SERDP PROJECT CU-1167

19 DEC 2003

Final Technical Report

December 2003

# AEROBIC AND ANAEROBIC TRANSFORMATION OF *cis*-DICHLOROETHENE (*cis*-DCE) AND VINYL CHLORIDE (VC): STEPS FOR RELIABLE REMEDIATION

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MAR 11 2004 9
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04-S-1022

# Report Documentation Page

*Form Approved*
*OMB No. 0704-0188*

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| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| **DEC 2003** | | **00-00-2003 to 00-00-2003** |

4. TITLE AND SUBTITLE

**Aerobic and Anaerobic Transformation of cis-Dichloroethene (cis-DCE) and Vinyl Chloride (VC): Steps for Reliable Remediation**

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

**Michigan State University,East Lansing,MI,48824**

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

**Approved for public release; distribution unlimited**

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | |
| **unclassified** | **unclassified** | **unclassified** | **Same as Report (SAR)** | **84** | |

**Standard Form 298 (Rev. 8-98)**
Prescribed by ANSI Std Z39-18

# 1. SUMMARY OF MAJOR FINDINGS AND CONCLUSIONS

- *Dehalococcoides* sp. strain FL2 was isolated in pure culture and characterized. Strain FL2 couples growth with the reduction of TCE, *cis*-DCE, and *trans*-DCE.

- The first isolate capable of growth with VC as electron acceptor was obtained in pure culture. Other growth-supporting electron acceptors include all DCE isomers, vinyl bromide, and 1,2-dichloroethane. The isolate, designated strain BAV1, belongs to the Pinellas group of the *Dehalococcoides* cluster.
  → Populations that grow with all DCE isomers and VC as metabolic electron acceptor exist.

- Numerous robust enrichment cultures that reduced polychlorinated ethenes and VC to ethene were obtained. *Dehalococcoides* populations were detected in all ethene-producing cultures.
  → The results from this research effort, along with findings from other groups, suggest a link between the presence of *Dehalococcoides* spp. and ethene formation.

- *Dehalococcoides* populations that reduce *cis*-DCE and VC are not rare in the environment, and were detected in 75% of the aquifer and sediment materials tested.
  → Biostimulation is a promising approach to enhance dechlorination rates.

- *Dehalococcoides* populations are not uniformly distributed throughout the contaminated zone at a site, and are not present at all sites.
  → Bioaugmentation is a promising approach to promote complete reductive dechlorination.

- Reductively dechlorinating populations, including *Dehalococcoides* populations, were found aquifer material collected from oxic zones.

- Chloroethene-reducing bacteria were enriched from pristine and contaminated sites. Enrichment with *cis*-DCE or VC resulted in similar communities, independent of whether samples were collected from pristine or chloroethene-contaminated sites.
  → The ability to dechlorinate choroethenes may have evolved prior to anthropogenic pollution.

- The in-depth analysis and comparison of *Dehalococcoides* 16S rRNA gene sequences suggests that the 16S rRNA molecule has insufficient information to infer dechlorination

activity. Future research efforts should focus on the identification of structural genes involved in the process of interest (e.g., *cis*-DCE and VC detoxification).

→ The 16S rRNA gene approach is powerful for detetcing and quantifying *Dehalococcoides* populations but the resolution of these approaches is insufficient to infer specific dechlorination activities.

- High Fe(III) concentrations did not preclude reductive dechlorination activity in microcosms.
  → Reductive dechlorination is feasible at sites that have high amounts of bioavailable ferric iron.

- High $NO_3^-$ concentrations shut down reductive dechlorination but ethene formation resumed following the complete consumption of oxidized nitrogen species.
  → Dechlorinating populations are resilient to changes in environmental conditions.

- Aerobic degraders were not found in anaerobic zones.
  → Biostimulation of aerobic degradation of the target contaminants is only promising in aerobic portions of a plume.

- All VC-assimilating aerobic enrichments also degraded *cis*-DCE, but only in the presence of VC as the primary substrate.

- The aerobic TRW enrichment assimilated VC efficiently at low oxygen concentrations (DO < 1 mg/L) suggesting microaerophilic growth
  → This is important for natural attenuation of VC because many VC plumes contain little DO.

- The TRW enrichment culture survives VC starvation periods exceeding 6 months without losing its ability to assimilate VC.
  → The ability to survive extended starvation periods without losing degradation activity suggests that aerobic VC oxidation can be a robust process.

- Based on site geochemical and microbial data, most sites have the potential for reductive dechlorination of *cis*-DCE and VC. However, *cis*-DCE and VC persist at many sites.
  → Biostimulation/bioaugmentation is often necessary to enhance degradation rates.

## 2. PROJECT BACKGROUND

Considerable research has focused on the anaerobic transformation of PCE and TCE, two of the most common chlorinated solvents found in groundwater. However, relatively little is known about the types of microorganisms and specific environmental conditions associated with the dechlorination of dichloroethenes (DCEs) and vinyl chloride (VC). For the successful remediation of a contaminated site, the complete reduction to the environmentally benign products (e.g., ethene and inorganic chloride), or complete mineralization must be achieved. Recent research identified four different microbial processes that determine the fate of these compounds in groundwater. These processes include:

- Anaerobic energy-yielding reductive dechlorination (chlororespiration) **(Process 2)**
- Anaerobic energy-yielding oxidation **(Process 3)**
- Aerobic cooxidation **(Process 4)**
- Aerobic energy-yielding oxidation **(Process 5)**

Although the anaerobic cometabolic reduction (Process 1) of PCE to TCE and *cis*-1,2-dichloroethene (*cis*-DCE) was demonstrated (Jablonski and Ferry 1992; Fathepure and Boyd 1987, 1988; Fathepure et al. 1988; Gantzer and Wackett 1991; Glod et al. 1997), Process 1 does not contribute to the fate of DCEs and VC, and hence, was not a focus of our studies. The major goals were to shed light on the microbial populations carrying out Processes 2-5, and to combine the microbial information with site geochemistry and activity information to produce criteria for site-specific recommendations. Specifically, the following objectives were addressed during the 3-year performance period:

- Identify the processes that result in the rapid degradation of *cis*-DCE and VC under aerobic and anaerobic conditions.
- Identify the important groups of organisms involved in *cis*-DCE and VC degradation/dechlorination.
- Determine mechanisms of enhancing the *cis*-DCE/VC degradation rates of the individual processes.

- Isolate *cis*-DCE/VC degrading/dechlorinating organisms in pure culture.
- Characterize the physiology and phylogeny of the most important *cis*-DCE/VC degrading/ dechlorinating organisms.
- Investigate how site-specific characteristics determine which of the above identified processes is the most promising bioremediation strategy.
- Develop a protocol to evaluate site-specific characteristics in order to facilitate decisions on which process is most promising for a particular site.

## 3. ACCOMPLISHMENTS

### Task 1: Sample Collection

To address these objectives, samples were collected from chloroethene-contaminated aquifers, including numerous military sites, and pristine river sediments. Site geochemical data were analyzed to decide on the most promising locations at a given site for sample collection and exploration of Processes 2-5. For example, locations where the geochemical data suggested active iron reduction associated with loss of *cis*-DCE and/or VC were targeted to explore Process 3. Microcosms were established to evaluate the occurrence of bacterial populations responsible for Processes 2-5. Tables 1, 2, and 3 summarize the sites from which sample materials for microcosm set up were obtained. Note that from most sites, sample materials from multiple locations were collected and tested.

**Table 1.** Site materials evaluated for reductive dechlorination of *cis*-DCE and VC (**Process 2**).

| Site | Dechlorination end product formed from | | Enrichment cultures | *Dehalococcoides* detected |
|---|---|---|---|---|
| | *cis*-DCE | VC | | |
| Alameda NAS, CA | Ethene | Ethene | No | Yes |
| Au Sable River, MI | Ethene | Ethene | Yes | Yes |
| B&J Industrial site, Nunica, MI | *cis*-DCE | VC | - | No |
| Bachman Road site, Oscoda, MI | Ethene | Ethene | Yes | Yes |
| Dobbins ARB (AFP6), GA | Ethene | Ethene | Yes | Yes |
| Fallon NAS, NV | *cis*-DCE | VC | - | No? |
| FMC Site, San Jose, CA | Ethene | Ethene | Yes | Yes |
| Hydrite Chemical Co., Cottage Grove, WI | Ethene | Ethene | Yes | Yes |
| Jacksonville site, FL | Ethene | Ethene | No | Yes |
| Long Branch Site, NJ | *cis*-DCE | VC | - | No |
| Northrop Grumman plant 66, GA | Ethene | Ethene | Yes | Yes |
| Père Marquette River, MI | Ethene | Ethene | Yes | Yes |
| Pine River, MI | Ethene | Ethene | Yes | Yes |
| Red Cedar River, MI | Ethene | Ethene | Yes | Yes |
| Rottenwood Creek, Marietta, GA | Ethene | Ethene | Yes | Yes |
| San Jose Site, CA | Ethene | Ethene | No | Yes |
| Savannah River site, SC | *cis*-DCE | VC | - | No |
| Schoolcraft site, Kalamazoo, MI | *cis*-DCE | VC | - | No |
| Suzi River, Korea | Ethene | Ethene | Yes | Yes |
| TRW Minerva Site, OH | Ethene | Ethene | Yes | Yes |

**Table 2.** Site materials evaluated for anoxic oxidation of *cis*-DCE and VC (**Process 3**).

| Site | Disappearance of | | Enrichment cultures |
|---|---|---|---|
| | *cis*-DCE | VC | |
| Au Sable River, MI | No | No | - |
| B&J Industrial site, Nunica, MI | No | Yes | No |
| Bachman Road site, Oscoda, MI | No | No | - |
| Fallon NAS, NV | No | No | - |
| Hydrite Chemical Co., Cottage Grove, WI | No | No | - |
| Long Branch Site, NJ | No | No | - |
| Père Marquette River, MI | No | No | - |
| Red Cedar River, MI | No | No | - |
| Savannah River site, SC | No | No | - |
| TRW Minerva Site, OH | No | No | - |

**Table 3.** Site materials evaluated for aerobic oxidation of *cis*-DCE and VC (**Processes 4 and 5**).

| Site | Oxidation of [a] | | Enrichment cultures | Zone of sample collection |
|---|---|---|---|---|
| | *cis*-DCE | VC | | |
| B&J Industrial site, Nunica, MI | Yes (c) | Yes (c) | Yes | Aerobic |
| Bachman Road site, Oscoda, MI | Yes (c) | Yes (c) | Yes | Aerobic |
| Hydrite Chemical Co., Cottage Grove, WI | No | No | No | Anaerobic |
| Savannah River site, SC | No | No | No | Anaerobic |
| Aberdeen Proving Ground, MD | Yes (c) | Yes (m) | Yes | Aerobic wetland |
| Landfill in Norman, OK | No | No | No | Anaerobic |
| Portsmouth, OH | No | No | No | Anaerobic |
| Stillwater, OK | No | No | No | Anaerobic |
| Tinker AFB, OK | No | No | No | Anaerobic |
| TRW Minerva Site, OH | Yes (c) | Yes (m) | Yes | Microaerophilic |
| Wichita Site, KS | - | No | No | Anaerobic |

[a] Indicated in parenthesis is the nature of the process (i.e., c, cometabolic; m, metabolic).

## Task 2: Microcosm Setup

### Process 2: Anaerobic energy-yielding reductive dechlorination (chlororespiration).

Procedures for sampling and microcosm setup were performed essentially as described by Fennell et al. (2001) and He et al. (2002), unless indicated otherwise. Aquifer materials were collected by geoprobing (Geoprobe Systems, Salinas, KS). The cores were capped, immediately stored at 4°C, and shipped. In the lab, the cores were immediately transferred inside an anoxic

chamber, and transferred to sterile Mason jars and mixed thoroughly. Reductive dechlorination potential of indigenous microorganisms and dechlorination endpoints were assessed in duplicate or triplicate microcosms amended with a single chloroethene (i.e., *cis*-DCE or VC) added as electron acceptor. $H_2$, acetate, or lactate was provided in excess (about five times the amount theoretically needed for complete reduction of the chlorinated electron acceptor to ethene) as a source of reducing equivalents. Hydrogen and acetate concentrations were monitored, and microcosms were fed additional electron donor (i.e., $H_2$ or acetate) to maintain an electron donor surplus.

Dependent on the amount of aquifer materials available, microcosms were established in 24-ml (nominal capacity) vials or 160-ml (nominal capacity) serum bottles by placing 2 g of aquifer or 40 g of aquifer or sediment materials into sterile 24-ml glass vials or 160-ml serum bottles, respectively. Anoxic groundwater collected from the sampling location, anoxic potassium phosphate buffer (10 mM, pH 7.2), or synthetic basal salts medium (Löffler et al. 1997, 1999; Sung et al. 2003) was added to a total volume of 10 ml or 100 ml. All manipulations were performed inside the anoxic chamber or under a stream of sterile $N_2$ gas. The culture vessels were sealed with sterilized Teflon-lined rubber stoppers. *cis*-DCE was added from anoxic aqueous stock solutions to initial aqueous concentrations ranging from 20 to 200 μM. Gaseous VC was added by syringe to intial aqueous concentrations ranging from 20 to 500 μM. To prevent contamination, aseptic technique was applied to the greatest extent possible during sample collection, material handling, and microcosm setup inside the anaerobic chamber. Aquifer materials from different sites were never handled simultaneously to avoid cross contamination. Microcosms were incubated stationary in horizontal position at 25°C in the dark.

**Process 3: Anaerobic, Energy-Yielding Oxidation**

Microcosms were established as described above in 160-ml serum bottles. Following removal of the serum bottles from the anaerobic chamber, $H_2$ was removed by purging the headspace with ultra pure nitrogen gas. Nitrate (5 mM), Fe(III) citrate (5 mM), Fe(III)-EDTA, αFeOOH (goethite), or amorphous $MnO_2$ were added as electron acceptors. Iron-EDTA was used in some of these studies because it does not supply a potential carbon source (as Fe(III) citrate), and it is

a readily bioavailable form of dissolved ferric iron. Amorphous $MnO_2$ was prepared according to a method described by Lovley and Phillips (1988), and crystalline $MnO_2$ was prepared according to the protocol by Nealson et al. (1991) Synthetic goethite (αFeOOH) was produced following the recipe by Atkinson et al. (1967) Total and reduced iron was measured using a modified version of the ferrozine assay (Stookey 1970), and nitrate was monitored as described (Sanford et al. 1996). Microcosms were established in triplicates, and loss of *cis*-DCE and VC was compared to heat-killed control microcosms and microcosms that were not amended with additional electron acceptor. *Shewanella putrefaciens* strain MR-4 reduces nitrate and many forms of solid and soluble iron and manganese with a variety of electron donors but cannot dechlorinate *cis*-DCE or VC. Microcosms containing sterilized site materials were inoculated strain MR-4, and served as (i) positive controls for metal and nitrate reduction not associated with *cis*-DCE or VC oxidation, and (ii) as negative controls for anoxic *cis*-DCE or VC oxidation.

