ABSTRACT

Title of Document: EVALUATION OF THE EFFECTS OF BIOAUGMENTATION AND BIOSTIMULATION ON NATURAL ATTENUATION AND BIODEGRADATION PATHWAYS OF CHLORINATED COMPOUNDS IN A TIDAL WETLAND

Emily N. Devillier, Master of Science, 2006

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The usefulness of bioaugmentation and biostimulation in enhancing the natural attenuation of chlorinated ethanes, ethenes, and methanes at a seep site at Aberdeen Proving Ground (APG), MD was tested. The biodegradation of (1) a mixture of 1,1,2,2-tetrachloroethane (TeCA), tetrachloroethene (PCE), and carbon tetrachloride (CT), or (2) TeCA alone was compared in sediment and groundwater microcosms amended with chlorinated substrates alone, chlorinated substrates and electron donor, or chlorinated substrates, electron donor and a TeCA-degrading enrichment culture. A third experiment evaluated the usefulness of H_2 thresholds in determining the importance of co-metabolic and metabolic processes in the biodegradation of chlorinated substrates. Biostimulation alone did not significantly affect chlorinated substrate removal. Biodegradation of TeCA was significantly enhanced by the addition of electron donors coupled with bioaugmentation. However, the presence of other contaminants, especially chlorinated methanes appeared to inhibit TeCA biodegradation, even in the presence of exogenous electron donors and the

enrichment culture. H_2 thresholds did not prove useful in determining the importance of metabolic and co-metabolic processes in the transformation of CT, PCE, and TeCA; however, evaluating the biodegradation of each chlorinated compound individually provided insight in regards to biodegradation pathways followed and the effects of electron donor substrates on degradation rates. Overall, the results provide evidence that when assessing a site contaminated with a mixture of chlorinated solvents, it is necessary to examine each contaminant individually and as a mixture, in order to develop a successful remediation plan.

Evaluation of the Effects of Bioaugmentation and Biostimulation on Natural Attenuation and Biodegradation Pathways of Chlorinated Compounds in a Tidal Wetland

By

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Dedication

This thesis is dedicated to the memory of my grandfather, Leonard N. Larson, a talented Ceramic Engineer, who showed me the power of engineering.

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Chapter 1: Introduction

Contamination of groundwater with chlorinated solvents, such as tetrachloroethene (PCE) and trichloroethene (TCE), is a serious problem at many areas within the United States. This research focuses on a study area at Aberdeen Proving Ground (APG, Maryland). Aberdeen Proving Ground is a military installation dedicated to the research and development of chemical warfare (Lorah et. al, 1997). However, up until two decades ago, the waste stemming from chemical manufacturing operations and chemical warfare training exercises led to extensive contamination of the site through spills, landfills, and discharge from sewers (Lorah et. al, 1997). Currently, the main contaminants of concern at the study areas are 1,1,2,2-tetrachloroethane (TeCA), tetrachloroethene (PCE), trichloroethene (TCE), chloroform (trichloromethane, CF), and carbon tetrachloride (CT) (Lorah et al., 1999). These parent compounds and their biodegradation daughter products are referred to here as chlorinated volatile organic compounds (CVOCs).

The groundwater containing these contaminants, and their biodegradation daughter products, discharges to freshwater wetlands and a tidal creek (Lorah et al., 1999). These chlorinated compounds are of great concern because they are highly toxic, thus posing a threat to the surrounding ecosystems. All of the parent compounds of concern at the site are suspected carcinogens; however, vinyl chloride, a potential daughter product, is particularly toxic and a known carcinogen (Lorah et al., 2003a).

Elevated concentrations of parent and daughter compounds have been observed at the West Branch Canal Creek study area (Lorah et al., 1997, Lorah et. al, 2003). In some cases, the

contaminant concentrations exceed the maximum contaminant level (MCL) of 5 ppb, as defined by the U.S. Environmental Protection Agency (U.S. EPA, 1998). At a majority of the locations within the study area, natural attenuation has been observed through continuous monitoring of both parent and daughter contaminant concentrations over time. Natural attenuation reduces the concentration of a groundwater contaminant as it migrates away from its source through a variety of physical, chemical, and biological processes (Weidemeier et al., 1999, McAllister and Chiang, 1994). Physical, chemical, and biological processes can be divided into two categories: destructive and non-destructive processes. Destructive processes, such as biodegradation, are preferred because they can result in the reduction of contaminant mass or toxicity, e.g. in the case of CVOCs via the reduction of the parent compound to daughter products. On the other hand, non-destructive processes (e.g., dispersion, sorption, and volatilization) reduce the overall concentration of the compound, but not the overall mass of the compound, i.e. no compounds are degraded. Both destructive and non-destructive processes may occur under anaerobic conditions of interest in this study.

Although natural attenuation is effective at reducing contaminant concentrations throughout most of the study areas at APG, a number of sites exist at which natural attenuation is not reducing the concentration of contaminants below the MCLs, making them of particular concern. These locations are known as seep sites. Therefore, the purpose of this research is to evaluate the effectiveness of engineered bioremediation methods, including bioaugmentation and biostimulation, which could potentially promote the degradation of the contaminants of interest at the APG seep sites.

The following chapter provides a review of literature pertinent to this research. Subsequently, Chapters 3 and 4 focus on the problem, the seep sites, and objectives for developing an appropriate engineered bioremediation method for the seep sites at WBC. Chapter 5 explains the methods and materials used in the three experiments to accomplish the goals and objectives. Chapter 6 describes the results obtained from a previous bioaugmentation and biostimulation experiment with sediment from site 3-4W. Chapters 7 and 8 explain the data obtained from the H_2 threshold and seep site experiments. Finally, Chapter 9 provides an overall conclusion about all three experiments, as well as future work to consider.

Chapter 2: Literature Review

2.1. Introduction

Contaminant remediation in wetlands is especially challenging because of the hydrogeological connection between ground water and surface water, which means if contaminants are present in the groundwater they could pose a threat to the wetland ecosystem (Lorah et. al., 1997). In fact, as previously mentioned, contaminant plumes have formed at APG due to extensive contamination from chemical manufacturing operations, and these plumes extend into wetland soils.

Although natural attenuation mechanisms, mainly biodegradation and sorption, have proven successful in remediating the groundwater at many sites before it reaches the soil surface (Lorah et. al, 1997), the concentration of contaminants in surface water is relatively high at a number of seep sites. Therefore, an engineered bioremediation method must be applied at these sites, in order to promote biodegradation of the contaminants. Engineered methods can either be applied in the aquifer or surface sediment where contamination occurs (*in-situ*) or they can be applied to groundwater that has been pumped to the surface or on excavated soil (*ex-situ*). *In-situ* methods are often preferred over *ex-situ* methods because removing contaminated sediment and groundwater from a wetland can negatively impact the ecosystem. However, there are also some physical *in-situ* methods, such as pump-and-treat, that can damage a wetland ecosystem through dewatering (Lorah et. al, 1997).

In this literature review, an overview of the physical, biological, and chemical processes involved in natural attenuation is given. This background is necessary to gain a better understanding of the natural attenuation processes occurring at APG, and their impact on the development of engineered bioremediation methods for cleaning up the seep sites.

2.2. Natural Attenuation

As stated previously, natural attenuation reduces the concentration of a groundwater contaminant as it is transported away from its source through a variety of physical, chemical, and biological processes (Weidemeier et al., 1999; McAllister and Chiang, 1994). Physical processes are non-destructive, whereas chemical and biological processes are destructive. Natural attenuation processes that could potentially be important in the remediation of sites contaminated with chlorinated solvents are briefly reviewed below.

2.2.1. Physical Processes

Although biodegradation has been identified as the primary process involved in reducing the concentration of chlorinated solvents, physical processes have also been shown to contribute to contaminant decreases (Lorah et al., 1997). Sorption, the partitioning of a compound from a liquid to a solid phase, is particularly important in soils with a high organic content, i.e. wetland sediment (Lorah et al., 1997). The hydrophobic nature of chlorinated compounds promotes their sorption to the organic matter. Although this reaction is reversible, the organic matter to which it is sorbed directly affects the desorption rate of the contaminant(s). Sediments with a higher organic content possess a lower desorption rate because of the CVOC's strong affinity for the

organic matter. Chemical and biological processes are, thereby, impeded because the potential for contaminant destruction in the aqueous phase is reduced (Seagren and Becker, 2002).

Dispersion is a mechanical mixing process that, like sorption, reduces the concentration of a contaminant, but not its mass (McAllister and Chiang, 1994). Molecular diffusion, the net movement of a compound from an area of higher concentration to one of lower concentration, can also affect the overall contaminant concentration in the plume, at low flow velocities. Dispersion promotes the contact of microorganisms with nutrients and substrates, including contaminants, thereby indirectly promoting biodegradation of these compounds, and the consequent formation of redox zones. Volatilization transfers contaminants from the aqueous phase to the gas phase. The volatilization of an aqueous phase contaminant is dependent on the depth of the water table, temperature, and contaminant and surface water chemistry (McAllister and Chiang, 1994). Many chlorinated hydrocarbons are highly volatile because of their low solubility in water. Therefore, volatilization of these compounds may be a significant attenuation process near the water-air interface.

2.2.2. Biological and Chemical Transformations

In wetland systems, anaerobic and aerobic conditions typically coexist. Anaerobic reactions dominate in bulk wetland sediment because of (1) the saturated conditions, which slows the introduction of O_2 compared with unsaturated soils, and (2) the presence of organic matter, which leads to the rapid utilization of O_2 as a terminal electron acceptor (Lorah et. al., 1997). At the surface, aerobic conditions may develop because O_2 can be introduced through dissolution at the air-water interface. In addition, wetland plant roots can release O_2 to the

surrounding soil through the rhizosphere. The presence of both anaerobic and aerobic conditions in wetland soils makes them ideally suited for the complete destruction of CVOCs. Specifically, the more highly chlorinated compounds are highly oxidized, which makes them susceptible to reductive transformations (Vogel et. al, 1987), which are favored by anaerobic (or highly reducing) conditions. In contrast, compounds with one or two chlorines are less oxidized and readily undergo either reductive or oxidative transformations. Thus, one scenario that could arise when groundwater containing CVOCs discharges to a wetland is that the highly chlorinated compounds could be transformed as the groundwater passes through the bulk anaerobic soil and lightly chlorinated compounds could undergo oxidative reactions as the contaminated water nears the soil surface.

2.3. Reductive Dechlorination and Other Anaerobic Dechlorination Reactions

Lightly chlorinated VOCs are likely to arise through the reductive dehalogenation of the parent compounds in the anaerobic bulk soils. In fact, reductive dechlorination is often the most important mechanism contributing to the destruction of chlorinated solvents by microorganisms under anaerobic conditions. As the name suggests, in a reductive dechlorination reaction, a chlorine is removed through the addition of electrons. Two types of reductive dechlorination reactions, hydrogenolysis and dichloroelimination, may be important for the CVOCs at APG. Hydrogenolysis is the replacement of a chlorine with a hydrogen atom. Sequential reductive dehalogenations of TeCA to ethane via 1,1,2-trichloroethane (TCA), 1,2-dichloroethane (DCA), and CA are examples of this type of reaction (Figure 2.1). Complete dechlorination of PCE to ethene, and CT to methane (Figure 2.2) via hydrogenolysis reactions are also theoretically feasible (Lorah et. al., 2003a).

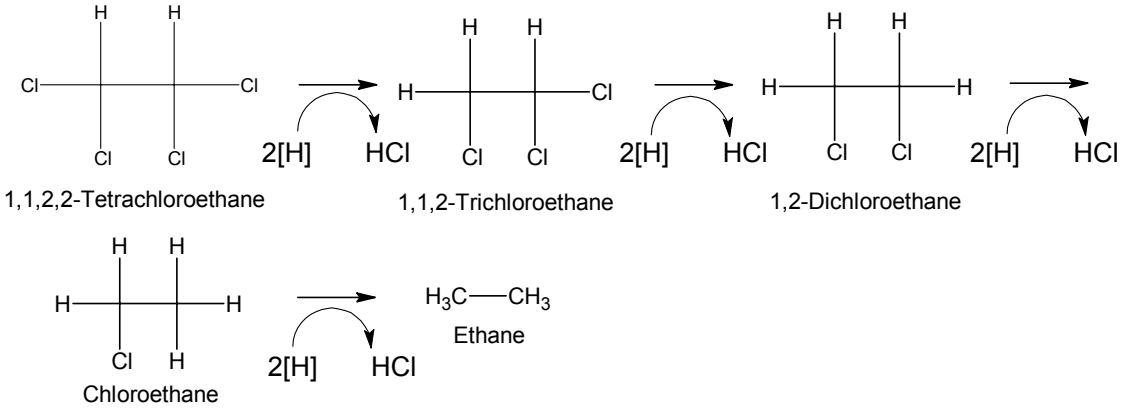


Figure 2.1. The conversion of TeCA to ethane through sequential hydrogenolysis reactions.

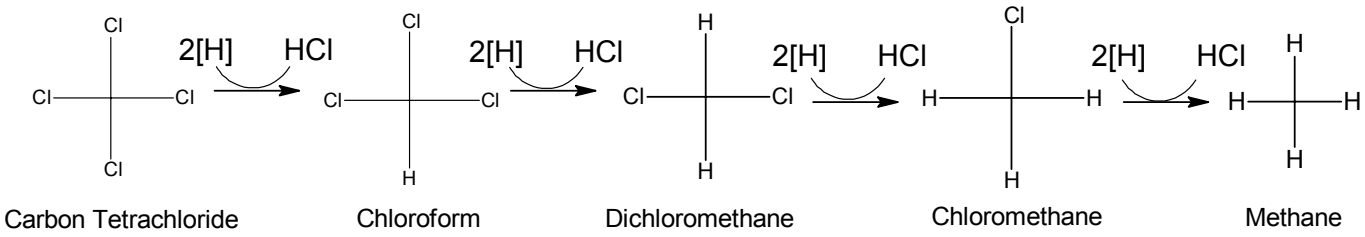


Figure 2.2. The conversion of CT to methane through the sequential hydrogenolysis reactions.

Along with hydrogenolysis, dichloroelimination, the removal of two chlorine atoms through the conversion of an alkane to an alkene, is also an important process for the conversion of saturated chlorinated compounds (Lorah et. al., 1999, 2003a). The reductive dechlorination of TeCA to *cis*-1,2-dichloroethene (*cis*-DCE) is an example of dichloroelimination (Figure 2.3). Conversion of TCA to vinyl chloride (VC), and DCA to ethene via dichloroelimination reactions are also possible (Lorah et. al., 2003a).

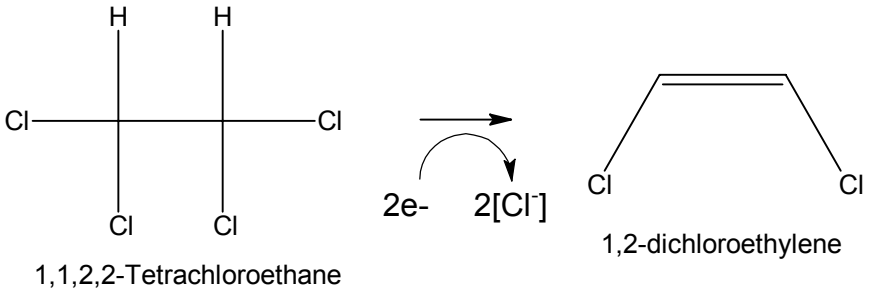


Figure 2.3. The reduction of TeCA to *cis*-DCE through the process of dichloroelimination.

Reductive dechlorination reactions may be biologically mediated, although abiotic transformations, e.g., in the presence of zero valent iron or certain metallocofactors, can also occur (Vogel et. al., 1987; Cookson, 1995). The focus here is on biological reductive dehalogenation, which can occur via metabolic or co-metabolic processes. At many contaminated sites, diverse microbial communities may exist that can carry out both co-metabolic and metabolic reductive dechlorinations. When reductive dechlorination occurs metabolically, chlorinated compounds are utilized as terminal electron acceptors in a form of anaerobic respiration known as dehalorespiration. Dehalorespiration has been demonstrated for a number of chlorinated hydrocarbons. Relatively few species that utilize chlorinated compounds as terminal electron acceptors have been isolated; however, the list of dehalorespiring organisms is rapidly growing. In particular, a number of different isolates that can respire PCE and/or TCE have been identified, including members of the genera *Dehalococcoides, Sulfurospirillum*, *Desulfuromonas*, *Dehalobacter,* and *Desulfitobacterium* (Major et. al., 2002; Lorah et. al., 2003a). Dehalorespiration of DCEs and VC has to date been observed only in *Dehalococcoides* strains (Becker, 2006). Therefore, bioaugmentation with cultures that contain DCE and VC-respiring *Dehalococcoides* strains is increasingly being

recommended as an engineered bioremediation strategy at sites where biodegradation of PCE and/or TCE is incomplete.

Many anaerobic bacteria, including many methanogens, can carry out co-metabolic reductive dehalogenation (Yang and McCarty, 1998). For example, the chlorinated methanes, CT and CF, can be co-metabolically transformed by some methanogens and anaerobic bacteria (Criddle et. al., 1990a,b; Galli and McCarty, 1989; Novak et. al., 1998a,b). In addition to the reductive dechlorination of CT via co-metabolism by methanogens, hydrolysis of CT has also been demonstrated with *Acetobacterium woodii* (Figure 2.4). Dehalorespiration of these compounds has not been demonstrated to date, although DCM has been shown to be used as a growth substrate (Freedman and Gossett, 1991).

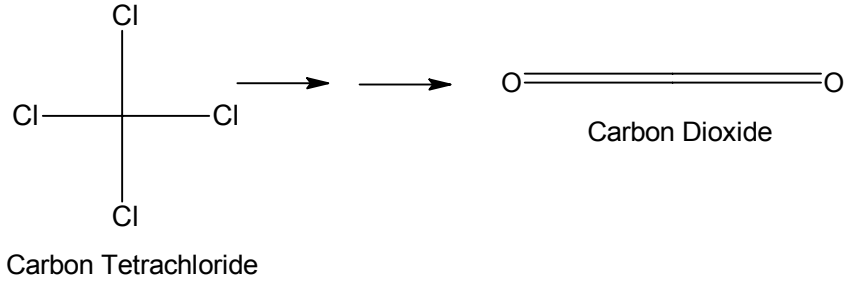


Figure 2.4. Hydrolysis of CT to CO_2.

In co-metabolic reactions, including reductive dechlorinations, enzymes and co-factors normally used in growth-related reactions probably act on and transform compounds in addition to their normal substrates. However, transformation of the "non-target" compounds do not contribute to the growth of the organism mediating the reaction. Although the ability to carry out co-metabolic reductive dehalogenation may be more broadly distributed compared with

dehalorespiration, the latter process probably has a much greater impact on the concentration of many CVOCs because of its faster and more specific kinetics compared with co-metabolic transformations. This means that bioremediation based on metabolic reductive dehalogenation occurs more rapidly and uses the electron donor required to fuel reductive dehalogenation more efficiently compared with co-metabolic transformations. Even more importantly, because the bacteria are growing as a result of metabolic transformations, they can be selectively enriched, which results in increasing rates of reaction and improved process control. In contrast, co-metabolism processes tend to be slow. Co-metabolic reductive dehalogenation also results in incomplete degradation, which can often lead to the accumulation of toxic end product(s) (Alexander, 1999). On the other hand, the involvement of broad-substrate enzymes in co-metabolism can be advantageous if a mixture of contaminants exists because multiple contaminants may be transformed by broad-specificity enzymes (Holliger and Schraa, 1994).

Whether reductive dehalogenation occurs metabolically or co-metabolically, it cannot be sustained without an electron donor. At most contaminated sites where natural attenuation is being utilized as the clean-up approach, reductive dechlorination is limited by the availability of the electron donor (Becker, 2006). Therefore, biostimulation, the addition of electron donors, is sometimes used to overcome electron donor limitation and enhance natural attenuation. Dehalogenating organisms vary with respect to their electron donor substrate ranges. However, many dehalorespirers can utilize H_2 as an electron donor and some chlorinated ethene-respiring organisms use H_2 exclusively. Therefore, when biostimulation is applied to chlorinated ethene contamination, it typically focuses on increasing H_2 concentrations within the contaminant plume (He et al., 2002).

H_2 is often supplied to chlorinated ethene contaminated sites through the addition of fermentable substrates including methanol, ethanol, lactate, propionate, and benzoate, as well as complex substrates such as whey or chitin (Yang and McCarty, 1998). However, in addition to dehalorespirers, a CVOC-contaminated site will likely be inhabited by methanogens and other populations that may compete with dehalorespirers for H_2. Differences in the free energy change associated with the fermentation of different organic compounds makes them thermodynamically feasible and allows them to occur at different H_2 partial pressures. The thermodynamics of fermentation of substrates like benzoate and lactate are relatively favorable and occur at fairly high H_2 partial pressures that promote the growth of methanogens, which can consume H_2 only at relatively high concentrations, as discussed below. Other substrates like propionate and butyrate are fermented only at very low H_2 partial pressures that are feasible through the activity of hydrogenotrophic populations (Fennell et al., 1997). Fermentation of propionate or butyrate helps to selectively deliver H_2 to dehalorespirers, which have lower H_2 thresholds compared to many other hydrogenotrophs, as discussed below.

In addition to reductive dechlorination, under anaerobic conditions, saturated chlorinated hydrocarbons may undergo dehydrochlorination, in which a proton and chloride ion are removed to form a chlorinated alkene. For example, dehydrochlorination of TeCA occurs abiotically and produces TCE (Figure 2.5). The potential pathways for dechlorination of TeCA, including their intersection with the chlorinated ethene degradation pathway are summarized in Figure 2.6.

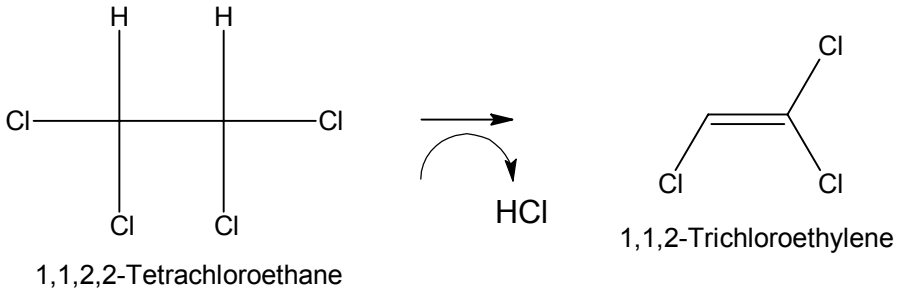


Figure 2.5. The transformation of TeCA to TCE through the process of dehydrochlorination.

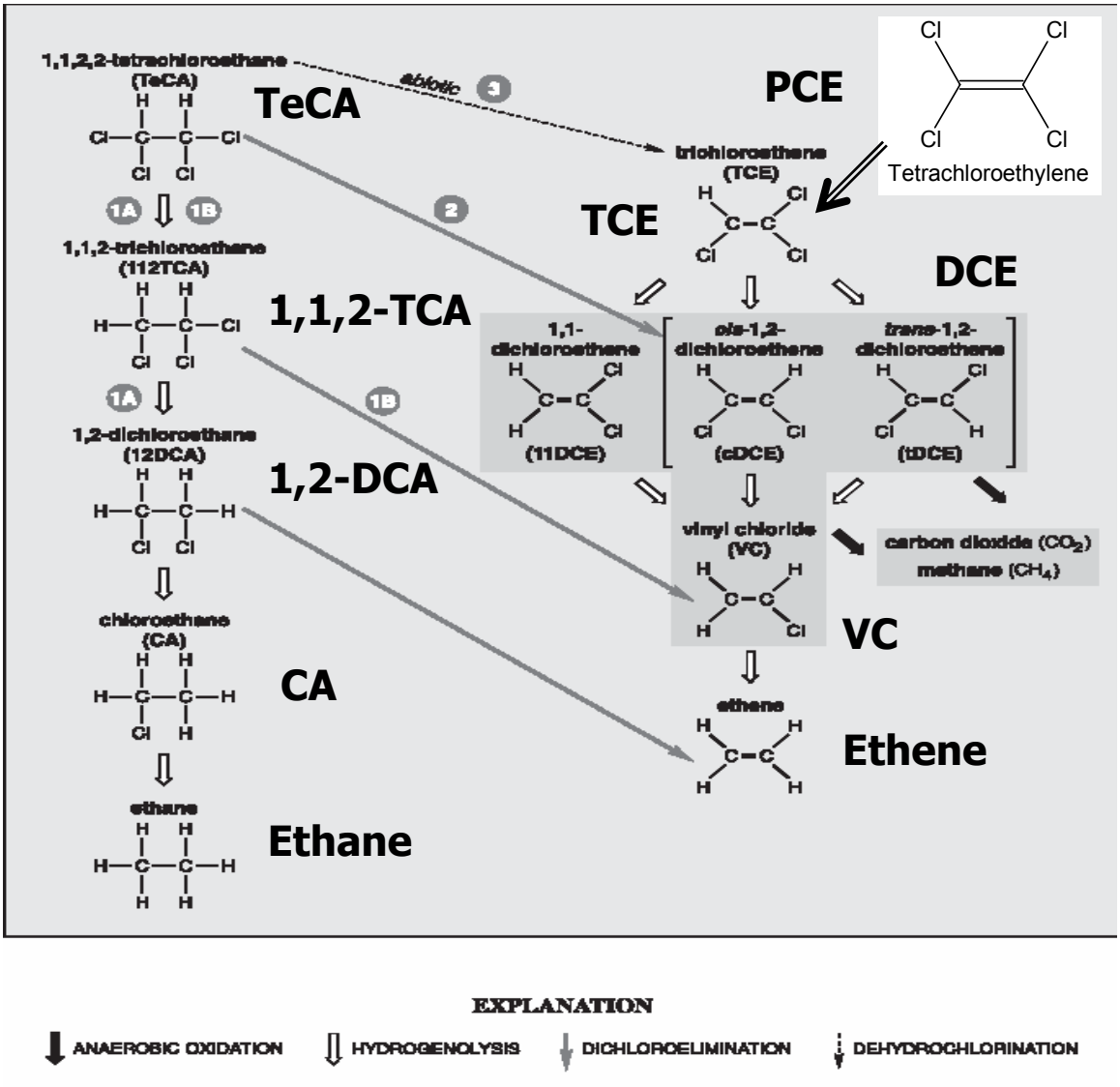


Figure 2.6. Anaerobic reductive dechlorination pathways for PCE and TeCA. (Lorah et. al., 2003a).

2.4. H_2 Thresholds as an indicator of metabolic and co-metabolic processes

One potentially useful measure of the relative importance of metabolic and co-metabolic processes in the removal of CVOCs is the H_2 threshold. Previous studies have shown that the H_2 threshold measured in sediment and groundwater is related to the dominant terminal electron-accepting process (TEAP).

The relationship between the H_2 threshold and the dominant TEAP can be understood by considering the equation for S_{min} (Eqn.1), the minimum concentration of a limiting substrate needed to maintain steady-state bacterial growth:

$$S_{\min} = \frac{K_s b}{q_{\max} Y - b} \qquad (1)$$

where S_{min} = the minimum substrate concentration [M L^{-3}], K_s = the half saturation constant [M_s L^{-3}], b = the decay coefficient, q_{max} = the maximum specific substrate utilization rate [M_s L^{-3} T^{-1}], and Y = the true yield coefficient [M_x M_s] (Rittmann et. al., 1994; Becker et. al., 2005).

Table 2.1. H_2 Concentrations characteristic of the following redox processes at West Branch Canal Creek (Löffler and Sanford, 2005).

Microbial Process	**Standard Free Energy Change (kJ/mol H_2)**	**H_2 Threshold (nM)[b]**
Methanogenesis	-33.9	5-95
Sulfate Reduction	-38.0	1-15
Iron Reduction	-108	0.1-0.8
Dehalogenation	-130 to -187	0.04-0.3

[a]Free energy values were calculated from free energies of formation or were taken from previously published works.

Thus, if H_2 is the limiting substrate, then its minimum concentration will be controlled by the kinetic characteristics of the dominant H_2 consuming organisms, which are reflected in the K_s, q_{max}, and b values, and by Y. Y is proportional to the free energy released by the coupling of H_2 oxidation to the reduction of a TEA. Therefore, the more energy that is released by an oxidation-reduction reaction, the greater the Y. According to Eqn. 1, if the kinetic characteristics of the H_2–consuming populations do not vary significantly for different dominant TEAPs, then S_{min} (the H_2 threshold concentration) should increase as the TEA becomes more reduced and the $\Delta G^{o'}$ resulting from H_2 metabolism decreases. As shown in Table 2.1, the H_2 concentrations measured in anaerobic sediments dominated by different TEAPs generally appear to follow this trend. The variability in these determined H_2 threshold concentrations is a result of environmental conditions, i.e. pH, electron acceptors, electron donors, and temperature, and the microbial communities present, i.e. the kinetic factors. However, although variability in the ranges do exist, previous studies with pure and mixed cultures have demonstrated that H_2 thresholds for dehalorespiration and methanogenesis are quite different (Löffler and Sanford, 2005).

Thus, if CVOCs are being transformed via metabolic (dehalorespiration) processes, then H_2 concentrations should be quite low (~1 nM or lower) (Table 2.1). In contrast, if CVOC removal is due to abiotic or co-metabolic processes, then H_2 should be expected to be in a range characteristic of methanogens or sulfate reduction (~1-95 nM). Kassenga et. al. (2004) used this approach to evaluate whether DCE and DCA were removed via co-metabolic or metabolic processes in wetland sediment column reactors. When DCE was added to the column reactors, H_2 concentrations were noted to decrease to approximately 2.71 nM, and methane production ceased. This indicated that dehalorespiration was responsible for DCE removal, not

methanogenesis. However, when DCA was added to the reactors, H_2 concentrations remained high, ranging from 31.7 to 43.4 nM, a range characteristic of methanogenesis.

2.5. Monitoring Natural Attenuation

Under regulatory policies enacted by the U.S. government, specifically the U.S. EPA, sites in which natural attenuation is the primary remediation method for removing contaminants must be continuously monitored (Weidemeier et. al., 1999). APG was declared a Superfund site, which is the term used to describe hazardous waste sites under the Comprehensive Environmental, Compensation, and Liability Act of 1980 (CERCLA) and, therefore, must follow environmental clean-up policies dictated by the EPA. (U.S. EPA, 2005)

In general, natural attenuation monitoring protocols recommend the collection of converging lines of evidence that suggest that the mass and/or concentration of the contaminant is decreasing and microorganisms are contributing to contaminant removal (e.g. Wiedemeier et al., 1999). The types of evidence collected in such a monitoring protocol include (1) observing fluctuations in contaminant concentration at the site with, hopefully, an overall decrease in contaminant concentration over time; (2) the generation of degradation products or change in geochemical factors, i.e., noting the prominent "microbial footprints"; and (3) performing laboratory experiments that evaluate the potential for biodegradation *in-situ*.

Monitored natural attenuation, like other remediation methods, has advantages and disadvantages. One important advantage is that it does not disrupt the environment. In addition, compared to many other *in-situ* or *ex-situ* remediation methods, natural attenuation is less

expensive and does not transfer contaminants to another phase that also requires treatment (Lee et. al., 1998). However, one disadvantage of natural attenuation is that it is a slow process. Therefore, continuous monitoring is often necessary for many years. This is important because not only will the site be a long-term liability for the responsible parties for years to come, but over time the hydrogeological and geochemical aspects of the site could change, which could either hinder or enhance natural attenuation. If natural attenuation becomes hindered at a site, engineered bioremediation methods can usually be applied to the site.

2.6. West Branch Canal Creek Study Site

West Branch Canal Creek is located on the Edgewood side of APG, and flows into the Gunpowder River (Figure 2.7). On the east and west sides, the creek is bordered by tidal wetlands, which have been shown to contain contaminated groundwater, which is a result of chemical warfare operations, as previously stated. The Canal Creek aquifer, which is 30 to 70 ft. thick, is the primary contaminated aquifer. The water that infiltrates the wetland is greatly influenced by the tide, which fluctuates between 0.5 and 2 feet. *Phragmites australis* dominates the vegetation found at the wetlands, although cattail, pickerelweed, and southern wild rice have also been observed (Lorah et. al., 1997).

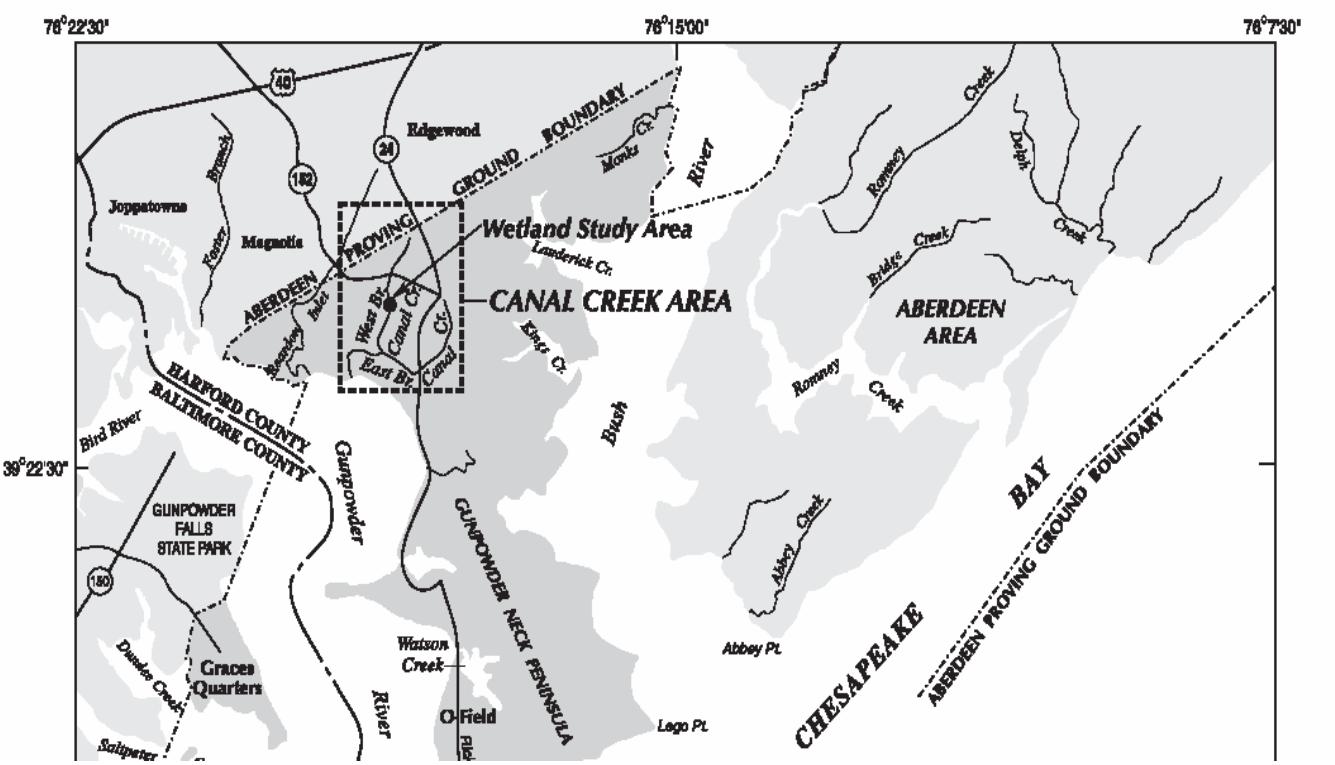


Figure 2.7. Location of West Branch Canal Creek in reference to APG.

