ACCLIMATION OF ANAEROBIC SYSTEMS TO BIODEGRADE CHLORINATED SOLVENTS

by

Michelle Lalonde

A thesis submitted in conformity with the requirements for the degree of Master of Applied Science.

Graduate Department of Civil Engineering
University of Toronto

Acclimation of Anaerobic Systems to Biodegrade Chlorinated Solvents
by Michelle Lalonde
Master of Applied Science, 1997
Department of Civil Engineering, University of Toronto

ABSTRACT

Microbial acclimation to tetrachloroethene (PCE), trichloroethene (TCE), and carbon tetrachloride (CT) in an anaerobic sand column and in microcosm studies was investigated. The column was inoculated with unacclimated organisms from an anaerobic sludge digester. Initially, ethanol, PCE, TCE, and CT were added to the column. All incoming CT was degraded to chloroform and dichloromethane, while ethanol was transformed to acetate and propionate. With CT in the influent, consumption of PCE, TCE, acetate and propionate did not occur. However, in the absence of other chlorinated compounds, PCE was completely degraded to *cis*-1,2-DCE in the column within 210 days of inoculation. Yeast extract addition stimulated degradation, with PCE degradation in microcosms occurring within 9 days with 100 mg/L yeast extract, but within 36 days with 2 mg/L. In practice, it appears that propionate degradation capability is essential for systems in which ethanol is used as the electron donor.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude and appreciation to my supervisor, Professor David M. Bagley, for his assistance, guidance, and patience throughout my work at the University of Toronto. Special thanks also to Professor Brent E. Sleep, for his assistance and comments.

Thank you to my fellow graduate students: Kevin, Mansour, Ken, Susan, Ron and Jerry. Your support and advice over the past year have been invaluable.

Acknowledgments go out to ESTAC and NSERC for financial support of this research.

I am grateful to my family and my roommate, Marnie, who had to deal with me on those days when nothing seemed to work. I'm sure you've learned more about gas chromatography than you ever wanted or needed to know.

And last, but certainly not least, thank you to Derek. You have always been there for me. You listened and offered advice when I was down, but most importantly, you believed in me. Thanks!

TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGMENTS iii
TABLE OF CONTENTS iv
LIST OF FIGURES vi
LIST OF TABLES vii
1. INTRODUCTION 1
2. BACKGROUND 3
2.1 Reductive Dechlorination 3
2.2 Reductive Dechlorination of PCE and TCE 3
2.2.1 Microbiology of PCE and TCE Degradation 5
2.2.2 Electron Donors for the Reductive Dechlorination of PCE and TCE 9
2.2.3 Yeast Extract for Enhanced Degradation 11
2.3 Biodegradation of CT 12
2.3.1 CT Degradation Under Denitrifying Conditions 13
2.3.2 CT Degradation Under Anaerobic Conditions 15
2.3.3 In vitro CT Degradation 16
2.3.4 Anaerobic Chloroform Degradation 18
2.4 Anaerobic Degradation of Ethanol 18
2.5 Acclimation of Anaerobic Systems 20
3. METHODS AND MATERIALS 23
3.1 Chemicals 23
3.2 Analytical Procedures 23
3.3 Standard Curve Procedures 25
3.4 Inoculum Source 27
3.5 Column Operation 29
3.6 Chlorinated Compound Solution 31

3.7 Microcosm Studies 32
4. RESULTS 35
4.1 Column 35
4.1.1 Delivery of Chlorinated Compounds 35
4.1.2 Pre-inoculation 35
4.1.3 Column Studies 36
4.1.3.1 Phase I 37
4.1.3.2 Phase II 41
4.1.3.3 Phase III 44
4.1.3.4 Phase IV 46
4.1.4 Pressure Profile 51
4.2 Microcosm Studies 53
4.2.1 PCE Microcosms 53
4.2.2 Ethanol/acetate/propionate Microcosms 59
5. DISCUSSION 63
5.1 CT Degradation 63
5.2 PCE Degradation in the Absence of CT and TCE 63
5.3 Recalcitrance of PCE 66
5.4 Recalcitrance of *cis*-1,2-DCE 68
6. CONCLUSIONS 71
7. RECOMMENDATIONS 73
REFERENCES 74
APPENDIX A 80

Figure 3.1 - Reactor Configuration 28
Figure 3.2 - Column Set-up 30
Figure 4.1 - PCE, TCE and CT breakthrough profiles 37
Figure 4.2 - EtOH/AA/PA profile 30 days after inoculation 39
Figure 4.3 - CT/CF/DCM profile 30 days after inoculation 39
Figure 4.4 - CT/CF/DCM profile 90 days after inoculation 40
Figure 4.5 - EtOH/AA/PA profile 90 days after inoculation 40
Figure 4.6 - CT profile at Port 7 42
Figure 4.7 - CF profile during the starvation study 43
Figure 4.8 - DCM profile during the starvation study 43
Figure 4.9 - PA profile during the starvation study 44
Figure 4.10 - CT/CF/DCM profile after CT reduction 45
Figure 4.11 - PCE/TCE/*cis*-DCE profile 210 days after inoculation 47
Figure 4.12 - EtOH/AA/PA profile 210 days after inoculation 48
Figure 4.13 - PCE/TCE/*cis*-DCE profile 225 days after inoculation 49
Figure 4.14 - EtOH/AA/PA profile 225 days after inoculation 50
Figure 4.15 - PCE/TCE/*cis*-DCE profile at initiation of degradation (Port 13) 52
Figure 4.16 - Pressure profile results 53
Figure 4.17 - Microcosm results for a bottle with PCE, etOH and 2 mg/L YE 55
Figure 4.18 - Microcosm results for a bottle with PCE, etOH and 100 mg/L YE 55
Figure 4.19 - Microcosm results for a bottle with PCE, meOH and 100 mg/L YE 56
Figure 4.20 - EtOH/AA/PA tracking study 140 days after inoculum source start-up .. 62
Figure 4.21 - EtOH/AA/PA tracking study 210 days after inoculum source start-up .. 62
Figure 5.1 - Sequence of events prior to the onset of PCE dechlorination in the column 65

LIST OF TABLES

Table 3.1 - Chlorinated compound retention times 24
Table 3.2 - Physical constants for PCE, TCE and CT 31
Table 4.1 - Solutions examined for chlorinated compound delivery to the column 36
Table 4.2 - Inflow conditions for column studies 37
Table 4.3 - Observed molar ratios for PA:etOH and AA:etOH 50
Table 4.4 - Microcosms examined 54
Table 4.5 - Acclimation time for different electron donors 57
Table 4.6 - Acclimation time for various YE concentrations 58
Table 4.7 - Mass balance for a bottle amended with PCE, etOH and 2 mg/L YE for the sampling interval between Day 57 and Day 60 60
Table 4.8 - Mass balance for a bottle amended with PCE, etOH and 100 mg/L YE for the sampling interval between Day 27 and Day 30 60
Table 4.9 - Mass balance for a bottle amended with PCE, meOH and 100 mg/L YE for the sampling interval between Day 42 and Day 45 60
Table 4.10 - Mass balance for a bottle amended with PCE, etOH and 10 mg/L YE for the sampling interval between Day 27 and Day 30 60
Table 5.1 - Concentrations for events prior to the onset of PCE dechlorination in the column 65

1. INTRODUCTION

Chlorinated aliphatic hydrocarbons with one or two carbon atoms are commonly used for industrial and domestic purposes. Their applications range from use as solvents, dry-cleaning and degreasing agents, fumigants, and feedstocks for the manufacture of plastics. The widespread use, inadequate disposal and accidental spillage of these chemicals has resulted in the contamination of groundwaters and soils. Since these compounds and/or their transformation products are suspected carcinogens, their presence in the environment poses a threat to public health. Accordingly, their fate in the environment is of special concern.

In industrialized nations, tetrachloroethylene (PCE), trichloroethylene (TCE) and carbon tetrachloride (CT) are among the most frequently found chlorinated contaminants in groundwater (Westrick *et al.*, 1984). All three were designated as priority pollutants by the U.S. Environmental Protection Agency in 1976 and are strictly regulated under the Safe Drinking Water Act Amendments of 1986 at maximum contaminant levels of 5 μg/L. These chlorinated solvents have been found in groundwaters and soils adjacent to or underlying many industrial sites. When spilled in the subsurface, these solvents may form a separate fluid phase that is more dense than water. Accordingly, these compounds are commonly referred to as dense nonaqueous phase liquids or DNAPLs. Because they have relatively high water solubilities, these compounds are highly mobile in soils and may move into groundwater supplies. In the subsurface, even small volumes of DNAPL may migrate large distances, acting as long-term sources for dissolved contamination (Mackay and Cherry, 1989).

The persistence of these compounds necessitates long-term approaches that contain the plume and destroy the contamination in place. Physical and chemical processes such as air stripping and carbon adsorption merely transfer the contaminants from one part of the environment to another. An alternative approach is the *in situ* anaerobic biodegradation of these solvents. The power of this approach is that biodegradation of the solvents can result in complete mineralization to nontoxic end products.

methane, carbon dioxide, and other inorganic products. This process is accomplished by a consortium of bacteria, which use an electron donor as a source of carbon and energy while the chlorinated contaminant serves as the electron acceptor. The most widely reported transformation of highly halogenated compounds under anaerobic conditions is reductive dechlorination. With this mechanism, highly chlorinated compounds, which are often toxic, can be dehalogenated, yielding less halogenated compounds. The less halogenated products are often less toxic and/or more amenable to further biodegradation.

Previous research has indicated that PCE, TCE and CT are all susceptible to reductive dechlorination (e.g., DiStefano *et al.*, 1991; Criddle *et al.*, 1990b). While much research has been conducted on the anaerobic degradation of these contaminants individually, very little work has been done to examine mixtures of the three compounds. Since most of the affected groundwaters and industrial effluents invariably contain a mixture of highly chlorinated, less chlorinated, and nonchlorinated compounds (Thomas and Ward, 1989), examining the biodegradation of mixtures is essential. Furthermore, an understanding of contamination by a mixture of chemicals is important since the interactions between the contaminants may affect the viability of reductive dechlorination.

While acclimation is a critical phase of *in situ* bioremediation, few studies have examined the acclimation characteristics of microbial communities in soils. Acclimation refers to the period prior to the onset of detectable degradation after a culture is exposed to a new chemical. A knowledge of this acclimation period is critical in understanding and predicting the transport and fate of these contaminants.

The objectives of the present work are to examine the degradation of a mixture of chlorinated compounds (namely PCE, TCE and CT), to gain an understanding of the acclimation characteristics of the system and to determine 'ideal' conditions for start-up of degradation. These objectives will be met by examining the biodegradation of PCE, TCE and CT in a sand column and in microcosms with ethanol as the electron donor.

2. BACKGROUND

2.1 Reductive Dechlorination

Reductive dechlorination of chlorinated methanes and ethenes consists of a series of sequential electron and hydrogen additions and chlorine eliminations that can result in complete dehalogenation. Compounds that are more highly substituted with chlorine are more likely to undergo reductive dechlorination under anaerobic conditions, forming less halogenated intermediates. The reductive dehalogenation mechanism is generalized as follows, with e^- representing electrons from an electron donor (Vogel *et al.*, 1987):

$$RCl + 2e^- + H^+ \rightarrow RH + Cl^- \qquad (2\text{-}1)$$

Almost all chlorinated methane and ethene compounds are susceptible to transformation by reductive dechlorination reactions in anaerobic environments. The speed and efficiency at which this reaction is carried out, however, differs considerably from compound to compound. Reductive dechlorination is relatively rapid for chemicals with a higher number of chlorine substituents such as PCE, TCE, and CT when compared to their less-chlorinated products, i.e., dichloroethylene (DCE) and dichloromethane (DCM) (Fathepure and Boyd, 1988a; Krone *et al.*, 1989a). Hence, less-chlorinated compounds persist longer in anaerobic environments than highly chlorinated compounds. It must be noted that the biodegradation of these chemicals can be considered complete only when they are converted to harmless metabolites.

The reductive dechlorination of chlorinated methanes and ethenes requires an electron donor (reductant) that supplies electrons to reduce the chlorinated hydrocarbons. The chlorinated contaminant serves as the electron acceptor (oxidant).

2.2 Reductive Dechlorination of PCE and TCE

Until the early 1980's, PCE and TCE were considered to be persistent in aquatic environments and resistant to microbial degradation. Studies showed that TCE could be cometabolized by a variety of aerobic microorganisms (Wilson and Wilson, 1985). PCE, however, was found to be recalcitrant under aerobic conditions. Investigations later

reductive dechlorination.

Transformation of PCE under anaerobic conditions proceeds by sequential reductive dechlorination to TCE, dichloroethene (DCE) isomers, and vinyl chloride (VC). In general, the rates of anaerobic reductive dechlorination decrease as chlorine is removed. Based on experimental evidence, the reductive dechlorination steps beyond DCEs are inherently slow (Freedman and Gossett, 1989).

Unfortunately, conversion of PCE and TCE to these less chlorinated alkenes is of little benefit (some mass reduction does occur) as most of these transformation products are also regulated under the Safe Drinking Water Act. Accordingly, anaerobic bioremediation of PCE and TCE must proceed to nonchlorinated, environmentally acceptable products such as ethylene (ETH).

PCE dechlorination to ETH was first observed by Freedman and Gossett (1989). Enrichment cultures converted PCE to ETH but with a significant accumulation of VC. Further work indicated that VC degradation to ETH was apparently inhibited by the presence of the more chlorinated precursors, i.e., PCE, TCE, *cis*-1,2-DCE, and 1,1-DCE (Tandoi *et al.*, 1994). Significant VC removal occurred only after all precursors were removed.

Other products of complete PCE dechlorination that have been observed include ethane (DeBruin *et al.*, 1992) and CO_2 (Vogel and McCarty, 1985). Conversion to ethene and ethane occurred in a continuous-flow, fixed bed column receiving lactate, and was quantitatively complete; essentially all chlorinated compounds were recovered as ethene and ethane. The production of CO_2 was verified with ^{14}C but accounted for only 24 % of the initial labelled PCE. Although a pathway was proposed, CO_2 production has not been confirmed in subsequent studies and should be considered at most a very minor degradation pathway for PCE.

While VC dechlorination to ETH leads to essentially complete detoxification of the chlorinated ethenes and is, therefore, a critical step, VC is resistant to anaerobic degradation. VC is, however, readily degraded aerobically. Sequential anaerobic/aerobic systems may, therefore be successful in the decontamination of polluted sites. By coupling

lower chlorinated compounds, it may be possible to attain complete decontamination (Fathepure and Boyd, 1988b). For instance, a sequential anaerobic/aerobic treatment process involving two columns was used for the dechlorination of PCE (Fogel *et al.*, 1995). PCE was converted to VC and ethylene with no detectable TCE or DCE. Anaerobic conditions were created in the first column, while aerobic conditions were created in the second column. The soil was first treated anaerobically to convert PCE completely to DCE, then, with the introduction of oxygen, treated aerobically to mineralize the DCE.

2.2.1 Microbiology of PCE and TCE Degradation

While the anaerobic dechlorination of PCE and TCE has been investigated for more than a decade, the microbiology involved is still not well understood. Early work suggested that methanogens played a key role in the dechlorination of PCE. For example, four acetate-utilizing methanogen cultures, a *Methanosarcina* sp., *Methanosarcina mazei*, *Methanosarcina acetivorans*, and a *Methanothrix* culture, were tested for their ability to convert PCE to TCE (Fathepure *et al.*, 1987). Significant dechlorination of PCE to TCE was observed in two *Methanosarcina* strains.

Methanosarcina sp. strain DCM was also observed to degrade PCE to TCE via reductive dechlorination (Fathepure and Boyd, 1988a). Reductive dechlorination occurred only during methanogenesis, suggesting a clear dependence of PCE dechlorination on the amount of methanogenic substrate, in this case methanol, consumed. Furthermore, in methanogenic anaerobic sewage sludge, 80% of the initially added PCE was reductively dechlorinated to TCE (Fathepure and Boyd, 1988b). The addition of bromoethane sulfonic acid (BES), a potent methanogenic inhibitor, significantly inhibited PCE dechlorination, providing 41-48% less PCE removal. This appeared to strengthen the hypothesis that methanogenesis and dechlorination were linked.

The hypothesis appeared further supported when the degradation of TCE and methane production ceased in the presence of BES in enrichment cultures (Freedman and Gossett, 1989). The fact that reductive dechlorination was stimulated by electron donors typically used by methanogens provided further proof that methanogens played a role.

However, these reductive dechlorination studies were conducted with mixed cultures, so it was not possible to specify the class of organisms responsible for PCE or TCE degradation.

Degradation of PCE in the absence of methanogens, however, indicated that other organisms may also be capable of effecting the dechlorination. Reductive dehalogenation of PCE under anoxic conditions occurred with benzoate as the electron donor (Sholz-Muramatsu *et al.*, 1990). Inhibition of methanogenesis by BES resulted in complete inhibition of benzoate degradation in the absence of PCE. However, benzoate was decomposed in the presence of BES if PCE was added to the cultures. Furthermore, biotransformation of PCE was nearly the same in cultures with and without methanogenesis. These results suggested that PCE could be dehalogenated under anaerobic conditions by reductive dehalogenation without methanogenesis, if a fermentable substrate was available.

Non-methanogenic, sulfate-reducing cultures also showed PCE dechlorination to TCE and *cis*-1,2-DCE (Bagley and Gossett, 1990). Low methane production in the cultures indicated very little methanogenic activity. Additionally, cultures inhibited with BES showed virtually no methane production, yet demonstrated PCE-dechlorinating capability equal to that of noninhibited bottles. Cultures inhibited with fluoroacetate (an acetate-consumer inhibitor) also showed insignificant methane production and enhanced PCE-dechlorinating capability versus the noninhibited bottles. Clearly, methanogenesis was not required for the PCE dechlorination. The organism(s) responsible was not identified.

Aerobic enrichment cultures from contaminated groundwaters reductively dechlorinated TCE and PCE to *cis*-1,2-DCE after the transition from aerobic to anaerobic conditions (Kästner, 1991). No methanogenic bacteria could be detected in the culture and no chlorinated products other than *cis*-1,2-DCE and 1,1-DCE were found. The hypothesis was presented that aerobic or facultative anaerobic bacteria may facilitate the reductive dechlorination of PCE and TCE to *cis*-1,2-DCE, but that the dechlorination to vinyl chloride was conducted by strictly anaerobic or methanogenic bacteria.

Although evidence has been provided for the dechlorination of PCE to ethene and ethane, ethane was not formed in the presence of BES (DeBruin *et al.*, 1992). The role of methanogenic bacteria in the reduction of ethene remained unclear, however, because

acetate production were absent in cultures provided with H_2 or methanol in the presence of PCE, indicating the complete absence of methanogenic and acetogenic bacteria (Maymó-Gatell *et al.*, 1995).

While some organisms dechlorinate PCE slowly and incompletely in what is believed to be a cometabolic process, of greater interest are the high rates of degradation exhibited by organisms that metabolically dechlorinate PCE (e.g., Holliger *et al.*, 1993; and Neumann *et al.*, 1994). These organisms gain energy directly from dechlorination of PCE by using it as a respiratory electron acceptor. For instance, concentrations of PCE were routinely dechlorinated to 80% ethene and 20% vinyl chloride with methanol as electron donor (DiStefano *et al.*, 1991). While the cultures effecting this degradation began as predominantly methanogenic cultures, methane production ceased and a shift in organism predominance apparently occurred as the PCE dose was increased. An acetogenic organism may have been directly responsible for the dechlorination, however it is also possible that methanogens were still the mediators of reductive dehalogenation and that when methanogenesis was inhibited by high levels of PCE, electron flow in the methanogens was diverted toward reductive dechlorination. Alternatively, other organisms may have been responsible for PCE dehalogenation, either using methanol directly as an electron donor or perhaps using hydrogen produced by acetogens.

More recent work done to elucidate the microbiology of PCE degradation suggests a role for methanogens in reductive dechlorination. Inhibitors were applied to PCE-MeOH and PCE-H_2 cultures to investigate the roles of different microbial populations in PCE reduction (DiStefano *et al.*, 1992). Both vancomycin, an inhibitor of cell wall synthesis in eubacteria, and BES were fed to the cultures. BES inhibited dechlorination beyond TCE in MeOH- and H_2-fed systems, providing evidence for the involvement of methanogens in reductive dechlorination. Sustained dechlorination in the presence of vancomycin suggested that acetogens were probably not the dechlorinators.

While the complete degradation of PCE to ethylene has been shown to occur with mixed cultures and sediments, no pure cultures which can carry out this reaction have been isolated. Several pure cultures can, however carry out the partial dehalogenation. These

transformations are metabolic since they occur at relatively high rates and are necessary for growth. *Dehalobacter restrictus*, a pure enrichment referred to as PER-K23, was isolated from an anaerobic packed-bed column in which PCE was reductively transformed to ethane (Holliger *et al.*, 1993). PER-K23 was shown to dechlorinate PCE via TCE to *cis*-1,2-DCE. The culture displayed a very narrow electron donor spectrum; the only substrates that supported growth were H_2 and formate, and only in the presence of PCE or TCE as an electron acceptor. Additionally, PCE or TCE could not be replaced by inorganic or organic electron acceptors used by other anaerobic bacteria. The dominating organism in the enrichment culture PER-K23 was the first bacterium described that depended completely on a chlorinated hydrocarbon as an electron acceptor.

Dehalospirillum multivorans, a strictly anaerobic bacterium, was also observed to dechlorinate PCE via TCE to *cis*-1,2-DCE as part of its energy metabolism (Neumann *et al.*, 1994). The dechlorination rate was dependent on the PCE concentration, however concentrations greater than 300 μM were inhibitory. The isolation of this organism represented the first report on the isolation of a pure culture of a PCE-dechlorinating bacterium capable of growing on mineral medium with H_2 plus PCE as sole energy sources. Compared to *Dehalobacter restrictus* described by Holliger *et al.* (1993), *D. multivorans* was versatile with respect to its substrate spectrum. The organism grew with pyruvate, formate, H_2, lactate, ethanol, and glycerol as electron donors, however, none of these substrates could be utilized in the absence of PCE or fumarate as electron acceptors (Scholz-Muramatsu *et al.*, 1995)

A freshwater anaerobe, strain TT4B, was isolated from anaerobic sediments known to be contaminated with a variety of organic solvents (Krumholz *et al.*, 1996). This bacterium grew with acetate as the electron donor and PCE or fumarate as the electron acceptor, with *cis*-1,2-DCE as the halogenated product. This strain did not grow fermentatively and used only acetate or pyruvate as electron donors.

2.2.2 Electron Donors for the Reductive Dechlorination of PCE and TCE

While the availability of a suitable electron donor is essential for dehalogenation of PCE and TCE, a wide variety of electron donors have been shown to support this reductive dechlorination. Researchers have shown that dechlorination can not be sustained unless an electron donor is provided (Freedman and Gossett, 1989).

Gibson and Sewell (1992) tested four fatty acids and three alcohols for their ability to act as the source of reducing equivalents for PCE dehalogenation. Samples from microcosms amended with lactate or ethanol had TCE present at the first sampling point of 6 days. Microcosms amended with compounds such as butyrate, crotonate, and propionate, which are more difficult to degrade anaerobically, had a longer incubation period before large amounts of dehalogenation products were present. Acetate, methanol, and isopropanol did not support dehalogenation above that observed in the unamended control during this time period. Investigation into the biologically mediated interactions of toluene and PCE also suggested that toluene could act as an initial source of reducing potential for the reductive dechlorination of chloroethenes under anaerobic conditions (Sewell and Gibson, 1991).

An anaerobic microbial consortium capable of dechlorinating chlorinated ethenes to ethylene was developed as anaerobic granules in a laboratory-scale upflow anaerobic reactor (Wu *et al.*, 1995). Hydrogen, formate, acetate, propionate, butyrate, lactate, benzoate, methanol, ethanol, glucose and cane molasses were tested to determine their feasibility in supporting PCE and TCE dechlorination. Rapid dechlorination was observed when hydrogen, formate, glucose and molasses were used. Dechlorination rates were slow but then became rapid when propionate, butyrate, lactate, benzoate, or ethanol were used. Dechlorination was slow with acetate and methanol as substrate.

In a similar study, Fogel *et al.* (1995) tested four electron donors (methanol, acetate, glucose, and hydrogen) for their effect on PCE degradation. When fed methanol, PCE was degraded to VC and then to ethylene, whereas feeding acetate resulted in the production of only TCE and DCE and some VC. Feeding hydrogen and acetate initially produced a mixture of VC and ethylene, however, with time, ethylene production ceased and TCE and

at a slower rate than methanol.

The fact that such a wide variety of substrates had been shown to support dechlorination in pure and mixed cultures, including more exotic reductants, such as toluene, suggested that it may be the simplest, hydrogen, that serves as the direct electron donor for dechlorination (DiStefano *et al.*, 1992). Accordingly, cultures amended with PCE and methanol in serum bottles were switched from methanol to H_2 as the electron donor. These cultures were shown to immediately use H_2 to convert PCE to ETH and small amounts of VC. While methanol was well suited for sustained dechlorination of PCE, H_2 could be readily substituted for several feedings, provided that proper nutritional supplements were present (Maymó-Gatell *et al.*, 1995).

While hydrogen may be the direct electron donor for dechlorination, hydrogen addition would likely be difficult for large-scale treatment systems. Accordingly, an electron donor which is fermented to hydrogen may be more practical. Studies were conducted with methanol, ethanol, lactic acid, and butyric acid to determine their suitability for maintaining reductive dechlorination by an anaerobic mixed culture (Fennell *et al.*, 1995). Results indicated that dechlorination was better sustained with butyric acid, lactic acid, or ethanol, than with methanol. Additionally, it was concluded that non-methanogenic substrates that produce a low and steady supply of hydrogen resulted in more complete, and better sustained dechlorination of PCE and lesser chlorinated ethenes than those that produced a higher amount of hydrogen in a shorter time.

In work done by Fennell *et al.* (1997), four H_2 donors, namely butyric and propionic acids, ethanol and lactic acid, were compared for their ability to sustain dechlorination of PCE. Butyric and propionic acids could only be fermented when the H_2 partial pressure was lower than $10^{-3.5}$ or $10^{-4.4}$ atm respectively, while ethanol and lactic acid were readily fermented at H_2 partial pressures 2 to 3 orders of magnitude higher. Previous work done by Smatlak *et al.* (1996) had indicated that dechlorinators utilize H_2 at lower concentrations than do methanogens. Therefore, fermentation of electron donors that result in a slow, steady, and low-level release of H_2 over time could maximize dechlorination potential while

(VFAs) such as propionic and butyric acids.

While for long-term operation, equally good establishment and maintenance of dechlorination was noted for all substrates, the low-H_2-producing substrates (butyric and propionic acids) favoured dechlorination over methanogenesis in the short term (Fennell *et al.*, 1997). Interestingly, ethanol conversion to acetic and propionic acid was noted in studies conducted. This finding might help account for the similarities noted for long-term operation since the accumulated propionic acid contributed to the pool of slowly degradable donor and facilitated continued dechlorination after ethanol was depleted. It was therefore postulated that adding ethanol or lactic acid might almost be equivalent to adding propionic acid.

Clearly, a wide range of cultures have been shown to dechlorinate PCE and TCE, with an even greater variety of electron donors shown to initiate this dechlorination. The reductive dechlorination of PCE and TCE has been observed repeatedly in both natural and laboratory environments by both pure and mixed cultures.

2.2.3 Yeast Extract for Enhanced Degradation

Previous research conducted seems to indicate that the organisms that conduct reductive dechlorination require micronutrients to effect degradation. For instance, Maymó-Gatell *et al.* (1995) noted that deleting YE from their growth medium did not allow sustained PCE dechlorination. They postulated that YE or some product derived from it was serving as a carbon source. As such, they tested whether YE could be replaced with acetate, a common carbon source that is used by many anaerobic organisms. Their findings showed that acetate greatly stimulated dechlorination by the culture when YE was not present, suggesting that YE served as a source of carbon for reductive dechlorination.

