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Use of Bioaugmentation To Stimulate Complete Reductive Dechlorination of Trichloroethene in Dover Soil Columns

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Soil columns were constructed in support of the Remediation Technologies Development Forum accelerated biodegradation study at Dover Air Force Base to evaluate the impact of amendments on the anaerobic reductive dechlorination of trichloroethene (TCE) in Dover soil. Dechlorination of TCE to *cis*-dichloroethene (c-DCE) was observed in the columns using lactate, lactate and methanol, butyrate, glutamate and 1,2-propanediol, or toluene as electron donors, in combination with vitamins and other supplemental nutrients. However, the c-DCE formed was not further dechlorinated using any of these amendments. Subsequent inoculation of two columns with a competent, non-native TCE-dechlorinating culture resulted in the dechlorination of TCE to ethene after 30 days. Once the culture was established, dechlorination of TCE to ethene was complete in the first several centimeters of the columns at TCE influent concentrations of 4 mg/L. The culture was also able to dechlorinate TCE to ethene when TCE influent concentrations were increased to 170 mg/L. These results suggest that a critical bacterial population was missing in these soils and that bioaugmentation is an appropriate remedial strategy under such circumstances.

Introduction

Tetrachloroethene (PCE) and trichlorethene (TCE) are ubiquitous groundwater contaminants at government and industrial sites across the nation. In the past decade, reductive dechlorination of PCE and TCE to ethene or ethane by anaerobic bacteria has been demonstrated both in the laboratory and in the field (*1*−*3*). These results, coupled with laboratory and field observations that dechlorination intermediates such as *cis*-dichloroethene (c-DCE) and vinyl chloride (VC) can be oxidized in groundwater under aerobic and anaerobic conditions (*4*−*10*), have created substantial interest in intrinsic biodegradation as a remedial option for chlorinated solvent-contaminated aquifers.

Although intrinsic biodegradation is appealing from a number of standpoints, there are some circumstances where it is not appropriate. These include situations where the reductive dechlorination of TCE or PCE does not proceed to completion or proceeds too slowly to reduce groundwater concentrations to acceptable levels before sensitive receptors are impacted. This may be due to a limitation in electron donors, electron acceptors, or other nutrients required to maintain the dechlorinating microbial population, or to an absence of appropriate dechlorinating bacteria. In any case, PCE, TCE, c-DCE, and/or VC remain at levels which cannot be mitigated by other natural attenuation processes, including dilution, sorption, and aerobic biodegradation.

In these cases, the potential exists to supply the missing component to initiate or enhance the biodegradation activity. This process is known as accelerated bioremediation, whereby electron donors, electron acceptors, or nutrients are added to the subsurface to stimulate biological activity. The approach could be used either to create a permeable bioactive barrier or for source remediation and offers considerable potential costs savings over comparable remedial technologies, such as pump and treat (*11*).

Several field demonstrations using accelerated bioremediation to stimulate the reductive dechlorination of PCE and TCE in groundwater have been performed to date. The first documented field test was carried out by DuPont workers in Victoria, TX (*12*), where benzoate and sulfate were used to enhance the reductive dechlorination of PCE. More recently, benzoate, lactate, and/or methanol have been used to accelerate the dechlorination of PCE and TCE in field tests in Florida, Texas, and The Netherlands (*13*−*15*).

While these results have been encouraging, several questions remain unanswered regarding the field application of this technology. The most perplexing question relates to the difficulty commonly encountered in stimulating complete dechlorination to ethene or ethane at all sites. On the basis of limited experience, the dechlorination of PCE or TCE appears to stop at c-DCE or VC in roughly half the soils tested in microcosm studies (*16*). This suggests either that dechlorination is sensitive to the electron donor and nutrient mix applied to the microcosms or that the appropriate microorganisms necessary for complete dechlorination do not exist in all soils. The latter issue is compounded by the possibility that dechlorinating microorganisms may lose viability during the collection and transport of soils from the field into the laboratory, where the microcosm studies are performed.

To address this issue, a soil column study was performed by GE in support of an accelerated biodegradation field study recently conducted at Dover Air Force Base (AFB), DE, by the Remediation Technologies Development Forum (RTDF) Bioremediation of Chlorinated Solvents Consortium. The objectives of this study were to (1) demonstrate that the complete dechlorination of TCE to ethene could be stimulated in Dover soil and (2) demonstrate mass balance of TCE, daughter products, and end products in a flow system. The study was carried out in three phases. Phase I examined the reductive dechlorination of TCE in Dover soil using lactic acid as the primary electron donor. Phase II examined dechlorination using additional electron donors and supplemental nutrients, while phase III examined dechlorination after addition of a non-native dechlorinating culture to the columns.

Materials and Methods

Description of Site and Soils. Dover AFB is located in Kent County, DE, and covers 4000 acres of land. Various military services have operated at the base since 1941, including units responsible for jet engine testing, repair, and maintenance. Since 1986, multiple sources of contamination to groundwater have been identified at the site. In many cases, the associated contaminated surface soils have been removed.

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TABLE 1. Typical Chemical Composition of Dover AFB Groundwater

compd (mg/L)	site 6	site 7
PCE	0.33	0.04
TCE	1.15	5.61
c-DCE	8.90	1.00
VC	0.71	0.03
ethene	0.15	0.02
TOC	4.4	3.3
nitrate-*N*	2.1	0.85
sulfate	2.7	<2
alkalinity (as $CaCO_3$)	26	19
chloride	21	32

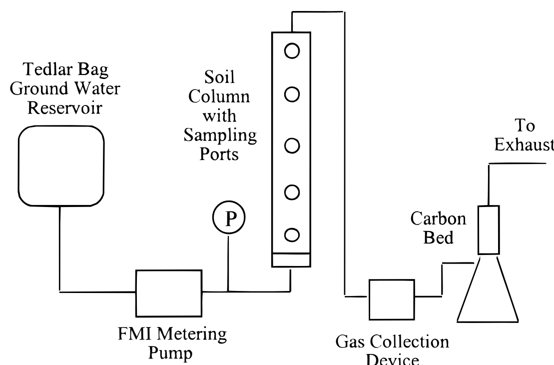

FIGURE 1. Schematic of soil column setup. The Tedlar bag is blanketed with nitrogen to minimize oxygen diffusion (not shown). A pressure gauge (P) was used to measure the pressure drop across the column.

However, groundwater under these source areas continues to contain elevated levels of chlorinated aliphatics, primarily TCE, forming a plume 2500 m long and 1000 m wide. The plume contains distinct regions, including site 6, where active and extensive intrinsic reductive dechlorination of TCE to ethene is occurring, and site 7, where dechlorination is limited to minimal conversion of TCE to c-DCE (Table 1).

The soil used in the study was obtained from the Columbia Formation, which is the major shallow water bearing formation in the region. The formation consists primarily of fine to medium sand with low (<0.1%) organic carbon content. Soil samples from 30 to 35 feet below ground surface were obtained at both sites 6 and 7 using 4 in. diameter steel Shelby tubes. The tubes were capped and sealed with wax immediately upon retrieval and then packed in coolers and sent to the GE Corporate Research and Development Center. Upon arrival, the soil was transferred from the tubes to glass jars in a tent under nitrogen and passed through a $^1/_4$ in. screen to remove stones and debris. Soil analyses indicated the soil from site 6 was marginally finer and had twice as much organic carbon, iron, and biomass as did the soil from site 7. Phospholipid fatty acid (PLFA) analyses revealed that the proportion of saturated, monoenoic, and branched fatty acids was similar in the two soils, suggesting that the microbial communities were similar.

