Evaluation of Enhanced Bioremediation for Reductive Dechlorination of Tetrachlorethene (PCE): Microcosm Study

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(ABSTRACT)

Laboratory microcosm experiments were conducted to assess the potential for biostimulation and bioaugmentation as source reduction measures in support of a monitored natural attenuation remedial strategy at Naval Amphibious Base (NAB) Little Creek. Previous work with laboratory microcosms conducted under simulated natural (unamended) conditions has demonstrated that indigenous dehalorespirators were capable of partial dechlorination of tetrachloroethene (PCE) to *cis*-dichloroethene (*cis*-DCE). This study attempts to achieve complete reductive dechlorination with amendments to static microcosms to test the hypotheses that nutrient-limited or microorganism-limited conditions exist in aquifer sediments obtained from the site. The enhanced bioremediation experiments were comprised of nutrient-amended microcosms receiving additions of electron donors, mineral medium, or anaerobic digester supernatant, and dechlorinating culture-amended microcosms were inoculated with a culture capable of transforming PCE to ethene. Reductive dechlorination in the nutrient-amended microcosms proceeded to *cis*-DCE over a 260-day study period, at slightly higher rates than in experiments conducted with aquifer sediments from the same location under natural conditions. Inoculation of aquifer sediments with a small amount of dechlorinating culture initiated rapid transformation of PCE to vinyl chloride (VC) by day 18 of the study. Zero-order rates of PCE dechlorination in unamended, propionate-, formate-, mineral medium-, digester supernatant-, and dechlorinating culture-amended microcosms were 0.24, 0.750, 1.30, 0.339, 0.177, and 1.75 μM/day, respectively. The results of this study suggest that an engineered biostimulation approach alone may not be as beneficial for PCE source reduction at NAB Little Creek, than bioaugmentation with competent dehalorespirators, along with the inclusion of supplemental nutrients which would be available to stimulate dechlorination activity of both indigenous and introduced microorganisms.

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CHAPTER 1
Literature Review

Introduction

Chlorinated solvents are among the most frequently reported groundwater contaminants in the United States. The favorable physical properties of chlorinated solvents have led to their widespread industrial application. They are used for metals and electronics degreasing, dry cleaning, textile processing, and serve many other purposes. As a consequence of their popularity, chlorinated solvents have been released into the environment from poor handling, storage, and disposal practices. A major chlorinated solvent is tetrachloroethene (PCE), which is also known by its common name perchloroethene. The compound and its associated degradation products, trichloroethene (TCE), the dichloroethene isomers (DCE), and vinyl chloride (VC) are classified as volatile chlorinated aliphatic hydrocarbons or chlorinated ethenes. All chlorinated ethenes are considered as known or probable carcinogens (2).

The physical and chemical properties of chlorinated ethenes are presented in Table 1. Chlorinated ethenes are alkenes with a carbon-carbon double bond. Since chlorinated ethenes (with the exception of VC) are denser than water, they will tend to migrate through the saturated zone of an aquifer until reaching a lower confining layer. The migration of these dense non-aqueous phase liquids (DNAPLs) is sensitive to variations in aquifer permeability and they can move laterally if aquifer heterogeneities are encountered. Chlorinated ethenes generally have low water solubilities and high octanol-water partition coefficients (i.e. hydrophobic) which retard their mobility in the saturated zone, thereby limiting the effectiveness of conventional pump-and-treat technologies that rely on advection processes for remediation.

The scientific understanding of how chlorinated ethenes degrade in the environment is of paramount interest because of the potential health implications from human exposure. Only in the past two decades have chlorinated ethenes been demonstrated to biodegrade in the natural environment, and knowledge of their specific transformation mechanisms continues to evolve. In comparison to petroleum hydrocarbons, chlorinated ethenes are much more recalcitrant to biodegradation processes. They have been shown to biodegrade under limited circumstances, and the extent and rates of transformation are usually restricted by environmental and microbiological factors. Nevertheless, there is great interest in exploiting microbiological processes for the bioremediation of chlorinated solvent contaminated sites.

Natural Attenuation

Natural attenuation, also referred to as intrinsic remediation, is a remedial approach that relies on natural attenuation processes to remediate soil and groundwater at contaminated sites. The U.S. EPA defines "natural attenuation processes" to include (3):

"a variety of physical, chemical, or biological processes that, under favorable conditions, act without human intervention to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in soil or groundwater. These in-situ processes include biodegradation; dispersion; dilution; sorption; volatilization; radioactive decay; and chemical or biological stabilization, trans*formation, or destruction of contaminants."*

Natural attenuation has distinct advantages and disadvantages in comparison to engineered remediation approaches (4). The potential advantages of natural attenuation are: (1) contaminants can be destroyed or transformed to innocuous end-products; (2) the approach is non-intrusive to the facility; (3) it allows conjunctive use with active remedial technologies; and (4) may have lower overall costs than other remedial approaches. The potential limitations of natural attenuation are: (1) long time frames may be necessary to achieve regulatory action levels; (2) site characterization and long-term monitoring is expected to be more intensive and expensive; (3) site conditions may change over time to become unfavorable to natural attenuation process; and (4) by-products of contaminant transformation may be more toxic than the parent compound.

The implementation of natural attenuation at contaminated sites has evolved from what was once viewed as a "no action" or "walk-away" approach to a remedial strategy that has been increasingly accepted by the scientific and regulatory communities when used in conjunction with source control and long-term monitoring. Natural attenuation processes can typically occur at all sites, but to varying degrees of effectiveness. Hence, comprehensive measures through site-specific characterization data and analysis have been recommended by the U.S. EPA to demonstrate the potential efficacy of natural attenuation at contaminated sites (3); these "lines of evidence" include:

- Historical trends demonstrating decreased contaminant mass and/or concentration in soil and groundwater.
- Indirect chemical and geochemical data demonstrating active natural attenuation process are occurring at the site, i.e. the presence of daughter compounds and depletion of electron donors or electron acceptors.
- Data from microcosm studies demonstrating that indigenous microorganisms are capable of degrading the contaminants of concern.

Biological Transformation Pathways

Bouwer et al. (5) were the first to demonstrate that chlorinate ethenes could be transformed by microorganisms present in the subsurface environment. In the two decades since Bouwer et al.'s findings, much has been learned about how chlorinated ethenes biodegrade. To date, chlorinated ethenes are known to biologically degrade via (i) reductive dechlorination where the chlorinated ethene is used as a primary substrate, termed as dehalorespiration (6-8); (ii) aerobic and anaerobic oxidation of the less chlorinated intermediate compounds, DCE and VC (9-11); and (iii) cometabolic pathways (12-14). These transformation pathways and the conditions under which they are applicable are illustrated in Figure 1. Dehalorespiration is widely accepted as the

predominant transformation pathway for the more highly chlorinated ethenes and will be discussed in detail in subsequent sections.

The less oxidized chlorinated ethenes DCE and VC are known to biodegrade by direct oxidation under both aerobic and anaerobic conditions. The chlorinated ethene serves as an electron donor and is coupled with the reduction of compounds such as oxygen, sulfate, iron(III), and manganese(IV), which act as electron acceptors. The microbially mediated reactions lead to the mineralization of the chlorinated ethene to CO_2. Under aerobic conditions with oxygen as the sole electron acceptor, Davis and Carpenter (9) investigated the aerobic biodegradation of ^{14}C-labeled VC in microcosms. They found that 99% of the labeled VC was degraded after 108 days, of which 65% was mineralized to $^{14}CO_2$. In several studies by Bradley and Chapelle (10, 11, 14-17), the microbial mineralization of VC or DCE was investigated under varying terminal electron-accepting conditions (aerobic, Mn(IV)-, Fe(III)-, and SO_4-reducing, and/or methanogenic conditions). They found that VC mineralization decreased as conditions became more reduced, i.e. mineralization was greatest and lowest in the aerobic and methanogenic microcosm treatments, respectively (11). In the case of DCE mineralization, recovery of [1,2-^{14}C] DCE as $^{14}CO_2$ did not vary considerably between methanogenic and Fe(III)-reducing conditions (14), however, microbial mineralization of DCE was significant among aerobic and Mn(IV)-reducing conditions (15, 16). Overall, VC mineralization was greater than that of DCE under all of the terminal electron accepting processes tested.

Chlorinated ethenes are also known to biodegrade via cometabolism. In this process, microorganisms gain energy for growth from the metabolism of a primary substrate other than chlorinated ethenes, yet chlorinated ethenes are biodegraded by enzymes and cofactors, which serve as a catalyst in a process that the metabolizing organism derives no known benefit from (55). Cometabolism is carried out in an accidental or incidental manner as the co-metabolizing organisms undergo their normal functions. The process can occur under either aerobic (with the exception of PCE) or anaerobic conditions resulting in oxidation or reduction, respectively, of the chlorinated ethene. Cometabolism is thought to occur at many chlorinated solvent sites, however such transformations are slow and incomplete thereby limiting its application as a successful natural attenuation strategy (13).

Reductive Dechlorination (Dehalorespiration)

Reductive dechlorination is a reaction where the chlorinated ethene serves as an electron acceptor (and not as an organic carbon source typically associated with the biodegradation of petroleum hydrocarbons) as chlorine atoms are sequentially replaced with hydrogen atoms to yield a step-wise transformation of PCE to TCE to DCE to VC, and ultimately to the innocuous end-products ethane and ethene. Vogel et al. (6) were the first to illustrate the pathways for the complete reductive dechlorination of PCE to ethene (Figure 2). For many years, it was thought that reductive dechlorination was restricted to cometabolic mechanisms, however beginning in the early 1990s, researchers demonstrated that microorganisms can dechlorinate in a respiratory process (8). These

microorganisms, known as dehalorespirators, use the chlorinated ethene as a primary growth substrate, and derive energy and growth from the reaction.