In work conducted with a strictly anaerobic bacterium, *Dehalospirillum multivorans*, that dechlorinated PCE, it was discovered that the organism required yeast extract for growth (Scholz-Muramatsu *et al.*, 1995). Cultures amended with yeast extract showed higher growth yields.

ethanol and PCE as a nutritional supplement in work done by Fennell *et al.* (1997). It was confirmed experimentally that FYE was serving as an important supplemental electron donor as well as a nutritional supplement. Apparently, it provided a slowly released pool of H_2 to fuel the continued dechlorination of PCE that remained after ethanol was depleted.

2.3 Biodegradation of CT

Carbon tetrachloride can be anaerobically biotransformed by at least two pathways: hydrolyzation to CO_2, and reductive dechlorination to chloroform (CF) and dichloromethane (DCM). For example, in work done by Bouwer and McCarty (1983a), the production of CO_2 from the decomposition of CT and the partial incorporation of carbon into cells indicated removal by biotransformation. CF was also detected in cultures with CT initially present, indicating that reductive dechlorination had also occurred. CT is transformed to CO_2 by hydrolysis and not by oxidation-reduction, therefore CT would not be expected to serve as a sole source of carbon and energy for heterotrophic bacterial growth (Bouwer and McCarty, 1983b). Since CF is more persistent than CT in many environments, pathways that do not produce CF are of interest.

It has been suggested that the reason that many chlorinated compounds undergo reductive dehalogenation is that these transformations are fortuitous and result from the inherent reactivity of reducing agents created by microorganisms (Criddle *et al.*, 1990b). Accordingly, many common and familiar microorganisms would be expected to bring about transformations when confronted with these chemicals. While CT is not degraded under aerobic conditions, dechlorination has been observed under denitrifying and sulfate-reducing conditions. Additionally, CT dechlorination has been catalyzed by cofactors.

Studies of *Escherichia coli* K-12 were initiated to determine whether the extent and rates of reductive dehalogenation could be related to the electron acceptor condition of a microbial culture (Criddle *et al.*, 1990b). This was accomplished by monitoring the fate of CT in cultures of *E. coli* grown and incubated under four different electron acceptor conditions: oxygen respiring (aerobic), nitrate respiring, fumarate respiring, and fermenting. Aerobic growth was found to be the least reduced (most oxidized)

which was the most reduced environment.

It was postulated and confirmed through experimentation that the reduced electron acceptor conditions were more conducive to higher rates of reductive dehalogenation. CT metabolism in *E. coli* resulted in a variety of reaction products, depending on the conditions of incubation. Under fumarate-respiring conditions, CT was recovered as CO_2, CF and a nonvolatile fraction, while fermenting conditions resulted in more CF and almost no CO_2. Similarly, Bouwer and Wright (1988), observed that more CF was found under sulfate-reducing conditions, compared to denitrifying conditions.

2.3.1 CT Degradation Under Denitrifying Conditions

In situ bioremediation of CT by denitrifying microorganisms has been tested at the U.S. Department of Energy's (DOE's) Hanford Site. This research has demonstrated that microbes from the Hanford aquifer are capable of transforming CT using nitrate with a variety of electron donors (Petersen *et al.*, 1993). The production of CF from CT has also been observed for other denitrifying consortia. For example, CT was transformed to CF at a rapid rate when a native population was biostimulated by acetate addition in the presence of nitrate and sulfate (Semprini *et al.*, 1992). A lower fraction of the CT transformed was observed as CF after more strongly reducing conditions were created by removing nitrate from the injected fluid. No methane production was observed in the test zone, suggesting that a methanogen was not responsible.

In the area of CT degradation, considerable work has been undertaken involving *Pseudomonas* sp. strain KC which has been shown to rapidly transform CT to CO_2 and nonvolatile end products. This pure culture effectively transforms CT under denitrifying conditions (Criddle *et al.*, 1990a). Unlike previous investigations, degradation of CT by *Pseudomonas* sp. produces little or no CF.

The inhibition of CT degradation by strain KC when reduced iron was provided in the growth medium indicated that transformation of CT depended on the absence of soluble reduced iron (Criddle *et al.*, 1990a). One possible explanation for the observed inhibition was that induction of a trace metal scavenging mechanism is associated with the

to the growth medium appeared to inhibit transformation of CT, enhanced growth of *Pseudomonas* strain KC was observed.

Pseudomonas sp. strain KC was grown on a medium with a low content of transition metals in order to examine the conditions for CT transformation (Lewis and Crawford, 1993). Additions of dissolved iron (II), iron (III), and cobalt (II), as well as an insoluble iron (III) compound, and ferric oxyhydroxide inhibited CT transformation. While the addition of ferric iron to *Pseudomonas* sp. strain KC inhibited CT transformation, it was found that inhibition increased with higher iron concentrations (Tatara *et al.*, 1993). Removal of iron from the trace metals solution resulted in faster rates of degradation, as did removal of iron containing precipitates from the growth medium.

The effect of copper on *Pseudomonas* sp. strain KC was also examined (Tatara *et al.*, 1993). Cultures grown at high pH without any added trace copper exhibited slower growth rates and greatly reduced rates of CT transformation, indicating that copper was required for CT transformation. The use of pH adjustment to decrease iron solubility, to avoid copper toxicity, and to provide a selective advantage for strain KC led to removal of CT. Additionally, the transformation capacity of strain KC was found to be high enough to enable removal of CT at concentrations that exceed those for most CT-contaminated sites (i.e., 1 to 5 mg/L).

Additional characterization of the nonvolatile fraction produced during transformation of CT by *Pseudomonas* sp. strain KC was provided by Dybas *et al.* (1995). It was demonstrated that electron transfer plays a role in the transformation. Experiments conducted with CT indicated that more than one nonvolatile product is produced during CT transformation by *Pseudomonas* sp. One of these products, accounting for about 20% of the CT transformed, was identified as formate. It seemed possible that CO_2 could be produced by the oxidation of formate, however, CO_2 was recovered even when CT transformation was conducted in the presence of a large pool of unlabelled formate, indicating that formate was not an intermediate in the pathway to CO_2. While the mechanisms of CO_2 production remained unclear, the findings suggested that the reaction was dependent upon some kind of energy input.

In work done by Lewis and Crawford (1995), several carbon sources, including acetate, glucose, glycerol, and glutamate, were able to support CT transformation under denitrifying conditions. The rate of CT removal was most rapid when acetate was used as the carbon and energy source.

2.3.2 CT Degradation Under Anaerobic Conditions

Degradation of CT under anaerobic conditions has been observed by many researchers. For example, an anaerobic organism identified as a *Clostridium* sp., was isolated from the effluent of an anaerobic suspended-growth reactor (Gälli and McCarty, 1989). The organism was able to biotransform CT to CF, with no lag time, which was further transformed to DCM and unidentified products. This isolation represented the first report of a microorganism from an anaerobic mixed culture that displayed the ability to transform halogenated C_1 and C_2 aliphatic compounds. While no methanogenic organisms were isolated, methane formation was observed.

Five anaerobic bacteria were tested for their abilities to transform CT (Egli *et al.*, 1988). Cultures of the sulfate reducer *Desulfobacterium autotrophicum* transformed CT to CF and a small amount of DCM. In comparison, the acetogens *Acetobacterium woodii* and *Clostridium thermoaceticum* degraded CT completely in 3 days. While CF accumulated as a transient intermediate, the only compounds recovered at the end of the incubation period were DCM and traces of CM. No transformation of CT was observed in a culture of *Desulfobacter hydrogenophilus*, whose heterotrophic and autotrophic growth was completely inhibited by the compound, or in a culture of nitrate-reducing bacterium, which was not affected by CT. Overall, reduction of CT was only observed in organisms with the acetyl-coenzyme A pathway, indicating that a possible correlation between the acetyl-Co A pathway and dechlorination may exist.

While dehalogenation of CT to CF and DCM was observed in *Desulfobacterium autotrophicum*, *A. woodii* degraded only a fraction of the CT via this sequence, with approximately 90% of the CT transformed to nonchlorinated products. This finding suggested that CT metabolism by *A. woodii* can proceed via two distinct pathways. First, there is the reductive branch catalyzed by corrinoid enzymes and leading to CF and DCM.

Second, the substitutive branch transforms CT into CO_2 by a series of unknown reactions, which do not cause a net change in the oxidation state of the carbon atom.

Many substrates have demonstrated their ability to initiate degradation of CT. The biotransformability of CT was investigated under low concentration of acetic acid as the electron donor to evaluate the concentration effect of acetic acid on degradation (Doong and Wu, 1996). It was demonstrated that the acetic acid concentration could change the dechlorination capability of the system and, thus, influence the biotransformation of CT. The rate of removal of CT was found to increase with an increase in the substrate concentration. Overall, results indicated that low concentrations of acetic acid could support the microbial dechlorination, however, better biotransformation efficiency was evident with higher concentrations of acetic acid. Thus, higher concentrations of acetic acid provided a higher intensity of available electrons for the anaerobic microbial communities to facilitate the dechlorination.

As with research conducted on PCE and TCE, hydrogen has also been found to be the ultimate electron donor for reductive dechlorination of CT. The formation of CF from CT, with hydrogen as the sole source of electrons, unequivocally demonstrated that electron transfer from hydrogen to CT had occurred (Odom *et al.*, 1995).

2.3.3 In vitro CT Degradation

A wide variety of cobalamins and cobinamides have been shown to serve as catalysts in the reductive dehalogenation of CT. For instance, aquocobalamin, methylcobalamin and (cyanoaquo)cobinamide were shown to be effective catalysts in the reductive dehalogenation of CT (Krone *et al.*, 1989b). CF, DCM, CM (trace amounts) and methane were all detected as intermediates. This reaction was also found to be mediated by the nickel-containing porphinoid, coenzyme F_{430}, found in methanogenic bacteria (Krone *et al.*, 1989a).

Laboratory studies, using bacterial enrichments from aquifers, showed that certain transition metal catalysts can effect partial dechlorination of chlorinated methanes. The mechanism for this activity appears to involve direct catalysis by the transition metal complexes. Work conducted by Odom *et al.* (1995) demonstrated the possibility of

electrochemically coupling dechlorination of CT by transition metal cofactors with bacterial electron transfers. When this combination was carried out with the *Desulfovibrio* species, dechlorination of CT to CF was observed.

The bacterial transition-metal coenzymes vitamin B_{12} (Co), coenzyme F_{430} (Ni), and hematin (Fe) were examined for their ability to catalyze the reductive dechlorination of CT and PCE (Gantzer and Wackett, 1991). Vitamin B_{12} and coenzyme F_{430} had the capacity to mediate the reduction of PCE to ethylene, while hematin catalyzed the reductive dechlorination of PCE to vinyl chloride. All three cofactors were able to dechlorinate CT.

Dechlorination of CT with an anaerobic enrichment culture grown on dichloromethane as the sole organic carbon and energy source proceeded to CF, carbon disulfide and CO_2 (Hashsham *et al.*, 1995). When cyanocobalamin (vitamin B_{12}) was added to the culture, a three-fold increase in the conversion of CT to CO_2 was noted. Furthermore, none of the CT was reduced to CF. Since CF formation is undesirable, cyanocobalamin addition appeared to offer promising results.

The biotransformation of CT and CF with lactate- and acetate-grown sulfate-reducing enrichment cultures was examined (Freedman *et al.*, 1995). Both cultures were found to transform CT, with approximately 50% reductively dechlorinated to CF and up to 10% to DCM. The addition of cyanocobalamin (vitamin B_{12}) increased the rate of CT and CF transformation more than 100-fold, while minimizing the formation of lesser chlorinated methanes. Furthermore, low levels of cyanocobalamin altered the distribution of products formed. A reduction in the accumulation of lesser-chlorinated methanes was observed when the culture was amended with cyanocobalamin.

Granular methanogenic sludge was found to readily degrade CT without any prior adaptation to this compound (van Eekert *et al.*, 1995). CF was formed as a transient intermediate which was rapidly degraded to DCM and traces of CM. Degradation by autoclaved controls indicated the involvement of biologically generated abiotic cofactors such as vitamin B_{12} and cofactor F_{430}.

Chloroform, formed from the degradation of carbon tetrachloride, can be degraded under anaerobic conditions, both through reductive dechlorination to DCM and by oxidation to CO_2. Many researchers have found that chloroform serves as an inhibitor of methanogenesis. For instance, Bagley and Gossett (1995) investigated the effects of methanol consumption on CF degradation and DCM production. Methanol consumption was found to be inhibited by CF in both methanogenic methanol enrichment cultures and *M. barkeri* 227 cultures. However, methanol additions did stimulate CF degradation in both cultures. Furthermore, methanol addition increased the molar ratio of DCM produced from CF degradation for both cultures. In enrichment cultures, the DCM production appeared to be a function of the initial CF concentration and/or the initial methanol consumption rate, while no such relationship was found to exist for *M. barkeri* 227 cultures.

Biotransformation of CF was studied in a methanogenic environment using acetic acid as the primary substrate (Gupta *et al.*, 1996). The inhibition due to CF was also examined at different concentrations of primary substrate to understand the role of primary substrate on inhibition. The findings suggested that the presence of CF at any concentration inhibited the utilization of acetic acid and for CF concentrations equal to and exceeding 2.7 μM, no acetic acid was utilized by the microorganism even after CF was completely degraded.

Yang and Speece (1986) also found CF to be inhibitory to methane fermentation. Inhibition of unacclimated cultures was noted at CF concentrations of 0.5 mg/L, while with acclimation, concentrations of 15 mg/L could be tolerated. There was a clear correlation between CF dose and inhibition, and the duration of CF exposure directly related to the degree of residual inhibition.

2.4 Anaerobic Degradation of Ethanol

Ethanol can be used by a number of anaerobic organisms as an electron and/or carbon source. A brief review of several ethanol removal pathways is necessary to predict how ethanol may be used in PCE-degrading systems.

The conversion of ethanol to propionate by *Desulfobulbus propionicus* and *Pelobacter propionicus* was observed to proceed via a randomizing pathway (Schink *et al.*, 1985). The enhanced exchange of the carbon atoms of ethanol compared to those of acetate indicated that propionate was involved as an intermediate in the ethanol degradation. Furthermore, propionate was not only produced during breakdown of more complex substrates, but also by the fermentation of C_2 compounds (such as acetate) with concomitant reduction of CO_2 (Schink *et al.*, 1987). It was also speculated that propionate could serve as a hydrogen sink in anoxic environments since the yield of utilizable energy for the organism involved was close to zero.

Laanbroek *et al.* (1982) found that ethanol was rapidly degraded to acetate and small amounts of propionate in anaerobic freshwater sediment slurries. *Desulfovibrio propionicus* was the dominant bacteria among the ethanol-degrading organisms. Whereas propionate formation from lactate or pyruvate is a well-known process, the formation of propionate from residues like ethanol or acetate is an unexpected phenomenon as it involves chain elongation as follows (Schink *et al.*, 1987):

$$3CH_3CH_2OH + 2HCO_3^- \rightarrow CH_3COO^- + 2CH_3CH_2COO^- + H^+ + 3H_2O \qquad (2\text{-}4)$$

The degradation of ethanol can only occur if the reducing equivalents derived can be transferred to suitable electron acceptors keeping the hydrogen partial pressure low. The energy available from ethanol degradation depends on the type of H_2-oxidizing species and on the terminal electron acceptor used. Sulfate reducing bacteria can reduce sulfate, as an electron acceptor, to sulfide during ethanol oxidation. In the absence of sulfate, ethanol oxidation can be coupled with hydrogen transfer to methanogens. For this latter case, ethanol is oxidized with a concurrent reduction of CO_2 to form acetate or propionate (Wu and Hickey, 1996).

While for reductive dechlorination ethanol is used as a source of hydrogen and electrons, the hydrogen may also be used by any competitive organism, including methanogens. For instance, under sulfate-free conditions, a *Desulfovibrio* strain was used as the ethanol-degrading species producing acetic acid and hydrogen (Tatton *et al.*, 1989).

in a two-membered mutualistic coculture, the hydrogen was converted to methane by a *Methanobacterium* sp. Methane production from ethanol by a coculture of *Desulfovibrio* and *Methanobacterium* strain in sulfate-free medium was carried out as follows:

$$2CH_3CH_2OH + CO_2 \rightarrow 2CH_3COOH + CH_4 \qquad (2\text{-}2)$$

The expected methane yield was 0.5 mol of CH_4 produced from each mole of ethanol under conditions when all the ethanol was used for energy.

Methane production from ethanol by a triculture of a *Desulfovibrio* sp., a *Methanobacterium* sp., and *M. mazei* in sulfate-free medium was carried out as follows:

$$2CH_3CH_2OH \rightarrow 3CH_4 + CO_2 \qquad (2\text{-}3)$$

The expected yield of methane was 1.5 mol of CH_4 per mole of ethanol.

2.5 Acclimation of Anaerobic Systems

The degradation of many organic compounds in different environments is preceded by an acclimation period. This acclimation period, which is the length of time between the addition of a compound and the onset of detectable mineralization, may be environmentally significant if the time for acclimation is long because the chemical may become widely dispersed.

The possible reasons for acclimation of microbial communities to initiate degradation of organic compounds in lake water and sewage have been investigated (Wiggins *et al.*, 1987). A number of mechanisms have been postulated to account for the acclimation period. This period may be a result of the time needed for enzymes to be induced or for mutation or genetic exchange to occur or the time required for small populations of mineralizing organisms to become sufficiently large to bring about a detectable loss of the chemical. Other possible explanations include an insufficient supply of inorganic nutrients, the preferential utilization of other organic compounds before the chemical of interest, the time needed for the mineralizing species to adapt to the presence of

toxins or for inhibitors that are present in the environment to be destroyed. Furthermore, the concentration and structure of the compound itself probably influences the acclimation period.

Research done in the field of reductive dechlorination of PCE, TCE and CT indicates that an acclimation period is often required before degradation is observed. For example, while CT is often reduced to low levels without a detectable lag time (Bouwer and McCarty, 1983a), an acclimation period was observed before CF was degraded in seeded cultures (Bouwer and McCarty, 1983b). A nearly complete transformation of CF and CT was detected after 3 weeks of incubation. Additionally, complete transformation of PCE occurred after 8 weeks. TCE, on the other hand was removed slowly, with a reduction of only 40% after 8 weeks.

Studies under aerobic and methanogenic conditions were performed for periods longer than a year to determine the transformability of several potentially hazardous halogenated organic compounds found in contaminated groundwaters (Bouwer and McCarty, 1984). A concentrated solution of CF and PCE (among other compounds) with acetate as the primary substrate, was pumped to an upflow column seeded with inoculum from a laboratory-scale methanogenic anaerobic filter. In continuous-flow column experiments, significant removals of CF and PCE did not occur until after a ten-week lag period. After 15 months of operation, CT was added to the influent. No acclimation period was required for the transformation of CT. Apparently, the acclimation of the biofilm culture to CF led to simultaneous acclimation to CT. Overall, the column studies indicated that the type of electron acceptor present was an important factor affecting biotransformability of the pollutants studied.

A two-stage anaerobic-aerobic biofilm reactor successfully degraded a mixture of chlorinated organic compounds to water-soluble metabolic intermediates (Fathepure and Vogel, 1991). Reductive dechlorination of PCE and CF occurred with glucose, methanol and acetate as carbon sources. The extent of dechlorination was maximum when the anaerobic biofilm column was fed acetate as a primary carbon source. PCE and CF were removed, resulting in trace amounts of their dechlorinated products, such as TCE, *cis*-1,2-DCE and DCM. Dechlorination commenced after an initial lag of 2 to 6 months, depending

a result of the lack of sufficient appropriate substrate, electron acceptor, or a small cell population.

The acclimation period before anaerobic dehalogenation was investigated by Linkfield *et al.* (1989). The acclimation time was confirmed by the appearance of a dehalogenated product that was representative of quantities greater than 3% of the initial substrate. Acclimation periods prior to significant substrate depletion and product formation ranged from about 3 weeks to 6 months and were not correlated with complete mineralization of the substrates. An extended acclimation period was observed at high substrate concentrations, compared with low concentrations.

Clearly, acclimation periods differ greatly in their length and vary from situation to situation. A knowledge of the acclimation characteristics of a microbial system is, however, essential. Because of its possibly lengthy nature, especially in anaerobic environments, the acclimation period cannot be ignored in understanding and predicting the transport and fate of chemicals which may reside in anaerobic environments.

3. METHODS and MATERIALS

3.1 Chemicals

PCE, TCE, CT, *trans*-1,2-DCE, *cis*-1,2-DCE, 1,1-DCE, CF and DCM were obtained in neat liquid form (1000- or 5000-mg ampoules; Supelco, Inc.) for use as analytical standards. VC (1000 ppm ● 2% in N_2; Scott Specialty Gases) and methane (≥ 99% pure; Scott Specialty Gases) were obtained as gases. N_2 and 90% N_2 /10% CO_2 (to maintain anaerobic conditions in the column) as well as compressed air, helium and oxygen (for operation of gas chromatography equipment) were obtained from BOC Gas. Other chemicals used were reagent grade and were purchased from VWR Scientific and Fisher Scientific.

3.2 Analytical Procedures

Identification and quantification of chlorinated methanes and ethenes was accomplished using a 5890 Series II Plus Hewlett Packard gas chromatograph with a purge and trap unit (Hewlett-Packard, Co.). The GC was equipped with a Vocol capillary column (30 m long x 0.53 mm i.d. x 3.0 μm film thickness; Supelco, Inc.) and a flame ionization detector (FID). Aqueous samples (1 or 2 mL) were purged onto a VOCARB 4000 trap (Supelco, Inc.) with helium for 11 min at 6 mL/min and desorbed at 250°C for 4 min. The oven temperature program started at 35°C for 4 min, then increased at 10°C/min to 100°C, where it was held for 2 min. Injector and detector temperatures were 240 and 250°C, respectively. Peak-area responses for samples were compared with standard curves to obtain concentrations.

For each of the chlorinated compounds examined, the retention times were found to be as listed in Table 3.1. Accordingly, detection of a chlorinated compound in the column or in microcosm studies was confirmed when a peak was observed at the corresponding retention time. It should be noted that some shifting in retention times took place over the course of this work.

Methane was quantified using an HP 5890 II Plus GC equipped with a thermal conductivity detector (TCD). Headspace samples (volume of 10 μL) were injected onto a

Compound	Retention Time, min
Tetrachloroethylene (PCE)	10.02-10.03
Trichloroethylene (TCE)	7.38-7.40
Carbon tetrachloride (CT)	6.24-6.25
Chloroform (CF)	5.34-5.36
cis-1,2-Dichloroethylene (cDCE)	5.11-5.14
trans-1,2-Dichloroethylene (tDCE)	3.54-3.59
Dichloromethane (DCM)	3.20-3.23
1,1-Dichloroethylene (1,1-DCE)	2.66-2.70
Vinyl Chloride (VC)	1.56-1.59

Carboxen fused-silica capillary column (30 m long x 0.53 mm i.d.; Supelco, Inc.) operated isothermally at 35°C for 6 min. Both injector and detector temperatures were 150°C. Helium was used as the carrier gas at a flow rate of 6 mL/min.

Ethanol, methanol, acetate and propionate concentrations were determined on an HP 5890 Series II Plus GC equipped with a flame ionization detector (FID) and a 15m x 0.53mm x 0.5 μm film thickness Nukol fused-silica capillary column (Supelco, Inc.). In total, three different methods were examined for sample analysis. With the first method, the column was operated at 35°C for 2 min and increased at 15°C/min to 120°C, where it was held for 2 min and increased again at 15°C/min to 180°C. This method was later rejected due to the poor quality of the peak area responses. It was felt that having the oven temperature below 100°C might allow for condensation of the aqueous samples onto the column. As a result, in the second method examined, the oven temperature program started at 110°C for 5 min and increased at 15°C/min to 180°C, where it was held for 2 min. Again, a diminishing quality in the peak area responses was observed. It was later determined that ramping the temperature programming might allow deposits on the column to pass further into the column.

In a similar study (Fennell *et al.*, 1997), an isothermal method was used in which the column was operated at 90°C for 9 min. A 5 m guard column was used in front of the

guard column was chopped off. Additionally, aqueous samples were preserved with H_3PO_4 instead of HCl and the inlet liner was replaced every 40 to 50 samples. Since some success was achieved with this method, it was adopted in the present work.

In all cases, the injector and detector temperatures were 200°C and helium was the carrier gas with a flow rate of 7 mL/min. Aqueous samples were prepared in 2 mL vials with Teflon-lined septa. All samples were diluted with 1.2 mL water. Prior to injection (volume of 1 μL), aqueous samples were filtered through a 0.45-μm-pore-size syringe filter (Gelman Sciences) and acidified to pH 4.5 with HCl or H_3PO_4. Peak area responses for samples were compared with standard curves to obtain concentrations. Water blanks were periodically run between samples to reduce and quantify carryover. Additionally, standards were frequently run to verify that the responses were reproducible.

Both total and volatile solids measurements were made on the culture whenever organisms were obtained for experiments. Replicate (3) solids measurements were obtained as outlined in Standard Methods for the Examination of Water and Wastewater (APHA, 1989) using a 10 mL sample volume.

3.3 Standard Curve Procedures

Mass fraction stock solutions for PCE, TCE, CT, CF and DCM were prepared by adding 1.5, 15 and 150 μL of each chlorinated compound to separate 20 mL vials (actual volume 22 mL) containing 20 mL of methanol (determined gravimetrically) and sealed with Teflon-lined rubber septa and aluminum crimp caps. The mass of the syringe plus the chlorinated compound as well as the empty syringe mass after addition were recorded. The vial mass before and after addition of the chlorinated compound was also determined to provide a second check on the amount added. Standard solutions were prepared by adding 5, 10, and 20 μL of each stock solution to each of three 20 mL vials containing 20 mL of distilled water (determined gravimetrically). The syringe used to extract the stock solution was weighed both before and after addition to provide a precise measure of mass in the standard. In total, therefore, nine standards were used to obtain points for each standard

and CT standard curves were obtained from the same solutions).

Standard curves for the three DCE isomers were produced in a similar manner with the exception that 2, 20, and 200 μL of the DCEs were added to methanol rather than the 1.5, 15, and 150 μL added previously.

For the vinyl chloride standard curve, Henry's Law was consulted. At the time of preparation, the lab temperature was approximately 22°C, giving a Henry's Law constant of 1.006 for H_c (Gossett, 1987). The vinyl chloride used was 1000 ppm ± 2% in N_2. The ideal gas law therefore gives a concentration of 0.0413 mol/m^3 as follows:

$$\text{concentration} = n/V = P/RT \qquad (3\text{-}1)$$

where: P = 101325 Pa, R = 8.314 Pa·m^3/mol·K, and T = 295 K.
Since this gives a total concentration, the value obtained was multiplied by a ratio to account for the fact that the vinyl chloride used was 1000 ppm (i.e. 1000/1,000,000).

Various volumes of vinyl chloride were injected to 20 mL vials (actual volume of 22 mL) containing 20 mL of water (determined gravimetrically). The molar amount of VC injected to each vial was determined by multiplying the calculated concentration by the volume of gas injected. Knowing the mass in moles and the liquid and gas volumes, the equilibrium water concentration was calculated from:

$$C_w = M/ (V_g/H_c + V_w) \qquad (3\text{-}2)$$

where: M = total moles of vinyl chloride added to the vial
V_g = volume of headspace in the bottle (L)
H_c = Henry's Law constant (dimensionless)
V_w = volume of water in the vial (L)

Once the standards were prepared, 2.5 mL of each standard solution was injected onto the purge and trap. The areas obtained from the chromatograms were plotted against the chlorinated compound mass to obtain the standard curves (Figures A1 through A9 in Appendix A). Concentrations in mg/L were obtained by dividing the chlorinated

that a non-zero intercept was obtained, when no peak was detected for a particular compound, a concentration of 0 was recorded (as opposed to the intercept value). Standards were periodically run on the GC throughout the course of this work to ensure that the responses remained the same.

Standards for the methane standard curve were prepared in a similar manner as those for the vinyl chloride standard curve. Various volumes of methane gas were injected to 2 mL vials containing 1 mL of water (verified gravimetrically). Since the number of moles of CH_4 injected to the vial was readily determined, and the air and liquid volumes were known, the concentration was determined from Henry's Law (Equation 3.2) where $H_c = 26.5$ for CH_4 at 22 °C (Bagley and Gossett, 1995). For each of the methane standards, a 10 µL headspace sample was injected to the gas chromatograph and the area count recorded. A plot of concentration versus area was then produced (Figure A10 in Appendix A).