Column Construction and Operation. The columns used in these experiments were glass chromatography columns (5 cm i.d. × 60 cm length) with Teflon endcaps. The columns were fitted with five equally spaced glass sampling ports (10 mm i.d.) sealed with Teflon-coated butyl rubber septa to allow for soil and groundwater sampling (Figure 1). Glass wool was used to keep soil from filling the sampling port extensions. The columns were covered with aluminum foil during operation to inhibit the growth of photosynthetic organisms. Groundwater was pumped into the bottom of the columns using FMI (Oyster Bay, NY) QG 6 metering pumps. A coarse fritted glass filter disk was fitted into the bottom endcap to ensure uniform distribution of the groundwater entering the columns. A 2 cm layer of coarse, clean beach sand was placed on top of the glass disk to keep soil from entering and potentially plugging the disk.

Soil was added to the top of the columns in small increments as anaerobic groundwater was slowly pumped up from the bottom. The soil was compacted and air bubbles removed by gently tapping the outside of the column with a plastic rod. The water-filled porosity in the columns ranged from 0.280 to 0.335, such that each soil-filled column also contained 340−400 mL of groundwater. After packing, tracer tests were performed on the columns to ensure that channeling of groundwater did not occur and to measure TCE retardation in the soil. The tracer tests were carried out by pumping groundwater amended with 5 mg/L TCE and 30 mg/L bromide through the column at a nominal flow rate of slightly less than 0.1 mL/min and measuring the breakthrough of these constituents down the length of the column. The tracer tests yielded residence times of 61−82 h for bromide in the columns. These values were close to calculated residence times based upon porosity measurements and indicated that excessive channeling was not occurring. Retardation factors of 1.0−1.3 were calculated by comparing the TCE and bromide breakthrough curves, indicating that sorption of TCE to matrix materials was minimal.

Groundwater was periodically obtained from wells adjacent to the soil boring locations and stored at 4 °C prior to use. The groundwater was filtered, autoclaved, sparged with nitrogen, and then pumped into black, 3.4 L Tedlar bags. The bags were placed in Plexiglas containers and spiked with electron donor, electron acceptor, bromide tracer, nutrients, and TCE solutions using a glass gas-tight syringe. The exterior surfaces of the bags were blanketed in nitrogen in order to minimize diffusion of oxygen through the bag and maintain anaerobic conditions. The bags were replaced every 15−35 days, depending upon the pumping rate. Pumping rates were initially established at 0.1 mL/min, resulting in a hydraulic residence time of about 60 h in the columns. Pumping rates were subsequently reduced to 0.05 mL/min on day 208 of the study. All tubing (0.12 cm i.d.), connections, and fittings were Teflon. Any gas evolved in the column was trapped in a specially designed gas collection device so that the volume could be quantified and the composition determined. The effluent was collected in a flask, where residual chlorinated aliphatics were absorbed onto carbon bed filters.

Experimental Design. Four soil columns (columns 1−4) were prepared using soil and groundwater taken from site 7, and one column (column 5) was prepared using soil and groundwater from site 6. Three amendment conditions were tested during phase I of the study (Table 2). Columns 1 and 5 were amended with 2.5 mM lactic acid (high lactate condition). Column 2 was amended with 2.5 mM lactic acid and 5.4 mM magnesium sulfate (high lactate/sulfate condition). Column 3 was amended with 0.75 mM lactic acid (low lactate condition). Column 4 was unamended.

For the first three weeks of the study, methanol was added to the amended columns that did not receive sulfate to accelerate the establishment of anaerobic conditions. The amended columns also received yeast extract, ammonium chloride, trimetaphosphate (TMP), and sodium bromide—the latter added as a conservative tracer (Table 2). Concentrated (1000×) solutions of lactic acid, magnesium sulfate, yeast extract, and nutrients were autoclaved prior to addition to the feed bags. Methanol was added to the bags in neat form, whereas TCE was added from a saturated water stock solution to a target concentration of 30 μM (4 mg/L).

During phase II of the study, additional electron donors and nutrients were used in an attempt to stimulate the

TABLE 2. Summary of Experimental Conditions in Dover Soil Columns

days	amendments				
	column 1	column 2	column 3	column 4	column 5
0–20	lactic acid [1] methanol	lactic acid [1] $MgSO_4$ [1]	lactic acid [2] methanol	none	lactic acid [1] methanol
21–68	lactic acid [1]	same	lactic acid [2]	same	lactic acid [1]
69–86	same	lactic acid [1] $MgSO_4$ [2]	same	same	same
87–105	Na lactate [1]	Na lactate [1] $MgSO_4$ [3]	Na lactate [2]	same	Na lactate [1]
106–118	same	Na lactate [1]	Na lactate [2] methanol	same	same
119–167	Na lactate [1] double YE vitamins	Na lactate [1] double YE vitamins	Na lactate [2] methanol double YE vitamins	Na glutamate propanediol double YE vitamins	Na lactate [1] double YE vitamins
168–185	Na butyrate double YE vitamins	toluene [1] double YE vitamins	same	same	Na butyrate double YE vitamins
186–234	same	toluene [2] double YE vitamins	same	same	same
235–360	same	terminated	same	terminated	terminated
361–511	Na lactate [1] vitamins		Na lactate [1] methanol vitamins		

[a] Notes: All columns except column 4 were amended with ammonium chloride (35 mg/L), trimetaphoshate (10 mg/L), and yeast extract (10 mg/L). On day 119, column 4 was also amended with these nutrients. Concentrations of lactic acid or sodium lactate used were [1] (2.5 mM) and [2] (0.8 mM). Concentrations of other electron donors used were methanol (5.0 mM), sodium butyrate (2.5 mM), sodium glutamate (0.67 mM), 1,2-propanediol (1.33 mM), toluene [1] (1.0 mM), and toluene [2] (0.33 mM). Concentrations of magnesium sulfate used were [1] (5.4 mM), [2] (2.7 mM), and [3] (1.4 mM). A vitamin solution (*17*) was added at a concentration of 2 mL/L of influent. All the columns were amended with sodium bromide (0.6 mM) and TCE (30 μM). The TCE concentration was increased in column 3 on day 208. Column 3 was bioaugmented on day 165, column 1 on day 371.

reductive dechlorination of c-DCE (Table 2). Sulfate was reduced in stepwise fashion from column 2 beginning on day 69 of the study and was completely eliminated on day 104. Lactic acid was replaced by sodium lactate in all the amended columns on day 87. At the same time, methanol was added to column 3. A vitamin solution (*17*) was added, and the yeast extract was doubled in all amended columns between days 119 and 124. At the same time, L-glutamic acid (0.67 mM) and 1,2-propanediol (1.33 mM) were added to previously unamended column 4. On day 168, sodium lactate was replaced by toluene in column 2 and by sodium butyrate in columns 1 and 5.