**Process 4: Aerobic Cooxidation**

**Process 5: Aerobic, Energy-Yielding Oxidation**

Aerobic biodegradation of *cis*-DCE and VC was evaluated using 160-ml serum bottles (Wheaton, Millville, NJ) sealed with 20-mm Teflon-lined rubber septa and aluminum caps (The West Co., Lionville, PA). Microcosm studies were initiated using 10 g (wet weight) aquifer material from the TRW site and 90-ml mineral salts medium (MSM). Enrichment cultures were established from active microcosms following repeated transfers (2% vol/vol) to fresh MSM amended with *cis*-DCE or VC with/without a primary substrate. All experiments using the enrichment culture were performed in 160-ml serum bottles with 50 ml MSM. The bottles were amended with VC, methane, ethane, and/or ethene using 1 or 5-ml disposable plastic syringes. Addition of TCE, *cis*-DCE, or *trans*-DCE to microcosms was accomplished with gas-tight glass syringes (Hamilton Co., Reno, NV). The amount of cis-DCE, VC and other volatile organic substrates added to microcosms were calculated using a dimensionless Henry's constant (Gossett 1987). Henry's constant at 25°C for TCE, *cis*-DCE, *trans*-DCE, VC, ethene, ethane, and methane was 0.372, 0.167, 0.294, 1.137, 7.24, 17.3 and 26.12, respectively. For each treatment, triplicate active and duplicate autoclaved

controls were used. Microcosms were incubated static in the dark at 30°C with the stoppers facing down. Prior to analysis, bottles were kept at room temperature for 30 min (23 ± 2°C).

## Task 3: Physiological and Phylogenetic Characterization

## Task 4: Enrichment and Isolation

**Process 2: Anaerobic energy-yielding reductive dechlorination (chlororespiration)**
Figures and Tables for this section are attached in Appendix I.

### (i) Isolation of *Dehalococcoides* sp. strain FL2.

A TCE-to-ethene-dechlorinating culture consisting of three populations was derived from a PCE-to-ethene dechlorinating enrichment derived from Red Cedar River sediment collected in Okemos, MI. A 16S rRNA gene-based community analysis of this culture revealed the presence of three distinct populations, a *Dehalococcoides* population and two spirochetal populations. The spirochetal populations could readily be obtained in pure culture whereas the isolation of the *Dehalococcoides* population was difficult and time-consuming. Dilution-to-extinction series in mineral salts medium with TCE as the only available electron acceptor eventually eliminated both spirochetal populations.

### (ii) Characterization of *Dehalococcoides* sp. strain FL2.

The isolate, designated *Dehalococcoides* sp. strain FL2, reductively dechlorinated TCE to stoichiometric amounts of VC (Figure 1) followed by slow reduction to ethene (not shown). Strain FL2 could be propagated in defined, completely synthetic mineral salts medium amended with TCE, *cis*-DCE, or *trans*-DCE as the electron acceptor, acetate as the carbon source, and hydrogen as the electron donor (Figure 2 a, b). 1,1-DCE, however, did not support growth and was not dechlorinated, even in the presence of a growth-supporting electron acceptor (Figure 3a). *Dehalococcoides* 16S rRNA gene-targeted real-time PCR confirmed growth of strain FL2 with TCE, *cis*-DCE, or *trans*-DCE as electron acceptors. VC accumulated in these cultures, and was slowly reduced to ethene, similar to what has been described for *Dehalococcoides ethenogenes* strain 195 (Maymó-Gatell 1997, 1999, 2001). VC reduction to ethene required the presence of a polychlorinated ethene (e.g., TCE, *cis*-DCE, *trans*-DCE). No growth or dechlorination occurred

in medium amended with VC as the only available electron acceptor (Figure 3b). In contrast to *Dehalococcoides ethenogenes*, strain FL2 failed to grow with PCE as electron acceptor but was able to cometabolically reduce PCE to TCE in the presence of TCE or *cis*-DCE. Hydrogen was required to support reductive dechlorination, and no other growth-supporting redox couples were identified. As shown in Figure 4, similar *cis*-DCE dechlorination rates were measured at $H_2$ partial pressures ranging from 0.05 to 1.5 atm. In the presence of a growth-supporting electron acceptor (i.e., TCE, *cis*-DCE, or *trans*-DCE), strain FL2 exhibited a hydrogen consumption threshold concentration of 0.078 ± 0.02 ppmv. This value is consistent with $H_2$ consumption threshold concentrations determined for other chlororespiring populations.

The phylogenetic analysis based on double stranded sequencing of nearly complete 16S rRNA genes placed strain FL2 within the Pinellas group of the *Dehalococcoides* cluster, and demonstrated that strain FL2 shared an identical 16S rRNA gene sequence with *Dehalococcoides* sp. strain CBDB1, a chlorobenzene-dechlorinating strain. The 16S rRNA gene sequence of *Dehalococcoides* sp. strain FL2 was submitted to GenBank (AF357918.2). Figure 5 shows a 16S rRNA gene-based phylogenetic tree demonstrating the relatedness of strain FL2 with other isolates and selected environmental clone sequences within the *Dehalococcoides* cluster.

Strain FL2 could be maintained in defined mineral salts medium amended with $H_2$ and TCE, and we dismissed our initial hypothesis that both spirochetal strains contribute to the nutrition of the dechlorinating *Dehalococcoides* population. This finding contrasts the observations made for *Dehalococcoides ethenogenes* strain 195, which cannot be grown in defined mineral salts medium. Since both spirochetal strains were isolated in pure culture, we tested culture supernatants, as well as cell fractions (e.g., whole cells, soluble cytoplasmic fraction, membrane fraction) to enhance reductive dechlorination activity in cultures of strain FL2. None of these additions increased the dechlorination rates relative to controls without amendments. The preparation of a manuscript describing the isolation and properties of *Dehalococcoides* sp. strain FL2 is in the final stage, and will be submitted shortly.

**(iii) Characterization of reductively dechlorinating enrichment cultures.**

Numerous sediment-free cultures that dechlorinated chloroethenes beyond *cis*-DCE and

produced ethene were derived from dechlorinating microcosms (Table 1). Dechlorination in all cultures was readily inhibited by air, and reducing conditions were a prerequisite for the initiation of the reductive dechlorination process. The inhibition of methanogenesis had no effect on the extent and the rates of reductive dechlorination. We focused on an enrichment derived from the Bachman Road site because of this culture exhibited robust dechlorination activity and dechlorinated VC readily to ethene with hydrogen provided as the electron donor. Interestingly, complete reductive dechlorination was supported by acetate as the only available electron donor, although the dechlorination rates with were lower compared to cultures that received hydrogen as electron donor. Neverthess, this was a relevant observation because all described cultures capable of producing ethene require $H_2$ as the electron donor. We investigated this phenomenon in more detail, and could demonstrate that the VC-to-ethene-dechlorinating Bachman enrichment culture contained hydrogenotrophic chlororespiring populations capable of complete reductive dechlorination to ethene, and syntrophic, acetate-oxidizing populations. In cultures that received acetate as the only electron donor, the syntrophs played a key role in supplying hydrogen generated in the oxidation of acetate to the hydrogenotrophic dechlorinators. Figure 6 shows the reduction of VC to ethene with acetate as the only available electron donor. The careful monitoring of reductive dechlorination, acetate consumption, and $H_2$ flux demonstrated that syntrophic acetate-oxidizing populations were present in the Bachman enrichment culture. As shown in Figure 6, the rates of VC dechlorination increased significantly when $H_2$ was added suggesting that $H_2$ is the preferred electron donor. A major conclusion of this work is that at sites that have syntrophic acetate-oxidizing populations and hydrogenotrophic chlororespiring populations, approaches that increase fluxes of either electron donor, acetate or $H_2$, may be sufficient to drive the reductive dechlorination process to completion. These findings were published in the September 2002 issue of Environmental Science & Technology, and a reprint is attached to this report.

The physiological characteristics of this highly enriched Bachman culture were studied in more detail. Growth occurred with *cis*-DCE, *trans*-DCE, 1,1-DCE, and VC as electron acceptors, and the reduction of DCEs to ethene is shown in Figure 7. VC was reduced at rates of

54.4 ± 3.5 μM $day^{-1}$, whereas DCEs were dechlorinated at about half this rate. Interestingly, PCE and TCE were not used as growth-supporting electron acceptors by the dechlorinating population(s) in the Bachman culture. In the presence of small amounts of a growth-supporting electron acceptor (e.g., *cis*-DCE, *trans*-DCE, 1,1-DCE, or VC), however, PCE and TCE were cometabolically reduced to *cis*-DCE followed by complete reduction to ethene. PCE and TCE were also reductively dechlorinated in cultures that were pregrown with *cis*-DCE, *trans*-DCE, 1,1-DCE, or VC, as shown in Figure 8. After several transfers to medium with lactate as the source of reducing equivalents, the culture had lost the ability to reduce VC with acetate as the only electron donor, presumably because the population(s) implicated in syntrophic acetate oxidation was lost in the enrichment process. Hence, no intrinsic $H_2$ formation from acetate occurred, and we were able to measure true $H_2$ consumption threshold concentrations rather than compensation concentrations for the reductively dechlorinating population(s). In the presence of VC, $H_2$ was consumed to a consumption threshold concentration of 0.12 (+/- 0.02) ppmv. This value was similar to $H_2$ threshold concentrations determined for *Dehalococcoides* sp. strain FL2, and is consistent with the findings for other hydrogenotrophic populations that use chloroorganic compounds as metabolic electron acceptors (Löffler et al. 1999).

In an effort to identify the population(s) responsible for DCE and VC reductive dechlorination in the Bachman culture, different 16S rRNA gene-based approaches were used. First, 16S rRNA gene primers targeting known chloroethene-dechlorinators were tested on genomic DNA extracted from the Bachman enrichment culture. No amplicons were obtained with primer pairs targeting 16S rRNA gene sequences of *Dehalobacter* and *Desulfuromonas* populations, even if the highly sensitive nested PCR approach was used. As shown in Figure 9, amplicons of the expected size (620 bp) were obtained in direct PCR with primers targeting 16S rRNA gene sequences characteristic for the *Dehalococcoides* group. Second, 16S rRNA gene clone libraries were established in *E. coli* using community DNA isolated from a VC-grown Bachman culture. The *Dehalococcoides* targeted primer pair identified five out of 82 clones in 16S rRNA gene clone libraries that contained inserts most similar to *Dehalococcoides* 16S rRNA genes sequences. Restriction fragment length polymorphism (RFLP) and partial sequencing (ca.

800 bp analyzed) of clones containing a *Dehalococcoides* 16S rRNA gene fragment identified two clones with chimeric 16S rRNA gene inserts. The three remaining clones could not be distinguished by RFLP and sequencing suggesting that the 16S rDNA inserts were derived from a single *Dehalococcoides* population. Nearly complete, double stranded sequence analysis of two cloned 16S rRNA genes yielded identical sequences most similar to *Dehalococcoides* sequences of the Pinellas subgroup. Quantitative PCR suggested that growth of one or more *Dehalococcoides* populations in the enrichment culture depended on the presence of VC as electron acceptor (Figure 10). The characterization of the ethene-producing Bachman enrichment culture was published in the May 2003 issue of Applied and Environmental Microbiology.

**(iv) Isolation of *Dehalococcoides* sp. strain BAV1.**

A major goal was to obtain a *cis*-DCE- and VC-dechlorinating population in pure culture. The isolation procedure took advantage of the organism's ability to derive all its energy required for growth from the reduction of VC to ethene, and continued transfers in mineral salts medium amended with VC, hydrogen, and acetate yielded a nonmethanogenic, ethene-producing culture. As mentioned above, dechlorination occurred with acetate as the sole electron donor, although at lower rates, apparently mediated in association with a syntrophic, acetate-oxidizing partner population. Consecutive transfers without hydrogen achieved further enrichment of the dechlorinating population. VC dechlorination activity was recovered repeatedly from $10^{-5}$ dilutions of consecutive dilution-to-extinction series in hydrogen-amended medium. Microscopic examination following this enrichment procedure revealed the presence of three morphotypes: a small, disc-shaped organism, as well as two rod-shaped organisms: one short and one long. Early attempts to tease out the VC-dechlorinating population in pure culture using the dilution-to-extinction principle as well as cultivation in semisolid medium containing 0.5% low melting agarose were unsuccessful. Similar to *Dehalococcoides* strains 195, CBDB1 and FL2, the addition of high concentrations of the peptidoglycan inhibitor ampicillin did not prohibit dechlorination, and after five consecutive transfers in liquid medium with 1 mg $ml^{-1}$ ampicillin, the rod-shaped organisms were no longer detectable by microscopic examination. Following ampicillin treatment, dechlorinating activity was recovered repeatedly from $10^{-7}$ dilutions in

defined basal salts medium amended with VC, hydrogen, and acetate. In addition, VC dechlorination occurred after transferring tiny opaque colonies that developed after 4 to 5 weeks in semisolid medium to liquid medium. No growth occurred in complex medium, and the culture appeared microscopically homogeneous. The isolate, designated BAV1, is a small disc-shaped organism of no more than 0.8 μm in diameter (Figure 11). Flagellar-based motility was not observed.

**(v) Characterization of *Dehalococcoides* sp. strain BAV1.**

Isolate BAV1 respired VC in defined, completely synthetic basal salts medium amended with acetate and hydrogen (Figure 12). At room temperature (22-25°C), BAV1 dechlorinated VC at rates of up to 134.2 ± 10 nmol $min^{-1}$ mg of $protein^{-1}$, and grew with a doubling time of 2.2 days to yield 239 ± 27 mg (mean ± SD, n = 6) of protein per mole of chloride released. Growth depended strictly on reductive dechlorination and the presence of hydrogen as an electron donor, which could not be replaced by organic substrates including formate, acetate, lactate, pyruvate, propionate, glucose, ethanol, and yeast extract. Besides VC, other growth-supporting electron acceptors included *cis*-DCE, *trans*-DCE, 1,1-DCE, or 1,2-dichloroethane and vinyl bromide, and stoichiometric amounts of ethene accumulated as the reduced end product. Notably, BAV1 is the first isolate capable of metabolic dechlorination of all DCE isomers. Chlorinated compounds not supporting growth included PCE, TCE, chlorinated propanes, 1,1,1-trichloroethane, 1,1-dichloroethane, and chloroethane. PCE and TCE, however, were cometabolized in the presence of a growth-supporting chloroethene, and ethene was produced. Other organic and inorganic electron acceptors such as nitrate, fumarate, ferric iron, sulfite, sulfate, thiosulfate, sulfur, or oxygen were not utilized, and no fermentative growth was observed. Respiratory growth was demonstrated conclusively by the chloroethene-dependent increase in cellular macromolecules (e.g., protein and DNA). Neither cell proliferation nor protein increase were detected in cultures lacking VC, acetate or hydrogen. Quantitative real-time (RTm) PCR demonstrated that the increase in cell numbers was concomitant with the consumption of VC (Figure 12). A linear increase in biomass (i.e., cells, as measured by the increase of 16S rRNA gene copies) occurred with increasing amounts of VC provided as electron acceptor, indicating a tight coupling between

reductive dechlorination and growth (Figure 13). The number of 16S rRNA gene copies measured in cultures without VC corresponded to the number of cells transferred with the inoculum, corroborating that no growth occurred in the absence of VC. Cultures that had consumed 80 µmoles of *cis*-DCE contained about twice as many 16S rRNA gene copies than cultures grown with 80 µmoles of VC (e.g., $9.28 \pm 0.41 \times 10^9$ versus $4.99 \pm 0.26 \times 10^9$ copies). These findings demonstrated that BAV1 captured energy from both dechlorination steps when grown with *cis*-DCE.

Phylogenetic analysis carried out with double stranded 16S rRNA gene sequencing affiliated isolate BAV1 (AY165308) with the Pinellas group of the *Dehalococcoides* cluster, a deep branch within the phylum Chloroflexi (green nonsulfur bacteria). The Pinellas group also includes *Dehalococcoides* sp. strains CBDB1 (AF230641) and FL2 (AF357918.2). Metabolic ethene formation is not restricted to members of the Pinellas group and was described in a mixed culture containing a *Dehalococcoides* population of the Victoria group. Table 4 displays known metabolic electron acceptors along with the phylogenetic grouping of identified *Dehalococcoides*-like populations.