Since reductive dechlorination is an oxidation-reduction process and is based on fundamental thermodynamic principles, the propensity for reduction of the chlorinated ethene decreases as the degree of chlorination decreases (18, 19). The highly chlorinated ethenes, PCE and TCE, have greater oxidation levels and are more susceptible to reduction. VC is the least-oxidized/most-reduced among the chlorinated ethenes (1 chlorine atom), and may be more prone to undergo aerobic or anaerobic oxidation than reductive dechlorination. Therefore, biodegradation rates are typically higher for the highly chlorinated compounds relative to those that are less chlorinated (19). Suarez and Rifai (20) performed a comprehensive review of the published first- and zero-order decay rates obtained from 138 field and laboratory chlorinated solvent studies and compiled the results into a series of tables; in addition to transformation rates, the review also includes Michaelis-Menton kinetic parameters and the biodegradability of chlorinated solvents under different transformation pathways. Haston and McCarty (21) conducted a kinetic analysis on an anaerobic mixed culture seeded with aquifer sediment from an PCE-contaminated site in Victoria, TX, and obtained results suggesting that zero-order kinetics may be more appropriate for chlorinated ethene concentrations above the few micromolar or low milligram per liter range.

The majority of reductive dechlorination studies have been conducted on mixed cultures containing a consortium of microorganisms. Several strains of PCE dehalorespirators have been isolated and include *Dehalobacter restrictus* (22), *Dehalospirillum multivorans* (23), *Dehalococcus ethenogenes* (24), and *Desulfitobacterium* (25). Of these, only one pure culture, *Dehalococcus ethenogenes*, can carry out the complete transformation of PCE to ethene (24). The remainder of the isolates produces *cis*-DCE as the end product.

Partial dechlorination of PCE or TCE to the intermediate products DCE and VC has been frequently observed in field and laboratory studies (6, 18, 26-29). Of the three possible DCE isomers (*cis*-DCE, *trans*-DCE, and 1,1-DCE), *cis*-DCE is the most commonly encountered intermediate product of reductive dechlorination (19, 56). Reasons for the accumulation of intermediate products may be due to less favorable oxidation-reduction conditions, depletion of key nutrients, or the lack of competent dehalorespirators in completing reductive dechlorination to ethene (13). Odom et al. (27) proposed that dehalorespirators capable of transforming PCE to *cis*-DCE are more common than those that can dechlorinate *cis*-DCE to ethene, suggesting that more than type of dehalorespirator is involved in the complete reductive dechlorination observed at some chlorinated solvent contaminated sites. Flynn et al. (30) have found through analysis of 16S rRNA gene sequencing, changes in community composition as reductive dechlorination shifted from PCE to *cis*-DCE/VC biodegradation, thereby supporting that that a consortium of specialized dehalorespirators is involved in complete reductive dechlorination. In the context of the environmental restoration of chlorinated solvent contaminated sites, reductive dechlorination can only be considered a success when complete dechlorination of PCE to ethene has been achieved, as VC poses a greater

health risk and is the only proven carcinogen among the chlorinated ethenes (2). Overall, the efficiency of reductive dechlorination has exhibited wide variability among different contaminated sites and even within locations at individual sites.

Role of Dissolved Hydrogen

Dehalorespiration requires not only the presence of competent microorganisms, but also the appropriate quantity and quality of electron donors, which serve as the driving force for dehalorespiration. A variety of electron donors have been shown to sustain reductive dechlorination (22, 23, 25, 26, 29, 31-33), however only recently, it has been recognized that dissolved hydrogen is the actual electron donor in dehalorespiration (31, 32, 34). The electron donors typically used in field and laboratory studies, e.g. acetate, lactate, benzoate, methanol, propionate, formate, etc., serve as the precursors to dissolved hydrogen generation via fermentation. Obligate proton reducers are required to ferment organic substrate present in the subsurface environment to waste products of acetate, formate, dissolved hydrogen, and carbon dioxide (35). After fermentation, dissolved hydrogen becomes available for subsequent use by other microorganisms, such as methanogens and dehalorespirators. This syntrophic relationship of hydrogen producers and consumers is known as interspecies hydrogen transfer. Dehalorespiration relies on the presence of fermentable organic substrates that produce dissolved hydrogen.

In addition to the quality of an electron donor, the quantity needs to be addressed as well. Since the dissolved hydrogen produced from the fermentation of organic substrates can be used by a variety of microorganisms (e.g. methanogens and dehalorespirators), it is important to consider the competition for dissolved hydrogen when assessing the potential for dehalorespiration (13). Researchers have used the Monod model to examine the uptake of dissolved hydrogen by competing bacteria groups. The Monod model is based on microbial growth under a limiting substrate (e.g. dissolved hydrogen) and is expressed as:

$$\mu = \hat{\mu} \frac{S_S}{K_S + S_S} \qquad \text{(Equation 1)}$$

where μ is the specific growth rate, û is the maximum specific growth rate, S_s is the substrate concentration, and K_s is the half-saturation constant (36). The parameter K_s gives an indication of how rapidly μ approaches û. A lower K_s suggests that a microorganism will reach its maximum specific growth rate at a lower substrate concentration than another microorganism with a higher K_s, and hence are better scavengers when competing for the same limiting substrate.

Smatlak et al. (34) compared the kinetics of dissolved hydrogen use by methanogens and dehalorespirators and obtained Monod-half saturation constants, K_s, of approximately 1.0 and 0.1 mM H_2 for methanogens and dehalorespirators, respectively. Their results suggest that dehalorespirators are better scavengers for dissolved hydrogen than methanogens, and that the choice of an electron donor that ferments to release dissolved hydrogen at slow, steady, and low levels, such as propionate or butyrate, would favor dehalorespirators over methanogens in the competition for hydrogen.

Natural Attenuation Indicator Parameters

Natural attenuation, as a remedial strategy for chlorinated solvent-contaminated groundwater requires that favorable microbiological and environmental conditions exist at a site; these generally include the presence of dehalorespirators capable of biodegrading the target compounds, suitable concentrations of electron donor (dissolved hydrogen or organic compounds which via fermentation generate dissolved hydrogen), a favorable oxidation-reduction potential, an organic carbon source, vitamins and nutrients, and an appropriate pH, and temperature (4, 37). Deficiencies in any of these conditions may result in either no dechlorination activity or partial dechlorination at a site thereby limiting the implementation of natural attenuation as a feasible remedial option.

In order to evaluate if natural attenuation processes are occurring at a site, detailed groundwater characterization is required in support of MNA approval (3). Some positive indicators for reductive dechlorination are (38):

- The presence of PCE daughter compounds.
- Elevated chloride concentration (>2 times background).
- Methane, ferrous iron (> 1 mg/L), and sulfide (> 1 mg/L) production.
- Dissolved hydrogen concentration greater that 1 nM.
- Low dissolved oxygen concentration (< 0.5 mg/L).
- Low oxidation-reduction potential (< 50 mV).

Dehalorespiration occurs as an oxidation-reduction reaction where microorganisms obtain usable energy for growth from the oxidation of dissolved hydrogen, the electron donor. The oxidation-reduction potential (ORP) is an indicator of a system's tendency to accept or give up electrons, and some biological processes can operate only within a given range of ORPs. The ORP may influence the rate of biodegradation, since greater energy-yielding biologically mediated reactions will succeed those of lower energy yield, thereby governing ecological succession (39). In the context of a contaminated site, aerobic bacteria will predominate in environments where the ORP in groundwater is in a range where oxygen is favored as the electron acceptor. As oxygen is depleted and ORP decreases, community succession will proceed to nitrate-, managanese-, iron-, sulfate-reducers, and onwards. The typical ORPs ranges for pertinent oxidation-reduction reactions are shown in Figure 3. The approximate ORPs for the reduction of chlorinated ethenes are: PCE to TCE, 430 mV; TCE to DCE, 420 mV, DCE to VC, 310 mV; and VC to ethene, 380 mV (39). Therefore, if sulfate-reduction or methanogenesis is occurring, the ORP conditions are suitable for dehalorespirators to obtain energy through reductive dechlorination.

The ORP parameter assumes thermodynamic equilibrium of the groundwater system, which is generally not the case (39). Chapelle et al. (40) have found an alternative method to evaluating oxidation-reduction processes, dissolved hydrogen measurement, to be superior to traditional ORP measurement. The premise of the dissolved hydrogen method is the hydrogen-utilization efficiency of a microorganism. Different terminal electron-accepting processes will have different hydrogen utilization efficiencies, and

thus maintain characteristic dissolved hydrogen concentration ranges (Table 2). Given that dissolved hydrogen is consumed as rapidly as it is produced, iron-reducers which are more efficient than methanogens at utilizing dissolved hydrogen can maintain lower steady-state dissolved hydrogen concentrations. Chapelle et al. note that this method is most reliable when concentrations of electron-acceptor and final products (e.g. sulfate and sulfide) agree with dissolved hydrogen results. Smatlak et al. (34) found that about 1 nM of dissolved hydrogen is required to support growth of dehalorespirators, so dechlorination can be expected in the sulfate-reducing and methanogenic dissolved hydrogen ranges.