Phase III of the study was initiated on day 165, when column 3 was inoculated with 15 mL of a soil slurry containing a TCE-dechlorinating bacterial culture originating from the Pinellas plant site, a Department of Energy site in Largo, FL (*18*). The slurry amendment was added to the second port, displacing about 4% of the column liquid volume. Soil microcosms were also inoculated with the slurry at the same time. These microcosms contained freshly collected Dover AFB soil and groundwater, reduced anaerobic mineral medium (RAMM) (*19*), vitamins, sodium lactate, methanol, and TCE. Column 1 was inoculated with a liquid culture of the Pinellas culture on day 371. In this case, 3 mL of inoculum was added through the third port, displacing about 1% of the column liquid volume.

Development of the Pinellas Culture. The Pinellas culture was enriched from soil columns containing Pinellas soil and groundwater amended with a combination of lactate, methanol, sulfate, supplemental nutrients, and TCE (*20*). The culture was later transferred to microcosm serum bottles consisting of Pinellas soil and RAMM amended with lactate and methanol, or lactate alone, and TCE. An aliquot of this soil slurry that was several months old and actively dechlorinating TCE to ethene was used to bioaugment column 3. The culture was subsequently transferred from soil slurries to RAMM liquid medium amended with lactate and TCE. During multiple transfers in liquid medium, the culture appeared to be oxygen tolerant, exhibited good growth characteristics, and maintained full dechlorination activity of TCE to ethene. A stationary phase culture (optical density, ~1.0) was obtained after 1 month of culturing in the amended liquid medium and was used to bioaugment column 1.

Microbial characterization of the Pinellas culture was done by both commercial and academic laboratories. A diagnostic test for coliform bacteria called Colilert (Idexx Laboratories, Westbrooke, ME) indicated that coliform bacteria were not present. The culture was also plated on trypticase soy broth agar, and isolated colonies were subcultured and extracted for PLFA analysis (*21, 22*). PLFA profiles of three bacterial genera were identified (*Alcaligenes*, *Hydrogenophaga*, and *Pseudomonas*) that accounted for greater than 1% of the Pinellas culture. No pathogenic microorganisms were found. PLFA analysis performed directly on the liquid culture indicated that there were approximately 10^7 cells/mL, consisting predominantly of Gram positive and some Gram negative bacteria. Specific fatty acid markers were present for sulfate reducers of the *Desulfovibrio* type.

Terminal restriction fragment length polymorphism analysis (*23*) of extracted DNA from Pinellas liquid culture resulted in six dominant bands that accounted for the greater part of the population diversity. Blast similarity searches of the bands with the GenBank database showed high similarity scores for the following bacteria: *Citrobacter freundii*, *Campylobacter spp.*, *Geospirillum spp.*, *Lactosphaera pasteurii*, *Clostridium spp.*, and *Zoogloea ramigera*.

Sampling and Analytical Procedures. All groundwater samples were taken through Teflon-lined septa located in the Tedlar bags and column sampling ports using glass gas-tight syringes and filtered (0.2 μm) prior to analysis. Effluent gas samples were taken through Teflon-lined septa in the gas collection devices, while gas that accumulated in the sampling ports was sampled through the port septa. With

the exception of the first weeks of the study, samples were taken at 14−35 day intervals, dependent upon the groundwater pumping rate.

TCE and c-DCE in water were measured by extracting a 0.5 mL water sample into 5.0 mL of hexane and analyzing the hexane using an Hewlett-Packard (HP) (Palo Alto, CA) 5890 gas chromatograph (GC) equipped with an electron capture detector and a DB624 (30 m × 0.53 mm i.d.) fused silica capillary column (J&W Scientific, Folsom, CA). Alternatively, TCE, c-DCE, VC, ethane, and ethene were measured by adding a 1.0 mL water sample to 6.0 mL of matrix-modifying solution and analyzing the headspace using a Tekmar 7000 headspace analyzer (Tekmar-Dohrmann, Cincinnati, OH) in tandem with a HP 5890 GC and a Fisons Trio 1000 mass spectrometer (MS) equipped with a GS-Q (30 m × 0.32 mm i.d.) fused silica capillary column (J&W Scientific). These two methods yielded comparable results in side-by-side analyses for TCE and c-DCE. Methanol was quantified using an HP 5890 GC equipped with a flame ionization detector and DB624 column.

Organic salts such as lactate, butyrate, acetate, and propionate were measured by high-pressure liquid chromatography (HPLC) under isocratic conditions using a Waters 490 UV Detector (Millipore Corporation, Bedford, MA) at 210 nm and Aminex HPX-87H ion exclusion column (Bio-Rad Laboratories, Hercules, PA). Anions such as chloride, bromide, phosphate, and sulfate were measured by HPLC under isocratic conditions using a Waters 431 conductivity detector and an IC−Pak Anion HR column (Millipore Corp.). Ammonia was measured by HPLC under isocratic conditions using a Waters 431 conductivity detector and an IC−Pak Cation M/D column. Soluble iron was quantified by colorimetric analysis using ferrozine (*24*). The absorbance at 562 nm was measured using a HP 8452A Diode-Array Spectrophotometer. The oxidation−reduction potential (ORP) was measured by passing a 1.0 mL water sample through a small section of polyethylene tubing containing a custom-made micro ORP probe (Microelectronics, Londonderry, NH). pH was measured in the same sample using a semi-micro pH probe (Orion, Boston, MA).

Gaseous concentrations of carbon dioxide, methane, ethane, and ethene were quantified using a Schimadzu GC-3BT GC equipped with a thermal conductivity detector and an Alltech 2701PC (6 ft x 1/8 in x 0.085) chromatography column packed with Porapak Q (80/100 mesh). The hydrogen concentration in the gas was quantified using RGA3 reduction gas analyzer (Trace Analytical, Menlo Park, CA) equipped with a mercury vapor detector.

Results

Biodegradation Results−Phase I. All the amended soil columns went anaerobic within 5−7 days of start up. Influent groundwater typically contained ca. 0.5 mg/L of dissolved oxygen, with a corresponding ORP of +150 to +200 mV. Column 5 (high lactate/site 6 soil) was the first column to turn anaerobic and was the most strongly reduced. Minimum ORP readings in this column reached −200 to −220 mV after 19 days. The ORP in column 2 (high lactate/sulfate/site 7 soil) also reached −200 mV while sulfate was present, whereas column 1 (high lactate/site 7 soil) and column 3 (low lactate/site 7 soil) were less reduced, with minimum ORP readings ranging from −50 to −150 mV. Unamended column 4 (site 7 soil) did not become anaerobic.

Dechlorination of TCE to c-DCE was nearly complete in the effluent of columns 1 and 5 by day 20 of the study (Figure 2). TCE dechlorination was initially evident in the uppermost region (port 5) of the columns and then spread down the column toward the influent over time (Figure 3). By day 45, dechlorination of TCE to c-DCE was complete by the third port, corresponding to a residence time of 35 h in the columns.