For ethanol, methanol, acetate and propionate, standards were prepared by injecting a known mass of each chemical into a 20 mL vial (actual volume of 22 mL) containing 20 mL of distilled water so as to give a concentration of 100 mg/L of each chemical. The syringe mass with the chemical, the empty syringe mass, and the mass of the vial both before and after addition of the chemical were recorded. Varying amounts of the stock solution were added to 2 mL vials with distilled water to obtain standards for the standard curve. Since the sample volume was consistently 1 µL, standard curves of concentration vs. area were produced (see Figures A11 to A14 in Appendix A for typical standard curves). Curves were adjusted when sample responses deviated by more than 10%.

3.4 Inoculum Source

A 3-L anaerobic ethanol enrichment culture was maintained as an inoculum source for the column and microcosm studies (~ 21°C). The source reactor was a stirred, 4-L bottle containing 3 litres of liquid and sealed with a rubber stopper. The reactor configuration is shown in Figure 3.1. The organism source for the 3-L culture was 1.5 litres of anaerobic

litres of basal medium.

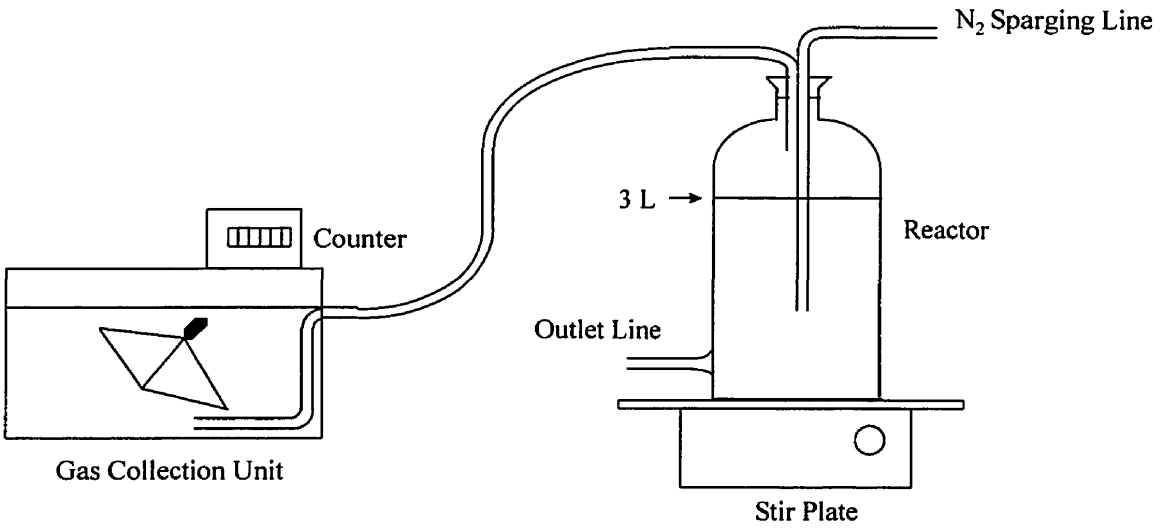


Figure 3.1: Reactor configuration

Unless otherwise stated, the basal medium contained per litre of distilled water: 60 mg NH_4Cl, 20 mg K_2HPO_4, 50 mg $CaCl_2{\cdot}2H_2O$, 1000 mg $NaHCO_3$, 100 mg $MgCl_2{\cdot}6H_2O$, 15 mg $FeSO_4{\cdot}7H_2O$, 30 mg $Na_2S{\cdot}9H_2O$, 1 mg resazurin, 5 mg nitrilotriacetic acid, 2 mg yeast extract, and 10 mL of a trace metal solution. The trace metal solution was composed of 0.86 mg $MnSO_4{\cdot}H_2O$, 1.70 mg $CoCl_2{\cdot}6H_2O$, 2.10 mg $ZnSO_4{\cdot}7H_2O$, 0.19 mg H_3BO_3, 0.5 mg $NiCl_2{\cdot}6H_2O$, and 0.2 mg $Na_2MoO_4{\cdot}2H_2O$. Note that resazurin addition permitted visualization of low-potential anaerobic conditions by the change in color from pink to colorless.

The enrichment culture was fed 300 mg of ethanol on a daily basis (to provide 100 mg/L). Methane production was monitored with a gas collection device. To feed the enrichment culture, N_2 purging was initiated, the stopper was removed, and the electron donor added. The hydraulic retention time of the reactor varied depending on the need for organisms but was on the order of 20 days. When culture was required for an experiment, the volume removed was replaced with basal medium. During replacement, anaerobic conditions were maintained by purging with N_2.

Theoretical methane production was calculated from reaction stoichiometry. However, actual gas production was lower than calculated theoretical values, likely due to

(Zitomer and Speece, 1993). Consumption of the ethanol was determined to have occurred once methane production was complete. This takes place as follows:

$$2CH_3CH_2OH \rightarrow 3CH_4 + CO_2 \qquad (3\text{-}3)$$

The theoretical methane production was approximately 219 mL. Since the gas collection unit measured gas production with a counter, with each increment representing a volume of 51.5 mL, methane production of 219 mL was equivalent to 4 to 5 increments on the counter. The gas measurement device indicated that gas production was as expected. Solids measurements were made and indicated that total solids were roughly 2500 mg/L, with volatile solids at approximately 1200 mg/L (note, these values changed depending on the need for organisms). Additionally, pH measurements were periodically made on the source reactor. A neutral pH of approximately 7.0 was always observed.

When the culture was first obtained, 200 mL of sludge was preserved for later analysis. In total, 5 bottles were preserved, with each containing 10 mL glycerol (to ensure that the sludge would not crystallize) and 40 mL sludge. Prior to the glycerol and sludge addition, the bottles were autoclaved for 15 minutes at 250°F (15 lb/in^2). The bottles were well shaken to ensure thorough mixing of the glycerol and sludge. They were then labelled and stored in the freezer at -30°C. The same procedure was followed after inoculation of the column. At this time, 400 mL (10 bottles) were preserved.

3.5 Column Operation

The experiments were performed in a stainless steel column (2 m length; 10 cm inside diameter) equipped with Teflon Mininert valves located every 10 cm beginning at 5 cm, with additional ports at the bottom and top of the column. These served as sampling ports for concentration profiles in the column. The column was packed with a natural screened sand obtained from a local sand supplier.

The column was operated in an upflow mode with anaerobic basal medium (Figure 3.2). Anaerobic conditions in the delivery vessel were ensured by maintaining a positive pressure with 90% N_2/10% CO_2 during delivery. The medium was pumped into the column

measurements indicated that the column porosity was 0.35 so the pore volume was 15.7 L x 0.35, providing a retention time of about 24 hours.

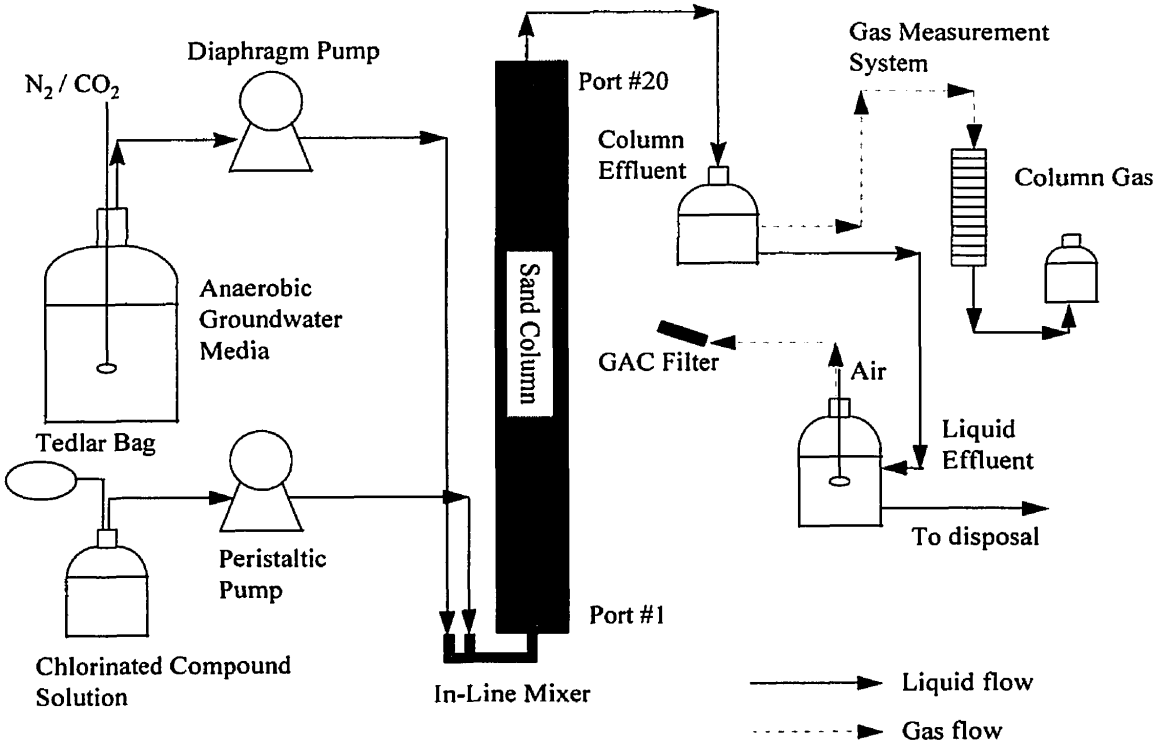


Figure 3.2: Column set-up

A stock solution containing the chlorinated compounds and ethanol (the electron donor) was delivered separately with a peristaltic pump (Pulsatron) at a rate of 300 mL/day. The chlorinated compound solution was held in a glass bottle, under N_2 purge with a Tedlar gas bag. Initially, 5 mg/L of PCE, TCE and CT and 100 mg/L of ethanol were delivered to the column. These concentrations were later changed.

Sampling of the column was carried out 2 to 3 times a week. For the most part, the bottom port as well as Ports 1, 2, 3, 5, 7, 10, 13, 15 and 17 were sampled to obtain column profiles. A gastight syringe equipped with a side port needle was used to withdraw samples from the Mininert valves on the column. For chlorinated compound measurement, a sample volume of 2 mL was withdrawn from the column, with 1.5 mL withdrawn for ethanol/acetate/propionate measurement. After each sampling interval, graphs of concentration vs. port were plotted.

Pressure profiles were obtained using a pressure gauge with a side port needle. The gauge was inserted into each of the ports and a pressure in psi units was recorded. Gas production in the column was observed as indicated in Figure 3.2.

To inoculate the column, organisms were obtained from the inoculum source by piercing the outlet tubing (Figure 3.1) with a syringe and withdrawing 5 mL The syringe was then inserted into the appropriate Mininert valve on the column and the organisms were injected into the column. In terms of total solids, a mass of roughly 15 mg was injected to onto each port, or approximately 7 mg in terms of volatile solids.

3.6 Chlorinated Compound Solution

When the chlorinated solution was initially prepared, it was determined that both partitioning of the compounds as well as solubility limitations needed to be taken into account. For PCE, TCE and CT, the physical constants of interest are listed in Table 3.2.

Table 3.2: Physical constants for PCE, TCE and CT[1,2]

Compound	H, $m^3 \cdot atm \cdot mol^{-1}$	Water solubility, mg/L
Tetrachloroethylene, PCE	0.0120	150
Trichloroethylene, TCE	0.0088	1100
Carbon tetrachloride, CT	0.0230	800

[1] Henry's Law constants at 20°C from Gossett (1987)
[2] Solubilities at 25°C from Mackay *et al.* (1993)

Partitioning of the compounds was taken into account with the following formula:

$$V_a/V_w = (C_o/C - 1)\,(RT/H) \qquad (3\text{-}4)$$

where: $V_a = 1.07L$ and $V_w = 1L$

Accordingly, once both partitioning and solubility had been examined, the solution mixed with the synthetic groundwater media had concentrations as follows:

$$[PCE] \approx 150 \text{ mg/L}$$
$$[TCE] \approx 120 \text{ mg/L}$$
$$[CT] \approx 170 \text{ mg/L}$$
$$[etOH] \approx 2000 \text{ mg/L}$$

basal medium. This was done under anaerobic conditions by purging with N_2. A concentrated solution containing the chlorinated compounds and ethanol was prepared in a 6 mL vial. The appropriate volume was then withdrawn with a gas-tight syringe and added to the 1-L vessel through the Mininert valve located at the top of the vessel.

3.7 Microcosm Studies

Microcosm studies were conducted in order to examine degradation of PCE as well as to determine ethanol, acetate, and propionate kinetics. The 100-mL bottles used in these studies were washed with a methanol-distilled water solution and dried at 105°C prior to the addition of organisms. Bottles were anaerobically inoculated by sparging with N_2 while transferring the culture directly from the reactor outlet tubing to the bottle. Once the bottle was filled to the desired volume (determined by filling the bottle with a volumetric flask and marking this volume on the bottle), the bottles were quickly capped with a Teflon-lined rubber septa and aluminum crimp cap.

All microcosm experiments were conducted with 100-mL culture suspensions in duplicate 100-mL serum bottles (actual volume of 122.5 mL). The bottles were sealed with Teflon-lined rubber septa and aluminum crimp caps, incubated under quiescent conditions at room temperature (~ 21°C) and protected from the light. Liquid was kept in contact with the septa to minimize losses of volatile compounds.

A total of 12 bottles was examined for PCE degradation: four bottles with 100 mg/L ethanol (etOH) as electron donor, four with 100 mg/L methanol (meOH) as electron donor, and four with 100 mg/L each of etOH and meOH. All bottles were amended with PCE at a concentration of approximately 1.5 mg/L.

For each electron donor examined, two bottles had a yeast extract (YE) concentration of 2 mg/L, and two had a YE concentration of 100 mg/L. One of each duplicate bottle was fed and sampled every 3 days, the other bottles were fed every 3 days but only sampled once every 12 days. At the time of feeding, samples were taken to confirm electron donor consumption and observe PCE degradation. Liquid volume was replaced with anaerobic feed solution. For those bottles sampled every 3 days, the feed

had feed volumes of 500 μL (i.e. a more concentrated solution). In this way, the volume of the bottles would remain essentially constant.

An additional six bottles were examined in order to determine the effect of yeast extract on the degradation of PCE. Of the six, two had a YE concentration of 10 mg/L, two had a YE concentration of 25 mg/L and the remaining two had a YE concentration of 50 mg/L. All bottles were amended with ethanol as the electron donor at a concentration of 100 mg/L and PCE at approximately 1.5 mg/L. Again, one of each duplicate bottle was sampled every 3 days, while the other three were sampled every 12 days.

Feed solutions were prepared with anaerobic basal medium. The electron donor was added to the feed solution so as to yield 100 mg/L electron donor in the microcosm. Therefore, one set of feed solutions contained 5000 mg/L ethanol (2 mL feed) and the others contained 20,000 mg/L (0.5 mL feed). YE in the feed solutions was always equivalent to the desired concentration in the microcosm (i.e. for a bottle with 100 mg/L YE, the feed solution would also contain 100 mg/L YE). PCE solutions were administered to the culture once the PCE mass in a bottle had been reduced to below 0.15 μmol. These solutions were made up in a similar manner, with the addition of PCE.

At the time of feeding, the expected methane produced was withdrawn with a syringe. For those bottles fed 100 mg/L ethanol, methane production of 7.3 mL was calculated. Similarly, for 100 mg/L methanol, methane production of 5.3 mL was calculated. Finally, for 100 mg/L of both ethanol and methanol, a combined methane production of 12.6 mL was expected.

In all cases, results from microcosm studies were plotted as mass of chlorinated compounds against time where mass represents the total mass of the chlorinated chemicals in the bottles. A liquid concentration was determined from GC analysis and was then converted to a total molar mass (through the use of Henry's Law) to represent the total mass of chlorinated compounds in a bottle (i.e. this includes both headspace and liquid mass).

The kinetics for ethanol, acetate and propionate consumption, as well as acetate and propionate formation were obtained through serum bottles. Experiments were conducted

122.5 mL). Approximately 10 mg ethanol (i.e. concentration of 100 mg/L) was injected to the serum bottles and samples were taken to examine the consumption of ethanol, and subsequent formation and consumption of acetate and propionate. Samples (volume of 1 mL) were withdrawn from the serum bottles with a Hamilton gastight syringe and preserved for analysis.

4. RESULTS

4.1 COLUMN

4.1.1 Delivery of Chlorinated Compounds

With the original column set-up, a 50-mL syringe pump was used to deliver the chlorinated solution to the column. Concentrated solutions of PCE, TCE and CT in ethanol were prepared and delivered to the column. However, upon mixing with the groundwater media, the instantaneous water solubilities of the chlorinated compounds were exceeded and two phases formed (even though the target concentrations of 5 mg/L were well below solubility limits). This led to the development of a pool of DNAPL at the column inlet, which was unacceptable.

To examine this problem, solutions containing ethanol, water, PCE, TCE and CT were prepared. While the solubilities of the chlorinated compounds in water are known (i.e. 150, 1100, and 800 mg/L for PCE, TCE and CT respectively; see Table 3.2), little data (both in terms of phase diagrams and published research) is available on 3-phase solutions (i.e. ethanol, water and either PCE, TCE or CT). Accordingly, various solutions were examined to determine where the envelope for a 1-phase system would lie. Table 4.1 summarizes the information developed for the solutions that were examined. As can be seen, while the chlorinated compounds are more soluble in ethanol, increasing the ethanol concentration only helped at very high levels (i.e. $\geq$ 400,000 mg/L).

As indicated in Table 4.1, the envelope for a single-phase 5 component system was very narrow, too narrow to provide appropriate operational flexibility. Therefore, the use of a syringe pump delivery system was abandoned and the peristaltic pump system shown in Figure 3.2 was used.

4.1.2 Pre-Inoculation

To determine the adsorption characteristics of the column, the chlorinated solution was introduced to the column 30 days prior to inoculation. Adsorption was gauged by the length of time to breakthrough (i.e. time required before inlet concentration = outlet concentration). The retention time of the column was approximately 24 hours. As seen in

Ethanol Concentration (mg/L)	PCE/TCE/CT Concentration (mg/L)	Observations[1]
20,000	1,000	1 phase
40,000	1,000	2 phase
40,000	2,000	2 phases
80,000	2,000	2 phases
125,000	2,000	2 phases
165,000	2,000	2 phases
210,000	2,000	2 phases
285,000	5,000	2 phases
400,000	5,000	1 phase
550,000	10,000	1 phase

[1] An observation of '2 phases' indicates that when the ethanol, chlorinated compounds and water were mixed, a 2-phase solution resulted. In contrast, an observation of '1 phase' indicates that the mixture resulted in one phase.

Figure 4.1, breakthrough of TCE occurred within about 80 hours, with breakthrough of CT taking approximately 130 hours. By contrast, breakthrough of PCE took weeks. In Figure 4.1, C_{20}/C_o (the concentration at Port 20 over the concentration at Port 0), was plotted against time.

Prior to inoculation, CF (at concentrations of 30 μg/L) was detected in the column. This was likely due to the initial presence of organisms within the pore structure of the sand that were degrading the incoming CT.

4.1.3 Column Studies

For clarity, the studies carried out in the column have been divided into four phases, with each phase representing different inlet conditions. Table 4.2 describes the target inflow conditions for each of the four phases in terms of the chlorinated compound, ethanol and yeast extract concentrations delivered to the column. Due to fluctuations in flow, the concentrations listed in Table 4.2 were not always achieved.

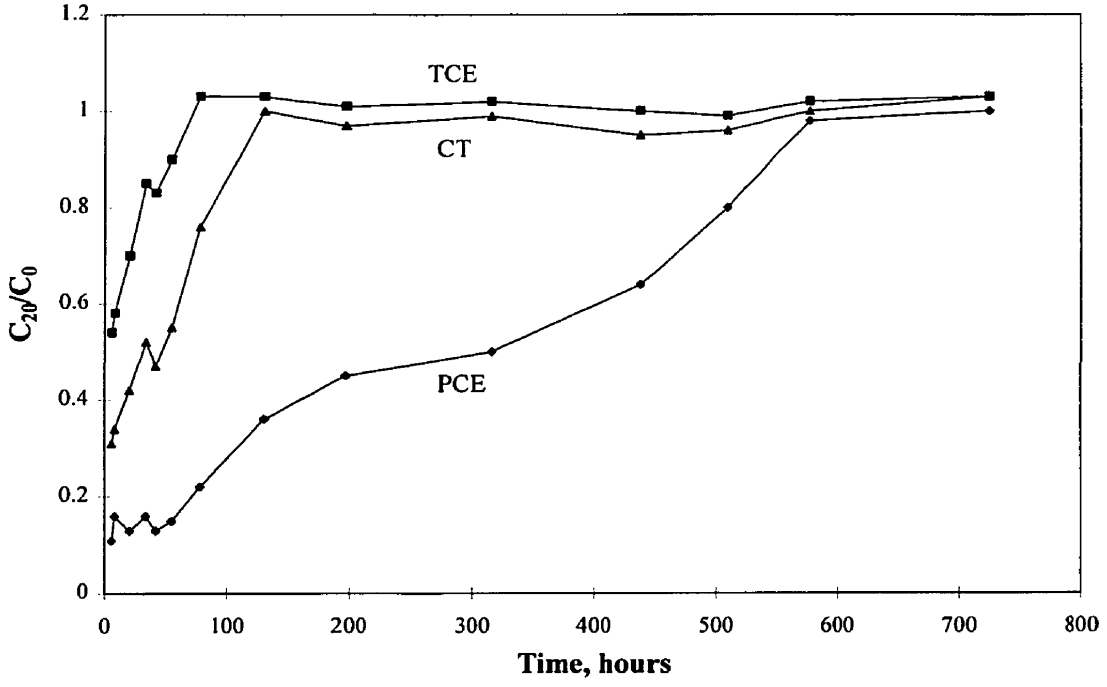


Figure 4.1: PCE, TCE and CT breakthrough profiles

Table 4.2 : Inflow conditions for column studies

Phase #	PCE (mg/L)	TCE (mg/L)	CT (mg/L)	ethanol (mg/L)	YE (mg/L)	Flow (L/day)
I	5	5	5	100	2	5.5
II	NA[1]	NA	NA	NA	NA	0
III	5	5	0.5	100	2	5.5
IV	5	0	0	100	100	5.5

[1] NA = not applicable because there was no flow.

4.1.3.1 Phase I

Initially, PCE, TCE and CT were delivered to the column at 5 mg/L, with ethanol at a concentration of 100 mg/L. Almost immediately following inoculation of the column, degradation of CT was observed with resulting CF and DCM formation. Within 2 days, CF as high as 1.6 mg/L and DCM at 0.05 mg/L were observed in the column, representing 41%

occurred, as expected, with subsequent formation of acetate and propionate.

Within 30 days of inoculation, ethanol (etOH) was no longer detectable above Port 5, while acetate (AA) and propionate (PA) concentrations remained high throughout the column (Figure 4.2). Molar ratios of propionate to ethanol and acetate to ethanol of 1.3 and 0.5 respectively were noted.

Although acetate production was expected (acetate is a product or by-product of virtually any electron donor added to an anaerobic system), propionate production was unexpected. While the formation of propionate from ethanol has been observed by other researchers (Schink *et al.*, 1987; and Wu and Hickey, 1996), Fennell *et al.* (1997) found that propionic acid could only be fermented when the H_2 partial pressure was lower than $10^{-4.4}$ atm while ethanol was readily fermented at much higher H_2 partial pressures. It is possible that the lack of propionate and acetate consumption in the column may have been due to a high H_2 partial pressure, although the measurements were not conducted. While Figure 4.2 seems to indicate that some acetate and propionate consumption were observed at the top of the column (above Port 13), no degradation of the chlorinated compounds was detected in the column during this phase.

After 30 days, carbon tetrachloride concentrations were 0.20 mg/L or less above Port 5, with 54% molar conversion to CF (concentrations at about 2.5 mg/L), and 13% molar conversion to DCM (concentrations of 0.50 mg/L) (Figure 4.3). The remaining 33% of the incoming CT was likely mineralized to CO_2 (Bouwer and McCarty, 1983a). No degradation of PCE or TCE was observed at this time (i.e. PCE and TCE concentrations remained at 5 mg/L).

Within 90 days of inoculation, CT was no longer detectable above Port 2. CF concentrations were as high as 1.4 mg/L at Port 2 (64% molar conversion), but stabilized at 0.7 mg/L further up in the column, with DCM at about 0.4 mg/L (24% molar conversion) (Figure 4.4). Again, no degradation of PCE or TCE was observed. Ethanol was completely consumed by Port 3, but acetate and propionate concentrations remained high (Figure 4.5). From Figure 4.5, values of 1.0 and 0.8 for the molar ratios of propionate to ethanol and acetate to ethanol were obtained.