**Table 4.** Electron acceptor utilization profiles of defined *Dehalococcoides*-like populations

| *Dehalococcoides* sp. | Group [a] | GenBank accession number | Electron acceptors |
|---|---|---|---|
| *Dhc. ethenogenes* strain 195 | Cornell | AF004928.2 | PCE, TCE, *cis*-DCE, 1,1-DCE, 1,2-dichloroethane, 1,2-dibromoethane |
| *Dhc.* sp. strain VS | Victoria | AF388550 | *cis*-DCE, VC |
| *Dhc.* sp. strain FL2 | Pinellas | AF357918.2 | TCE, *cis*-DCE |
| *Dhc.* sp. strain BAV1 | Pinellas | AY165308 | *cis*-DCE, *trans*-DCE, 1,1-DCE, VC, vinyl bromide, 1,2-dichloroethane |
| *Dhc.* sp. strain CBDB1 | Pinellas | AF230641 | 1,2,3-TCB [c], 1,2,4-TCB, 1,2,3,4-TeCB [c], 1,2,3,5-TeCB, 1,2,4,5-TeCB, PCDD [c] |

[a] Group designations according to Hendrickson et al. (2002); C/Cornell, V/Victoria, P/Pinellas sequence subgroups

[b] Bacterium VS was identified in a mixed culture (Cupples et al. 2003)

[c] TCB, trichlorobenzene; TeCB, tetrachlorobenzene, PCDD, polychlorinated dibenzo-*p*-dioxins

Isolate BAV1 shares a highly similar 16S rRNA gene sequence with *Dehalococcoides* populations that failed to dechlorinate chloroethenes or grow with VC as a metabolic electron acceptor (e.g., strains CBDB1 and FL2). This high degree of 16S rRNA gene sequence similarity among members of the *Dehalococcoides* cluster implies that analysis of 16S rRNA gene sequences cannot distinguish between populations of this group exhibiting different physiological activities. Hence, focusing only on 16S rRNA gene sequence analysis is not sufficient for characterizing the dechlorinating community, or to predict reliably the dechlorination potential associated with a particular environment.

BAV1 is the first isolate capable of metabolic reductive dechlorination of all DCE isomers and VC. The end products are nontoxic ethene and inorganic chloride. The isolation and characterization of *Dehalococcoides* sp. strain BAV1 was published in the July 2003 issue of the journal Nature.

A mixed culture containing strain BAV1 was used for a pilot demonstration in a hydraulically-controlled recirculation test plot at the chloroethene-contaminated Bachman Road site in Oscoda, Michigan (Lendvay et al. 2003). This pilot study validated that bioaugmentation with BAV1 is a promising approach to achieve detoxification at sites where the *in situ* microbiology to drive the reductive dechlorination process to completion is absent or the rates for contaminant removal are insufficient. Regenesis Bioremediation Products (www.regenesis.com) is marketing a mixed culture containing BAV1 as the Bio-Dechlor Inoculum.

**(vi) Community analysis of VC-dechlorinating enrichment cultures.**

**Detection of dechlorinating populations in ethene-producing microcosms and enrichment cultures.** Most of the ethene-producing microcosms listed in Table 1 were monitored for the presence of known dechlorinating populations (e.g., *Dehalococcoides*, *Desulfuromonas* and *Dehalobacter*) using 16S rRNA gene-based approaches. Importantly, *Dehalococcoides* populations were detected in all ethene-producing cultures, corroborating that members of the *Dehalococcoides* group are key players in complete reductive dechlorination to ethene (Ritalahti et al. 2001; Hendrickson et al. 2002). *Dehalobacter* populations were never detected in cultures that were enriched with *cis*-DCE or VC suggesting that members of this

group are not involved in reductive dechlorination in any of the ethene-producing microcosms listed in Table 1. Ethene-producing enrichment cultures were obtained from numerous dechlorinating microcosms. Four VC-to-ethene dechlorinating enrichment cultures (including the Bachman enrichment culture described above) were selected for a detailed analysis of the community structure using 16S rRNA gene-targeted PCR-based tools was performed. The four cultures were selected because they were obtained first, exhibited robust dechlorination activity, and were repeatedly transferred, and hence, more highly enriched. In addition, the cultures were derived from sites with different contamination histories, allowing us to compare dechlorinating communities derived from pristine and contaminated environments. The Bachman culture was derived from a chloroethene-contaminated site, the Red Cedar River (RC) culture from a site impacted by agricultural runoff, and the Au Sable River (AuS) and Père Marquette River (PM) cultures were obtained from pristine river sediment.

Community DNA was extracted from all four cultures, and 16S rRNA gene clone libraries were established. Amplified ribosomal DNA restriction analysis (ARDRA), PCR amplification with dechlorinator-targeted primer pairs, sequencing of 16S rRNA gene fragments of selected clones, and terminal restriction fragment length polymorphism (T-RFLP) profiles were performed to thoroughly characterize and compare communities. In addition, these techniques were evaluated and compared for their applicability for reliable identification of *Dehalococcoides* populations in the dechlorinating enrichment cultures.

A total of 291 individual clones were analyzed with primer pairs targeting known dechlorinating populations (e.g. *Dehalococcoides, Desulfuromonas, and Dehalobacter*). A single *Dehalococcoides* clone was found in the clone library established with the VC-dechlorinating culture derived from the Red Cedar River, and no clones with *Dehalococcoides* 16S rRNA gene inserts were detected in the clone libraries established with community DNA from the other cultures. This was an unexpected result because *Dehalococcoides*-specific amplification products were obtained from all four cultures when community DNA was used as template. Following two transfers to medium amended with ampicillin, an additional 31 *Dehalococcoides* clones were obtained (12 from the Au Sable culture, five from the Père Marquette culture, and 24

from Bachman Road Site culture). Apparently, enrichment in the presence of ampicillin selectively enriched for *Dehalococcoides* populations, and clones containing a *Dehalococcoides* 16S rRNA gene insert were among the dominating clones types.

Cloned 16S rRNA gene fragments of other community members from four of the constructed libraries were analyzed using the 4-base cutting enzyme *Msp*I. ARDRA analyses revealed highly enriched communities with only 5-8 different populations. A representative cloned 16S rRNA gene fragment that generated each dominant *Msp*I pattern was sequenced (approximately 500 bases of the 5' end) to determine the phylogenetic relationships of the corresponding populations. In addition to the *Dehalococcoides* species, members of the gamma-Proteobacteria (*Pseudomonas, Serratia, Acinetobacterium, Dechloromonas,* and *Lysobacter*), and members of the Firmicutes (Clostridiales) (*Clostridium, Acetobacterium, Acidaminobacter, Anaerovibrio, Sporomusa* and *Denitrobacterium*) were detected in the enrichment cultures. Despite differences in composition, *Clostridium* species were detected in all cultures, either as members of the clone libraries and/or by isolation of pure cultures. Surprisingly, 16S rRNA gene sequences 99% similar to that of *Pseudomonas lundensis* were also consistently found in all of the libraries, yet pure cultures could not be recovered under aerobic growth conditions.

Despite subtle differences, prolonged enrichment with VC as terminal electron acceptor resulted in similar bacterial communities, independent if the samples were collected from pristine sites or a chloroethene-contaminated aquifer. T-RFLP analysis using three different restriction enzymes corroborated the findings of the clone library analyses. No more than 4-10 different populations were detected in all four VC-dechlorinating enrichment cultures. T-RFLP, however, failed to consistently detect a peak (T-RF) indicative of a *Dehalococcoides* population in every DNA preparation from the VC-dechlorinating enrichment cultures, which is consistent with what we observed in the clone library analyses. Therefore, the T-RFLP approach cannot be expected to reliably identify specific *Dehalococcoides* T-RFs in a mixture of 16S rRNA gene amplicons obtained from community DNA of naturally diverse sediment or aquifer samples. *In silico* digests of known *Dehalococcoides* 16S rRNA gene sequences suggested that *Dehalococcoides* populations belonging to the Cornell group are not detectable using a T-RFLP approach under

certain conditions. For instance, *Dehalococcoides ethenogenes* strain 195 yielded a T-RF of 1,073 bases in length when digested with enzyme *Hha*I, a fragment too large to be resolved with T-RFLP. A missing *Hha*I restriction site in strain 195's 16S rRNA gene is consistent with the sequence difference that places strain 195 in the Cornell group of the *Dehalococcoides* cluster (Hendrickson 2002). Another problem arises when DNA is obtained from samples harboring complex microbial communities. Then, T-RFLP analysis may not unambiguously assign T-RFs to unique populations. For example, *in silico* digestion of 16S rRNA gene sequences of several *Clostridium* species resulted in T-RFs of 514 or 518 bp with *Msp*I, and 445 bp with *Rsa*I, which are very similar to the T-RF sizes of the known *Dehalococcoides* populations of the Pinellas group (i.e., T-RFs of 514 and 442 bp). *Clostridia* are common members of anaerobic microbial communities, and thus, clostridial T-RFs might mask or be interpreted erroneously as *Dehalococcoides* T-RFs, leading to a false-positive interpretation of T-RFLP profiles. *Hha*I digestion distinguishes *Clostridia* (T-RFs of 223 or 233 bp) from populations of the Pinellas group, which generate 202 bp T-RFs.

Due to the difference in the number of target 16S rRNA genes among different populations, the screening of clone libraries and T-RFLP profiles is not recommended to either validate or exclude the presence of *Dehalococcoides* populations, unless the community DNA was extracted from highly enriched dechlorinating cultures. Neither approach unambiguously detects the presence of *Dehalococcoides* populations, whereas the nested PCR approach was always reliable.

Two manuscripts summarizing the findings of the community analysis of the dechlorinating cultures and describing the application of 16S rRNA gene -based tools to detect *Dehalococcoides* populations have been prepared. One manuscript is currently under review for publication in Applied and Environmental Microbiology, and a second manuscript will be submitted to Microbial Ecology shortly.

**Process 3: Anaerobic oxidation of *cis*-DCE and VC.**

An extensive screening of the site materials listed in Table 2 for the anaerobic oxidation of *cis*-DCE and VC coupled to the reduction of ferric iron, manganic ion, or nitrate was performed.

Several different chelated forms of ferric iron including ferric citrate, iron-EDTA, and αFeOOH were tested as electron acceptors. Increased VC disappearance relative to controls occurred in a single microcosm established with aquifer material from B&J Industrial site. All microcosms were established in triplicate, and VC disappearance occurred in only one of three identical microcosms established with B&J material. Ferrous iron formation occurred in the microcosm that also showed significant loss of VC. Despite extensive efforts, we were unable to establish secondary enrichments that degraded VC under iron-reducing conditions or with any other electron acceptor tested (i.e., nitrtate, manganese oxide). When the original 'active' microcosm was amended with more VC, no further disappearance occurred. We established additional microcosms with B&J aquifer material and VC. None of the microcosms showed any significant loss of VC relative to killed control microcosms.

One of the promising materials tested from the Minerva site contained physiologically relevant concentrations of bioavailable ferric iron, and field measurement suggested the disappearance (e.g., anoxic mineralization linked to Fe(III) reduction) of VC in this zone. In microcosms we readily detected reductive dechlorination (Process 2) and aerobic metabolic oxidation (Process 5) of VC, but we never observed VC degradation linked to the reduction of different forms of iron and manganese in any of the microcosms.

Interestingly, in several microcosms reductive dechlorination was not inhibited in the presence of ferric iron as an alternate electron acceptor suggesting that reductive dechlorination is a competitive terminal electron-accepting process. In contrast, reductive dechlorination was never observed in the presence of nitrate, however, reductive dechlorination started and ethene was produced in microcosms that had completely depleted nitrate and nitrite. This observation suggests that reductively dechlorinating populations (e.g., *Dehalococcoides* populations) endure unfavorable redox conditions in the bulk environment, and resume activity once geochemical conditions are suitable.

**Process 4, 5: Aerobic (co)metabolism of *cis*-DCE and VC.**

Several stable enrichment cultures were derived from VC and/or *cis*-DCE degrading

microcosms (Table 2, page 7). The following pages summarize the major findings, and include all Tasks and Milestones addressing Process 4 (co-metabolic aerobic oxidation) and Process 5 (metabolic aerobic oxidation). Figures and Tables for this section are attached in Appendix II. The Bachman and B & J Industrial aquifer materials yielded enrichments that degraded *cis*-DCE and/or VC only in the presence of ethene or methane as primary substrates. Further experiments involving these enrichment cultures were terminated due to poor degradation rates. The aquifer materials obtained from the TRW and Aberdeen sites yielded enrichments that degraded VC a growth substrate. Therefore, most of the kinetic and optimization work was carried out using the TRW and the Aberdeen enrichments. The report below only summarizes experiments carried out with the TRW enrichment. Two pure cultures of VC-assimilating organisms were isolated from the above enrichments. Preliminary characterization based on 16S rRNA sequence analysis indicated that these isolates belong to the genus *Mycobacterium* and *Ochrobacterium*..

**(i) Characterization of VC degradation by the TRW enrichment.**

Many anaerobes including chlororespiring bacteria catalyze reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) in a step-wise fashion to ethene via *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC). Although a large body of information exists concerning the reductive dechlorination of PCE and TCE, there is great uncertainty about the environmental fate of *cis*-DCE and VC, two of the most commonly detected dechlorination products. Microorganisms at many contaminated sites seem unable to completely dechlorinate PCE and TCE; consequently *cis*-DCE and VC often accumulate (McCarty 1977; Milde et al, 1998; Parson et al. 1984). VC can also be produced from 1,1,2,2-tetrachloroethane and 1,1,1-trichloroethane (Fathepure et al. 1999; Lorah & Olsen 1999; Vogel & McCarty 1987; Vogel et al. 1987). Both *cis*-DCE and VC are US EPA priority pollutants and VC is a known human carcinogen (Kielhorn 2000). Hence, their accumulation in the environment poses an important health risk. The maximum contaminant level for VC in drinking water (2 μg/liter) is lower than for any other volatile organic compound (Pontius 1999).

Numerous studies have demonstrated that VC can be oxidized readily to $CO_2$ under aerobic conditions. In addition, a few studies have implicated anaerobic oxidation of *cis*-DCE and

VC under iron-reducing, manganese-reducing, sulfate-reducing, humic acid-reducing, and methanogenic conditions (Bradley et al. 1998a, 1998b, 1997). However, the significance of anaerobic oxidation as a natural attenuation process in groundwater is unknown. Similarly, little is known about the ubiquity and diversity of organisms capable of anaerobic oxidation. Studies have shown that *cis*-DCE or VC can be readily degraded cometabolically by many pure and mixed cultures expressing mono and dioxygenase when grown on numerous primary substrates including methane (Dolan & McCarty 1995; Han et al. 1999), ethene (Freedman & Herz 1996; Koziollek et al. 1999), ethane (Freedman & Herz 1996; Verce & Freedman 2001), propane (Malachowsky et al. 1994; Phelps et al. 1991), propene (Ensign et al. 1992), isoprene (Ewers et al. 1990), isopropylbenzene (Dabrock et al. 1992), butane (Hamamura et al. 1997), 3-chloropropanol (Castro et al. 1992), ammonia (Rasche et al. 1991; Vannelli et al. 1990) or toluene (Schafer & Bouwer 2000). For each of these processes, oxidation of *cis*-DCE and VC was apparently a fortuitous occurrence with no clear advantage to the responsible organisms, and consequently required a sufficient concentration of a primary substrate to support microbial growth. Also, studies have shown that many of the cometabolic degradations are inherently slow, resulting in incomplete degradation of the target compounds. This is due to substrate competition, enzyme inactivation, and toxicity to the organism (Dolan & McCarty 1995; Rasche, et al. 1991; Alvarez-Cohen & McCarty 1991; Arp et al. 2001; Newman & Wackett 1997; Oldenhuis at al. 1991). Therefore, for maximal and sustained rates of cometabolic degradation of chloroethenes, a careful balancing of non-growth (chloroethene) and growth substrate concentrations is essential, a difficult task for *in-situ* processes (Arp et al. 2001). Further, the primary substrate may support the growth of non-degrading populations leading to decreased degradation efficiency and increased cost resulting from the addition of auxiliary substrates and nutrients.