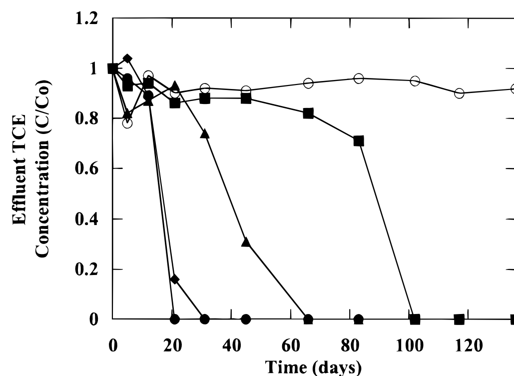

FIGURE 2. Effluent TCE concentration over the first 140 days of the study under high-lactate conditions in column 1 (●); high-lactate conditions in column 5 (◆); low-lactate conditions in column 3 (▲); high-lactate/sulfate conditions in column 2 (■); and unamended conditions in column 4 (○). Influent TCE (ca. 30 μM) was reductively dechlorinated to c-DCE, but no further. The sulfate concentration in column 2 was reduced from 5.0 to 0.0 mM in stepwise fashion between days 67 and 105.

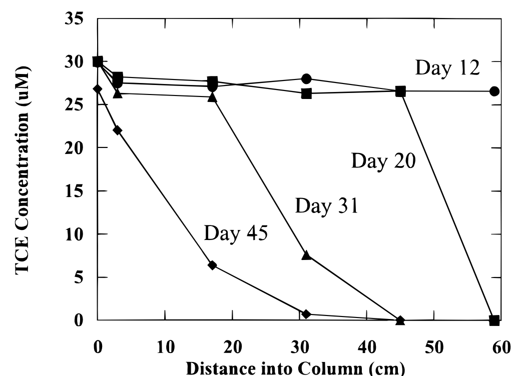

FIGURE 3. Change in the TCE profile in high-lactate column 1 over time. TCE was reductively dechlorinated to c-DCE. Lactic acid was the electron donor. Similar progressions were also observed in high-lactate column 5 and low-lactate column 3.

The onset of TCE dechlorination was slower in column 3, suggesting that the time to the onset of dechlorination was a function of the electron donor concentration. c-DCE was first noted in the effluent of column 3 on day 32, although dechlorination was not complete at that location until day 66. No TCE dechlorination was observed in unamended column 4.

Conversion of TCE to c-DCE was not observed in column 2 when sulfate was present. Beginning on day 67, the influent concentration of sulfate was reduced in stepwise fashion, resulting in the eventual depletion of sulfate in the upper regions of the column and the concomitant production of c-DCE in this depleted zone (Figure 4). Further reductions in sulfate resulted in more extensive c-DCE production, suggesting that sulfate may have been inhibitory to TCE dechlorination in Dover soil.

Lactic acid was degraded to acetate, propionate, hydrogen, methane, and CO_2 in columns 1, 3, and 5 and to acetate, hydrogen, and CO_2 in the presence of sulfate in column 2. An example of the carbon and electron flow for column 1 is illustrated in Figure 5. After an initial lag period, lactate was typically degraded in the first 15 cm of the columns. Initially, acetate and propionate were formed in near stoichiometric amounts relative to the lactate consumed. However, as the study progressed both compounds were ultimately degraded to CO_2 and methane. Methanol was degraded more slowly than lactate and persisted in the columns until it was removed

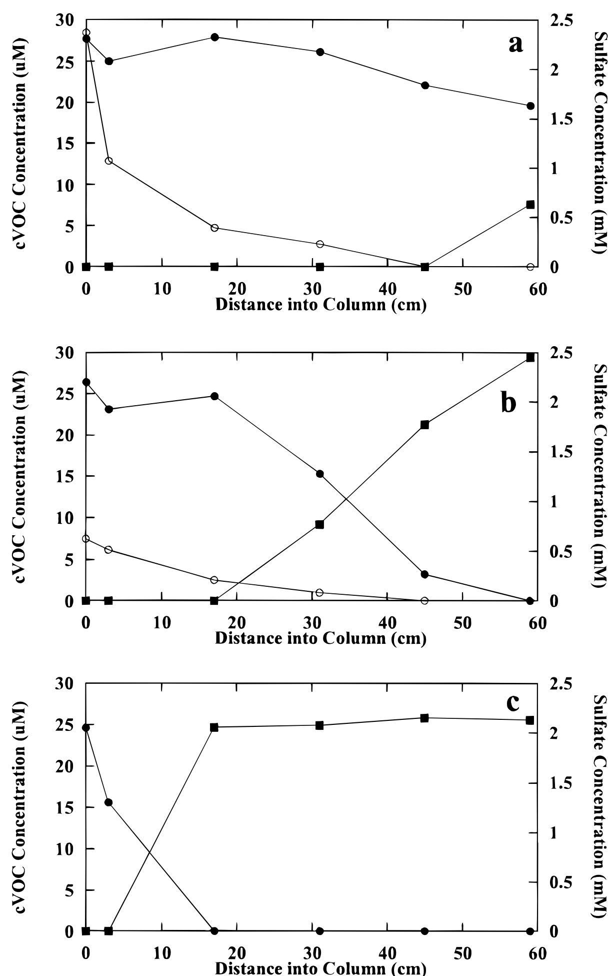

FIGURE 4. Reductive dechlorination of TCE (●) to c-DCE (■) in column 2 in the presence of sulfate (○) at (a) 82, (b) 102, and (c) 117 days. The sulfate concentration in column 2 was reduced in stepwise fashion over this period and totally removed from the influent on day 105.

from the influent on day 21.

Iron reduction, methanogenesis, and sulfate reduction were all present in the amended columns. Soluble Fe(II) was produced in all the amended columns (Figure 4). Effluent levels of Fe(II) increased to a maximum of 1.4 mM in columns 1 and 5 and to a maximum of 0.5 mM in column 3 during days 45−86. The observed Fe(II) was derived from the microbial reduction of Fe(III) minerals, and possibly from the dissolution of Fe(II) from the soil under the acidic feed conditions. The latter mechanism was subsequently eliminated when the lactic acid in the influent was replaced by sodium lactate on day 87.

Gas production was noted in the effluent of column 5 on day 14 of the study. The gas contained both CO_2 and methane, indicating that methanogenesis had begun in that column. Methane was measured in the effluent of column 1 on day 69 and in the effluent of column 3 on day 95. Fe(II) continued to be produced in these columns after the onset of methanogenesis, suggesting that both iron reduction and methanogenesis were operating simultaneously in these soils. Column 4 did not produce gas during this period.

The onset of sulfate reduction in column 2 occurred between days 6 and 10 and was accompanied by the formation of a black, iron sulfide precipitate in the soil. By day 32, virtually all of the lactate was being degraded to CO_2 in the column, while 71−84% of the electrons produced by this degradation were used for sulfate reduction. Column 2 did not became methanogenic until after all the sulfate was removed from the feed.

After gas production was established in the columns, hydrogen, CO_2, and methane were measured along the length of the column by sampling small quantities of gas trapped in the sampling ports over time. It was assumed that this gas was in equilibrium with the water flowing nearby. Elevated levels (>10 nM) of hydrogen generally coincided with the presence of electron donors, including acetate and propionate, at any point in the column (Figure 5). When these electron donors were depleted, the hydrogen concentration rapidly dropped to 1−2 nM. Similarly, the concentrations of CO_2 and methane increased with distance up the column while electron donors were present and then reached a steady state value after they were depleted.