To monitor the fluctuations in parent and daughter compound concentrations, as contaminated groundwater moved up through the sediment at the West Branch Canal Creek site, two transects (A-A' and C-C') were placed perpendicular to the creek, in the direction of groundwater flow (Figure 2.8) (Lorah and Olsen, 1999). Groundwater was observed to flow from the aquifer vertically through the wetland at an average linear velocity of 2.3 ft/yr (.006 ft/d) (Lorah et. al., 1997). Sediment samples were collected during well installation and groundwater was collected from various wells (piezometers) at different depths along the transects to evaluate total organic carbon (TOC) content, and to observe changes in CVOC concentrations and redox conditions throughout the contaminant plume. To determine the organic content, soil types were evaluated. Soils along the transects were primarily composed of medium- to coarse-grained sand and gravel

with layers of clay, fine sand, and silt. Organic-rich clay (TOC = 1%) and peat (TOC ranged from 6.9% and 32.6%) were observed near the land surface (Lorah et. al.,1997).

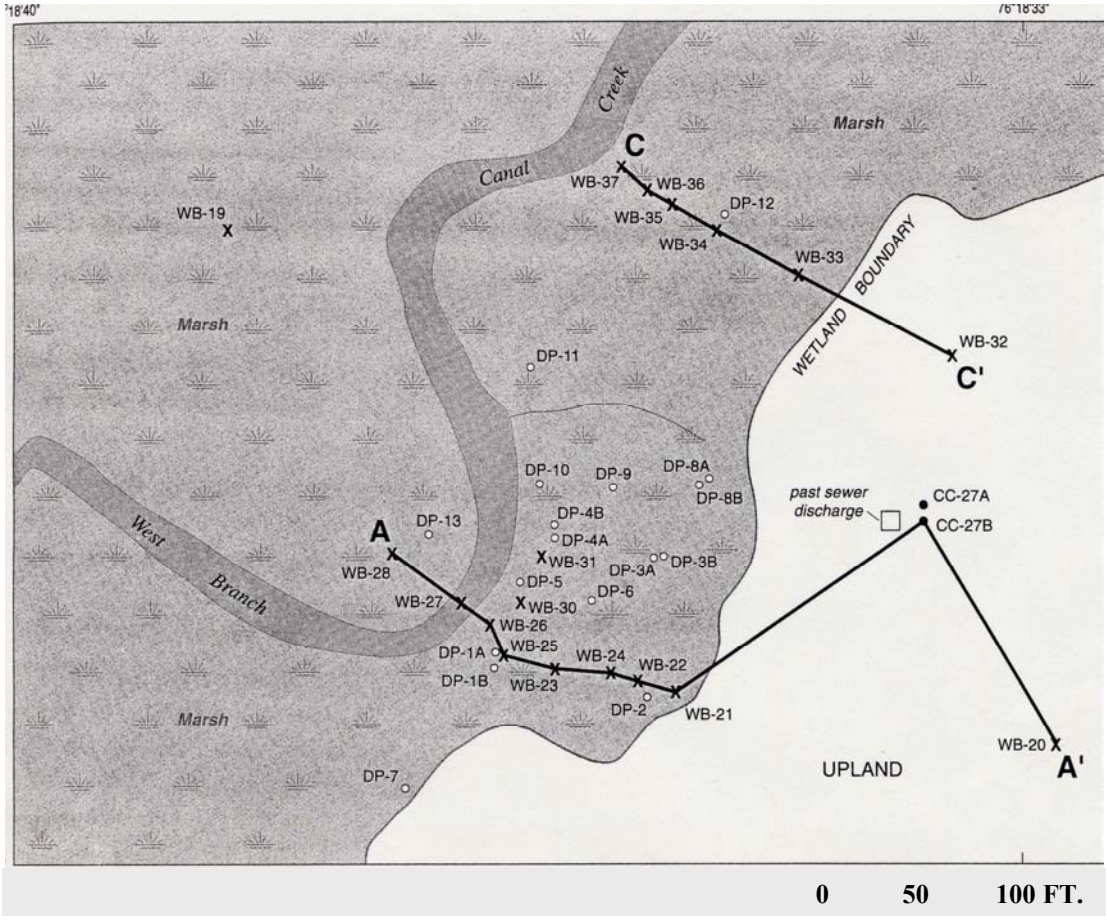


Figure 2.8. A map of transects A-A' and C-C' perpendicular to WBCC. "CC" and "DP" are piezometers installed from a previous study, and "WB" piezometers are located along the transects. Both are/were used to monitor CVOC concentrations, and/or to characterize the redox processes at the sites. (Lorah and Olsen, 1999)

As contaminated groundwater moved upward towards the sediment surface, the concentrations of the parent contaminants, TeCA and TCE, were higher in the aquifer compared to the concentrations detected near the surface along both transects. Near the aquifer, the total concentrations of CVOCs were 5000 and 2100 µg/L along transects A-A' and C-C', respectively, and decreased to less than 10 µg/L near the surface (Figure 2.9) (Lorah et. al., 1997). As parent

concentrations decreased, the daughter products of TCE and TeCA increased in concentration, indicating that biological and/or chemical transformations were occurring at the site. DCE and VC were the dominant daughter products, although TCA and DCA were also detected in low concentrations.

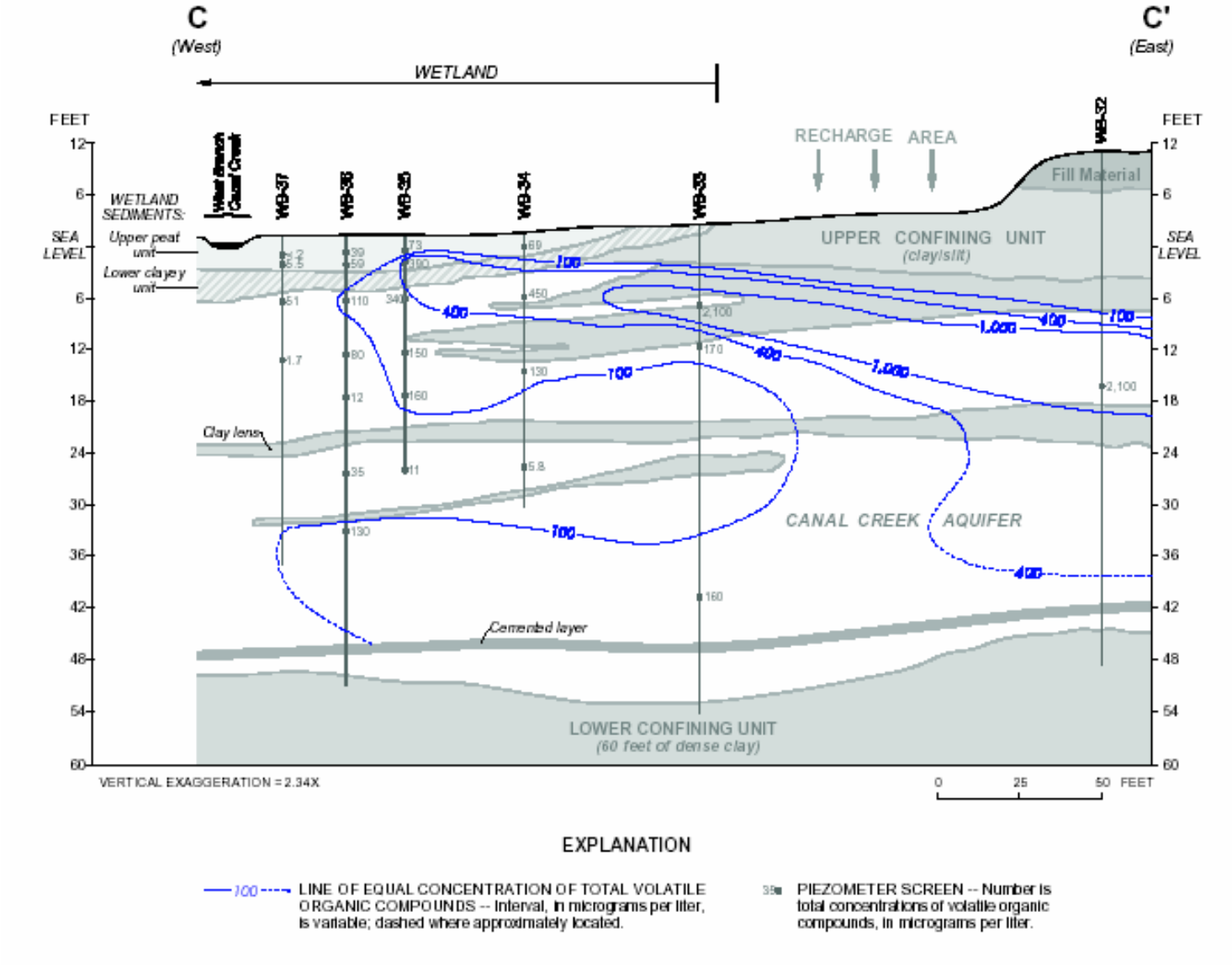


Figure 2.9. Distribution of the total CVOC concentration throughout transect C-C'. (Lorah et. al., 1997)

These data suggest that dichloroelimination and hydrogenolysis were the main degradative pathways involved in the removal of TCE and TeCA, respectively (Figure 2.6, Lorah and Olsen, 1997). Dichloromethane (DCM) was also detected in low concentrations, indicating that CT and CF were being transformed by hydrogenolysis as well. Dehydrohalogenation of TeCA to TCE was also observed; however, it was not considered a major degradation pathway and contributed to less than 2% of the TCE observed *in situ* (Lorah et. al., 2003a). Geochemical analyses of the groundwater at different heights along the transects provided evidence of iron- and sulfate-reducing, and methanogenic conditions (Lorah et. al., 1997). The highest rate of CVOC removal was observed under methanogenic conditions, compared to reduction of CVOCs under iron- and sulfate-reducing conditions. In particular, an increased rate of conversion of TeCA and TCE was observed under methanogenic conditions. The detection of highly reduced conditions, which are conducive to abiotic and biological reductive dechlorination of CVOCs supports the idea that biological and/or chemical transformation of CVOCs occurred along the transects.

To compliment the field data, laboratory experiments were also conducted to evaluate the feasibility of natural attenuation in wetland sediment collected along the transects (Lorah et. al., 1997). As expected based on the field data, degradation of TCE and TeCA primarily produced DCE and VC as daughter products. TCA and DCA were minor products. 16S rRNA gene-based fingerprinting of the microbial communities in laboratory microcosms revealed that *Dehalococcoides* and *Desulfuromonas* strains were present at site WB-30. This is significant because characterized members of both genera are known to carry out dehalorespiration (Sung et. al., 2003; Maymo-Gatell, et. al., 1999). Therefore, these data indicate that organisms with the ability to carry out metabolic degradation of certain CVOCs were present in the field. Again, the

laboratory data support the idea that biodegradation of CVOCs in the field was feasible, although they do not provide evidence that certain microorganisms or processes were active *in situ*.

The laboratory microcosm studies also provided information on TeCA and TCE removal mechanisms (Lorah and Olsen, 1999). Sterile microcosms amended with TeCA produced DCE, demonstrating that the dichloroelimination of TeCA is not primarily a biological reaction in the sediment. Approximately fifty percent of the aqueous phase TCE and TeCA from sterile and non-sterile microcosms was lost four days after adding the CVOCs to the microcosms (Lorah et. al., 1997). However, after CVOC sorption reached an equilibrium, aqueous CVOC concentrations remained constant in the sterile microcosms, but decreased quickly in the live microcosms indicating that in addition to sorption, biodegradation was also an important natural attenuation mechanism.

Field and laboratory observations of the concomitant decreases in parent CVOCs and increases in daughter CVOCs, highly reducing conditions that promote critical reductive dechlorination reactions, and evidence of sorption and chemical transformations of CVOCs demonstrated that natural attenuation may be a suitable remediation approach at West Branch Canal Creek. This conclusion was further supported by the detection of members of genera that include known dehalorespirers at West Branch Canal Creek.

2.6.1. Seep Sites

Seep sites are areas where the groundwater discharges at the wetland surface. At seep sites 3-4W, 3-1E, and 3-7E, the following maximum parent concentrations were detected in the

surface water in 2002 and 2003: TeCA, 476 µg/L; PCE, 914 µg/L; and CT, 7920 µg/L, which exceed the maximum contaminant level of 5 µg/L (U.S. EPA, 1998; Lorah et. al., unpublished data). There are three likely explanations for the high concentrations of contaminants at seep sites. One, the high porewater velocities limit the amount of biodegradation that can occur because bacteria do not have enough time to transform contaminants. Two, bacteria that possess the ability to biodegrade the chlorinated pollutants at the locations where natural attenuation is successful are absent, or present in insufficient numbers, at the seep sites. Three, indigenous bacteria that are able to transform the parent compounds may be limited by the availability of electron donors.

The third hypothesis is supported by the results of a preliminary laboratory experiment conducted with seep sediment from site 3-4W in West Branch Canal Creek as part of this study. The 23-day experiment evaluated the availability of methanogenic substrates in the unamended seep site sediment and groundwater. Low concentrations of methane (less than 0.05 µM) were produced, suggesting that the levels of endogenous degradable substrates were low and the addition of electron donors would be needed to promote methanogenic conditions and, presumably, sustain reductive dechlorination.

Chapter 3: Problem Statement

An engineered bioremediation approach is needed to enhance natural attenuation processes at certain seep sites within the West Branch Canal Creek area(s), because of the high CVOC concentrations being discharged in the groundwater. However, before an appropriate clean-up approach can be identified, it must first be determined whether the availability of electron donors, the number of CVOC-transforming bacteria, or both electron donors and bacteria is (are) limiting CVOC removal at the seep sites. Further, the relative importance of metabolic and co-metabolic transformations in bringing about the transformation of various CVOCs in the wetland sediment and a potential bioaugmentation culture is not well understood. This lack of information limits our ability to develop effective bioremediation strategies for enhancing natural attenuation.

Chapter 4: Experimental Goals and Objectives

The goals of this project are to understand the importance of co-metabolic and metabolic processes in bringing about the transformation of CVOCs in the wetland sediment and a bioaugmentation culture, and to evaluate the effectiveness of bioaugmentation and biostimulation for overcoming limitations in the availability of electron donors and/or bacteria that are able to transform CVOCs. Specifically, the experimental objectives are to:

1. Use wetland sediment and groundwater microcosms to evaluate (a) the effectiveness of biostimulation and/or bioaugmentation at enhancing natural attenuation of TeCA alone and TeCA in the presence of other CVOCs at two seep sites, and (b) the impact (i.e. competition between bacterial populations) of these engineered bioremediation approaches on the indigenous microbial community.
2. Use H_2 concentrations to evaluate the relative importance of metabolic and co-metabolic processes in the transformation of CVOCs in the wetland sediment and a bioaugmentation culture.

Chapter 5: Materials and Methods

5.1.Experimental Approach

5.1.1. Microcosm Experiments

To assess the potential for success of biostimulation and/or bioaugmentation in the field, laboratory-scale studies were conducted using sediment collected from seep sites of interest, sites 3-4W and 3-1E (Figure 5.1), and groundwater collected from a nearby well, site WB-24B. All microcosms were amended with TeCA alone or a mixture of CVOCs. For the site 3-4W experiment, the mixture consisted of PCE, TCE, CT, CF, and TeCA. The mixture was simplified to PCE, CT, and TeCA for the site 3-1E experiment because it was anticipated that TCE and CF would be produced during the degradation of the parent compounds. For the site 3-4W experiment, large numbers of microcosms were prepared without headspace and sacrificed in duplicate at each sampling interval. This was done because TeCA has a relatively low Henry's constant that does not favor partitioning into the headspace. Therefore, aqueous samples had to be heated in order to drive sufficient amounts of TeCA into the headspace for quantification using a gas chromatograph (GC) equipped with an old electron capture detector (ECD). After the completion of the 3-4W experiment, the ECD was replaced, and the new detector was much more sensitive than the old one. As a result, it was possible to quantify TeCA in the gas phase without heating the aqueous samples. Therefore, the 3-1E microcosms were constructed in duplicate with a headspace and sampled repeatedly at each sampling interval.

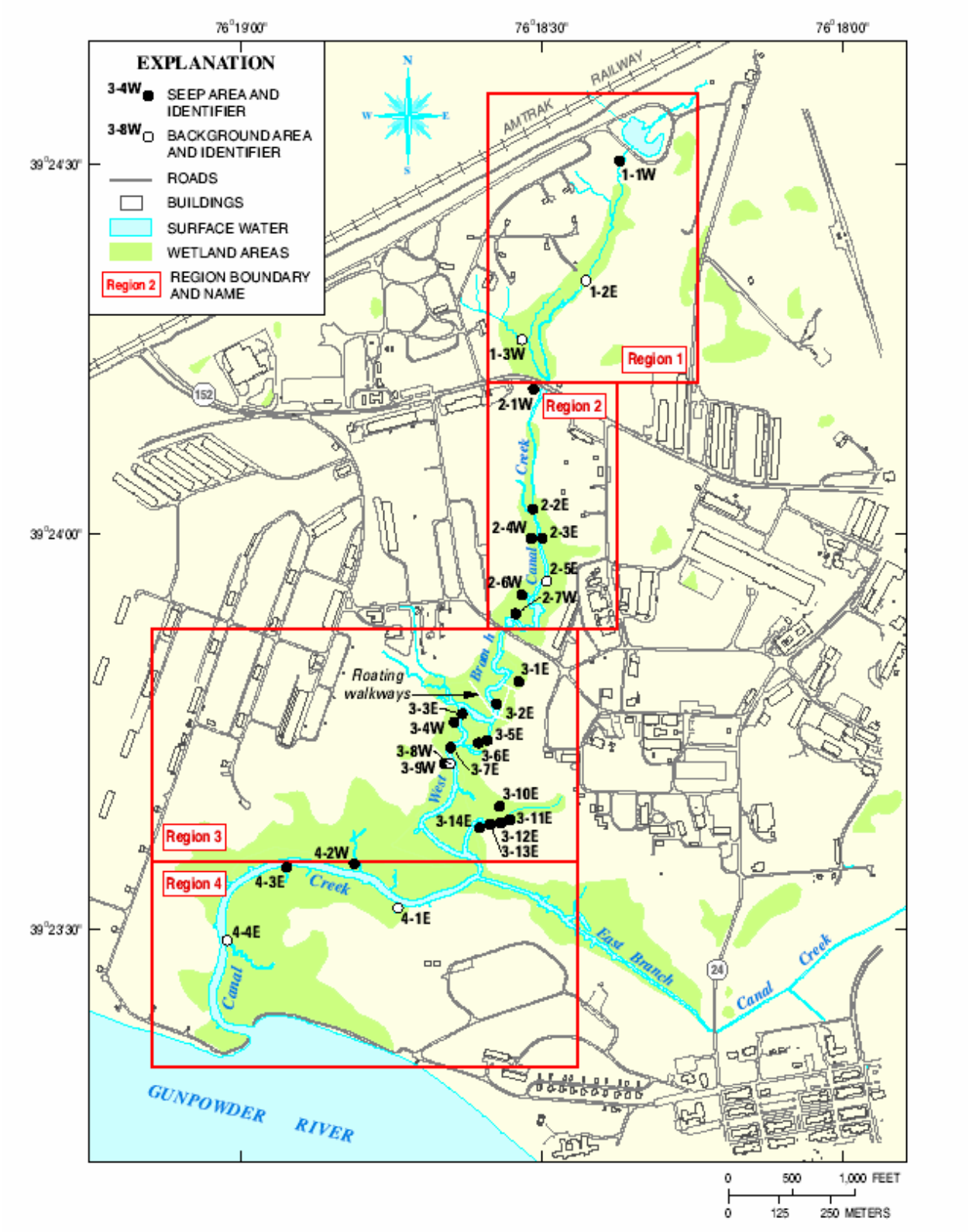


Figure 5.1. Location of the Seep Sites at Canal Creek (Majcher et. al., 2006).

To assess the effectiveness of biostimulation at enhancing natural attenuation for sites 3-1E and 3-4W, the CVOC removal rate and extent of transformation in microcosms amended with electron donors (DMIX treatment, Table 5.1) were compared to microcosms treated only with the VOC mix (MIX treatment, Table 5.1). The effectiveness of bioaugmentation was evaluated by comparing the rate of CVOC removal and the extent of transformations in microcosms amended with electron donors and a TeCA-degrading enrichment culture (DMIX and DMWBC microcosms, Table 5.1). To assess the effects of CT, its dechlorination product, CF, and PCE on TeCA removal, analogous microcosm treatments were prepared with TeCA only, instead of the VOC mix (Table 5.1). Finally, the contributions of abiotic losses were assessed by comparing CVOC removals in these viable microcosms with CVOC losses in water controls (WC), and microcosms in which microbial activity was inhibited by physical and chemical treatments before amending them with a CVOC mixture (SMIX and SWBCMIX).

5.1.2. Use of H_2 threshold concentrations to evaluate the relative importance of metabolic and co-metabolic processes in the removal of CVOCs in the wetland sediment.

In order to assess the relative importance of co-metabolic and metabolic processes in the removal of individual parent contaminants, sediment and groundwater microcosms, as well as culture tubes containing anaerobic culture media and the augmentation culture (see *Section 5.1.3*), were amended with either TeCA, CT, or PCE only (Table 5.2). The concentrations of the parent compounds and any daughter products were monitored along with H_2 and CH_4 concentrations. Previous researchers have used this approach to correlate high concentrations of H_2 and concomitant CH_4 production and removal of a CVOC, with a co-metabolic CVOC degradation process (Kassenga et al., 2004). On the other hand, CVOC removal in the absence

of methanogenesis and low H_2 levels may indicate that CVOC removal was due to dehalorespiration.

Table 5.1. Treatment Design for Seep Sites.

Treatment Name	Treatment: D = electron donors added[1]; MIX = CVOC mixture[2]; T = TeCA[3]; WBC-2 = West Branch Consortium; S = sterile control
MIX[a,b]	CVOC MIX + Sediment + Groundwater
DMIX[a,b]	CVOC MIX + Sediment + Electron Donors + Groundwater
DMWBC[a,b]	CVOC MIX + Sediment + WBC-2 + Electron Donors + Groundwater
TeCA-Only[b]	T + Sediment + Groundwater
TDONOR[b]	T + Sediment + Electron Donors + Groundwater
TDWBC[a,b]	T + Sediment + Electron Donors + WBC-2 + Groundwater
TeCA-$FeCl_2$ & Na_2S [b,6]	T + Sediment + $FeCl_2$ & Na_2S + Groundwater
TeCA- Na_2S[b,6]	T + Sediment + Na_2S + Groundwater
SMIX[a,b,5]	VOC MIX + Sediment + Groundwater
SWBCMIX[a,5]	VOC MIX + Sediment + Groundwater + WBC-2
WC[a,b]	DI-Water control with VOC MIX (day 0)
Methane[a]	Sediment + Groundwater + Electron Donors

[a]Treatments were conducted for Site 3-4W.

[b]Treatments were conducted for Site 3-1E.

[1]Ethanol and lactate will be added at 5 mM. Chitin was also added in addition to these donors for site 3-4W.

[2]CVOC Mixture: (**3-1E:** TeCA, 5 mg/L (30 µmol/L); PCE, 3 mg/L (18 µmol/L); CT, 4 mg/L (26 µmol/L)); (**3-4W:** TeCA, 5 mg/L (30 µmol/L); PCE, 3 mg/L (18 µmol/L); TCE, 3 mg/L (23 µmol/L); CT, 5 mg/L (33 µmol/L); CF, 5 mg/L (42 µmol/L))

[3]TeCA added alone at 5 mg/L (30 µmol/L).

[4]WBC-2 added at 10% of the groundwater volume.

[5]Killed by autoclaving for 1 h on each of three consecutive days and adding formaldehyde at 1% of the total volume. Site 3-4W was killed by formaldehyde only.

[6]To promote the use of iron as a reducing agent, $FeCl_2$ and Na_2S were added at 0.1 mg/L and 0.5 mg/L, respectively. Bottles containing Na_2S only were also given a concentration of 0.5 mg/L.

Table 5.2. Treatment design for H_2 experiment.

Treatment Name	Treatment: Electron Donor and Culture Media (WBC)[2]; PCE = 3 mg/L; CT = 4 mg/L; TeCA = 5 mg/L; WBC = West Branch Consortium
PCE	PCE + Sediment + Groundwater
CT	CT + Sediment + Groundwater
TeCA	TeCA + Sediment + Groundwater OR WBC
Sterile Controls:	
SPCE	PCE + Sediment + Groundwater
SCT	CT + Sediment + Groundwater
STeCA	TeCA + Sediment + Groundwater
No CVOC Control:	
NOCVOC[1]	Sediment + Water
Water Controls:	
WC-PCE	DI Water + PCE (~3 mg/L; 26 µmol/L)
WC-CT	DI Water + CT (~4 mg/L; 18 µmol/L)
WC-TeCA	DI Water + TeCA (~5 mg/L; 30 µmol/L)

[1]No CVOC controls were prepared for the sediment microcosms only.
[2]Only TeCA was examined in the augmentation culture experiment.

5.1.3. Using T-RFLP to evaluate the effectiveness of bioaugmentation and biostimulation.

To monitor the effect of bioaugmentation on sediment community structure, terminal restriction fragment length polymorphism (T-RFLP), a common molecular fingerprinting technique, was used. Currently, T-RFLP is being used to assess microbial diversity and identify potentially important populations at multiple sites at West Branch Canal Creek (Lorah et. al., 2003a,b; Lorah and Voytek, 2004). This genetic technique was a compliment to the geochemical data collected, and provided additional evidence for variable degradation rates and predominant redox conditions. (Lorah and Voytek, 2004)

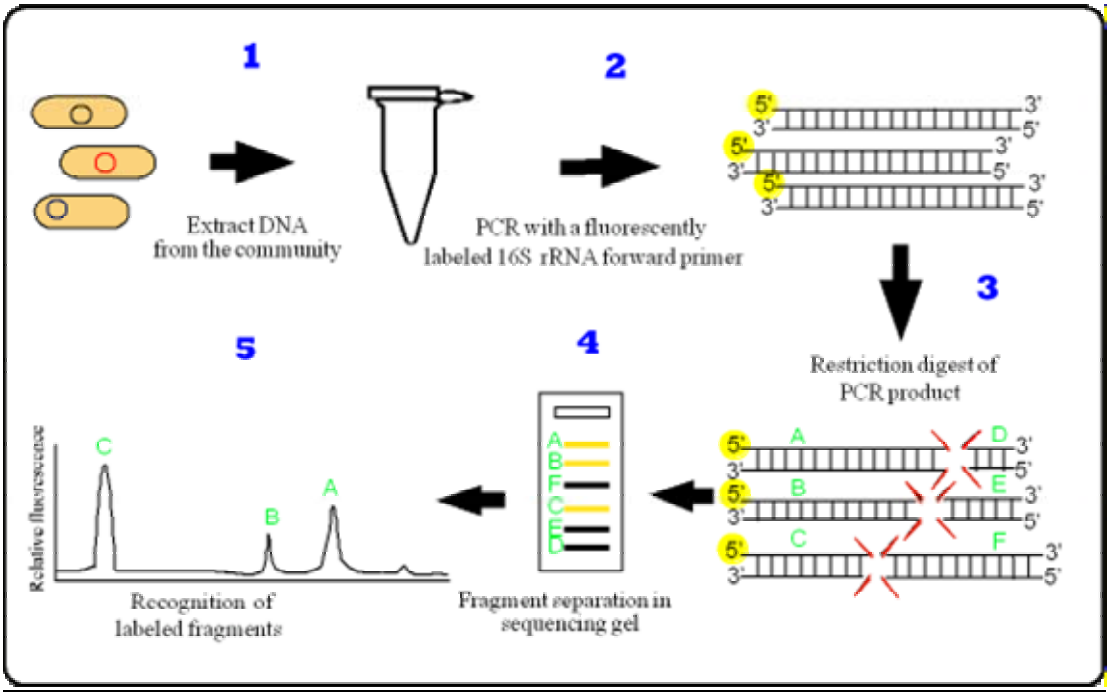


Figure 5.2. Illustration of key steps in T-RFLP analysis: (1) DNA extraction, (2) Amplification via PCR, (3) Enzymatic digestion, and (4) Fractionation by electrophoresis (step 4 and 5) (Gruntzig et. al., 2002).

Isolation of the DNA from the microbial community was the first step in performing a T-RFLP analysis (Figure 5.2, Step 1). To isolate the DNA, a series of chemicals were added to a sediment sample to lyse the cells and extract DNA. The polymerase chain reaction (PCR) was then used to amplify a conserved region of the DNA, i.e. the gene encoding the 16S rRNA molecules using specific primers, including one with a fluorescent label. The 16S rRNA gene was a useful molecule for analyzing the structure of microbial communities because it is highly conserved, due to its importance in the translation of proteins. However, some regions of the 16S rRNA gene are more variable than others. Sequences within these regions may be characteristic of different phylogenetic groups, such as specific genera or even species.

In the next step of the T-RFLP process, the amplified 16S rRNA gene sequences were then cut by restriction enzymes that recognize specific DNA sequences (Figure 5.2, Step 3). Because of sequence differences in the variable regions of the 16S rRNA gene, the restriction enzymes generated fragments of different lengths from the 16S rRNA genes derived from different populations. The restriction fragments were then separated and analyzed using automated capillary electrophoresis, along with a size standard, to determine the fragment lengths and generate the T-RFLP chromatogram or fingerprint. For each peak in the chromatogram, the area was calculated and used to infer the abundance of a given target group in the sample (Figure 5.2, Osborn et. al., 2000).

5.2. Materials

5.2.1. Bioaugmentation Culture and Electron Donors

The enrichment culture used to test the effectiveness of bioaugmentation, WBC-2, was developed by USGS researchers. It was derived from sediment obtained from two APG sites (WB-23 and WB-30) that were diluted with groundwater and initially individually supplied with TeCA for one month. 100 mLs of WB-23 and WB-30 were then aliquoted out into serum bottles, each of which was supplied with a different daughter product of TeCA (TCA or cDCE) for one month. TCA and cDCE supplied cultures were diluted with a mineral medium described by Jones et. al. (2006) and fed TeCA again. From this new enrichment culture, several sub-cultures were then established, including WBC-2B, which is able to degrade TeCA and the dominant daughter products. This was demonstrated by enriching sub samples of WBC-2 on TCA and DCE and observing high rates of degradation (1-1.5 $\mu M\ day^{-1}$) (Jones et. al., 2006).

Lactate and ethanol were selected as electron donors for these experiments because previous experiments conducted by USGS researchers demonstrated that microcosms amended with these donors have a high rate of CVOC removal, compared to unamended microcosms. A concentration of 5 mM for both ethanol and lactate was chosen because it provides enough reducing equivalents to completely reductively dehalogenate TeCA, PCE, TCE, CT, and CF at the concentrations used in the experiments and to deplete sulfate and ferric iron in anaerobic respiration so that the microcosms become methanogenic. (Lorah and Voytek, 2004)

5.2.2. Reagents and Compressed Gases

TeCA (99% pure), DCM (99.5% pure) and CT (99.9% pure) were purchased from Fisher Scientific, Inc. (Chicago, IL.). PCE (99% pure) was obtained from Spectrum (Gardena, CA). TCE (99% pure), CT (99% pure), CF (99% pure), TCA (99% pure), DCA (99% pure), 1,1-DCE (200 μg/mL in MeOH), and DCE (97% pure) were purchased from Sigma Chemical Co. (Milwaukee, WI). VC (2000 μg/mL), chloroethane (CA, 2000 μg/mL), and chloromethane (CM, 2000 μg/mL) were obtained from Restek (Bellefonte, CA). Sodium lactate (99% pure, Fisher), and ethanol (100% pure, Sigma Chemical Co.) were used as electron donors. A 37% w/w formaldehyde solution in water stabilized by 10-15% methanol was obtained from Fisher. 2,2-dipyridyl (Bipyridine) (99% pure, Sigma Chemical Co.) and sodium acetate trihydrate (Crystalline, Fisher) were used in determining ferrous iron concentrations.