Thermodynamic calculations suggest that sufficient energy is available for microbial growth with VC or *cis*-DCE under aerobic conditions (Dolfing and Janssen 1994). In addition, recent investigations have revealed that VC is naturally produced in soil in a reaction between humic substance, chloride ion, and an oxidant such as $Fe^{3+}$ or $OH^-$ (Keppler et al. 2002), and hence may

have provided long-term evolutionary selection for microbes with the ability to grow with VC. Despite these favorable conditions, only a few organisms belonging to different phyla or genera that utilize VC as the sole carbon source have been isolated, and hence, the ecological and biochemical diversity of this oxidation is still poorly understood (Hartmans 1995; Hartmans et al. 1985; Hartmans and de Bont, 1992; Hartmans et al. 1992; Verce et al. 2000; Coleman et al. 2002a). Similarly, only one organism capable of utilizing *cis*-DCE as a carbon source was recently isolated (Coleman et al. 2002b). Work by Coleman et al (2002a) showed VC degradation in 23 of 37 samples from 22 sites. Phylogenetic analysis of the VC-degrading isolates obtained from the various sites revealed that the majority of isolates belonged to the genus *Mycobacterium*. Therefore, isolation of novel microbes belonging to various groups or genera that assimilate VC is needed for understanding the diversity, ecology, and mechanism of VC degradation. On the other hand, studies with mixed or highly enriched cultures can provide important information about VC oxidation under competing environments. Most importantly, the data will be helpful in comparing rates and stability of the process with pure cultures, which is important for field applications. In the present study, we describe VC metabolism by a highly enriched consortium obtained from a chloroethene-contaminated site (TRW-Minerva, OH).

***Aquifer material and chemicals.*** The VC degrading culture was enriched from aquifer material obtained from a chloroethene plume at the TRW facility in Minerva, OH. The soil core was retrieved using a Geoprobe from 10 feet below ground surface at a location downstream from a TCE source area. The groundwater at the sample location mainly contained *cis*-DCE and VC and low concentrations of dissolved $O_2$, (<1 mg/l), $Fe^{2+}$ (<3 mg/l), $Mn^{2+}$, and organic carbon

Gaseous compounds including VC, ethene, ethane, and methane were 99%+ pure and obtained from Aldrich Chemical Co., Milwaukee, WI. TCE, *cis*-DCE, and *trans*-DCE were 98% pure and also purchased from Aldrich Chemical Co., Milwaukee, WI. All other chemicals used were of reagent grade. The following mineral salts medium (MSM) was used for the development of enrichment cultures and growth studies. The MSM contained (g/l): NaCl, 1.0; $NH_4Cl$, 1.0; KCl, 0.1; $KH_2PO_4$, 0.45; $K_2HPO_4$, 0.9; $MgSO_4.7H_20$, 3.0 and $CaCl_2.2H_2O$, 0.04. No vitamins or trace metals were added. The pH was adjusted to 7.0 with 1 N HCl or 1 N NaOH.

*Microcosm Setup.* Aerobic biodegradation of VC was evaluated using 160-ml capacity serum bottles (Wheaton, Millville, NJ) closed with 20-mm Teflon-lined rubber septa and aluminum caps (The West Co., Lionville, PA). Microcosm studies were initiated using 10 g (wet weight) aquifer material from the TRW site and 90-ml MSN medium. All experiments using the enrichment culture were performed in 160-ml capacity serum bottles with 50-ml MSM. The bottles were amended with VC, methane, ethane, and/or ethene using 1 or 5-ml disposable plastic syringes. Addition of TCE, *cis*-DCE, or *trans*-DCE to microcosms was accomplished with dedicated glass syringes (Hamilton Co., Reno, NV). The amount of VC and other volatile organic substrates added to microcosms were calculated using a dimensionless Henry's constant (Gossett 1987). Henry's constant at 25°C for TCE, *cis*-DCE, *trans*-DCE, VC, ethene, ethane, and methane was 0.372, 0.167, 0.294, 1.137, 7.24, 17.3 and 26.12, respectively. For each treatment, triplicate active and duplicate autoclaved controls were used. Microcosms were incubated static in the dark at 30°C with stoppers facing down. Prior to analysis, bottles were kept at room temperature for 30 min (23 ± 2°C).

*Optimization experiments.* Biodegradation of VC, ethene, *cis*-DCE, *trans*-DCE, and/or TCE was tested in serum bottles containing 50-ml MSM inoculated with 2.5 ml (5%) active TRW enrichment culture. The effect of oxygen levels on the biodegradation of VC was evaluated by first sparging microcosms with nitrogen for 20 to 25 min to remove dissolved oxygen (DO) from the growth medium. Bottles were crimp sealed and inoculated with active TRW culture. Different amounts of oxygen in the headspace were achieved by replacing a known volume of headspace with pure oxygen. The volume of oxygen needed to achieve desired concentrations of oxygen in the liquid was calculated using the Henry's constant of 31.5 for oxygen at 25°C. The potential for the TRW-enrichment to degrade VC after various periods of VC -starvation was assessed. A set of three active and two autoclaved microcosms were established with VC as the sole carbon source and the degradation was monitored. To determine long-term viability without VC, the cultures were consecutively fed VC three times in order to generate a sufficient amount of active biomass. After the third feeding, when no detectable VC remained, the culture was incubated in the absence of VC for various periods of time. At the end of each starvation period,

the bottles were purged with air and spiked with VC and the degradation was monitored.

*Analytical methods.* Biodegradation of VC and other substrates was monitored using an Agilent 6890 gas chromatograph equipped with a micro-electron capture detector (μECD) and a flame ionization detector (FID). The instrument control, data acquisition, and data analysis were accomplished using a ChemStation (Agilent Technologies). The chlorinated hydrocarbons including TCE, *cis*-DCE, and *trans*-DCE were separated on a DB-624 capillary column of 30 m x 0.323 mm x 1.8 μm film thickness (J & W Scientific, Folsom, CA) and the peaks were detected using the μECD. Similarly, VC, ethene, ethane, and methane were separated on a 30 m x 0.53 mm x 15 μm HP-Plot/AL203 capillary column (Agilent Technologies) and the peaks detected using the FID. Nitrogen (zero grade) was used as a carrier gas for both columns. The operating GC conditions for μECD were: carrier flow rate of 2.0 ml/min, make-up gas of 40 ml/min, oven temperature of 70°C for 5 min, and inlet temperature and pressure of 200°C and 10 psi, respectively. The detector temperature was 325°C. Samples were injected in split mode with a 10:1 ratio. The operating GC conditions for FID were: carrier flow rate of 10.9 ml/min, make-up gas of 5 ml/min, flow rates of $H_2$ and air 40 ml/min and 450 ml/min, respectively. The column was equilibrated at 100°C for 5 min before each run, inlet temperature and pressure were 150°C and 10 psi, and the detector temperature was 235 °C. Samples were injected in splitless mode.

For quantification of volatiles, 20 or 30 μl of headspace gas from microcosms were withdrawn using a 50-μL capacity gas-tight glass syringe and analyzed by GC. The GC response for each compound was calibrated and the peak areas were compared to the standards. Standards were prepared for each chlorinated and non-chlorinated hydrocarbons separately in 160-ml serum bottles filled with 50-ml distilled water and closed with Teflon-faced septa and aluminum caps. After equilibration at room temperature, the GC response for a range of mass of each compound was plotted and aqueous concentrations were calculated using Henry's constant, the slopes were used to quantify the unknown. The detection limit for aqueous VC was < 0.1μM. (much lower concentrations of VC could be detected when larger volumes were injected).

***Development of enrichment cultures.*** Aquifer microcosms that consistently degraded VC in the absence of added co-substrates were selected for further enrichment. A 5% (vol/vol)

sediment-free slurry was transferred to a new set of serum bottles containing fresh MSM spiked with approximately 300 μM aqueous VC. Degradation was monitored and the bottles were re-spiked with VC, once previously added VC was degraded. This was repeated at least six more times, before 5% of the sediment-free mixed culture was transferred to 1-L capacity bottles containing 500-ml MSM. The larger bottles were closed with rubber bungs containing Balch tubes (Bellco, Biotechnology) inserted in the center and closed with 20-mm Teflon-faced septa and aluminum caps. The culture was further enriched by repeatedly feeding with VC and transferring 5 % of the enrichment culture to fresh MSM after 7 or 8 additions of 200-250 μM VC in liquid. The headspace was purged with air after every 3 or 4 VC spikes and the pH of the culture medium was adjusted to 7.0. After 12 months of enrichment, the culture seemed to contain at least 2 or 3 morphologically distinct bacteria. All experiments were carried out using this highly enriched and stable culture, which degraded VC consistently within 2-3 days. Unless otherwise stated, experiments were initiated by transferring 2.5 ml of the enrichment from the 1-L bottles to 160-ml serum bottles containing 50-ml MSM.

***Analysis of microbial community by Denaturing Gradient Gel Electrophoresis (DGGE).*** The microbial community analysis was performed by Microbial Insights, Inc (Rockford, TN). Approximately 10-ml of the enrichment, maintained on VC as the sole carbon source, was pelletted by centrifugation and genomic DNA was extracted using the bead-beating procedure (Stephen et al. 1999). The DNA was purified by a glass-milk DNA purification protocol using a Gene Clean™ kit as described by the manufacturer (Qbiogene, Inc). PCR amplified bacterial 16S rRNA gene fragments were obtained by using a nested PCR approach. The first primer set corresponded to *Escherichia coli (E. coli)* bp positions 27f and 1492r of the 16 S rRNA gene. The second set of primers targeted a region of the 16S rRNA gene corresponding to *E. coli* positions 341f - 534r (forward primer contained a 40 bp GC-clamp). PCR product from the second amplification was separated on a DGGE gel as described by Muyzer et al. (1993). Amplicons were analyzed using a Bio-Rad D-Code 16/16 cm gel system maintained at a constant temperature of 60°C in 6 L of 0.5 x TAE buffer (20mM Tris acetate, 0.5 mM EDTA, pH 8.0). Denaturing gradients ranged from 30% to 65% denaturant (with 100% denaturant defined as 7 M

urea, 40% v/v formamide). A secondary size gradient was imposed on the denaturing gradient by including an 8-10% acrylamide gradient as described by Cremonesi et al. (1997). Each prominent band was excised and purified using QiaQuick purification kits (Quigen). Purified DNA was then sequenced. Sequence identifications were performed using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/Blast) and the "Sequence Match" facility of the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.htm)

***Biodegradation of VC by the TRW enrichment.*** Complete removal of VC occurred within 33 days with a 2-week lag time in slurry microcosms established with aquifer material from the TRW site. Additional amendments of VC resulted in immediate and rapid degradation (data not shown). Enrichment cultures were established from slurry microcosms that degraded 250 to 300 μM aqueous VC within 3 days over a 12-month period, suggesting that a population that grows on VC had been selected. Microscopic observations as well as colony morphology on agar plates suggested that the enrichment was mainly composed of 2 or 3 morphologically distinct organisms. The results in Figure 1 demonstrate the performance of the TRW enrichment culture for 200 days after the initial 12 months of enrichment process. The partial identity of dominant members of the enrichment was obtained by DGGE profiles of 16S rDNA amplified PCR products (Figure 2). The DGGE analysis of the enrichment culture shows three distinct bands (many faint bands also appeared). Band A did not yield usable sequence, while bands B and C were associated with two different organisms, *Mycobacterium* and *Rhodococcus* (>94% sequence similarity).

Experiments were conducted to evaluate degradation potential of the enrichment at various aqueous concentrations of VC ranging from 50 μM to 2500 μM. The initial rate of VC biodegradation increased linearly with increasing aqueous VC ranging from 50 μM to 250 μM (Figure 3), yielding a first-order rate constant of 0.214 day$^{-1}$. The rates further increased with increasing initial aqueous VC from 500 μM to 1,500 μM, but this removal rate could not be adequately described by Michaelis-Menten kinetics. At concentrations ≥1000 μM VC, removal occurred following a lag of 2 days. At the highest concentration tested (2,500 μM), only 70% of

the added VC was removed before degradation stopped, perhaps due to toxicity of VC or toxic metabolites.

***Biodegradation of VC at different oxygen concentrations.*** Degradation of VC occurred over a wide range of oxygen in the headspace, spanning 0.02 mmol/bottle to 4.47 mmol/bottle (Figure 4). Our results have shown that removal of VC occurred even at < 0.08 mmol of $O_2$ / bottle (0.7 mg/L DO). However, degradation was relatively rapid in microcosms containing 0.2 mmol of $O_2$ /bottle (1.8 mg/L DO). Repeated additions of VC to these bottles (no additional $O_2$ added) showed continued removal of VC suggesting that the TRW culture can degrade VC in the presence low levels of DO (see inset, Figure 4). Addition of higher amounts of oxygen in the headspace (0.2 to 4.47 mmol) did not result in an enhanced rate of VC degradation (data not shown). The incomplete removal of VC at $\leq$ 0.04 mmol/bottle may be due to $O_2$ limitation.

***Biodegradation of ethene, ethane, cis-DCE, trans-DCE and TCE.*** Experiments were initiated to evaluate the metabolic versatility of the TRW enrichment culture. The TRW enrichment readily degraded approximately 125 μM ethene equally well in the presence or absence of added VC (Figure 5), and could be maintained on ethene as the sole carbon source for an extended period of time (data not shown). The culture failed to degrade *cis*-DCE when added alone even after incubating for longer periods. However, *cis*-DCE was degraded in the presence of VC as a primary carbon source (Figure 6). Our data also shows that the culture did not degrade *trans*-DCE or TCE either in the presence or absence of VC. Similarly, the culture did not degrade methane or ethane when added alone or in conjunction with VC. Biodegradation of VC was also tested in the presence of easily assimilated compounds including acetate, glucose, pyruvate, yeast-extract, or trypticase soy. VC degradation occurred in the presence of all the substrates except glucose and pyruvate (Table 1 in Appendix II). However, when a 5% inoculum from these bottles was transferred to new bottles that lacked glucose or pyruvate, VC degradation proceeded normally (data not shown).

***Starvation experiment.*** The TRW culture was able to resume biodegradation of VC after various periods of VC starvation (Figure 7). The data demonstrated that VC degradation resumed with a minor lag after each starvation period. For example, a lag of roughly 3 to 5 days was seen

when the culture was incubated at 30°C without VC for a period ranging from 15 to150 days. Interestingly, the lag time did not increase proportionally with increased starvation times.

A stable microbial consortium capable of using VC as the sole source of carbon was developed using the TRW site material. The TRW enrichment culture differs in many respects from previously reported VC-degrading enrichments or pure cultures (Coleman et al. 2002a; Hartmans 1995; Hartmans et al. 1985; Hartmans and de Bont, 1992; Hartmans et al. 1992; Verce et al. 2000; Verce, et al. 2002). The culture's ability to survive and degrade VC at extremely low dissolved $O_2$ concentrations and the ability to withstand long starvation periods sets this enrichment apart from the other VC degrading cultures.

The highly enriched culture consistently degraded 250 μM aqueous VC to below the detection limit within 1 to 3 days of incubation (Figure 1). However, the rate declined markedly after 6 or 7 additions of VC. This could be due to decreased $O_2$ levels, accumulation of toxic intermediates, and/ or decreasing pH. Our tests have shown that after 6 or 7 repeated spikes of VC, the pH of the culture decreased to < 5.0. Adjusting the pH to 7.0 and replenishing the headspace with fresh air effectively restored the activity. The drop in pH indicates the production of HCl due to mineralization of added VC (Hartmans et al. 1985). DGGE analysis revealed the presence of *Mycobacterium* and *Rhodococcus* as dominant populations. Although several strains of *Mycobacterium* have been isolated and were shown to assimilate VC, no report of a pure culture exists in the literature showing the ability of *Rhodococcus* to degrade VC. From this mixed culture study, the role of *Rhodococcus* cannot be inferred. However, *Rhodococci* are a remarkable group of bacteria known for their ability to inhabit a diverse range of habitats and degrade a variety of compounds including haloalkanes (Parales et al 2002).