HPLC analyses indicated that ammonia was present at 5−15 mg/L throughout the amended columns. Phosphate was typically measured at 1−3 mg/L in the feed bags, but was only intermittently observed above detection limits (0.5 mg/L) in the columns, probably because phosphates sorb readily to aquifer matrix material.

Biodegradation Results—Phase II. During the initial phase of the study, TCE was dechlorinated solely to c-DCE using lactic acid as the electron donor. Therefore, a number of steps were undertaken in an attempt to stimulate the dechlorination of c-DCE. These steps included replacing lactic acid with sodium lactate, adding a vitamin solution previously shown to be beneficial for sustaining complete dechlorination of PCE (*25*), doubling the yeast extract concentration, doubling the residence time in the columns, and adding a number of different electron donors to selected columns. However, none of these steps resulted in the dechlorination of c-DCE.

The use of lactic acid caused the influent to be acidic, resulting in pH readings of 3.5−4.1 in the first several centimeters of the columns. This acidity generally did not persist past port 1. However, between days 66 and 82 the low-pH zone began to expand in column 1. Although the trend was only apparent in column 1, lactic acid was replaced with sodium lactate in all the amended columns at that point. Lactate consumption and TCE dechlorination to c-DCE were both more rapid under pH-neutral conditions. For example, TCE was dechlorinated in the first 15 cm of the columns, instead of in the first 20 to 30 cm, while lactate was partially degraded in the feed bags unless great care was taken in sterilizing the feed solutions. The levels of Fe(II) produced in the effluent of columns 1, 3, and 5 were reduced by 40−75% under pH-neutral conditions.

The addition of methanol to column 3 promoted methanogenesis in that column, whereas the addition of vitamins and the doubling of yeast extract facilitated the mineralization of acetate and propionate into methane, hydrogen, and CO_2 and increased gas production by 50−100% in all the amended columns.

The addition of glutamate and 1,2-propanediol to previously unamended column 4 rapidly produced anaerobic conditions there. TCE dechlorination began between 14 and 34 days after this amendment. By day 34, TCE was completely converted to c-DCE in the first quarter of the column. Acetate, propionate, and CO_2 were produced from the degradation of glutamate and 1,2-propanediol. Methane was noted in the sampling ports after 52 days, but the column never generated a significant amount of gas in the effluent in 106 days of operation.

Sodium butyrate was added in place of sodium lactate in columns 1 and 5 because it was previously shown to be a more efficient electron donor than lactate for TCE or PCE dechlorination (*26*). Butyrate was degraded to acetate and CO_2 more slowly than was lactate and did not degrade in the feed bag, unlike lactate and glutamate. However, butyrate

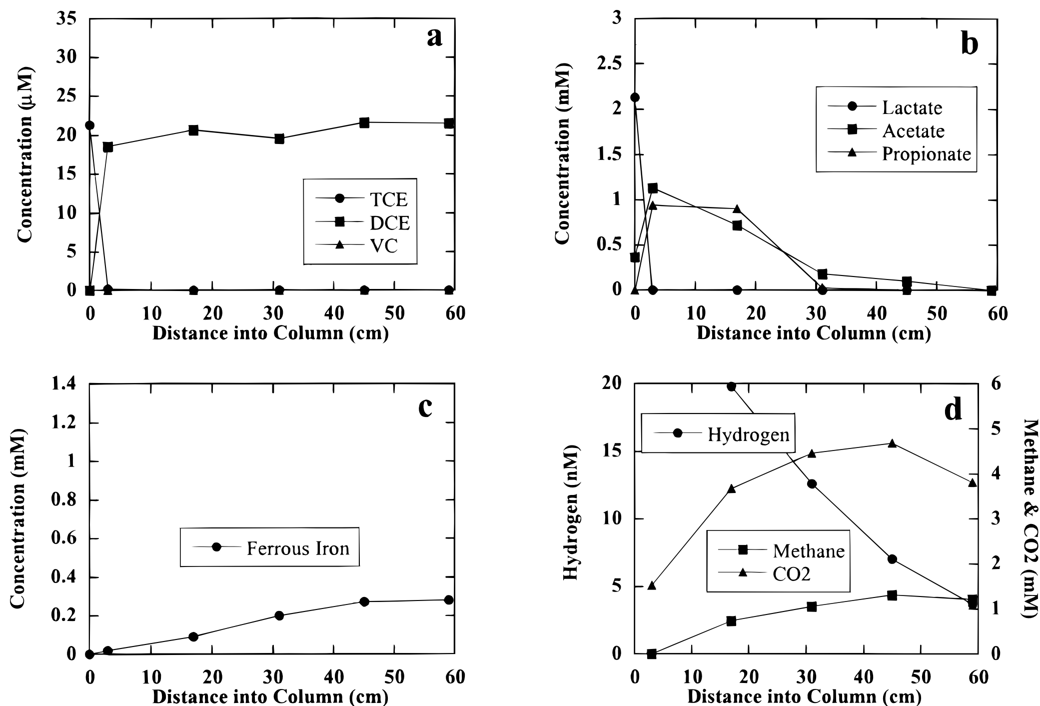

FIGURE 5. TCE dechlorination (a), electron donor utilization (b), and Fe(II) (c) and hydrogen, CO_2, and methane production (d) as a function of distance into the column in column 1 on day 157. Sodium lactate is the electron donor. Concentrations shown for hydrogen, methane, and CO_2/carbonate reflect aqueous phase concentrations only.

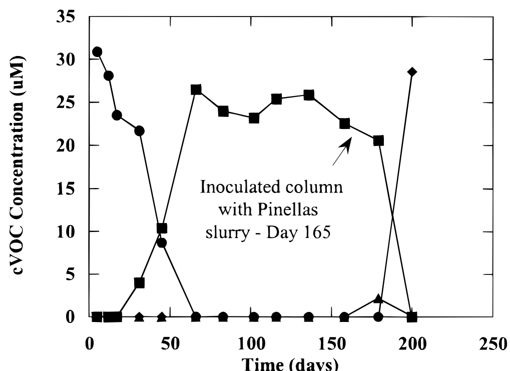

FIGURE 6. TCE dechlorination in column 3 before and after bioaugmentation with the Pinellas culture slurry on day 165. Effluent concentrations of TCE (●), c-DCE (■), VC (▲), and ethene (◆) are shown. The ethene shown is the sum of both gas and liquid concentrations.

did not change the rate of TCE dechlorination compared to lactate and did not elicit dechlorination of c-DCE in 200 days of column operation. TCE dechlorination to c-DCE was markedly slower using toluene as the electron donor in column 2 but was complete by the end of the column. Only 0.04–0.13 mM of the toluene was degraded in the column, producing hydrogen and low levels of CO_2 and methane.

Biodegradation Results—Phase III. Bioaugmentation of column 3 with a 4% inoculum of the Pinellas soil slurry resulted in the rapid dechlorination of c-DCE. Ten days after bioaugmentation, 2.5–4.6 μM of VC was evident in the upper two-thirds of the column, along with c-DCE and trace amounts of ethene. Twenty days later, TCE and c-DCE appeared only in the first port and ethene was the predominant degradation product in the rest of the column, accompanied by trace amounts of VC (Figure 6). Other microbial processes, such as lactate degradation, iron reduction, or methanogenesis did not appear to be impacted by the addition of the non-native culture to the column.