Enhanced Bioremediation

The U.S. EPA acknowledges that environmental conditions favorable to intrinsic bioremediation of chlorinated solvent contaminated sites within a reasonable time frame will occur only in limited circumstances (3). Under conditions where natural attenuation alone will not meet the site remediation objectives of a contaminated site, the U.S. EPA's Monitored Natural Attenuation (MNA) directive recommends active remediation, or source control, to be used in conjunction with long-term performance monitoring.

The subsurface conditions at chlorinated solvent contaminated sites can be viewed as heterogeneous systems that may have fluctuating influxes of oxygen and organic carbon, aerobic and anaerobic microenvironments, and variable distributions of dehalorespirators. In-situ engineered enhanced bioremediation strategies such as biostimulation and bioaugmentation have emerged as technologies to address sites where environmental and microbiological conditions limit the implementation of natural attenuation. Biostimulation provides electron donors and nutrients to the impacted zone in attempts to stimulate the biological activity of indigenous dehalorespirators. Bioaugmentation involves inoculating the impacted zone with exogenous dehalorespirators, along with any required nutrients, to complement or replace native dehalorespirators in biodegrading the target contaminants. Field and laboratory studies conducted to assess the impacts of biostimulation (28, 29, 41-44) and bioaugmentation (29, 45, 46) for the treatment of chlorinated ethenes have encountered varying degrees of success.

Biostimulation

Natural attenuation processes at chlorinated solvent contaminated sites may be limited by the amount of electron donors and nutrients available to indigenous dehalorespirators, and spatial variations in aerobic and anaerobic conditions. Under these circumstances, supplying the missing components may passively stimulate dehalorespiration and address the key inadequacies preventing complete reductive dechlorination. In addition to stimulating dehalorespirators, the introduction of electron donors and nutrients can potentially stimulate the activity of other microorganisms that carry out syntrophic roles or depress ORP (by population succession) to ranges amenable for reductive dechlorination (47).

Dehalorespiration requires the presence of adequate quantities of suitable electron donors. As discussed earlier, numerous studies have demonstrated that many organic electron donors can support reductive dechlorination, however dissolved hydrogen is the

actual electron donor used by dehalorespirators. Kinetic studies of reductive dechlorination have indicated that slowly fermentable substrates are most effective for sustaining reductive dechlorination. Yang and McCarty (48) found that steady-state dissolved hydrogen levels between 2 and 11 mM were optimum for dehalorespirators to out-compete methanogens and homoacetogens; this can be achieved in the field by either selecting slowly fermentable electron donors or by adjusting the delivery rate to maintain dissolved hydrogen concentrations within the target range. Wiedemeier et al. (4) recommend that organic substrate concentrations should be 25 to over 100 times greater than chlorinated solvent concentrations to achieve complete reductive dechlorination. This will provide sufficient amounts of organic carbon to drive the system anaerobic (hence, lower the ORP), and compensate for hydrogen utilization efficiencies resulting from competition of dissolved hydrogen among dehalorespirators and other microorganisms.

The application of proprietary products that release organic electron donors into contaminated aquifers has become increasingly popular for bioremediation approaches. The most well documented is Hydrogen Release Compound (HRC™) manufactured by Regenesis of San Juan Capistrano, CA. The product is a polylactate ester that slowly degrades to lactic acid upon hydration. The lactic acid subsequently degrades to produce dissolved hydrogen. HRC™ is typically pressure injected into the treatment area, but since it is also available in a semi-solid form, the product can be placed within a well for passive flow-through treatment. Koenigsberg (co-inventor) and Farone (49) highlight the application of HRC™ at a field site where 240 pounds of HRC™ were injected into 12 delivery points in a 60 ft^2 area. After eight months, PCE mass was reduced by 80%. The authors do not provide data for ethene production, however, 160 grams PCE was converted to approximately 35 grams PCE, 0.8 grams TCE, 25 grams DCE, and 0.02 grams VC.

Newell et al. (50) suggest direct hydrogen addition (as opposed to organic compounds that ferment to produce dissolved hydrogen) into contaminated aquifers to stimulate reductive dechlorination, and describe preliminary pilot testing involving a two-well extraction/injection system. Extracted groundwater can be saturated with hydrogen at the surface and injected upgradient of the test area. Only considering hydrogen costs and assuming 100% hydrogen utilization by dehalorespirators, they estimate treatment costs of less than $0.000001 per liter of groundwater contaminated with 1 mg/L PCE.

In addition to electron donors, deficiencies of available vitamins and nutrients can also limit dehalorespiration; such nutrients may include organic carbon, nitrogen, phosphorous, amino acids, trace elements, and vitamin B_{12}. The complexity of undefined microbial communities makes the understanding of specific nutritional requirements difficult. Yeast extract, a complex substrate, has been shown to increase dechlorination rates to those greater than of simpler substrates (27). Nutrient amendments to a contaminated aquifer may also benefit reductive dechlorination by stimulating the activity of non-dehalorespirators, which for example, prevent the accumulation of an inhibitory product (47). Maymo et al. (51) investigated the nutritional requirements of an anaerobic enrichment culture competent at transforming PCE to ethene. Their results

suggested that the dehalorespiring culture was dependent on other microorganisms to satisfy some nutritional requirements, and that yeast extract and vitamin B_{12} played unknown roles in dechlorination activity. Vitamin B_{12} was also shown to be a factor in sustaining dehalorespiration by *Dehalospirillum multivorans* (23).

An engineering challenge in applying biostimulation in the field is ensuring adequate mixing of nutrients, contaminants, and dehalorespirators. The nutrient medium is typically mixed at the surface and injected into the contaminated aquifer. Once introduced into the subsurface, the medium has a tendency to displace contaminated water near the injection point, thereby preventing mixing of the target contaminants and nutrient medium. The potential for this can be minimized if the aquifer sediments exhibit strong desorption properties where sorbed contaminants will be released into aqueous solution to maintain equilibrium (52). An injection and recovery well configuration can be implemented upgradient and downgradient, respectively, of the impacted zone, thus allowing for treatment in the aquifer between the well points. An alternative configuration is to locate injection and recovery within the same well. Groundwater is recirculated in the subsurface, thereby eliminating the need to transport contaminated water above ground. Another consideration is biofouling near the injection point. Since substrate and nutrient concentrations will be highest near the points of introduction, excessive microbial growth is likely to occur at these locations. To alleviate this, the nutrient medium can be delivered in a series of alternating pulses to distribute the microbial growth away from the injection point.

Bioaugmentation

The nature and distribution of indigenous dehalorespirators may also limit natural attenuation of a chlorinated solvent contaminated site. The appropriate consortium of dehalorespirators capable of transforming PCE to ethene may not exist in the contaminated aquifer. In addition, differences in the hydrogeological properties of an aquifer may alter the spatial distribution of dehalorespirators by channeling chlorinated ethenes, electron donors, and other nutrients away from dehalorespirators, or conversely, bring oxygenated recharge water to the anaerobic microorganisms. Bioaugmentation of the contaminated zone with exogenous dehalorespirators competent of dechlorinating PCE to ethene may facilitate an MNA remedy.

A considerable number of bioaugmentation studies have been conducted to investigate the bioremediation of petroleum hydrocarbons (53). In contrast, there have been only a few bioaugmentation studies conducted for the reductive dechlorination of chlorinated solvents (29, 46) and they have both used cultures originating from the Pinellas plant site located in Largo, FL. Harkness et al. (29) performed laboratory soil column studies to assess the effects of amendments made to aquifer sediments from Dover Air Force Base (AFB), DE. For 165 days into the study, partial dechlorination of TCE to *cis*-DCE was observed even after various attempts were made to stimulate further dechlorination such as using several electron donors, adding a vitamin solution, and increasing yeast extract concentrations. However, after an amendment of a 4% inoculum of soil slurry containing competent dehalorespirators from the Pinellas plant site, dechlorination of TCE to predominantly ethene was achieved after 28 days.

As a follow-up to the Harkness soil column study, Ellis et al. (46) conducted a field-scale bioaugmentation study at Dover AFB. The Pinellas culture was grown in a 20-L container on a mineral salts medium, and scaled-up to 180 L through a series of 10% transfers. Since the impacted zone was a naturally aerobic aquifer, lactate was injected to drive the aquifer anaerobic prior to bioaugmentation. The site was inoculated with about 35 g (dry) of exogenous bacteria via an upgradient injection well on day 269 and day 284 of the study; VC and ethene first appeared on day 360 and 367 of the study. After about 625 days into the study, 75 to 80% of the initial TCE and *cis*-DCE were recovered as ethene.

Steffan et al. (45) conducted a bioaugmentation pilot study using *Burholderia cepacia* ENV435, a specialized strain that cometabolically degrades TCE under aerobic conditions. The cells were grown in a 750-L fermentor at the surface, and were delivered into the treatment zone by a series of six injection wells located in a 4.6 m by 12 m test plot. A recovery well located 12 m downgradient recirculated groundwater back to the treatment zone. The study demonstrated rapid degradation of TCE, DCE, and VC in the test plot, by as much as 78% (of the initial total mass of TCE, DCE, and VC) within 2 days.

For sites with competent dehalorespirators but low populations or poor distribution, an alternative is to inoculate the impacted zone with microorganisms native to the site. Enrichment cultures can be maintained ex-situ and once suitable populations of competent dehalorespirators are grown, the culture can be re-introduced into the contaminated zone. Although previous work is not known to have been conducted, there are apparent economic and environmental advantages to inoculating the site with indigenous microorganisms. In addition, ex-situ fermentation may be superior to biostimulation since there is better control of maintaining optimum growth conditions.