For maintenance of the WBC-2 culture, the nutrient solution contained (g/L): $NaHCO_3$ (2.5), NH_4Cl (0.5), NaH_2PO_4-H_2O (0.5), and KCl (0.1). The trace vitamin solution contains (g/L): Nitrilotriacetic acid (1.5; 99% pure, Sigma), $MgSO_4$–$7H_2O$ (3.0; Sigma), $MnSO_4$–H_2O (0.5;

Fisher), NaCl (1.0; Fisher), $FeSO_4$–$7H_2O$ (0.1; J.T. Baker, Phillipsburg, NJ), $CaCl_2$–$2H_2O$ (0.1; 99% pure, Sigma), ZnCl (0.13; Fisher), $CuSO_4$–$5H_2O$ (0.01; Crystalline, Fisher), $AlK(SO_4)$-$12H_2O$ (0.01; Sigma), H_3BO_3 (0.01; 99.95% pure, Sigma), Na_2MoO_4-$2H_2O$ (0.025; 99% pure, Sigma), $NiCl_2$-$12H_2O$ (0.024; J.T.Baker), and Na_2WO_4-$2H_2O$ (0.025; Sigma) The trace vitamin solution contained (mg/L; Balch et.al.,1979): Biotin (2), Folic acid (2), pyridoxine hydrochloride (10), thiamine hydrochloride (5), riboflavin (5), nicotinic acid (5), $_{DL}$- calcium panthothenate (5), vitamin B_{12} (0.1), *p*-aminobenzoic acid (5), and lipoic acid (5). Vitamins were purchased from Sigma.

Hydrogen (Ultra Pure Carrier Grade), helium (Ultra Pure Carrier Grade), nitrogen (Ultra Pure Carrier Grade), air (Ultra Pure Carrier Grade), a 20% CO_2/80% N_2 gas mixture (Certified Standard), and a 20 ppm H_2 balance nitrogen mix (Certified Standard) were purchased from Air Gas (Hyattsville, MD). Methane (CH_4, 99% pure), ethane (99% pure), and ethene (99% pure) were purchased from Sigma Chemical Co.

5.3. Microcosm Preparation for Seep Sites

Microcosms were prepared using sediment collected at a depth of 12-16″ (Lorah et al., 2003a). Groundwater was collected from a productive well at WB-24B (Figure 2.4) that yielded groundwater with biological and chemical characteristics typical of the overall wetland environment. After collection, the groundwater was purged with nitrogen for an hour, and then dispensed into mason jars. Collected seep sediment, purged groundwater, and clean 72-mL serum bottles (Fisher Scientific, Atlanta, GA) were then placed in an anaerobic chamber (85%

N_2/10% CO_2/5% H_2, Coy Laboratories, Model A, Grass Lake, MI) overnight to ensure anaerobic conditions were fully established in all materials.

For the seep 3-4W experiment, serum bottles were completely filled with slurry. For the seep 3-1E experiment, serum bottles were filled with 45-mLs of slurry, leaving 27-mLs of headspace. Each slurry consisted of groundwater and sediment combined in a 1.5:1 (v/v) ratio and sieved through a U.S. Standard Sieve (No. 4, Size 4.76-mm, W.S. Tyler Company, Mentor, Ohio). All 3-4W serum bottles were capped with black butyl septa (Fisher) and sealed with aluminum crimp caps (Fisher). All 3-1E serum bottles were capped with grey teflon septa (Fisher) and sealed with aluminum crimp caps. Black butyl septa were not used for the 3-1E experiment, as it was observed that a high percentage of TeCA sorbed to them. Inhibited controls were prepared by first autoclaving the microcosms for 1 h on each of three consecutive days, and then adding 0.5-mLs (1% by groundwater volume) of formaldehyde before adding CVOCs. Water controls were made by adding 45-mLs of DDI water to a serum bottle before adding CVOCs.

To evaluate the effectiveness of bioaugmentation and biostimulation at seep site 3-4W and 3-1E in the West Branch Canal Creek area, two different treatment designs were followed (Table 5.1). In regards to bioaugmentation and biostimulation, the only differences between the construction of site 3-4W and 3-1E experiments were the technique used to add the bioaugmentation culture and the use of chitin as an electron donor substrate. For site 3-4W, the bioaugmentation culture was added to the necessary treatments at 5 mLs using a sterile 10-ml glass pipette (Fisher). For site 3-1E, the bioaugmentation culture was prepared for addition to the necessary treatment bottles by first transferring a determined volume of the culture, which was contained in a 1000-

mL Wheaton bottle (Fisher), to an autoclaved 25-mL serum bottle (Fisher) flushed with 80% N_2/ 20% CO_2. From the 25-mL serum bottle, 2.7-mL volumes (10% of the groundwater volume) were removed using a 3-mL B&D Syringe (Fisher) flushed with 80% N_2/ 20% CO_2 and a 22-gauge 1" needle (Fisher), and added to the necessary treatments. Lactate was added at 0.5-mL volumes from a stock solution at a concentration of 50.4 g/L (450 mM) to provide a final concentration of 5 mM in the slurry.

After the culture and substrate additions were made, site 3-4W received 10-µL of a stock solution prepared in ethanol containing a mixture of CVOCs or TeCA-Only (Table 5.1). For site 3-1E, microcosms that were not to receive any electron donors were amended with 1 µL of neat TeCA and flushed for 40 min. with 80% N_2/ 20% CO_2. Through a trial-and-error approach, this method was found to result in an aqueous TeCA concentration of 5 mg/L (30 µM). For those requiring electron donor substrate, 13 µL volumes of a TeCA stock solution (1.67×10^{-6} g TeCA/µL) were added to the specific treatments, resulting in the desired final concentrations of ethanol (5 mM) and TeCA (30 µM). PCE and CT were added to microcosms requiring CVOC mixtures by using gas-tight syringes to transfer 7-mLs and 1.5 mLs volumes, respectively, of the headspace in equilibrium with neat compound to the treatment bottle.

In the site 3-1E experiment, $FeCl_2$ and Na_2S were added to certain microcosms at concentrations of 0.1 mg/L and 0.5 mg/L, respectively. These treatments evaluated the importance of abiotic reductions involving reduced iron and/or sulfide in the removal of TeCA in the wetland sediment. Previous studies with FeS as a reducing agent have shown that abiotic reduction of TeCA by FeS is feasible (Butler and Hayes, 2000).

5.4. Microcosm Preparation for the CVOC H_2 Experiment

Microcosm slurries were prepared with groundwater from site WB-24B and sediment from background site WB-35, where CVOCs are known to be below their MCLs. Serum bottles were filled with 45-mLs of slurry, leaving 27-mLs of headspace. PCE and CT microcosms were then capped with black butyl septa, whereas TeCA microcosms were capped with grey teflon septa. All microcosms were sealed with aluminum crimp caps. Sterile and water controls for this experiment were prepared as described above.

After microcosm preparation, treatments were then amended with CVOCs. PCE and CT additions were done following the above protocol. TeCA was added following the protocol described above for treatments that were not amended with electron donors.

5.5. WBC-2 H_2 Experiment Preparation

Mineral medium was prepared by adding 1-mL of the trace mineral medium described above to 100-mLs of the nutrient solution described above. 10-mL of the medium was added to each of three anaerobic culture tubes (Fisher), flushed with 80% N_2/ 20% CO_2, autoclaved, and amended with 0.1 mL of trace vitamin solution (described above).

All culture tubes were then amended with 1-mL of 10 mM lactate for a final concentration of 1 mM. Previous studies conducted by USGS researchers have shown that the WBC-2 culture is not effective unless it is provided with a suitable amount of electron donor (Jones et. al., 2006). 1-mL of the culture provided by Elizabeth Jones (USGS, Reston, VA) was added to the

anaerobic culture medium, which contained the necessary nutrients, trace vitamins and minerals, and an electron donor substrate, lactate, using sterile technique.

5.6. Analytical Methods

5.6.1. CVOCs and Hydrocarbons

Quantification of all parent and daughter CVOCs were completed using two headspace gas chromatography (GC) methods. PCE, TCE, *cis*- plus *trans*-1,2-DCE isomers, 1,1-DCE, VC, CT, CF, DCM, CA, VC, CM, CH_4, ethane (ETA), and ethene (ETE) were monitored on a Hewlett-Packard 5890 Series II Plus GC with a flame ionization detector (FID) and a 1% SP-1000 on 60/80 Carbopak-B (Supelco) (2.44-m x 3.2 mm) packed column. CT was quantified with a GC-FID (as opposed to GC-ECD) because the high CT concentrations used in this study overload the ECD detector, which is very sensitive to CT. The injector and detector temperatures were set at 200 ^{o}C and 250 ^{o}C, respectively. Helium was the carrier gas at a flow rate of 40 mL/min. Air and hydrogen fueled the FID at flow rates of 400 and 40 mL/min, respectively. The temperature program is as follows: (1) hold isothermally at 60 ^{o}C for 2.00 min, (2) ramp at 20 ^{o}C/min to 150 ^{o}C, and (3) ramp at 10 ^{o}C/min to 200 ^{o}C for 4.2 min. VC, CM, and CA are analyzed using a packed column at 20˚C because their boiling points are too low to detect using a 30 m x .53 mm (ID) capillary column.

TeCA, TCA, and DCA were monitored using a Hewlett-Packard 5890 Series II Plus Gas Chromatograph (GC) equipped with an electron capture detector (ECD) and a DB-624 (30 m x .53 mm (ID) x 3 um film thickness) capillary column (Agilent Technologies, New Castle, DE). The GC injector and detector temperatures were 250^{o}C and 300 ^{o}C, respectively. Helium and nitrogen were provided as carrier and make-up gases at flow rates of 6 and 60 mL/min,

respectively. The temperature program used initially was: (1) ramp at 4.0 C/min. to 60ºC and hold for 5 min., and (2) ramp at 15.0ºC/min. to 200°C and hold for 2 min. The current temperature program is: (1) ramp at 40ºC/min. to 200°C and hold for 30 min.

To determine the CVOC concentrations in samples collected, external calibration curves were prepared. For the CVOC calibration curves, methanol stock solutions of all CVOCs were prepared gravimetrically. Aqueous standards were prepared in vials that were identical to those used in the actual experiment.

The concentration of CVOCs in the headspace was determined by accounting for the partitioning of the added CVOC between the headspace and liquid phases according to the equation:

$$M_T = C_g V_g + C_w V_w \tag{2}$$

Headspace samples, 500-µL and 300-µL for GC-FID and GC-ECD were injected using a 1-mL gas tight syringe equipped with a push valve (Supelco, Pressure-Lok ®, Series A-2). These samples were used to determine a relationship between peak area and the µmol of the CVOC in the gas phase. Aqueous phase concentrations were calculated according to:

$$C_w = \frac{\left(\frac{\mu mol\ in\ headspace\ sample}{L\ injected} \right)}{H_c} \tag{3}$$

where μmol in headspace sample was determined from the calibration curve. Calibration curves for CH_4, ETA, and ETE were prepared by injecting different volumes of the pure gases on to the GC-FID directly. Dilution of the pure gases in 160-mL serum bottles that had been flushed with N_2, and contained glass beads to improve mixing, were used to provide lower concentration standards. The moles of gas injected were calculated from the ideal gas law and measured using laboratory temperatures. All injections were made using a 1-mL gas tight syringe equipped with a push-valve (Pressure-Lok ®, Series A-2).

5.6.2. Analysis of Ferrous Iron

A dissolved ferrous iron colorimetric bipyridine method (I-1388-78, Techniques of Water Resources Investigations; Baedecker and Cozzarelli, 1992) was used to analyze the aqueous Fe(II) concentrations in the 3-4W microcosms. A 1-mL plastic syringe (B&D, Fisher) and an IC Acrodisc 25-mm syringe filter with a 0.2-μm Supor (PES) membrane (Filter) was used to collect and filter a 1-mL of ground water sample. The filtered sample was placed in a 15-mL plastic test tube (Fisher) containing 0.5-mL of 2.0 g/L of bipyridine and 1-mL of DDI water. After 30 minutes, 1-mL of 350 g/L of sodium acetate solution was added to fix the iron and prevent the formation of additional ferrous iron. Samples and standards were analyzed in round glass cuvets (Fisher) at 520-nm using a Spectronic 20 spectrophotometer (Spectronic Instruments, Rochester, NY). A ferrous iron calibration curve was prepared by USGS by adding different volumes of an iron standard solution to a glass culture tube (Fisher) and adjusting the total volume of each to 25-mLs using DDI water. These standards were then treated with 1.0-mL of 2.0 g/L bipyridine solution, 2.0-mL of hydroxylamine-hydrochloric acid, and 2.0-mL of 350 g/L sodium acetate solution.

5.6.3. H_2 Analyses

H_2 was quantified using a headspace GC method and monitored on a Peak Performer 1 GC equipped with a reducing compound photometer (RCP) detector and two columns, a 31" UNI 1S guard column, to filter out the CVOCs, and a 31" Molecular Sieve 13X analytical column that employs clay as the adsorbent (Peak Laboratories, Mountain View, CA). The column and detector temperatures were set at 105 ^{o}C and 265 ^{o}C, respectively. The carrier gas was nitrogen at a flow rate 20 mL/min. The temperature program was isothermal at 265°C. After the sample was injected, the instrument was programmed to run for 210 seconds. The retention time of H_2 is 48 seconds with an error of ± 4 seconds.

To determine the H_2 concentration in samples collected, an external calibration curve was prepared. Five different headspace volumes, ranging from 50 to 500 µL, from a concentrated standard was removed using both a 100-µL and 1-mL gas tight syringe equipped with a push valve (Supelco, Pressure-Lok ®, Series A-2). The concentrated standard was prepared by flushing a 60-mL serum bottle containing three glass beads, to ensure adequate mixing, with 18.7 ppm of H_2. The moles of H_2 in each headspace volume injected was calculated by using the ideal gas law.

The following equation was used to determine a relationship between peak area and the nmol per liter of H_2 in the aqueous phase (Löffler et. al., 1999):

$$H_2\ (aq.\ conc.) = \frac{LP}{RT} \tag{4}$$

where H_2 = aqueous concentration (moles per liter), L = 0.01941 (Ostwald coefficient for H_2 solubility at 20ºC, P = H_2 pressure (atm), R = universal gas constant (.0821 L-atm/K-mol), and T = 293 K (20°C). To convert H_2 concentrations in mol/L to atm, the mol/L units was first converted to ppmv using the ideal gas law. The ppmv concentration was then converted to atm using the conversion factor: 1 ppmv = 0.1 Pa = 10^{-6} atm (Löffler et. al., 1999).

5.6.4. Acetate Analyses

Quantification of acetate was completed by using an enzymatic method that used acetyl CoA synthase to convert acetate, ATP, and coenzyme A to acetyl-CoA, AMP, and PP_i (King, 1991):

$$Acetate + ATP + CoA \xrightarrow{acetylCoAsynthase} AcetylCoA + AMP + PP_i \quad (5)$$

AMP, which is related stoichometrically to acetate by a 1:1 ratio, was monitored with a high-pressure liquid chromatograph (HPLC, Waters Corporation, Milford, MA), which employs a carbamate analysis system equipped with a 600E Multisolvent pump (Waters), a 717 Autosampler (Waters), a 996 Photodiode Array Detector (Waters) at a wavelength of 254 nm, and a silica C18 reverse phase column (Supelcosil LC-18), 25 cm x 4.6 mm with 5 μm particles. The mobile phase was 50 mM of HPLC grade potassium phosphate monobasic (Fisher, 99% pure), and 10% methanol at a flow rate of 1.0 mL/min. To determine the concentration of AMP in the microcosms, standard curves were prepared from a 1-mM acetate stock. A 1-mL volume of each standard and sample was then transferred to 1-mL microcentrifuge tubes (Fisher) where all reactants and the acetyl CoA enzyme were added at 10-μL volumes from the following stock solutions: acetyl CoA synthase (Sigma, A1765), 20 U/mL, CoA disodium salt (Fluka), 10 mM,

ATP (Sigma), 100 mM, and bovine serum albumin (BSA, Sigma), 200 μg/mL. All standards and samples were then shaken and incubated in a 37°C water bath (Neslab, Marietta, OH) for 12 h. The reaction was halted by boiling all standards and samples for 2 min, and then centrifuging (Eppendorf, Centrifuge 5415C) them at 8000 rpm for 10 min to remove spent reagents. 800-μL of the supernatant from all samples and standards were then added to 1-mL HPLC vials and analyzed for AMP.

5.7. Determining Partitioning Coefficients for All Parent and Daughter Compounds

A 30 g sample of wet sediment from each site was air-dried according to standard methods (APHA, 1999) and then portions of the dried sediment were added to triplicate 10-mL glass vials (Fisher) containing groundwater. Selection of the appropriate sediment to water ratio was critical in order to ensure good mixing and detectable amounts of CVOC in the headspace. Following the guidelines outlined in the EPICS (Garbarini and Lion, 1985) and ASTM methods (ASTM: D 5285-03, 2004) appropriate sediment to water ratios were experimentally determined. Different sediment to water ratios were needed for all highly chlorinated parent compounds (TeCA, PCE, TCE, CT, and CF), except TCA, which was grouped with the lightly chlorinated compounds (VC, CM, CA, DCE, DCM), because they have a much greater tendency to sorb to organic matter. Thus, a sediment-to-water ratio of 1:8 yielded an appropriate amount of parent (highly-chlorinated) compounds in the headspace, and a sediment-to-water ratio of 1:1 was used for the daughter (lightly chlorinated) compounds. The average CVOC peak area was determined in triplicate 10-mL glass vials containing groundwater only or groundwater plus sediment that were shaken on a S/P Rotator V orbital shaker (VWR) at 120 rpm at 20ºC for 24 hours. The measured peak area for the water-only, and water and sediment samples were then used along

with the measured values of M, V_{gt}, V_{geq}, V_l, and eqn. (6) to calculate the K_d value for a given combination of sediment and CVOC.

5.8. DNA Analyses

5.8.1. DNA Extraction

For extraction and analysis of total sediment microbial community DNA, 1-mL slurry samples were obtained from microcosms, and their DNA extracted using a FastDNA® SPIN Kit (Qbiogene, Carlsbad, CA). However, DNA extractions were not done until the experiment was completed. Therefore, slurry samples were stored in 15-mL plastic centrifuge tubes (Fisher), and frozen (-20°C). They were thawed at room temperature for 30 minutes and vortexed briefly, to ensure adequate mixing of the sample, prior to beginning the extraction process. The first step was to remove 1-mL volumes from all soil samples collected and transfer them to individual 1.5-mL microcentrifuge tube containing a lysing matrix (Qbiogene). Microcentrifuge tubes were then placed in a Mini-Beadbeater 8 (BioSpec ®, Bartlesville, OK) for 45 seconds and set to homogenize to lyse the cells. A series of buffers and a protein precipitation solution (PPS, Qbiogene) were then added to the individual samples to precipitate out proteins and other unwanted cellular material. To obtain only the DNA, a binding matrix was used. The binding matrix solution was then filtered, using SPIN filters (Qbiogene) and 1.5-mL microcentrifuge tubes supplied by the kit, and purified using an ethanol wash solution. The DNA was eluted using 50-µLs of DNase/Pyrogen Free Water, and collected in a clean 1.5-mL microcentrifuge tube (Qbiogene). The final volume of DNA was 50-µLs.

To confirm that the DNA extraction was successful, a 5 µL sample of extracted DNA was run on a 0.7% Low-Electroendosmosistype (Low-EEO) Agarose (Fisher) gel, providing a sharper resolution of bands, with Tris-Borate-EDTA (TBE) buffer (Fisher) in a Mini-Sub Horizontal Gel Electrophoresis system (BioRad, Hercules, CA). A 1-kb DNA ladder (Promega, Madison, WI) was used as a positive control. A 5-µL water sample was used as a negative control. The gel electropherogram was illuminated on a UV Transilluminator (Model 20-E, VWR).

5.8.2. Polymerase Chain Reaction (PCR) Amplification

PCR was used to amplify 16S rRNA extracted from the sediment community. The following was added to a 50-µL PCR mixture: 1-µL of DNA; 5-µL of 10X PCR buffer (0.3M Tricine pH 8.4, 0.5M KCl, 15mM $MgCl_2$; Fisher); 5-µLs of 12 mg/mL non-acetylated bovine serum albumin (BSA) (Ambion, Austin, TX); 2.5-µLs of 1% IgePal (Sigma Chemical Co.); 1-µL of 1U Taq polymerase (Promega, Madison, WI); 30.5-µLs of sterile DDI water; 4-µLs of 2.5 mM of all dNTPs (Promega, Madison, WI); 0.5-µLs of 20 µM of a fluorescent tagged 46F primer (FAM), S-D-Bact-0046-a-S-20 (Qiagen, Valencia, CA); 0.5-µLs of 20 µM of a 519R primer (Qiagen, Valencia, CA), S-*-Univ-0536-a-A-20. The 473 bp DNA fragments were amplified in a PTC-100 thermocycler (MJ Research, Watertown, MA) programmed as follows: Hold at 94 ^{o}C for 3 min., followed by 30 cycles of 30 sec. at 94 ^{o}C, 30 sec at 56 ^{o}C, 1.5 min. at 2 ^{o}C, and conclude with 7 min. at 72 ^{o}C, before cooling to 4 ^{o}C (Lorah et. al, 2003a).

5.8.3. Digestion with restriction enzymes

Amplified 16S rRNA gene sequences were digested using the *MnlI* restriction enzyme (New England Biolabs, Ipswitch, Mass.; Lorah et. al, 2003a), which targets recognition sites with the following leading strand: 5'…CCTC$(N)_7$…3'. A 20-µL reaction mixture was prepared using 6-µLs of amplified DNA, 2-µLs of 10X enzyme-specific buffer (New England Biolabs), 0.5-µLs of 5,000 U/mL of *MnlI* (New England Biolabs), 0.2 µLs of 100X BSA (New England Biolabs), and 11.3-µLs of sterile DDI water. The reaction mixture was then placed in a 37˚C incubator (Fisher) and incubated overnight.

To remove the used reagents, 2-µLs of 3M sodium acetate and 45-µLs of 100% ethanol was added to the restriction digest mixture. This mixture was then vortexed briefly and the spent reagents precipitated out at -20°C for 2 h. Separation of the supernatant from the DNA fragments was conducted using a microcentrifuge (Eppendorf, Centrifuge 5415C) at 14,000g for 15 minutes at 4°C. After removing the supernatant, the DNA was washed again with 500-µLs of 70% ethanol, vortexed, and spun down at 14,000g for 5 min. Any remaining supernatant was removed. The remaining DNA pellet was then dried by placing for 2 min. at 93ºC on a heat block (Multi-Blok, Lab-Line) to drive off residual ethanol. The final product was resuspended in 10-µL of 10X Tris-EDTA buffer (Fisher).

5.8.4. T-RFLP Analysis

T-RFLP analyses were performed using an ABI310 Sequencer (Applied Biosystems, Inc., Foster City, CA). A master mix containing 12-µLs of deionized formamide, which keeps the sample denatured (Applied Biosystems, Inc.) and 0.5-µLs of ROX500, a size standard,

(Applied Biosystems, Inc.) were prepared and aliquoted into 0.5-mL ABI tubes (Applied Biosystems, Inc.). 2-µLs of the resuspended digest was then added to each tube. The T-RFLP samples were then vortexed briefly, denatured for 3-5 min. in a 93°C heat block (Multi-Blok, Lab-Line), and cooled on ice for 2 min. These samples were then loaded into the sequencer and run overnight. Chromatograms were analyzed with Genescan.

Chapter 6: Seep Site 3-4W Results

6.1. K_d (Partitioning Coefficient) Values

K_d values were obtained for the parent CVOCs of interest and all potential chlorinated daughter products at two seep sites (3-4W and 3-1E). In this study, K_d values were determined using a variation on the equilibrium partitioning in closed systems (EPICS) method (Garbarini and Lion, 1985), which compares CVOC headspace concentrations in two pairs of serum bottles that contain the same volume of water, but differ in that one pair of bottles contains sediment, and one does not. The results are summarized in Table 6.1 and can be used to estimate the total mass of a CVOC in a microcosm based on aqueous- or gas-phase concentrations, according to Eqn. 6.

$$K_d = \frac{\left\{\left[\left(\frac{PA_{gt}}{PA_{geq}}\right)\left(H_C V_{gt} + V_l\right)\right] - \left(H_C V_{geq} + V_l\right)\right\}}{M} \qquad (6)$$

where PA_{gt} is the GC peak for a VOC in the gas phase in equilibrium with water only, PA_{geq} is the GC peak for a VOC in the gas phase in equilibrium with water and sediment, K_d is the partitioning coefficient (L^3M^{-1}); M (M) is the air-dried mass of sediment; V_l (L^3) is the volume of water, V_{gt}(L^3) is the headspace volume in the bottle containing only water, and $V_{g\,eq}$(L^{-3}) is the headspace volume in the bottle containing water and sediment.

Table 6.1. Kd values for all CVOCs.

Compound	Site 3-4W	Site 3-1E
CM	1.73	0.74
VC	3.80	1.77
CA	2.57	2.48
DCM	0.65	0.98
DCE	2.52	2.77
CF	2.13	4.60
CT	12.55	18.51
DCA	1.44	1.68
TCE	9.96	16.03
TCA	2.97	3.66
PCE	32.07	50.77
TeCA	9.09	14.32

6.2. Seep 3-4W Experiment

Highly reduced methanogenic conditions are conducive to the reductive dechlorination of CVOCs (Cookson, 1995). In fact, previous studies conducted at APG sites have suggested that methanogenic conditions promote faster rates of CVOC removal (Lorah et. al., 2003a). Therefore, methane monitoring bottles (Methane, Table 5.1) were prepared to ensure that methanogenic conditions developed before CVOCs and the WBC-2 culture were added to the microcosms.

The methane monitoring bottles were amended with donors and contained 45-mLs of sediment slurry. This left 27-mL of headspace for repeated sampling of methane concentrations. By day 1, aqueous concentrations of greater than 400 μM (data not shown) were detected. This high concentration of methane meant that the conditions in the microcosms were already highly reduced and there was no need to delay the addition of CVOCs to the microcosms.

In addition to methane, total ferrous iron was measured as (1) a measure of the microcosm redox conditions, and (2) because studies have shown the presence of iron-reducing conditions can influence dechlorination pathways followed by these CVOCs (Lorah and Voytek, 2004). Iron reduction occurred to a much greater extent in the inhibited controls, especially in the SMIX bottles, which were not amended with WBC-2, compared to in the live microcosms (Figures 6.1 and 6.2). Ferrous iron concentrations were approximately 20 times higher in the inhibited controls than in the viable microcosms. Methane production was inhibited in the controls. This suggests that methanogens were more sensitive to the formaldehyde compared to the iron reducers. However, if Fe(III) reduction was an important terminal electron-accepting process in the 3-4W sediment, then it is somewhat surprising that the addition of large amounts of electron donor to the DMIX and DMWBC microcosms did not result in the consumption of Fe(III) and detection of significant amounts of Fe(II) at the start of the experiment. One possibility is that significant Fe(III) utilization did occur in the DMIX and DMWBC microcosms before day 0, but the Fe(II) produced was converted to forms that were not detectable with the analytical method used in this study, e.g., through precipitation with sulfides. The possibility that Fe(III) reduction in the sterile controls was linked to the removal of some compounds is discussed below.

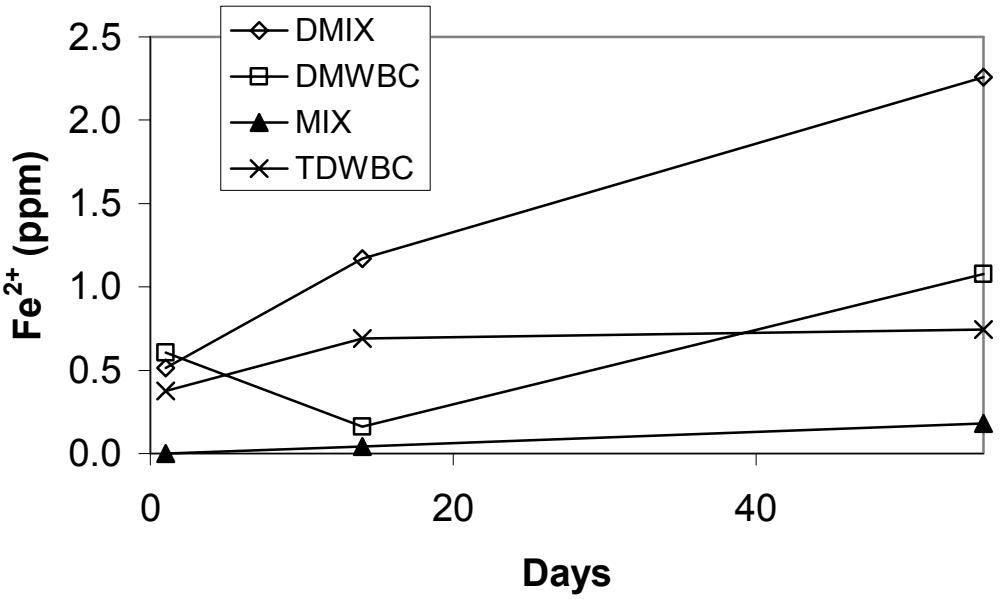


Figure 6.1. Concentration of ferrous iron (ppm) in the live microcosms.

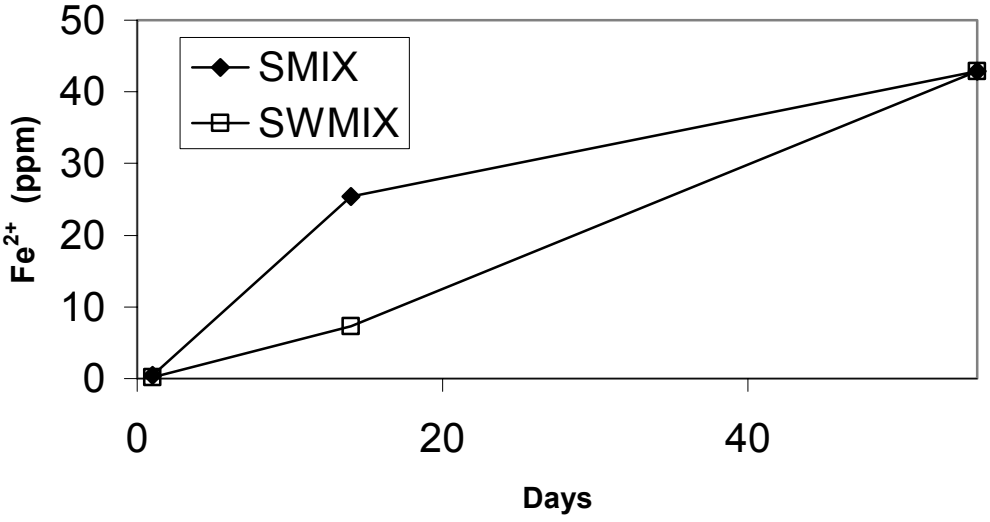


Figure 6.2. Concentration of ferrous iron (ppm) in the sterile microcosms.

In regards to chlorinated ethane removal, simultaneous iron-reducing and methanogenic conditions have been shown to promote the dichloroelimination of TeCA and TCA under sterile conditions in live treatments (Lorah and Voytek, 2004). In the TDWBC microcosms (Table 5.1),

TeCA removal occurred rapidly and was largely depleted by day 11 (Figure 6.3). However, it should be noted that the initial TeCA aqueous concentrations in these microcosms was less than half the target concentration. The detection of the daughter products TCA and DCA indicates that at least some of the TeCA was transformed and that transformation occurred exclusively via hydrogenolysis. Interestingly, past studies at West Branch Canal Creek have shown that both hydrogenolysis and dichloroelimination are involved in TeCA removal, although dichloroelimination was the dominant pathway (Lorah et. al., 1997). TCA and DCA were essentially depleted between days 11 and 14. This relatively high rate of removal of TeCA and its daughter products might have been due to the intrinsic capacity of the 3-4W sediment or to the addition of WBC-2.

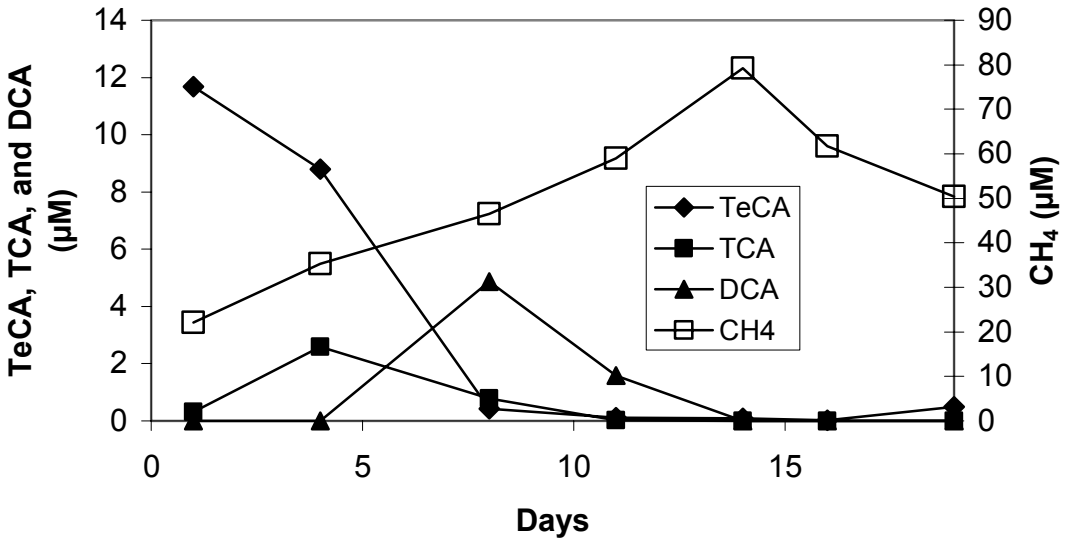


Figure 6.3. Aqueous TeCA, TCA, DCA, and CH_4 (µM) in the TeCA-only microcosms. Data points represent averaged concentrations in duplicate bottles.

In addition to TeCA transformation, some methane production was observed in the TDWBC microcosms. The decrease in methane levels after day 13 suggests that methane production eventually stopped, and that total methane levels were depleted due to repeated sampling of the

headspace. In contrast, methane concentrations in microcosms amended with a mix of VOCs did not increase over time (Figure 6.4). This suggests that one or more of the other compounds contained in the VOC mix inhibited methanogenesis. CF has been shown to inhibit methanogenesis (Becker and Freedman, 1994) and previous studies conducted with APG wetland sediment have also observed inhibition of methanogenesis by CF and/or CT (Lorah et., al., 1997).