TCE dechlorination in the bioaugmented microcosms containing fresh Dover soil proceeded at a rate similar to that observed in the columns. TCE dechlorination commenced 7–14 days after bioaugmentation, producing c-DCE, VC, and a small amount of ethene. By 21 days, the microcosms contained only VC and ethene, in near equal amounts. By 35 days, the predominant degradation product was ethene, accompanied by trace amounts of VC.

Column 1 was subsequently bioaugmented with a 1% inoculum of the Pinellas culture grown in liquid medium. Twenty-six days after bioaugmentation, VC and ethene were the only degradation products present in the column above port 2. Sixty days later, VC and c-DCE were measured only in the first port, whereas the rest of the column contained only ethene. Since the column was bioaugmented at the third port, these results suggest that dechlorination activity moved down the column, counter to the direction of groundwater flow.

After bioaugmentation in column 3, the influent TCE concentration was gradually increased to test the tolerance of the culture to higher TCE levels. The feed concentration was increased from 30 to 1300 μM in stepwise fashion between days 200 and 500. During that time the microbial community within the column was consistently able to degrade the TCE to stoichiometric amounts of ethene (Figure 7). VC was measured in the column effluent after TCE increases on day 360, where it appeared that the capacity of the electron donors to support the complete dechlorination of TCE was exceeded. This hypothesis is supported by the hydrogen measurements, which indicated that hydrogen levels fell to below 1.0 nM in the upper third of the column at this time. The lactate concentration in the influent was tripled thereafter, resulting in the return to higher (>1.0 nM) hydrogen levels in the upper part of the column and the dechlorination of VC to ethene. VC was also measured in the column effluent on day 460. In this case, it appeared the system was kinetically limited because the 120 h of residence time in the column was not long enough to facilitate the complete conversion of VC to ethene (Figure 8). Acetate and propionate were observed in

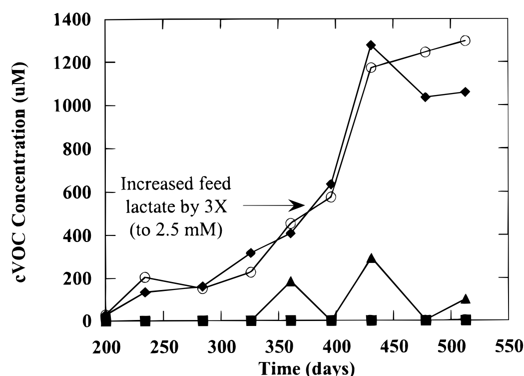

FIGURE 7. Dechlorination of TCE in column 3 with increasing influent TCE concentration. Shown are influent TCE (○) and effluent c-DCE (■), VC (▲), and ethene (◆) concentrations. No TCE or c-DCE was observed in the effluent in this time period. The ethene concentration shown is the sum of ethene leaving the column in both the gas and liquid phases.

the effluent during this period, suggesting that the high levels of influent TCE were inhibitory to the bacterial populations degrading these organic acids. The pH in the column began to fall during this period due to hydrochloric acid buildup.

Mass and Electron Balances. Molar balances of chlorinated aliphatics were calculated by comparing influent TCE concentrations to concentrations of dechlorination products in the effluent. TCE was converted to stoichiometric quantities of c-DCE prior to bioaugmentation and to stoichiometric quantities of ethene thereafter (Table 3). Chloride balances could not be determined at nominal TCE influent levels due to high background chloride concentrations, but they could be calculated at the higher TCE concentrations used in column 3. Chloride recoveries in column 3 ranged from 70 to 94% (data not shown). Carbon mass balances ranging from 73 to 137% were calculated by comparing the electron donor concentrations measured in the feed bags to the concentrations of acetate, propionate, or metabolic gases produced in the column effluent (Table 4). Once the microbial populations in columns were fully acclimated, roughly half the carbon ended up as methane. The rest was degraded to CO_2, which was present either as free CO_2 or in carbonate form.

TABLE 3. Example Molar Balances for Chlorinated Aliphatics in Columns 1, 3, and 5[a]

day	column	influent TCE (μM)	effluent c-DCE (μM)	effluent VC (μM)	effluent ethane (μM)	recovery (%)
157	1	21.3	29.3			137
	3	21.7	22.6			104
	5	29.2	21.5			74
200	1	30.7	25.1			82
	3	29.3		0.1	28.6	98
	5	29.3	24.1			82
284	1	26.5	24.2			91
	3	151.8			161.2	106
397	1	33.5			38.9	86
	3	576			635	110
478	3	1245			1036	83

[a] Note: The ethene concentrations shown represent the sum of ethene leaving the column in both the gas and liquid phases.

In the absence of sulfate, methanogenesis was the primary electron-accepting process in this study, accounting for 89 to 99% of the transferred electrons (Table 5). Iron reduction, as measured by the production of soluble iron, accounted for only 1% of the electrons transferred. TCE dechlorination accounted for roughly 0.25% of the available electrons when the TCE concentration was low (30 μM) but increased to 5.9% of the available electrons at high TCE concentrations (1300 μM) (Figure 9). Thus TCE dechlorination operated more efficiently at higher TCE concentrations, at least as measured by electron transfer.

Kinetic Analysis. Kinetic analysis was performed on the TCE dechlorination just described and suggested that the

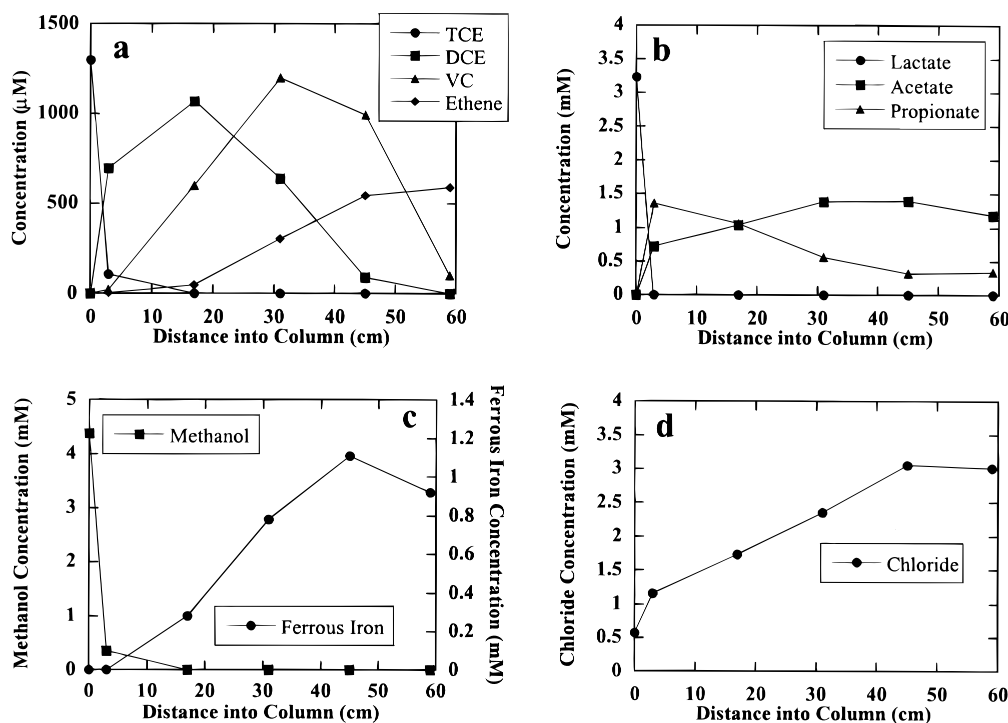

FIGURE 8. TCE dechlorination (a), electron donor utilization (b & c), and Fe(II) (c) and chloride production (d) as a function of distance into the column in column 3 under high TCE loading on day 511. Concentrations shown for ethene reflect aqueous phase concentrations only.