The same fundamental engineering considerations of biostimulation exists for bioaugmentation. Adequate mixing in the treatment zone is essential to assure direct contact of the contaminant and introduced catalyst, and aquifer plugging near the injection points can lead to poor distribution of introduced bacteria into the treatment zone. Niche adjustment is also an important consideration in a successful bioaugmentation approach. Exogenous dehalorespirators will need to compete with indigenous microorganisms for common substrates and nutrients in a foreign environment. Since there will usually be many uncertainties regarding microbial ecology and its potential response to endogenous microorganisms, the "brute force" approach can sometimes be effective (54). In this approach, large numbers of bacteria are added with expectations that the microorganisms will perish shortly thereafter. Another strategy is that microorganisms are added with the goals of long-term survival, where the introduced dehalorespirators achieve growth from the biodegradation of chlorinated ethenes. Since bacteria mobility may be limited once introduced into the aquifer, a permeable biological barrier design with groundwater recirculation through the treatment zone may be an effective implementation approach.

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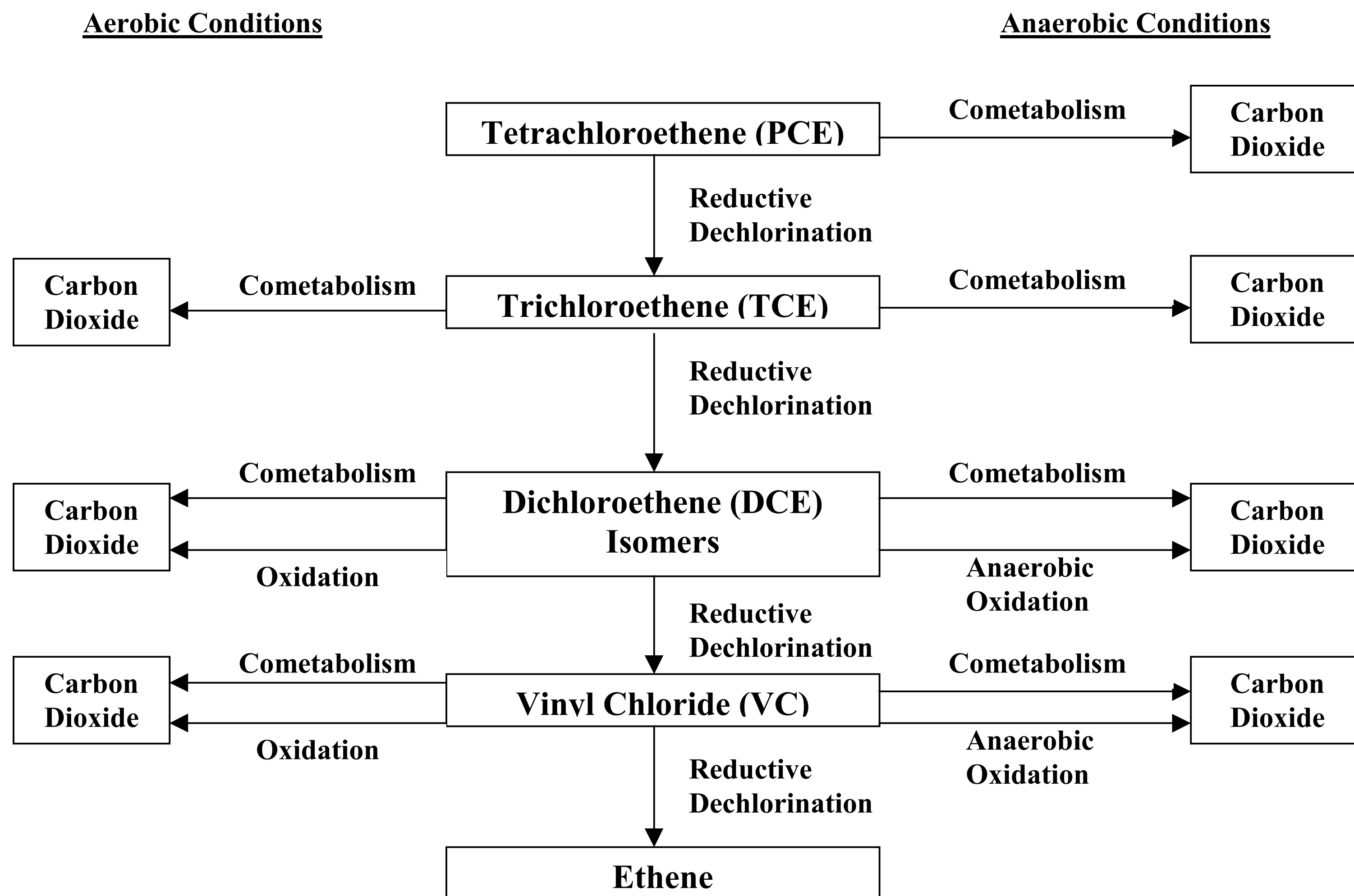

Figure 1. Biological pathways for chlorinated ethenes degradation. Adapted from Smith and Vogel (2000).

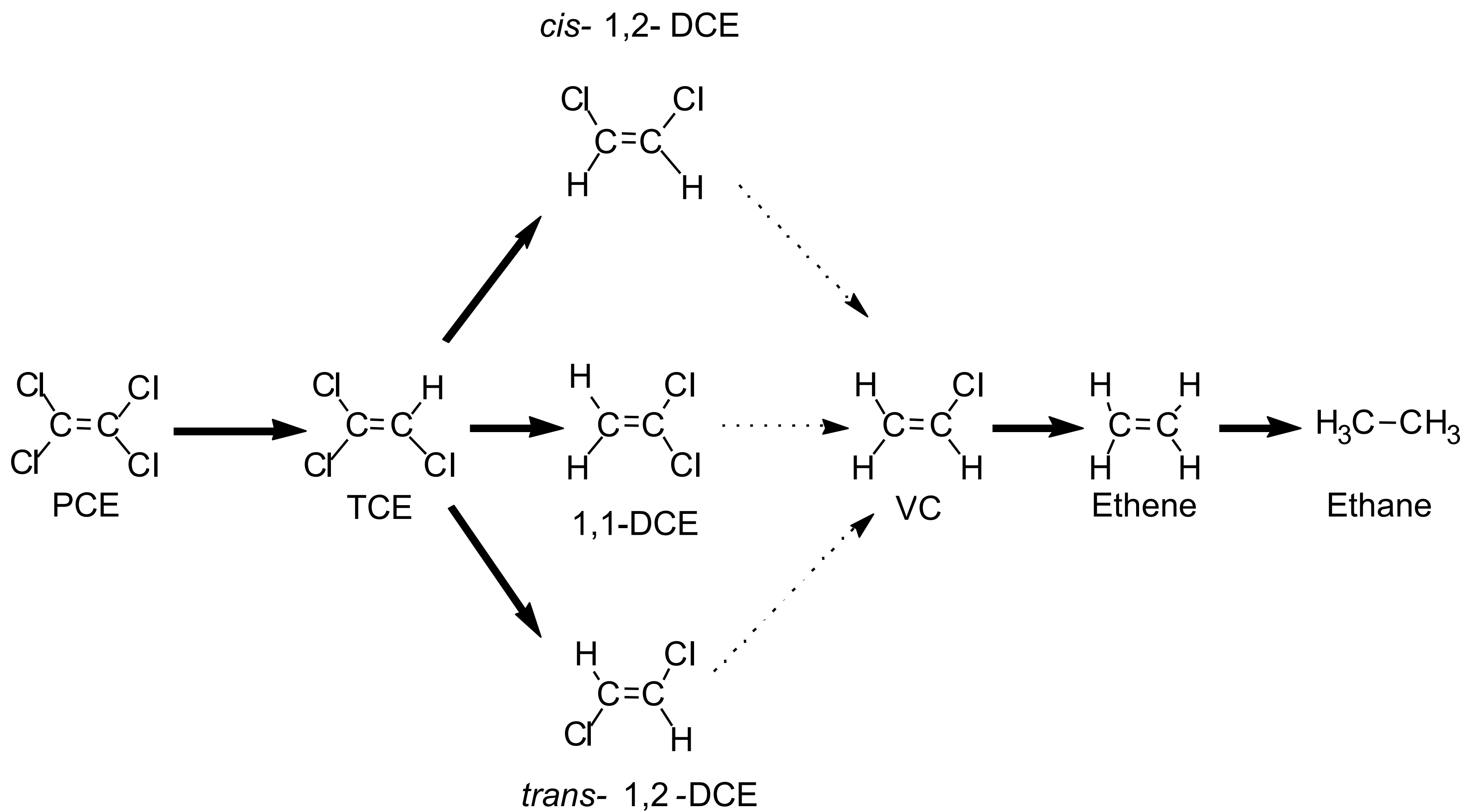

Figure 2. Complete reductive dechlorination. Adapted from Vogel et al. (1987).