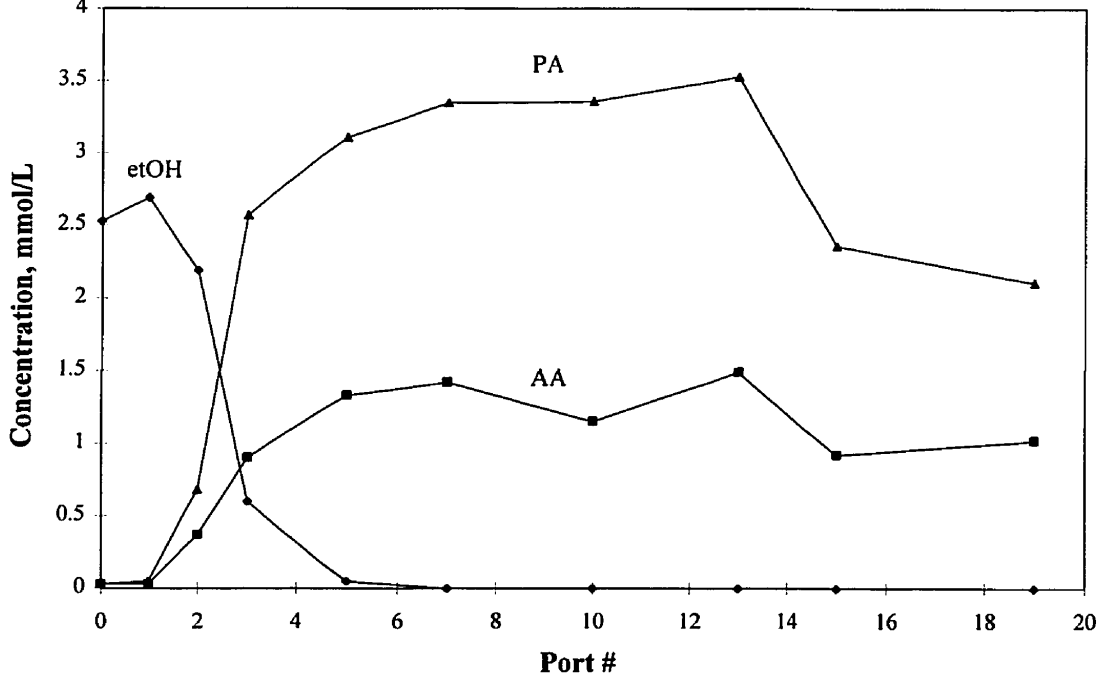


Figure 4.2: EtOH/AA/PA profile 30 days after inoculation

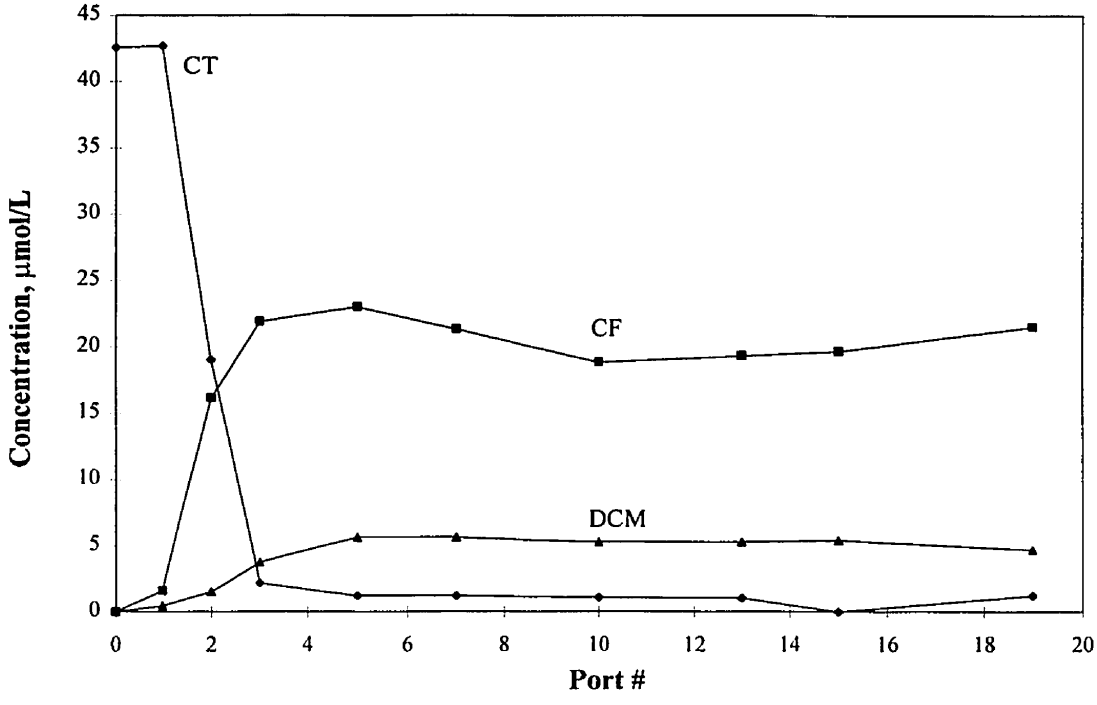


Figure 4.3: CT/CF/DCM profile 30 days after inoculation

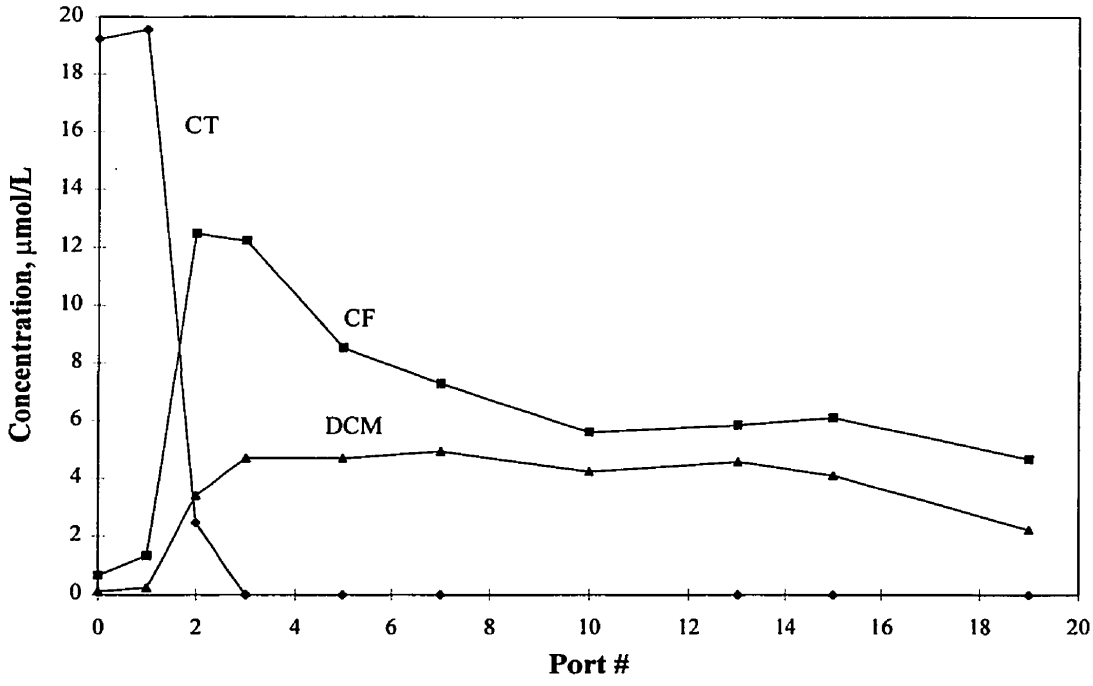


Figure 4.4: CT/CF/DCM profile 90 days after inoculation

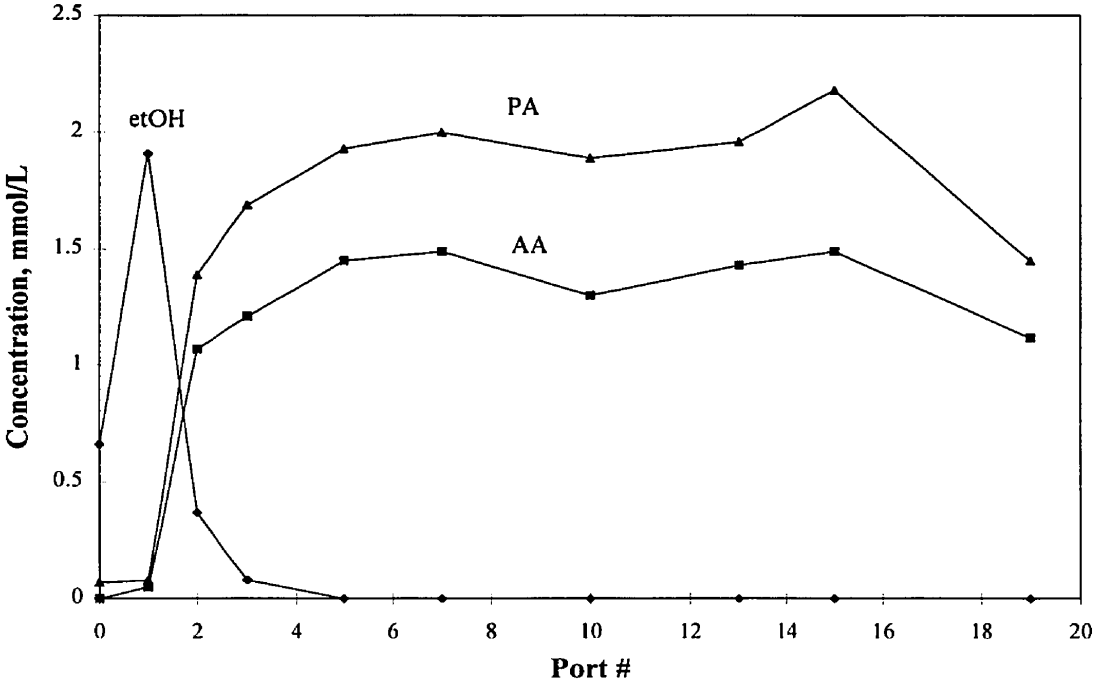


Figure 4.5: EtOH/AA/PA profile 90 days after inoculation

complete by Port 3. Since the culture was not acclimated to consume acetate or propionate at this time, very little electron donor was available for reductive dechlorination of CF higher in the column.

Clearly, the culture was rapidly acclimated to degrade CT. If all of the incoming CT went to CF and DCM, stoichiometric degradation of 5 mg/L CT (i.e. 32.51 mmol/L) would produce 3.88 mg/L CF which would be degraded to 2.76 mg/L DCM. While conversion to CF and DCM was observed, this only accounted for 88% of the incoming CT (at 90 days after inoculation). The remaining 12% was likely mineralized to CO_2 (Bouwer and McCarty, 1983a). Since the basal medium fed to the column contained $NaHCO_3$, however, measurement of the CO_2 formed from CT and its degradation products was not possible (since radiolabelled CT was not used). No PCE or TCE degradation was observed during this phase of the study.

Plots of concentration versus time at various ports were also constructed for CT, CF and DCM (Figure 4.6 shows this type of plot for CT at Port 7) in order to examine the degradation and formation of these compounds with respect to time. The degradation of CT and formation of DCM were observed. The formation and degradation of CF was more difficult to quantify since formation and degradation were concurrent. Results obtained at Ports 7, 10, 13 and 15 were the most readily analyzed. Degradation of CT occurred at an approximate rate of 1.6×10^{-3} mmol·L^{-1}·d^{-1}. CF formation and degradation rates of roughly 1.5×10^{-3} mmol·L^{-1}·d^{-1} and 2×10^{-4} mmol·L^{-1}·d^{-1} were observed with DCM formation observed to occur at approximately 1.6×10^{-4} mmol·L^{-1}·d^{-1}.

4.1.3.2 Phase II

To determine whether PCE, TCE, AA and PA degradation were rate limited, or otherwise inhibited, flow to the column was ceased starting 105 days after inoculation for 20 days. It was postulated that the lack of PCE and TCE degradation was due to inhibition from CF. If so, then degradation of CF during the starvation period may eliminate the

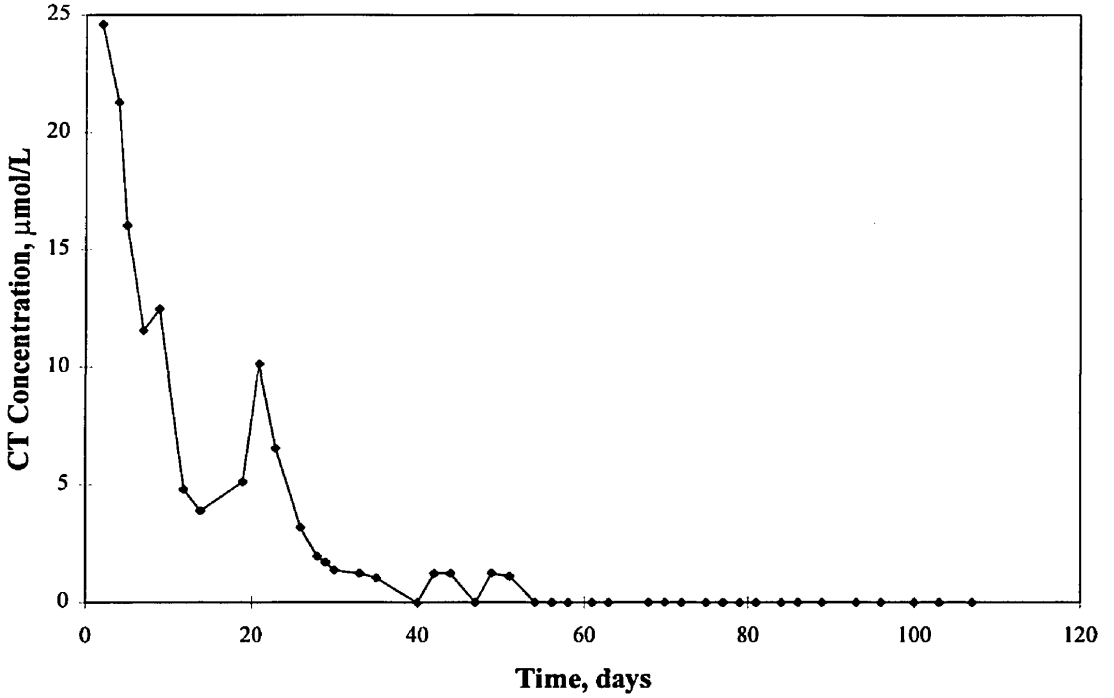


Figure 4.6: CT profile at Port 7

source of inhibition. Alternatively, the recalcitrance of PCE and TCE may have been due to the lack of acetate and propionate consumption. Since both acetate and propionate could serve as electron sources, consumption of acetate and propionate during starvation may initiate degradation of PCE and TCE.

Samples were taken prior to shut down (105 days), mid-way through the study (115 days) and prior to start-up (125 days). Complete degradation of CT occurred in the column during shut-down. Increased CF degradation was also observed, although residual CF remained (Figure 4.7). Prior to shut down, there was approximately 1 mg/L CF in the column. During starvation, CF concentrations reached 0.10 mg/L at the inlet where the microbial population was believed to be the most active. Higher in the column, however, CF values remained virtually unchanged. DCM concentrations increased during starvation (from the increased CF degradation), again, with the most marked difference close to the inlet (Figure 4.8). No degradation of PCE or TCE was observed.

Complete consumption of ethanol in the column was observed during the starvation study. Ethanol was detected at the inlet to the column and at Ports 1 and 2 prior to shut

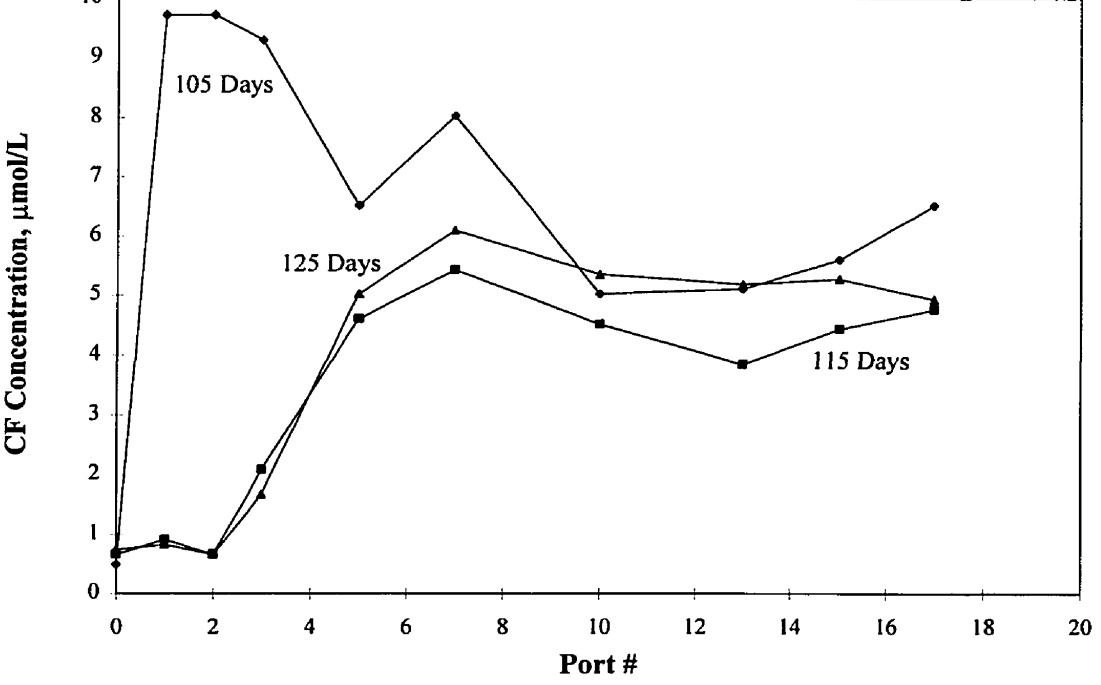


Figure 4.7: CF profile during the starvation study

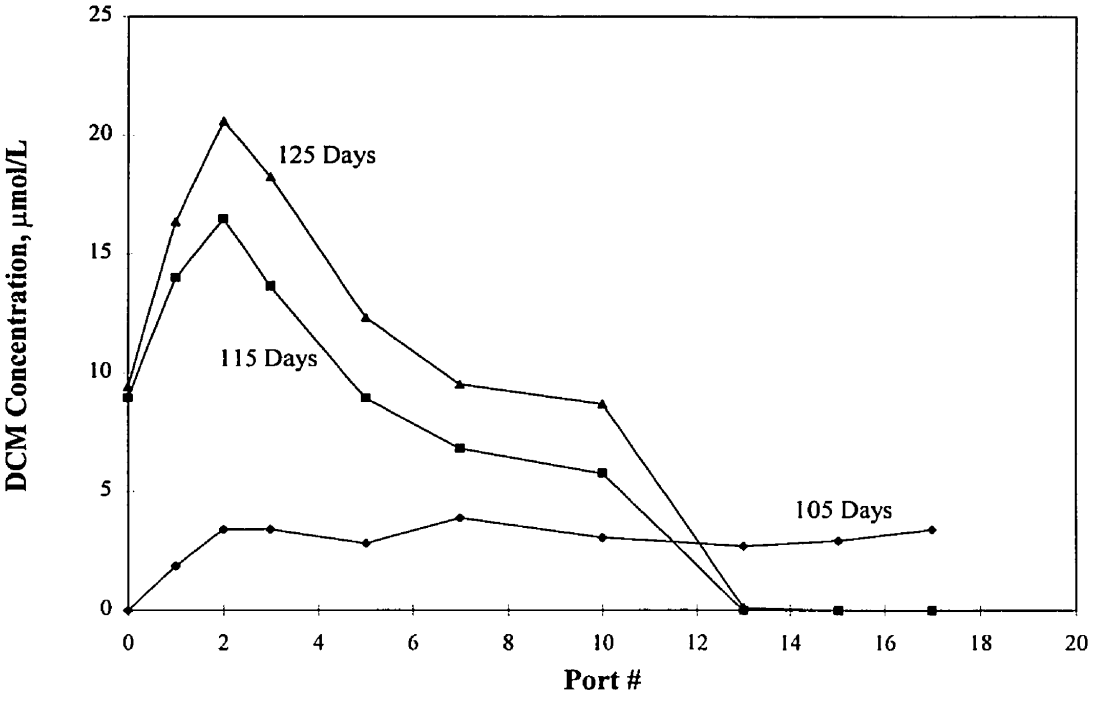


Figure 4.8: DCM profile during the starvation study

down, and was completely consumed throughout the column both mid-way through the study and prior to start-up (data not shown). It is likely that this increased ethanol usage led to the increased CF degradation observed during the starvation study. Additionally, it appeared that a small amount of propionate consumption had occurred at the upper end of the column (Figure 4.9). No acetate consumption was detected during the starvation study.

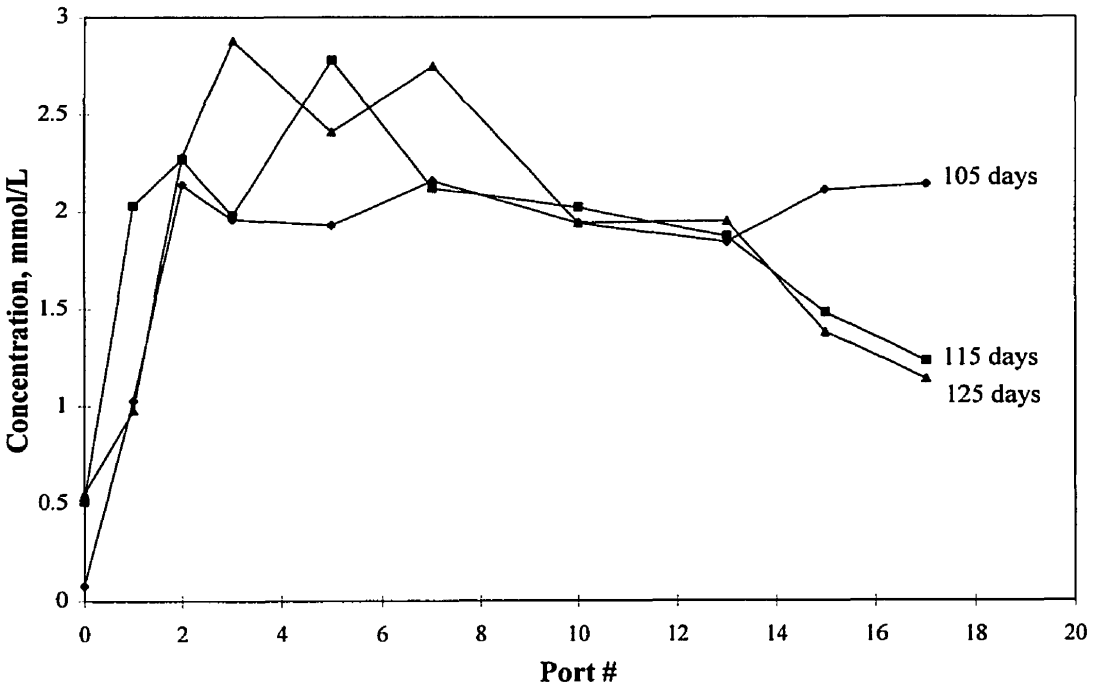


Figure 4.9: PA profile during the starvation study

4.1.3.3 Phase III

After starvation, the column was re-started with a reduced CT concentration of 0.5 mg/L. If CF was inhibiting PCE and TCE degradation in the column, a diminished CT concentration would result in less CF formed, which presumably would cause less inhibition or be degraded close to the inlet. Ethanol presence up to Ports 2 or 3 may provide sufficient electrons for the dechlorination of CF. PCE and TCE were added at 5 mg/L as before with ethanol at 100 mg/L.

The reduced CT concentration had little effect, as neither PCE nor TCE degradation were observed. While CF concentrations were lower, CF was still detected in the column at

respectively). Figure 4.10 depicts the column profile for CT and its degradation products, 130 days after inoculation (5 days after re-starting). Ethanol was not detected past Port 2 (data not shown). No consumption of acetate or propionate was observed which may have explained the recalcitrance of CF in the column. Furthermore, no degradation of PCE or TCE was observed. PCE and TCE degradation would only be seen higher in the column if the organisms were able to consume acetate and propionate as electron donors.

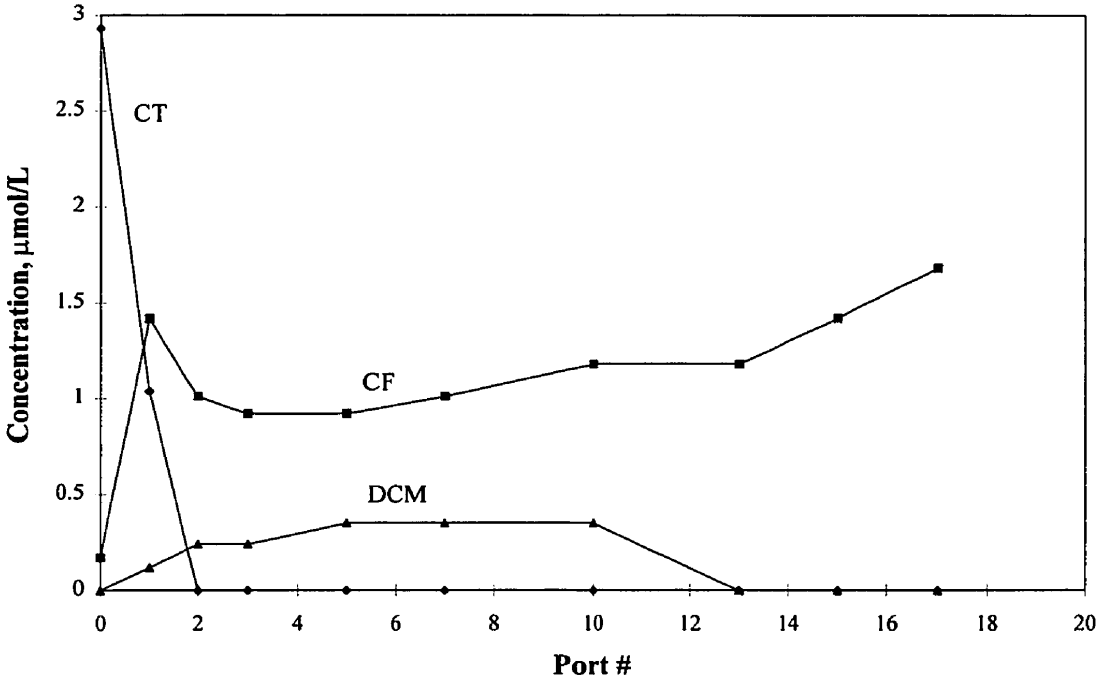


Figure 4.10: CT/CF/DCM profile after CT reduction

After 130 days of operation, acetate and propionate consumption in the absence of chlorinated compounds was observed in microcosm studies (described in Section 4.2.2). Reinoculation of Ports 10, 11, 12, 13 and 15 with inoculum source organisms (160 days after initial inoculation), however, did not stimulate acetate or propionate removal (at least, not in the presence of CF). These compounds represent an untapped source of electrons that could be used for reductive dechlorination. Previous studies have shown that ethanol, acetate and propionate can directly or indirectly support reductive dechlorination of PCE

Fennell *et al.* (1997) observed more sustained dechlorination of PCE in cultures amended with propionic acid over those fed ethanol, as propionic acid provided a slow, steady release of H_2. If the column organisms would consume acetate and propionate, perhaps degradation of PCE and TCE would be initiated.

4.1.3.4 Phase IV

To ensure that CF was not inhibiting acetate and propionate consumption or PCE and TCE degradation, CT was removed from the chlorinated solution after 170 days of column operation. Additionally, to determine the onset of PCE degradation, TCE was also removed from the column inlet since TCE in the influent might mask any PCE degradation occurring.

During initial start up, TCE was seen to break through the column in about 80 hours indicating that sorption of TCE to the sand was relatively low (since the retention time of the column was 24 hours). However, after removal of TCE from the chlorinated feed solution, residual TCE was still detected up to 65 days after its removal from the column inlet. TCE was removed from the chlorinated solution at Day 170. By Day 175, the TCE concentration was down to 1.0 mg/L and by Day 185, the concentration was down to 0.4 mg/L. TCE removal was occurring but much more slowly than expected. Complete removal (to non-detectable levels, i.e., less than 4 μg/L) occurred only where PCE degradation took place in the column, otherwise concentrations were on the order of 0.10 mg/L.

Microcosm studies (described in Section 4.2.1) indicated that an increase in the yeast extract concentration from 2 to 100 mg/L led to faster degradation of PCE. Accordingly, on Day 185, the YE concentration in the basal media delivered to the column was increased to 100 mg/L.

Since acetate and propionate were consumed in microcosm studies in the absence of chlorinated compounds (described in Section 4.2.2), Ports 2 through 9 were reinoculated with inoculum source organisms on Day 195. It was hoped that these organisms would consume acetate and propionate in the column, thereby serving as an increased electron

organisms known to consume acetate and propionate during phase III).

On Day 205, degradation of PCE was observed at the upper end of the column (i.e. above Port 10). Small amounts of *cis*-1,2-DCE (0.20 mg/L) were detected. Within five days of this initial detection, *cis*-1,2-DCE as high as 3.5 mg/L was observed, representing 95% conversion of PCE. Figure 4.11 represents the column profile for PCE and its degradation products at 210 days after inoculation. At no point during this phase was accumulation of TCE observed and no degradation beyond *cis*-1,2-DCE was detected. The TCE observed at the lower end of the column was not a result of PCE degradation, but rather residual TCE desorbing from the sand.

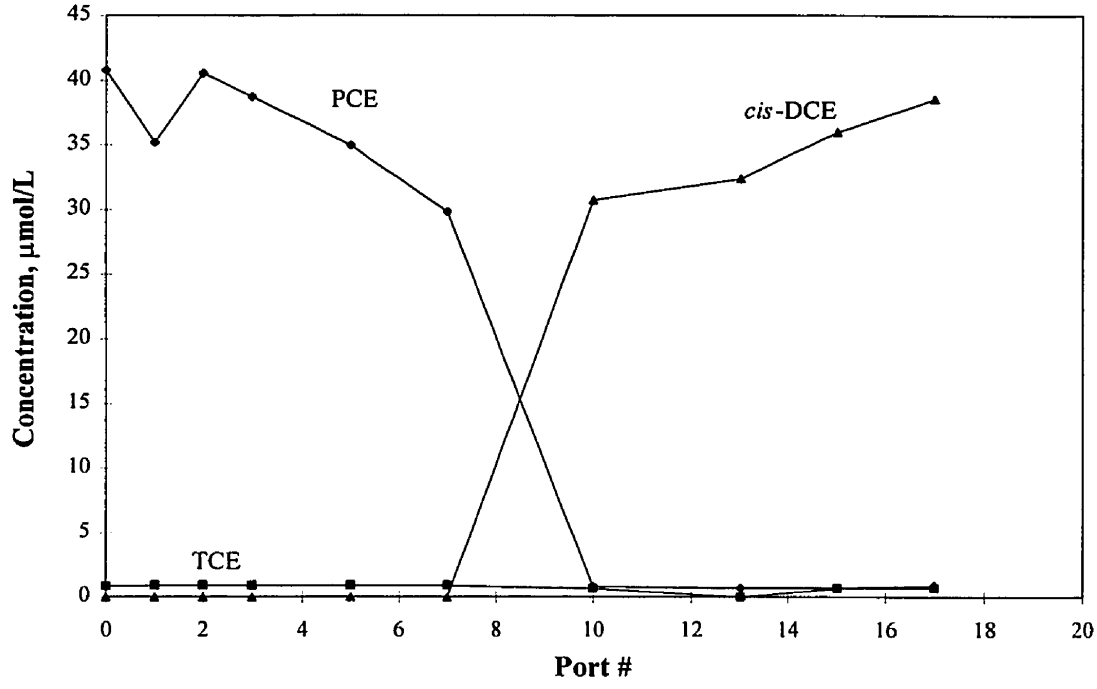


Figure 4.11: PCE/TCE/*cis*-DCE profile 210 days after inoculation

Samples taken from the column for ethanol/acetate/propionate measurement when PCE degradation was observed indicated that some acetate and propionate consumption was taking place (Figure 4.12). Whereas at 90 days after inoculation, PA concentrations of 2 mmol/L were observed (Figure 4.5), only 1 mmol/L was observed at 210 days after inoculation. However, complete removal of acetate and propionate was not observed.