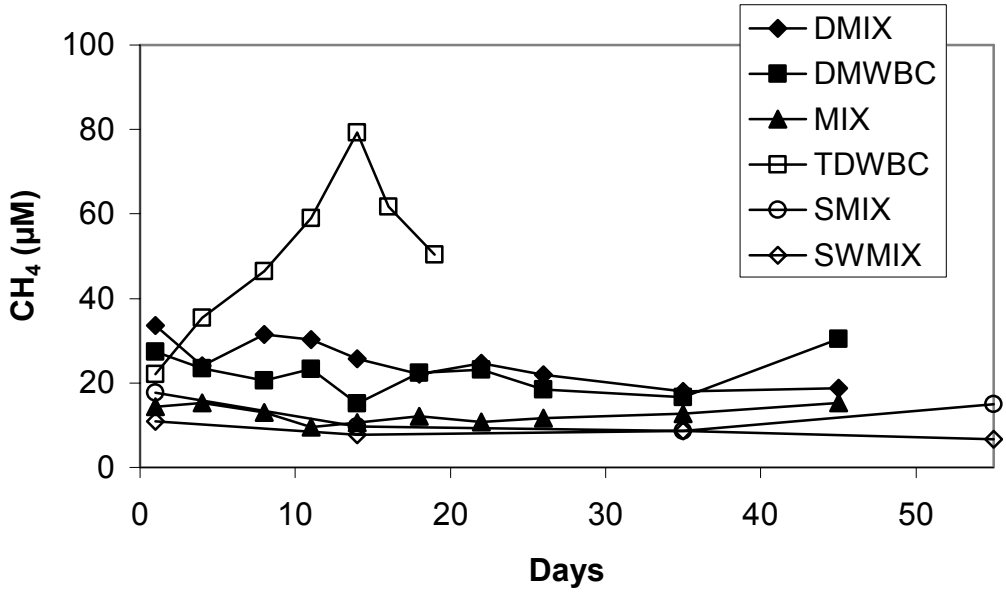


Figure 6.4. Aqueous CH_4 (µM) in the live and sterile microcosms. Data points represent averaged concentrations in duplicate bottles.

In the other live treatments, TeCA removal occurred more slowly than in the TDWBC microcosms (Figure 6.5), and it is possible that TeCA removal in these microcosms was inhibited by one or more CVOCs. However, it should be noted that the initial concentrations of TeCA in the VOC-mix-amended bottles were about twice as high as in the TDWBC microcosms. The higher initial concentration of TeCA in the VOC-mix amended bottles may have contributed to the slower rate of removal.

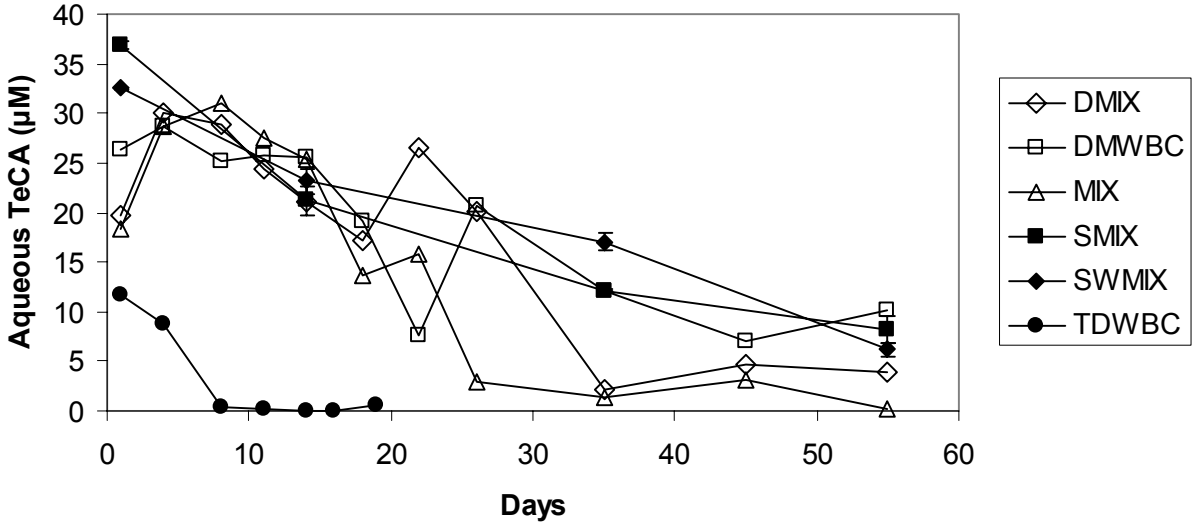


Figure 6.5. Aqueous TeCA (µM) in live microcosms and sterile controls. Data points represent averaged concentrations in duplicate bottles.

Through day 22, the amount of TeCA removal in the sterile controls (SMIX and SWMIX) was generally similar to that observed in the VOC mix-amended live microcosms (DMIX, DMWBC, MIX; Figure 6.6). After day 22, the TeCA remaining in the MIX bottles was depleted fairly rapidly. Therefore, it is likely that TeCA removal after day 22 in the MIX bottles probably was due, at least in part, to biological activity. However, it not clear whether or not most of the TeCA removal that occurred in the DMIX and DMWBC microcosms (especially through day 22) was due to biological activity. One possibility is that a significant amount of the observed TeCA removal in both the live microcosms and the sterile controls was due to abiotic reactions.

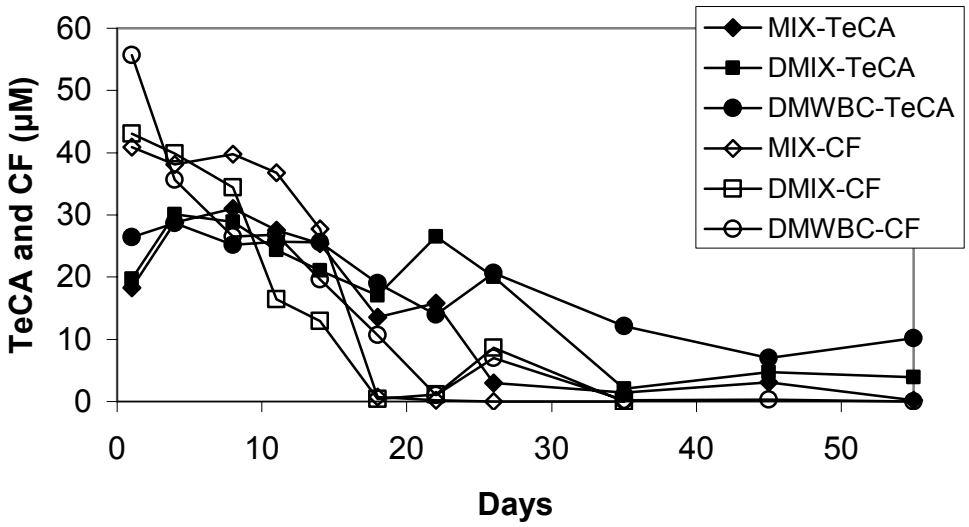


Figure 6.6. Aqueous CF and TeCA (µM) in live treatments. Data points represent averaged concentrations in duplicate bottles.

It is also quite likely that the formaldehyde added to the sterile controls did not fully inhibit microbial activity, including perhaps, TeCA biodegradation. Previously, it was mentioned that CVOCs, such as CF and CT, that were inhibitory to methanogenesis might have also inhibited TeCA biodegradation in the microcosms that were amended with the mix of CVOCs. However, the presence of CF and CT did not appear to be closely related to TeCA removal. The initial concentration of CT in the live microcosms (<1.53 mg/L or 10 µM) was significantly lower than intended (5 mg/L; 33 µmol/L) perhaps due to extensive sorption to the sediment and/or septa (Figure 6.7). This small amount of CT was removed by day 4, well before the rate of TeCA removal in the MIX and DMIX bottles began to increase slightly relative to the rate of removal in the sterile microcosms (after day 22; Figure 6.5).

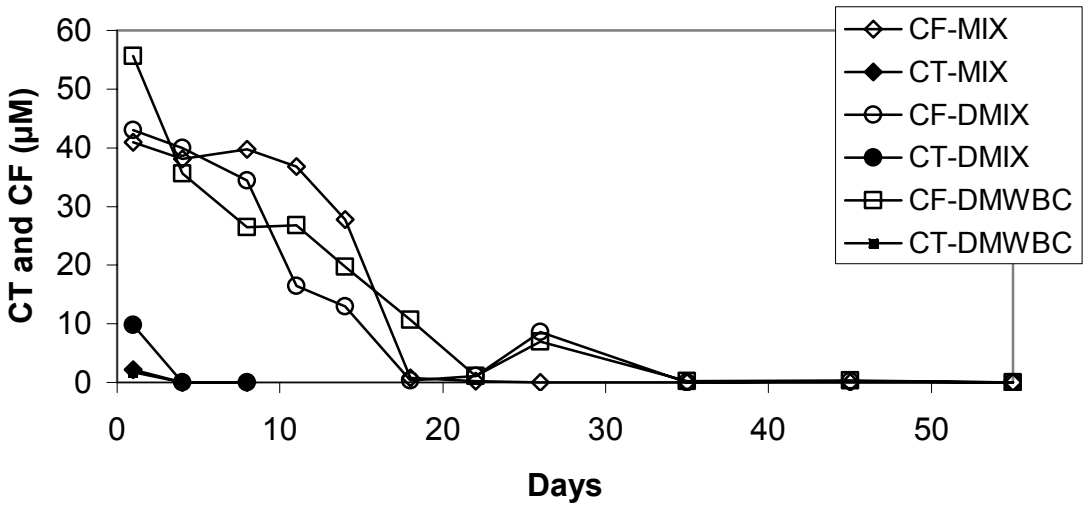


Figure 6.7. Aqueous CT and CF (µM) in live microcosms. Data points represent averaged concentrations in duplicate bottles.

CF was degraded by day 18 in the MIX and DMIX microcosms (Figure 6.7), and it is tempting to speculate that the removal of CF in these microcosms stimulated TeCA biodegradation after day 21. CF was also largely depleted in the DMWBC microcosms by day 8 and 22 and no substantial increase in the rate of TeCA removal was observed after day 22 in these microcosms. Therefore, the removal of CF and enhanced TeCA removal may or may not have been linked.

The sterile controls also showed some removal of CF (data not shown). However, as CF was degraded in the live microcosms, DCM was produced, indicating that at least some of the CF removal was due to reductive dehalogenation (Figure 6.8). Significant amounts of DCM also accumulated in the sterile controls. Based on these data alone, it cannot be distinguished whether CF transformation in the live microcosms was due to abiotic processes or whether biological transformation of CF in the "sterile controls" was not sufficiently inhibited by the addition of formaldehyde. However, as discussed below, data obtained in a subsequent

experiment support the latter explanation. In any case, there were clear differences in the fate of DCM in the various live microcosms.

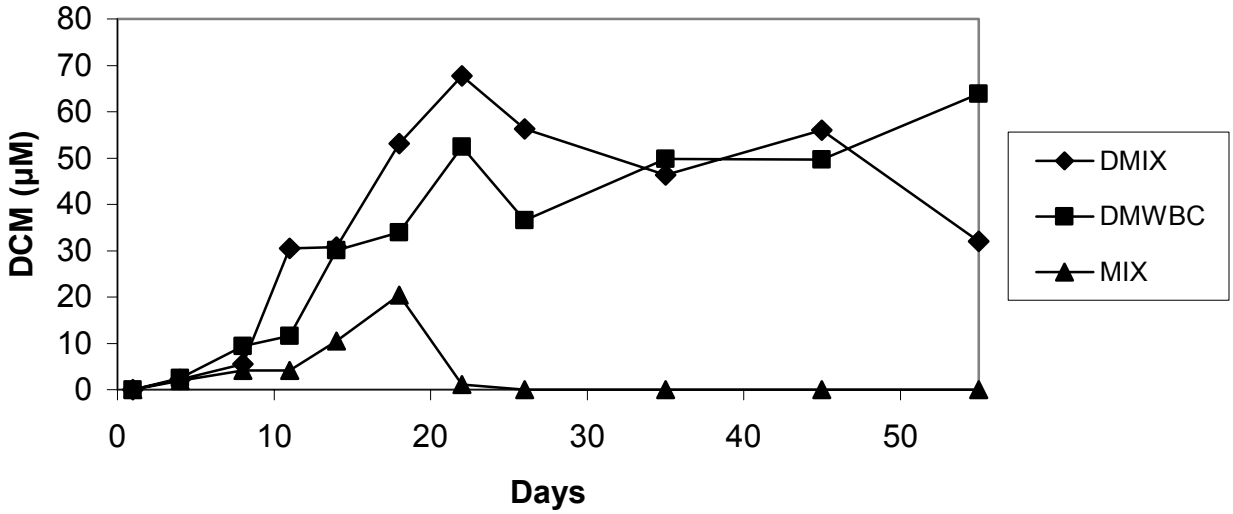


Figure 6.8. Aqueous DCM (µM) in the live microcosms. Data points represent averaged concentrations in duplicate bottles.

In the DMIX and DMWBC treatments, DCM accumulated to relatively high concentrations, but was degraded by day 22 in the MIX microcosms. Further, although DCM was degraded in the MIX microcosms, CM did not accumulate. One possible explanation for this observation is that in the MIX bottles, DCM was not utilized as a terminal electron acceptor in a reductive process in the MIX microcosms, and instead, was used as a source of carbon and energy in an anaerobic process previously observed in an acetogen (Mägli et al., 1995). It is tempting to speculate that utilization of DCM as an electron donor might have been favored in the MIX microcosms because of the limited availability of endogenous electron donors in these microcosms. A small volume of ethanol was added to the MIX microcosms along with the CVOC mixture. However, the amount of ethanol added was too low to provide enough donor for the complete degradation of all the parent CVOCs present. PCE and TCE were also added the microcosms. The initial

aqueous concentrations of PCE and TCE were supposed to be 3 mg/L (18 μM (PCE) and 23 μM (TCE)). However, when the initial concentration of PCE was measured on day 1, it ranged from 0.57 μM (.09 mg/L) to 8 μM (1.33 mg/L). Because there concentrations were so much lower than the desired concentration, no definite conclusion can be made about the degradation of PCE. The initial aqueous TCE concentrations were also lower than intended. However, they were higher and more consistent than the PCE concentration. TCE concentrations remained nearly constant in the sterile controls (Figure 6.9). Therefore, the removal of TCE was primarily biotic. TCE was degraded the fastest in the MIX microcosms and the TCE concentration was below the detection limit by day 18 (Figure 6.10). The temporary increase in TCE concentrations observed in the MIX bottles between days 8 and 11 may have been due to variability in the initial TCE concentration intervals. It is also possible that abiotic transformation of TeCA to TCE via dehydrohalogenation contributed to the observed increase in TCE concentration.

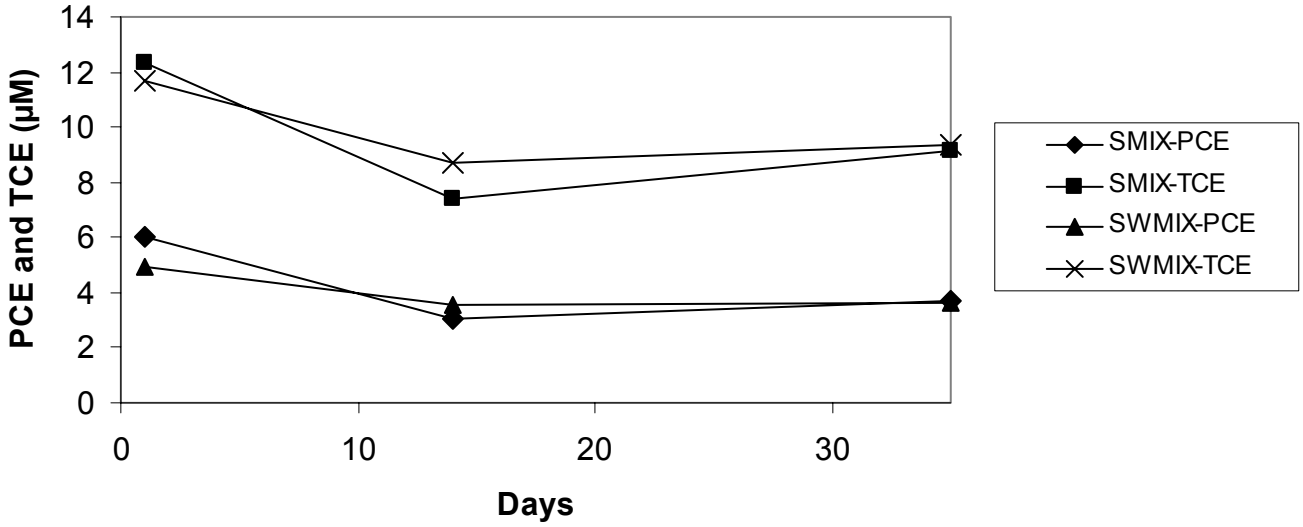


Figure 6.9. Aqueous PCE and TCE (µM) in the sterile controls. Data points represent averaged concentrations in duplicate bottles.

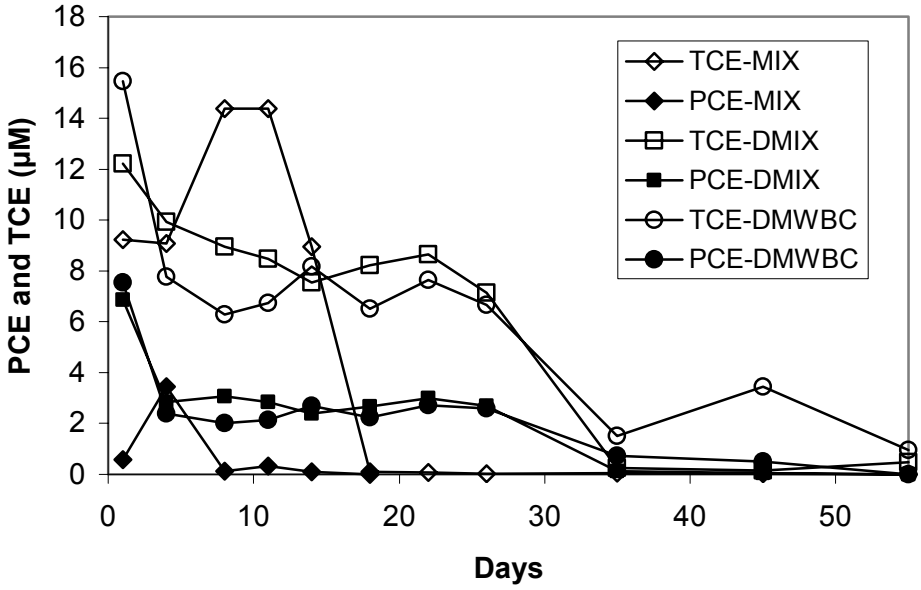


Figure 6.10. Aqueous PCE and TCE (µM) in the live microcosms. Data points represent averaged concentrations in duplicate bottles.

After day 10, DCE began accumulating in all of the live microcosms (Figure 6.11). Presumably, the DCE was primarily the result of TCE hydrogenolysis, although dichloroelimination of TeCA also leads to the formation of DCE (Figure 2.2). DCE persisted for a relatively long period in the MIX treatment microcosms. However, once DCE removal began in these microcosms it was transformed more rapidly than in the other treatments.

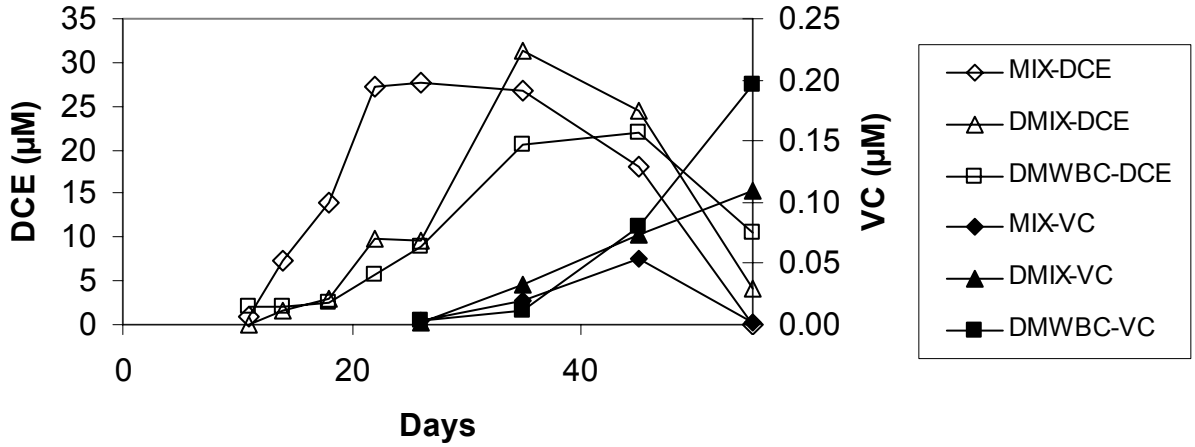


Figure 6.11. Aqueous DCE and VC (µM) in the live microcosms. Data points represent averaged concentrations in duplicate bottles.

Even though the bioaugmentation culture was enriched and able to degrade DCE quickly, DCE removal in the DMWBC microcosms was relatively slow. Only very small amounts of VC were detected in the live microcosms. However, consistent with the patterns observed for the other CVOCs, VC was completely removed in the MIX microcosms, but not in the DMIX and DMWBC microcosms.

The removal of the parent and daughter compounds in all three sets of microcosms amended with the CVOC mix and the TeCA-only microcosms is summarized in Table 6.2. Table 6.2 reports the last day that a given compound was detected in a set of microcosms. If the compound

was still detected on day 45 of the experiment, then the concentration on day 45 was given in parentheses (in μM). The microcosms in which each parent or daughter compound was removed most rapidly or reached the lowest concentration is highlighted in Table 6.2. As previously noted, initial PCE and CT concentrations were significantly lower than intended, so it is difficult to draw any conclusions in terms of PCE and CT. However, it is clear that overall, the most rapid and extensive biodegradation occurred in the MIX treatment microcosms. The rapid degradation of the VOCs in the MIX treatment could be due to the addition of VOCs via ethanol. 5 μLs of an ethanol stock solution were added to all microcosms. Initially, it was assumed that the volume of ethanol added was not sufficient to promote the complete degradation of the CVOCs; however, 5 μLs of 100% ethanol is equivalent to a concentration of 1190 μM, which is 2x the required concentration, as previously stated in Chapter 5. Therefore, this high concentration of ethanol most likely could have promoted the rapid degradation of CVOCs that was observed through the 55-day experiment. This possibility suggests that these CVOCs should be added in pure form to controls in order to gain an accurate conclusion in regards to the success of bioaugmentation and/or biostimulation.

There are a couple of possible explanations for the failure of the subculture to enhance biodegradation. (1) The concentration of added microorganisms was relatively small compared to the total community biomass, and, therefore, any activity of the added organisms was not noticeable. Presumably the addition of large amounts of electron donor resulted in significant biomass production before WBC-2 was added, so this is a likely possibility. (2) Key populations within the subculture were inhibited by certain CVOCs that they had not been extensively exposed to (e.g., CF). (3) Dechlorinating populations were inhibited by chitin, its by-products,

or populations that grew up as a result of the chitin. The first and second explanations seem most reasonable, but do not explain why the electron donor amended microcosms (DMIX) did not perform as well as CVOC mix-amended bottles (MIX).

There are undoubtedly other interesting trends that we have not yet identified while reviewing the data. Overall, the results may indicate that biodegradation by the native sediment microorganisms *in situ* is limited by electron donors, as the MIX treatment, which was amended with an equivalent concentration of ethanol, could promote the complete degradation of all CVOCs. In addition, the ability of bioaugmentation and/or biostimulation to enhance natural attenuation cannot be ruled out, as data show that a combination of electron donors and CVOCs could have placed these engineered methods at a disadvantage. Since methane production was significant within the methane monitoring bottles, which were amended with electron donor, it would be interesting to investigate the inhibitory effect of CVOCs. There is also the possibility that the rate at which the CVOCs are advecting through the wetland sediments is faster than the rates of CVOC biodegradation (one of the previously stated hypotheses for the formation of the seep sites). This would suggest that the use of a biomat to increase the hydraulic residence time of the groundwater in the biologically active zone might be effective at site 3-4W.

Table 6.2. Last day of detection of parent and daughter compounds in live microcosms or compound concentration in μM on day 45 (in parentheses).

	Chlorinated ethanes				Chlorinated ethenes				Chlorinated methanes			
Treatment	**TeCA**	**TCA**	**DCA**	**CA**	**PCE[a]**	**TCE**	**DCE**	**VC**	**CT[b]**	**CF**	**DCM**	**CM**
MIX	(0.19)	22	(0.45)	ND[c]	55	55	55	55	4	55	22	ND
DMIX	(3.89)	(0.14)	(0.85)	ND	55	(0.47)	(4.13)	(0.11)	4	(.02)	(31.93)	ND
DMWBC	(10.12)	(0.63)	(0.36)	ND	(0.01)	(0.96)	(10.42)	(0.20)	4	(.02)	(63.81)	ND
TDMWBC	19	11	11	14								

[a]Initial PCE concentrations were significantly lower than expected. The average concentration in the DMIX and the DMWBC bottles was around 1 mg/L, but was 0.5 mg/L or less in the MIX microcosms. This may have been due in part to sorption of PCE to the sediment, and/or errors in preparation of the stock solution and analysis.

[b]Initial CT concentrations were significantly lower than expected (~0.4 to 1.5 mg/L). Again, this may have been due to sorption or errors in stock preparation and analysis.

[c]ND=not detected at any time.

[d]CF levels were essentially 0 in the DMIX and DMWBC bottles by day 22, but rebounded slightly on day 25.

Chapter 7: H_2 Threshold Experiments: Results and Discussion

In the H_2 threshold experiments, microcosms containing sediment from site WB-35 were amended with PCE, CT, or TeCA. H_2 concentrations were monitored along with CVOC concentrations to assess whether characteristic H_2 concentrations were associated with the biodegradation of any of the parent compounds or their daughter products. Relatively constant H_2 concentrations characteristic of dehalorespiration processes could signify that biodegradation of a particular CVOC occurred via a metabolic process, whereas higher H_2 concentrations commonly found in methanogenic systems could indicate that co-metabolism was largely responsible for the removal of a given CVOC. In addition, H_2 concentrations were measured in diluted WBC-2 cultures that were amended with TeCA to gain insight into the roles of co-metabolic and metabolic processes in the biodegradation of TeCA and its daughter products in the enrichment culture. This information could aid in the design of an effective bioremediation plan because different approaches may be needed to stimulate and maintain co-metabolic and metabolic processes.

7.1. PCE

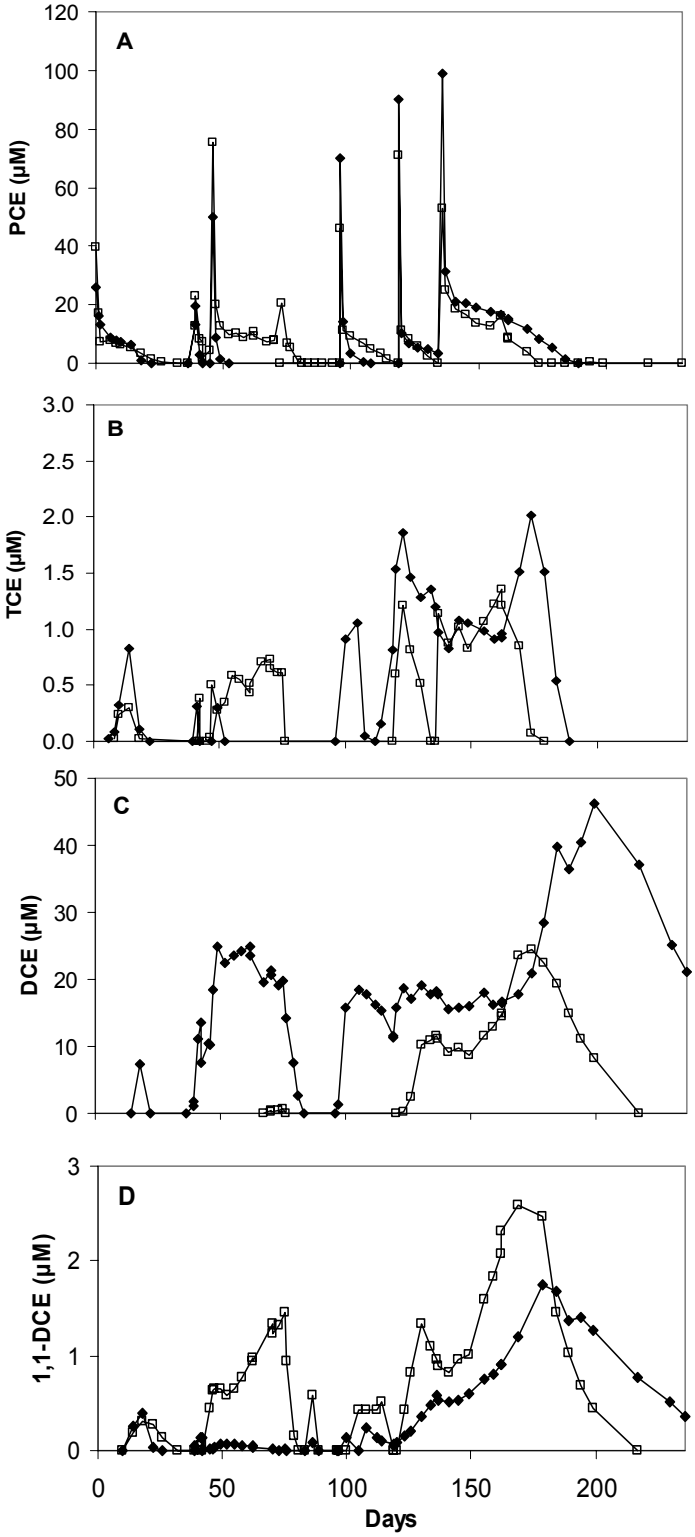
Duplicate WB-35 sediment microcosms were amended with PCE on days 0, 39, 46, 96, 119, and 136 and were monitored for 199 d. PCE concentrations after these additions ranged from 11 to 25 μM (Figure 7.1). H_2 (35 kPa) and acetate (100 μM) were added to both live microcosms on days 75 and 174, respectively. Removal of the first addition of PCE began without delay. This removal was due in part to abiotic processes because an

average loss of 13 μM PCE was also observed in the water controls over 37 d (data not shown). A similar rate of loss of 16.5 μM over 36 d was also observed in the inhibited controls (Figure 7.2).

However, the production of small amounts of TCE in both live microcosms (Figure 7.1b) and larger amounts of DCE in PCE-1 suggest that biological reductive dechlorination processes played an important role in PCE removal in the microcosms. In contrast, no reductive dechlorination of PCE to TCE or DCE was observed in the first 35 d in the inhibited controls (Figure 7.2). Minor amounts of TCE were detected after day 50. This suggests that either some biological reductive dechlorination activity recovered from the physical and chemical inhibition or some abiotic reductive dehalogenation capacity was present in the sediment.

Transformation of a second dose of PCE, which was added to both microcosms on day 36, occurred more rapidly compared with the first dose and was completed by day 42. The increasing rate of PCE removal during the first 42 days suggests that the reductive dechlorination capacity in the microcosms increased due to transformation of the first PCE dose and thus was due to a biological process. While the two microcosms performed similarly during transformation of the first two PCE doses, differences were observed in the biodegradation of the third PCE dose. PCE concentrations were undetectable in PCE-1 within six days. In contrast, the concentration of PCE in PCE-2 leveled off at approximately 7 μM for around 20 days in PCE-2. TCE also persisted at

low concentrations, and, although there was no production of DCE, 1,1-DCE accumulated during this time period in PCE-2.



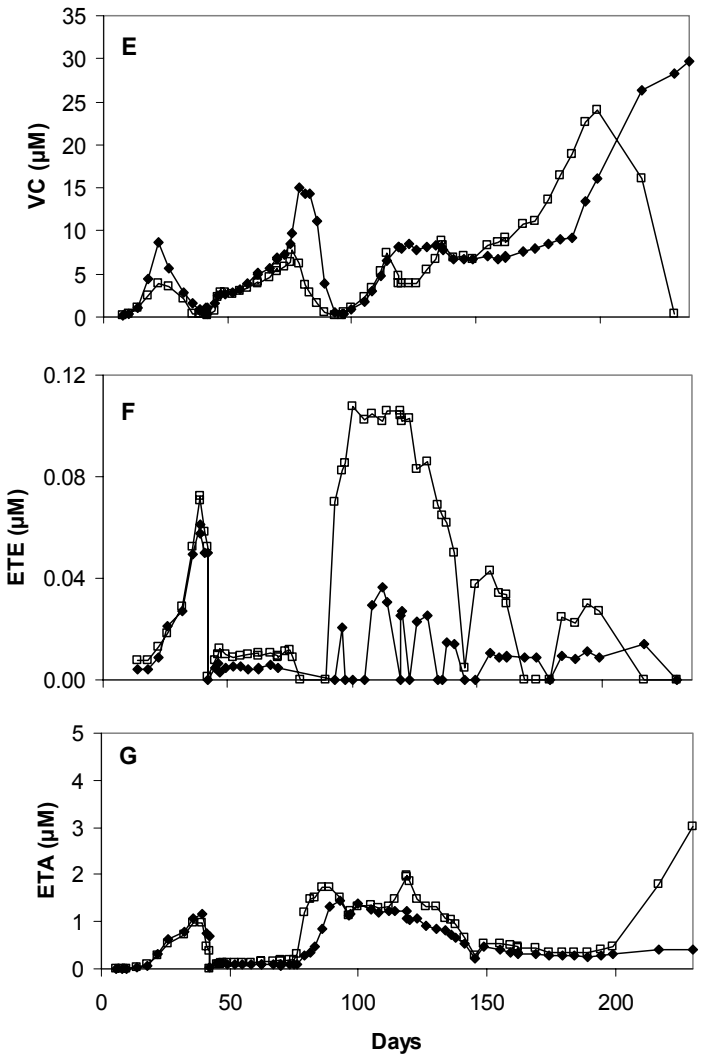


Figure 7.1. Aqueous concentrations of (A) PCE, (B) TCE, (C) DCE, (D) 1,1-DCE, (E) VC, (F) ETE, and (G) ETA in PCE-1 (diamonds) and PCE-2 (squares). Arrows indicate PCE additions on days on days 0, 39, 46, 96, 119, and 136. The septa were changed on day 42. H_2 was added on day 75. Acetate was added on day 174.