At low initial concentrations, biodegradation of VC proceeded with a first-order rate constant of 0.214 $day^{-1}$. However, at higher concentrations, the rate increase was not linear and the removal could not be accurately described by either the first-order or zero-order rate kinetics. Therefore, it is conceivable that the enrichment may have harbored more than one species of VC degrader, each with a different affinity for VC. In addition, other factors such as diffusion barriers or accumulating intermediates at high concentrations may have suppressed the VC

degradation rates. Kinetic equations have been developed to describe the degradation of one organic compound when transformation of pollutants involves both the uptake of the primary substrate and further metabolism of intermediates (Alexander, 1999).

The culture efficiently degraded VC in the presence of extremely low DO. Addition of as low as 0.06 to 0.08 mmoles of $O_2$ per bottle (0.5 to 0.7 ppm DO) resulted in the degradation of all 42 µmoles of VC (15 ppm aqueous VC). Therefore, these results clearly indicate that the enrichment is capable of degrading VC efficiently at much lower DO. Similarly, 150 mmoles of VC were consumed in the presence of only 0.2 mmole of $O_2$ (see inset Figure 4). Based on stochiometric calculations, complete mineralization of 1 mole of VC would require 2.5 moles of $O_2$. Therefore, the complete removal of 150 µmoles of VC requires 375 µmoles of $O_2$, suggesting that incomplete mineralization and thus accumulation of metabolic intermediates had occurred. Continued degradation of VC at low DO contraindicates toxicity expected due to the accumulation of first metabolite, VC- epoxide (chlorooxirane) implying that VC may have been metabolized to intermediates past chlorooxirane in the metabolic pathway (Hartmans 1995; Hartmans and de Bont, 1992). Additional research is needed to fully understand the pathways and specific intermediates that accumulate under microaerophilic conditions. The ability of the TRW culture to sustain growth and degrade VC at low DO is relevant because many contaminated sites are low in DO. A recent study by Coleman et al (2002a) showed that several strains of *Mycobacterium* possess a low Ks for oxygen during growth on VC, indicating that these strains can metabolize VC at a low oxygen threshold. VC did not degrade under alternative electron accepting conditions, such as denitrifying and sulfate-reducing (data not shown) indicating that $O_2$ is needed for VC metabolism.

The enrichment's ability to metabolize ethene and *cis*-DCE is important for natural attenuation. Both ethene and *cis*-DCE are predominant end-products formed during reductive dechlorination of higher chloroethenes (e.g., PCE, TCE) and chloroethanes (e.g., 1,1,1-TCA) and commonly can be found in the leading fringes of most contaminated plumes (Lee, et al 1998). Interestingly, the culture did not degrade methane or ethane even when supplied with VC. These observations suggest that perhaps alkene-monoxygenase-like enzymes may be involved in the

degradation of both VC and ethene (Arp et al. 2001; Hartmans and de Bont, 1992). Alkene monooxygenases from *Xanthobacter* Py2 and *Rhodococcus corallinus* B-276 (formerly, *Nocardia corallina*) degraded alkenes and chloroalkenes but not alkanes nor their chlorinated derivative (Ensign 1992; Saeki et al 1999).

The culture's ability to cometabolize *cis*-DCE in the presence of VC is consistent with a recently isolated VC metabolizing *Pseudomonas* sp, strain MF1 (Verce et al. 2000; Verce at al. 2002). However, the strain MF1 did not begin to degrade *cis*-DCE until nearly all of the provided VC was consumed (Verce, et al 2002). On the other hand, degradation of *cis*-DCE by the TRW culture began even before all the VC was consumed, suggesting that perhaps the same organism or enzyme system(s) may be involved in the degradation of both compounds. The culture's ability to degrade VC in the presence of methane, ethane, acetate, trypticase soy or yeast-extract is important for predicting natural attenuation of VC in organic rich environments; methane, ethane and acetate are produced anaerobically upstream or source area. The reason why VC utilization was inhibited in the presence of glucose or pyruvate is not known. However, the degradation of VC proceeded normally when the culture was transferred to bottles devoid of glucose or pyruvate. Therefore, we hypothesize that perhaps these compounds are favored over VC as growth substrate by the culture.

The potential for the TRW culture to survive and initiate VC degradation with a minimal lag period, even after 5 months of starvation, is noteworthy and of practical significance. A recently isolated VC degrading strain, MF1 also displays similar capabilities. Strain MF1 degraded VC after 15 days of lag when incubated without VC for 24 days (Verce et al. 2000). On the other hand, *Mycobacterium aurum* L1 completely lost its ability to degrade VC after a brief interruption in the supply of VC to the reactor, and this loss of activity was attributed to the accumulation of VC-epoxide, chlorooxirane or chloroacetaldehyde (Hartmans 1995; Hartmans and de Bont 1992). The exact mechanism by which the TRW culture could survive in the absence of VC is not known. It is conceivable that lysate from dead cells could provide a source of nutrients for the sustenance. In addition, the absence of apparent chlorooxirane toxicity to the TRW culture was also evident in the *M. aurum* strain L1 following a brief starvation is

remarkable and warrant additional studies to identify the exact mechanism and breakdown pathways operative in the culture.

Overall, the TRW culture is robust and is capable of degrading VC below GC detection limit when tested over a wide range of dissolved VC concentrations. The culture's ability to degrade *cis*-DCE with VC as a primary carbon source avoids the necessity of introducing another substrate for remediation. The culture's ability to degrade VC uninhibited in the presence of easily assimilated substrates is important for natural attenuation. In addition, the ability to survive extended starvation periods and be able to metabolize VC at low DO is important for intrinsic remediation and the development cost-effective treatments.

### (ii). Isolation and characterization of aerobic VC-assimilating populations.

Vinyl chloride (VC) can be degraded efficiently under aerobic conditions by both metabolic and cometabolic processes. VC-degrading populations belonging to only two genera, *Mycobacterium* and *Pseudomonas*, have been obtained in pure cultures (Coleman, et al. 2002a; Verce, et al. 2000). Among these, *Mycobacterium* species are widely distributed and found at many chloroethene-contaminated sites (Coleman, et al. 2002a). Isolation of novel organisms that degrade VC as the sole carbon source is important for a greater understanding of VC-degradation mechanisms and pathways. In addition, information on the diversity and abundance of VC-degrading bacteria at contaminated sites is important for understanding their contribution to natural attenuation of VC.

Isolation of VC degraders. Two pure cultures that degrade VC as the sole source of carbon have been isolated from the TRW and Aberdeen enrichments. Repeated isolation attempts using serial 10-fold dilution series of the enrichments, followed by streaking to agar plates and incubating the agar plates in a desiccator filled with 1 to 2 % (v/v) of VC as the sole carbon source were unsuccessful. Hence, another approach that involved the dilution-to-extinction technique was used. Vials were amended with VC as the sole source of carbon and energy, and additional dilution-to-extinction series were established with the highest dilution of culture that showed VC degradation activity. This was repeated at least three times for each enrichment culture before

streaking aliquots onto agar plates. The agar plates were prepared with mineral salts medium (MSM) and glucose as the carbon source.

Individual colonies were aseptically transferred to different 160-ml serum bottles containing 50-ml of MSM amended with VC and air. The bottles were crimped with Teflon-coated septa and aluminum caps. Degradation of VC was monitored by injecting 20-mL of headspace gases into a gas chromatograph equipped with an FID detector. The cultures that degraded VC were further purified using standard serial 10-fold dilutions and streaking on MSM/glucose agar plates. This was repeated at least three times before further characterization of the isolates was performed.

Identification and characterization of pure cultures: Phylogenetic characterization of the VC-degrading isolates was done by partial sequencing of amplified 16S rRNA gene fragments and 96-well BIOLOG substrate-utilization plates. Genomic DNA from each isolate was extracted from 50-ml of a liquid culture that was consecutively fed 120 mM VC at least three times. The cells were collected by centrifugation, and the cell pellet was washed twice with sterile phosphate buffer (5 mM) prior to DNA extraction using the bead-beating procedure (Stephen et al. 1999). DNA was purified by a glass-milk DNA purification protocol using a Gene Clean™ kit as described by the manufacturer. Amplified 16S rRNA gene fragments were obtained by PCR using the universal bacterial primer set targeting *(E. coli)* positions 27f and 1492r. The amplicons were purified and sequenced. Phylogenetic analysis was perfomed with tools provided by the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.htm). The VC-assimilating pure cultures were identified as *Mycobacterium* sp. (Sequence Match S_ab score of 0.99) and *Ochrobacterium* sp. (Sequence Match S_ab score of 1.0).

Biodegradation of VC and other compounds by VC-assimilating isolates. Biodegradation of VC, ethene, *cis*-DCE, *trans*-DCE, and/or TCE was tested in serum bottles containing 50-ml MSM inoculated with a 2.5 ml of pure culture grown on VC (Table 2 in Appendix II). Bottles were crimp sealed with Teflon-coated septa and aluminum caps and incubated in an inverted position at 30°C. A set of three active and two autoclaved microcosms were established for each of the substrates tested and degradation was monitored. Both isolates used VC or ethene as the

sole carbon and energy source (Figure 8a, b in Appendix II). In addition, both populations degraded *cis*-DCE, but only in the presence of VC as the primary substrate. On the other hand, *cis*-DCE was not degraded when ethene was added as the primary carbon source. These results suggest that cometabolic degradation of *cis*-DCE only occurs in the presence of VC. None of the isolates degraded *trans*-DCE or TCE in either the presence or absence of VC. Neither *trans*-DCE nor TCE, however, inhibited VC degradation.

Bioaugmentation with the new isolates. The isolates were individually tested for their ability to survive and degrade VC in sterile and non-sterile microcosms established with aquifer materials obtained from the chloroethene-contaminated Wichita site in Kansas. Microcosms (160-ml capacity) were constructed with 10 g (wet weight) of the aquifer material and 45-ml of MSM as described above. Both sterile and non-sterile bottles were inoculated with a VC-assimilating pure culture at varying cell densities. VC depletion was monitored by headspace analysis. VC degradation occurred with both isolates in the absence and presence of native populations associated with the Wichita aquifer material. However, VC was degraded at faster rates in the microcosms containing sterile aquifer material (Figure 9a in Appendix II). This could be due to the absence of competition for available nutrients and oxygen. Significantly lower VC degradation rates were observed in the presence of native bacteria (Figure 9b in Appendix II). This was particularly true for the microcosms inoculated with the *Ochrobacterium* species. This population was able to degrade VC only at the highest cell density tested even in the absence of competition by native populations, while no or little degradation occurred in the presence of native bacteria (Figure 10a, 10b in Appendix II).

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## Task 5: Site Characterization

Whenever feasible, geochemical and contaminant data were collected from sites tested for *cis*-DCE/VC degradation activity with the most extensive data collection obtained from the Hydrite Chemical site and the Bachman Road site. Aquifer materials from both sites were collected from distinct locations within the plumes to obtain samples from different redox zones. Whenever feasible, samples were retrieved from zones with varying contaminant concentrations and varying redox conditions, e.g., aerobic portions of the plume, oxic/anoxic transition zones, iron-reducing zones, and anaerobic zones.

The degradation of *cis*-DCE and VC is dependent on several site characteristics, including hydrogeology, groundwater geochemistry and microbiology. Determining the redox (aerobic or anaerobic) state of the aquifer is one of the most important factors in determining the relevance of Process 2 or Process 5. Additional site assessment criteria that follow can be used to further evaluate the potential for Process 2 or Process 5. A Site Screening Protocol was developed to guide practitioners in evaluation of site assessment data (Appendix III). The protocol contains a site screening matrix to evaluate the potential for Process 2 or Process 5. Case studies using the matrix correlating contaminant and site geochemical data with the microbial activities are presented in Appendix III.

## Task 6: Probe Development

### Process 2: Anaerobic energy-yielding reductive dechlorination (chlororespiration)

**(i) Refine 16S rRNA gene-based probes and PCR primers.**

More than 25 nearly complete 16S rRNA gene sequences of *Dehalococcoides* populations were retrieved from PCE-, TCE,- *cis*-DCE-, VC-, and 1,2-dichloropropane (1,2-D)-dechlorinating

cultures. Interestingly, the 16S rRNA gene sequences of two *Dehalococcoides* populations implicated in the reductive dechlorination of 1,2-D were identical to the 16S rRNA gene sequence of the DCE- and VC-dechlorinating isolate BAV1derived from the Bachman culture. Two other *Dehalococcoides* 16S rRNA gene sequences obtained from other VC-dechlorinating cultures were identical to and *Dehalococcoides* sp. strain CBDB1 and strain FL2. Both strains, CBDB1 and FL2, cannot grow with VC as electron acceptor. Although the 16S rRNA gene method is a reliable approach for detecting and monitoring *Dehalococcoides* populations, the information contained in the 16S rRNA molecule is insufficient to distinguish *Dehalococcoides* populations with distinct dechlorinating abilities. Therefore, additional targets (i.e., functional genes) must be identified that provide information beyond that of the 16S rRNA gene to unambiguously distinguish *Dehalococcoides* populations with different dechlorination activities and to predict the potential for complete microbial detoxification of chloroethenes (i.e., ethene formation) at contaminated sites.

**(ii) Develop a quantitative Real-Time PCR (RTm PCR) approach.**

*Dehalococcoides* populations have been found to be associated with anaerobic reductive dechlorination of chlorinated ethenes in mixed cultures and at chloroethene contaminated sites. More accurate site assessment and modeling tools are improved if the cell numbers of *Dehalococcoides* and its functional (e.g. the reductive dehalogenase) gene copy numbers can be detected in natural environments. Some methods have been developed to quantify microbes such as most-probable-number measurement. Since *Dehalococcoides* populations are very difficult to culture in the laboratory, this method would require months of incubation and would likely still be unreliable. Hence, alternative molecular biology methods, such as RTm PCR, should overcome the inability to grow *Dehalococcoides* populations. This system requires the design of a forward and a reverse primer and a specific probe that hybridizes between them. The probe is labeled at both ends. The fluorescent dye at the 5' end serves as a reporter, and its emission spectra are quenched by the molecule attached to the 3' end of the probe. Once separated from the quencher, the reporter fluorescence is detected. Repeated rounds of PCR result in an increase in fluorescence. The copy number of the target DNA is determined by comparison to a standard

curve. The RTm PCR method is quantitative since it focuses on the logarithmic phase of product accumulation rather than on the end product abundance.

The RTm PCR approach has advanced to a level that justifies applying this method to quantifying *Dehalococcoides* populations at contaminated sites. Standard curves were developed using pure culture genomic DNA and plasmid DNA containing a cloned *Dehalococcoides* 16S rRNA gene. The RTm PCR approach was tested with samples collected from different locations inside a pilot test plot at the Bachman Road site. The RTm PCR data suggested that *Dehalococcoides* populations were present in high numbers at locations where complete dechlorination to ethene occurred. On the other hand, *Dehalococcoides* populations were present in low numbers, or were not detectable in locations where ground water measurements indicated incomplete dechlorinating activity. This experiment showed that RTm PCR was able to detect and reliably quantify populations in the Bachman aquifer at cell numbers greater than $10^3$ per gram of solids. Quantitative assessment of the *Dehalococcoides* community was instrumental for establishing cause-effect relationships following biostimulation and bioaugmentation at the Bachman Road site. Application of the RTm PCR approach demonstrated that the *Dehalococcoides* population increased in size following treatment. In other words, we used the RTm PCR approach to show that augmented *Dehalococcoides* populations grew under field conditions (Lendvay et al. 2003).