0.7 and 0.9 respectively.

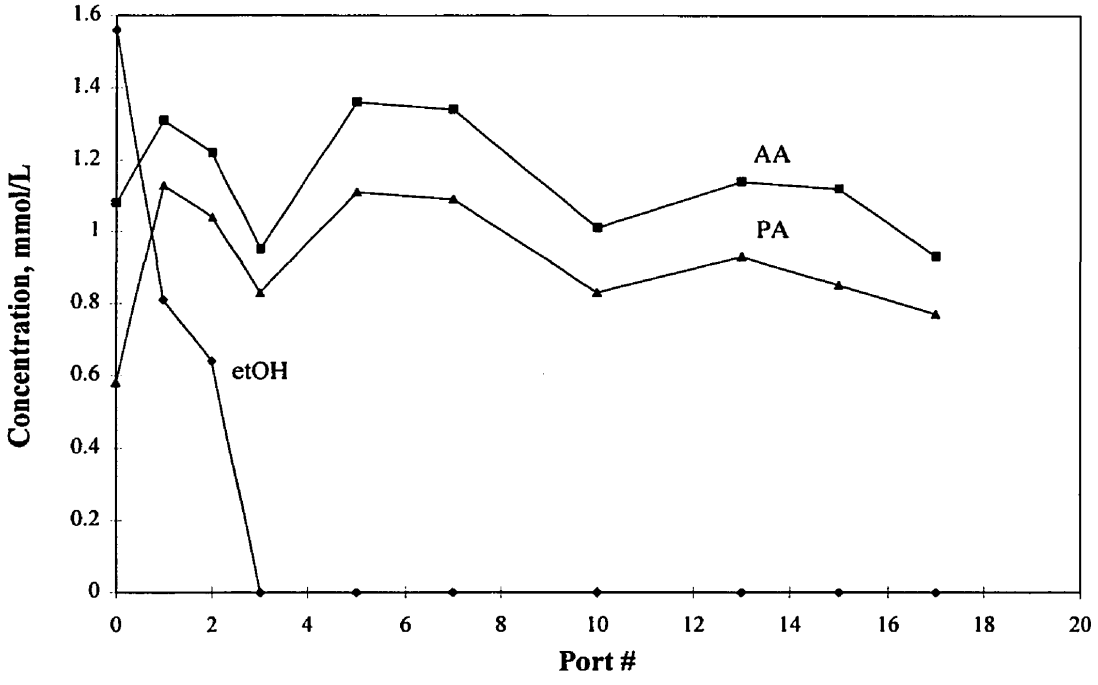


Figure 4.12: EtOH/AA/PA profile 210 days after inoculation

Reductive dechlorination requires the gain of 2 moles of electrons per mole of chloride ion removed. Therefore, for the dechlorination of PCE to *cis*-1,2-DCE, a transfer of 4 moles of electrons from the electron donor to PCE is required in order to provide sufficient reducing equivalents. With approximately 30 μmol/L of PCE present in the column, 120 μmol/L of electrons from the electron donor is required for the degradation to *cis*-1,2-DCE. The column influent contains approximately 2200 μmol/L of ethanol, with roughly 1400 μmol/L acetate and 2000 μmol/L propionate formed in the column. While it is not understood why *cis*-1,2-DCE degradation was not observed, the lack of dechlorination could not have been due to insufficient electron donor since both acetate and propionate were still observed throughout the column.

For 5 mg/L PCE (i.e. 30.15 mmol/L), stoichiometric degradation leads to 3.96 mg/L TCE which is then degraded to 2.92 mg/L DCE. Observed PCE degradation to *cis*-1,2-

PCE concentration fluctuated between 4.5 and 6.5 mg/L, resulting in corresponding DCE values between 2.6 and 3.8 mg/L.

Within 225 days of initial inoculation (i.e., 15 days after initial degradation of PCE was observed), degradation of PCE was observed at Port 6 and above (Figure 4.13). This was either due to movement of organisms from higher in the column or acclimation of the organisms in this region. Either way, there seemed to be a moving 'front' of PCE degradation down the column. No accumulation of TCE was observed. Degradation appeared to proceed directly from PCE to *cis*-1,2-DCE. Degradation beyond *cis*-1,2-DCE was not observed.

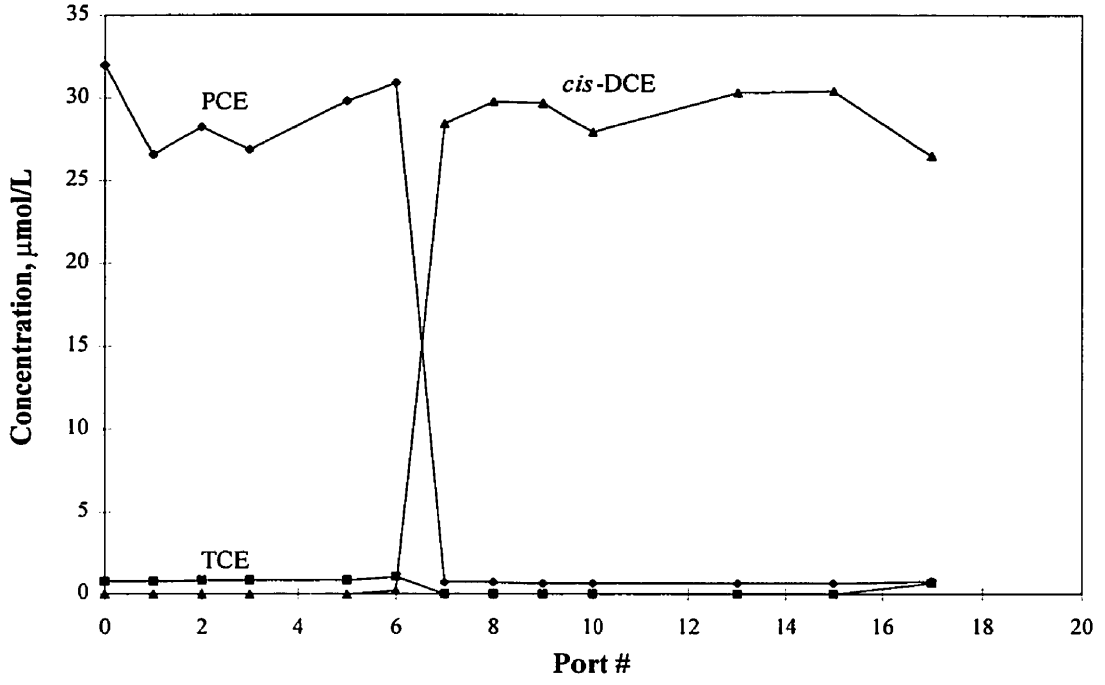


Figure 4.13 : PCE/TCE/*cis*-DCE profile 225 days after inoculation

Figure 4.14 illustrates the ethanol, acetate and propionate profile at 225 days. As can be seen, significant consumption of propionate was observed. Whereas at 210 days, propionate concentrations of roughly 1 mmol/L were detected (Figure 4.12), at 225 days only 0.5 mmol/L of propionate was observed. Note too, that the inlet ethanol concentration was much higher at 225 days than at 210 days. In other words, more acetate and propionate

were likely formed and subsequently consumed. Molar ratios of propionate to ethanol and acetate to ethanol revealed values of 0.2 and 0.4 respectively.

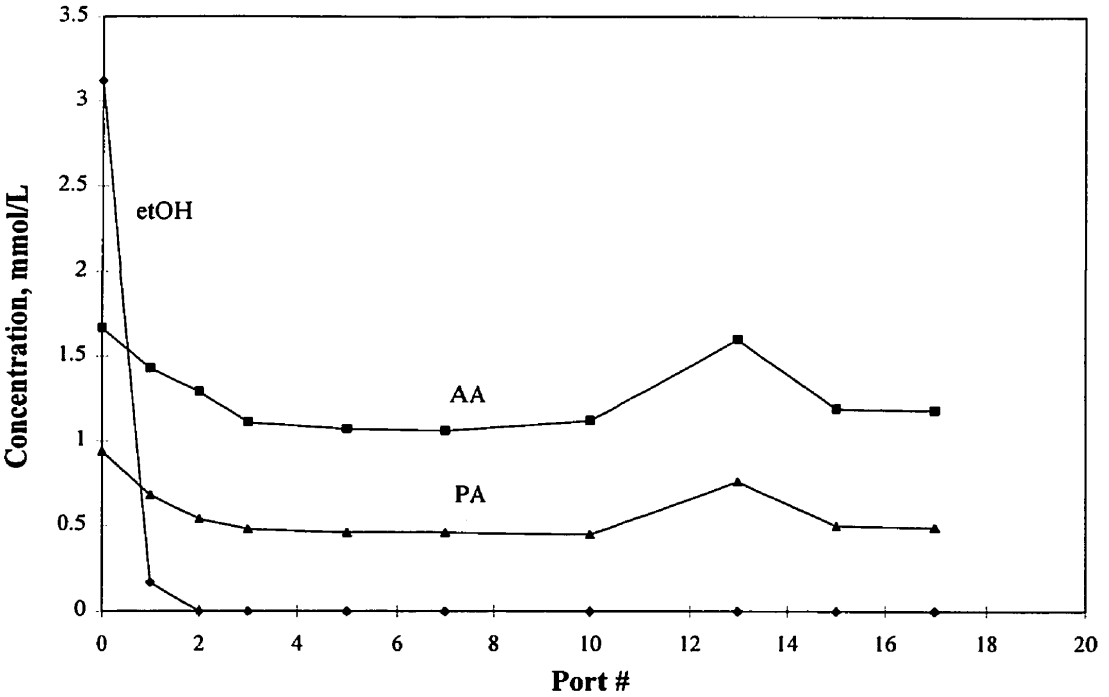


Figure 4.14: EtOH/AA/PA profile 225 days after inoculation

Overall, the molar ratios of propionate to ethanol and acetate to ethanol have been seen to shift throughout operation of the column. Table 4.3 summarizes the observed ratios.

Table 4.3: Observed molar ratios for PA:etOH and AA:etOH

Days of Column Operation	PA:etOH	AA:etOH
30 (Figure 4.2)	1.3	0.5
90 (Figure 4.5)	1.0	0.8
210 (Figure 4.12)	0.7	0.9
225 (Figure 4.14)	0.2	0.4

Clearly, throughout the 225 days of column operation, the amount of propionate and acetate produced from ethanol have shifted. This may be due to either inhibition of the culture, or a shifting in organism dominance.

From the time that CT was removed from the column inlet, traces of CT and CF were detected at the lower end of the column (i.e. below Port 5). Apparently there was some CT contamination in the PCE used in the column. Perhaps CT degradation to CF was inhibiting organisms in the lower portion of the column, thus explaining the recalcitrance of PCE in this region. It should be noted that CT was not always detected, but that, when observed, concentrations did not exceed 0.15 mg/L or 0.05 mg/L for CT and CF respectively.

Plots of PCE, TCE and *cis*-1,2-DCE concentration versus time were constructed for various ports in order to examine the rates of degradation and formation for these compounds. In Figure 4.15, PCE, TCE and *cis*-1,2-DCE results at Port 13 are plotted against time. As was previously mentioned, no accumulation of TCE was observed in the column. This phenomenon could be described as an almost instantaneous switching on of PCE degrading capability. As can be seen, after initial detection of *cis*-1,2-DCE, there seemed to be complete conversion of PCE to DCE. As such, it is difficult to come up with a rate of formation. Again, no degradation of *cis*-1,2-DCE was observed.

4.1.4 Pressure Profile

To track microbial growth in the column, pressure profiles were taken. An increase in pressure indicates growth of the culture (some of the pressure increase may have been due to sulfide precipitation from the groundwater media although this was likely a minor source). Significant growth was not observed until 195 days after initial inoculation of the column. Until this time, a pressure of about 3.0 psi (equivalent to the height of sand) was recorded at the bottom (Port 1), of the column, with a roughly linear decrease throughout the column. Within 195 days of inoculation, however, a considerable increase in pressure was observed at the bottom of the column, with further increases noted with time. The pressures measured at the other ports remained virtually unchanged during this time period (minor fluctuations were noted). Figure 4.16

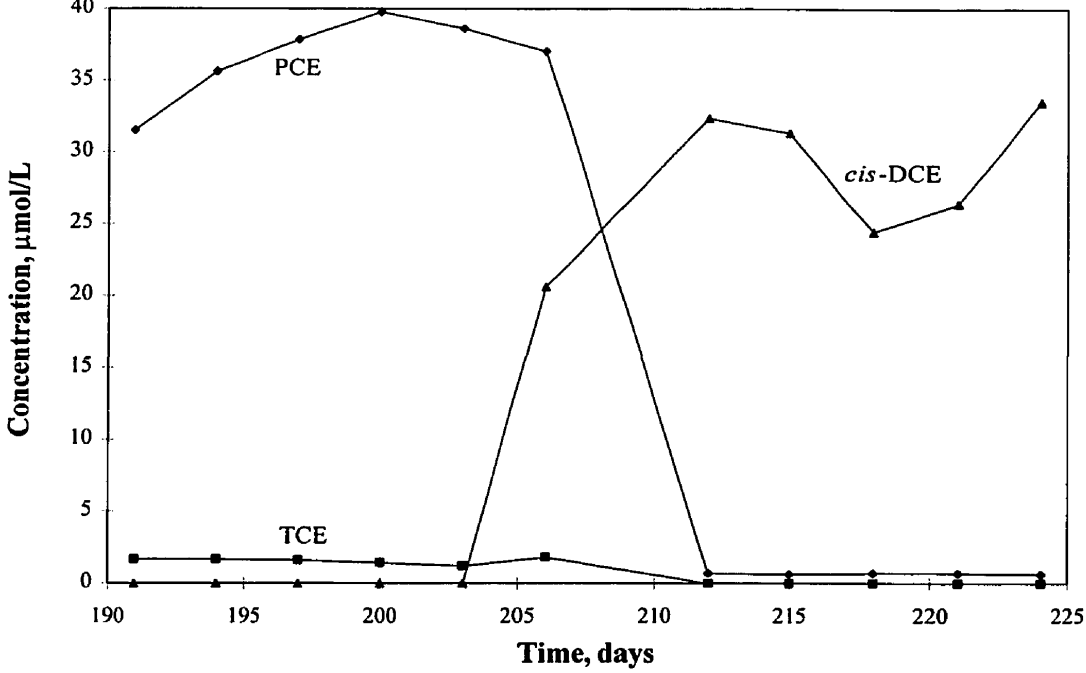


Figure 4.15: PCE/TCE/*cis*-DCE profile at initiation of degradation (Port 13)

illustrates pressure profiles observed in the column once the pressure started to increase.

The observed increases were likely due to one of two reasons. First, since ethanol consumption was complete by Port 2, it would seem reasonable that the highest rate of growth would occur at the bottom of the column. In other words, since these organisms were receiving the most substrate, it follows that the growth rate in this region of the column should have been the greatest. However, since the amount of organisms initially present was so small, growth of the population took a long time. Second, perhaps CT or CF was inhibiting growth of the culture. CT was removed from the column inlet 170 days after inoculation, shortly before the increases in pressure were observed. Removal of CT might have allowed for a growth rate not previously observed.

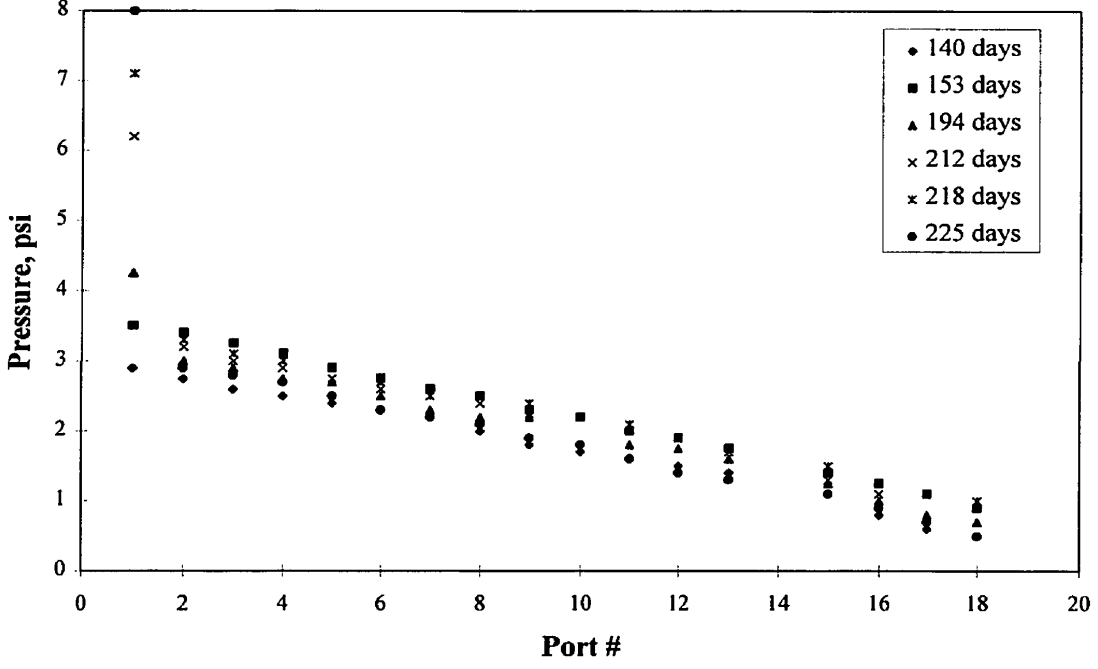


Figure 4.16 : Pressure profile results

4.2 MICROCOSM STUDIES

4.2.1 PCE Microcosms

Microcosm studies were conducted to determine the ability of the inoculum source to degrade PCE in the absence of CT and TCE and to examine the acclimation rate as a function of electron donor and yeast extract concentration. Prior to column study phase IV, no degradation of PCE was observed in the column. It was postulated that perhaps ethanol was not suitable for the initiation of degradation. Since degradation of PCE with methanol has been observed by other researchers (e.g., Freedman and Gossett, 1989), methanol was examined for its ability to initiate degradation with the present culture. Table 4.4 describes the various microcosms that were studied in terms of the electron donor and yeast extract concentration examined. The table also indicates that duplicate analysis was performed, with one bottle sampled every 3 days, and the other every 12 days. In total, 18 bottles were examined. For all of the bottles, the PCE concentration was roughly 1.5 mg/L. Fluctuations from this 1.5 mg/L were due to the fact that concentrated feed solutions were

dependent, the PCE concentration in the different feed solutions varied somewhat. Total and volatile solids measurements were made when each bottle was filled with culture. In all cases total solids were between 2050 and 2150 mg/L, with volatile solids between 750 and 850 mg/L.

Table 4.4: Microcosms examined

Condition #	Electron Donor (100 mg/L)	YE Conc. (mg/L)	Replicates	Sampling Interval (days)[1]
1	ethanol	2	2	3 (12)
2	methanol	2	2	3 (12)
3	ethanol/methanol	2	2	3 (12)
4	ethanol	100	2	3 (12)
5	methanol	100	2	3 (12)
6	ethanol/methanol	100	2	3 (12)
7	ethanol	10	2	3 (12)
8	ethanol	25	2	3 (12)
9	ethanol	50	2	3 (12)

[1] Sampling interval in parentheses is for second replicate

Small amounts of TCE (≤ 0.20 mg/L) were detected in all serum bottles within 3 days of PCE and electron donor addition. Acetate and propionate were also consumed in the presence of PCE. Bottles containing 2 mg/L YE showed only slow TCE production with repeated feeding during the first 30 days (Figure 4.17). The observed decrease in PCE in this initial stage is largely due to sampling and methane removal. Bottles containing 100 mg/L YE showed only slightly higher TCE concentrations initially (first 10 days) (Figure 4.18). After 12 days, however, all bottles amended with 100 mg/L YE showed substantial degradation (i.e. both TCE and *cis*-1,2-DCE were detected) with 88% removal of PCE In Figures 4.17 and 4.18, degradation is noted to proceed much faster with the increased YE concentration.

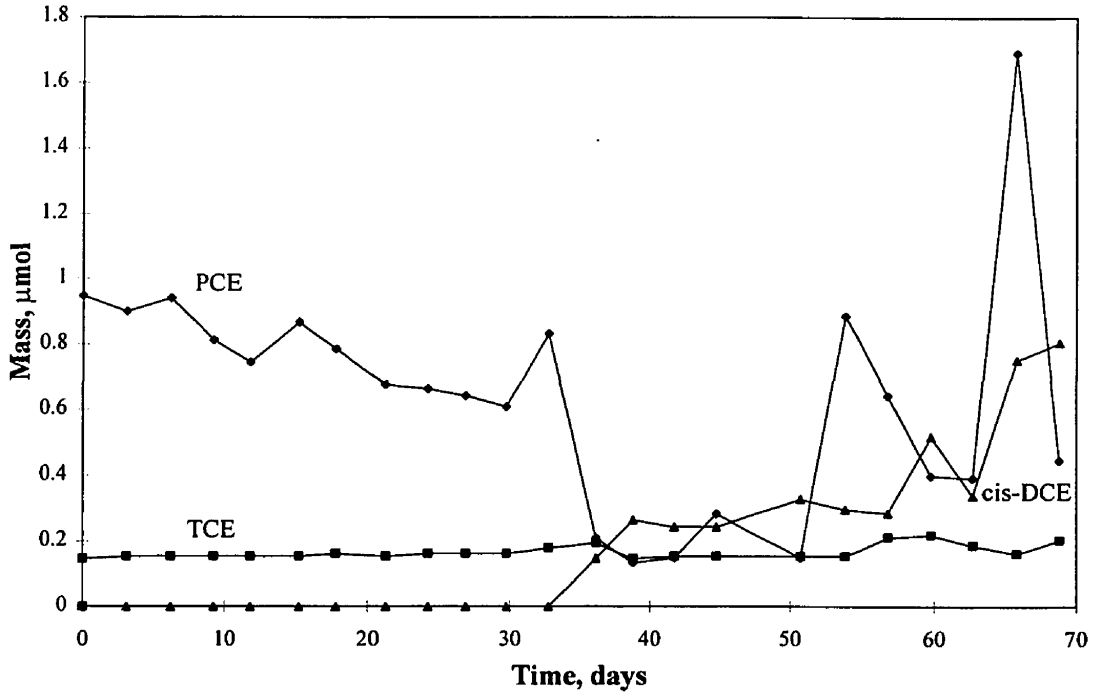


Figure 4.17: Microcosm results for a bottle with PCE, etOH and 2 mg/L YE

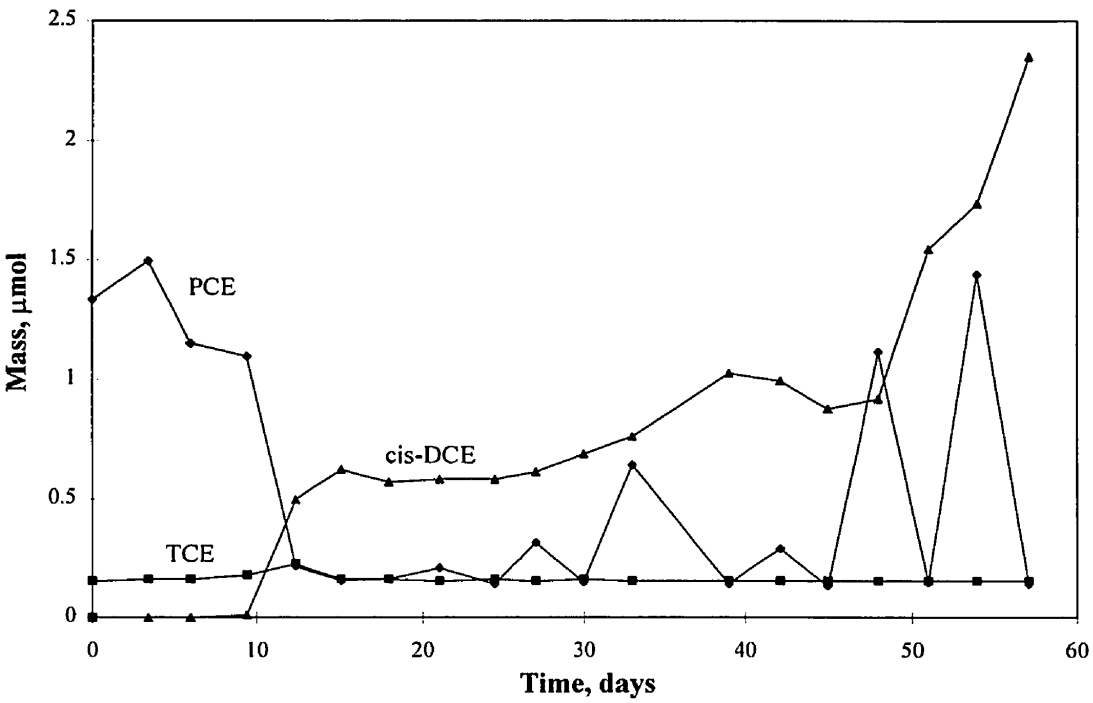


Figure 4.18: Microcosm results for a bottle with PCE, etOH and 100 mg/L YE

to the fact that feed solutions containing PCE were administered when the PCE mass in a bottle was found to be below 0.15 μmol. This was done to ensure that the culture could sustain dechlorination of PCE.

Detection of *cis*-1,2-DCE was observed after 9 days in the bottles with ethanol and the ethanol/methanol combination and after 12 days in bottles amended with methanol, suggesting that ethanol was as suitable, if not more suitable than methanol for the initiation of degradation in this culture. Figures 4.18 and 4.19 indicate similar results for degradation of PCE in bottles amended with ethanol and those with methanol. Table 4.5 compares the acclimation times for the various electron donors with 2 and 100 mg/L YE. While there are some anomalies, all electron donors appeared to perform the same.

