN- $\Sigma a_i \ln a_i$, where a_i is the proportional abundance of the ith RFLP).

The unique RFLPs were sequenced as described below and compared to the NCBI database to determine genus and species. Unique species from each treatment were compared and phylogeny was determined.

DNA Sequencing and Analysis:

PCR product from each clone generating unique RFLP patterns was purified using the Qiagen Purification Kit (Qiagen, CA) following manufacturer's instructions. Purified product was visually compared to a DNA size standard (DNA Biomarker Low, Invitrogen, CA) in a gel electrophoresis to determine the quantity of DNA template to be added to the DNA sequencing reaction.

The DNA sequencing reaction was prepared using the CEQ 2000 Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter Inc., CA) according to manufacturer's instructions. PCR dye labeling was amplified following the manufacturer's program (Beckman Coulter Inc.) using either primer 27f or 1492r. Within twenty-four hours of amplification, the DNA sequencing reaction was ethanol-precipitated following manufacturer's instructions (Beckman Coulter Inc.), resuspended in 40µl sample loading solution (supplied with kit) and stored at –20°C. Samples were sequenced on a Beckman Coulter CEQ2000XL Dye Terminator Cycle DNA Sequencer.

DNA Sequence Analysis:

Sequence chromatograms were converted to text files using Chromas (http://www.technelysium.com.au/chromas14x.html). Sequences were analyzed using the Check Chimera program of the Ribosomal Database Project (RDP). Sequences

determined to be chimeras were removed from further analysis. The sequence from primer 27f and the reverse compliment of sequence from primer 1492r were assembled by pairwise alignment and a consensus sequence (approximately 1400bp) was generated using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Consensus sequences were opened in ClustalX 1.8 for multiple sequence alignment. Phylogenetic trees were generated in ClustalX 1.8 and were manipulated using the neighbor-joining method in TreeView. *Methanosarcina lacustera*, an Archaebacterium, was used as an out-group to root the trees ensuring proper depiction of phylogenetic distances.

Chapter 3 – Results And Discussion:

^{14}TCE Mineralization:

On Day 0, the phenol treatment, groundwater treatment and their respective killed controls all supported approximately 1.7% mineralization (Figure 13). This background level of ^{14}C-TCE bound to the wick is present in all treatments and does not represent $^{14}CO_2$, as described previously. The groundwater treatment and killed controls remained at background levels throughout the incubation. The phenol treatment supported a maximal level of 14% mineralization after five days of incubation. The apparent drop in mineralization observed on day 7 does not represent a loss of mineralization, but rather is an artifact of natural variability between the discrete samples sacrificed at each time point. The level of TCE mineralization supported by this optimized phenol culture is lower than described in literature, however remains consistent with results previously obtained from this site.

Analysis of phenol concentration demonstrated that the amended groundwater culture fed 100ppm phenol contained 55ppm after four hours of culture growth. This loss in

Figure 13: Extent of TCE mineralization supported by an optimized phenol culture. This culture was subsequently manipulated to assess shifts in microbial community structure.

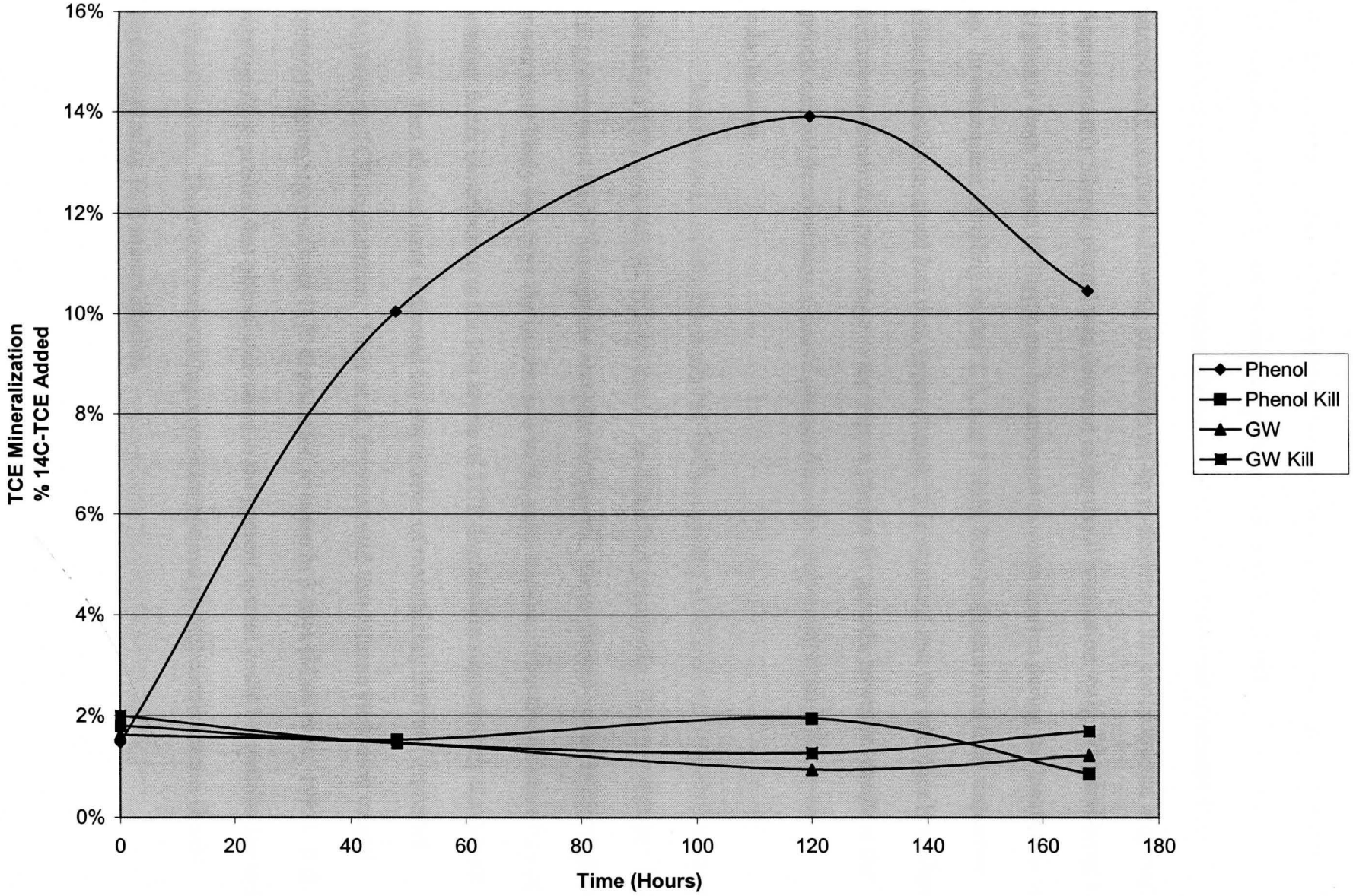
Legend:

Phenol – represents the percent of ^{14}C-TCE added to the phenol treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

Phenol Kill – represents the percent of ^{14}C-TCE added to the abiotic phenol treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

GW - represents the percent of ^{14}C-TCE added to the unamended groundwater treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.

GW Kill- represents the percent of ^{14}C-TCE added to the abiotic unamended groundwater treatment that was mineralized and trapped on the basic wick as $^{14}CO_2$.