**(iii) Expand the PCR approach to specifically detect and quantify structural genes involved in the dechlorination of *cis*-DCE and VC**

Although a powerful tool to detect, monitor and quantify *Dehalococcoides* populations, the 16S rRNA gene-based approach is limited by its inability to distinguish *Dehalococcoides* populations with similar or identical 16S rRNA genes but different dechlorinating activities. Hence, additional targets that contain information beyond that provided by the 16S rRNA gene must be identified. Obvious targets are functional genes involved in the reductive dechlorination process. The complete genome of the PCE-to-VC/ethene-dechlorinator *Dehalococcoides ethenogenes* strain 195 was sequenced (http://tigrblast.tigr.org/ufmg/). Magnuson et al. (1998, 2000) described primers targeting the *tceA* gene encoding the TCE reductase responsible for

dechlorination of TCE to VC in *D. ethenogenes* strain 195. Like other reductive dehalogenases, the TCE reductase is anchored to the membrane by a B-protein encoded by *tceB* located immediately downstream of *tceA* (Magnuson et al. 1998; Neumann et al. 1998; van de Pas et al. 1999; Smidt et al. 2000). Using the available *Dehalococcoides* genome information, we successfully PCR-amplified, cloned and sequenced the complete *tceAB* operon from six different TCE-dechlorinating cultures. We used this information together with sequence information of reductive dehalogenase genes from other organisms available in public databases to design several sets of degenerate primers to PCR-amplify putative reductive dehalogenase genes from *Dehalococcoides* sp. strain BAV1. Seven putative reductive dehalogenase gene fragments from BAV1 have been amplified to date. All fragments were cloned in *E. coli* and sequenced. Reverse transcription PCR was performed with mRNA extracted from VC-grown BAV1 cells identified a putative VC reductive dehalogenase gene. This is a very promising approach to identify relevant functional genes involved in the reductive dechlorination process, and to ascribe gene function. An expansion of this work could provide a set of targets and tools that would allow a comprehensive site assessment and monitoring of the key *Dehalococcoides* populations.

A manuscript describing the approach to PCR-amplify and to clone putative reductive dehalogenase genes from *Dehalococcoides* has been submitted to Applied and Environmental Microbiology. Another manuscript describing the identification of the putative VC reductive dehalogenase gene in *Dehalococcoides* sp. strain BAV1 will be submitted shortly.

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## 4. DELIVERABLES

Published, peer-reviewed journal publications, manuscripts currently under review, and manuscripts that will be submitted within the next 3 months are listed below. Also listed are posters, proceeding papers, and oral presentation stemming from this SERDP-supported research effort.

### Peer-Reviewed Publication (Journal Articles)

1. **He, J., K. M. Ritalahti, K.-L. Yang, S. S. Koenigsberg, and F. E. Löffler. 2003. Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature 424:62-65.**

2. Sung, Y, K. M. Ritalahti, R. A. Sanford, J. W. Urbance, S. J. Flynn, J. M. Tiedje, and F. E. Löffler. 2003. Characterization of two tetrachloroethene (PCE)-reducing, acetate-oxidizing anaerobic bacteria, and their description as *Desulfuromonas michiganensis* sp. nov. Appl. Environ. Microbiol. 69:2964-2974.

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4. He, J., Y. Sung, M. E. Dollhopf, B. Z. Fathepure, J. M. Tiedje, and F. E. Löffler. 2002. Acetate versus hydrogen as direct electron donors to stimulate the microbial reductive dechlorination process at chloroethene-contaminated sites. Environ. Sci. Technol. 36:3945-3952.

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7. Ritalahti, K. M., and F. E. Löffler. 2003. Populations implicated in the anaerobic reductive dechlorination of 1,2-dichloropropane in highly enriched bacterial communities. Appl. Environ. Microbiol. Submitted.

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10. Krajmalnik Brown, R., K. M. Ritalahti, J. He, and F. E. Löffler. 2003. Cloning and expression analysis of a putative vinyl chloride (VC) reductive dehalogenase from *Dehalococcoides* sp. strain BAV1. Appl. Environ. Microbiol. In preparation.

## Poster Presentations

1. Ritalahti, K. M., F. E. Löffler, and S. S. Koenigsberg. 2003. Improved tools for initiating and monitoring reductive dechlorination at chloroethene-contaminated sites. Abstr. 254, p. 190. In Partners in Environmental Technology Technical Symposium & Workshop. Dec. 2-4, Washington, DC.

2. Krajmalnik-Brown, R., J. He, F. M. Saunders, and F. E. Löffler. 2003. Identification of structural genes implicated in complete detoxification of chlorinated ethenes. *In* Abstracts of the 7th International Symposium In situ and on-site Bioremediation, Orlando, FL.

3. He, J., and F. E. Löffler. 2003. Isolation of a vinyl chloride-respiring population in pure culture, abstr. Q-016. *In* Abstracts of the 103rd Annual Meeting of the American Society for Microbiology 2003, Washington, D.C. Published on CD-ROM.

4. Krajmalnik-Brown, R., J. He, M. Saunders, and F. E. Löffler. 2003. Identification and cloning of a gene implicated in complete vinyl chloride detoxification, abstr. Q-063. *In* Abstracts of the 103rd Annual Meeting of the American Society for Microbiology 2003, Washington, D.C.

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8. Löffler, F. E., B. M. Griffin, and R. Krajmalnik-Brown 2002. Complete Microbial detoxification of *cis*-1,2-dichloroethene (*cis*-DCE) and vinyl chloride (VC). 6th International Symposium on Environmental Biotechnology, June 9-12, Veracruz, México.

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10. Krajmalnik-Brown, R., K. M. Ritalahti, Y. Sung, J. He, F. M. Saunders, and F. E. Löffler (2002). Detection and Comparison of the *tceAB* Operon from *Dehalococcoides*-Containing Reductively Dechlorinating Cultures. 6th International Symposium on Environmental Biotechnology, June 9-12, Veracruz, México.

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12. Sung, Y., J. He, R. Krajmalnik-Brown, E. Padilla, K. M. Ritalahti, and F. E. Löffler. 2002. Distribution and ecoclogy of chloroethene-dechlorinating populations in subsurface environments, abstr. Q-15, p. 381. *In* Abstracts of the 102nd Annual Meeting of the American Society for Microbiology 2002, Salt Lake City.

13. Ritalahti, K. M., R. Krajmalnik-Brown, J. He, Y. Sung, and F. E. Löffler (2002). Microbial communities contributing to the degradation of *cis*-1,2-dichloroethene (*cis*-DCE), vinyl chloride (VC) and 1,2-dichloropropane (1,2-D). 6th International Symposium on Environmental Biotechnology, June 9-12, Veracruz, México.

14. Fathepure, B. Z., H. Singh, F. E. Löffler, and J. M. Tiedje. 2002. Aerobic Biodegradation of *cis*-dichloroethene and vinyl chloride by highly enriched consortia: optimization studies. 6th International Symposium on Environmental Biotechnology, June 9-12, Veracruz, México.

15. Fathepure, B.Z., H. Singh, M. E. Dollhopf, F. E. Löffler, and J. M. Tiedje. 2001. Metabolic and cometabolic oxidation of vinyl chloride (VC) and *cis*-dichloroethene (*cis*-DCE) in aquifer materials from contaminated sites. ISME 9, August 26-31, 2001. Amsterdam, Netherlands.
16. Petrovskis, E., Fredrickson, C., Burnell, D. and Adriaens, P. 2001. Full-scale Design of a Biobarrier: Bioaugmentation or Biostimulation? Poster presentation at Battelle Conference on Remediation of Chlorinated and Recalcitrant Compounds.

### Oral Presentations

1. Löffler, F. E. 2003. Microbial degradation of chlorinated ethenes under different redox conditions. Partners in Environmental Technology, Technical Symposium & Workshop, Dec. 2-4, Washington, D.C.
2. Löffler, F. E. 2003. Complete reductive dechlorination of chlorinated ethenes: characterization of the key players and implications for their specific detection and enumeration. 7th International Symposium In Situ and On-Site Bioremediation, Orlando, FL.
3. Ritalahti, K. M. 2003. Biostimulation or bioaugmentation? Decision making based on scientific data: remediation decisions guided by real time PCR quantification of reductively dechchlorinatin *Dehalococcoides* populations. 19th International Conference on Soils, Sediments and Water, Amherst, MA.
4. Löffler, F. E. 2003. Advances in the identification and quantification of *Dehalococcoides* from field samples. 7th International Symposium In Situ and On-Site Bioremediation, Orlando, FL.
5. Löffler, F. E. 2003. Anaerobic bioremediation of chlorinated solvent sites. 103rd General Meeting of the American Society for Microbiology, Washington, D.C.
6. Löffler, F. E. 2003. Status of molecular approaches for monitoring bioremediation efforts at chloroethene-impacted sites. 13th Annual West Coast Conference on Contaminated Soils, Sediments and Water, San Diego, CA.
7. Löffler, F. E. 2003. Innovative strategies for chloroethene plume control. Georgia Environmental Protection Division, Hazardous Waste Management Branch.
8. Löffler, F. E. 7/2003. Anaerobes - new allies for site restoration. Westinghouse Savannah River Company. Aiken, SC.
9. Petrovskis, E., Loffler, F., and Henry, B. 2004. Tools For Evaluating Enhanced Anaerobic Bioremediation for Chlorinated Solvents. Platform presentation at Battelle Conference on Remediation of Chlorinated and Recalcitrant Compounds.
10. Petrovskis, E. Bioaugmentation to Remediate Chlorinated Solvents. 2003. Seminar to Delphi Corporation.
11. Petrovskis, E. and Henn, K. 2003. Guidelines for Anaerobic Bioremediation: Biostimulation and Bioaugmentation. Seminar to Tetra Tech, Inc.
12. Petrovskis, E. 2003. Bioaugmentation to Remediate Chlorinated Solvents. Seminar to US Navy Southern Division.

13. Löffler, F. E. 2002. Novel Approaches for Bioremediation of Chlorinated Solvent Sites. Environmental Engineering and Science Seminar Series. Department of Civil and Environmental Engineering. University of Illinois at Urbana/Champaign, August 2002.
14. Löffler, F. E. 2002. Bioremediation of Chlorinated Solvent Sites: From Lab Studies to Field Applications. Environmental Science and Engineering Seminar Series, California Institute of Technology, June 2002.
15. Löffler, F. E. 2002. The Role of Microbial Biomarkers in Site Characterization. First Annual EICE On-Line Conference in Environmental Biotechnology. Environmental Institute for Continuing Education, October 2002.
16. Tiedje, J. M. 2002. Biology and use or halorespiring microbes for environmental clean-up. University of Connecticut, April, 2002.
17. Tiedje, J. M. 2002. Ecology of halorespiring microbes and their application in site remediation. Rutgers University, May, 2002.
18. Petrovskis, E. 2002. Bioremediation of Chlorinated Solvents. Seminar to Borden Chemical Company.
19. Petrovskis, E. 2002. Bioaugmentation to Remediate Chlorinated Solvents. Seminar to Tetra Tech, Inc.
20. Petrovskis, E. 2001. Bioaugmentation to Remediate Chlorinated Solvents. Presentation to GeoTrans, Inc.
21. Petrovskis, E. 2001. Bioaugmentation to Remediate Chlorinated Solvents. Seminar to Dow Chemical Company.