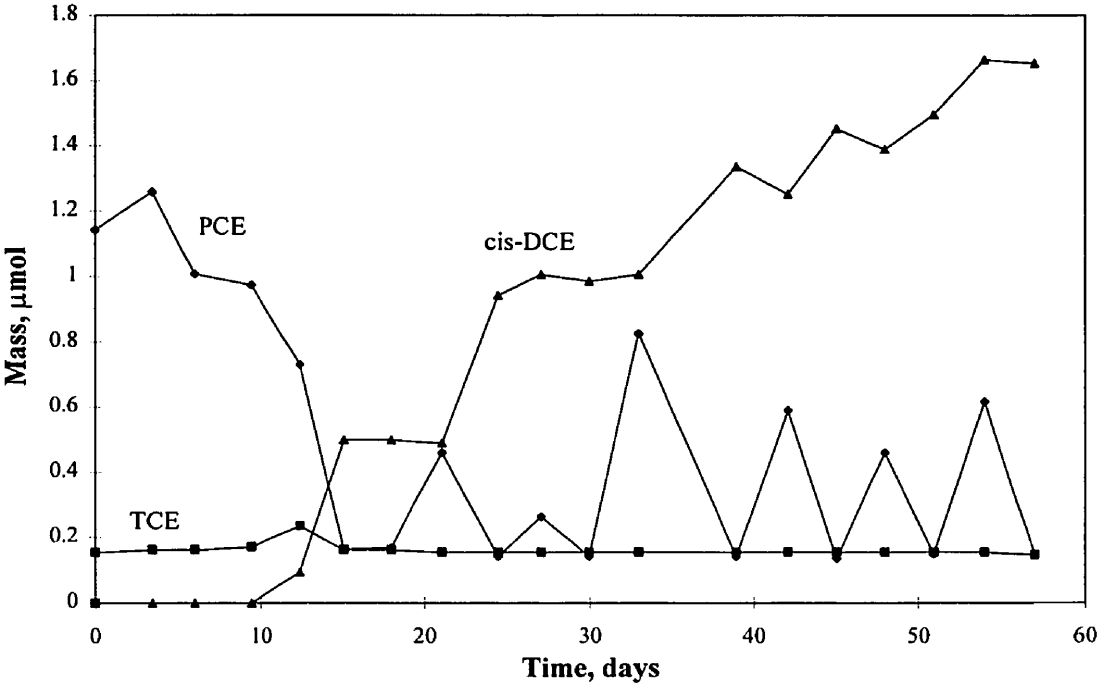


Figure 4.19: Microcosm results for a bottle with PCE, meOH and 100 mg/L YE

As can be seen in Table 4.5, duplicate bottles did not always behave in a similar manner as demonstrated by the difference in acclimation times. Differences in bottles, however, are likely due to the different sampling periods. While some bottles were

Electron Donor	YE Concentration (mg/L)	Acclimation Time (days)[1]	
		3-Day	12-Day
ethanol	2	33-36	36-48
methanol	2	45-48	12-24
ethanol/methanol	2	-[2]	12-24
ethanol	100	6-9	12-24
methanol	100	9-12	12-24
ethanol/methanol	100	6-9	12-24

[1] The number of days from the start-up of a bottle before 65 % removal of PCE was observed during a sampling interval.
[2] No degradation was observed during study duration.

sampled every 3 days, other bottles were only sampled every 12 days. The discrepancies observed in the acclimation times therefore, were likely due to the infrequency of sampling in the latter bottles (i.e., degradation might have occurred at Day 13, however after Day 12, the next sampling interval was Day 24). The discrepancy may also have been due to disturbances in bottles, for instance the presence of oxygen.

In all the bottles examined, acclimation was said to have occurred when 65% removal of PCE had occurred during a sampling interval. While TCE was detected in all of the bottles examined, the TCE concentration was quite low, with no accumulation of TCE once degradation was initiated. A decrease in the PCE concentration by 65% was always coincident with the presence of *cis*-1,2-DCE, and as such was used as the benchmark for the onset of degradation. No significant degradation beyond *cis*-1,2-DCE was observed at the time of write-up (i.e. accumulation of *cis*-1,2-DCE was observed), however several samples run on the mass spectrometer indicated that small amounts of VC were present. These amounts were not quantified.

Clearly, the unacclimated inoculum source was capable of PCE dechlorination using ethanol as an electron donor. However, 2 mg/L YE was not as effective as the higher concentrations. A significantly higher YE concentration, for instance 100 mg/L, was required for rapid acclimation.

Additional bottles were also set up to examine the effect of yeast extract on the degradation of PCE. Yeast extract concentrations of 10, 25, and 50 mg/L (in addition to

the 2 and 100 mg/L) were examined with ethanol at a concentration of 100 mg/L. While a 50-fold increase in YE seemed to lead to a faster rate of degradation, it is likely that 100 mg/L was higher than necessary for the initiation of degradation in a suitable time frame. Accordingly, these other concentrations were examined so that a relationship between YE and degradation could be determined. Table 4.6 compares the acclimation times for various YE concentrations with ethanol as electron donor.

Table 4.6: Acclimation time for various YE concentrations

YE Concentration (mg/L)	Acclimation Time (days)	
	3-Day	12-Day
2	33-36	36-48
10	15-18	12-24
25	24-27	12-24
50	12-15	12-24
100	6-9	12-24

Although degradation of PCE was observed in bottles containing 2 mg/L YE, the time frame required for initiation of degradation was much longer than in bottles with higher YE concentrations. As can be seen in Table 4.6, for bottles with 2 mg/L YE, degradation was observed at roughly 36 days. By comparison, in bottles containing higher YE concentrations, degradation was observed between 6 and 24 days. It would seem plausible that the YE encouraged growth of PCE degraders, thereby leading to faster rates of degradation.

While degradation of PCE was observed after 15 days for a bottle amended with 50 mg/L YE, and after 18 days for a bottle amended with 10 mg/L YE, degradation of PCE in a bottle amended with 25 mg/L YE was not observed until after 24 days. This was likely due to one of two reasons. First, perhaps there was some disturbance to the culture (i.e., oxygen entered the bottle) which slowed degradation. Second, while YE serves to increase the population of PCE degraders, perhaps there were fewer PCE degraders present in this bottle initially.

Overall, in terms of the yeast extract concentration, the results appear to indicate that the higher yeast extract concentrations lead to faster initiation of degradation of PCE.

The relationship does not appear to be linear, however. Since duplicate bottles did not always behave in a similar manner, it seems likely that a limiting factor is the number of PCE degraders present initially. It appears that YE may serve to allow more rapid growth, however, the amount of organisms initially dispensed from the source reactor must be considered.

Mass balances were performed for several bottles. Since the data presented in Figures 4.17 through 4.19 does not account for dilution from the feed solution or methane withdrawal, a mass balance was conducted to appropriately account for these removals. As can be seen from Tables 4.7 through 4.10, mass balances of between approximately 90 and 105% were achieved. As was expected, the mass balances indicated that the major by-product of PCE degradation was *cis*-1,2-DCE.

Analysis of samples for ethanol, methanol, acetate and propionate indicated that both ethanol and methanol were completely consumed within the 3 day sampling interval. Significant acetate and propionate consumption was also observed during the sampling intervals, however complete consumption did not always occur. Concentrations on the order of 20-50 mg/L were sometimes observed. As previously mentioned, only 120 μmol/L of electrons from the electron donor are required for the degradation of PCE to *cis*-1,2-DCE. More than enough donor was consumed to facilitate this reaction. Furthermore, additional electron donor was added every 3 days.

4.2.2 Ethanol/acetate/propionate Microcosms

The ability of the inoculum source organisms to consume ethanol in the absence of chlorinated compounds was also investigated in microcosms. Results indicated that ethanol was rapidly removed, with acetate removal also occurring, but at a slower rate. However, while propionate was rapidly formed, degradation was not observed until after approximately 200 days of inoculum source operation.

the sampling interval between Day 57 and Day 60

Compound	Actual Mass Day 57 (μmol)[1]	Amount Withdrawn (μmol)[2]	Total Mass Day 60 (μmol)[3]	Actual Mass Day 60 (μmol)[4]
PCE	0.64	0.02	0.62	0.40
TCE	0.21	0.01	0.20	0.22
cis-DCE	0.29	0.01	0.28	0.52
		Total:	1.10	1.14

Table 4.8: Mass balance for a bottle amended with PCE, etOH and 100 mg/L YE for the sampling interval between Day 27 and Day 30

Compound	Actual Mass Day 27 (μmol)[1]	Amount Withdrawn (μmol)[2]	Total Mass Day 30 (μmol)[3]	Actual Mass Day 30 (μmol)[4]
PCE	0.32	0.02	0.30	0.15
TCE	0.15	0.01	0.14	0.16
cis-DCE	0.62	0.02	0.60	0.69
		Total:	1.04	1.00

Table 4.9: Mass balance for a bottle amended with PCE, meOH and 100 mg/L YE for the sampling interval between Day 42 and Day 45

Compound	Actual Mass Day 42 (μmol)[1]	Amount Withdrawn (μmol)[2]	Total Mass Day 45 (μmol)[3]	Actual Mass Day 45 (μmol)[4]
PCE	0.59	0.03	0.56	0.14
TCE	0.15	0.01	0.14	0.15
cis-DCE	1.25	0.03	1.22	1.45
		Total:	1.92	1.74

Table 4.10: Mass balance for a bottle amended with PCE, etOH and 10 mg/L YE for the sampling interval between Day 27 and Day 30

Compound	Actual Mass Day 27 (μmol)[1]	Amount Withdrawn (μmol)[2]	Total Mass Day 30 (μmol)[3]	Actual Mass Day 30 (μmol)[4]
PCE	0.35	0.02	0.33	0.14
TCE	0.21	0.01	0.20	0.15
cis-DCE	0.38	0.01	0.37	0.57
		Total:	0.90	0.86

[1] Actual mass indicates the total mass (both liquid and headspace) of each chlorinated compound in a bottle.

[2] Amount withdrawn was calculated from the liquid volume withdrawn for sampling purposes and the gas sample withdrawn for pressure balancing.

[3] Total mass indicates the mass of each chlorinated compound that should be present at the next sampling interval without degradation (i.e. this only takes the withdrawals into account, however the total should remain constant for each sampling interval).

[4] Actual mass indicates the mass of each chlorinated compound as measured through gas chromatography and converted to total mass with Henry's Law.

examine the consumption of ethanol as well as the subsequent formation and consumption of acetate and propionate. In the first of these studies, intensive sampling was undertaken to determine ethanol consumption and acetate formation and consumption rates. As can be seen, complete ethanol degradation was observed within approximately 5 hours in this tracking study. Acetate consumption, on the other hand, was not complete until 12 hours. Finally, propionate consumption is seen to be slow in the initial tracking study with no removal observed in the time frame of the study (12 hrs.). In the second study, propionate consumption was found to be complete in roughly 50 hours.

For the initial tracking study conducted, ethanol consumption of approximately 40 $mg \cdot L^{-1} \cdot hr^{-1}$ was observed, with acetate formation and consumption rates of roughly 30 $mg \cdot L^{-1} \cdot hr^{-1}$ and 4 $mg \cdot L^{-1} \cdot hr^{-1}$ respectively. While propionate removal was not observed in these earlier studies, by 210 days after inoculum source start-up, propionate consumption had reached a rate of 1.3 $mg \cdot L^{-1} \cdot hr^{-1}$.

Note that the starting residual is high for propionate in Figure 4.20 whereas the starting residual in Figure 4.21 is quite low. This indicates that the source reactor was consuming propionate better at 210 days than at 140 days.

The lag period observed before the onset of propionate consumption could be due to the fact that propionate consumers were not present in great enough numbers to initiate appreciable consumption of propionate. Since the initial inoculum source was a sludge digester, propionate may have been present but would not be rapidly generated. Accordingly, there wouldn't necessarily be a lot of propionate degraders. Furthermore, since the formation of propionate from ethanol occurs very rapidly, it is possible that the propionate degraders just couldn't keep up. Propionate degraders are also very slow growers, which supports the observed slow increase in the propionate consumption rate.

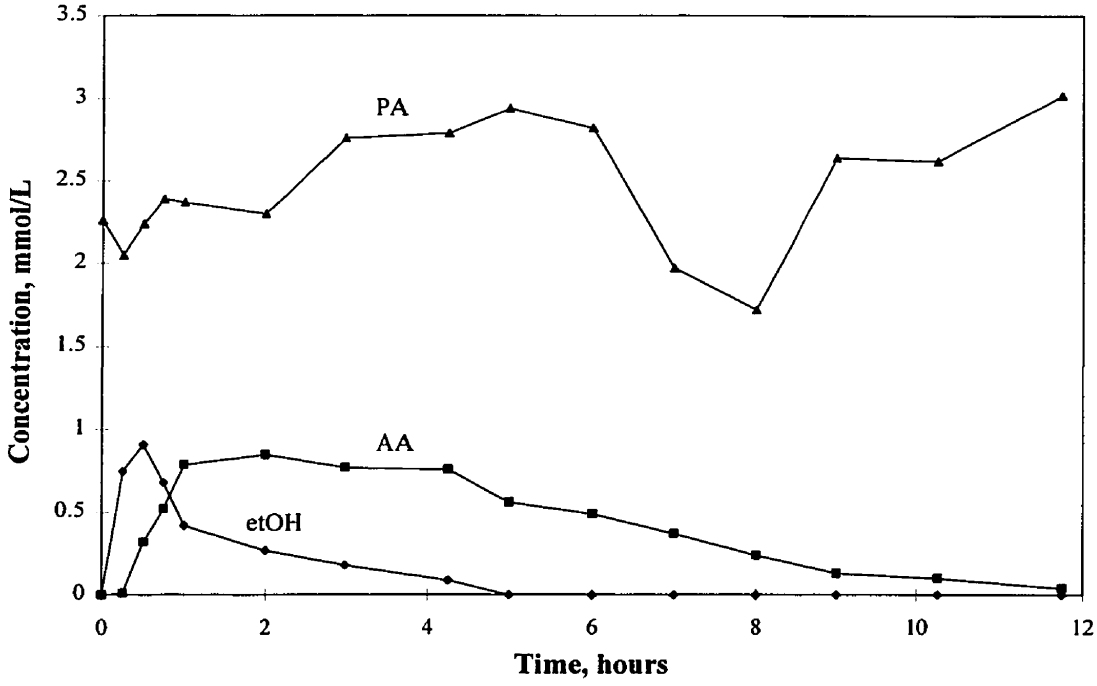


Figure 4.20: EtOH/AA/PA tracking study 140 days after inoculum source start-up

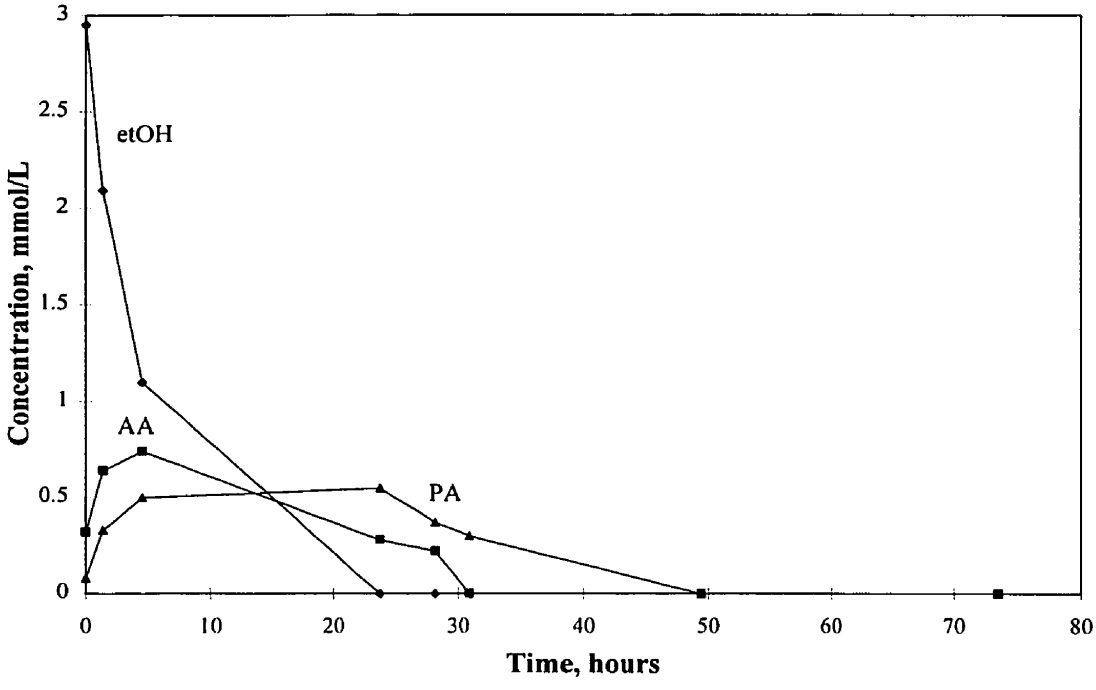


Figure 4.21: EtOH/AA/PA tracking study 210 days after inoculum source start-up

5. DISCUSSION

5. 1 CT Degradation

The results from column study phase I clearly indicated that unacclimated biomass from an anaerobic digester can readily degrade CT. Immediate conversion of CT to CF and DCM indicated that acclimation of the culture to degrade CT was very rapid.

Previous research has indicated that CF and DCM can be degraded by an oxidative pathway leading directly to the formation of carbon dioxide (Egli *et al.*, 1988). Freedman and Gossett (1991) reported that the principal mode of DCM degradation under methanogenic conditions was oxidation to carbon dioxide. As such, since the presence of CF and DCM only accounted for 88% of the incoming CT, the remaining 12% was likely mineralized to CO_2.

The persistence of CF through the column in phases I, II and III was possibly due to insufficient electron donor in the upper part of the column. During the starvation study (phase II), increased electron donor consumption resulted in increased CT and CF degradation. Since the culture was not acclimated to consume acetate and propionate, and ethanol was only available up to Port 3, no degradation beyond that which was observed was possible. Additionally, the recalcitrance of CF may have been due to the lack of reaction sites.

5.2 PCE Degradation in the Absence of CT and TCE

In the present work, dechlorination of PCE to *cis*-1,2-DCE in the absence of CT and TCE was observed both in the column and in microcosm studies. While stoichiometric degradation of PCE to *cis*-1,2-DCE was observed during phase IV of the column studies and in microcosm work, kinetic rates for this process could not be calculated. No accumulation of TCE was observed. This can be viewed as an almost instantaneous switching on of PCE degrading capability.

Increased yeast extract concentrations were found to lead to higher rates of degradation both in the column and in microcosm work. While the initiation of degradation for a culture amended with 2 mg/L YE was slower than for those cultures amended with 10, 25, 50 and 100 mg/L YE, dechlorination of PCE was equally sustained in

concentrations initiated degradation faster, it is suggested that initially a high YE concentration (100 mg/L) be used to initiate degradation. Once it is observed, however, the YE concentration can be lowered to 2 mg/L.

While research has been conducted on the topic of yeast extract as it applies to the dechlorination of PCE, it seems that many different conclusions have been drawn. In work conducted by Fennell *et al.* (1997), pre-fermented yeast extract, prepared by inoculating the yeast extract with the source culture and waiting a period of time before use (this FYE is presumed to be a nutrient source with less significant electron-donor content than unfermented yeast extract), served as a source of slowly fermented volatile fatty acids and a required micronutrient supplement. While FYE resulted in more sustained dechlorination of PCE, the researchers were not sure if the difference was simply nutritional or that a fraction of the FYE served as slowly available electron donor that fuelled dechlorination after the initial burst from donor degradation.

Deleting YE from the growth medium did not allow sustained PCE dechlorination in another study (Maymó-Gatell, 1995). Since it was possible that YE or some product derived from it was serving as a carbon source, it was tested whether YE could be replaced with acetate, a common carbon source that is used by many anaerobes but is not utilizable as an energy source by most anaerobes. Their findings showed that acetate greatly stimulated dechlorination by the H_2-PCE culture when YE was not present, suggesting that YE served as a source of carbon.

Strain MS-1 dehalogenated PCE via TCE to *cis*-1,2-DCE when grown anaerobically in yeast extract-containing medium (i.e. 50 mg/L YE) (Sharma and McCarty, 1996). Yeast extract was metabolized mainly to acetate. It was postulated that since yeast extract contains many different electron donors and growth factors, its presence may ensure the growth of strain MS-1 under anaerobic conditions.

Since ethanol, acetate and propionate were all present in the microcosms, it seems unlikely that the yeast extract was serving as a significant electron donor. It has already been pointed out that propionate serves as a slowly released source of H_2 (Fennell *et al.*, 1997). As such, it is doubtful that it was the electrons from the yeast extract that led to

Rather, it is more probable that micronutrients were supplied by yeast extract.

Microcosm work revealed that ethanol and methanol both performed the same in terms of their ability to initiate degradation of PCE.

While microcosm work was initiated to look at the issue of PCE degradation on its own, many steps were taken in the column before dechlorination was observed. Figure 5.1 and Table 5.1 summarize the various events that took place prior to the onset of PCE dechlorination.

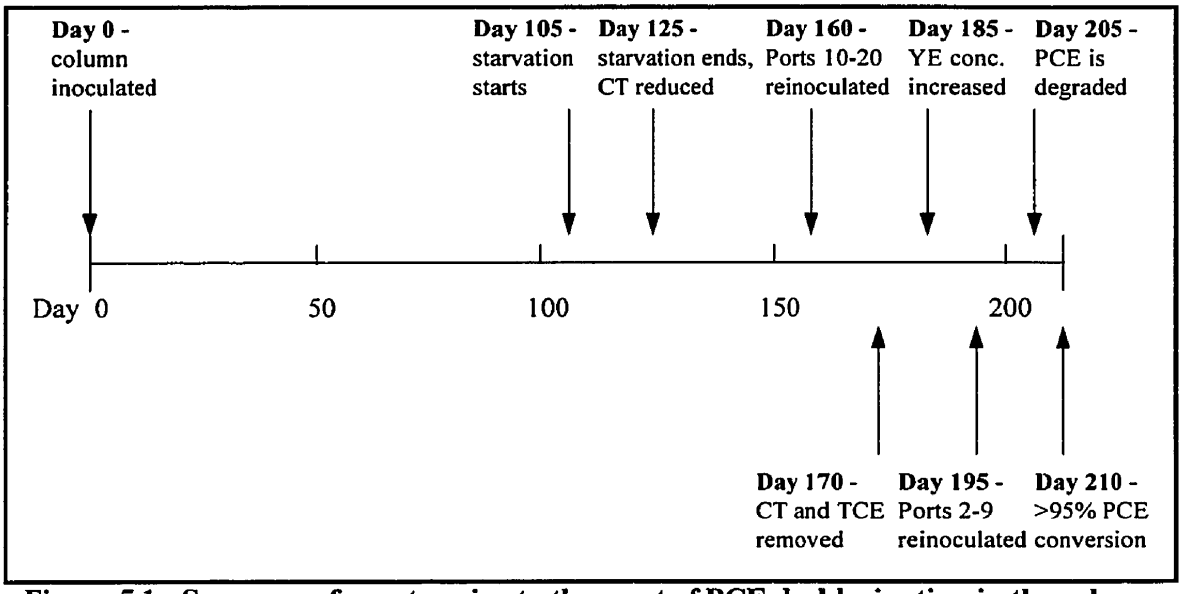


Figure 5.1: Sequence of events prior to the onset of PCE dechlorination in the column

Table 5.1: Concentrations for events prior to the onset of PCE dechlorination in the column

Chemical	Concentration (mg/L)				
	Day 0-105	Day 105-125	Day 125-170	Day 170-185	Day 185-210
PCE	5	NA	5	5	5
TCE	5	NA	5	0	0
CT	5	NA	0.5	0	0
etOH	100	NA	100	100	100
YE	2	NA	2	2	100

While it has been postulated that the presence of CF inhibited PCE degradation, there was clearly an acclimation period required once CT was removed from the column. CT was removed from the feed solution at 170 days, whereas degradation of PCE was only observed at 205 days. During this time, the column was reinoculated with source organisms known to consume acetate and propionate, and the yeast extract concentration was increased. Since microcosm studies revealed that PCE dechlorination was equally sustained in bottles with 2 and 100 mg/L YE, it was likely not this added micronutrient source that initiated degradation. It is, however, possible that the yeast extract increased the rate of acclimation. Most likely it was the availability of electrons from acetate and propionate that encouraged dechlorination of PCE.

The acclimation period prior to PCE degradation may have been due to either a "true" acclimation, a period of no biodegradation followed by initiation and acceleration of degradation, or an "apparent" acclimation, in which biodegradation proceeds from the time of addition but at rates so slow as to be nondetectable. A true acclimation could be caused by the time required for genetic changes, induction of new protein synthesis, or exhaustion of preferential substrates, whereas an apparent acclimation would probably be the case if the initial population was very small but grew continuously until, at some point, it was large enough to result in detectable biodegradation (Linkfield *et al.*, 1989).

5.3 Recalcitrance of PCE

In column study phases I through III, representing 170 days of column operation, no PCE or TCE degradation was observed. As mentioned, this was likely due to inhibition from CF. It has been reported that CF can inhibit unacclimated cultures at very low concentrations, while with acclimation, higher concentrations of CF can be tolerated (Yang and Speece, 1986). A direct relation was also found between CF concentration and inhibition, with the recovery of biological systems from inhibition depending on biomass concentration (with higher biomass concentrations leading to faster recovery from inhibition).

Since the culture was introduced to the column with CT already present, it is likely that there was never an opportunity for the culture to adapt to these inhibitory conditions.

did not seem to consume acetate and propionate, it is also possible that the acetate and propionate consumers were just not present in sufficient numbers. Since the degradation of PCE requires a transfer of electrons from the electron donor to PCE, and the electrons were trapped in acetate and propionate, this might also explain why no degradation was observed.

While there are few reports on the conversion of ethanol to propionate as it applies to reductive dechlorination, this phenomenon serves to make ethanol an even more attractive substrate since propionate provides a slow and steady release of H_2. In one study, enrichment cultures amended with propionic acid resulted in less methanogenesis than did amendment with ethanol, which generated much higher H_2 levels for reductive dechlorination (Fennell *et al.*, 1997). While ethanol did not support complete dechlorination during short-term tests, it was a viable donor over long-term testing because a portion was converted to a pool of slowly degraded propionic acid. The accumulated propionic acid in these cultures contributed to the pool of slowly degradable donor and facilitated continued dechlorination after the primary donors were depleted. It was therefore concluded that adding ethanol might be almost the equivalent of adding propionic acid, a slow-release H_2 donor.

In column study phases I through IV, ethanol consumption was observed with acetate and propionate formation resulting. While some acetate and propionate consumption was observed in phase IV, no consumption was observed in any of the phases prior to this. Since both have been shown to serve as electron donors for reductive dechlorination they were essentially serving as unutilized sources of electrons. It was speculated that the presence of chloroform in the present work inhibited consumption of acetate and propionate, thus explaining the recalcitrance of PCE.

Other researchers have also found that CF inhibits the consumption of electron donor. In work done by Bagley and Gossett (1995), chloroform concentrations as low as 2.5 μmol/L inhibited methanol enrichment cultures with 0.8 μmol/L chloroform inhibiting pure cultures. The presence of chloroform was also seen to decrease the rate of acetic acid utilisation in a mixed methanogenic culture studied by Gupta *et al.* (1996). Furthermore,

when CF concentrations exceeded 2.7 μmol/L, the culture was completely inhibited even after chloroform had been completely degraded.

While other research conducted appears to support the idea that acetate and propionate consumption were inhibited by chloroform, no direct evidence of this was gathered in the present work. Results gathered from the column indicated that 32.5 μmol/L CT was converted to approximately 20 μmol/L CF. Since such small amounts of CF were found to be inhibitory in other studies, inhibition of the column culture by CF was a strong possibility given the high concentration.

Pressure profiles taken over the course of column study phases I through IV demonstrated that the pressures remained virtually constant until CT was removed from the chlorinated solution delivered to the column. An increase in pressure at the bottom of the column was observed after CT was removed from the column inlet, providing further proof that CF inhibited the culture. While consumption of ethanol was observed when CT was fed to the column, perhaps the presence of CT (or most probably CF) impeded growth of the culture. Its removal, therefore, lead to significant growth of the culture, with pressures as high as 8.0 psi recorded.

Since degradation of PCE in microcosm studies was observed with all of the YE concentrations examined, it is unlikely that the low YE concentration had any adverse effect. Perhaps the PCE degraders were present but at such a small number so as to make degradation beyond detection. According to this theory, therefore, detection of PCE dechlorination would represent growth of the PCE degraders.

5.4 Recalcitrance of *cis*-1,2-DCE

In both the column and microcosm work conducted, degradation of PCE to *cis*-1,2-DCE was observed. Similar to the present work, other researchers have also found *cis*-1,2-DCE to be the major intermediate in reductive dechlorination of chlorinated ethenes (DeBruin *et al.*, 1992; Kästner, 1991; Tandoi *et al.*, 1994). While the complete degradation of PCE to ethylene has been shown to occur with mixed cultures and sediments, to date, no pure cultures which can carry out this reaction have been isolated. Several pure cultures which can carry out the partial dehalogenation have, however, been described. These

1995), which couples the oxidation of H_2 to the reduction of PCE to *cis*-1,2-dichloroethylene, *Dehalobacter restrictus* (Holliger *et al.*, 1993), which carries out a similar reaction, and strain TT4B, which couples the oxidation of acetate to the reductive dechlorination of PCE, again with *cis*-1,2-DCE as the product (Krumholz *et al.*, 1996).