16%
14%
12%
10%
8%
6%
4%
2%
0%
TCE Mineralization
% 14C-TCE Added
0
20
40
60
80
100
120
140
160
180
Time (Hours)
Phenol
Phenol Kill
GW
GW Kill

detectable phenol is due to both volatilization of phenol from the system and consumption by the microbes. On day 0, culture containing a detected level of 55ppm phenol was pipetted into incubation vials and sealed. Vials from each treatment were immediately sampled following incubation set-up to determine the concentration of phenol. Approximately 30ppm phenol was detected in the day 0 incubation vials. The additional loss of phenol from 55ppm to 30ppm can be attributed to volatilization during the incubation set-up. In subsequent sampling on day 2, 5, and 7 vials, both treatments and their respective killed controls contained less than 1ppm phenol. It is expected that the microbes in the biotic treatments acquired a percentage of the 30ppm phenol for growth, however loss from the abiotic control demonstrates a loss of phenol from the system and is most likely due to volatilization.

Mass balance results demonstrated 100% recovery of TCE from the system on day 0 but only a 57% recovery of TCE on day 7. As described previously, TCE is volatilized from the system most likely through the compromised septa. Since phenol is also highly volatile, it was most likely lost from the incubations in the same fashion. This loss of phenol could be a major factor contributing to the low levels of TCE degradation supported by the current system. Past studies have indicated the importance of maintaining actively expressed enzymes on TCE degradation. Mars et al. demonstrated that toluene starvation caused cultures expressing maximal TCE degradation to cease in 3 days (Mars et al., 1995). It is reasonable to predict that phenol starvation in the present system could be contributing to low mineralization. Therefore, maintaining a constant optimal phenol concentration should further optimize TCE mineralization.

Plate Count Results:

The unamended groundwater supported an average of 10^5 CFU/ml throughout the incubation. After amending the groundwater with 100ppm phenol three times per week, the phenol culture supported 10^8 CFU/ml. The phenol treatment supported 10^8 CFU/ml throughout the incubation even after spiking with TCE. The killed controls did not support growth.

The difference in biomass between the biotic phenol treatment and the biotic groundwater treatment could be a factor which led to the increased rate of TCE degradation. The groundwater treatment amended with phenol was compared to the unamended groundwater culture to determine the increase in mineralization supported by the augmented indigenous microbial community. It is important to differentiate between increased mineralization as a result of increased biomass and increased mineralization as a result of stimulation of enzymes capable of degrading TCE. In previous results documented in Chapter 1, phenol cultures supporting greater than 10^7 CFU/ml were shown to support less mineralization than the optimized culture used in the current experiment. Increased levels of TCE mineralization are a result of optimization of culture preparation and the maximal expression of TCE degrading enzymes rather than increased levels of biomass.

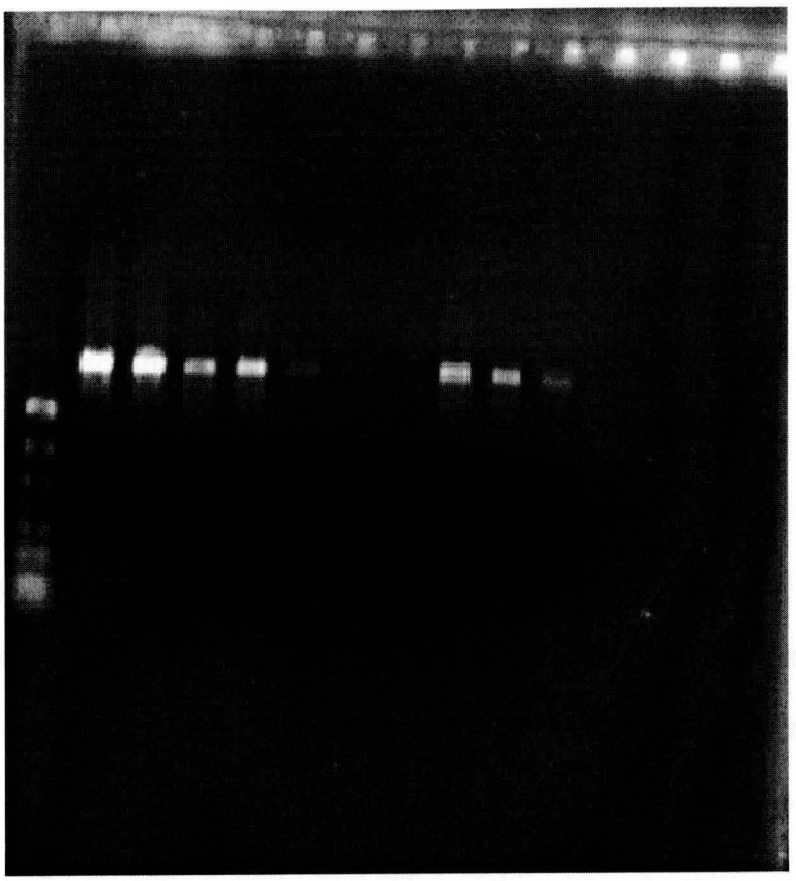
DNA Extraction Results:

Due to the presence of inhibitors in biological samples it is frequently necessary to dilute the extracted DNA and thereby dilute out any inhibitors that may be present. DNA extracted from the groundwater treatment was diluted to several concentrations, PCR amplified, and analyzed on a gel to determine the optimal concentration for PCR amplification (Figure 14). Concentrations of PCR product from the groundwater treatment

Figure 14: PCR amplification of 16s rDNA from varying concentrations of community extracted Groundwater DNA used to determine optimal PCR conditions.

Legend:

Lane 1 – 100bp DNA Ladder [(Invitrogen) 2072, 1500, 600, 100bp, respectively]
Lane 2 – + Control
Lane 3 – + Control
Lane 4 – Extracted Groundwater DNA diluted 1:10
Lane 5 – Extracted Groundwater DNA diluted 1:100
Lane 6 – Extracted Groundwater DNA diluted 1:1000
Lane 7 – Extracted Groundwater DNA diluted 1:10000
Lane 8-15 – Not Applicable



were undetectable at dilutions of 1:1000 and 1:10,000 (Figure 14, Lanes 6 and 7, respectively); however, was optimal at a dilution of 1:100 (Figure 14, Lane 5). Likewise, the phenol culture and phenol + TCE treatments were optimized at ratios of 1:100 (gels not shown).

Restriction Enzyme Digestion Results:

For each treatment, 80-100 clones were PCR amplified and digested with restriction enzymes. Restriction Fragment Length Polymorphisms (RFLPs) within each treatment were visually compared to a molecular size standard and were identified for uniqueness. To facilitate inter and intra-treatment comparison, unique RFLP patterns from each clone library were run side by side on common gels (Figure 15a-e). Although initial screening determined each RFLP to be unique in each of their respective treatments, secondary analysis on a common gel, provided evidence of identical RFLP patterns. For example, in the phenol + TCE treatment, RFLP patterns 1, 4 and 5 were initially determined to be unique, but upon further visual comparison on a common gel, have been determined to be identical (Figure 15e, Lane 2, 4 and 5 respectively). Likewise, phenol culture RFLP patterns 1, 2B, 2D and 4 (Figure 15c, Lane 2, 4, 5 and Figure 15d, Lane 2, respectively) were also determined to be identical upon further comparison.

After secondary comparisons, the occurrence of unique RFLPs in each treatment was documented and histograms of RFLP frequency were generated (Figure 16a-c). Statistical analysis determined the percent of each community sampled and the diversity of each clone library.

Figure 15a: RFLP's 2-6 generated from restriction digestion with HaeIII and CfoI of PCR amplified 16S rDNA groundwater community.