## ENCLOSED:

**APPENDIX I (FIGURES PROCESS 2)**

**APPENDIX II (FIGURES PROCESS 4 AND PROCESS 5)**

**APPENDIX III (SITE CHARACTERIZATION PROTOCOL AND CASE STUDIES)**

# Appendix I

**Figure 1.** Reductive dechlorination of TCE to VC by *Dehalococcoides* sp. strain FL2

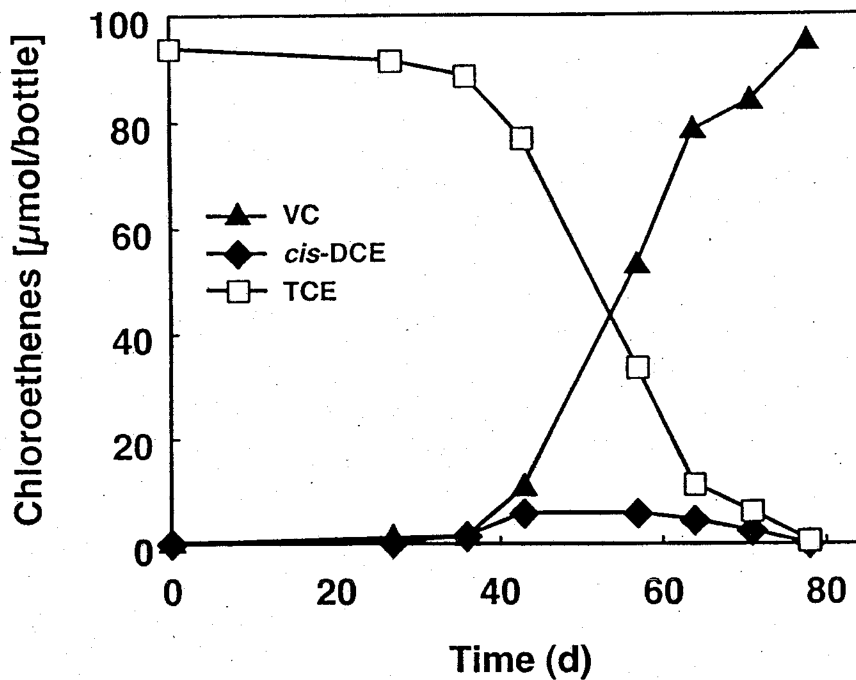


**Figure 2a.** Dechlorination of *cis*-DCE in cultures of *Dehalococcoides* sp. strain FL2

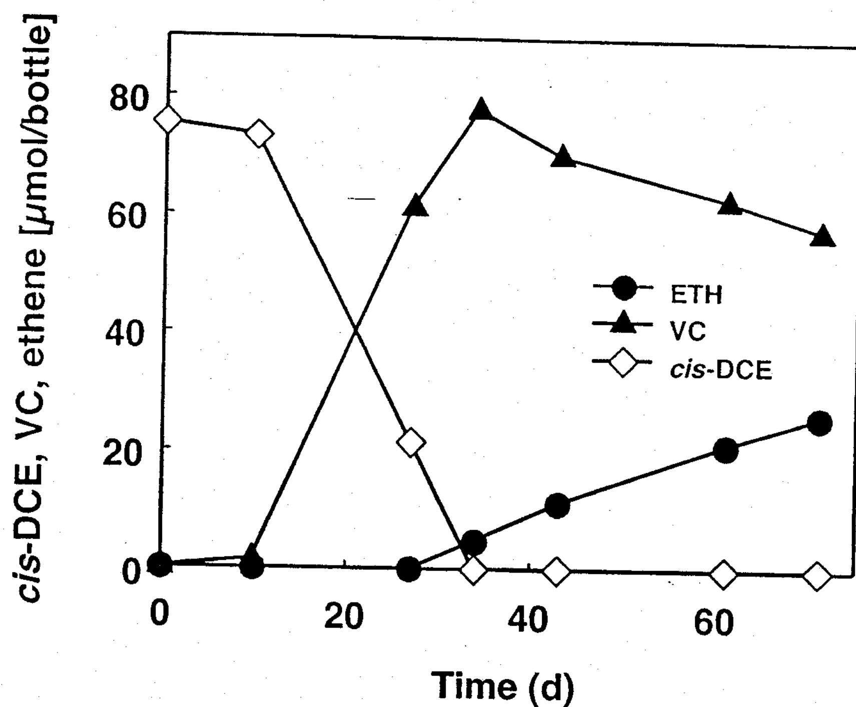



**Figure 2b.** Dechlorination of *trans*-DCE in cultures of *Dehalococcoides* sp. strain FL2

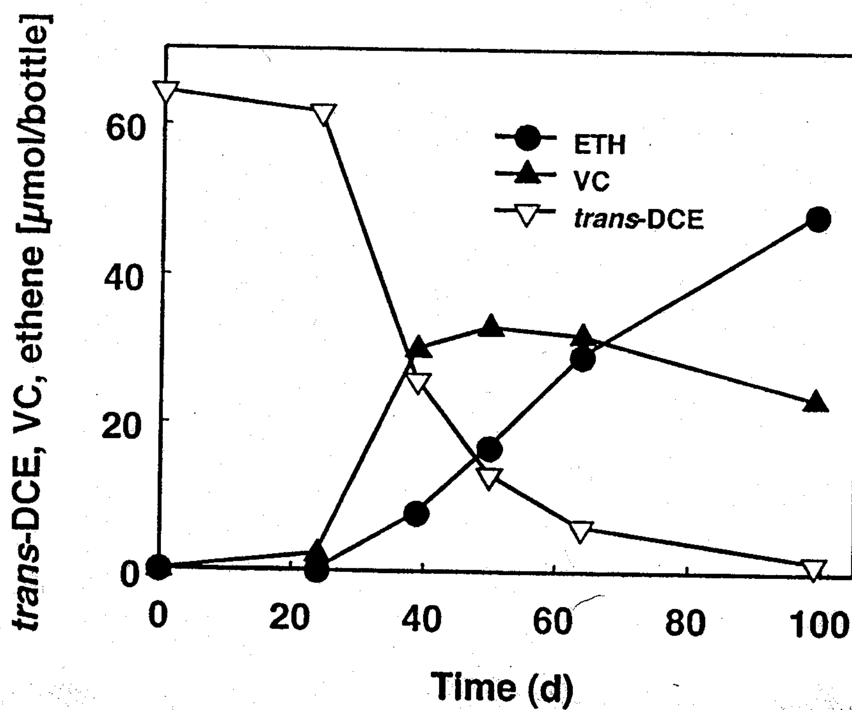


**Figure 3a.** *Dehalococcoides* sp. strain FL2 failed to grow with 1,1-DCE as the electron acceptor

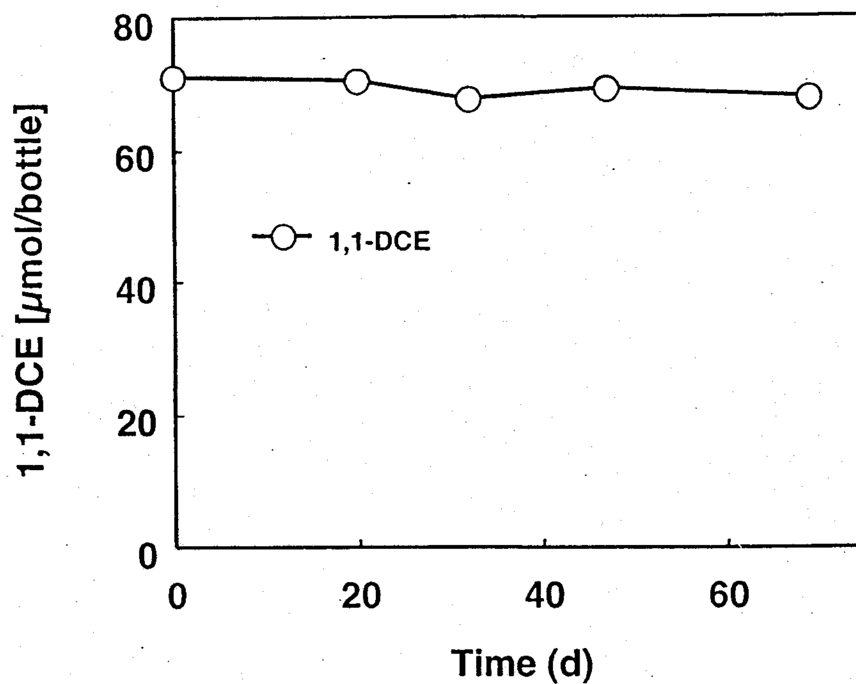



**Figure 3b.** *Dehalococcoides* sp. strain FL2 failed to grow with VC as the electron acceptor

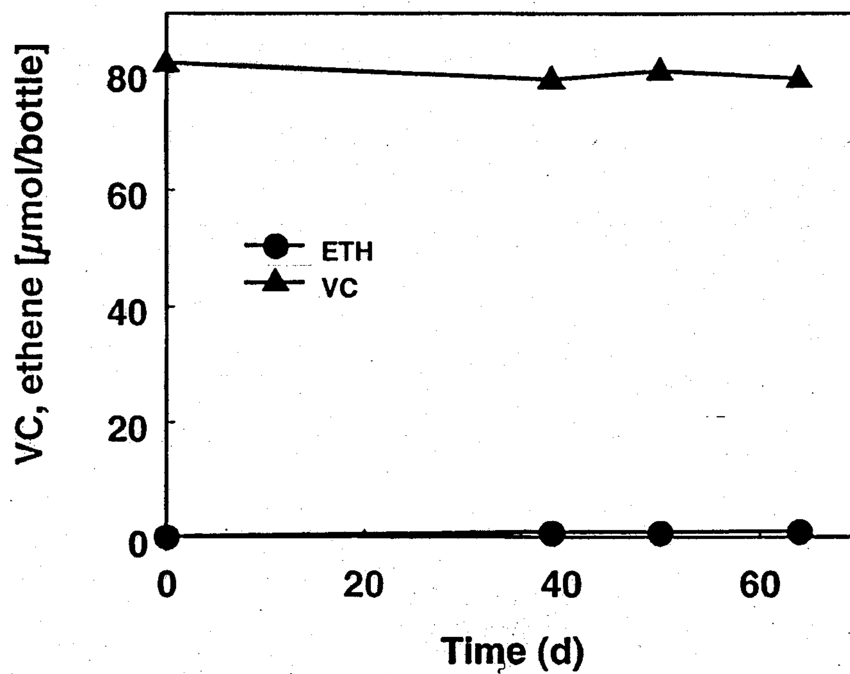


Figure 4. Reductive dechlorination of *cis*-DCE by *Dehalococcoides* sp. strain FL2 at different hydrogen partial pressures

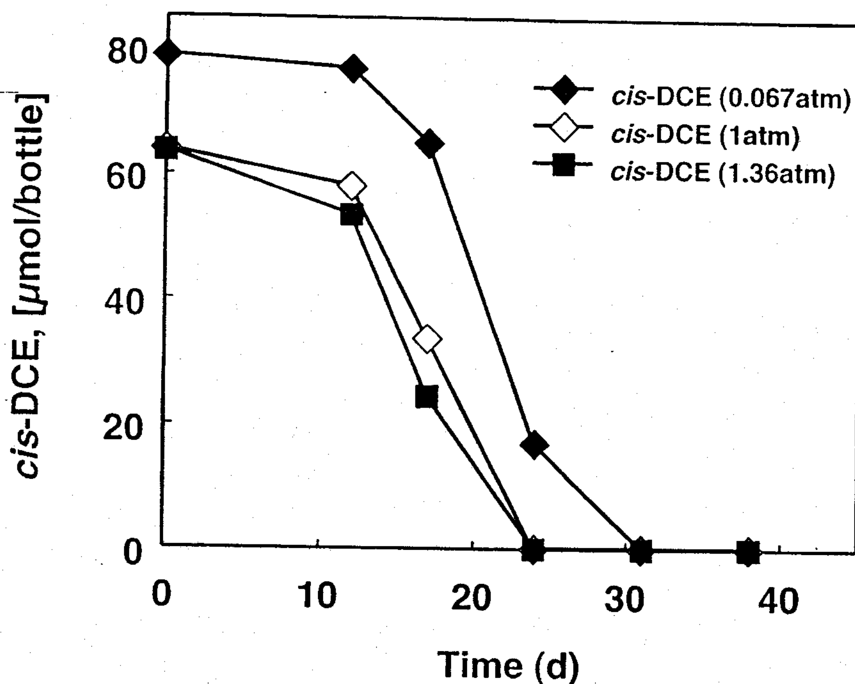



Figure 5. Phylogenetic tree generated using Phylip (rdp.cme.msu.edu) based on 1037 bases. Scale bar represents 10% sequence difference

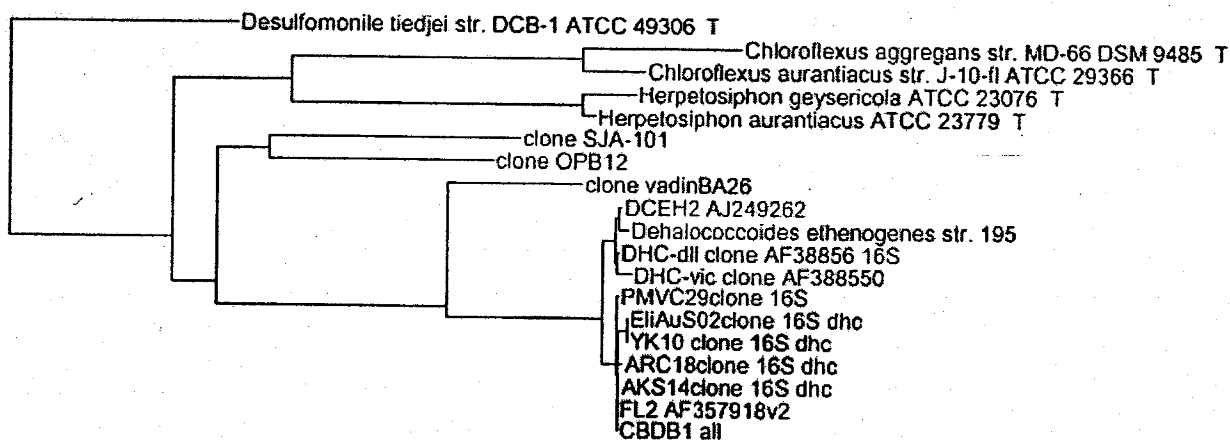


**Figure 6.** Reductive dechlorination of VC (triangles) to ethene (circles) under different electron donor conditions. Acetate was provided as the only electron donor, and $H_2$ concentrations (dashed line) were monitored (solid symbols). Another set of acetate-amended cultures received 223 μmol $H_2$ at day 42 (arrow) as additional electron donor (open symbols). Data points were averaged from triplicate cultures, and no error bars are shown when the standard deviations were to small to be illustrated.

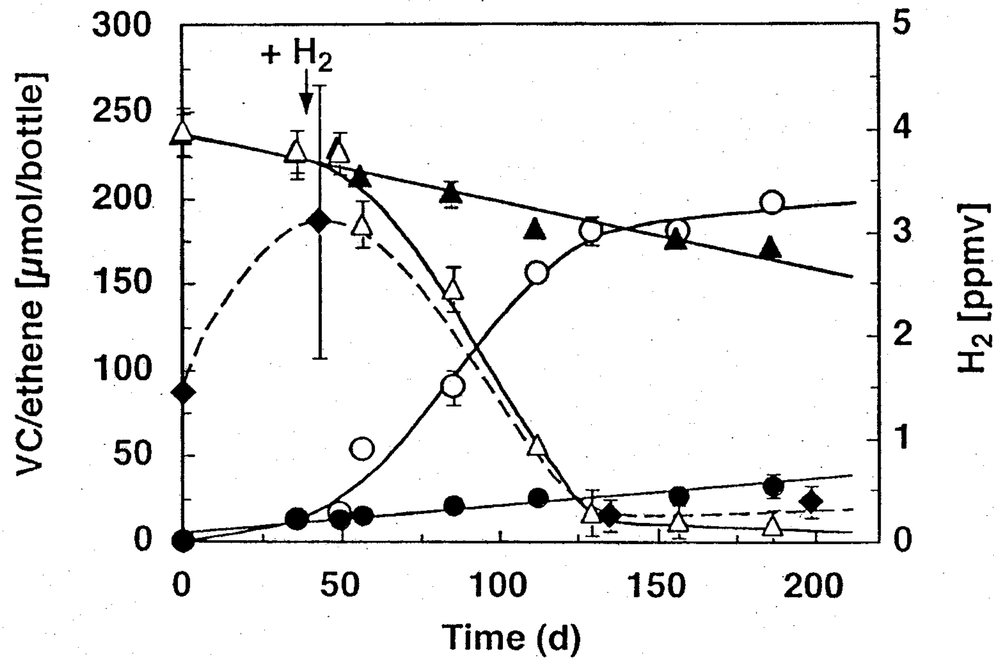


**Figure 7.** Reductive dechlorination of DCEs by the VC-enriched culture. (A) Dechlorination of *cis*-DCE (open diamonds) to VC (closed triangles) and ethene (closed circles). (B) Dechlorination of *trans*-DCE (open triangles) to VC and ethene. (C) Dechlorination of 1,1-DCE (open circles) to VC and ethene. Pyruvate was provided as electron donor to the *cis*- and *trans*-DCE-amended cultures, and acetate plus formate was added to the cultures containing 1,1-DCE. Inocula were derived from cultures grown with the same chlorinated electron acceptor, except for the 1,1-DCE experiment, which was initiated with a VC-grown inoculum.

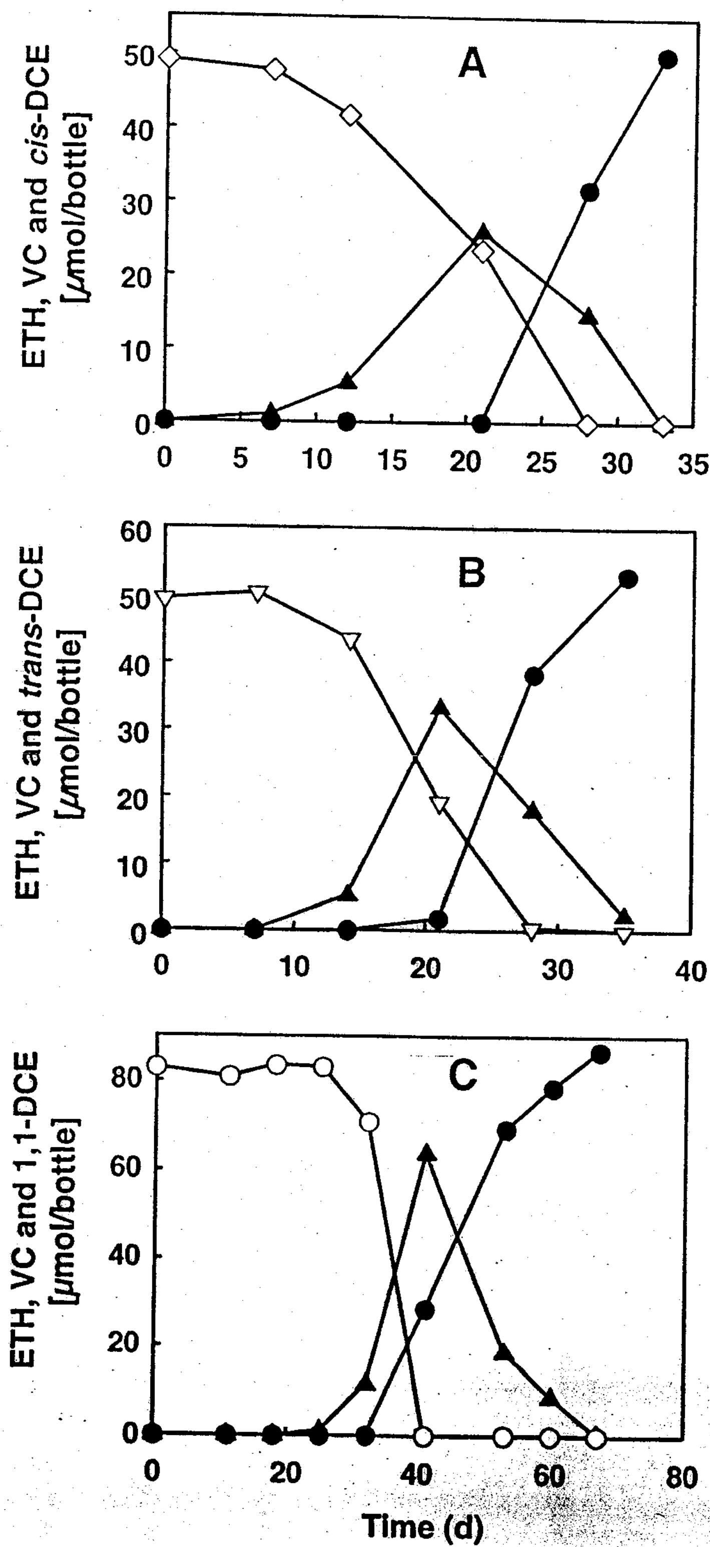


**Figure 8.** Reductive dechlorination of TCE (open squares) in VC-grown cultures with pyruvate as the electron donor (VC, closed triangles; ethene, closed circles). TCE was added on day 36 immediately after the initial amount of VC was dechlorinated. The inset shows that no TCE dechlorination occurred in cultures grown under the same conditions without VC.