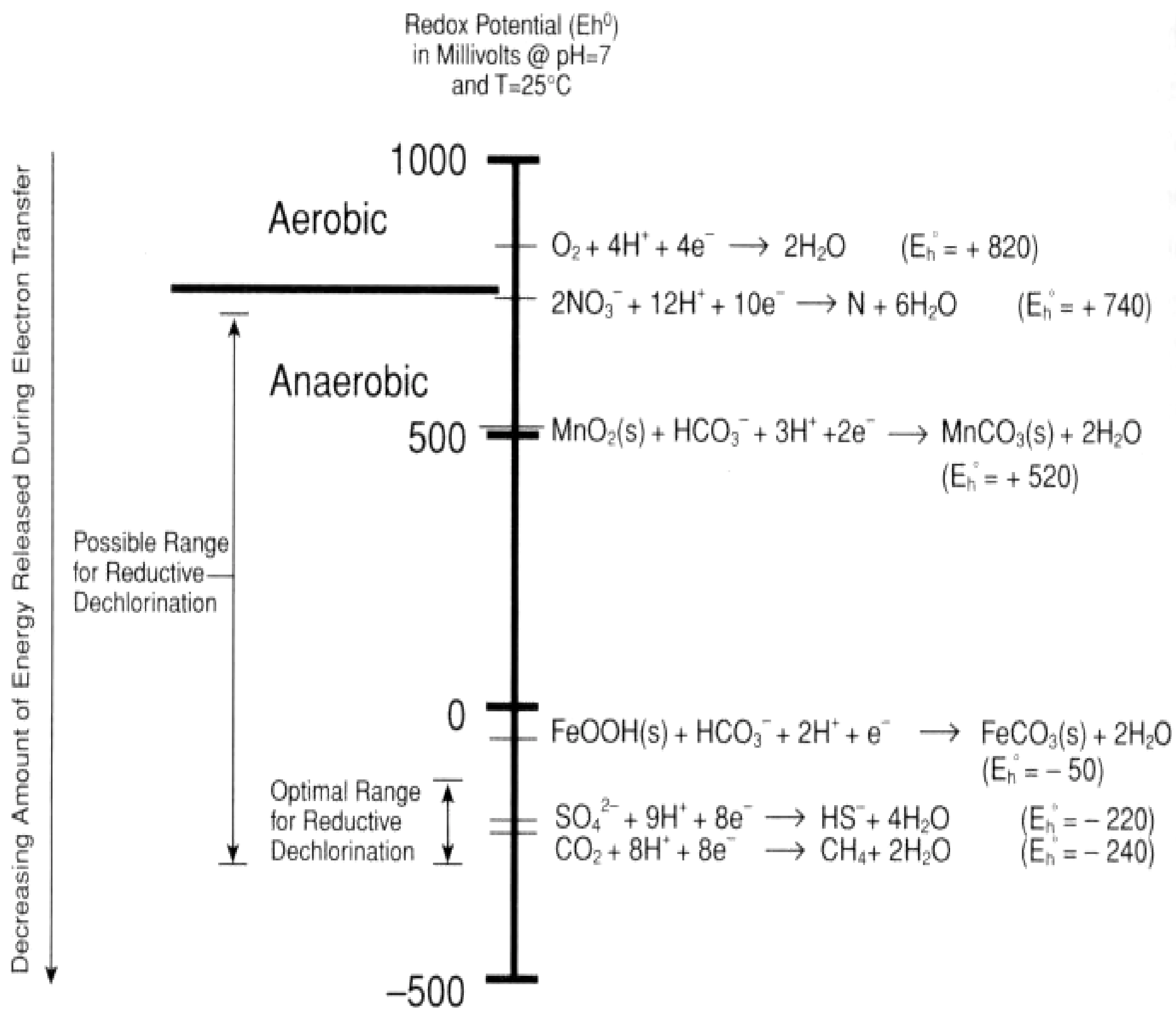

Figure 3. Oxidation-reduction potentials for selected reactions and ranges for reductive dechlorination. Taken from Wiedemeier et al. (1999).

Table 1. Properties of chlorinated ethenes.

Characteristic/Property	PCE	TCE	*cis*-1,2-DCE	VC
Molecular Formula	C_2Cl_4	C_2HCl_3	$C_2H_2Cl_2$	C_2H_3Cl
CAS Registry No.	127-18-4	79-01-2	156-59-2	75-01-4
Approx. Annual World Production[a] (metric tons)	1,100,000	600,000	NA	NA
MCLG[b] (µg/L)	0	0	70	0
MCL[b] (µg/L)	5	5	70	2
Human Carcinogenity[c]	Probable	Probable	Probable	Known
Molecular Weight (g/mol)	165.83	131.39	96.94	62.50
Density[d] (g/cm^3)	1.626	1.46	1.28	0.91
Water Solubility[d] (mg/L at 25°C)	150	1,100	800	1.1
Vapor Pressure[e] (mm at 25°C)	14	60	200	2,660
Henrys' Law Constant[f] (dimensionless at 24.8°C)	0.723	0.392	0.167	1.137
Octanol/Water Partition Coefficient[d], log K_{OW}	2.88	2.29	1.86	1.38
Sorption Partition Coefficient[d], log K_{OC}	2.56	2.03	1.69	1.76
Sorption Partition Coefficient[d], log K_{OM}	2.32	2.78	NA	NA

NA - Data not available.
[a] Source: Schwarzenbach et al. (1993).
[b] MCLG- Maximum Contaminant Level Goal, MCL- Maximum Contaminant Level; U.S. EPA National Primary Drinking Water Contaminant Standards. Source: American Water Works Association (1999).
[c] Source: American Water Works Association (1999).
[d] Source: Mackay, D., et al. (1993).
[e] Source: Fetter, C.W. (1999).
[f] Source: Gossett, J.M. (1987).

Table 2. Terminal electron-accepting process for given range of dissolved hydrogen concentrations. Data from Chapelle et al. (1996).

Terminal Electron-Accepting Process	Dissolved Hydrogen Concentration	
	nM	µg/L
Denitrification	< 0.1	$< 0.2 \times 10^{-3}$
Iron (III) Reduction	0.2-0.8	$0.4\text{-}1.6 \times 10^{-3}$
Sulfate Reduction	1-4	$2.0\text{-}8.0 \times 10^{-3}$
Methanogenesis	5-15	$10\text{-}30 \times 10^{-3}$

Chapter 2

Evaluation of Enhanced Bioremediation for Reductive Dechlorination of Tetrachlorethene (PCE): Microcosm Study

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Abstract

Laboratory microcosm experiments were conducted to assess the potential for biostimulation and bioaugmentation as source reduction measures in support of a monitored natural attenuation remedial strategy at Naval Amphibious Base (NAB) Little Creek. Previous work with laboratory microcosms conducted under simulated natural (unamended) conditions has demonstrated that indigenous dehalorespirators were capable of partial dechlorination of tetrachloroethene (PCE) to *cis*-dichloroethene (*cis*-DCE). This study attempts to achieve complete reductive dechlorination with amendments to static microcosms to test the hypotheses that nutrient-limited or microorganism-limited conditions exist in aquifer sediments obtained from the site. The enhanced bioremediation experiments were comprised of nutrient-amended microcosms receiving additions of electron donors, mineral medium, or anaerobic digester supernatant, and dechlorinating culture-amended microcosms were inoculated with a culture capable of transforming PCE to ethene. Reductive dechlorination in the nutrient-amended microcosms proceeded to *cis*-DCE over a 260-day study period, at slightly higher rates than in experiments conducted with aquifer sediments from the same location under natural conditions. Inoculation of aquifer sediments with a small amount of dechlorinating culture initiated rapid transformation of PCE to vinyl chloride (VC) by day 18 of the study. Zero-order rates of PCE dechlorination in unamended, propionate-, formate-, mineral medium-, digester supernatant-, and dechlorinating culture-amended microcosms were 0.24, 0.750, 1.30, 0.339, 0.177, and 1.75 μM/day, respectively. The results of this study suggest that an engineered biostimulation approach alone may not be as beneficial for PCE source reduction at NAB Little Creek, than bioaugmentation with competent dehalorespirators, along with the inclusion of supplemental nutrients which would be available to stimulate dechlorination activity of both indigenous and introduced microorganisms.

Introduction

In the last 20 years, the biodegradation of chlorinated ethenes has evolved into an intense area of research driven by continued successes in the field and laboratory. The favorable physical properties of chlorinated ethenes have led to their widespread industrial application. They are used for metals and electronics degreasing, dry cleaning, textile processing, and serve many other purposes. As a consequence of their popularity, chlorinated ethenes such as tetrachloroethene (PCE) and trichloroethene (TCE) have been released into the environment from improper handling, storage, and disposal practices, and are among the most frequently reported groundwater contaminants. All chlorinated ethenes are either known or probable carcinogens (1).

The scientific understanding of how chlorinated ethenes degrade in the environment is of paramount interest because of the potential health implications from human exposure. Only in the past two decades have chlorinated ethenes been demonstrated to biodegrade in the natural environment, and knowledge of their specific transformation mechanisms continues to evolve. In comparison to petroleum hydrocarbons, chlorinated ethenes are much more recalcitrant to biodegradation processes. They have been shown to biodegrade under limited circumstances, and the extent and rates of transformation are usually restricted by environmental and microbiological factors. Nevertheless, there is great interest in exploiting microbiological processes for the bioremediation of chlorinated solvent contaminated sites.