Since mixed cultures have been shown to carry out the complete dechlorination, it would seem that another organism, not present in the culture examined in this study, carries out the dechlorination from DCE to ethylene. DeBruin *et al.* (1992) came to a similar conclusion in work done with a mixture of anaerobic sediment from the Rhine river and anaerobic granular sludge. They observed degradation of PCE to TCE, *cis*-1,2-DCE, VC and ethene followed by a reduction of ethene to ethane. However, in a column with only ground anaerobic granular sludge as inoculum, PCE was initially converted to both TCE and *cis*-1,2-DCE. After 2 months of operation, *cis*-1,2-DCE was the only end product. VC, ethene, and ethane were never detected. Interestingly, in an earlier column study with only Rhine river sediment, mainly *cis*-1,2-DCE and small quantities of VC were observed as intermediates (DeBruin *et al.*, 1992). Complete dechlorination of PCE could be achieved only by a combination of Rhine river sediment and anaerobic granular sludge. Apparently, organisms from both inoculum sources were necessary for the full dechlorination process. It was therefore hypothesized that several different microbial species are needed to achieve complete dechlorination of PCE.

It is also possible that an acclimation period greater than that observed in this study is required before the onset of DCE dechlorination. Perhaps the population of organisms that carry out the dechlorination of DCE was so small that VC dechlorination was not detectable. Alternatively, since the culture had never before been exposed to DCE, perhaps an adaptation period was required before the onset of DCE dehalogenation.

It must be noted that the transformation of PCE to *cis*-1,2-DCE does reduce the environmental hazard because, unlike PCE or TCE, *cis*-1,2-DCE is not believed to be carcinogenic and has a higher U.S. Environmental Protection Agency drinking water maximum contaminant level (75 μg/L for *cis*-1,2-DCE versus 5 μg/L for PCE and TCE) (Federal Register, 1989). Since dechlorination involves the removal of chlorine atoms,

However, since all of the intermediate products of biotransformation between PCE and vinyl chloride that have been observed in laboratory studies have been found in groundwater (Westrick *et al.*, 1984), it is important to determine the required conditions for degradation of all of the by-products.

In the present work, rapid acclimation was observed for the degradation of CT, whereas acclimation was lengthy for PCE, acetate and propionate, and not observed for *cis*-1,2-DCE. Since there were so many interrelating factors affecting the adaptation period, it is difficult to determine the conditions under which acclimation is most rapid. The results seem to indicate that the presence of CF inhibited PCE, acetate and propionate degradation, with high yeast extract concentrations shortening the period before PCE dechlorination was observed. Because of its lengthy nature, especially in anaerobic environments, the acclimation period cannot be ignored in understanding and predicting the transport and fate of pollutant chemicals which may reside in anaerobic environments.

The following conclusions can be drawn from the present work:

(1) Unacclimated biomass from an anaerobic digester can readily degrade CT. Immediate conversion of CT to CF and DCM indicated that acclimation of the culture to degrade CT was rapid. The persistence of CF and DCM throughout the column was possibly due to insufficient electron donor consumption, as confirmed by the starvation study which indicated that increased electron donor consumption increased CT and CF degradation.

(2) The production of TCE and *cis*-1,2-DCE from PCE in microcosm studies and in the column, in the absence of CT, demonstrated the ability of an unacclimated culture to degrade PCE. Equal establishment of degradation was noted using both ethanol and methanol as electron donors.

(3) More rapid degradation of PCE was noted in bottles with increased YE concentrations. It is likely that increased YE concentrations may be required to initiate rapid degradation, however dechlorination of PCE was equally sustained for all of the yeast extract concentrations examined. Accordingly, it is suggested that a high concentration of YE (i.e., 100 mg/L) be used to initiate dechlorination, but that once established, a lower concentration be examined (i.e., 2 mg/L).

(4) It is possible that CF inhibited PCE and TCE degradation in the column. Support of this hypothesis comes from the pressure profile data which indicated that very little growth of the culture was observed prior to removing CT from the column inlet. No experiments were run to support this hypothesis, so it remains conjecture at the present time.

(5) Although acetate and propionate consumption were not observed until after 200 days of inoculum source operation, both have been shown to serve as electron donors for the

inoculating the column with organisms known to consume acetate and propionate, it is possible that there simply were not enough organisms present to facilitate reductive dechlorination of PCE prior to this addition.

7. RECOMMENDATIONS

While many valuable conclusions were drawn from the present work, there are still issues that need to be examined. The following are recommended as further areas of research:

(1) Determine how the addition of TCE affects the removal of PCE in microcosm studies and in the column.

(2) Determine how the addition of CT and/or CF affects the removal of PCE in microcosm studies and in the column.

(3) Determine how the addition of CT and/or CF affects the consumption of ethanol, acetic acid and propionic acid.

(4) Determine the kinetic constants required to describe PCE/TCE degradation.

(5) Characterize the microbial community involved in the degradation process.

REFERENCES

American Public Health Association. *Standard Methods for the Examination of Water and Wastewater*. 17th edition. Baltimore:1989.

Bagley, D.M., and J.M. Gossett. 1990. Tetrachloroethene transformation to trichloroethene and *cis*-1,2-dichloroethene by sulfate-reducing enrichment cultures. Appl. Environ. Microbiol. 56:2511-2516.

Bagley, D.M., and J.M. Gossett. 1995. Chloroform degradation in methanogenic methanol enrichment cultures and by *Methanosarcina barkeri* 227. Appl. Environ. Microbiol. 61:3195-3201.

Bouwer, E.J., and P.L. McCarty. 1984. Modeling of trace organics biotransformation in the subsurface. Ground Water. 22:433-440.

Bouwer, E.J., and P.L. McCarty. 1983a. Transformations of halogenated organic compounds under denitrification conditions. Appl. Environ. Microbiol. 45:1295-1299.

Bouwer, E.J., and P.L. McCarty. 1983b. Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl. Environ. Microbiol. 45:1286-1294.

Bouwer, E.J., and J.P. Wright. 1988. Transformations of trace halogenated aliphatics in anoxic biofilm columns. J. Contamin. Hydrol. 2:155-169.

Criddle, C.S., J.T. DeWitt, D. Grbic-Galic, and P.L. McCarty. 1990a. Transformation of carbon tetrachloride by *Pseudomonas* sp. strain KC under denitrification conditions. Appl. Environ. Microbiol. 56:3240-3246.

Criddle, C.S., J.T. DeWitt, and P.L. McCarty. 1990b. Reductive dehalogenation of carbon tetrachloride by *Escherichia coli* K-12. Appl. Environ. Microbiol. 56:3247-3254.

DeBruin, W.P., J.J. Kotterman, M.A. Posthumus, G. Schraa, and A.J.B. Zehnder. 1992. Complete biological reductive transformation of tetrachloroethene to ethane. Appl. Environ. Microbiol. 58:1996-2000.

DiStefano, T.D., J.M. Gossett, and S.H. Zinder. 1991. Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. Appl. Environ. Microbiol. 57:2287-2292.

dechlorination of tetrachloroethylene by an anaerobic mixed culture. Appl. Environ. Microbiol. 58:3622-3629.

Doong, R., and S. Wu. 1996. Effect of substrate concentration on the biotransformation of carbon tetrachloride and 1,1,1-trichloroethane under anaerobic condition. Wat. Res. 30:577-586.

Dybas, M.J., G.M. Tatara, and C.S. Criddle. 1995. Localization and characterization of the carbon tetrachloride transformation activity of *Pseudomonas* sp. Strain KC. Appl. Environ. Microbiol. 61:758-762.

Egli, C., T. Tschan, R. Scholtz, A.M. Cook and T. Leisinger. 1988. Transformation of tetrachloromethane to dichloromethane and carbon dioxide by *Acetobacterium woodii.* Appl. Environ. Microbiol. 54:2819-2824.

Fathepure, B.Z., J.P. Nengu, and S.A. Boyd. 1987. Anaerobic bacteria that dechlorinate perchloroethene. Appl. Environ. Microbiol. 53:2671-2674.

Fathepure, B.Z., and S.A. Boyd. 1988a. Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* sp. strain DCM. Appl. Environ. Microbiol. 54:2976-2980.

Fathepure, B.Z., and S.A. Boyd. 1988b. Reductive dechlorination of perchloroethylene and the role of methanogens. FEMS Microbiol. Lett. 49:149-156.

Fathepure, B.Z., and T.M. Vogel. 1991. Complete degradation of polychlorinated hydrocarbons by a two-stage biofilm reactor. Appl. Environ. Microbiol. 57:3418-3422.

Federal Register. 1989. National primary and secondary drinking water regulation; proposed rule. 54:22062-22160.

Fennell, D.E., M.A. Stover, S.H. Zinder, and J.M. Gossett. 1995. Comparison of alternative electron donors to sustain PCE anaerobic reductive dechlorination. In: Bioremediation of Chlorinated Solvents. Eds., R.E. Hichee, A. Leeson and L. Semprini. Battelle Presse. Columbus. v. pp. 9-16.

Fennell, D.E., J.M. Gossett, and S.H. Zinder. 1997. Comparison of butyric acid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene. Environ. Sci. Technol. 31:918-926.

Fogel, S., R. Lewis, D. Groher, and M. Findlay. 1995. PCE treatment in saturated soil columns: methanogens in sequence with methanotrophs. In: Bioremediation of Chlorinated Solvents. Eds., R.E. Hichee, A. Leeson and L. Semprini. Battelle Presse. Columbus. v. pp. 153-160.

Freedman, D.L., and J.M. Gossett. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl. Environ. Microbiol. 55:2144-2151.

Freedman, D.L., and J.M. Gossett. 1991. Biodegradation of dichloromethane and its utilization as a growth substrate under methanogenic conditions. Appl. Environ. Microbiol. 57:2847-2857.

Freedman, D.L., M. Lasecki, S. Hashsham, and R. Scholze. 1995. Accelerated biotransformation of carbon tetrachloride and chloroform by sulfate-reducing enrichment cultures. In: Bioremediation of Chlorinated Solvents. Eds., R.E. Hichee, A. Leeson and L. Semprini. Battelle Presse. Columbus. v. pp. 123-138.

Gälli, R., and P.L. McCarty. 1989. Biotransformation of 1,1,1-trichloroethane, tichloromethane, and tetrachloromethane by a *Clostridium* sp. Appl. Environ. Microbiol. 55:837-844.

Gantzer, C.J., and L.P. Wackett. 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. Environ. Sci. Technol. 25:715-722.

Gibson, S.A., and G.W. Sewell. 1992. Stimulation of reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by addition of short-chain organic acids or alcohols. Appl. Environ. Microbiol. 58:1392-1393.

Gossett, J.M. 1987. Measurement of Henry's Law constants for C_1 and C_2 chlorinated hydrocarbons. Environ. Sci. Technol. 21:202-208.

Gupta, M.D., D. Sharma, M.T. Suidan, and G.D. Sayles. 1996. Biotransformation rates of chloroform under anaerobic conditions - I. methanogenesis. Wat. Res. 30:1377-1385.

Hashsham, S.A., R. Scholze, and D.L. Freedman. 1995. Cobalamin-enhanced anaerobic biotransformation of carbon tetrachloride. Environ. Sci. Technol. 29:2856-2863.

Holliger, C., G. Schraa, A.J.M. Stams, and A.J.B. Zehnder. 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl. Environ. Microbiol. 59:2991-2997.

Kästner, M. 1991. Reductive dechlorination of tri- and tetrachloroethylenes depends on transition from aerobic to anaerobic conditions. Appl. Environ. Microbiol. 57:2039-2046.

Krone, U.E., K. Laufer, R.K. Thauer, and H.P.C. Hogenkamp. 1989a. Coenzyme F_{430} as a possible catalyst for the reductive dehalogenation of chlorinated C_1 hydrocarbons in methanogenic bacteria. Biochemistry. 28:10061-10065.

Krone, U.E., R.K. Thauer, and H.P.C. Hogenkamp. 1989b. Reductive dehalogenation of chlorinated C_1-hydrocarbons mediated by corrinoids. Biochemistry. 28:4908-4914.

Krumholz, L.R., R. Sharp, and S.S. Fishbain. 1996. A freshwater anaerobe coupling acetate oxidation to tetrachloroethylene dehalogenation. Appl. Environ. Microbiol. 62:4108-4113.

Laanbroek, H.J., T. Abbee, and I.L. Voogd. 1982. Alcohol conversions by *Desulfobulbus propionicus* Lindhorst in the presence and absence of sulfate and hydrogen. Arch. Microbiol. 133:178-184.

Lewis, T.A., and R.L. Crawford. 1993. Physiological factors affecting carbon tetrachloride dehalogenation by the denitrifying bacterium *Pseudomonas* sp. Strain KC. Appl. Environ. Microbiol. 59:1635-1641.

Linkfield, T.G., J.M. Suflita, and J.M. Tiedje. 1989. Characterization of the acclimation period before anaerobic dehalogenation of halobenzoates. Appl. Environ. Microbiol. 55:2773-2778.

Mackay, D.M., and J.A. Cherry. 1989. Groundwater contamination: pump-and-treat remediation. Environ. Sci. Technol. 23:630-636.

Mackay, D.M., W.Y. Shiu, and K.C. Ma. 1993. Illustrated Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals. Vol. III. Chelsea, Michigan. Lewis Publishers.

Maymó-Gatell, X., V. Tandoi, J.M. Gossett, and S.H. Zinder. 1995. Characterization of an H_2-utilizing enrichment culture that reductively dechlorinates tetrachloroethene to vinyl chloride and ethene in the absence of methanogenesis and acetogenesis. Appl. Environ. Microbiol. 61:3928-3933.

Neumann, A., H. Scholz-Muramatsu, and G. Diekert. 1994. Tetrachloroethene metabolism of *Dehalospirillum multivorans*. Arch. Microbiol. 162:295-301.

Odom, J.M., E. Nagel, and J. Tabinowski. 1995. Chemical-biological catalysis for *in situ* anaerobic dehalogenation of chlorinated solvents. In: Bioremediation of Chlorinated Solvents. Eds., R.E. Hichee, A. Leeson and L. Semprini. Battelle Presse. Columbus. v. pp. 35-43.

Petersen, J.N., R.S. Skeen, K.M. Amos, and B.S. Hooker. 1993. Biological destruction of CCl_4: I. Experimental design and data. Biotechnol. and Bioeng. 43:521-528.

Schink, B. 1984. Fermentation of 2,3-butanediol by *Pelobacter carbinolicus* sp. nov. and *Pelobacter propionicus* sp. nov., and evidence for propionate formation from C_2 compounds. Arch. Microbiol. 137:33-41.

ethanol in *Pelobacter propionicus*. Arch. Microbiol. 147:321-327.

Schink, B., T.J. Phelps, B. Eichler, and J.G. Zeikus. 1985. Comparison of ethanol degradation pathways in anoxic freshwater environments. J. Gen. Microbiol. 131:651-660.

Scholz-Muramatsu, H., A. Neumann, M. MeBmer, E. Moore, and G. Diekert. 1995. Isolation and characterisation of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Arch. Microbiol. 163:48-56.

Scholz-Muramatsu, H., R. Szewzyk, U. Szewzyk, and S. Gaiser. 1990. Tetratchloroethylene as electron acceptor for the anaerobic degradation of benzoate. FEMS Microbiol. Lett. 66:81-86.

Semprini, L., G.D. Hopkins, P.L. McCarty, and P.V. Roberts. 1992. In-situ transformation of carbon tetrachloride and other halogenated compounds resulting from biostimulation under anoxic conditions. Environ. Sci. Technol. 26:2454-2461.

Sewell, G.W., and S.A. Gibson. 1991. Stimulation of the reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by the addition of toluene. Environ. Sci. Technol. 25:982-984.

Sharma, P.K., and P.L. McCarty. 1996. Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenates tetrachloroethene to *cis*-1,2-dichloroethene. Appl. Environ. Microbiol. 62:761-765.

Smatlak, C.R., J.M. Gossett, and S.H. Zinder. 1996. Comparative kinetics of hydrogen utilization for reductive dechlorination of tetrachloroethene and methanogenesis in an anaerobic enrichment culture. Environ. Sci. Technol. 30:2850-2858.

Tandoi, V., T.D. DiStefano, P.A. Bowser, J.M. Gossett, and S.H. Zinder. 1994. Reductive dehalogenation of chlorinated ethenes and halogenated ethanes by a high-rate anaerobic enrichment culture. Environ. Sci. Technol. 28:973-979.

Tatara, G.M., M.J. Dybas, and C.S. Criddle. 1993. Effects of medium and trace metals on kinetics of carbon tetrachloride transformation by *Pseudomonas* sp. Strain KC. Appl. Environ. Microbiol. 59:2126-2131.

Tatton, M.J., D.B. Archer, G.E. Powell and M.L. Parker. 1989. Methanogenesis from ethanol by defined mixed continuous cultures. Appl. Environ. Microbiol. 55:440-445.

Thomas, J.M., and C.H. Ward. 1989. In situ biorestoration of organic contaminants in the subsurface. Environ. Sci. Technol. 23:760-765.

Van Eekert, M.H.A., M.C. Veiga, J.A. Field, A.J.M. Stams, and G. Schraa. 1995. Removal of tetrachloromethane by granular methanogenic sludge. In: Bioremediation of Chlorinated Solvents. Eds., R.E. Hichee, A. Leeson and L. Semprini. Battelle Presse. Columbus. v. pp. 139-145.

Vogel, T.M., C.S. Criddle, and P.L. McCarty. 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. 21:722-736.

Vogel, T.M., and P.L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. 49:1080-1083.

Westrick, J.J., W. Mello, and R.F. Thomas. 1984. The groundwater supply survey. J. Am. Water Works Assoc. 76:52-59.

Wiggins, B.A., S.H. Jones, and M. Alexander. 1987. Explanation for the acclimation period preceding the mineralization of organic chemicals in aquatic environments. Appl. Environ. Microbiol. 53:791-796.

Wilson, J.T., and B.H. Wilson. 1985. Biotransformation of trichloroethylene in soil. Appl. Environ. Microbiol. 49:242-243.

Wu, M.M., and R.F. Hickey. 1996. n-Propanol production during ethanol degeradation using anaerobic granules. Wat. Res. 30:1686-1694.

Wu, M.M., J. Nye, R.F. Hickey, M.K. Jain and J.G. Zeikus. 1995. Dechlorination of PCE and TCE to ethene using an anaerobic microbial consortium. In: Bioremediation of Chlorinated Solvents. Eds., R.E. Hichee, A. Leeson and L. Semprini. Battelle Presse. Columbus. v. pp. 45-52.

Yang, J., and R.E. Speece. 1986. The effects of chloroform toxicity on methane fermentation. Wat. Res. 20:1273-1279.

Zitomer, D.H., and R.E. Speece. 1993. Sequential environments for enhanced biotransformation of aqueous contaminants. Environ. Sci. Technol. 27:227-244.