Legend:
Lane 1- 100bp DNA Ladder [(Invitrogen) 2072, 1500, 600, 100bp, respectively]
Lane 2- Groundwater RFLP pattern 2
Lane 3- Groundwater RFLP pattern 3
Lane 4- Groundwater RFLP pattern 3A
Lane 5- Groundwater RFLP pattern 5
Lane 6- Groundwater RFLP pattern 6
Lane 7- 100bp DNA Ladder
Lane 8- Blank

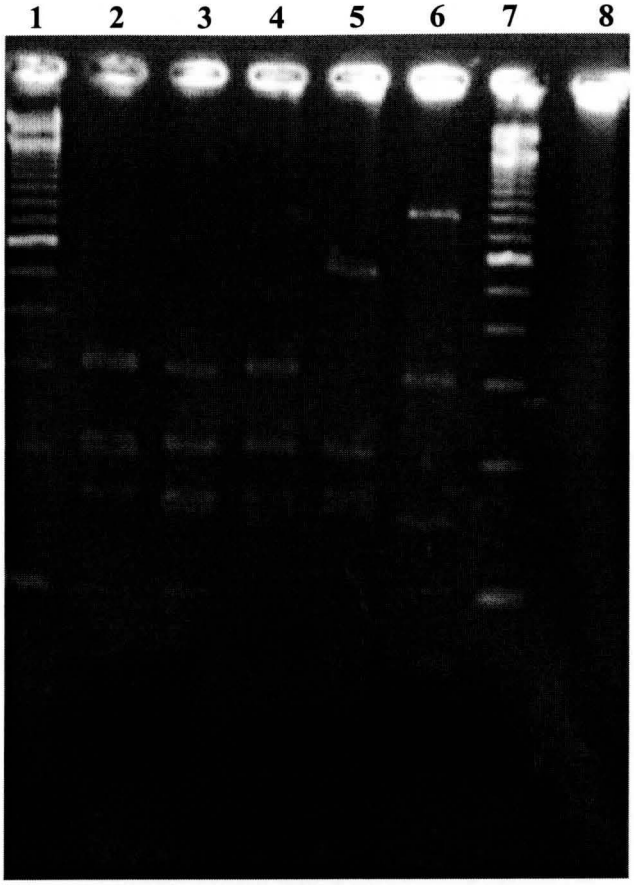

1
2
3
4
5
6
7
8

Figure 15b: RFLP's 9-13 generated from restriction digestion with HaeIII and CfoI of PCR amplified 16S rDNA groundwater community.

Legend:
Lane 1- 100bp DNA Ladder [(Invitrogen) 2072, 1500, 600, 100bp, respectively]
Lane 2- Groundwater RFLP pattern 9
Lane 3- Groundwater RFLP pattern 10
Lane 4- Groundwater RFLP pattern 11
Lane 5- Groundwater RFLP pattern 13
Lane 6- Biomarker
Lane 7- Blank
Lane 8- Blank

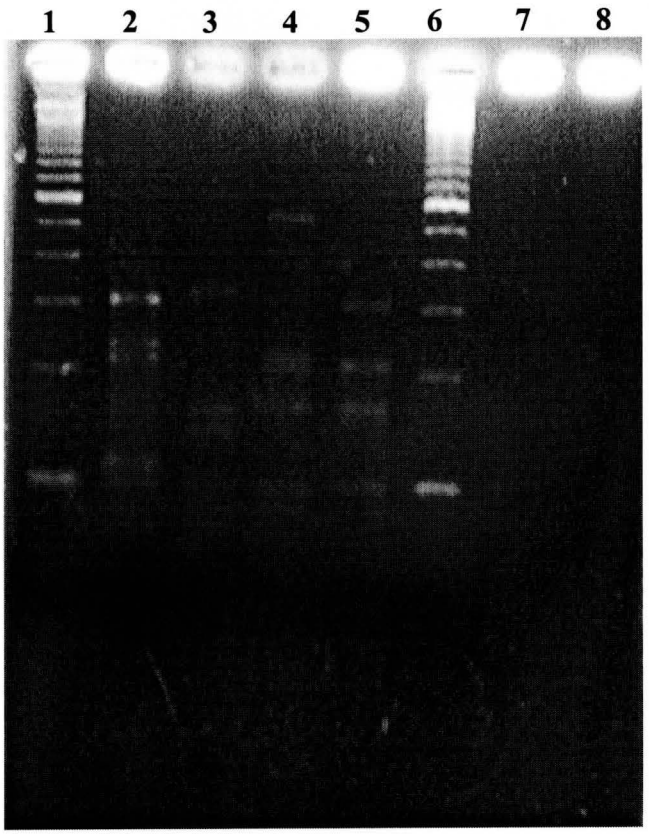

1
2
3
4
5
6
7
8

Figure 15c: RFLP's 1-2D generated from restriction digestion with HaeIII and CfoI of PCR amplified 16S rDNA phenol culture community.

Legend:
Lane 1- 100bp DNA Ladder [(Invitrogen) 2072, 1500, 600, 100bp, respectively]
Lane 2- Phenol Culture RFLP pattern 1
Lane 3- Phenol Culture RFLP pattern 2
Lane 4- Phenol Culture RFLP pattern 2B
Lane 5- Phenol Culture RFLP pattern 2D
Lane 6- Biomarker
Lane 7- Blank
Lane 8- Blank

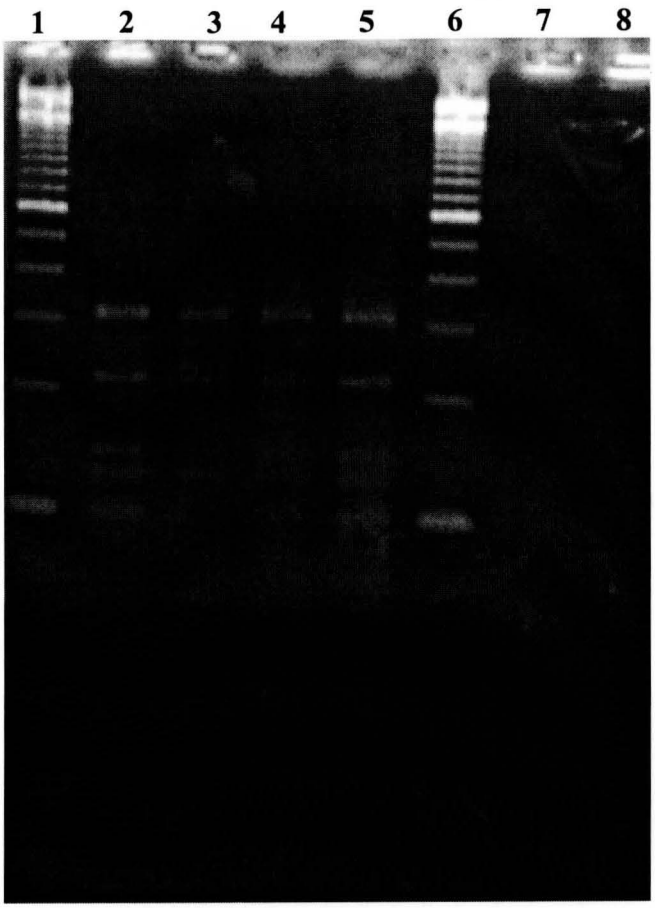

1
2
3
4
5
6
7
8

Figure 15d: RFLPs 4-10 generated from restriction digestion with HaeIII and CfoI of PCR amplified 16S rDNA phenol culture community.