During this same time period in PCE-1, much lower levels of 1,1-DCE were measured, TCE was not detected, and high levels of DCE accumulated in PCE-1. Similar trends were observed between 150 and 170 days. During this period, higher concentrations of 1,1-DCE accumulated in PCE-2 than in PCE-1, while DCE levels were generally much greater in PCE-1 than in PCE-2.

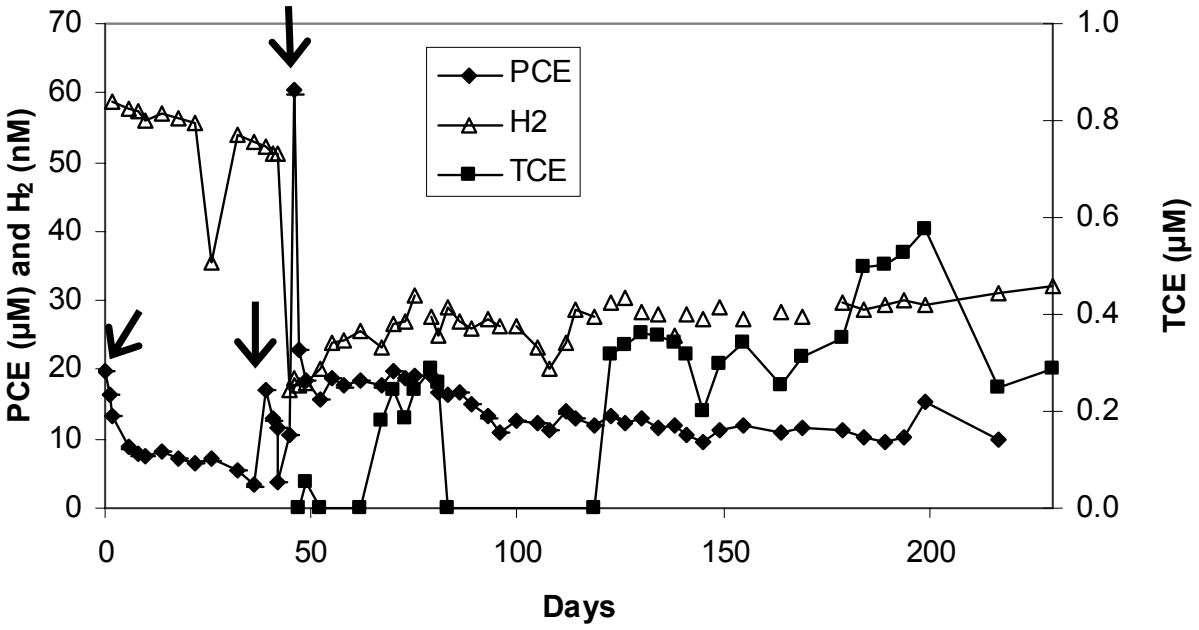


Figure 7.2. Aqueous PCE,H_2, and TCE in the inhibited controls. Each data point represents the average concentration in duplicate controls. Arrows indicate PCE additions on days 0, 39, and 46. The septa were changed on day 42.

The accumulation of DCE observed in PCE-1 is common at PCE-contaminated sites (Harkness et al. 1999) and is generally attributed to: (1) limitation by a suitable electron donor, and/or (2) inadequate capacity for reductive dehalogenation of DCE to VC and ethene, which currently appears to be limited to closely-related *Dehalococcoides* strains (Becker, 2006). Production of 1,1-DCE during the biodegradation of highly chlorinated ethenes is less common. However, 1,1-DCE was the dominant intermediate in a TCE-to-ethene dechlorinating culture that was enriched from contaminated groundwater emanating from a landfill and maintained on ampicillin (Zhang et al., 2006). Conversion of TCE to 1,1-DCE in the enrichment culture was apparently carried out by a *Dehalococcoides* strain, because ampicillin interferes with the synthesis of peptidoglycan, a key component of cell walls in many bacteria, but not in

Dehalococcoides species. Several characterized *Dehalococcoides* strains including strains 195, BAV1, VS, and GT, also utilize 1,1-DCE as a metabolic electron acceptor (Sung et al., 2006). It is possible that a *Dehalococcoides* strain was also responsible for the conversion of TCE to 1,1-DCE in the PCE-2 microcosm. The 1,1-DCE-producing strain was apparently also present in the PCE-1 microcosm, but appeared to be less active than in the PCE-2 microcosm. 1,1-DCE accumulated in the PCE-1 microcosm primarily after day 120, and the 1,1-DCE levels were generally lower in PCE-1 than in PCE-2. In addition, it seems likely that one (or more) additional *Dehalococcoides* strain(s) that did not produce significant amounts of 1,1-DCE were present and degrading 1,2-DCEs in the PCE-1 microcosm.

Electron donor availability is one factor that could have potentially affected the structures of the microbial communities or the activities of the key dehalogenating populations in the PCE-1 and PCE-2 microcosms. In particular, H_2 is an electron donor that is commonly used by several chlorinated ethene-respiring strains and several of these organisms use H_2 exclusively as an electron donor in dehalorespiration. The concentrations of H_2 measured in PCE-1 and PCE-2 on individual days were plotted (Figure 7.3a) to evaluate whether H_2 concentrations were related to the chlorinated ethene biodegradation patterns shown in Figure 7.1. H_2 concentrations varied significantly in samples taken from a given microcosm at intervals ranging from one to three days (Figure 7.3a). This was somewhat surprising because in an earlier study, the H_2 concentrations measured in anaerobic wetland sediment microcosms during reductive dechlorination of DCE or DCA were relatively constant (Kassenga et al., 2004). On the

other hand, the turnover rate of H_2 in anaerobic sediments is very high and thus H_2 concentrations are very susceptible to perturbations in substrate availability (Löffler and Sanford, 2005). In PCE-1 and PCE-2, the availability of potential metabolic electron acceptors (PCE, TCE, DCE, 1,1-DCE, and VC) varied from day to day and probably contributed to the dynamic nature of the H_2 measurements. Nevertheless, Figure 7.3a suggests that the highest H_2 concentrations occurred more frequently in PCE-2 than in PCE-1. The trends in the H_2 data are more apparent in Figure 7.3b, in which average H_2 concentrations in the most recent 10 samples is plotted for PCE-1 and PCE-2. Based on Figure 7.3b, it appears that the H_2 threshold in PCE-2 was 5-6 nM from day 36 through 67. The H_2 threshold was lower (2 to 4 nM) during the same time period in PCE-1. Similarly, after the large amount of added H_2 added on day 75 was consumed, the H_2 threshold in PCE-2 leveled off at 4—6 nM until day 162, and then decreased slightly to 2—3 nM for the remainder of the experiment. The H_2 threshold was more variable in PCE-1 following the addition and consumption of H_2. It typically ranged from 2 to 5 nM but reached nearly 7 nM on day 154.

When *Dehalococcoides ethenogenes* is present along with a second dehalorespiring population, higher electron donor concentrations theoretically favor *D. ethenogenes*, and, in some cases, allow it to out compete the other dehalorespiring population for all of its metabolic electron acceptors (PCE, TCE, and the DCE isomers) (Becker, 2006). When electron donors such as H_2 are limiting, PCE-to-DCE respiring populations with faster kinetics tend to out compete *D. ethenogenes* for PCE and TCE, forcing it to specialize in dechlorination of DCEs.

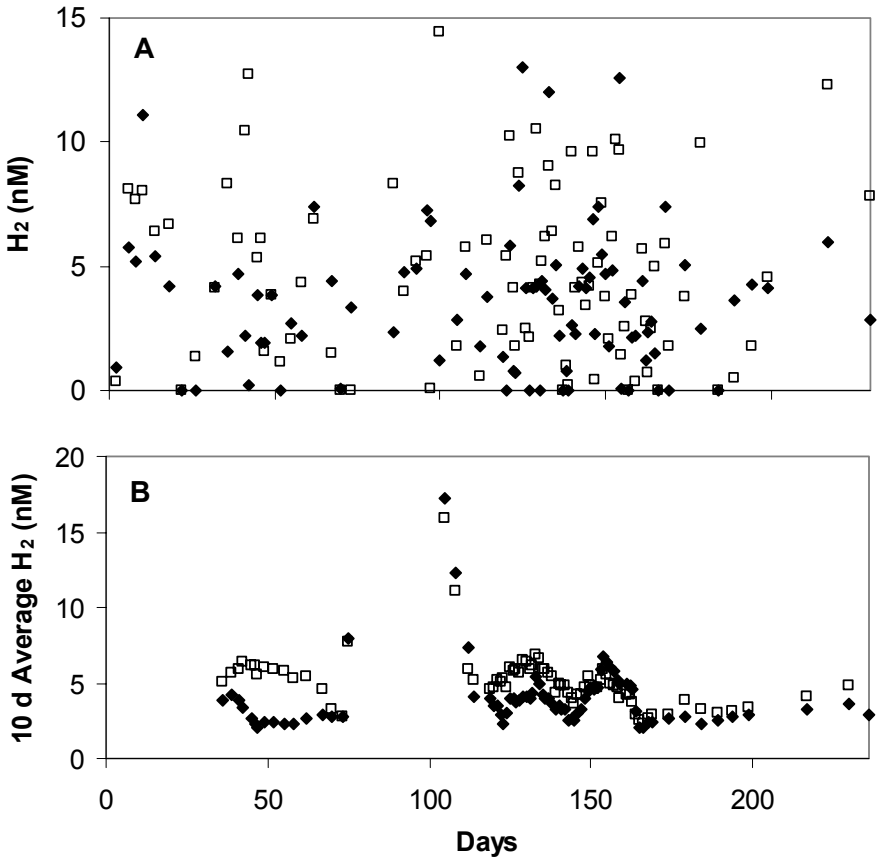


Figure 7.3. (A) H_2 concentrations in PCE-1 (diamonds) and PCE-2 (squares) individual data points and (B) moving 10 sample averages. H_2 (35 kPa) was added on day 75. High H_2 concentrations resulting from the addition of H_2 are not shown. Acetate was added on day 174.

Thus, it is interesting to speculate that the somewhat higher concentrations of H_2 observed in PCE-2, especially early in the experiment, enabled a *Dehalococcoides* species to grow on the highly chlorinated ethenes and produce 1,1-DCE, which was subsequently converted to VC and ethene. In contrast, because of the lower H_2 concentrations in PCE-1, a heterotrophic dehalorespiring population may have been able to out compete any *Dehalococcoides* for the PCE and TCE. Because *Dehalococcoides* strains apparently have slower kinetics compared with PCE-to-DCE dehalogenators,

DCE periodically accumulated in this culture before being dehalogenated by a *Dehalococcoides* population.

The H_2 thresholds associated with various TEAPs are not fixed and probably vary from site to site depending on environmental factors and microbial kinetic characteristics. However, H_2 concentrations in systems undergoing dehalorespiration often range from 0.04 to 0.3 nM (as summarized by Löffler and Sanford, 2005). H_2 thresholds in methanogenic systems can range from 5 to 95 nM. Most of the H_2 concentrations measured in this study were intermediate between these two ranges or at the low end of the methanogenic range.

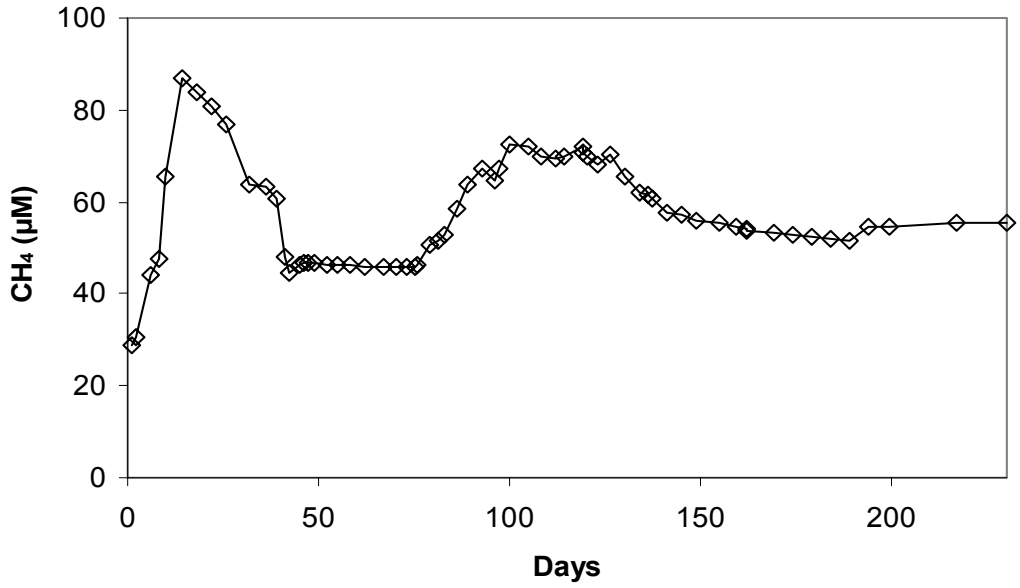


Figure 7.4. Aqueous CH_4 (µM) in the microcosms in the viable microcosms constructed with sediment collected from site WB-35. Each data point represents the average concentration in duplicate microcosms. The septa were changed on day 42.

Methanogenesis was clearly an important redox process in the sediment microcosms between days 0 and 14, and following the addition of H_2 (days 79 to 105) (Figure 7.4).

The production of CH_4 during these time periods is consistent with the measurement of H_2 concentrations that are at the lower end of the range characteristic of methanogens. However, it is unlikely that methanogens in PCE-1 or PCE-2 contributed significantly to the removal of the chlorinated ethenes through co-metabolic processes. Several observations support this idea. First, co-metabolic processes do not increase in rate (Alexander, 1999), as was observed for the removal of PCE in the microcosms. Second, involvement of known dehalorespiring organisms, specifically *Dehalococcoides* sp., in CVOC removal in the microcosms was suggested due to the production of 1,1-DCE and the biodegradation of DCEs. Finally, H_2 concentrations between days 14 and 26 decreased to levels that ranged from undetectable to 0.68 nM, which are more characteristic of dehalorespiration. Kassenga et. al. (2004) observed simultaneous dechlorination of DCA and methanogenesis and concluded that DCA was most likely transformed co-metabolically by methanogens. However, the H_2 threshold measured during DCA transformation (~38 nM) was significantly higher than in this study, and repeated additions of DCA were apparently not made to assess whether the rate of removal increased or decreased over time. Presumably in the current study, the chlorinated ethene concentrations were too low relative to the concentrations of methanogenic substrates to drive H_2 thresholds to the low levels typically associated with dehalorespiration. Thus, the results of this experiment suggest that H_2 threshold concentrations do not assist in determining the importance of co-metabolic and dehalorespiration processes in chlorinated ethene removal.

Although the primary goal of this experiment was to evaluate whether H_2 thresholds could provide insight into the importance of metabolic and co-metabolic processes in the removal of CVOCs, it is interesting to note that the transformation of DCE and VC was faster when H_2 was added to the microcosms compared with biodegradation of these compounds following the addition of acetate. DCEs and VC concentrations in both microcosms decreased rapidly after the addition of H_2 on day 75; however, the addition of acetate on day 174 only appeared to have an effect on PCE-2, not PCE-1, as DCE and VC were completely removed in PCE-2 by day 236 (Figure 7.1). In a previous study conducted by He et. al. (2002), acetate and H_2 were examined for their ability to sustain complete reductive dechlorination of PCE in aquifer material derived from two different sites. Although both electron donors were able to completely reduce PCE, the addition of H_2 resulted in higher rates of DCE and VC removal, as observed in our experiment. Ultimately, it was found that acetate could promote reductive dechlorination through the action of homoacetogens, which oxidize acetate to H_2. In the study by He et. al. (2002), 5 mM acetate was added to the sediment microcosms. In the current study, 100 μM was added to the microcosms, which should have provided ten times the electron equivalents needed to completely dechlorinate all of the DCE and 1,1-DCE to non-toxic daughter products.

7.2. CT

CT was added to duplicate viable microcosms on days 0, 32, 42, 55, 110, 113, 144, and 150 (Figure 7.5). In each case, CT was degraded rapidly and without delay. CT was also added to a pair of inhibited controls on days 0, 32, and 42 (Figure 7.6). Removal of CT

in the controls was initially as fast as in the viable microcosms. Removal of the second two CT additions was slower than in the viable microcosms. Thus, it seems likely that CT transformation in the WB-35 sediment was due at least in part to abiotic processes, although biological processes may have enhanced its removal in the viable microcosms.

As previously discussed, two major degradation pathways are known for CT. For example, *Acetobacterium woodii*, an acetogen, can carry out net hydrolysis of CT to CO_2 and reductive dechlorination of CT, which leads to the production of CF, DCM, and CM via corrinoid co-factors. Hydrolysis to CO_2 was the dominant CT transformation pathway in *Acetobacterium woodii*, as 67% of ^{14}CT was converted by this organism to CO_2, acetate, and other end products. It was suggested that the CO_2 produced from the hydrolysis of CT was cycled through the acetyl-CoA cycle and converted to acetate (Egli et. al., 1988, 1990).

Both the CT hydrolysis and reductive dechlorination pathways were apparently active in the viable microcosms and inhibited controls because, initially, the percentage of transformed CT recovered as CF and DCM during the first 60 d ranged from 10% to 35% in the viable microcosms and from 6% to 57% in the inhibited controls. Most of the CT that was not accounted for in CF and DCM was presumably converted to CO_2 because CT losses in the water controls were minor (data not shown).

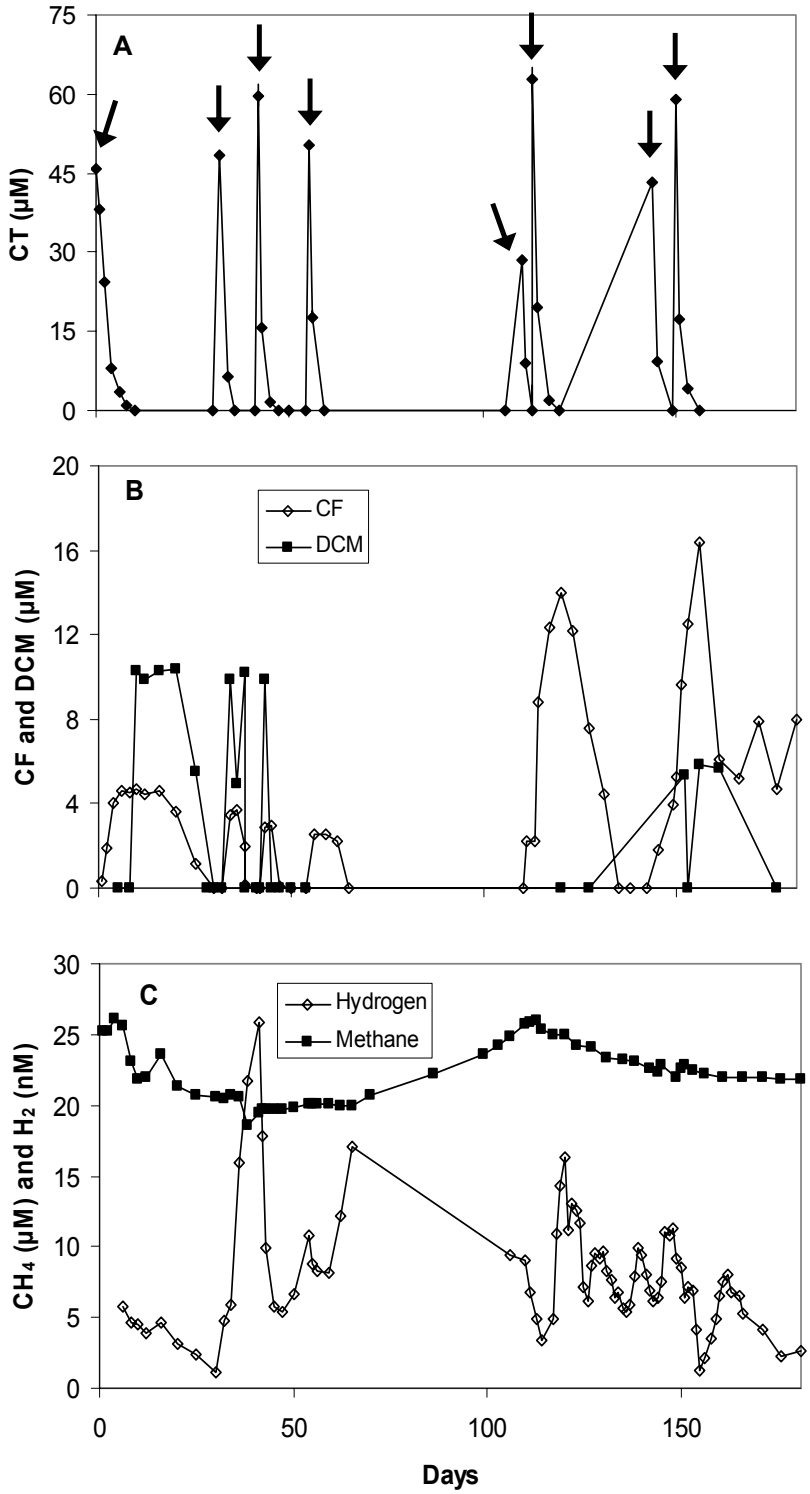


Figure 7.5. Average aqueous (A) CT, (B) CF and DCM, and (C) H_2 and CH_4 in viable microcosms amended with CT on days 0, 32, 42, 55, 110, 113, 144, and 150. H_2 is graphed using a moving 3 sample average. Septa were changed on day 38.

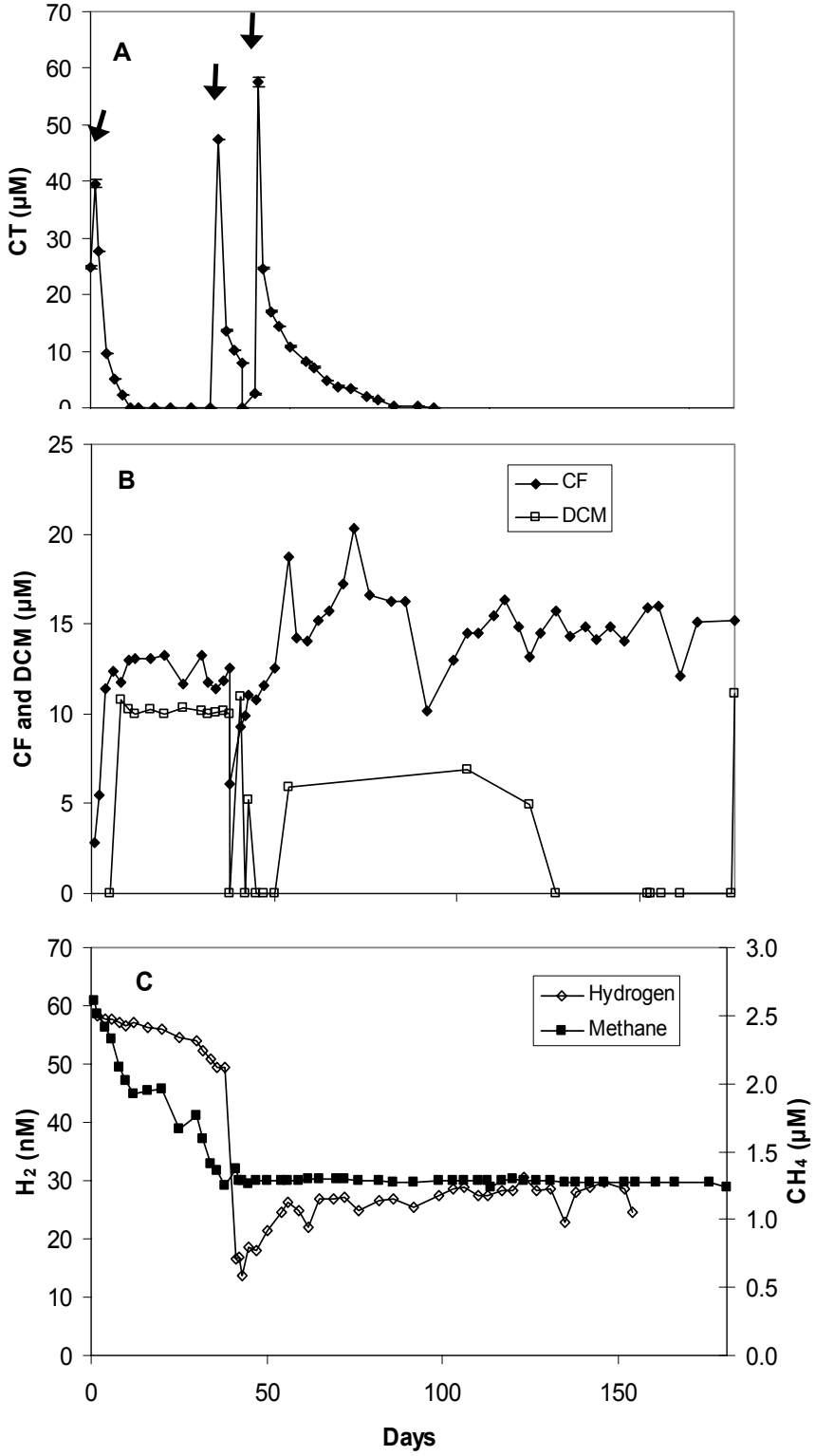


Figure 7.6. Average aqueous (A) CT, (B) CF and DCM, and (C) H_2 and CH_4 in inhibited microcosms amended with CT on days 0, 32, and 42. H_2 is graphed using a moving 3 sample average. Septa were changed on day 38.

It is interesting that, initially, reductive dechlorination of CT occurred to a greater extent in the heat-treated controls compared with the viable microcosms because it has been demonstrated that autoclaving cells and cell extracts from *A. woodii* almost completely inhibited reductive dechlorination reactions (Egli et. al., 1988, 1990). Genetic analyses conducted on the WBC-2, which was derived from sites WB-23 and WB-30, have demonstrated the presence of *Acetobacterium* spp. (Jones et. al., 2006). In addition, although acetate levels were not monitored in the CT-amended site WB-35 microcosms, analysis of the site WB-35 microcosms amended with TeCA showed acetate levels as high as 500 μM, which suggests acetogens were present at site WB-35. Thus, while the autoclaving regimen used in this study was specifically designed to inactivate spore formers like *A. woodii*, it is possible that some of them survived and were able to carry out CT transformation in the inhibited controls.

Although CT followed similar patterns in the viable microcosms and inhibited controls, different trends in CF and DCM levels were observed in the two sets of bottles. In the viable microcosms, the concentration and/or persistence of CF and DCM decreased during removal of the first three CT amendments. Presumably this indicates that the organisms or biological molecules (e.g. extracellular cofactors) that transformed CF and DCM were being enriched over time. CF concentrations were about three times higher and accumulated in the inhibited controls compared to the live microcosms, in which degradation was observed. However, DCM apparently was transformed to a certain extent in the inhibited controls. Thus, it seems likely that although specific organisms or

cofactors were able to degrade DCM, it is obvious that certain biological factors are involved in CF removal.

After a 47-day period during which no CT was added, the percentage of CF produced from a new spike of CT increased approximately 4-fold compared with CF production during the first 38 d. Presumably, the amount of CT converted to CO_2 decreased correspondingly. These results suggest that during the period of no CT feeding, the agents responsible for net hydrolysis were diminished and/or the bacteria or other cofactors carrying out the CT reductive dechlorination reactions recovered from inhibition due to CT. Methane production also recovered when CT was withheld. This provides additional evidence that the microbial communities at site WB-35 were able to recover after being exposed to high concentrations of chlorinated methanes. This is significant because chlorinated methanes, especially CF, are well-known methanogenic inhibitors (Bagley and Gossett, 1990). Interestingly, the increased CF production after day 127 did not lead to increased DCM production. In fact, higher concentrations of DCM were exhibited between days 0 and 50 compared to days 127 and 161. The rate of CF removal remained high after 127 days. Thus, the decrease in DCM production could mean that only some of the CF was transformed through reductive dechlorination. In fact, pathways involving the transformation of CF to CO_2 have been proposed based on the pathway used to transform MeOH to CO_2 in *A. woodii* and other bacteria.

H_2 concentrations were measured in the viable microcosms and inhibited controls to gain insight into the mechanisms involved in the transformation of CT and its daughter

products. H_2 concentrations were quite stable in the inhibited controls and ranged from 50 to 60 nM before the septa were changed. After the septa were changed, H_2 concentrations hovered around 30 nM. The relatively constant H_2 concentration observed in the inhibited controls is significant because it shows that the operation of the reduction gas analyzer was stable for several months. In contrast, H_2 concentrations in the viable microcosms were extremely variable, especially after day 127 (Figure 7.5). Because of this variability, H_2 concentrations did not prove useful in determining the importance of metabolic and co-metabolic processes in the transformation of CT.

7.2.1. CT T-RFLPs

Differences in the chromatogram scales makes direct comparison of the T-RFLP fingerprints for the two CT-amended site WB-35 microcosms on days 62 and 161 challenging, but overall they appear to be quite similar (Figure 7.7). This makes sense because CT, CF, and DCM also followed similar dechlorination patterns in the two viable microcosms. Comparison of T-RFLP fingerprints of the CT-amended microcosms on day 161 with day 178 fingerprints of the microcosms that were not amended with any CVOCs reveals that CT and its daughter products influenced the WB-35 sediment community. For example, a significant 100 bp peak is apparent in the CT-amended microcosms, but does not appear to be important in the no-substrate microcosms. Similarly, the 168 bp peak in the CT-amended microcosms appears to be enriched compared to the no substrate bottles.

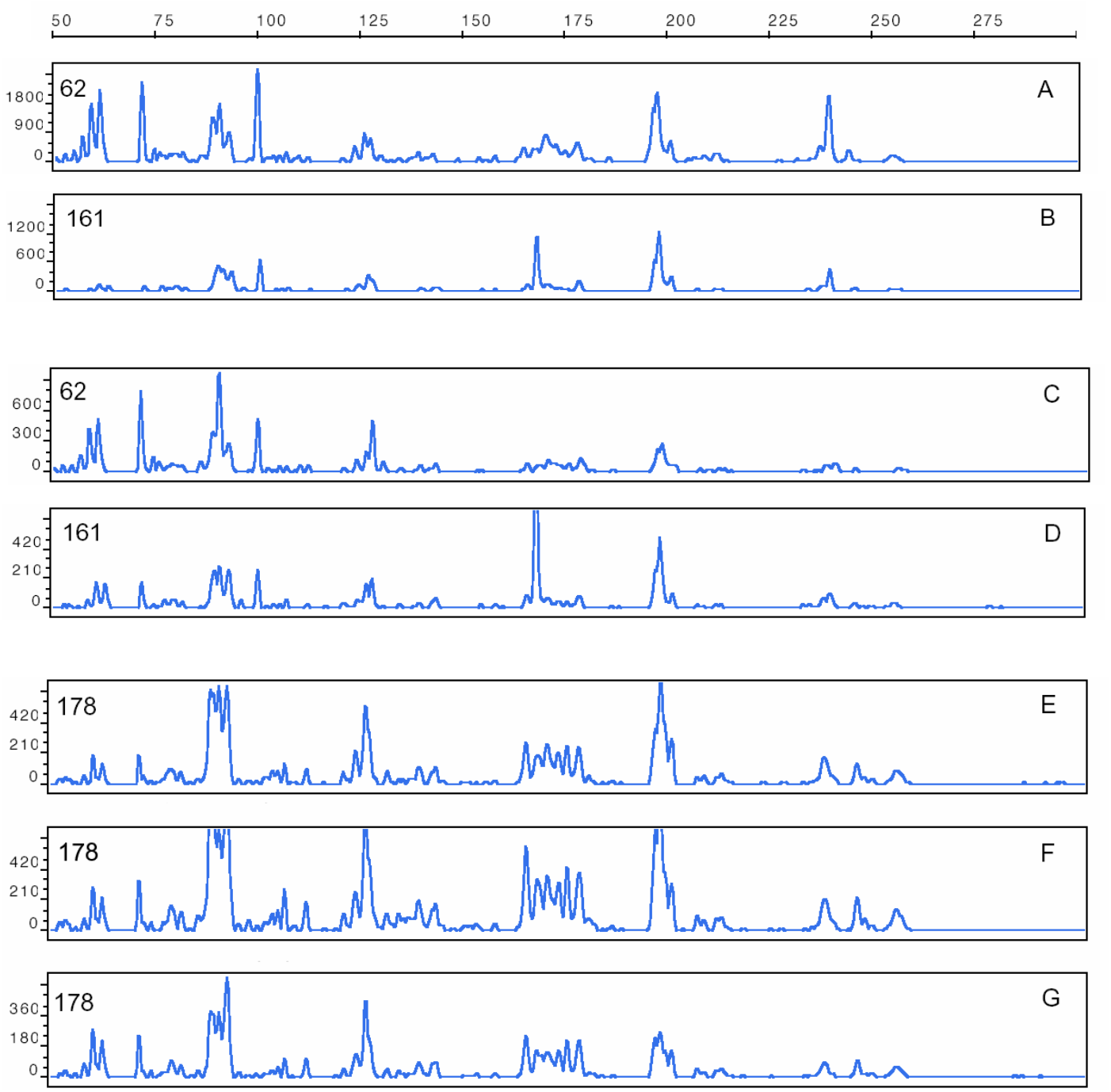


Figure 7.7. Terminal restriction fragment length polymorphism profiles of microcosms CT-1 on A) day 62, and B) day 161; CT-2 on C) day 62 and D) 161; and triplicate microcosms that were not amended with any substrates and were sampled on E-G) day 178. The fragment sizes in bp are given by the scale above in panel A.