To date, chlorinated ethenes are known to biologically degrade via (i) reductive dechlorination where the chlorinated ethene is used as a primary substrate, termed as dehalorespiration (2-4); (ii) aerobic and anaerobic oxidation of the less chlorinated intermediate compounds, dichloroethene (DCE) and vinyl chloride (VC) (5-7); and (iii) cometabolic pathways (8-10). Dehalorespiration is widely accepted as the predominant mechanism for the transformation of the highly chlorinated ethenes, PCE and TCE. In this process, the chlorinated ethene serves as an electron acceptor as chlorine atoms are sequentially replaced by hydrogen atoms to transform PCE to TCE to DCE to VC to the innocuous end products ethene and ethane. As reductive dechlorination of PCE proceeds, the oxidation-reduction reactions become less thermodynamically favorable and electron donors are depleted by dehalorespirators, frequently leading to the accumulation of the intermediate products *cis*-DCE and VC (2, 11-13). Partial dechlorination may also be attributed to a deficiency in the appropriate consortium of dehalorespirators capable of transforming PCE completely to ethene (2, 4, 15). The efficacy of reductive dechlorination has exhibited wide variability among different contaminated sites and even within locations at individual sites. In the context of the environmental restoration of chlorinated solvent contaminated sites, reductive dechlorination can only be considered a success when complete dechlorination of PCE to ethene has been achieved, as VC poses a greater health risk and is the only proven carcinogen among the chlorinated ethenes (1).

Natural attenuation, or intrinsic bioremediation, as a remedial strategy for chlorinated solvent-contaminated groundwater requires that favorable microbiological and environmental conditions exist at a site; these generally include the presence of

dehalorespirators capable of biodegrading the target compounds, suitable concentrations of electron donor (dissolved hydrogen or organic compounds which via fermentation generate dissolved hydrogen), a favorable oxidation-reduction potential, an organic carbon source, vitamins and nutrients, and an appropriate pH and temperature. Deficiencies in any of these conditions may result in either no dechlorination activity or partial dechlorination at a site thereby limiting the implementation of natural attenuation as a feasible remedial option. Under circumstances where natural attenuation alone will not meet the site remediation objectives of a contaminated site, the U.S. EPA's Monitored Natural Attenuation (MNA) directive recommends active remediation, or source control, to be used in conjunction with long-term performance monitoring (16).

In-situ engineered enhanced bioremediation strategies such as biostimulation and bioaugmentation have emerged as technologies to address sites where environmental and microbiological conditions limit the implementation of natural attenuation. Biostimulation provides electron donors and nutrients to the impacted zone in attempts to stimulate the biological activity of indigenous dehalorespirators. Bioaugmentation involves inoculating the impacted zone with exogenous dehalorespirators, along with any required nutrients, to complement or replace native dehalorespirators in biodegrading the target contaminants. Field and laboratory studies conducted to assess the impacts of biostimulation (14, 17-21) and bioaugmentation (14, 22, 23) for the treatment of chlorinated ethenes have encountered varying degrees of success.

Site Description and Characterization

The feasibility of MNA as a remediation strategy is being evaluated at Naval Amphibious Base (NAB) Little Creek, located in Virginia Beach, VA. The base is an active military facility that provides logistic and support services for amphibious warfare operations. NAB Little Creek was commissioned in 1945 and covers over 2,000 acres of land. The location of the site is shown in Figure 1. The area of concern is a former laundry and dry cleaning facility located on NAB Little Creek. It is estimated that approximately 200 gallons of PCE were dumped into an on-site storm sewer during the period of 1973 to 1978 (24). The impacted zone is in an unconfined aquifer of the Columbia Group, primarily comprised of fine to medium sand and some fine clay and silt. The depth to groundwater is approximately 2 m below ground surface (bgs). A 9- to 12-meter confining clay aquitard separates the impacted aquifer from the Yorktown Aquifer, a drinking water source for the region. Groundwater samples collected from the underlying confined aquifer have not detected contaminants above drinking water standards confirming that contamination is limited to the upper aquifer.

A series of conventional and multi-level sampler (MLS) monitoring wells were installed at the site to determine the extent of contamination. Groundwater samples were collected in June 1999 and submitted to a certified commercial laboratory for analysis. The presence of PCE biodegradation products that are not associated with the source discharge, and favorable environmental conditions (Table 1 and Figure 2) elucidate that reductive dechlorination by indigenous microorganisms is occurring at the site (25). Yet, the high concentrations of PCE remaining in the source area, with respect to those of its degradation products, indicate that the extent of these processes may be limited.

Dissolved hydrogen concentrations of groundwater samples collected in June 1999 suggest that sulfate reduction is the predominant terminal electron-accepting process (26) and are above the minimum levels required for dehalorespirator growth (27). However, sulfate and sulfide measurements in the background and contaminated zones do not support the occurrence of active sulfate reduction. Chapelle et al. (26) note that sulfate can be continuously replenished by geologic sources, and sulfides can readily precipitate from solution in the presence of metals, so the observed accumulation of sulfides does not necessarily need to occur for sulfate reduction to be an active process.

The purpose of this study was to investigate the factors that may restrict the complete reductive dechlorination of PCE to ethene, and to assess the potential for biostimulation and bioaugmentation as source reduction measures in support of an MNA approach at NAB Little Creek. Berry, et al. (25) conducted laboratory microcosm experiments under simulated natural (unamended) conditions, and demonstrated that indigenous dehalorespirators were capable of partial dechlorination of PCE to *cis*-DCE. This study attempts to achieve complete reductive dechlorination with amendments to static microcosms to test the hypotheses that nutrient-limited or microorganism-limited conditions exist at the site. The enhanced bioremediation experiments were comprised of nutrient-amended microcosms receiving additions of electron donors, mineral medium, or anaerobic digester supernatant, and dechlorinating culture-amended microcosms were inoculated with a culture capable of transforming PCE to ethene.

Materials and Methods

Aquifer Sampling

The aquifer sediments used in the laboratory microcosm experiments were obtained by split spoon sampling to depths of 2.5 to 3.5 m bgs in the immediate vicinity of well location MLS-12. Aquifer core material was extracted in sterile acetate liners, capped, immediately placed in anaerobic glove bags (AtmosBag; Aldrich Chemical Co., Milwaukee, WI) under a N_2 atmosphere, and transported to the laboratory on ice. The groundwater used in the microcosm experiments were collected from MLS-12-Shallow (2.5 m bgs), transported to the laboratory in 20-L plastic containers, and stored at 4°C until use. The aquifer sediments and groundwater were used for microcosm establishment within 48 hours of sample acquisition.

Chemicals and Solutions

Liquid PCE and TCE (Fisher Scientific, Pittsburgh, PA) were used for preparing stock feed solutions and analytical standards. *Cis*-DCE, 2000 µg/mL in methanol and VC, 200 µg/mL in methanol (Supelco, Bellefonte, PA), were used as analytical standards. Methane, ethane, ethene, and acetylene were obtained as gasses (Scotty II; Supelco) for use as analytical standards. Sodium propionate (Aldrich Chemical Co.) and sodium formate (Fisher Scientific) were used as electron donors. Yeast extract (Sigma Chemical Company, St. Louis, MO) was used as a supplemental nutrient source.

A laboratory-prepared trace metal and mineral salt medium (hereon referred to as mineral medium) (modified from 29) was used in the mineral medium- and dechlorinating culture-amended microcosm experiments, and consisted of: 270 mg/L KH_2PO_4, 350

mg/L K_2HPO_4, 530 mg/L NH_4Cl, 100 mg/L $MgCl_2 \bullet 2H_2O$, 20 mg/L $FeCl_2 \bullet 4H_2O$, 1 mL/L stock trace metal solution, 200 mg/L yeast extract, 120 mg/L $Na_2S \bullet 9H_2O$, and 1,200 mg/L $NaHCO_3$ (pH buffer) in distilled deionized water. The mineral medium was adjusted to pH 7 with NaOH or HCl, and resazurin (1 mL/L) served as a redox indicator. The mineral medium was sparged with deoxygenated N_2 using modified Hungate techniques (30) until anaerobic conditions were attained.

Sludge samples used in the digester supernatant microcosm experiments were obtained from the Pepper's Ferry Regional Wastewater Treatment Plant (WWTP) located in Radford, VA. Fresh sludge was collected from the primary anaerobic digesters in 4-liter jars, purged with N_2, loosely stoppered to accommodate gas evolution, and stored in the dark at 4°C until use. The sludge samples were centrifuged at 3,000 rpm for 1 hour and decanted. The supernatant was filtered through 0.45 µm glass fiber filters (Fisherbrand; Fisher Scientific) prior to microcosm amendment.

Microcosm Preparation

Laboratory microcosm experiments were prepared in an anaerobic chamber under an N_2 atmosphere using modified Hungate techniques and conducted in 15-mL amber bottles (Wheaton, Millville, NJ) containing 7 grams of saturated aquifer sediment and 10.5 mL of aqueous medium. A solids to water ratio of 67.7% (wt./vol.) was maintained to simulate aquifer conditions, and the headspace volume was minimized to approximately 0.5 mL. Prior to microcosm establishment, aquifer sediments were blended to mute the effects of spatial heterogeneities. Each microcosm was spiked with 25 µM PCE via direct injection. The bottles were sealed with Teflon-lined rubber butyl septa and aluminum crimp caps, and incubated at 20°C under quiescent conditions in an inverted position to minimize potential loss of volatile organics. The batch microcosms were prepared in duplicate (propionate-amended microcosms were prepared in triplicate) and sacrificed for each analysis in a 260-day study period.