APPENDIX A

STANDARD CURVE RESULTS

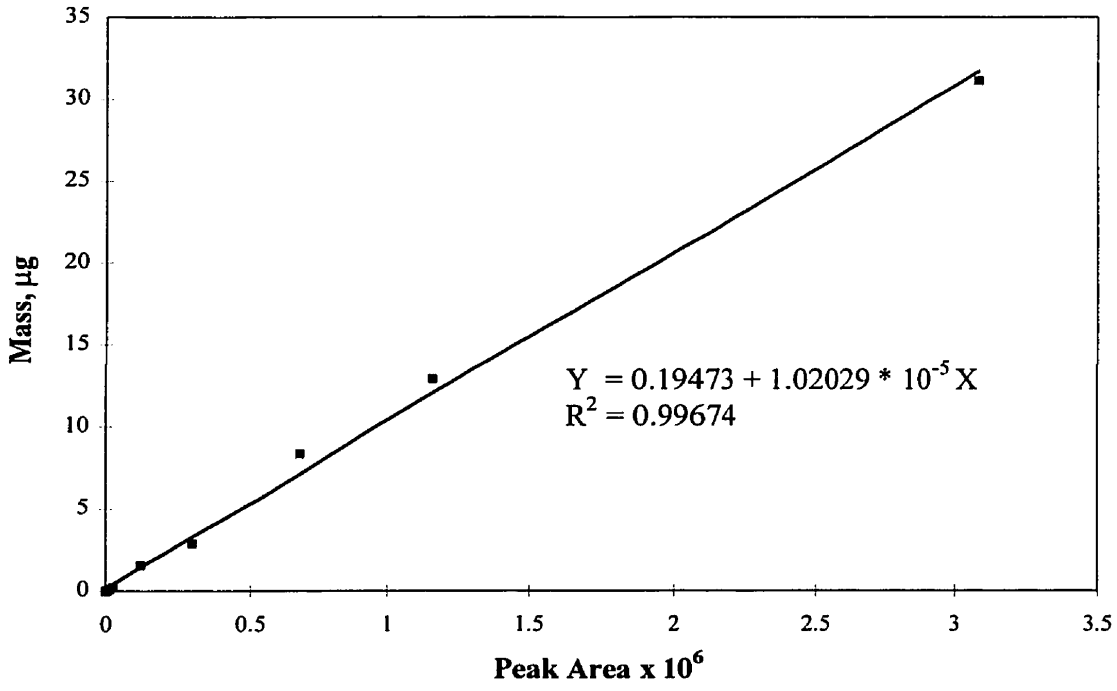


Figure A1: Tetrachloroethylene (PCE) standard curve

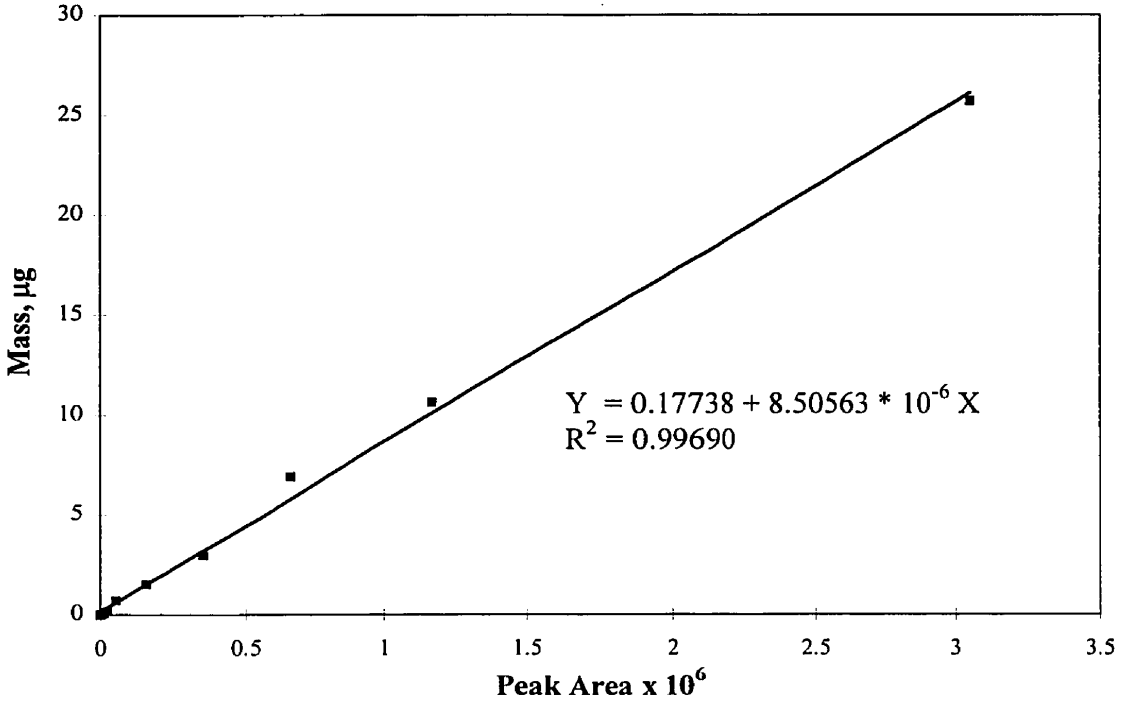


Figure A2: Trichloroethylene (TCE) standard curve

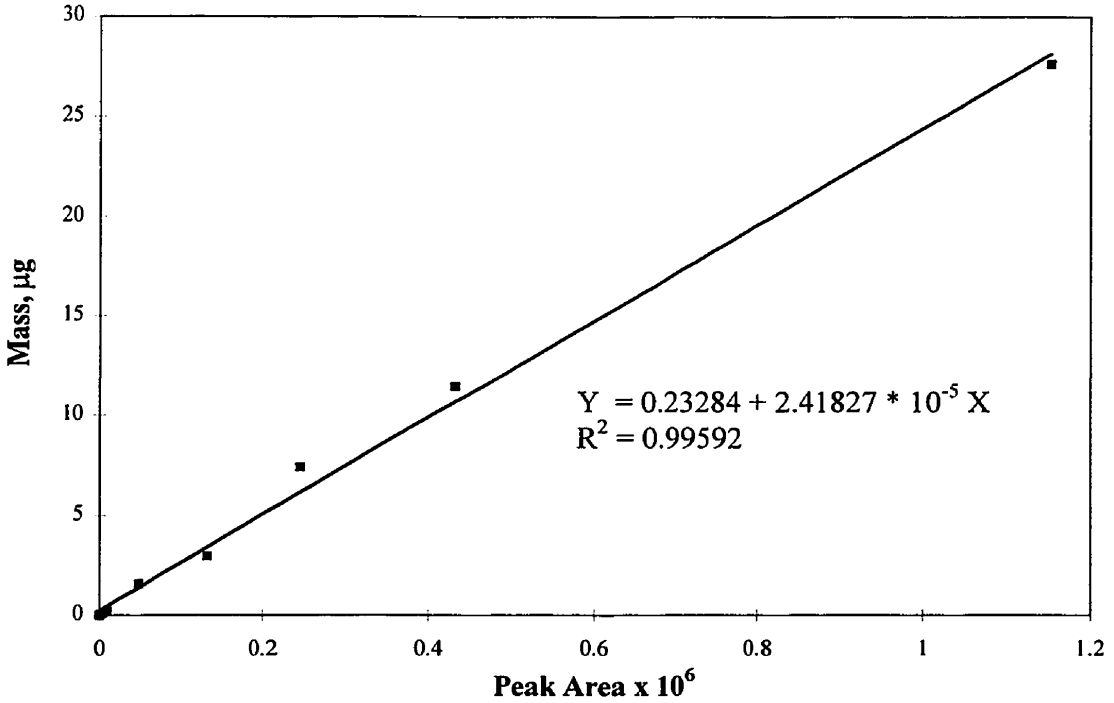


Figure A3: Carbon tetrachloride (CT) standard curve

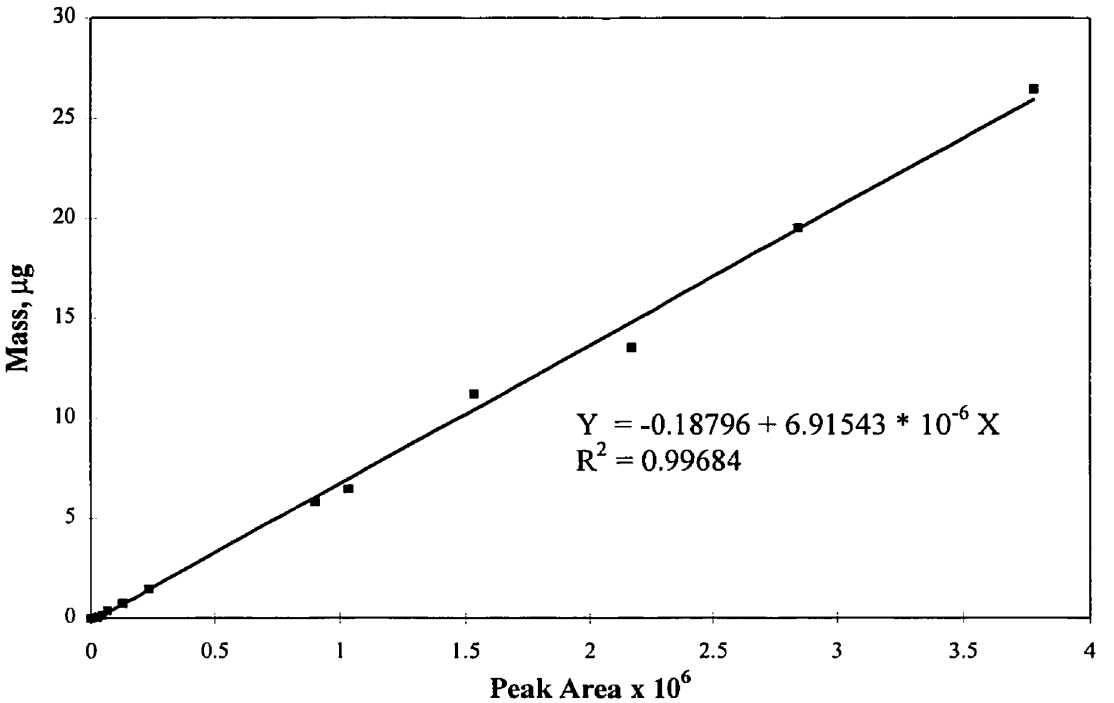


Figure A4 : 1,1-dichloroethylene (1,1-DCE) standard curve

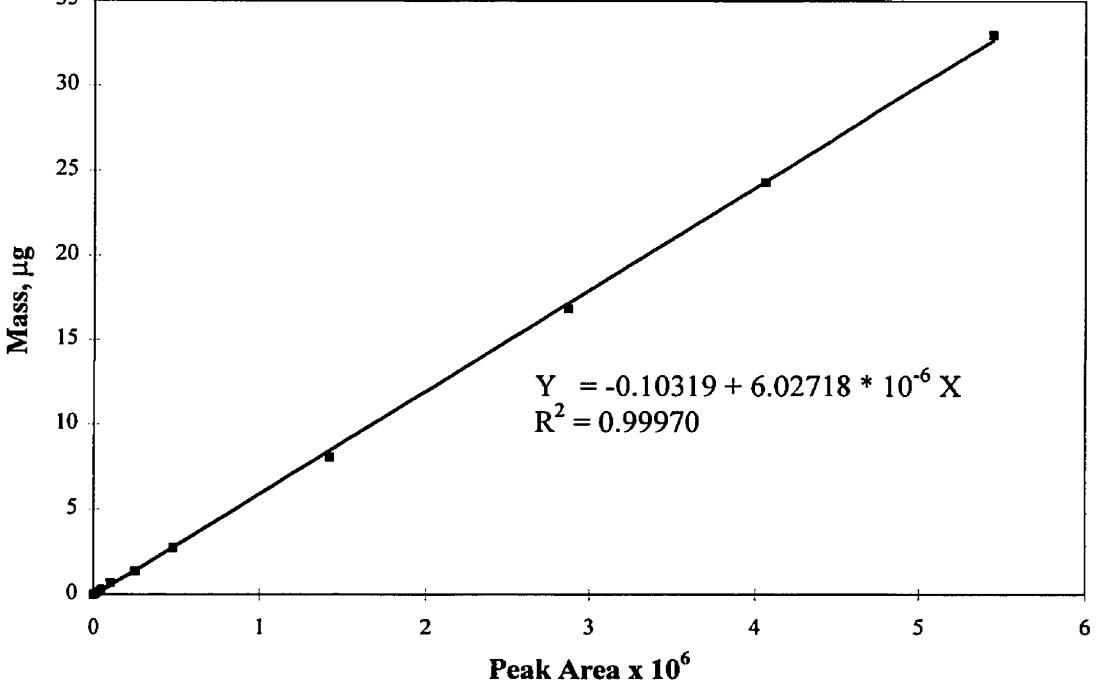


Figure A5: *cis*-dichloroethylene (*cis*-DCE) standard curve

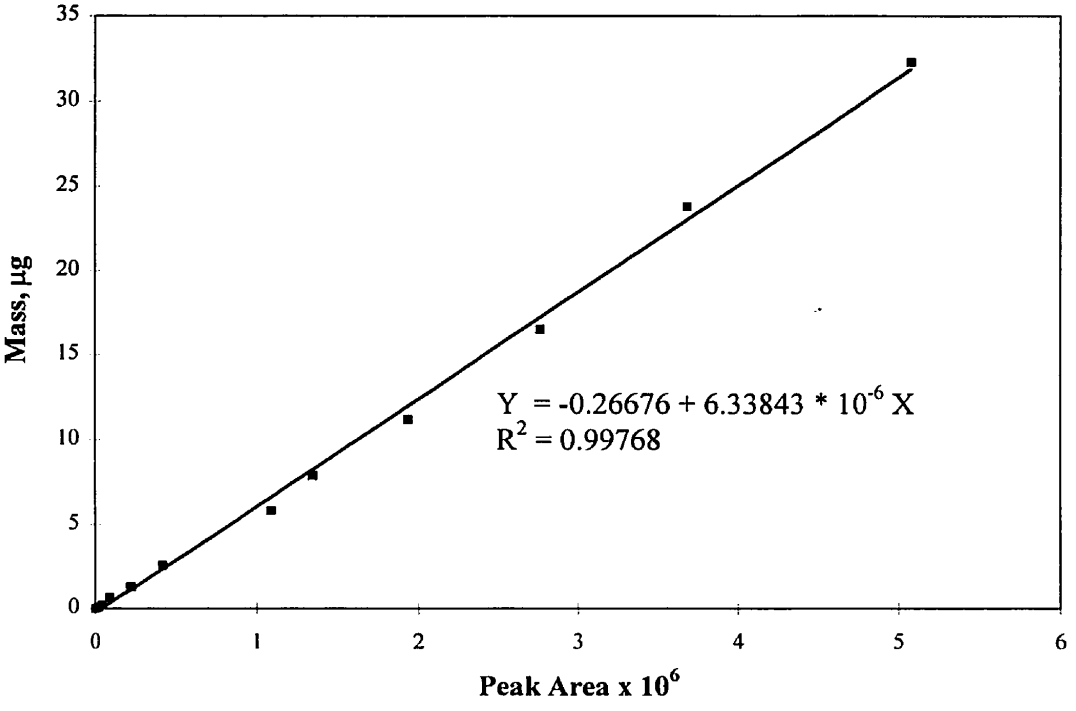


Figure A6: *trans*-dichloroethylene (*trans*-DCE) standard curve

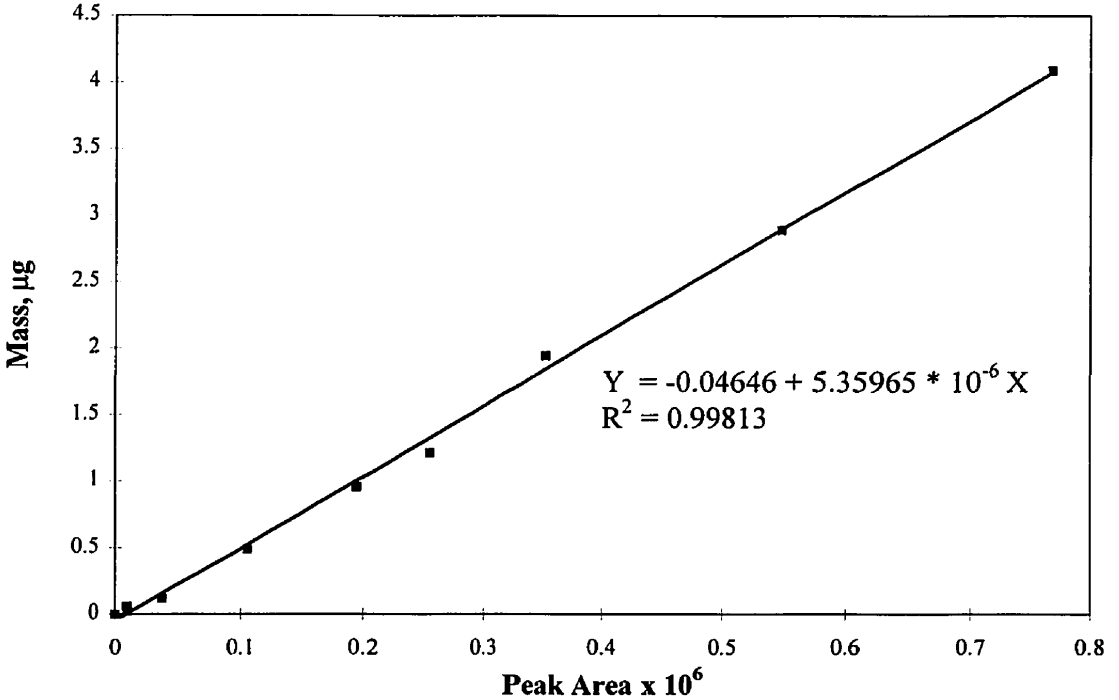


Figure A7: Vinyl chloride (VC) standard curve

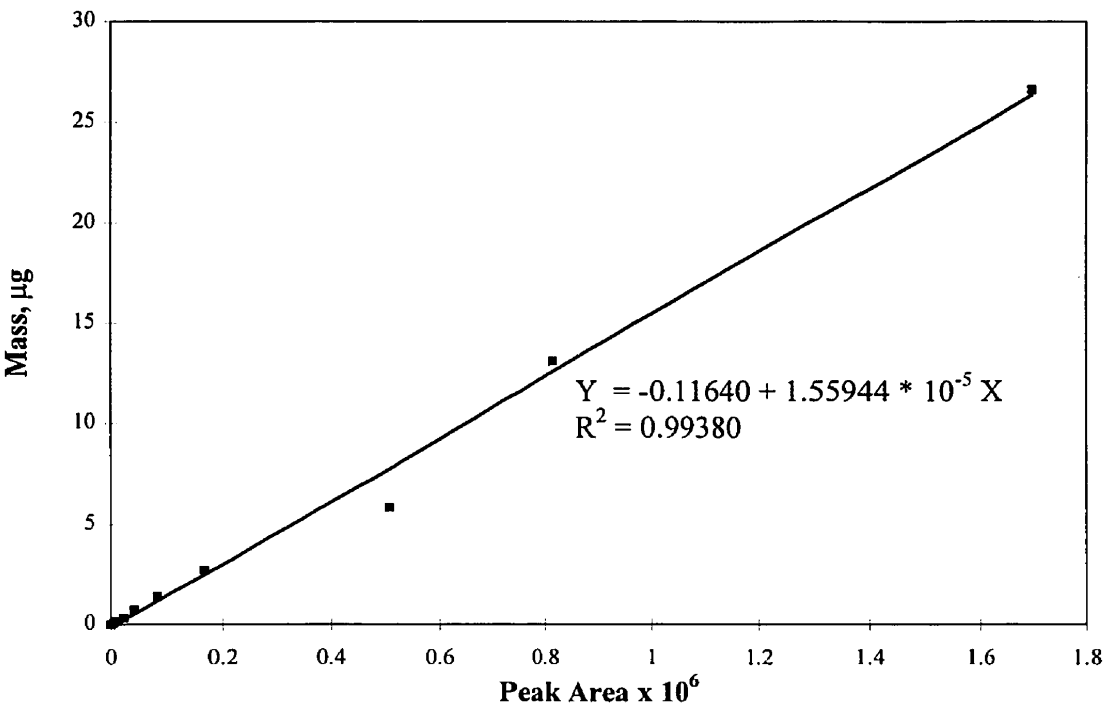


Figure A8: Chloroform (CF) standard curve

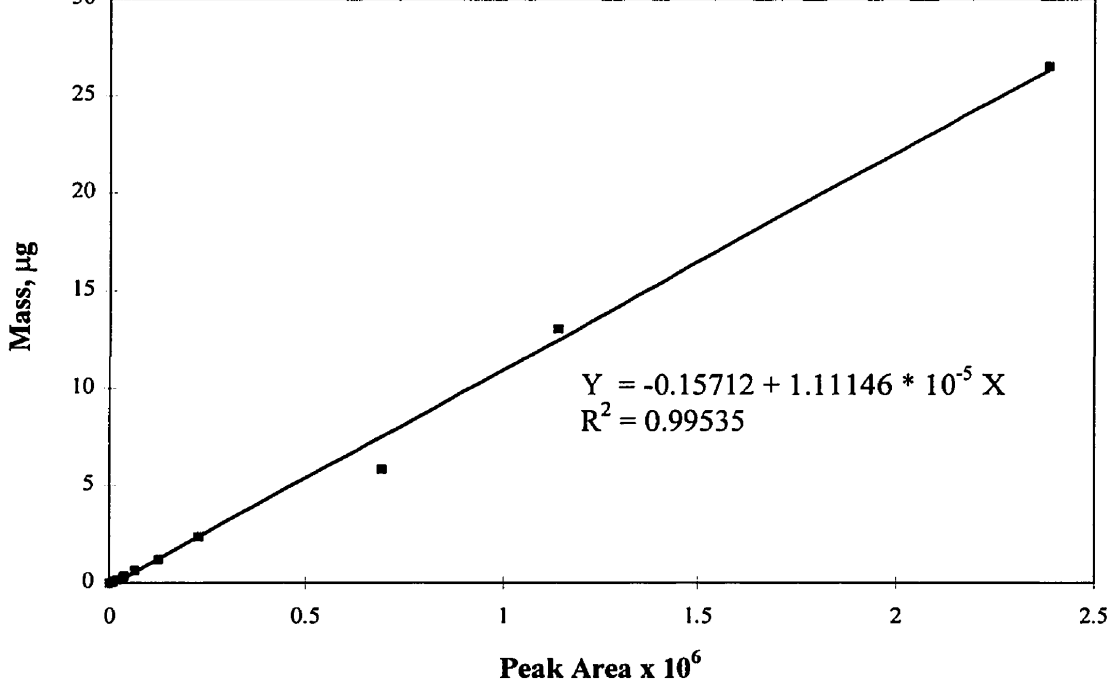


Figure A9: Dichloromethane (DCM) standard curve

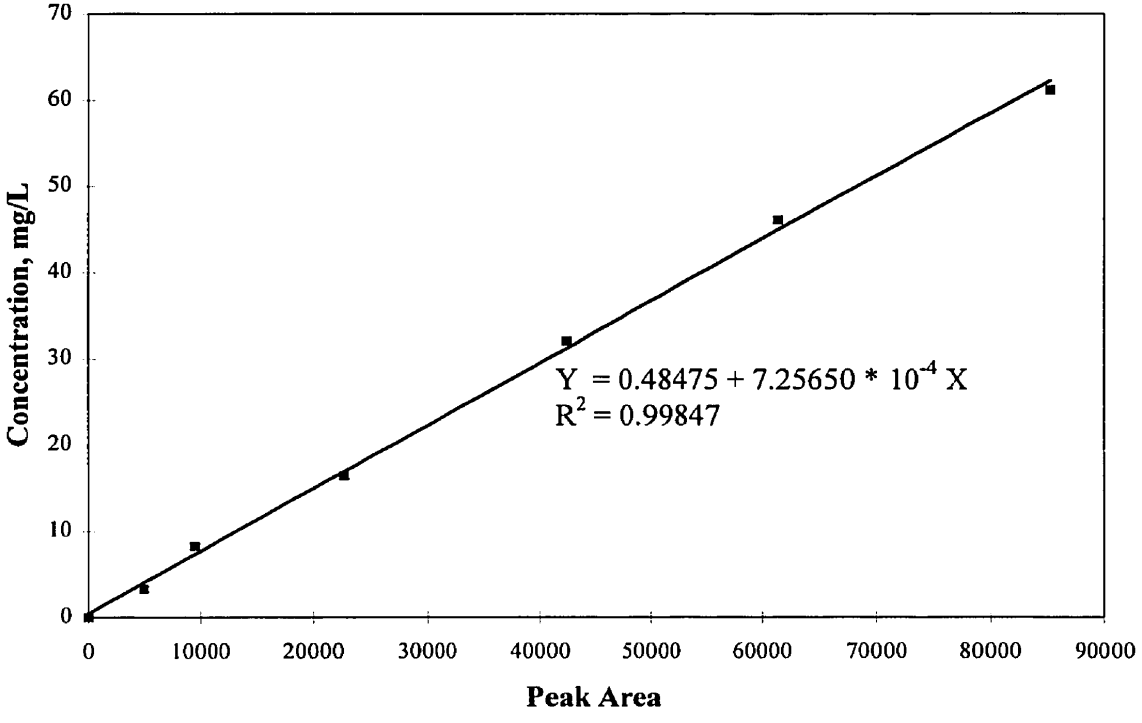


Figure A10: Methane standard curve

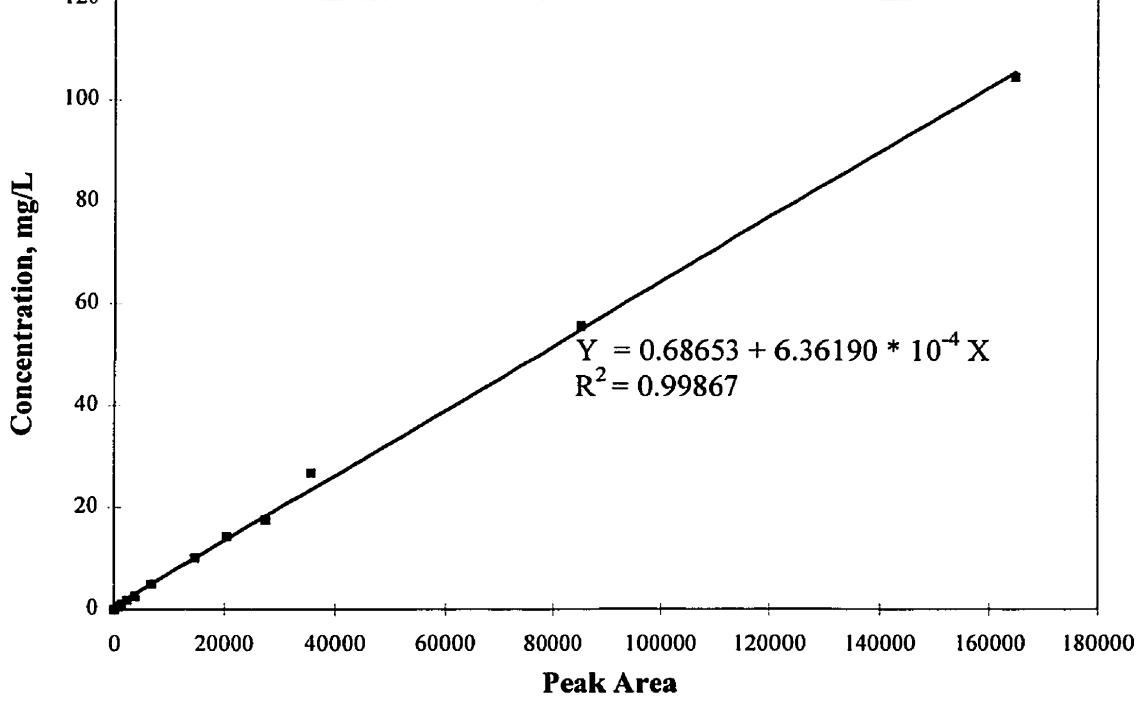


Figure A11: Typical ethanol (etOH) standard curve

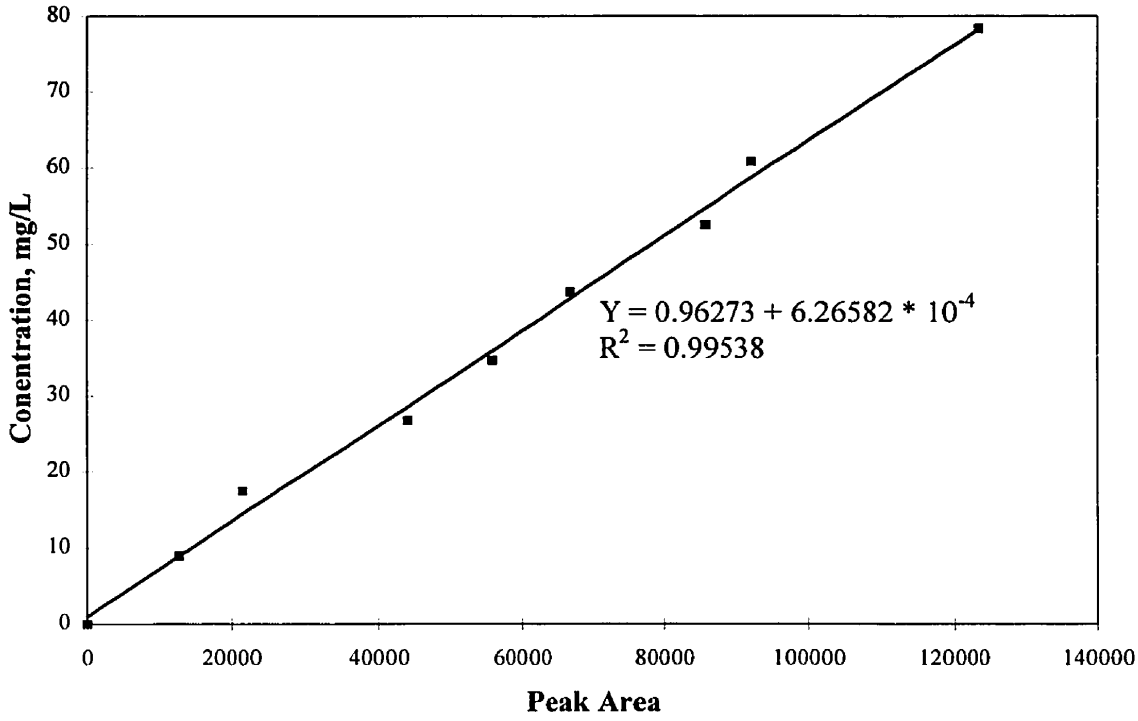


Figure A12: Typical methanol (meOH) standard curve

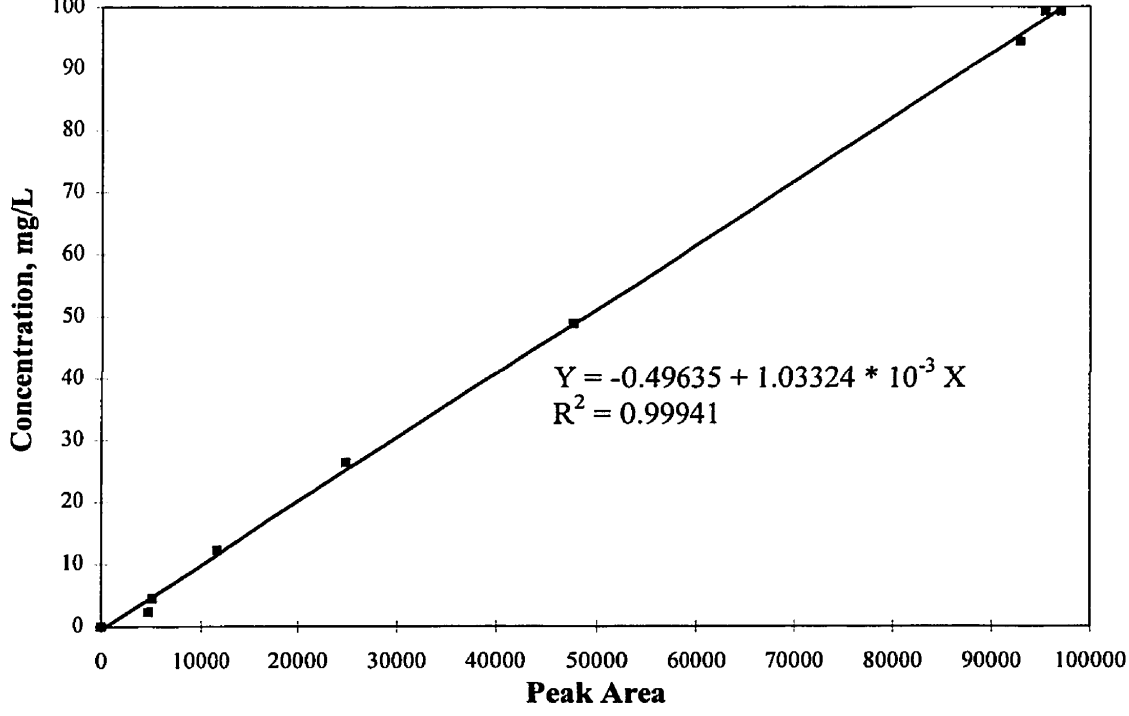


Figure A13: Typical acetic acid (AA) standard curve

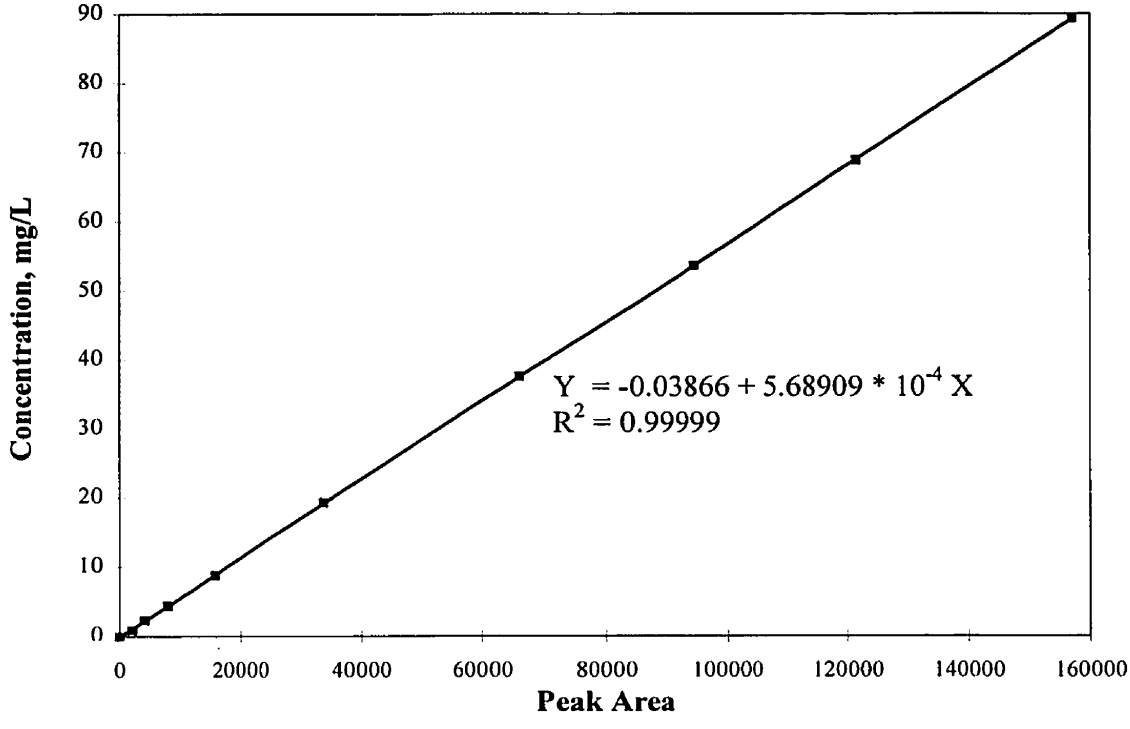
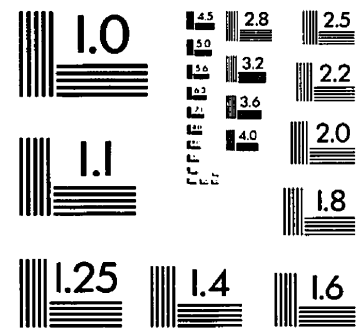
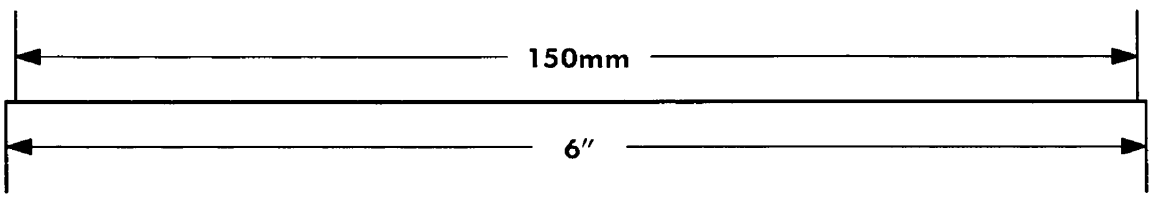


Figure A14: Typical propionic acid (PA) standard curve





APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989