7.3.1 TeCA

Replicate microcosms (TeCA-1 and TeCA-2) were constructed with site WB-35 sediment and amended with TeCA. The data from the two microcosms are graphed and discussed separately because they behaved differently and, consequently, were not amended with TeCA at the same times. The first dose of TeCA rapidly degraded in TeCA-1, in the absence of methanogenesis (Figure 7.8). Initially, TeCA was transformed predominantly via hydrogenolysis to TCA. Although DCA has been observed at the transects at West Branch Canal Creek (Lorah and Voytek, 2004), none was observed in the TeCA-amended microcosms constructed from sediment at site WB-35. When TCA became depleted, production of DCE began. Thus, TeCA removal followed patterns similar to those observed in the site 3-1E sediments as discussed below. Another phenomenon common to the TeCA-amended microcosms constructed with sediment from site 3-1E or WB-35 is that the DCE appeared to be produced at least in part, from sorbed TeCA.

This differs from the results of previous studies with microcosms constructed with sediment collected from transect locations in the West Branch Canal Creek Study Site, which generally showed that DCE was produced concomitantly with TCA (Lorah and Olsen, 1999). Significant methane production began after day 24, just as aqueous TCA and TeCA were depleted. It is possible that TeCA and/or TCA inhibited methanogens. However, an alternative explanation is that the electron donor(s) used in reductive dechlorination of the chlorinated ethanes was limiting and methanogens were out competed for electron donor(s) by reductive dechlorination. H_2 concentrations were

relatively high (~35 nM) during the period of TeCA and TCA dechlorination and decreased to around 10 nM when these substrates were depleted. Similarly, H_2 concentrations peaked during the initial period of TCA production in TeCA-2 and decreased while VC and DCE were produced. This suggest that an organic electron donor, rather than H_2, was used in reductive dechlorination of TeCA and TCA. Acetate concentrations in site WB-35 microcosms were quite low (29 μM to 49 μM) on day 1 (data not shown). These concentrations would limit reductive dechlorination of ~96 μM of TeCA. However, it was observed that H_2 concentrations decreased as DCE increased. This could mean that H_2 served as the electron donor for dichloroelimination of TeCA to DCE. DCE and VC concentrations decreased during the period of active methanogenesis. Several potential explanations for the apparent association between methane production and transformation of VC and DCE are discussed in the section on CVOC degradation and H_2 concentration in the WBC-2 culture.

In TeCA-2, complete removal of the aqueous TeCA was never observed. In particular, only a very small amount of DCE was produced (Figure 7.9) relative to the DCE levels in TeCA-1. Methane levels in TeCA-2 stopped increasing within a few days of the TeCA addition. The fact that little DCE was produced in the absence of methane production also suggests that the dichloroelimination reaction and methanogenesis is in some way limited. On day 56, TeCA-1 was amended with a second dose (5 mg/L; 20 μM) of TeCA. Some of this TeCA was converted to TCA; however, aqueous TeCA concentration remained relatively high suggesting that TeCA degradation slowed down. In fact, some desorption of TeCA from the sediment apparently occurred because

aqueous TeCA concentrations periodically increased. This pattern was also observed throughout the duration of the experiment in the TeCA-2 microcosm. The drop in the rate of TeCA removal and the lack of methane production in both microcosms could have been signs of electron donor limitation. Therefore, 17.5 kPa of H_2 was added to both microcosms on day 114 (Figures 7.8 and 7.9). Interestingly, the addition of H_2 stimulated production of TCA, DCE, and VC in both microcosms. However, aqueous TeCA was not depleted, perhaps due to continued desorption from the soil. Further, the addition of H_2 did not stimulate methanogenesis in either bottle. It is possible that methanogens were inhibited by TeCA and/or TCA, which were present during times when methane production was not occurring in TeCA-1 and TeCA-2.

7.3.1. TeCA T-RFLPs

The TeCA-1 and TeCA-2 microcosms performed quite different with respect to TeCA biodegradation, their T-RFLP fingerprints were fairly similar on day 151 (Figure 7.10). However, 136 bp and 200 bp peaks were significantly larger in TeCA-2 compared to TeCA-1 on day 151. Comparison of the T-RFLP fingerprints from TeCA-1 and TeCA-2 to the chromatograms of microcosms that received no substrates suggests that the 136 bp peak may play an important role in TeCA biodegradation in TeCA-2.

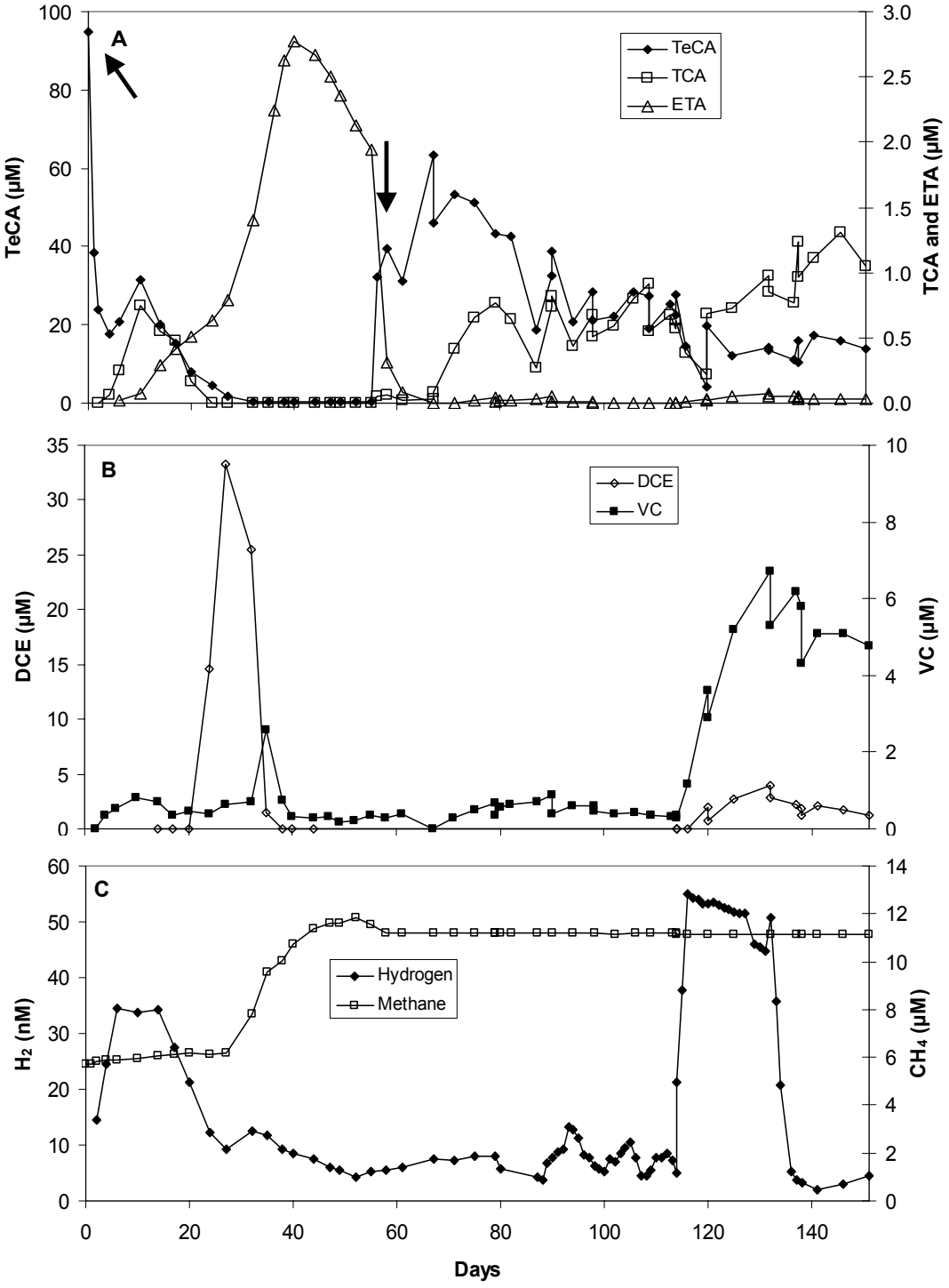


Figure 7.8. Aqueous concentrations of a) TeCA, TCA and Ethane (ETA); b) DCE and VC, and c) H_2 and CH_4 in TeCA-1. Septa were changed on days 98, 109, 114. TeCA was added on days 0 and 56 (indicated by arrows). H_2 was added on day 114 and graphed using a moving 3 sample average.

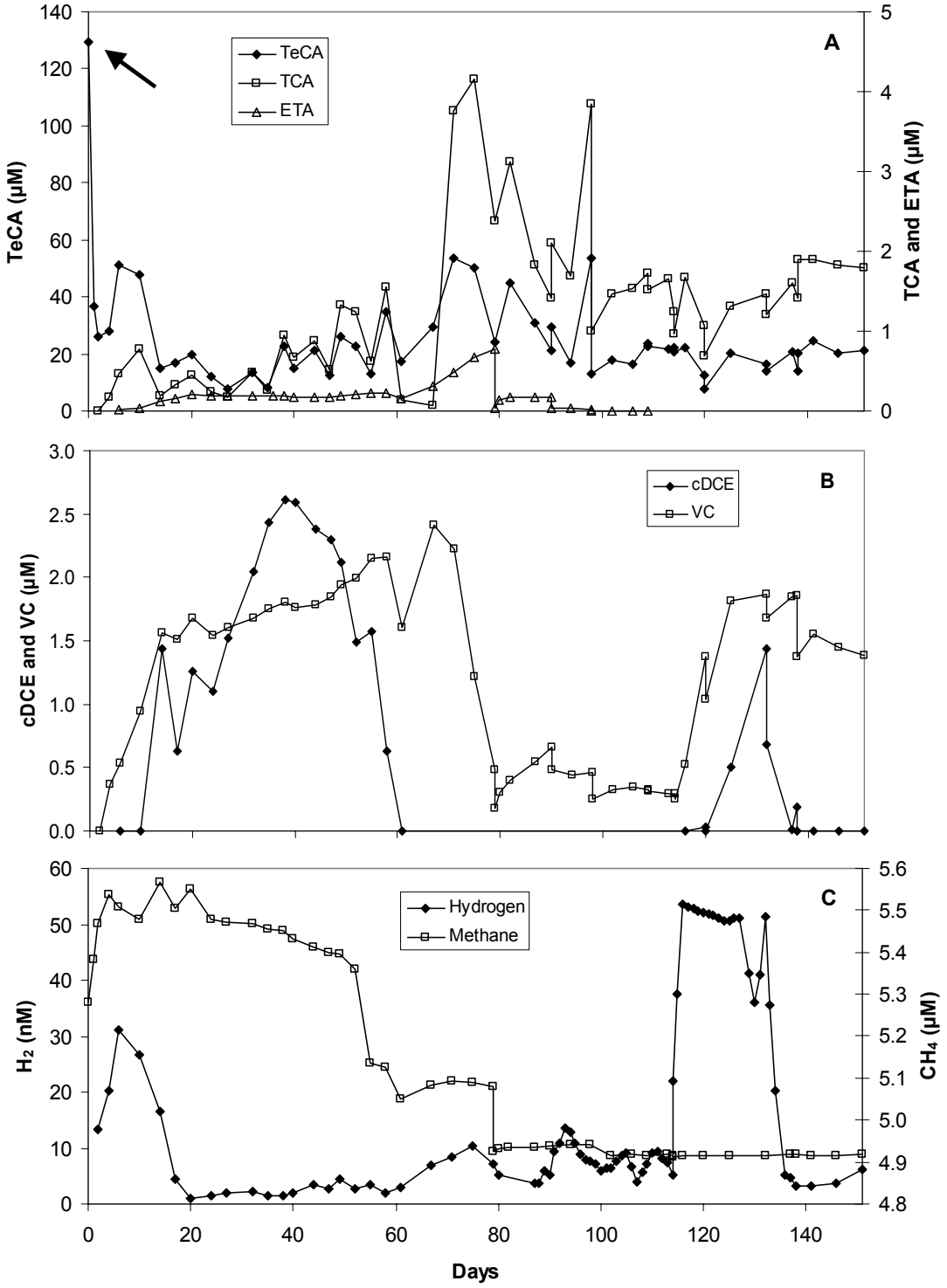


Figure 7.9. Aqueous concentrations of (A) TeCA, TCA and Ethane (ETA); (B) DCE and VC, and (C) H_2 and CH_4 in TeCA-2. Septa were changed on days 98, 109, 114. TeCA was added on day 0 (indicated by arrow). H_2 was added on day 114 and graphed using a moving 3 sample average.

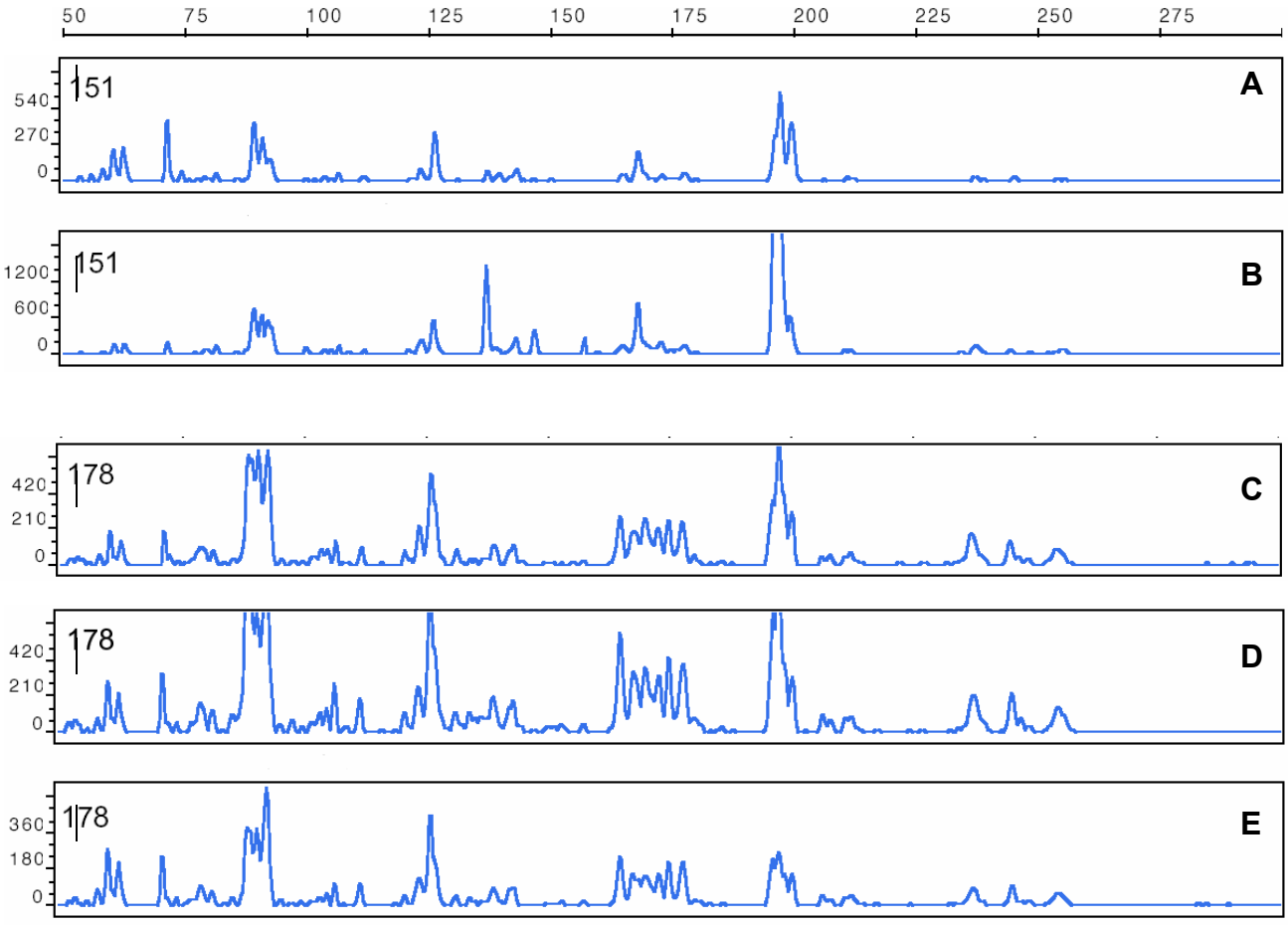


Figure 7.10. Terminal restriction fragment length polymorphism profiles of microcosms TeCA-1 and TeCA-2 on A-B) day 151; triplicate microcosms that were not amended with any substrates and were sampled on C-E) day 178. The fragment sizes in bp are given by the scale above in panel A.

7.4. WBC-2

The ability of the WBC-2 to degrade TeCA and its daughter products has been extensively studied (Jones et. al., 2006). However, little is known about the H_2 concentrations maintained by this culture. WBC-2 (Jones et. al., 2006) was diluted ten-fold in triplicate culture tubes containing growth medium and amended with TeCA (55 to 101 μM) on day 0. Removal of TeCA began without delay in all three cultures (Figure 7.11).

Dilution of cultures slowed down the transformation of the daughter products, which do not accumulate in the undiluted culture (Jones et. al., 2006). TCA and DCE began accumulating in the diluted cultures by day 3. Therefore, hydrolysis and dichloroelimination of TeCA apparently occur concomitantly in the culture. Simultaneous conversion of TeCA to TCA and DCE has also previously been observed at transect sites WB-23 and WB-30 (Lorah and Voytek, 2004). However, at the 3-1E seep site (discussed below), TeCA transformation shifted from being dominated by hydrogenolysis to dichloroelimination. By day 25, TeCA and all of its chlorinated daughter products (TCA, DCE, and VC) were completely reduced to non-chlorinated end products in two of the cultures. However, TCA and to a greater extent, VC, persisted in a third culture (data not shown).

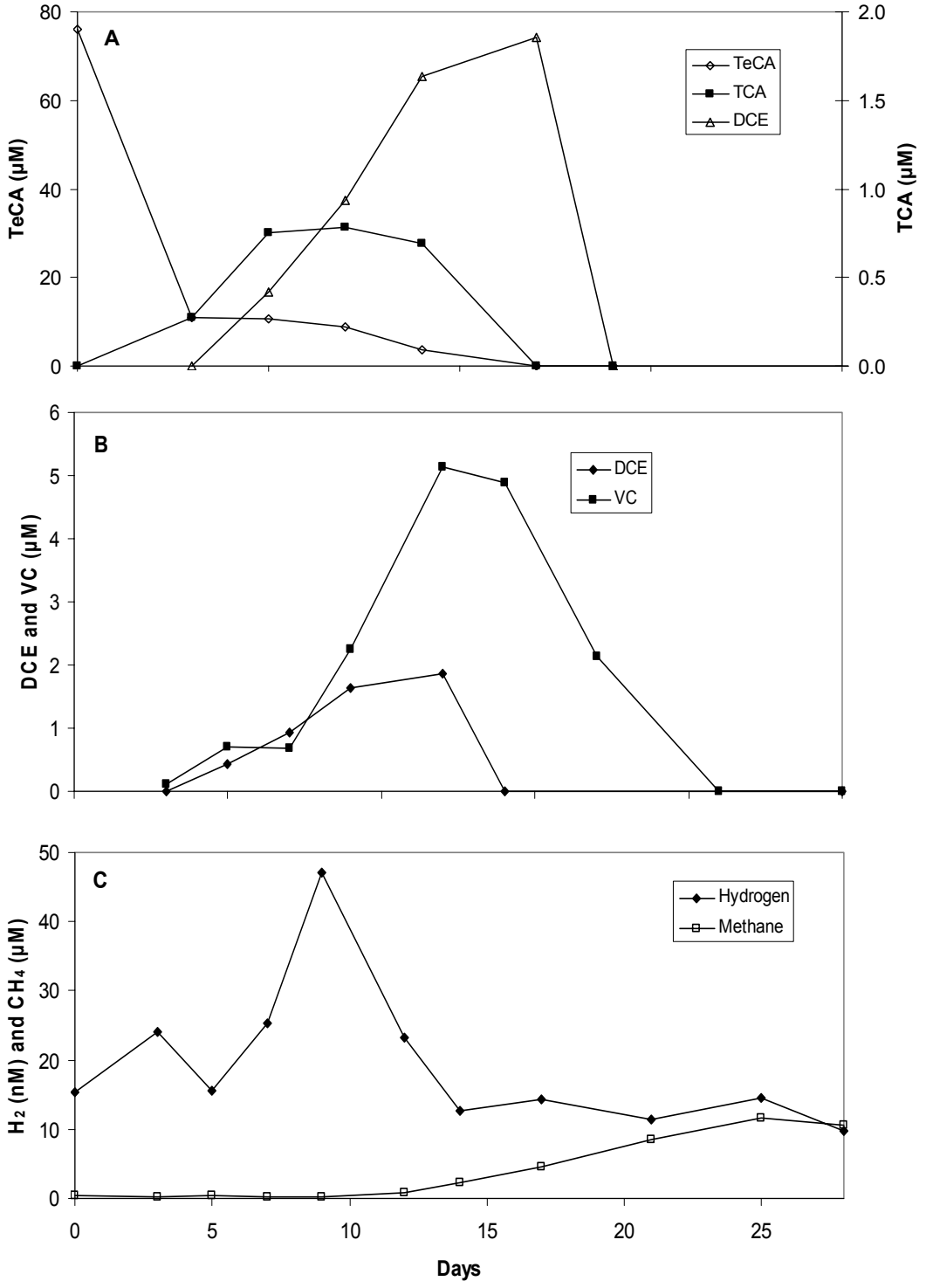


Figure 7.11. Aqueous concentrations of (A) TeCA, TCA and ETA; B) DCE and VC, and C) H_2 and CH_4.

VC removal and methane production occurred concomitantly in the two cultures that dechlorinated TeCA completely (Figure 7.11), where as methane production stopped after day 14, in the culture with persistent VC (data not shown). Thus, methanogenesis and VC transformation appear to be linked in the diluted WBC-2 cultures. Genetic analyses and prior experiments evaluating TeCA removal in this enriched subculture have also demonstrated the importance of methanogens in the overall removal of TeCA and its daughter products (Jones, E.J., personal communication; Jones et. al., 2006). In contrast, in experiments conducted with seep sediment from site 3-1E (this study) and transect sites (Lorah and Voytek, 2004), accumulation of VC appeared to coincide with CH_4 production. There are several possible explanations for the apparent association between VC removal and CH_4 production in the WBC-2 culture. First, it is possible that methanogens co-metabolized VC. H_2 concentrations were in a range characteristic of methanogens during VC degradation (and throughout the 40 day experiment), which is consistent with co-metabolic VC transformation. Second, it is possible that acetotrophic methanogens converted acetate to H_2, which was subsequently used as an electron donor in VC dechlorination. In fact, H_2 concentrations were a bit elevated around day 9, which could have been the result of acetate oxidation to H_2. This peak proceeded methane production and VC removal. A third possibility is that dechlorination of TeCA and TCA out competed methanogens and DCE and VC degraders for shared electron donors. Once TeCA and TCA were depleted, it may have been possible for methanogens and dechlorination of VC and DCE to proceed.

It is not entirely clear why methanogenesis was inhibited, as a result, culture maintenance procedures killed some or all of the methanogenic population, although anaerobic techniques were followed. Unfortunately, the H_2 concentrations measured in this study do not provide much insight into the importance of metabolic and co-metabolic processes in the dechlorination of TeCA, TCA, DCE, and VC by WBC-2. It is possible that electron donors in general and H_2 levels in particular were provided in excess and thus did not approach threshold concentrations. Another possibility is that H_2 was not the direct electron donor in the dechlorination of TeCA and some or all of its daughter products. H_2 concentrations in the diluted WBC-2 cultures were generally in the methanogenesis range. A spike in H_2 concentrations was noted around day 9. The increase in H_2 concentrations coincided with decreasing TeCA concentration. H_2 concentration decreased along with TCA concentrations. At the same time, DCE and VC increased, but the significance of these results is not clear. Future studies should evaluate H_2 and acetate concentrations in electron donor limited WBC-2 cultures amended with TeCA, TCA, DCE and VC to improve our understanding of substrate thresholds and their relationship to metabolic and co-metabolic transformations of CVOCs.

Chapter 8: Seep Experiments: Results and Discussion

8.1. Introduction

As previously stated, to evaluate the effectiveness of bioaugmentation and biostimulation, the biodegradation of a mixture of CVOCs was evaluated in three treatment sets (Table 5.1). It was also of interest to investigate the effects of chlorinated ethenes and methanes on the degradation of chlorinated ethanes, as it has been suggested that these compounds, especially CF, can inhibit their removal. The results for the site 3-1E microcosm experiment are described below.

8.2. MIX Seep Site Experiment

8.2.1. Electron Donor Availability

CVOC masses added to microcosms accounted for partitioning into the sediment. The desired initial CVOC concentrations in all microcosms were 18 μM PCE, 26 μM CT, and 30 μM TeCA; however, the measured initial concentrations were approximately 21 μM (PCE), 17 to 23 μM (CT), and 15 to 25 μM (TeCA).

As previously described, electron donors were added to the DMIX and DMWBC treatments one day before TeCA, PCE, and CT were administered to all of the microcosms on day 0. Metabolism of these electron donors apparently proceeded without delay, because by day 0, acetate concentrations in the DMIX and DMWBC microcosms exceeded 1000 μM (data not shown). In contrast, approximately 100 μM of

acetate (data not shown) was initially present in the MIX microcosms, which were not amended with electron donors.

The average methane concentrations on day 0 in the DMIX and DMWBC microcosms, 71 and 22 μM, respectively, were much higher than the initial methane levels in the MIX microcosms (<1 μM). These initial methane concentrations are consistent with the acetate data and suggest that electron donor metabolism in the DMIX and DMWBC microcosms began before the CVOCs were added. There are a number of potential explanations for the lower initial methane concentration in the DMWBC microcosms compared with the DMIX microcosms. For example, *Methanosarcina* populations comprised a large fraction of the sequences cloned in the WBC-2 (Jones et al., 2006). *Methanosarcina* populations can carry out oxidation of acetate to H_2 and CO_2 (reverse homoacetogenesis; Heimann et al., 2006). Thus, it is possible that the large numbers of *Methanosarcina* added with the WBC-2 to the DMWBC microcosms diverted reducing equivalents from methanogenesis to H_2 production. Similarly, the addition of the WBC-2 to the DMWBC microcosms presumably increased the abundance of dechlorinators relative to the DMIX microcosms. It is possible that these organisms consumed a relatively large fraction of reducing equivalents in the DMWBC microcosms, which reduced the availability of substrates for methanogenesis through day 0. Methane concentrations did not increase in any of the treatments after day 0 (data not shown), indicating that the addition of CVOCs completely inhibited methanogenesis. Numerous studies have shown that CT and CF are inhibitory to methanogenesis (Becker and Freedman, 1994; Koons et. al., 2000), and presumably CT and/or CF also caused

methane production to cease in the seep sediment microcosms. Significant methane loss in the DMIX treatments can be attributed to the changing of the septum on days 21 and 60. H_2 concentrations in the MIX treatment microcosms were quite low throughout the experiment and ranged from approximately 1-9 nM (Figure 8.1). The relatively low H_2 concentrations measured in the MIX microcosms are consistent with their low initial methane concentrations and suggest that the MIX microcosms were electron donor limited. Perhaps because methanogenesis was shut down, the availability of reducing equivalents in the form of H_2 remained high in the DMIX and DMWBC microcosms throughout the experiment. H_2 concentrations ranged from 31 to 54 nM in the DMIX microcosms and from 25 to 52 nM in the DMWBC microcosms (Figures 8.2 and 8.3).

8.2.2. Biodegradation of TeCA, PCE, and CT in the Sediment Microcosms

Significant differences were detected in the biodegradation of the three parent compounds—TeCA, PCE, and CT—in the three microcosm treatments.

8.2.2.1. TeCA

As noted above, significant reduction in the aqueous TeCA concentration was observed in the DMWBC and DMIX microcosms (Figure 6.5). However, a mass balance performed on TeCA and its potential daughter products (TCA, TCE, DCE, VC, ETA, and ETE) indicated that the accumulated daughter products accounted for only a portion of the TeCA removal. Therefore, some of the observed TeCA removal apparently was due to sorption to the sediment. As a result, subtracting the TeCA concentration measured on the final day of the experiment from the day 0 concentration

probably gives a liberal estimate of TeCA removal in the microcosms. The percent TeCA removed calculated using this approach was 14.4% for the MIX treatment, 52.9% for the DMIX treatment, and 93.3% for the DMWBC microcosms.

In the DMWBC and DMIX treatments, TCA and VC were the first daughter products detected and produced. This suggests at least some of the TeCA underwent hydrogenolysis to TCA, which subsequently underwent dichloroelimination to VC. In the MIX treatment, which was not amended with any electron donors or culture, TCA and VC production was delayed until days 21 and 42, respectively (Figure 8.1). Thus, it is possible that the addition of the culture and/or substrates enhanced hydrogenolysis of TeCA and VC production. It makes sense that addition of the WBC-2 may have stimulated TCA and VC production because it was derived from sediment collected from the two sites (WB-23 and WB-30) in the West Branch Canal Creek wetland study site where hydrogenolysis to TCA and dichloroelimination of TCA to VC were important reactions in the TeCA biodegradation pathway (Jones et. al., 2006; Lorah and Voytek, 2004).

Production of DCE through dichloroelimination of TeCA was also an important TeCA biotransformation at sites WB-23 and WB-30 (Lorah and Voytek, 2004), but not in the DMWBC and DMIX treatments in this study. DCE was observed in all of the MIX treatments; however, the increase in DCE in the DMIX and DMWBC treatments appeared to be mainly due to biodegradation of PCE (and TCE) (Figures 8.1, 8.2, and 8.3).

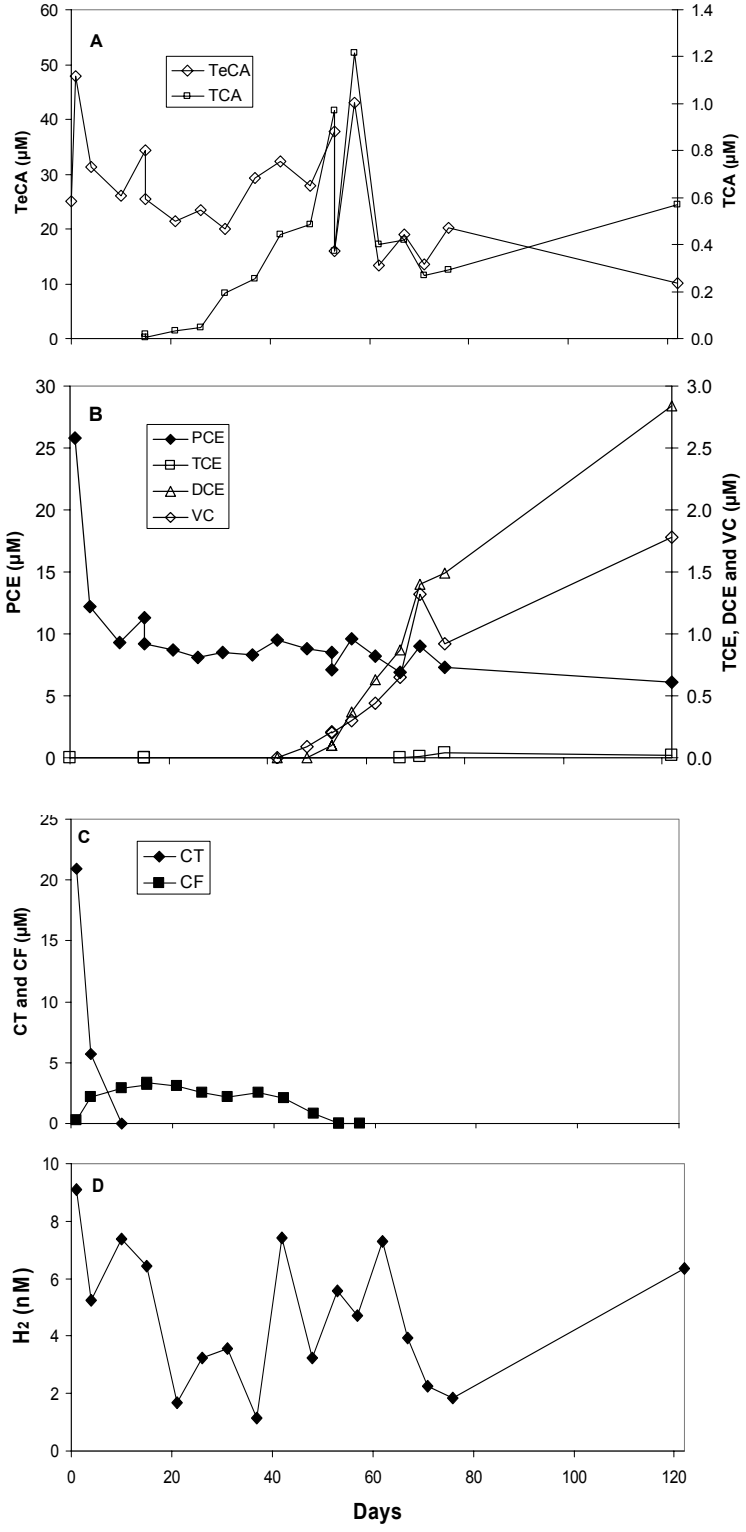


Figure 8.1. Aqueous (A) chlorinated ethanes, (B) chlorinated ethenes, and (C) chlorinated methanes, and (D) H_2 in the MIX microcosms. Data points are averaged for each sampling day. Septa were changed on days 15 and 53.