The enhanced bioremediation experiments were comprised of nutrient-amended microcosms receiving additions of electron donors, mineral medium, or anaerobic digester supernatant, and dechlorinating culture-amended microcosms were inoculated with a culture shown capable of transforming PCE to ethene. The composition of the aqueous medium differed among the separate experiments (Table 2) and consisted of the following: electron donor microcosms were amended with 1 mM propionate or 45 mM formate added to 10.5 mL groundwater and 200 mg/L yeast extract; mineral medium microcosms were amended with 45 mM formate and 10.5 mL mineral medium; digester supernatant microcosms were amended with 45 mM formate and 200 mg/L yeast extract added to 10.5 mL filtered digester supernatant; and the dechlorinating culture microcosms were amended with 45 mM formate and 200 mg/L yeast extract added to 1 mL dechlorinating culture, 4.5 mL mineral medium, 5.0 mL groundwater. The culture used in the dechlorinating culture microcosm experiments was initially obtained from an anaerobic digester at a municipal WWTP and developed by researchers at Bucknell University (28). The experimental microcosms, with the exception of the propionate-amended microcosms, were replenished with electron donors (20 mM formate) approximately 90 and 180 days into the study.

A set of abiotic control microcosms was prepared to distinguish chlorinated ethene losses from biological and physical/chemical processes. The aquifer sediments and groundwater used in the control experiments were sterilized by repeated autoclaving (8 cycles of 15-minutes under pressure at 121°C) and were subsequently spiked with 25 µM PCE.

Analytical Techniques

Chlorinated ethenes (PCE, TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE, and VC) were analyzed in the aqueous phase using a Model 3000 (Tekmar Co., Cincinnati, OH) purge-and-trap concentrator followed by a Model 9001 (Tremetrics Inc., Austin, TX) gas chromatograph (GC) equipped with a Model 1000 Hall detector (Tracor Instruments, Austin, TX) and capillary column (30 m × 0.53 mm, 2.0 µm film thickness, Rtx-Volatiles; Restek Corp., Bellefonte, PA). The oven temperature was held isothermal for 5 min. at 35°C, followed by temperature ramps of 6°C/min. up to 95°C and 25°C/min. up to 200°C.

Methane, ethane, ethene, and acetylene were analyzed by injecting 100 or 150 µL of headspace gas into a Model 5890 (Hewlett Packard, Wilmington, DE) GC equipped with a flame ionization detector and packed column (1.8 m × 2.1 mm, Carbosieve S-III; Supelco). The oven temperature was increased to 125°C, followed with temperature ramps of 25°C/min. up to 175°C and 10°C/min up to 200°C, where it was held isothermal for 10 min. Gas samples were extracted from microcosms using a gas-tight, locking, glass syringe (Hamilton Co., Reno, NV).

Microbial Enumeration

A five-tube MPN procedure was used for the enumeration of dehalorespirators (31). The tubes (40 mm × 18 mm) were inoculated with 1 mL of the appropriate soil dilution containing aquifer sediments obtained from MLS-12-Shallow, filled with groundwater obtained from the site, spiked with 25 µM PCE, 45 mM formate, and 200 mg/L yeast extract, and sealed with Teflon-lined butyl rubber septa and aluminum crimp caps. A dilution series of 10^{-1} to 10^{-9} of each sample was prepared by serially transferring 1 mL of the sediment slurry. The tubes used for the MPN tests were inoculated with 1-mL portions of the appropriate soil dilutions and incubated for 12 weeks at 20°C. A positive reading for dehalorespirators was recorded if PCE biodegradation was observed after the 12-week incubation period. The MPN was calculated from a table based on a 5-tube and 10-fold dilution analysis (31).

Results and Discussion

Microcosm Experiments

In all of the enhanced bioremediation experiments, PCE biodegradation led to the accumulation of *cis*-DCE or VC (Figures 3-6). The *trans*-DCE and 1,1-DCE isomers were not observed which support the findings of other researchers that *cis*-DCE is the predominant DCE isomer to accumulate in reductive dechlorination (32, 33). Headspace gas analysis did not detect ethene or ethane during the course of this study, however, moderate to vigorous methanogenesis was observed in the microcosms. The development of black, iron sulfide precipitates in aquifer sediments were observed

throughout the study and were associated with active sulfate reduction. Methanogenic and sulfate-reducing conditions confirmed that the strictly reduced environment required for reductive dechlorination and fermentation of electron donors prevailed in the microcosms. The initial PCE concentrations measured in the microcosms were approximately 20 to 25% less than the added concentration of 25 μM PCE. Reasons for the lower initial PCE concentrations may be attributed to adsorption of aqueous-phase PCE onto aquifer sediments and to a much lesser extent, aqueous-gas equilibrium partitioning into the headspace. Henry's constant (34) calculations indicate that gas-phase PCE would account for only about 2% of the initial PCE concentration. Abiotic control microcosms (data not shown) measured PCE losses of approximately 20% within 25 days of the study, which supports observations in live microcosms. PCE daughter compounds were not detected in any of the control microcosms, thereby confirming that the dechlorination observed in live microcosms can be associated with biological activity.

The electron donor microcosm experiments were conducted to provide an external source of dissolved hydrogen for use by indigenous dehalorespirators in biodegradation of PCE. A variety of electron donors have been shown to stimulate reductive dechlorination, however there have been conflicting reports on their respective efficiencies. Propionate and formate were selected as the electron donors; these compounds serve as the indirect electron donors to dehalorespiration via fermentation to produce dissolved hydrogen (3, 27, 35). The propionate-amended microcosms (Figure 3a) demonstrated dechlorination of PCE to *cis*-DCE within the first month of the study. Dechlorination beyond *cis*-DCE was not observed throughout the remainder of the experiment (through day 260). Although TCE was not detected, it is probable that TCE was produced and consumed between the time that the microcosms were established and the first sampling event on day 29.

Considerable variability in dechlorination activity was encountered in the formate-amended microcosms (Figure 3b). PCE was transformed to TCE and *cis*-DCE within 2 weeks. Throughout the remainder of the study, PCE was transformed to inconsistent amounts of TCE, *cis*-DCE, and VC, although *cis*-DCE was the predominant end product of the experiment. *Cis*-DCE transformation to an appreciable amount of VC was detected on day 225 of the study. The observed disparities in dechlorination activity may be attributed to the sacrificial nature of microcosm sampling and heterogeneities in aquifer sediments. Although thorough attempts were made to homogenize the aquifer sediments prior to microcosm construction, the presence of clay aggregates and fine- to medium-sized gravel in the samples made complete mixing difficult. The inconsistent degradation patterns presumably highlight the heterogeneous distribution of dehalorespirators in the aquifer sediments used in this study. Individual microcosms containing aquifer solids with high populations of active dehalorespirators exhibited considerably more dechlorination activity. Microcosm construction with larger amounts of aquifer sediments (than the 7 g aquifer sediment used in this study) may have reduced such effects of spatial variations on biodegradation. Soil MPN results for aquifer sediments obtained from MLS-12-Shallow and amended with formate estimated a very low population of native dehalorespirators (90 cells/kg of aquifer sediment), which supports that microorganisms are sparsely distributed in aquifer sediments.

The subsurface conditions at contaminated sites can be viewed as heterogeneous systems that may have fluctuating inflows and outflows of essential nutrients that at times may limit dehalorespiration. Mineral medium microcosms were established to provide an external source of electron donors trace metals and mineral salts that may be required to maintain the health of indigenous dehalorespirators. In addition to stimulating dehalorespirators, the introduction of supplemental nutrients can potentially stimulate the activity of other microorganisms that carry out syntrophic roles (e.g. prevent the accumulation of an inhibitory product) or depress the oxidation-reduction potential to ranges amenable for reductive dechlorination (36). Data from the mineral medium microcosms (Figure 4) show that PCE was converted to mostly *cis*-DCE by day 61. Low levels of VC (up to 1.4 μM) were detected early in the study and are likely to be attributed to desorption from previously contaminated aquifer sediments. The absence of color change in the mineral medium (which contained resazurin) confirmed that anaerobic conditions were maintained in the microcosms.

The digester supernatant microcosms received additions of an unknown mixture of complex substrates and nutrients associated with healthy anaerobe populations found in anaerobic digesters. Sludge supernatant may also be a possible source for unknown quality and quantity of required vitamins (37) not available in site habitats. Methanogens comprise a large population of the microbial consortium found in anaerobic digesters and were responsible for the vigorous gas evolution encountered early into the study (day 14). The excess pressure generated by methanogenesis necessitated periodic venting of the microcosms beginning on day 37, resulting in losses of gaseous-phase chlorinated ethenes; at the time of gas release, headspace gas analysis was performed. To address mass losses from venting, the data are presented on a mole fraction basis, the molar mass of the individual compounds relative to the total molar mass of chlorinated ethenes for each sampling period (Figure 5). Reductive dechlorination of PCE commenced to *cis*-DCE, with no VC or ethene detected. A distinct lag phase, or acclimation period, was observed for PCE biodegradation, where dechlorination did not occur until day 77 of the study. Possible explanations for the observed acclimation period may be the presence of preferential substrates, competing microbial populations, or toxins and inhibitors in the digester supernatant (36). The anaerobic digesters from which the sludge samples were obtained were also not known to have previous exposure to chlorinated ethenes, which may have contributed to the acclimation period.