Legend:
Lane 1- 100bp DNA Ladder [(Invitrogen) 2072, 1500, 600, 100bp, respectively]
Lane 2- Phenol Culture RFLP pattern 4
Lane 3- Phenol Culture RFLP pattern 6
Lane 4- Phenol Culture RFLP pattern 9
Lane 5- Phenol Culture RFLP pattern 10
Lane 6- Biomarker
Lane 7- Blank
Lane 8- Blank

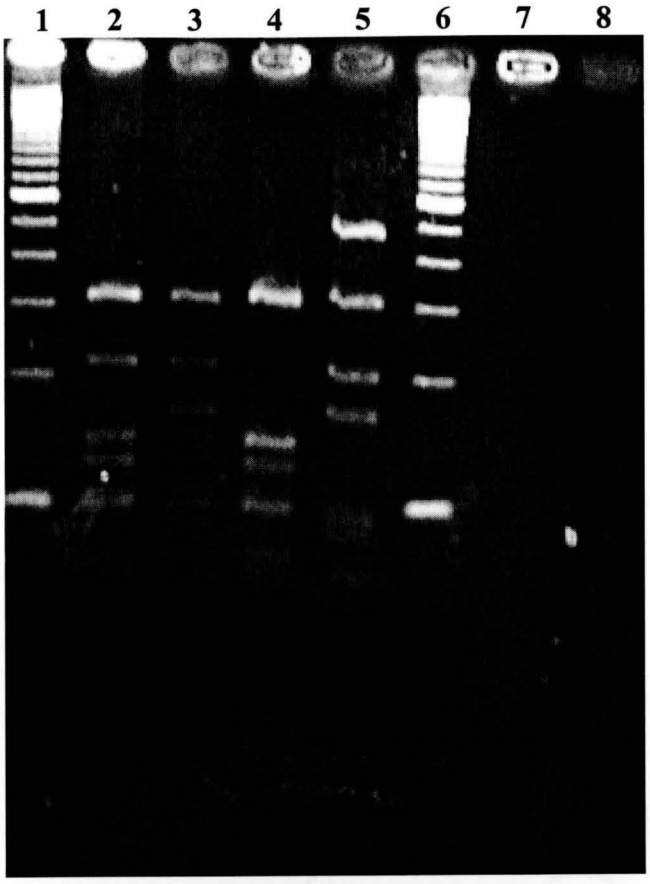

1
2
3
4
5
6
7
8

Figure 15e: RFLP's 1-8 generated from restriction digestion with HaeIII and CfoI of PCR amplified 16S rDNA phenol + TCE community.

Legend:
Lane 1- 100bp DNA Ladder [(Invitrogen) 2072, 1500, 600, 100bp, respectively]
Lane 2- Phenol + TCE Culture RFLP pattern 1
Lane 3- Phenol + TCE Culture RFLP pattern 2
Lane 4- Phenol + TCE Culture RFLP pattern 4
Lane 5- Phenol + TCE Culture RFLP pattern 5
Lane 6- Phenol + TCE Culture RFLP pattern 6
Lane 7- Phenol + TCE Culture RFLP pattern 8
Lane 8- Biomarker

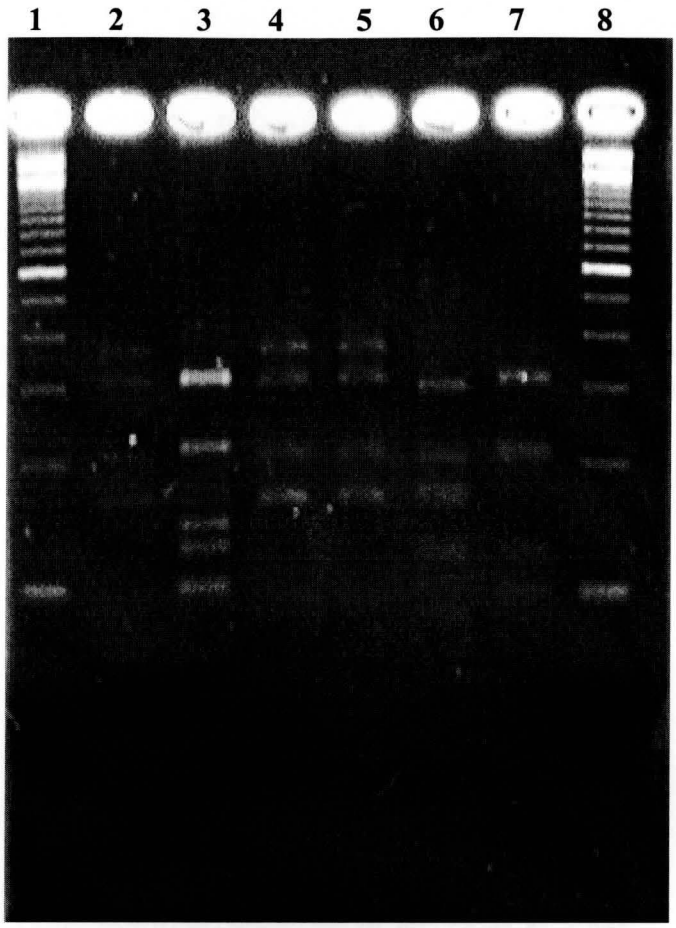

1
2
3
4
5
6
7
8

Figure 16a: Histograms of RFLP frequency illustrating the distribution of unique clones of PCR amplified ribosomal DNA extracted from the Groundwater community.