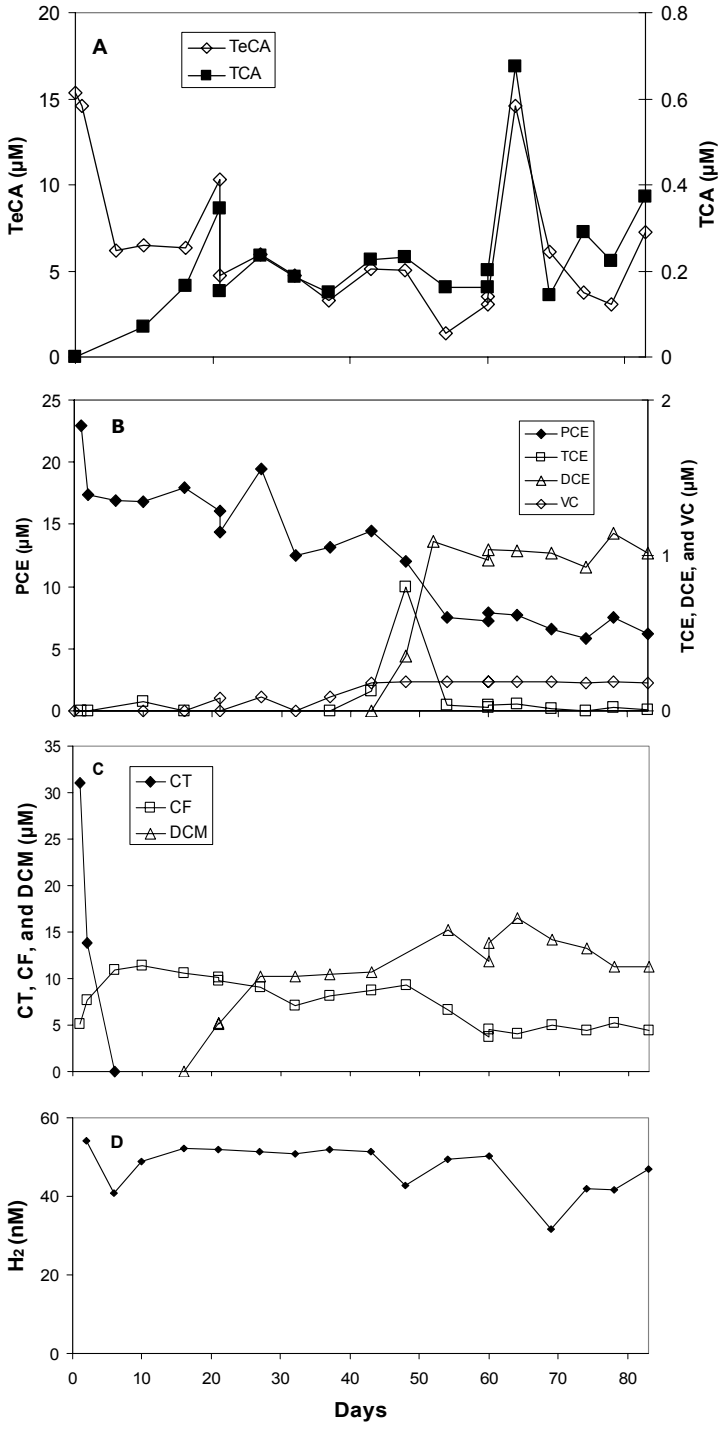


Figure 8.2. Aqueous (A) chlorinated ethanes, (B) chlorinated ethenes, and (C) chlorinated methanes, and (D) H_2 in the DMIX microcosms. Data points are averaged for each sampling day. Septa were changed on days 21 and 60.

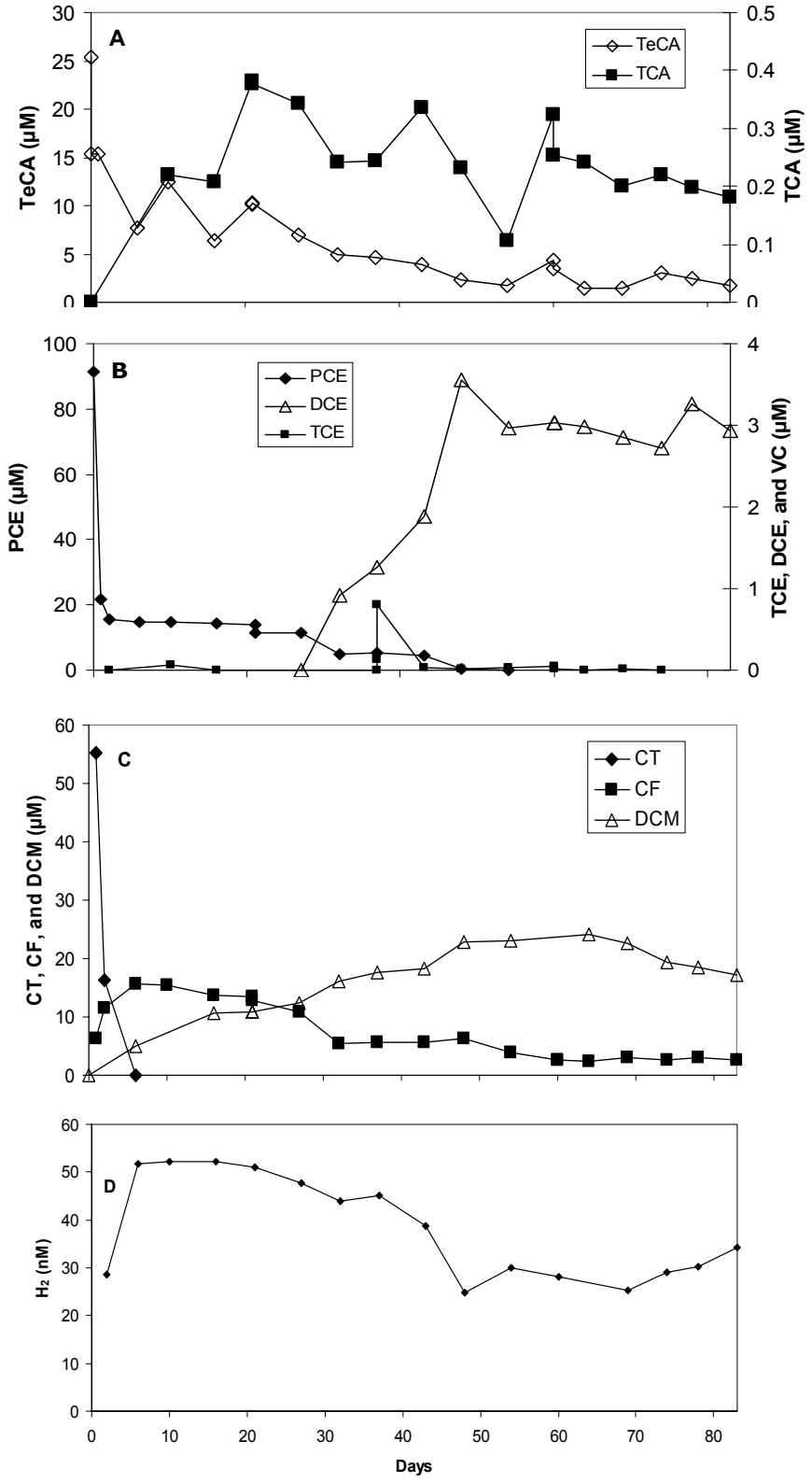


Figure 8.3. Aqueous (A) chlorinated ethanes, (B) chlorinated ethenes, and (C) chlorinated methanes, and (D) H_2 in the DMWBC microcosms. Data points are averaged for each sampling day. Septa were changed on days 21.

In the MIX treatment after day 10, no significant PCE removal occurred; therefore, TeCA most likely was the source of the DCE that accumulated in the MIX microcosms. Given that reductive dechlorination of TeCA to DCE was important in the sediment used to develop the WBC-2, it is a little surprising that the addition of the enrichment culture did not appear to increase TeCA to DCE reduction relative to the unamended sediment. Perhaps dichloroelimination of TeCA in this study and that conducted by Lorah and Voytek (2004) was an important process only in unamended sediment microcosms.

8.2.2.2. PCE

As previously stated, some removal of PCE was evident in all of the seep sediment treatments. However, in the MIX and DMIX treatments, removal was not much different than in the inhibited (SMIX) controls (Figure 8.4).

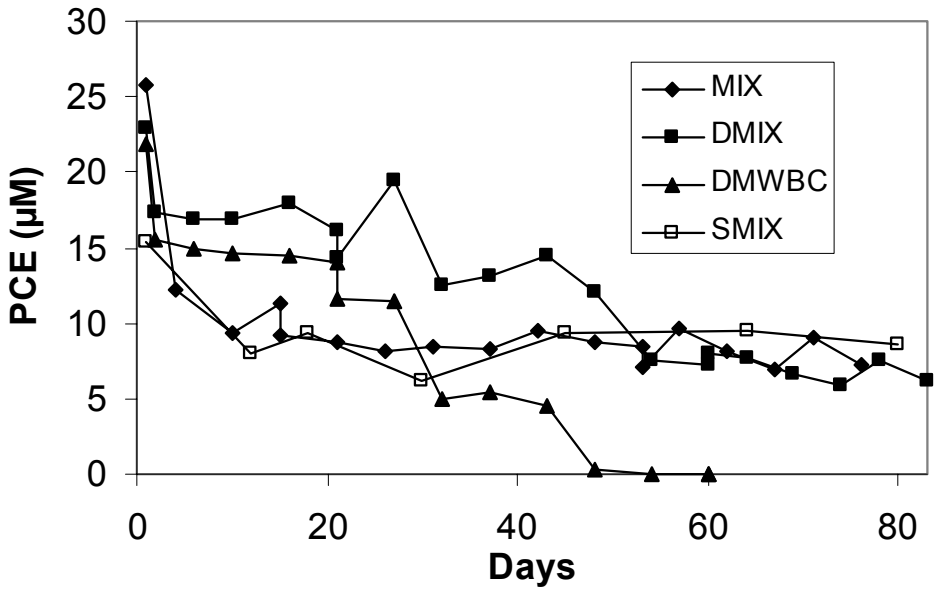


Figure 8.4. Aqueous PCE (µM) in all viable and inhibited microcosms. Data points are averaged for each treatment for each sampling day.

In contrast, complete removal of PCE was observed in the DMWBC microcosms by day 54 and DCE production occurred concomitantly, indicating that PCE underwent reductive dechlorination to TCE and DCE (Figure 8.3). The enhanced removal of PCE in the DMWBC microcosms was most likely due to the addition of the WBC-2. This is a somewhat surprising result because the WBC-2 was not enriched on PCE or TCE (Jones et. al., 2006), instead it was routinely supplied with TeCA, TCA, and DCE. The finding that bioaugmentation with the WBC-2 enhanced PCE removal could suggest that the DCE-degrading organism enriched in this culture was also able to dechlorinate PCE. To date, only *Dehalococcoides ethenogenes* strain 195, is known to respire PCE and DCE (Maymó-Gatell et. al., 1997), although a *Dhc.* strain (GT) that can respire TCE and DCE isomers was recently isolated (Sung et. al., 2006), and it is possible that other organisms that can respire both PCE and DCE exist. DCE accumulated in all of the microcosm treatments (Figure 8.2-8.4). The accumulation of DCE is common at sites undergoing clean-up of PCE contamination. However, the accumulation of DCE in the DMWBC microcosms, which were amended with the WBC-2, is somewhat surprising for two reasons. First, as discussed above, the addition of the WBC-2 appeared to enhance PCE removal. Second, the WBC-2 was routinely enriched and degraded DCE without delay. One likely explanation for the persistence of DCE in all of the cultures is that the DCE-dechlorinating organism was inhibited by CF. In fact, a recent study demonstrated that the reductive dechlorination of DCE by *Dehalococcoides ethenogenes* was inhibited by CF, which was present as an impurity in commercially prepared DCE (Maymó-Gatell et. al., 2001). An interesting implication of these observations is that the PCE-dechlorinating population (or enzyme) in the WBC-2 appears to be less sensitive to CF

than the DCE-dechlorinating population(s) or enzyme(s) present in either the seep sediment or WBC-2.

As noted above, CF is also a potent inhibitor of methanogenesis. A recent study of VC dechlorination in a mixed culture demonstrated that inhibition of aceticlastic methanogens within the genus *Methanosarcina* reduced VC dechlorination by inactivating acetate oxidation to H_2 (Heimann et. al., 2006). However, it seems unlikely that CF-mediated inhibition of H_2 production from acetate can explain the persistence of DCE in the sediment microcosms because H_2 concentrations in the DMIX and DMWBC microcosms were actually quite high (Figures 8.2 and 8.3).

8.2.2.3. CT

CT removal began in all of the live treatments and inhibited controls almost immediately. However, complete removal of CT was observed by day 10 in all of the live treatments, and by day 64 in the inhibited controls (Figure 8.5). These results suggest the CT removal was due to abiotic processes that may have been enhanced by microbial activity or heat labile extracellular components in the live microcosms (Novak et. al., 1998b). Under anaerobic conditions, CT can be removed through two different pathways, reductive dechlorination to CF or net hydrolysis to CO_2. Hydrolysis of CT to CO_2 may proceed abiotically via a CS_2 intermediate. Alternatively, reduction of CT to carbon monoxide and/or formate is possible, and carbon monoxide and formate can subsequently be oxidized to CO_2 (Hashsham et al., 1995).

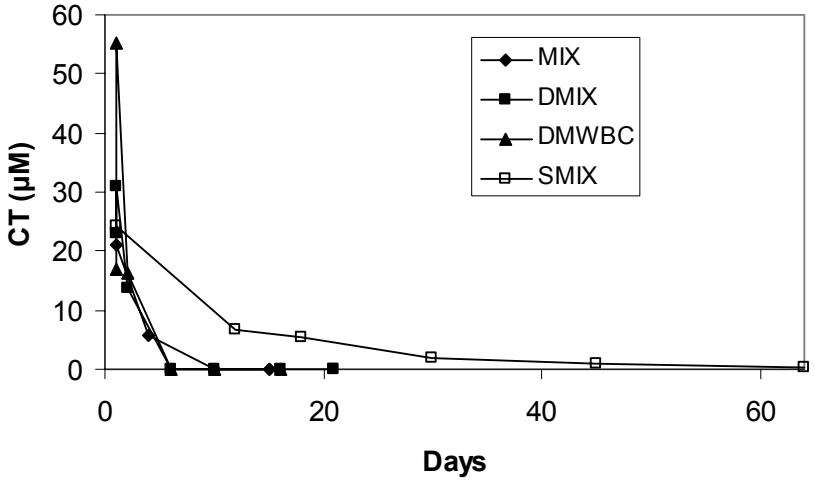


Figure 8.5. Aqueous CT (µM) in all viable and inhibited microcosms. Data points are averaged for each treatment for each sampling day.

It is possible that different CT biotransformation pathways may be dominant in the different live treatments and this could explain why CF levels varied so much among treatments. In the DMIX and DMWBC treatment microcosms, which contain excess electron donors, CF reached maximum concentrations of 11.4 and 15.6 µM, respectively, compared with CF levels of 3.4 µM or less in the MIX microcosms (Figures 8.2 and 8.3). In addition, DCM accumulated in the DMIX and DMWBC treatments, but not in the MIX microcosms (Figures 8.1, 8.2, and 8.3). These data suggest that there may have been a greater tendency for reductive dechlorination in the DMIX and DMWBC treatments due to the high concentrations of electron donors in the microcosms.

On the other hand, two observations suggest that CT removal in the MIX microcosms was primarily due to abiotic hydrolysis to CO_2 via a CS_2 intermediate. First, the potential for abiotic transformation was clearly present in the sediment because CT was removed with minimal CF production in the inhibited controls. Second, conversion of CT to CS_2

and CO_2 would not have consumed electrons in the electron donor-limited MIX microcosms. From a remediation standpoint, net hydrolysis to CO_2, the presumptive dominant CT removal pathway in the MIX microcosms, is preferable to the accumulation of CF and DCM in a reductive dechlorination pathway, as observed in the DMIX and DMWBC microcosms. However, CS_2, which was not detected in any of the microcosms, is considered hazardous and possesses neurotoxic properties (Hashsham et al., 1995). Differences in electron donor availability and dominant CVOC removal mechanisms in the various microcosm treatments are summarized in Table 8.1. The addition of electron donors to the DMIX and DMWBC microcosms appeared to promote conversion of TeCA to TCA and VC, whereas dichloroelimination of TeCA to DCE was only observed in the unamended MIX microcosms. Increased electron donor availability, however, was not sufficient to achieve complete removal of TeCA and PCE. Bioaugmentation with the WBC-2 appeared to be necessary to eliminate these parent compounds, although bioaugmentation did not stimulate DCE removal, perhaps because the DCE removing population was inhibited by CF. While bioaugmentation with the WBC-2 coupled with biostimulation using lactate and ethanol as electron donors appears to be an appropriate engineered bioremediation approach for TeCA and PCE at the seep site, it appeared to negatively impact mineralization of CT. Therefore, additional research is needed to determine whether the WBC-2's ability to biodegrade CT can be improved or whether another type of treatment, e.q., addition of cobalamin-type cofactors (Becker and Freedman, 1994; Hashsham et. al., 1995) should be used along with bioaugmentation and biostimulation to enhance CT hydrolysis.

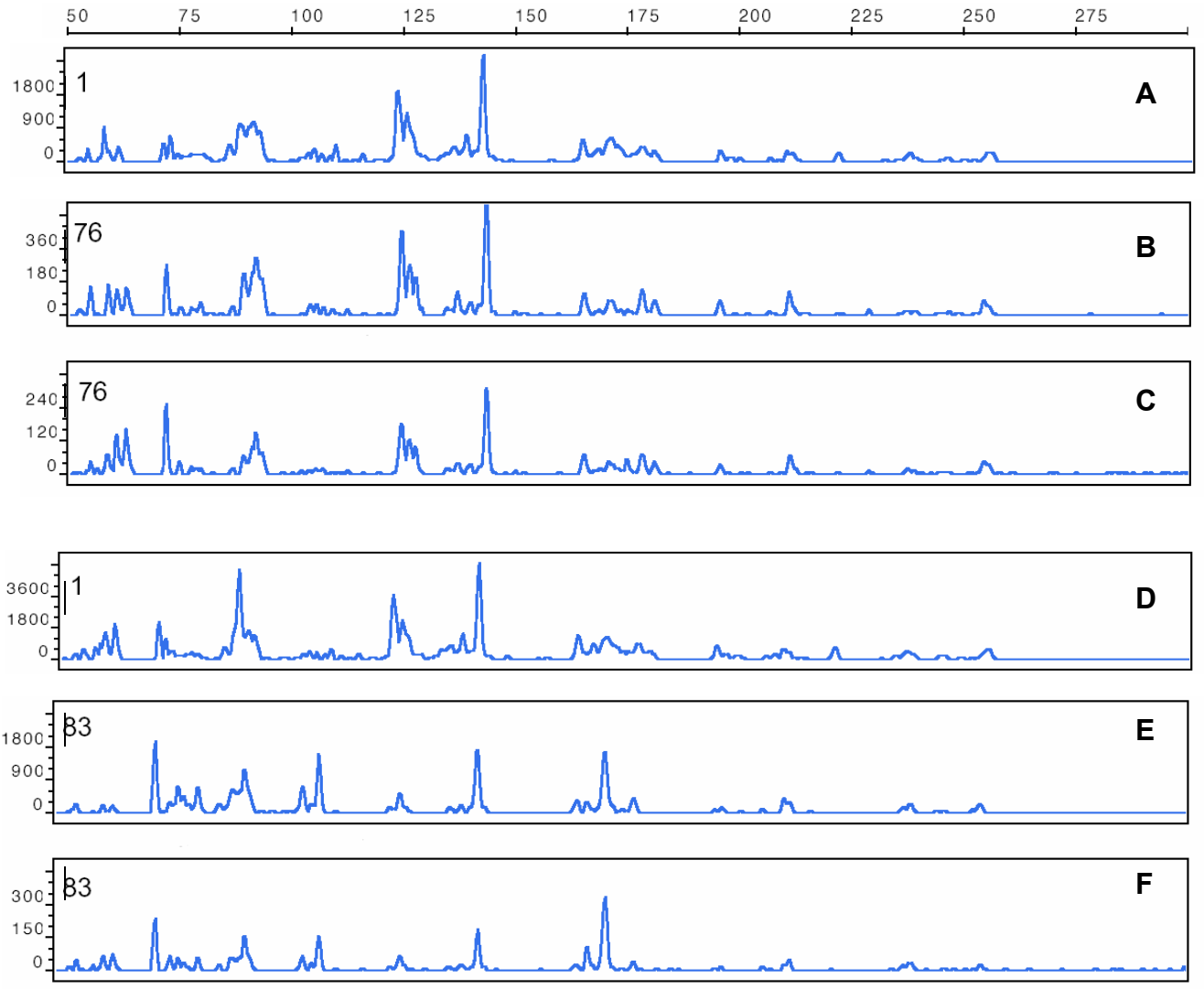
Table 8.1. Comparison of electron donor availability and dominant CVOC removal mechanisms in the seep site microcosms.

Live Treatment	Initial aqueous methane concentration (μM)	Initial acetate concentration (μM)	Range of aqueous H_2 concentrations (nM)	Conversion of TeCA to TCA and VC?	Conversion of TeCA to DCE?	Complete removal of PCE?	Dominant CT removal mechanism
MIX	<1 μM	~100 μM	1–9	delayed	seems likely	no	hydrolysis to CO_2[a]
DMIX	71 μM	>1000 μM	31–54	immediate	seems unlikely	no	reductive dechlorination
DMWBC	22 μM	>1000 μM	25–52	immediate	seems unlikely	yes	reductive dechlorination

[a]Presumptive.

8.2.2.4. MIX T-RFLPs

Comparison of T-RFLP fingerprints from the various site 3-1E treatments suggests that the addition of electron donors did not significantly affect the overall bacterial community structure (Figure 8.6). In contrast, the day 83 T-RFLP patterns from the DMIX and DMWBC microcosms are noticeably different from each other and from the MIX microcosm. For example, 103 bp and 115 bp peaks are apparent in the fingerprints of both treatments. However, in the DMIX microcosms, the 115 bp peak is bigger than the 103 bp fragment, whereas the 103 bp peak is larger than the 115 bp peak in the DMWBC microcosms. There are also numerous small peaks in the DMWBC microcosms that were not evident in either the DMIX and MIX microcosms. Still other peaks in the DMWBC chromatograms appear to be more highly resolved compared with the MIX and the DMIX chromatograms. For example, a distinct 238 bp peak is readily apparent in the chromatograms from the DMWBC, but is not evident in the other chromatograms.


50
75
100
125
150
175
200
225
250
275
1800
900
0
1
A
360
180
0
76
B
240
120
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C
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D
1800
900
0
83
E
300
150
0
83
F

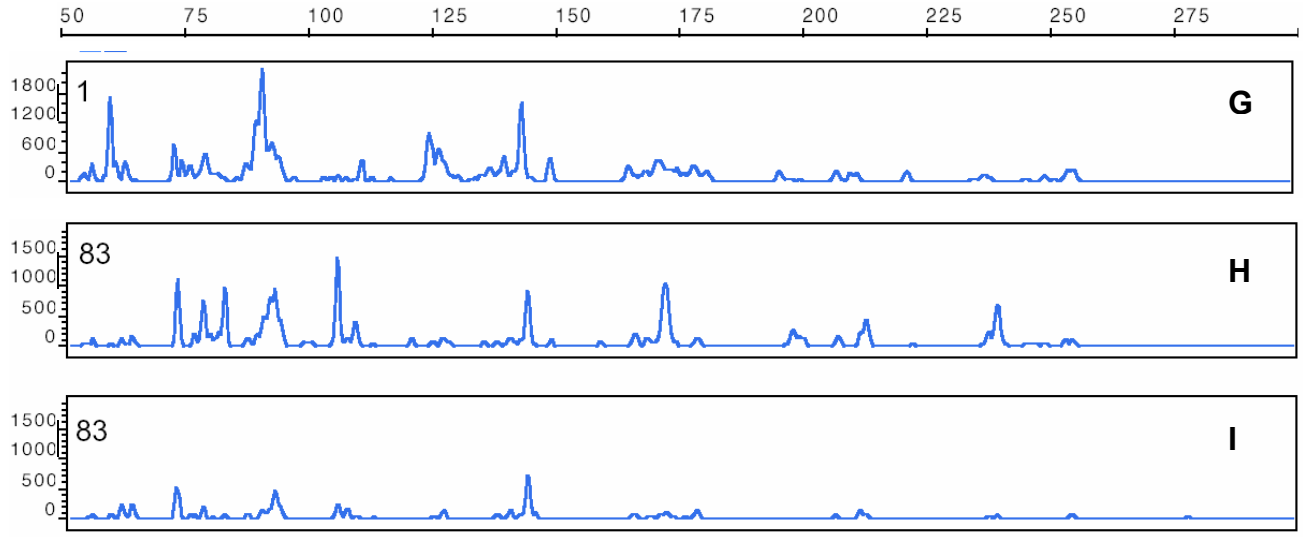


Figure 8.6. Terminal restriction fragment length polymorphism profiles of the MIX treatment on A) day 1 and B-C) day 76, the DMIX treatment on D) day 1 and E-F) day 83, and the DMWBC treatment on G) day 1 and H-I) day 83. The fragment sizes in bp are given by the scale above in panel A.

8.3. TeCA Seep Site Experiment

Five sets of duplicate microcosms were amended with TeCA as the sole CVOC to examine the effects of adding electron donor substrates with or without an augmentation culture or reducing agents on TeCA biodegradation in a mixture of seep sediment and groundwater collected from sites 3-1E and WB-24, respectively, as previously described. The results from this experiment provide some interesting insight into the dominant TeCA degradation pathways. By comparing the results obtained with the TeCA-amended microcosms to those amended with a mixture of contaminants, the effects of other CVOCs on TeCA removal and appropriate treatment approaches for enhancing natural attenuation of TeCA could also be evaluated.

TeCA was added to all of the treatments on day 0 at initial aqueous concentrations ranging from 21 to 39 μM after equilibration with sorbed TeCA. The septa on the serum bottles were changed on days 24 and 62 for the treatments amended with TeCA-Only (TeCA-Only #1 and #2; Figures 8.7 and 8.8), TeCA plus electron donors (Figure 8.9), and TeCA, electron donors and WBC-2 (TDWBC; Figure 8.10) to prevent O_2 from leaking through the septa, which were repeatedly punctured. The septa on the microcosms amended with TeCA and $FeCl_2$ and Na_2S (Figure 8.11) were changed on day 31. Following a septum change, the concentration of CVOCs in a microcosm were measured again so that decreases in the concentrations of CVOCs due to septum replacement could be accounted for. Methane was also lost from the bottles when septa were changed, but the amount of CH_4 stripped out during this procedure was accounted for in the cumulative CH_4 concentration plotted in Figures 8.7 through 8.11.

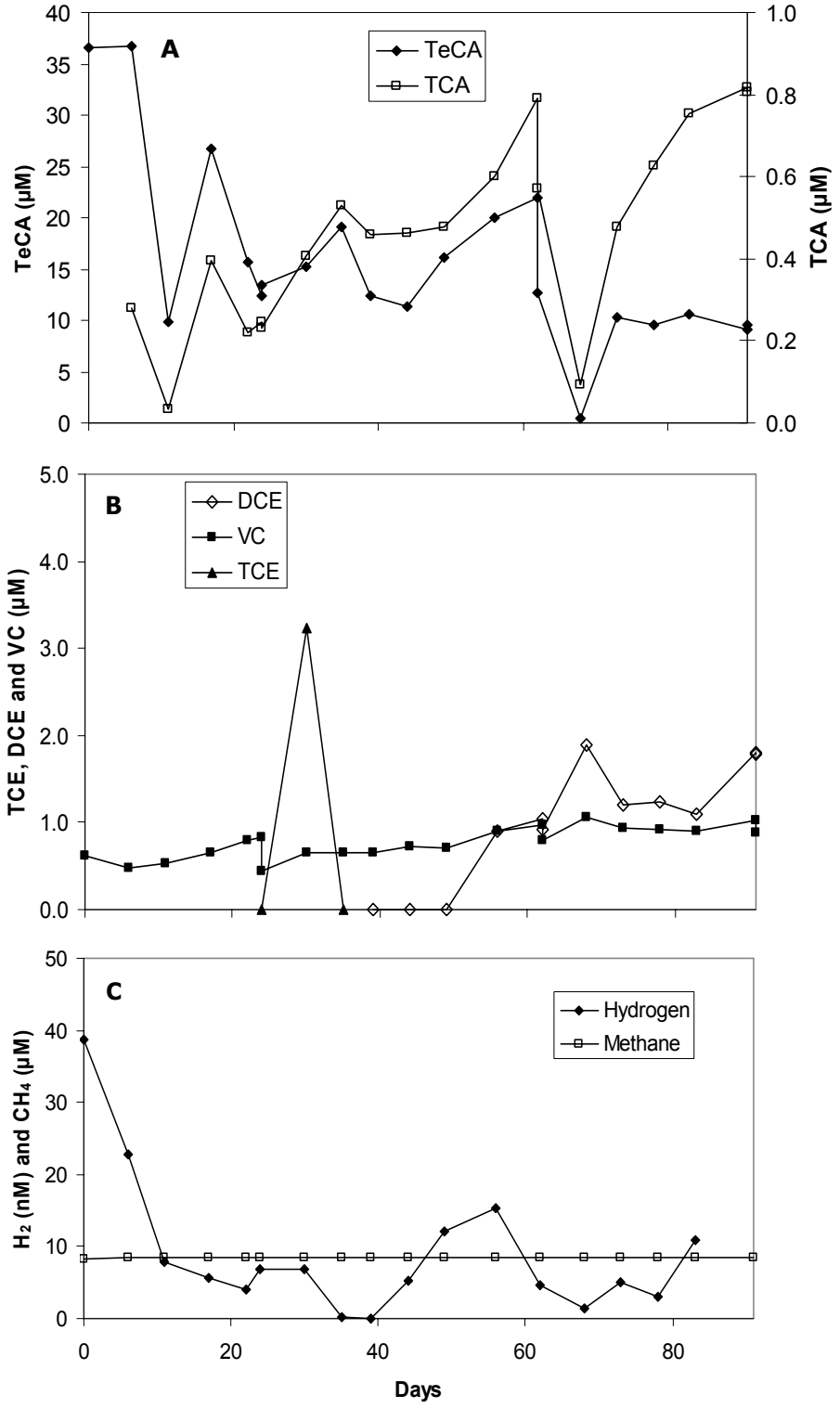


Figure 8.7. Aqueous (A) TeCA, TCA, and ETA, (B) TCE, DCE and VC, and (C) H_2 and CH_4 in TeCA-Only #1. Septa were replaced on days 24 and 62.

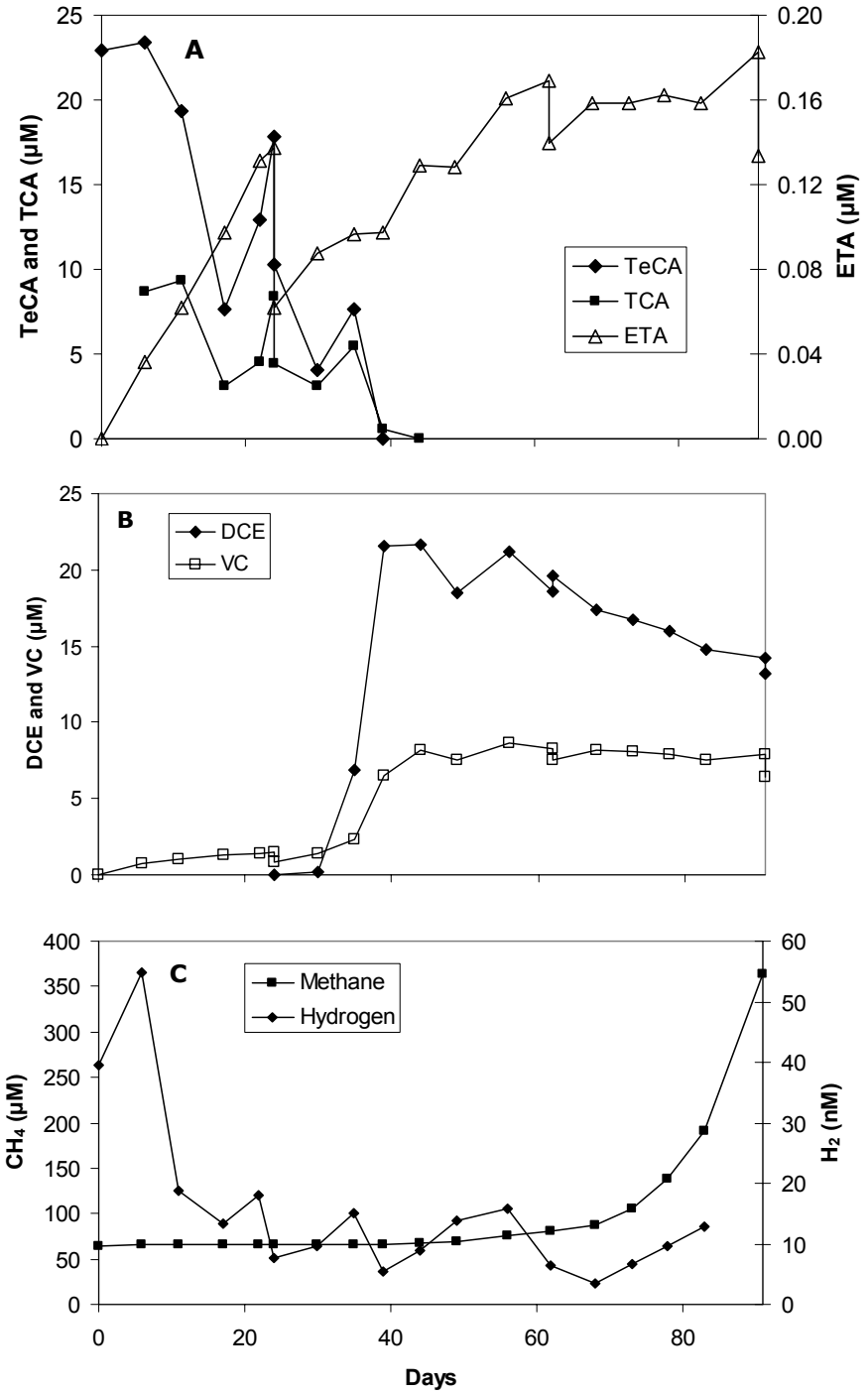


Figure 8.8. Aqueous (A) TeCA, TCA, and ETA, (B) DCE and VC, and (c) H_2 and CH_4 in TeCA-Only #2. Septa were replaced on days 24 and 62.