There are several reasons theorized for why complete reductive dechlorination of PCE to ethene could not be achieved in the biostimulated microcosm experiments. Flynn et al. have found through analysis of 16S rRNA gene sequencing, changes in community composition as reductive dechlorination shifted from PCE to *cis*-DCE/VC biodegradation (15). Their findings suggest that a consortium of specialized dehalorespirators is involved in complete reductive dechlorination. The dehalorespirators competent in degrading *cis*-DCE and VC may have been absent either in the field site or from the aquifer sediments used to establish the microcosm experiments. Field data have shown appreciable amounts of PCE, ethene, and ethane in the impacted zone, without significant levels of intermediate products detected. Key nutrients may have been depleted to levels below that which are required to sustain PCE dehalorespirators at the site. Furthermore,

cis-DCE and VC dehalorespirators may be more sensitive than PCE and TCE dehalorespirators to environmental stresses imposed during sample collection and transport, thereby either weakened or dead by the time of microcosm establishment. The latter could be explained by the 225 days required for the *cis*-DCE dehalorespirators to establish in the formate-amended microcosms. Carr and Hughes have reported similar results where approximately two years were required for an enrichment culture to dechlorinate beyond *cis*-DCE (20).

The dechlorinating culture microcosms were inoculated with exogenous microorganisms to assess the potential for bioaugmentation at the NAB Little Creek site. In addition to evaluating possible microorganism deficiencies, the experiments served to investigate for the presence of toxins and inhibitors that may be limiting reductive dechlorination by indigenous dehalorespirators. In previous work, the mixed methanogenic culture has been shown to be capable of dechlorinating PCE to 80% VC and 20% ethene when fed dissolved hydrogen or methanol as electron donors (28). The microcosms produced considerable amounts of methane gas, thus requiring microcosm venting and a subsequent loss of volatilized chlorinated ethenes; at the time of gas release, headspace gas analysis was performed. The data are presented on a mole fraction basis (Figure 6) and demonstrate rapid dechlorination of PCE to *cis*-DCE and VC by day 18, and complete transformation to VC by day 53. A small amount of inoculum (less than 10%; 1 mL culture: 4.5 mL mineral medium: 5 mL groundwater) was sufficient to bring about rapid transformation of PCE to VC. Similar to Dudiak and DiStefano's results with the culture, very low levels of TCE were observed during the study (28). Ethene was not detected in the dechlorinating culture microcosms, however, previous work with this culture has indicated that the culture might not be very efficient at the complete biodegradation of PCE, as only 20% ethene was produced at the conclusion of Dudiak and DiStefano's experiments.

A reason for the lack of complete dechlorination in either the nutrient- or dechlorinating culture-amended microcosms may be the failure to replenish PCE once biotransformed to intermediate products. DiStefano (38) found that VC dechlorination required the presence of higher chlorinated ethenes (PCE, TCE, and/or DCE) to possibly stimulate production of enzymes that catalyze VC dechlorination or support the growth of other microorganisms that may serve unknown roles. In another case, laboratory observations by Tandoi, et al. (39) suggested that VC dechlorination could actually be inhibited by the presence of PCE. The implications of not replenishing PCE in our study is unknown, however in the context of the NAB Little Creek MNA investigation, the addition of PCE to the field site would not be warranted. It is important to note that as PCE in the aqueous medium is degraded by dehalorespirators, sorbed contaminants can be re-released from the aquifer sediments to maintain equilibrium, however, these amounts are considered negligible.

Mass Balance

The average percent recovery of aqueous PCE and dechlorination products on days 90 and 260 for the individual microcosm experiments is shown in Figure 7. The final mass balance (day 260) of total chlorinated ethenes was between 19.2 and 81.3% (for dechlorinating culture and propionate-amended microcosms, respectively). Control

microcosms exhibited 63.2% recovery at the end of the study. Non-stoichiometric chlorinated ethenes recovery can be generally attributed to adsorption onto aquifer solids. The digester supernatant and dechlorinating culture microcosms also experienced losses of volatilized chlorinated ethenes from microcosm venting. Vinyl chloride is the most volatile compound among the chlorinated ethenes so as a result of gas release, the actual amounts of VC produced in the dechlorinating culture microcosms were likely to be greater than those measured. It is also possible that chlorinated ethenes may have been converted to products that were not analyzed (e.g. carbon dioxide or carbon monoxide).

Biodegradation Rates

A kinetic analysis was conducted to compare the relative rates of chlorinated ethenes biodegradation among the individual enhanced bioremediation treatments and to experiments conducted on aquifer sediments from MLS-12-Shallow under simulated natural conditions (25) (Table 3). Consistent with Berry et al.'s findings, biodegradation was observed to occur at near-constant rates and could be represented by zero-order kinetics. The rates were determined by linear regression of concentration vs. time data of chlorinated ethene formation and disappearance. Figure 8 plots the data points used in determining the zero-order chlorinated ethene dechlorination and production rates in the dechlorinating culture-amended microcosm experiments. Given the published half-saturation constants, K_s, values for dehalorespirators with chlorinated ethenes serving as the growth limiting substrate (40), Monod kinetics was the preferred approach. However, the biodegradation patterns in the microcosm experiments best followed a zero-order model.

The nutrient- and dechlorinating culture-amended microcosms generally exhibited higher rates of biodegradation than microcosm experiments conducted on aquifer sediments from the same location under natural conditions. The rate of PCE dechlorination in the unamended microcosms was 0.24 μM/day in comparison to those of the propionate, formate, mineral medium, digester supernatant, and dechlorinating culture microcosms of 0.750, 1.30, 0.339, 0.177, and 1.75 μM/day, respectively. The dechlorinating culture microcosms demonstrated the greatest dechlorination activity, in terms of rate and extent, among the enhanced bioremediation treatments. Rates of PCE dechlorination and *cis*-DCE production in the dechlorinating culture microcosms (1.75 and 1.40 μM/day, respectively) were higher than those in the nutrient-amended experiments, and dechlorination proceeded the furthest, with VC first observed very early into the study (day 18).

The rate of *cis*-DCE production in the propionate-amended microcosms was greater than in the other nutrient-amended microcosms (formate, mineral medium, and digester supernatant) that received formate as the electron donor. The competitive effects for hydrogen between dehalorespirators and other dissolved hydrogen utilizers may have been responsible for the observed differences in reductive dechlorination among these treatments. The propionate-amended microcosms received 1 mM propionate, whereas the other nutrient-amended microcosms received 45 mM formate and were replenished with 20 mM formate on days 90 and 180; these experiments were conducted in separate stages of the investigation and excess electron donor, as formate, was provided to ensure anaerobic conditions and compensate for hydrogen utilization inefficiencies resulting

from competition of dissolved hydrogen among dehalorespirators and other microorganisms. Smatlak et al. (27) measured $K_s(H_2)$ values of 100 nM for dehalorespirators and 1,000 nM for methanogens, and suggested that dehalorespirators would out-compete methanogens for electron donors only at low dissolved hydrogen concentrations. This implies that reductive dechlorination by dehalorespirators will be optimal when the amount of available electron donor is low, in order to minimize the direction of electron donors to methanogenesis.

The mineral medium microcosms generally exhibited lower transformation rates than the electron donor experiments. The reason for such differences are unknown since the mineral medium microcosms also received additions of formate and yeast extract at equal concentrations. One possibility may be that sodium sulfide was used as the reducing agent in the mineral medium; sulfides have been shown to be potentially toxic to microorganisms (41). Conversely, iron sulfide has also been shown to transform PCE and TCE, however the primary product of this abiotic reaction is acetylene, which was not observed in our experiments (42). The lowest rates of biodegradation were observed in the digester supernatant microcosms and can be attributed to the acclimation period previously discussed; also, the unknown amounts of organic substrates present in the digester supernatant, in addition to the 45 mM formate amended at the onset of the microcosm experiment, may have provided excess electron donors to favor the succession of methanogens over dehalorespirators.

Implications for Site Remediation

The results from the enhanced bioremediation microcosm experiments have provided valuable information for the MNA investigation at NAB Little Creek. In this study, an attempt was made to compare and contrast the rates and extent of PCE biodegradation in nutrient- and microorganism-amended microcosm treatments. Reductive dechlorination in the nutrient-amended microcosms (electron donor, mineral medium, and digester supernatant) proceeded to *cis*-DCE at slightly higher rates than in experiments conducted with aquifer sediments from the same location under natural conditions (25). Although complete reductive dechlorination was not observed in the microorganism-amended microcosms experiments, the most rapid and complete PCE biodegradation was achieved with inoculation of a small amount of dechlorinating culture.

In terms of an MNA remedy for NAB Little Creek, the results suggest that the addition of nutrients and exogenous microorganisms to the contaminated field site may enhance reductive dechlorination by supplying components that are not available in the existing natural environment. Nutrient amendments were of marginal success in stimulating the dechlorinating activity of indigenous microorganisms, as partial dechlorination to *cis*-DCE was encountered in both natural and nutrient-amended laboratory experiments, hence, there appears to be a greater need for competent dehalorespirators at the site. The variability of dechlorination activity observed in the field and the microcosm experiments are indicative of dispersed pockets of microbial activity and a macro-scale deficiency in competent indigenous dehalorespirators at the site.

The implementation of an engineered biostimulation approach alone may not be as beneficial for source reduction at NAB Little Creek, than bioaugmentation of the impacted zone with a culture of competent dehalorespirators. Since the application of endogenous dehalorespirators would also require the inclusion of electron donors and nutrients in the injection medium to supplement their growth, the amended nutrients can be available at the same time to stimulate the dechlorination activity of indigenous dehalorespirators. Differences in dehalorespiration rates were observed between microcosms experiments conducted with low levels of propionate (1 mM) and high levels of formate (45 mM), suggesting that the selection of the quantity and quality of electron donor will need to be examined prior to field application.

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