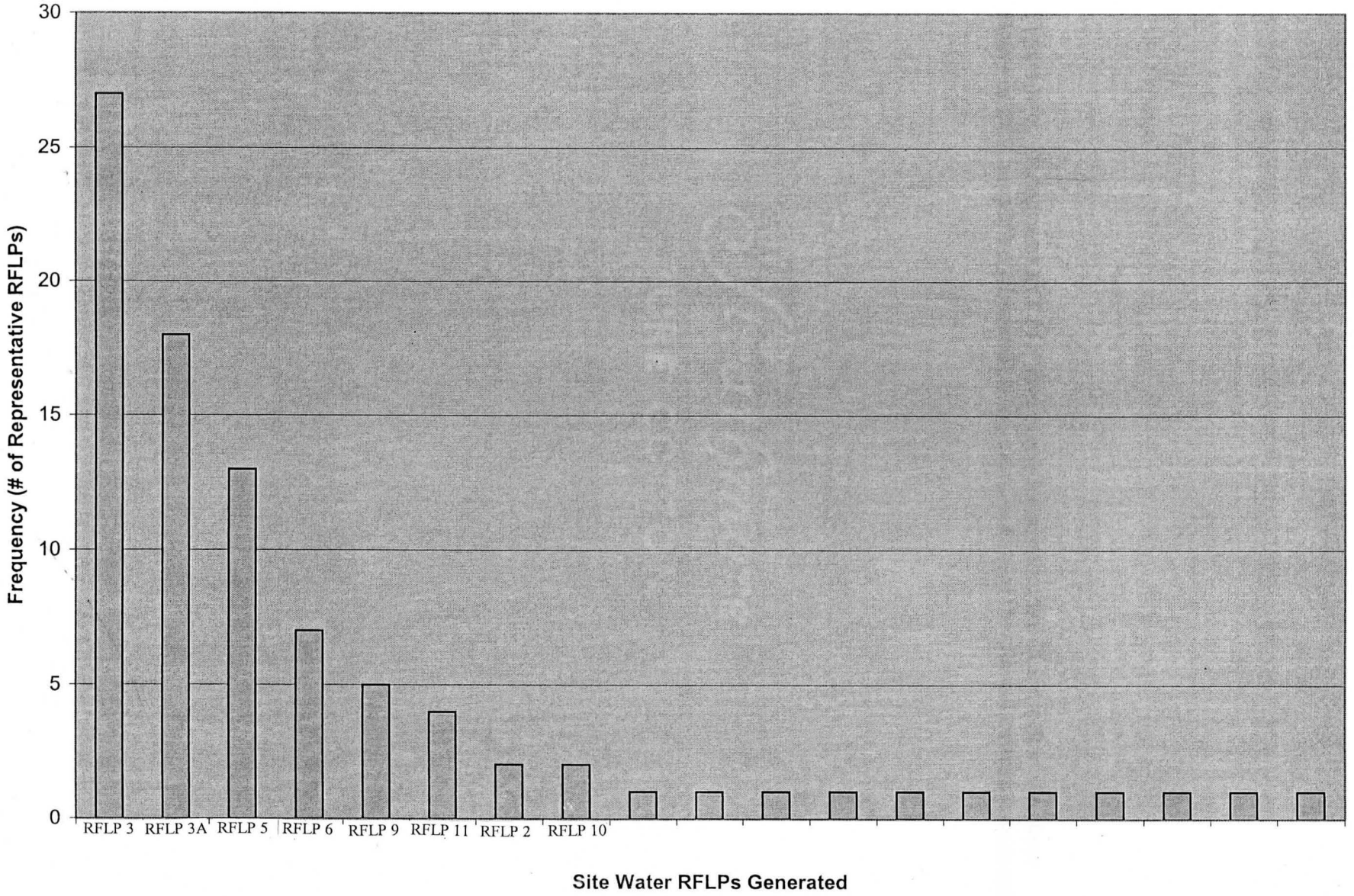

30
25
20
15
10
5
0
Frequency (# of Representative RFLPs)
RFLP 3
RFLP 3A
RFLP 5
RFLP 6
RFLP 9
RFLP 11
RFLP 2
RFLP 10
Site Water RFLPs Generated

Figure 16b: Histograms of RFLP frequency illustrating the distribution of unique clones of PCR amplified ribosomal DNA extracted from the phenol culture community.

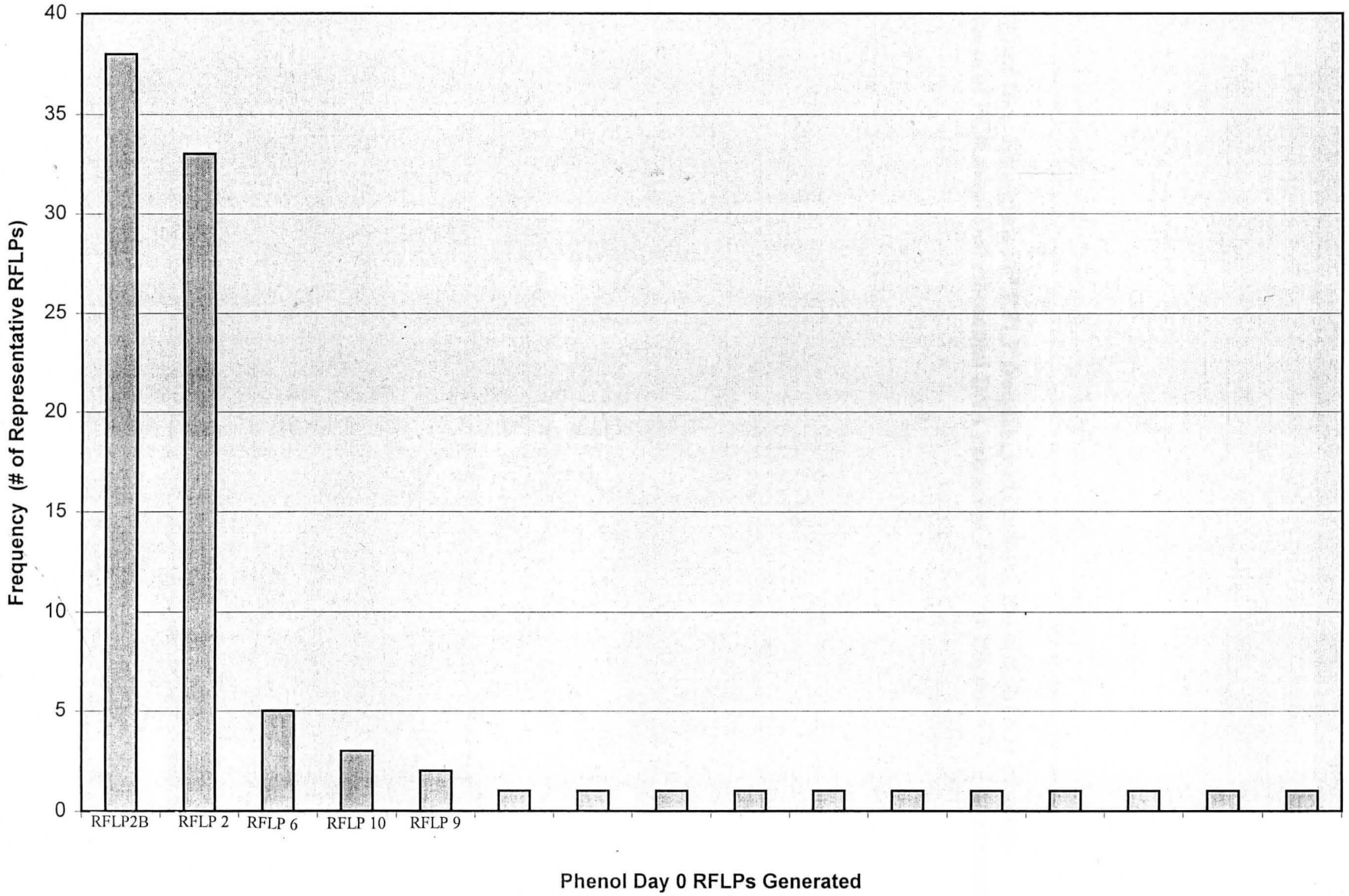

40
35
30
25
20
15
10
5
0
Frequency (# of Representative RFLPs)
RFLP2B
RFLP 2
RFLP 6
RFLP 10
RFLP 9
Phenol Day 0 RFLPs Generated

Figure 16c: Histograms of RFLP frequency illustrating the distribution of unique clones of PCR amplified ribosomal DNA extracted from the Phenol + TCE community.