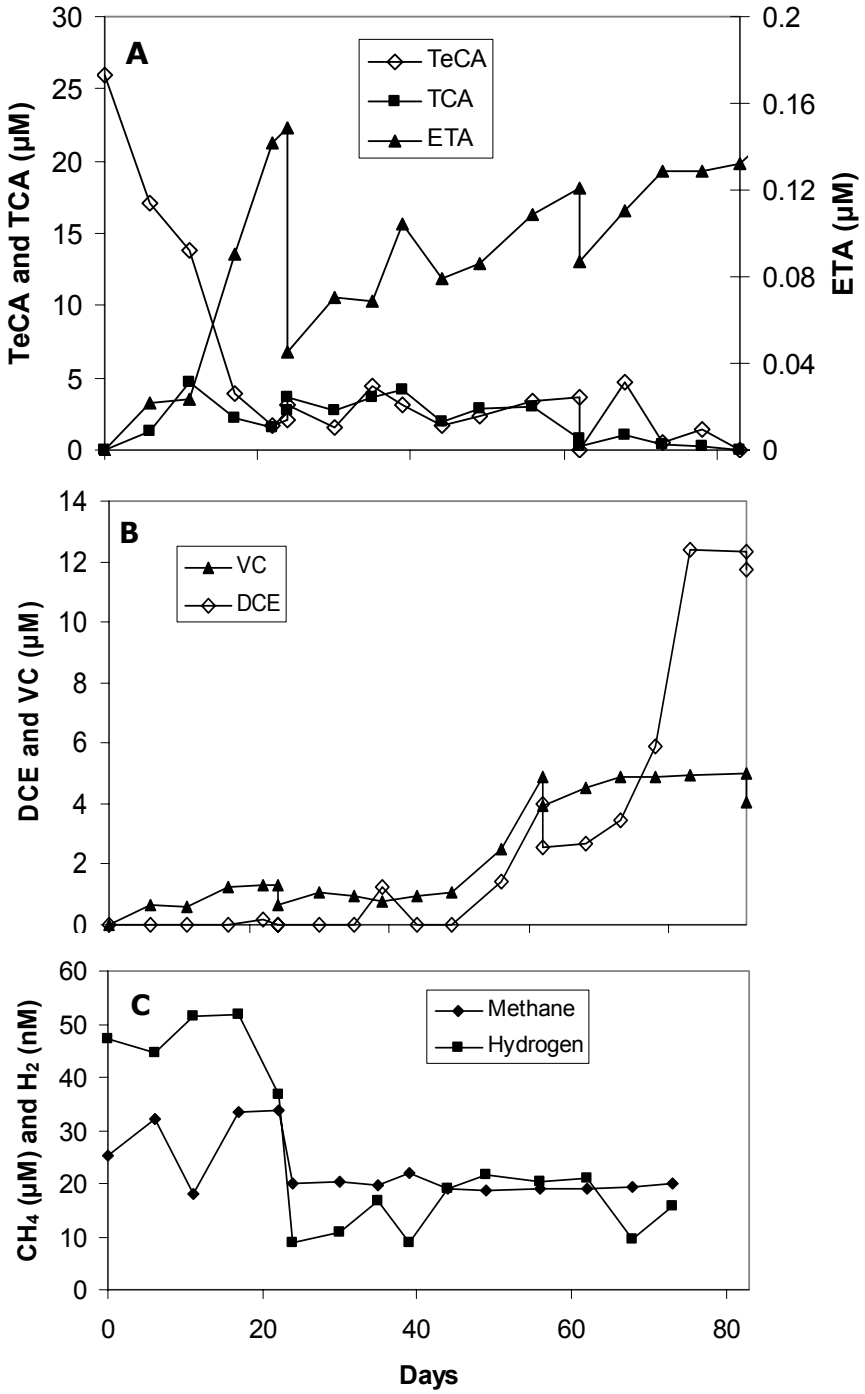


Figure 8.9. Aqueous (A) TeCA, TCA, and ETA, (B) DCE and VC, and (C) H_2 and CH_4 in TeCA-Donor. Data points are averaged for each sampling day. Septa were replaced on days 24 and 62.

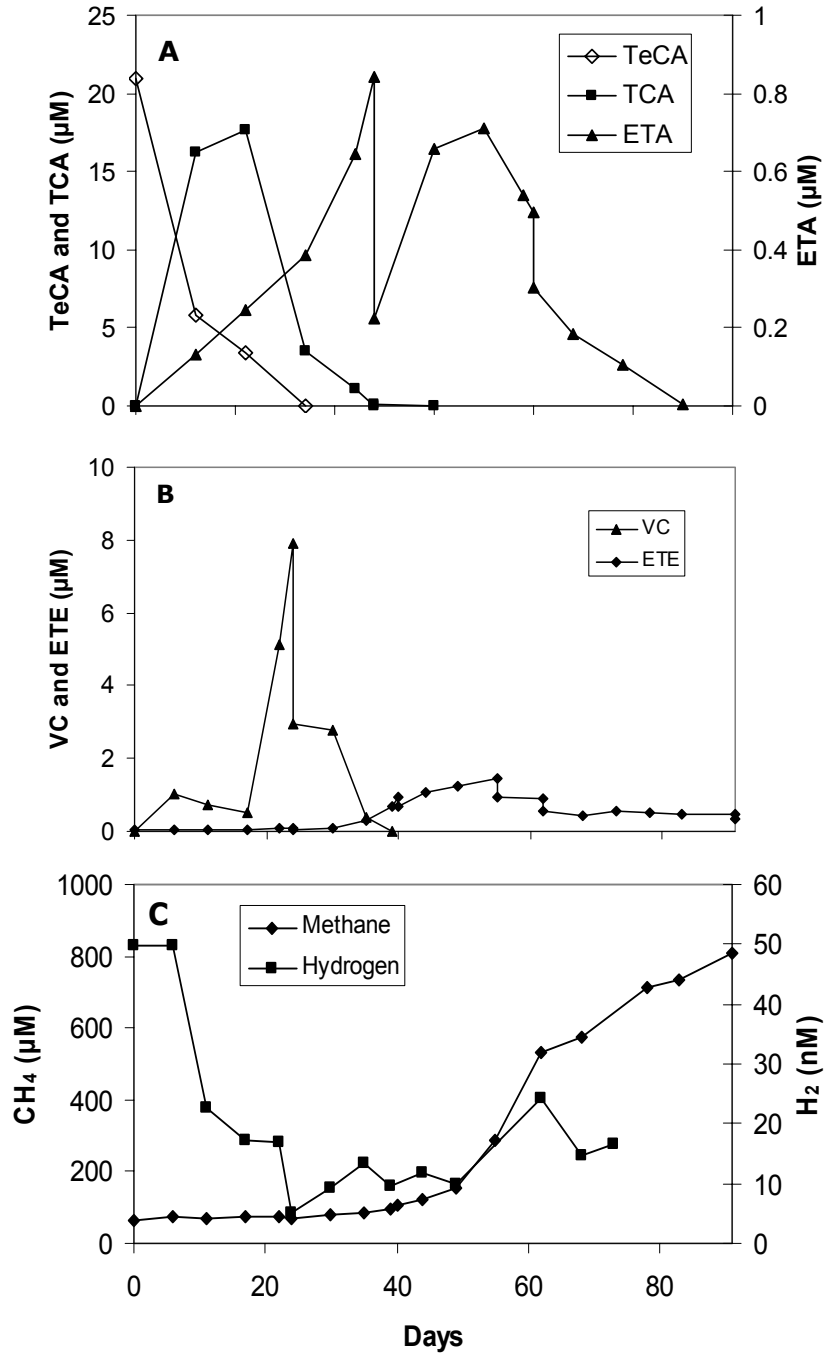


Figure 8.10. Aqueous (A) TeCA, TCA, and ETA, (B) VC and ETE, and (c) H_2 and CH_4 in TDWBC. Data points are averaged for each sampling day. Septa were replaced on days 24 and 62.

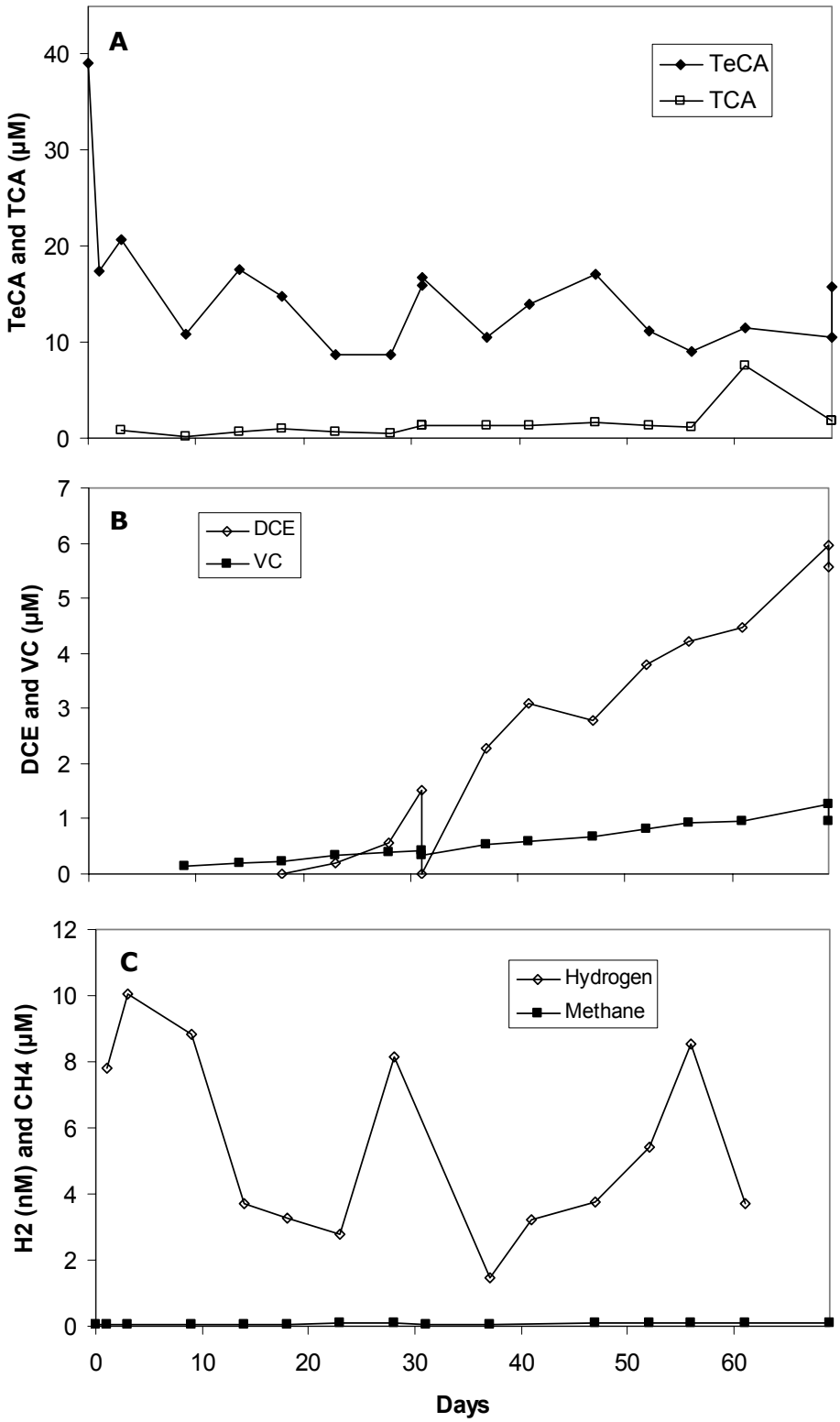


Figure 8.11. Aqueous (A) TeCA and TCA, and (B) VC and DCE, and (C) H_2 and CH_4 in Na_2S and $FeCl_2$. Data points are averaged for each sampling day. Septa were replaced on day 31.

In past studies conducted with sites WB-23 and WB-30 sediment at APG, approximately 3% of the total TeCA was transformed to TCE (Lorah and Olsen, 1999). In the present study, TCE was detected at a low concentration on a single day in only one microcosm (Figure 8.7). Therefore, abiotic dehydrochlorination to TCE does not appear to be a major removal mechanism at site 3-1E. Instead, dichloroelimination and hydrogenolysis of TeCA appeared to be the dominant pathways of TeCA removal at the 3-1E site. However, there were significant differences in the amount and timing of TeCA transformation via these two pathways among the various treatments.

In fact, different removal trends were observed in the two microcosms that were amended with TeCA alone (Figures 8.7 and 8.8). Therefore, they are discussed separately here. In the first replicate (Figure 8.7), relatively high TeCA concentrations were observed in the aqueous phase throughout the experiment. TCA and VC were detected immediately and persisted, suggesting that hydrogenolysis of TeCA followed by dichloroelimination of TCA to VC was a minor pathway *in situ*. In contrast, removal of aqueous TeCA was rapid in the second replicate (Figure 8.8). Aqueous TeCA was not detectable in this bottle after day 35 and apparently was converted primarily via hydrogenolysis to TCA. VC was detected immediately suggesting that a fraction of the TCA underwent dichloroelimination. At around day 35, a shift in the dominant TeCA removal mechanism apparently occurred. Prior to day 35, hydrogenolysis to TCA was dominant. After day 35, dichloroelimination to DCE was dominant. By day 39, the concentration of DCE was approximately equal to the initial TeCA concentration. Because a significant amount of the aqueous TeCA was apparently converted to TCA, it is likely that at least a

portion of this DCE was produced from TeCA that was sorbed to the sediment particles. VC levels also increased significantly on day 35, which suggests that some of the DCE underwent hydrogenolysis to VC. However, the concentration of VC remained fairly constant at around 8 μM for the remainder of the experiment, while DCE levels gradually decreased. The onset of DCE and greater VC production at around 35 d coincided with the onset of methanogenesis. Interestingly, Lorah and Voytek (2004) observed that VC accumulation was associated with the onset of methanogenesis in microcosms constructed with sediment from sites WB-23 and WB-30. However, in those samples conversion of TeCA to DCE and TCA occurred concomitantly. Methane production in the TDWBC microcosms followed a pattern similar to that observed in TeCA-Only #2 (Figure 8.10). However, TeCA and its daughter products were degraded below the detection limit by day 39 in the WBC amended microcosms (Figure 8.10).

There are several potential explanations for the apparent association between methanogenesis and the shift to a dichloroelimination mechanism of TeCA removal in TeCA-Only #2. One possible explanation is that acetotrophic methanogens converted acetate to H_2 that was subsequently utilized as the electron donor (Heimann et. al., 2006) by the TeCA-to-DCE dechlorinating population. However, this reaction is thermodynamically favorable only at extremely low H_2 concentrations. H_2 concentrations typically ranged from 10 to 20 nM in the electron donor amended microcosms and the TeCA-only replicate during the period of DCE accumulation and this may not have been sufficiently low enough to drive acetate oxidation to H_2. Another possibility is that the apparent relationship between methanogenesis and the TeCA dichloroelimination

pathway is linked to the redox potential of the microcosm. It is possible that methanogenesis was delayed in the microcosms until preferable terminal electron acceptors, e.g. Fe(III), were depleted, and these highly reduced conditions were necessary for the TeCA dichloroelimination, but not the TeCA hydrogenolysis reaction. In fact, Dolfing (2000) evaluated the potential role that thermodynamics play in controlling the biodegradation pathways of TeCA and other CVOCs and suggested that highly reducing conditions favored dichloroelimination reactions over hydrogenolysis. The fact that accumulation of DCE was observed in the microcosms amended with reducing agents by day 22 in the absence of methanogenesis also seems consistent with this explanation.

Still other possible reasons for the apparent association between methanogenesis and the accumulation of DCE and VC exist. For example, Lorah and Voytek (2004) noted that VC accumulation and increasing CH_4 concentration may have coincided in the WB-23 and WB-30 microcosms because VC was oxidized by iron-reducing bacteria until Fe(III) was depleted. It is possible that oxidation of VC and even DCE was also carried out by iron-reducers (Bradley and Chappelle, 1998) in the present study. It is also conceivable that methanogens were carrying out co-metabolic transformation of TeCA to DCE, particularly because organisms that can respire TeCA have not yet been identified.

Comparison of H_2 and TeCA levels in the various treatments may provide some insight into the nature of the TeCA removal mechanism. In the microcosms that were amended with electron donors (Figures 8.9), H_2 levels were relatively high (~10-50 nM) while the majority of the aqueous TeCA was removed. These high H_2 concentration could indicate

that TeCA is a co-metabolic, rather than a dehalorespiring, process. However, it is unlikely that methanogens are involved in the removal of TeCA or its daughter products before day 50 because significant methane production was not observed during this period in most of the microcosms. It is possible that co-metabolic degradation of the CVOCs during this period was attributable to acetogens or other populations within the wetland sediment microbial community. Alternatively, it is possible that the addition of electron donors to the two sets of microcosms resulted in high concentrations of H_2 that exceeded the H_2 demands of reductive dechlorination. In that case, the H_2 levels would not be expected to decrease to the levels characteristic of dehalorespiration under H_2-limited conditions. Finally, it is possible that H_2 did not serve as the electron donor for TeCA reductive dechlorination. In fact, Jones et. al. (2006) found that H_2 did not stimulate TCA reductive dechlorination in WBC-2. If an organic substrate served as the direct electron donor for reductive dechlorination of TeCA and its initial daughter products then again, H_2 levels would not necessarily approach the threshold concentrations characteristic of the dehalorespiration process.

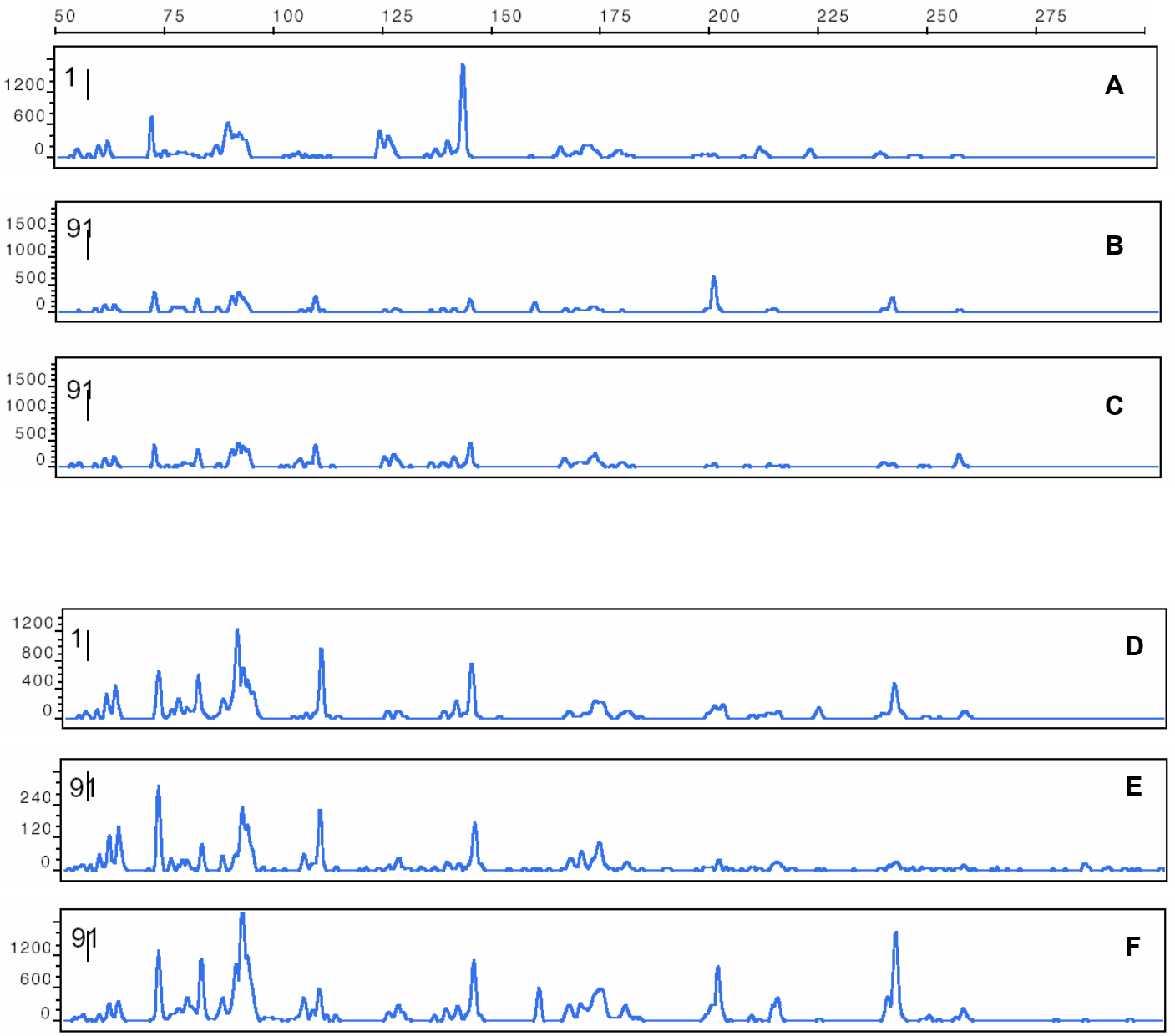
Compared to the microcosms amended with TeCA alone or TeCA plus electron donors, the TDWBC microcosms performed the best, both in terms of the rate of TeCA removal and the extent of transformation. It took only 39 days to completely degrade TeCA to non-toxic end products in the microcosms amended with WBC-2. No DCE was ever detected in these microcosms, which could suggest that the hydrogenolysis pathway alone is responsible for the complete removal of TeCA (Figure 8.10). However, WBC-2 was enriched on both DCE and TCA, and it could routinely degrade 240 μM of TeCA

within 2 days without the accumulation of DCE. Therefore, it is possible that DCE was produced, but did not accumulate in the bioaugmented site 3-1E sediment. Thus, the results of this experiment demonstrate that bioaugmentation of seep sites with WBC coupled with the addition of suitable electron donors (lactate and ethanol) may be an effective approach for enhancing natural attenuation processes if TeCA is the only contaminant present. Biostimulation of the sediment by adding electron donors alone did not appear to enhance TeCA removal. The fact that rapid removal of TeCA was observed in one microcosm that was not amended with WBC, suggests that key dechlorinating populations are present in the site 3-1E sediment. However, they are probably present in very low levels. Thus, bioaugmentation is needed to ensure consistent performance.

The results of the experiment conducted with TeCA suggest that bioaugmentation and biostimulation may be less effective in the presence of other contaminants such as PCE and CT. In the absence of these co-contaminants, removal of TeCA occurred quickly within 39 days, whereas low concentrations of chlorinated ethanes and ethenes still remained on day 83 of the DMWBC treatment. Thus, even in the presence of a culture enriched on TCA, DCE, and VC and sufficient amounts of electron donor, chlorinated ethane removal was inhibited by the presence of other CVOCs. Thus, if other contaminants are present, steps must be taken to reduce the concentration of these compounds (especially CF) before engineered bioremediation approaches targeting TeCA are implemented.

8.3.1. TeCA T-RFLPs

Despite the dramatic differences in the performances of the TeCA-only #1 and TeCA-only #2 microcosms, the day 91 T-RFLP fingerprints from these microcosms were fairly similar (Figure 8.12a-c). However, there were several minor peaks in the TeCA-only #2 chromatogram that were not present in the TeCA-only #1 chromatogram. It is possible that the populations associated with these peaks were responsible for the improved performance of TeCA-only #2. Interestingly, a 199 bp peak is evident in the TeCA-only #1 microcosm, but not in the TeCA-only #2 microcosm. Several peaks in the day 91 TDONOR microcosms were larger compared with the TeCA-only microcosms, suggesting that several populations were enriched by the addition of electron donors.


50
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150
175
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600
0
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1200
600
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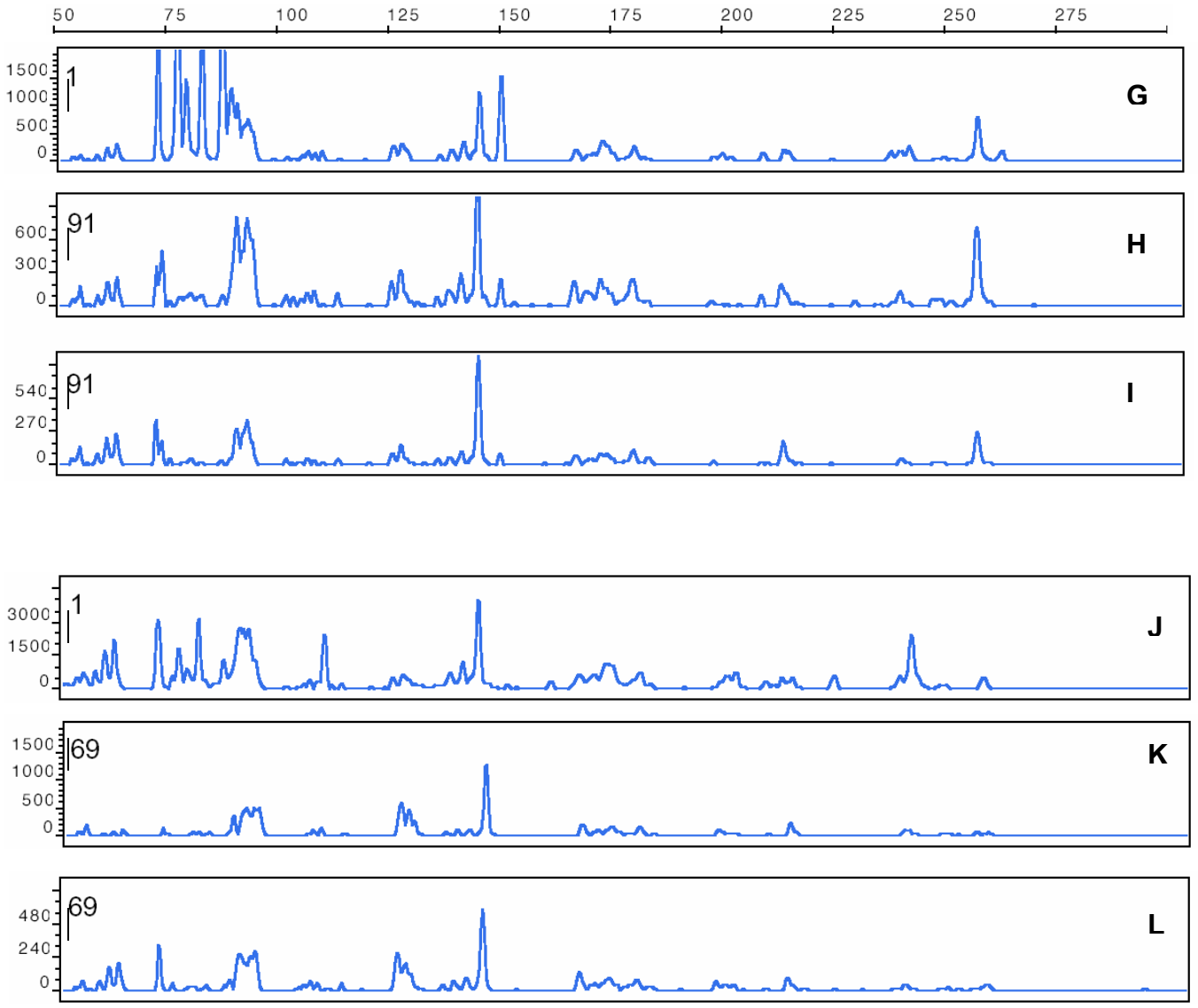

Figure 8.12. Terminal restriction fragment length polymorphism profiles of the TeCA-Only treatment on A) day 1 and B-C) day 91, the TDONOR treatment on D) day 1 and E-F) day 91, the TDWBC treatment on G) day 1 and H-I) day 91, the Na_2S and $FeCl_2$ treatment on J) day 1 and K-L) day 69. The fragment sizes in bp are given by the scale above in panel A.

Chapter 9: Conclusion and Future Work

This study was designed to evaluate the effectiveness of bioaugmentation and biostimulation to enhance the natural attenuation of CVOCs, and to assess whether characteristic H_2 concentrations were associated with the biodegradation of the parent compounds or their daughter products. H_2 concentrations characterizing dehalorespiration and methanogenesis were of great interest, as these redox processes have shown to be very important in the removal of these contaminants.

These hypotheses were evaluated using microcosms containing sediment from seep site 3-1E or the transect site, WB-35, and groundwater from site WB-24B. The relative amount of error among all of the replicates in the various replicates was between 0.01 and 16.17 μM. The overall results of these experiments demonstrated that the application of both bioaugmentation and biostimulation to a seep site(s) contaminated with TeCA is an appropriate engineered bioremediation method. However, when TeCA is in the presence of co-contaminants, PCE and CT, daughter products are observed to accumulate and, in some cases, removal of the parent compounds is incomplete. In the MIX treatment, complete removal of all chlorinated methanes was observed.

In addition to bioaugmentation and biostimulation, the results of this study suggest that when mixtures of contaminants are present additional enhancement techniques should be considered. Because CT and CF appeared to inhibit CVOC removal, it might be necessary to increase the CT removal rate, in order to promote the biodegradation of PCE and TeCA. A common solution is to add cyanocobalamin (Vitamin B-12) to the

treatment, as it has been shown to enhance CT removal (Novak et. al. 1998b). Chlorinated methanes are known to react with naturally-occurring corrinoids. Therefore, the addition of cyanocobalamin may prevent key metabolic pathways from being interrupted, thereby, alleviating CT toxicity and promoting abiotic reductive dechlorination (Becker and Freedman, 1994). Aside from considering additional enhancement techniques, it also appears that biodegradation of the contaminants should be evaluated individually and as a mixture to determine what co-metabolic and metabolic processes should be promoted within the particular seep site to develop a successful engineered bioremediation method.

Based on the results of this study, several recommendations for future studies can be made to increase our understanding of the factors controlling natural attenuation of CVOCs in the wetland sediment and engineered methods of enhancing these processes. Specifically, future experiments should examine biodegradation of mixtures of CF and TeCA, PCE and TeCA, and CT and PCE to better understand how biodegradation of individual compounds is influenced by the presence of contaminants. Based on the results of the current study, it appears that CT and CF may be inhibiting TeCA biodegradation, even in the presence of suitable amounts of electron donors and the WBC-2 enrichment culture. However, it has also been suggested that chlorinated ethanes can inhibit chlorinated ethene removal (Aulenta et. al., 2006). Therefore, it would be interesting to evaluate all of the combinations listed above in the presence of WBC-2, electron donors, and vitamin B-12 (if CT is present). The removal of CT and PCE

individually by the diluted WBC-2 culture would also be of interest, as the degradation of these compounds by the culture has not been extensively studied.

H_2 measurements did not fully resolve the issue of whether metabolic or co-metabolic processes were responsible for the degradation of the various CVOCs. Therefore, it would be useful to perform additional genetic analyses of the microcosm microbial communities to assess the relative abundance of dehalorespiring populations such as *Dehalococcoides* strains, as well as acetotrophic methanogens. The T-RFLP profiles produced by USGS Microbiology Lab utilized DNA primers that target the 16S rDNA sequence of all Bacteria. In order isolate *Dehalococcoides* sp. DNA, primers that recognize a DNA sequence specific to the species would be needed. In addition to genetic analyses, it would also be interesting to evaluate biostimulation and bioaugmentation at different seep sites to see if the same conclusions can be drawn in regards to the degradative pathways and the effectiveness of bioaugmentation and biostimulation. If similar results were demonstrated, then it is possible that these engineered bioremediation methods could be widely applied to other locations at APG.

Appendix A: List of Abbreviations

APG	—	Aberdeen Proving Ground
CA	—	Chloroethane
CERCLA	—	Comprehensive Environmental, Compensation, and Liability Act of 1980
CF	—	Chloroform
CM	—	Chloromethane
CT	—	Carbon Tetrachloride
CVOC	—	Chlorinated Volatile Organic Compound
DCA	—	1,2-Dichloroethane
11DCE	—	1,1-Dichloroethene
*c*DCE	—	*cis*-1,2-Dichloroethene
*t*DCE	—	*trans*-1,2-Dichloroethene
DCM	—	Dichloromethane
DMIX	—	VOC MIX + Donor + Sediment + Water
DMWBC	—	VOC MIX + Sediment + Water + WBC-2
EPA	—	Environmental Protection Agency
ETA	—	Ethane
ETE	—	Ethene
MIX	—	VOC MIX + Sediment + Water
SMIX	—	VOC MIX + Sediment + Water + Formaldehyde + Autoclaving
SWBCMIX	—	VOC MIX + Sediment + Water + Formaldehyde + Autoclaving +WBC-2
TCA	—	Trichloroethane
TCE	—	Trichloroethene
TDONOR	—	TeCA-Only + Donor + Sediment + Water
TDWBC	—	TeCA-Only + Donor + WBC-2 + Sediment + Water
TeCA	—	1,1,2,2-Tetrachloroethane
TeCA-Only	—	TeCA-Only + Sediment + Water
VC	—	Vinyl Chloride
VOC MIX	—	**3-1E:** TeCA, 5 mg/L (30 µmol/L); PCE, 3 mg/L (18 µmol/L); CT, 4 mg/L (26 µmol/L); **3-4W:** TeCA, 5 mg/L (30 µmol/L); PCE, 3 mg/L (18 µmol/L); TCE, 3 mg/L (23 µmol/L); CT, 5 mg/L (33 µmol/L); CF, 5 mg/L (42 µmol/L)
WBC	—	West Branch Consortium

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