TABLE 4. Example Carbon Mass Balances in Columns 1, 3, and 5

day	column	electron donor	carbon in (mM)	methane out[a] (mM)	CO_2 out[a] (mM)	carbon balance (%)
157	1	lactate	7.13	2.49	3.02	77
	3	lactate/methanol	5.31	2.87	3.11	113
	5	lactate	5.83	2.90	3.46	109
200	1	butyrate	6.63	3.66	5.42	137
	3	lactate/methanol	8.51	3.12	3.05	73
	5	butyrate	5.90	3.64	3.14	115
284	1	butryate	5.52	3.26	2.63	107
	3	lactate/methanol	6.86	2.81	2.29	74
397	1	lactate	6.34	3.10	3.26	106[b]
	3	lactate/methanol	9.10	3.44	6.13	104
478	1	lactate	5.85	3.79	2.14	101
	3	lactate/methanol	13.05	6.64	4.59	94[b]

[a] Notes: Methane and CO_2 values reflect a summation of these constituents in both the gas and water phases leaving the columns. CO_2 includes both free CO_2 and carbonate. Carbonate levels were calculated from gas-phase CO_2 measurements, assuming equilibrium between phases and equilibrium between dissolved CO_2 and carbonate in solution at the pH of the water phase. [b] Includes carbon associated with acetate in the effluent.

data is best described by Michaelis–Menten kinetics. Zero- or first-order kinetic models did not fit the data as well. Michaelis–Menton kinetics for TCE dechlorination take the form

$$d[\mathrm{TCE}]/dt = -V_{max}[\mathrm{TCE}]/(K_m + [\mathrm{TCE}]) \quad (1)$$

where [TCE] is the molar concentration of TCE in the aqueous phase (μM), V_{max} is the maximum rate of TCE dechlorination (μM/h), and K_m is the half velocity constant for TCE usage (μM). V_{max} can also be expressed in terms of a specific dechlorination rate, such that

$$V_{max} = K_{TCE}X \quad (2)$$

where K_{TCE} is the biomass specific maximum rate of TCE dechlorination (μM/mg of biomass·h) and X is the concentration of TCE dechlorinating bacteria in the column (mg of biomass).

Because the dechlorinating biomass in the columns was unknown, the experimental data were modeled using eq 1. A Lineweaver–Burke plot was constructed using the TCE dechlorination data from column 3, but it was not linear, displaying curvature at higher TCE concentrations. However, the Michaelis–Menton model fit the data very well at individual time points, as observed using Scientist data-fitting software (Micromath Scientific Software, Salt Lake City, UT). This suggests that the number of TCE dechlorinators may have been changing in the column over the time period in which the data was collected.

To adjust for the potential for increasing dechlorinating biomass in the column, a K_m of 136 μM was estimated using the TCE dechlorination data generated at early time points, when the TCE concentration was relatively low. With the K_m value kept constant, V_{max} values for TCE were generated for all time points using the data fitting software. V_{max} values calculated in this way from column 3 data were generally consistent through day 360 of the study, averaging about 45–50 μM/h (Table 6), and were similar to values calculated from the other lactate or butyrate amended columns (data not shown). However, the V_{max} values in column 3 increased continuously from day 360 onward, up to 210 μM/h on day 511. This increase in TCE dechlorination rate after day 360 corresponded to both a 3-fold increase in the amount of sodium lactate fed to the column on day 365 and to a series of increases in the column inlet TCE concentration over that period.

The procedure previously described was also used to calculate V_{max} values for c-DCE and VC dechlorination in column 3 after bioaugmentation. Lacking any better information, the same K_m used for TCE was assigned to c-DCE and VC and the V_{max} values for these compounds were generated simultaneously with those for TCE using Michaelis–Menton kinetics and the data-fitting routine. In contrast to TCE, the V_{max} values for c-DCE and VC appeared to be highest shortly after bioaugmentation and then diminish to a constant value with time and increasing TCE concentrations (Table 6). The V_{max} for c-DCE was found to be 78.3 μM/h at 200 days and then declined rapidly to ~25 μM/h thereafter. Similarly, the V_{max} for VC was 275 μM/h at day 200 and then declined more gradually to 10–15 μM/h for the last 100 days of the study.

It is probable that some of the higher biodegradation rates appearing in Table 6 for c-DCE and VC are overstated in this analysis. The fitting routine is very sensitive to peak concentrations of these contaminants. In some cases, the peak concentrations of c-DCE and VC may have occurred between sampling ports, and therefore they would have been missed in the analysis. This may partially explain the high V_{max} values calculated for c-DCE on day 200 and for VC on days 200 and 396.

Discussion

It was initially hypothesized that bacteria capable of dechlorinating TCE or PCE to ethene were ubiquitous in soil and that this activity was largely a function of the electron donor and supplemental nutrients used. On the basis of the results of this study performed in Dover soil, this hypothesis appears to be false. Dechlorination of TCE to c-DCE was established using a variety of electron donors, including lactate, lactate and methanol, butyrate, glutamate and 1,2-propanediol, and toluene, but none of these amendments stimulated c-DCE dechlorination. Supplemental nutrients and longer residence times in the columns also did not produce c-DCE dechlorination. Dechlorination of c-DCE to ethene was initiated only after the inoculation of the soil columns with a competent TCE-dechlorinating bacterial consortium.

These results support the alternate hypothesis that microorganisms capable of dechlorinating TCE to ethene are not present in all habitats. Bacteria that dechlorinate TCE to c-DCE appear to be robust and relatively ubiquitous, whereas bacteria that dechlorinate c-DCE to ethene appear more infrequently (*16*). In this study, TCE dechlorination to c-DCE was observed 20 days after adding lactate to columns 1 and 5, and in similar time after adding glutamate and 1,2-propanediol to column 4 following 4 months of unamended operation. These TCE-dechlorinating bacteria did not appear to be associated with methanogenesis because in most columns TCE dechlorination preceded methanogenic activity. The aquifer at Dover is poised between aerobic and anaerobic conditions. A facultative aerobic bacterium capable of dechlorinating TCE to c-DCE was recently isolated from a PCE-contaminated site in Victoria, TX (*27*), suggesting that similar microorganisms may catalyze this activity at Dover as well.

Microorganisms capable of dechlorinating c-DCE to ethene did not appear to be present in the Dover soil columns. These microorganisms were either never present in the Dover soil or were lost during collection and transport of the soil from the field to the laboratory. The latter may be the case for the site 6 soil, where dechlorination activity to VC and ethene was observed in the field but not in the laboratory (Table 1).