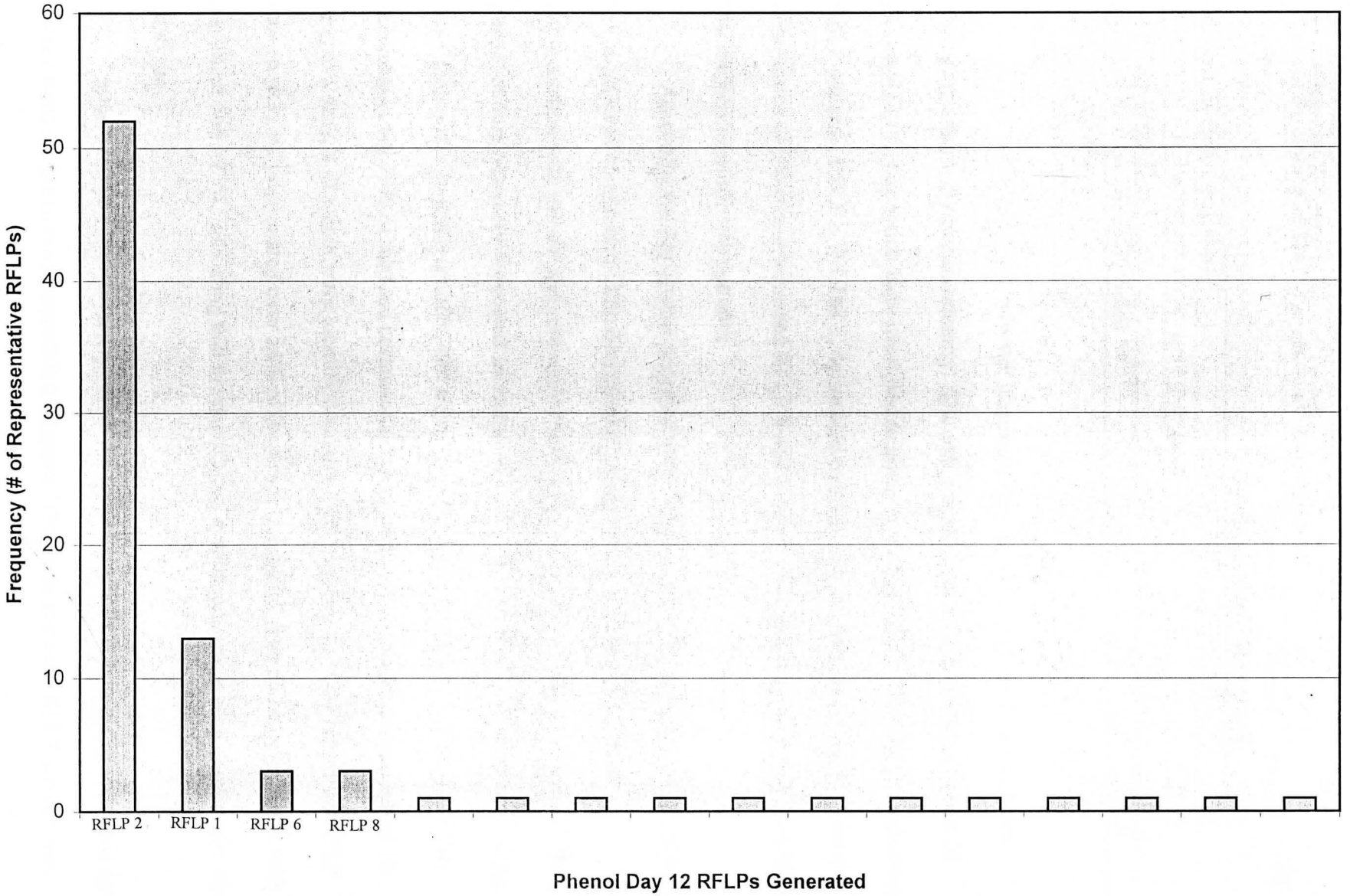

60
50
40
30
20
10
0
Frequency (# of Representative RFLPs)
RFLP 2
RFLP 1
RFLP 6
RFLP 8
Phenol Day 12 RFLPs Generated

Table 6: Summary of RFLP analysis of clone libraries

Treatment	Unamended Groundwater	Phenol (Day 0)	Phenol + TCE (Day 7)
Number of clones analyzed	89	99	96
Number of RFLPs that occurred more than once	8	5	4
Clone coverage (%) *	77.5	80.1	81.2
Shannon Weaver Diversity Index **	2.2	2.0	2.0

* equation c
** equation d

The unamended groundwater treatment supported the highest diversity index indicating a more diverse community. The indigenous groundwater community had 20 unique RFLPs, 9 of which were identified to occur more than once. Three of the repeating RFLP patterns, RFLP 3, 3A and 5, dominated over 65% of the total community. Visual comparison of patterns determined RFLP 3 and 3A to be very similar. Sequencing validated the uniqueness of the two patterns.

Amending the indigenous population with phenol served to decrease the diversity of the augmented community. The decreased diversity was expected as a result of augmentation with phenol and subsequent selection of phenol degrading organisms. The phenol culture treatment supported 16 unique RFLP's, 5 of which were identified to occur more than once. In this treatment, two of the repeating RFLP patterns (RFLP 2 and 2B) dominated over 70% of the total community. The phenol + TCE treatment supported 16 unique clones, 4 of which were identified to occur more than once. One repeating RFLP pattern (RFLP 2) dominated over 54% of the total community. The clone coverage in each treatment was >77%, indicating that the majority of species present in each community was analyzed.

Inter-treatment comparison revealed the majority of RFLP patterns generated in each library were unique to their respective treatments. Only one common RFLP pattern was

detected in each of the three treatments. The predominant RFLP pattern in the unamended groundwater, RFLP 3 (Figure 15a, Lane 3), dominated 30% of the community and was determined to be identical to RFLP 2B (Figure 15c, Lane 4), which dominated 33% of the phenol culture community and RFLP 2 (Figure 15e, Lane 3), which dominated 54% of the phenol + TCE community.

DNA Sequencing Results:

Representative clones were sequenced and closest relatives were determined using NCBI's BLAST. According to Bakerman and Madsen, RFLPs generated using the simultaneous digestion with two restriction enzymes will identify 96% of the taxonomic units (Bakermans and Madsen, 2002). With this in mind, one representative clone was sequenced when identical RFLPs were obtained from multiple clones. However, for the most predominant RFLPs generated from each treatment, multiple clones were sequenced to verify that identical RFLPs from the same treatment would generate identical sequences. Sequences obtained from each respective treatment were used to construct a phylogenetic tree (Figure 17). Each sequence in the dendogram is represented by the RFLP number, an identifier (GW, PH and PH+TCE), and a color (Red, Blue, and Green) to represent the groundwater, phenol culture and phenol + TCE treatments, respectively.

The sequence data generated from the unique groundwater clones demonstrated the decreased overall diversity of the phenol culture and phenol + TCE treatments compared to the unamended groundwater. Sequences from the groundwater community obtained from the TCE contaminated aquifer in Ohio were more diverse and matched closely with unexpected organisms, including arctic sea ice bacteria (GW10).

Figure 17: Phylogenetic tree of 16s rDNA derived from clones from three treatments; unamended groundwater, phenol culture and phenol + TCE.
Methanosarcina lacustera served as the outgroup. The scale is equal to 0.10 changes per nucleotide position.

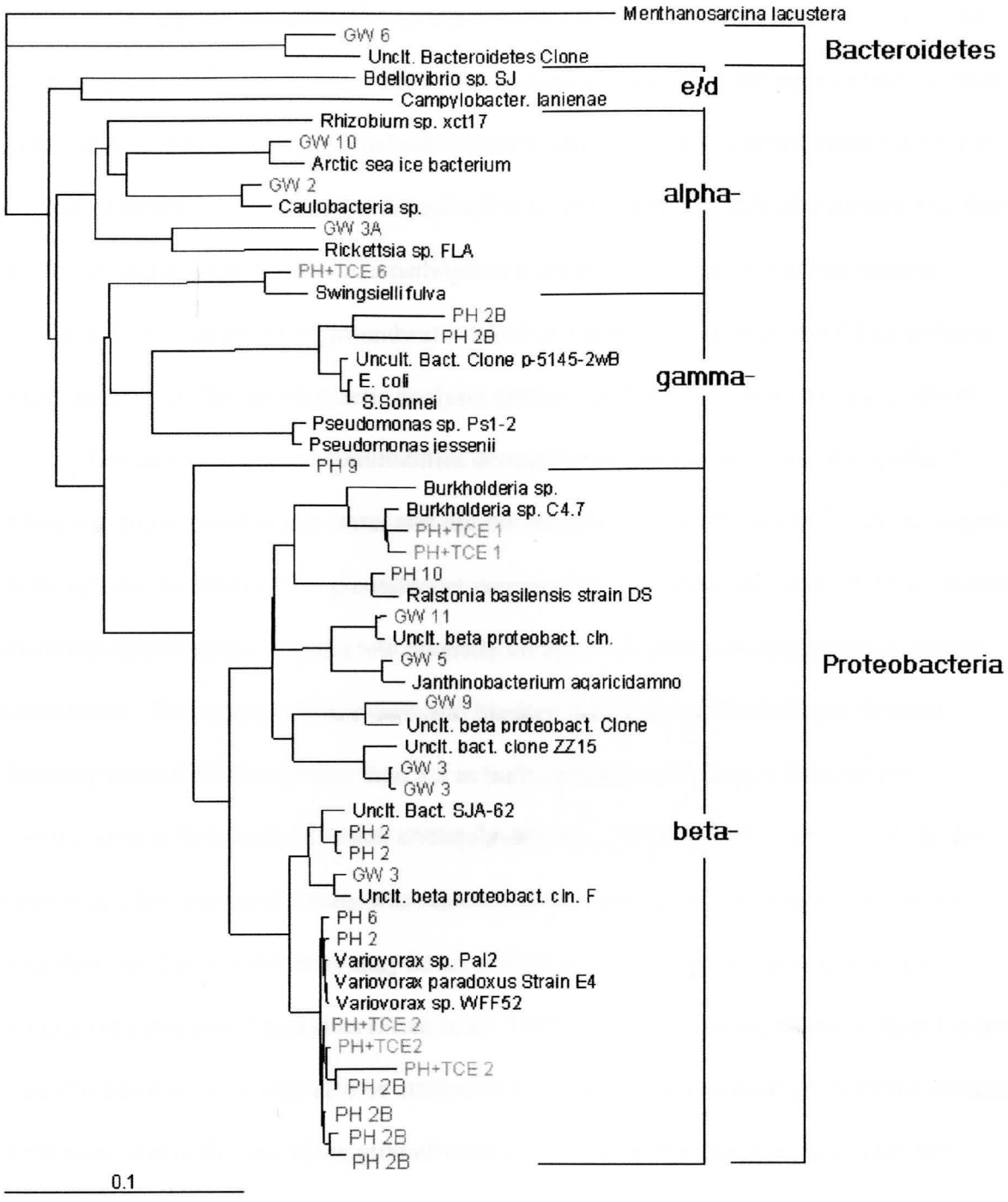
Legend:

GW (Red) – RFLP generated from unamended groundwater treatment.

PH (Blue) - RFLP generated from the phenol culture treatment.

PH + TCE (Green) – RFLP generated from the phenol + TCE treatment

***Numbers represent RFLP number from each respective treatment.**


Methanosarcina lacustera
GW 6
Uncit. Bacteroidetes Clone
Bdellovibrio sp. SJ
Campylobacter. lanienae
Rhizobium sp. xct17
GW 10
Arctic sea ice bacterium
GW 2
Caulobacteria sp.
GW 3A
Rickettsia sp. FLA
PH+TCE 6
Swingsiellifulva
PH 2B
PH 2B
Uncult. Bact. Clone p-5145-2wB
E. coli
S.Sonnei
Pseudomonas sp. Ps1-2
Pseudomonas jessenii
PH 9
Burkholderia sp.
Burkholderia sp. C4.7
PH+TCE 1
PH+TCE 1
PH 10
Ralstonia basilensis strain DS
GW 11
Uncit. beta proteobact. cln.
GW 5
Janthinobacterium agaricidamno
GW 9
Uncit. beta proteobact. Clone
Uncit. bact. clone ZZ15
GW 3
GW 3
Uncit. Bact. SJA-62
PH 2
PH 2
GW 3
Uncit. beta proteobact. cln. F
PH 6
PH 2
Variovorax sp. Pal2
Variovorax paradoxus Strain E4
Variovorax sp. WFF52
PH+TCE 2
PH+TCE2
PH+TCE 2
PH 2B
PH 2B
PH 2B
PH 2B
0.1
Bacteroidetes
e/d
alpha-
gamma-
beta-
Proteobacteria

Additionally, groundwater clone generating RFLP 6 (GW6) was identified which was closely related to Bacteroidetes, which characteristically reside in the gastrointestinal tracts of ruminants. Several other groundwater clones were related to different classes within the phylum proteobacteria. RFLP3a, the second most prominent groundwater pattern was found to closely match with Rickettsia, a pathogenic α-proteobacterium. Sequence analysis confirmed the uniqueness of groundwater RFLP's 3 and 3A. Although the RFLP patterns were visually similar, phylogenetic analysis determined them to be only distantly related.

The amended phenol communities demonstrated a more localized phylogenetic relationship compared to site water and did not include a preponderance of outlying species. Although the diversity of the groundwater community was higher than that of the amended treatment communities, it had a low diversity compared to other documented indigenous populations. For example Bakermans and Madsen detailed that the Shannon-Weaver diversity index (H') was greater than 3.5 in both contaminated and uncontaminated communities at their coal-tar-waste contaminated site. Although H' was not calculated by Fries et al., low species diversity was reported at their previously described site and they hypothesized that low diversity may be an artifact of several closely related organisms sharing the same restriction sites (Fries et al., 1997). The low species diversity found in our groundwater treatment may also be attributed to the selective bias associated with molecular techniques and to the age of the groundwater used in the molecular analyses. Due site availability, groundwater was sampled in early February and held a 4°C until needed for culture preparation. Molecular analysis was preformed on this groundwater sample after being housed at 4°C for over six months. The age of the groundwater used for the molecular

studies may have lost viable species during the hold time and may account for the low species diversity observed.

Inter-treatment comparisons of RFLP patterns revealed RFLP 3, the dominant groundwater clone was identical to the RFLP 2B, from the phenol culture treatment, and RFLP 2 from the phenol + TCE treatment, however these RFLPs did not generate identical DNA sequences (Figure 17, GW3, PH2B and PH+TCE2 respectively). Although not identical, sequences were closely related (all β-proteobacteria). Both PH 2B and PH+TCE 2 were most closely related to *Variovorax,* a β-proteobacteria, whereas GW3 was most closely related to an uncultured β-proteobacteria clone. Although clones from each treatment that generated the same RFLP pattern did not generate identical sequences, phylogenetic analysis demonstrates a close relationship between them. The unique but related sequences suggest that the RFLP analysis did not differentiate between the different β-proteobacteria strains as was seen by Fries et al.

There are no well-documented reports of bacterial growth on TCE as a carbon source (Newman and Wackett, 1999; Mars et al., 1995), however naturally occurring microorganisms are capable of degrading TCE when stimulated with phenol and other aromatics (Nelson et al., 1987). *Burkholderia cepacia* and *Methylosinus trichosporium* will oxidize TCE via the toluene 2-monooxygenase pathway and methane monooxygenase pathways, respectively (Newman and Wackett; Henry and Grbic-Galic, 1991). Additionally, *Pseudomonas* species have been documented to completely degrade TCE when induced by phenol (Kastner, 1991) and are reportedly well-suited for environmental remediation (Sun and Wood, 1996). In 1986, *P. putida* strain G4 was identified to degrade TCE when in the presence of a then unidentified component of the site water. This unidentified component

was later determined to be phenol (Nelson et al. 1987). A *Pseudomonas* species was found to be closely related to several clones from the phenol + TCE treatment although they did not represent a majority of the community.

More recently, Fries et al. determined a predominance of β-proteobacteria including *Variovorax* and *Burkholderia* from a TCE contaminated site remediated with successive treatments of phenol. The microbial succession in groundwater was monitored at this site to determine the site-specific impact of phenol and TCE on the indigenous microbial community. The predominance of *Variovorax* strains in the amended phenol treatments at this current site supports the findings of Fries et al. and suggests an importance for phenol degradation.

Since competition among populations during the enrichment process results in dominance of one or a few populations (Dunbar et al., 1997) the addition of phenol as a carbon source presumably selects for microorganisms capable of degrading phenol for energy. It is also presumed that bacteria capable of degrading phenol also fortuitously degrade TCE using the expressed PMO enzyme and are therefore essential for the degradation of TCE. Determination of PMO expression in the dominant species was beyond the scope of this study. Further research should include examining expression of TCE degrading enzymes.

Summary:

The goal of this study was to effectively determine the optimal culture and cometabolic parameters supporting maximal TCE degradation by indigenous microbes. Although previous field and laboratory studies showed the experimental cometabolite Compound C™ to be effective, current studies determined it to be ineffective at stimulating degradation in the indigenous microbes at the present site. The lack of mineralization induced by Compound C™ is thought to be due to a lack of suitable microorganisms. Phenol was determined to be the superior cometabolite, however supported lower levels of TCE mineralization than previously reported (Nelson et al., 1986; Fan et al., 1992). After optimization of culture conditions and phenol addition, a maximal level of 14% mineralization was achieved. Recent studies demonstrating the decrease in phenol concentrations in the incubations and abiotic control may explain the low rates of TCE degradation observed. As described previously, loss of phenol from the system will lead to starvation and may decrease the amount of phenol degraders actively expressing the appropriate enzymes.

As groundwater is augmented with phenol, a resultant shift in the indigenous population was anticipated as phenol degraders were selected for in the enrichment process. The microbial community was monitored by molecular techniques to demonstrate and assess the shift in microbial community structure after amending with both phenol and site concentrations of TCE. Communities were phylogenetically compared to determine the effect of manipulating the community with phenol and TCE. DNA analysis of approximately 300 clones (~100 from each treatment) revealed one RFLP pattern to be present and predominated in all three treatments. Although the RFLP pattern was identical

between the treatments, DNA sequencing analysis determined the clones generating this common RFLP were several unique but phylogenetically related species. With the exception of this one common RFLP, the populations were predominantly unique to each treatment. The unamended groundwater community appeared to be slightly more diverse than the amended treatments and supported unexpected and genetically diverse species. Both the amended phenol treatment and the phenol + TCE treatment supported a less diverse community and were more phylogenetically similar.

Prior to sequencing, the low levels of degradation supported by indigenous cultures was thought to be due to a lack of active phenol degraders, however molecular comparisons reveal a predominance of clones related closely to *Variovorax*. *Variovorax* was also was found to predominate at a TCE contaminated site that was remediated with successive phenol treatments (Fries et al., 1997). This suggests *Variovorax* was selected for as a phenol degrader and may express the PMO enzyme needed for TCE degradation.

The actual role of the dominant bacteria on phenol and TCE degradation is unknown, but the selection for and predominance of these microbes in the augmented treatments suggests they are capable of using phenol as an energy source. To degrade phenol, the microbes most likely possess the PMO enzyme described previously, which will fortuitously degrade TCE. Therefore, these predominant species observed may play an essential role in TCE mineralization. The actual role of these predominant microbes in expressing enzymes required in the TCE degradative pathway is beyond the scope of this research. Further research would include analysis of the enzymatic viability of these predominant species to determine via isolation of organisms and subsequent analysis enzyme expression may determine their role in phenol degradation. Additional optimization could be to assess the

effect of applying constant optimal concentrations of phenol on enzyme expression. Furthermore, cultures could be optimized and manipulated to maximize PMO expression and TCE degradation while minimizing the amounts of phenol needed as growth substrate. As already discussed, phenol is a proven human health hazard and is regulated by the EPA. Therefore, maximizing PMO expression in the presence of minimal phenol would make phenol cometabolic degradation more applicable to in-situ remediation.

Appendix A – Basal Salts Media (Makes 1 L)

Chemical Name	Chemical Formula	Mass (g)
DiAmmonium Phosphate	$(NH_4)_2HPO_4$	1.0
Magnesium Sulfate	$MgSO_4$	0.0125
Trace Elements	See Below	500µl

APPENDIX A – TRACE ELEMENTS 2000X (Makes 2 L)
(Original BRT Formula)

Chemical Name	Chemical Formula	Mass (g)
Boric Acid	H_3BO_3	4.64
Zinc Sulfate	$ZnSO_4*7H_2O$	3.48
Cobaltous Sulfate	$CoSO_4*7H_2O$	1.92
Cupric Sulfate	$CuSO_4*5H_2O$	0.16
Manganous Sulfate	$MnSO_4*H_2O$	0.12
Ferric Ammonium Sulfate	$Fe(NH_4)_2(SO_4)_2*12H_2O$	2.98
Ammonium Molybdate	$(NH_4)_6Mo_7O_{24}*4H_2O$	0.44

Appendix B - Preparation of ^{14}C-TCE Stock Solutions

100μCi of ^{14}C-TCE was received from Sigma Aldrich in a vacuum tight ampoule. The open-topped ampoule was glass sealed halfway down, keeping the small amount of ^{14}C-TCE contained. ^{14}C-TCE was dissolved in methanol as follows. The separating glass was broken while 5000μl of MeOH was added through the open top of the ampoule allowing mixing with the ^{14}C-TCE . The ^{14}C-TCE and methanol mix was pipetted into a 20ml serum vial and sealed with a PTFE-faced stopper and an aluminum crimp cap. Since 100uCi of ^{14}C-TCE was received and there are $2.2x10^6$ cpm/μCi, the resulting solution contained 44000cpm/μl. To make a working stock solution, 1ml of the concentrated stock solution was removed and mixed with 3.4ml of methanol, creating a 10,000cpm/μl solution. 10μl of this new diluted solution was used to spike the incubations, providing each vial with 100,000cpm.

Appendix C: EPA METHOD 551.1

Run Conditions:
30m, 0.25mm ID, 1.0um Rtx-5 (cat#10253)
1µl split injection.
1ng on-column concentration

Oven temp: 35°C (hold 22min)
To 200°C @ 10°C /min
(hold 3 min.)

Injector/Detector temp: 200°C/290 °C
Carrier gas: Helium
Linear velocity: 30cm/sec. @50°C
ECD sensitivity: 20kHz full scale
Split ratio: 10:1

Appendix D – DNA Solutions

Use Tris compatible electrodes for first three solutions.

Buffer 1
1% SDS (Sodium Dodecyl Sulfate)
0.1M NaCl
10mM Tris-HCl
1mM EDTA
pH = 8.0

Buffer 2
0.1M NaCl
10mM Tris-HCl
1mM EDTA
pH = 8.0

Buffer 3
10mM Tris-HCl
1mM EDTA
pH = 7.5

Buffer 4
3M NaOAC
pH = 5.2

Appendix E – LB (Luria-Bertani) Plates

Composition: 1.0% Trypone
0.5% Yeast Extract
1.0% NaCl
15g/L bactiAgar
100ug/ml Ampicillin
pH 7.0

1. For 1 liter, dissolve 10g tryptone, 5g yeast extract, 10g NaCl in 950ml deionized water and add 15g BactiAgar.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 Liter.
3. Autoclave for 20 minutes at 15lbs/sq. inch. Allow solution to cool to 55°C, add ampicillin and pour into 10cm plates.
4. Let harden and store inverted at 4°C.

X-gal stock Solution

To make a 40mg/ml stock solution, dissolve 400mg X-Gal in 10ml Dimethylformamide. Protect from light by storing in brown bottle at –20°C.

Thirty minutes prior to spreading cloned cells onto LB+ampicillan plates, pipette 40ul of X-gal solution onto plates, spread onto surface with a sterile glass rod, and allow to dry.

References:

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3. **Agency for Toxic Substances and Disease Registry (ATSDR).** 1989. Toxicological Profile for Phenol. U.S. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA.

4. **Bakermans, C., and E.L. Madsen.** 2002. Diversity of 16S rDNA and Naphthalene Dioxygenase Genes from Coal-Tar-Waste-Contaminated Aquifer Waters. Microbial Ecology. 44: 95-106.

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