TABLE 5. Example Electron Balances in Column 3[a]

day	inlet [TCE] (μM)	total donor electrons (meq/L)	electrons to methane production (meq/L)	electrons to iron reduction (meq/L)	electrons to dechlor. (meq/L)	unused donor electrons (meq/L)	total electron balance (%)
200	29.3	51.7	57.4	0.40	0.13		112%
284	152	36.7	34.1	0.51	0.54		96%
320	227	32.6	34.0	0.64	0.32		109%
360	455	32.6	33.9	0.76	1.60		111%
396	576	60.5	49.2	0.54	1.91		85%
431	1173	74.2	46.2	1.17	4.45	10.32	84%
478	1245	62.2	53.1	1.00	3.10	4.30	99%
511	1293	68.9	34.6	0.92	3.38	14.2	75%

[a] Notes: Electron milliequivalents of influent and unused electron donors were calculated assuming complete mineralization to CO_2 and hydrogen. Total electron balance reflects recovery of influent electrons.

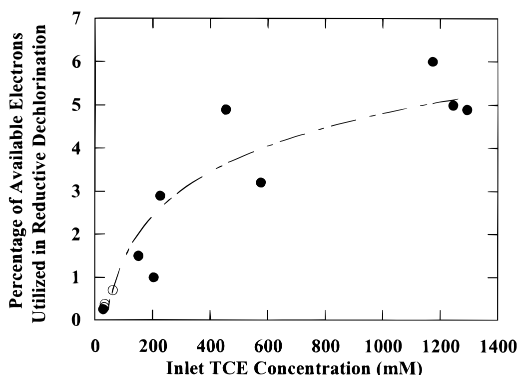

FIGURE 9. Efficiency of the biodegradation process as measured by the percentage of electrons utilized for reductive dechlorination as a function of influent TCE concentration in column 3. The system appeared to be electron donor limited when the TCE concentration was initially raised to 400 μM, resulting in incomplete dechlorination. Lactate was subsequently increased 3-fold, and dechlorination once again proceeded to completion.

TABLE 6. V_{max} for TCE, c-DCE, and VC as a Function of Time and TCE Concentration in Column 3[a]

day	inlet [TCE] (μM)	TCE V_o (μM/h)	TCE V_{max} (μM/h)	DCE V_{max} (μM/h)	VC V_{max} (μM/h)
134	28.0	5.07	40.6		
157	21.7	6.03	87.5		
178	28.6	6.63	60.6		
200	29.3	5.70	45.5	78.3	275.8
234	204.5	22.4	46.4	47.4	65.4
285	151.8	18.0	47.3	22.5	
325	227.0	24.2	46.9	21.8	27.8
360	455.0	47.3	75.1	14.9	4.1
396	575.6	69.1	97.8	39.7	500.0
431	1173.5	127.7	160.2	26.5	8.9
478	1244.7	169.9	176.4	23.0	15.6
511	1297.3	198.3	210.4	23.5	10.6

[a] Notes: TCE V_o values are the initial TCE dechlorination rates observed in the column. V_{max} values were calculated by fitting the TCE, c-DCE, and VC biodegradation data at each time point to a Michaelis–Menton model using a K_m value of 136 μM.

Dechlorination of c-DCE in Dover soil occurred only after bioaugmentation with the Pinellas culture. Once the inoculated bacteria had adapted in the columns, dechlorination of c-DCE was rapid and stable and persisted in column 3 for more than 1 year. The Pinellas culture has subsequently been used to bioaugment microcosms containing soils from three other sites where dechlorination was incomplete. In all of these soils, bioaugmentation stimulated complete dechlorination to ethene. Similar results have also been achieved by workers at DuPont, utilizing a different anaerobic culture (*28*).

These results suggest that a non-native c-DCE-dechlorinating culture can coexist with indigenous TCE-dechlorinating microorganisms in a soil matrix under amended conditions, perhaps by occupying a vacant niche in the microbial community. This indicates that bioaugmentation is a viable strategy for remediating chlorinated solvent sites where dechlorination has stopped at c-DCE or VC. These may represent as many as half of the chlorinated solvent sites currently under study. The Pinellas culture was recently applied in the field as part of the accelerated biodegradation pilot performed at Dover AFB (*29*). In that pilot, electron donor and nutrient addition alone stimulated the conversion of TCE to c-DCE, whereas complete dechlorination of c-DCE to ethene was observed only after bioaugmentation.

The results of the column study also demonstrated that dechlorinating bacteria can tolerate high levels of TCE and still maintain dechlorination activity. The microorganisms tolerated TCE concentrations up to 1300 μM (170 mg/L), or 13% of water saturation for TCE, without shutting down activity. A different anaerobic bacterial culture enriched from soil has been shown to dechlorinate 55 mg/L of PCE to ethene, which is 36% of water saturation for PCE (*30*). In a more recent column study, a third anaerobic culture was able to dechlorinate up to 100 mg/L of PCE to ethene and small amounts of VC (*31*). This level of PCE represents 66% of water saturation.

Dechlorination rates calculated in the present study were similar to those calculated by Tandol and co-workers (*32*) in methanol-amended microcosm studies of PCE, TCE, c-DCE, and VC dechlorination, where chlorinated ethenes were present at levels between 450 and 550 μM. These workers applied Michaelis–Menton kinetics to their data and estimated V_{max} values of 40, 30, and 45 mM/h for TCE, c-DCE, and VC dechlorination, using K_m values of 50, 10, and 400 μM, respectively. The agreement of these values with those from the present study is quite good, given that the level of biomass in the microcosms was probably dissimilar to that in the Dover columns and that the assignment of K_m values was rather arbitrary in both cases.

In the present study, faster TCE dechlorination rates were noted at longer times and higher TCE inlet concentrations. This increase in rate may have resulted from an increase in the numbers of TCE dechlorinators present due to higher levels of electron donor (sodium lactate) in the column, or perhaps from a shift in the microbial population to favor dechlorinators at the higher TCE concentrations. This increase in biodegradation rate with time and TCE concentration was not noted for c-DCE or VC dechlorination. It is possible that VC and c-DCE dechlorination may have been inhibited by the higher concentrations of TCE and its transformation products, as was observed in the Tandol study (*32*).

The concentrations of TCE and PCE tolerated by these cultures are well above those indicative of the presence of nonaqueous phase liquid (NAPL) in the subsurface. This suggests that accelerated biodegradation and bioaugmentation may represent an effective remedial strategy for source areas where residual NAPL reside. The high degradation rates observed in this and other studies are sufficient to remove levels of dissolved phase contaminants typically observed in the vicinity of NAPLs. In addition, biodegradation in proximity to NAPLs has the potential to create enhanced contaminant concentration gradients in the aqueous phase surrounding NAPL surfaces, resulting in more rapid NAPL dissolution and reduction in source life. Because source life is usually the single most important parameter in determining the remediation time, processes which accelerate source removal have the potential to substantially impact remedial economics.

In general, electron donor and nutrient costs at the levels used in this study are not a substantial component of the overall process cost for accelerated anaerobic biodegradation systems. Electron donor and nutrient costs were previously estimated to be 5−10% of the total system costs (*11*), although data from the pilot system at Dover AFB suggest this percentage may in fact be substantially lower. Capital, labor, and analytical costs are typically more significant in driving the process economics of these systems.

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