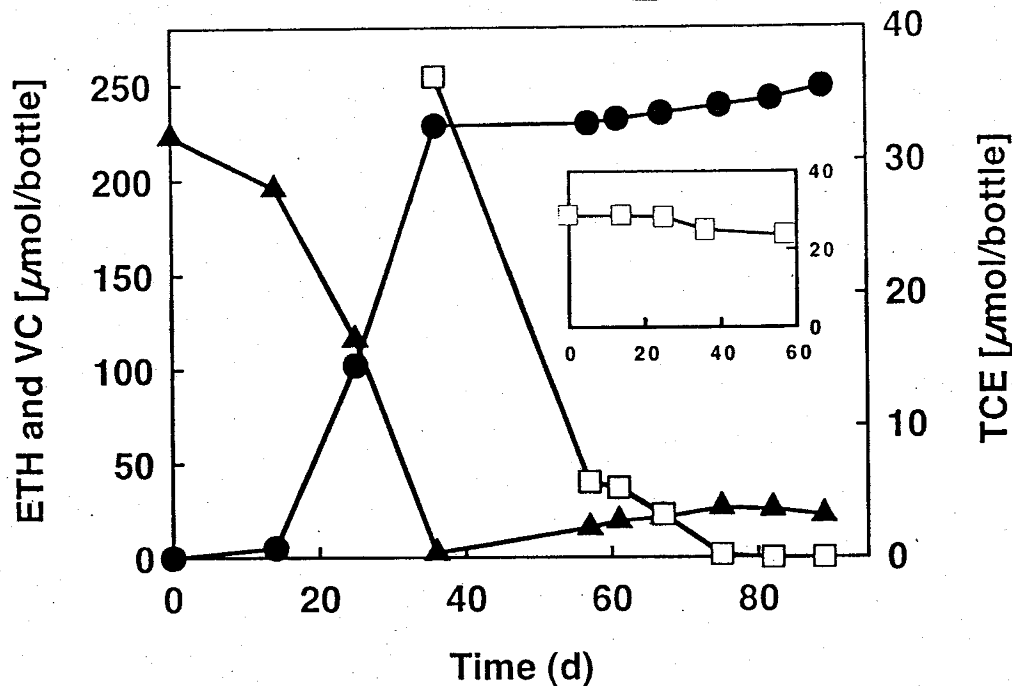



**Figure 9.** Detection of *Dehalococcoides* populations in the VC-dechlorinating culture with *Dehalococcoides* 16S rDNA-targeted PCR primers. Lane 1, 50-2,000 bp ladder (Bio-Rad); lane 2, VC-dechlorinating culture with lactate as electron donor; lane 3, VC-dechlorinating culture with pyruvate as electron donor; lane 4-6, negative controls (*Acetobacterium* sp., $H_2O$, *Desulfuromonas michiganensis* strain BB1); lane 7, *Dehalococcoides* sp. strain FL2.

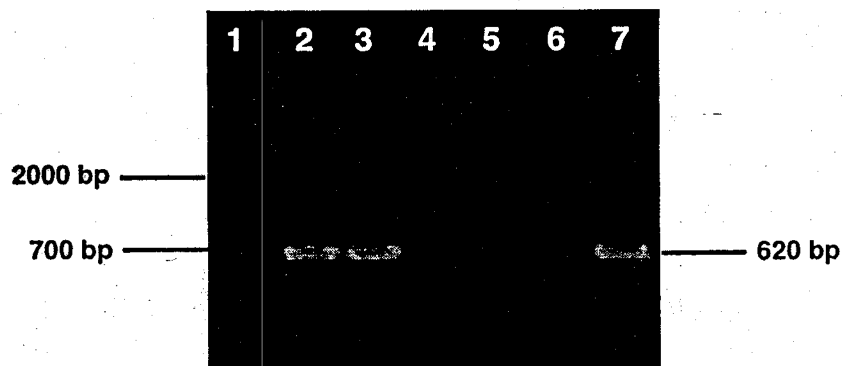


**Figure 10.** Increase of *Dehalococcoides* 16S rRNA genes dependent on the presence of VC as electron acceptor. Lane 1 and lane 8, 50-2,000 bp ladders (Bio-Rad); lanes 2-7, dilution series of template DNA (25 ng $\mu l^{-1}$) from pyruvate/VC-grown cultures (1:1, 1:10, 1:50, 1:100, 1:500, 1:1000); lanes 9-14, dilution series of template DNA (37.5 ng $\mu l^{-1}$) from pyruvate-grown cultures; lane 15, $H_2O$; lane 16, *Desulfuromonas michiganensis* strain BB1; lane 17, *Dehalococcoides* sp. strain FL2.

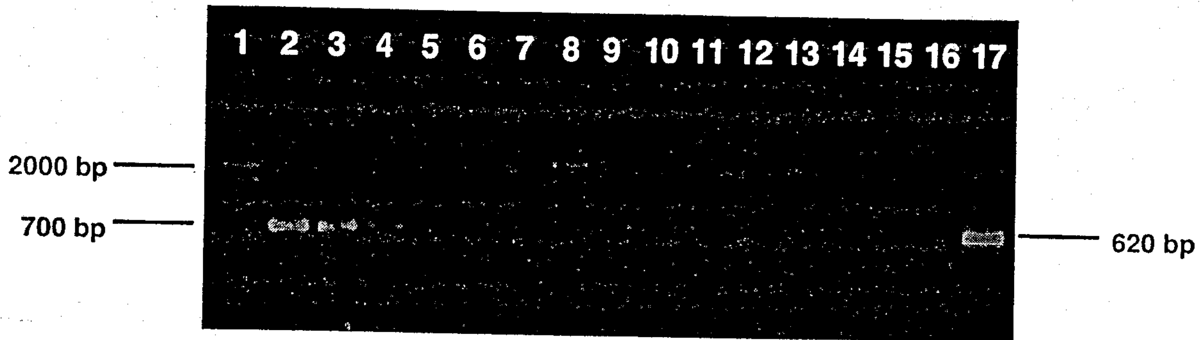



**Figure 11.** Scanning electron micrograph of isolate BAV1.

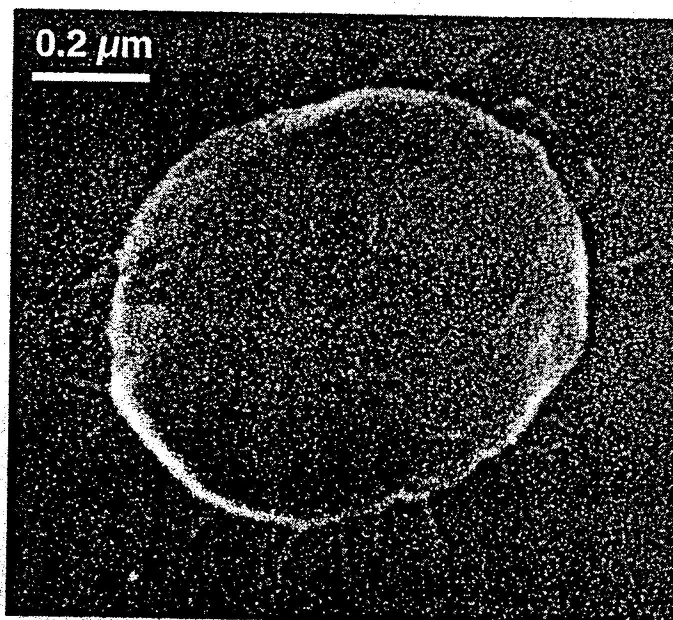


**Figure 12.** An increase in 16S rRNA gene copies as determined by RTm PCR (closed circles) during the reductive dechlorination of VC (closed triangles) to ethene (not shown) by culture BAV1.

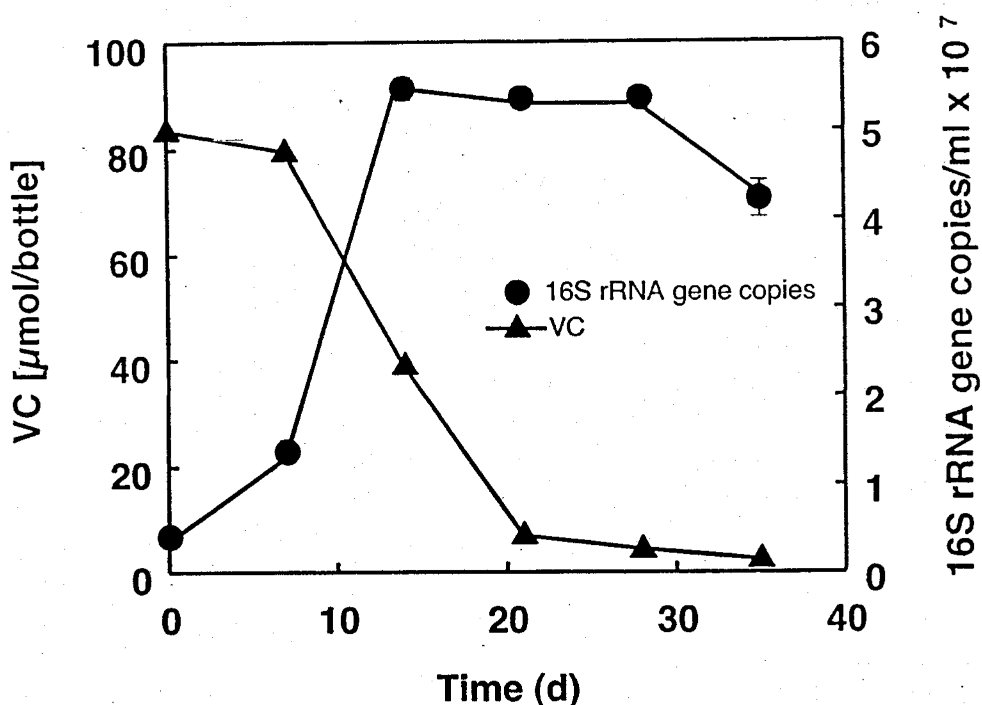
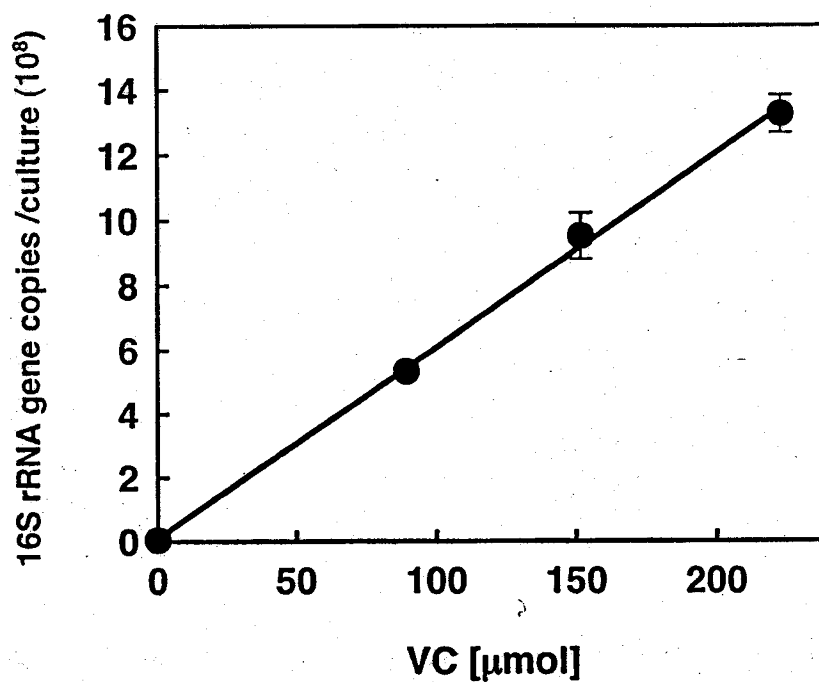



**Figure 13.** 16S rRNA gene copies of isolate BAV1 after dechlorinating different amounts of VC. Data points were averaged from triplicates, and error bars are not shown if they are hidden by the symbol.



# Appendix II

**Table 1.** Biodegradation of VC in the Presence of Alternate Substrates by the TRW Enrichment [1]

| Alternate substrate | Conce. | [2]Degradation of alternate substrate | [3]Degradation of VC |
|---|---|---|---|
| VC | 250 ?M | None added | + |
| Ethene | 125 ?M | + | + |
| Ethane | 220 ?M | - | + |
| Methane | 120 ?M | - | + |
| *cis* -DCE | 210 ?M | + | + |
| *trans* -DCE | 225 ?M | - | + |
| TCE | 192 ?M | - | + |
| Acetate | 5 mM | ND | + |
| Pyruvate | 5 mM | ND | - |
| Glucose* | 5 mM | ND | - |
| Trypticase soy | 0.10% | ND | + |
| Yeast-extract | 0.10% | ND | + |

[1]Results are averages of three bottles. ND; Not Determined.
[2]Degradation of alternate substrate was evaluated in the presence of VC.
[3]Degradation of VC was evaluated in the presence alternate substrate.
*Glucose was filter sterilized.

**Table 2.** Biodegradation of Polychlorinated ethenes by VC-Assimilating Pure Cultures[a]

| Organism | VC[b] | ETH[b] | *c*-DCE[b] | *t*-DCE[b] | TCE[b] | *c*-DCE (+VC)[c] | c-DCE (+ETH)[d] | *t*-DCE (+VC)[c] | TCE (+VC)[c] |
|---|---|---|---|---|---|---|---|---|---|
| *Ochrobacterium* | + | + | - | - | - | + | - | - | - |
| *Mycobacterium* | + | + | - | - | - | + | ND | - | - |

[a] Microcosms were amended with 200 to 250 µM aqueous VC and/or other chloroethenes.

[b] Microcosms were amended with VC, ETH, *c*-DCE, *t*-DCE, or TCE only. The *Mycobacterium* sp and *Ochrobacterium* sp degraded only VC or ETH as the carbon source.

[c] Biodegradation of *cis*-DCE, *trans*-DCE, or TCE was tested in the presence of VC. Only *cis*-DCE was degraded in the presence of VC. No degradation of *trans*-DCE or TCE occurred. VC was degraded in all bottles.

[d] Biodegradation of *cis*-DCE was assessed in the presence of ethene (ETH). No degradation of *cis*-DCE occurred.

ND = Not done

**FIGURE 1**

Sustained use of VC as a growth substrate by the TRW enrichment culture, indicated by the repeated depletion of VC. The data are shown for the last 250 days of the enrichment maintained on VC as the sole carbon source.

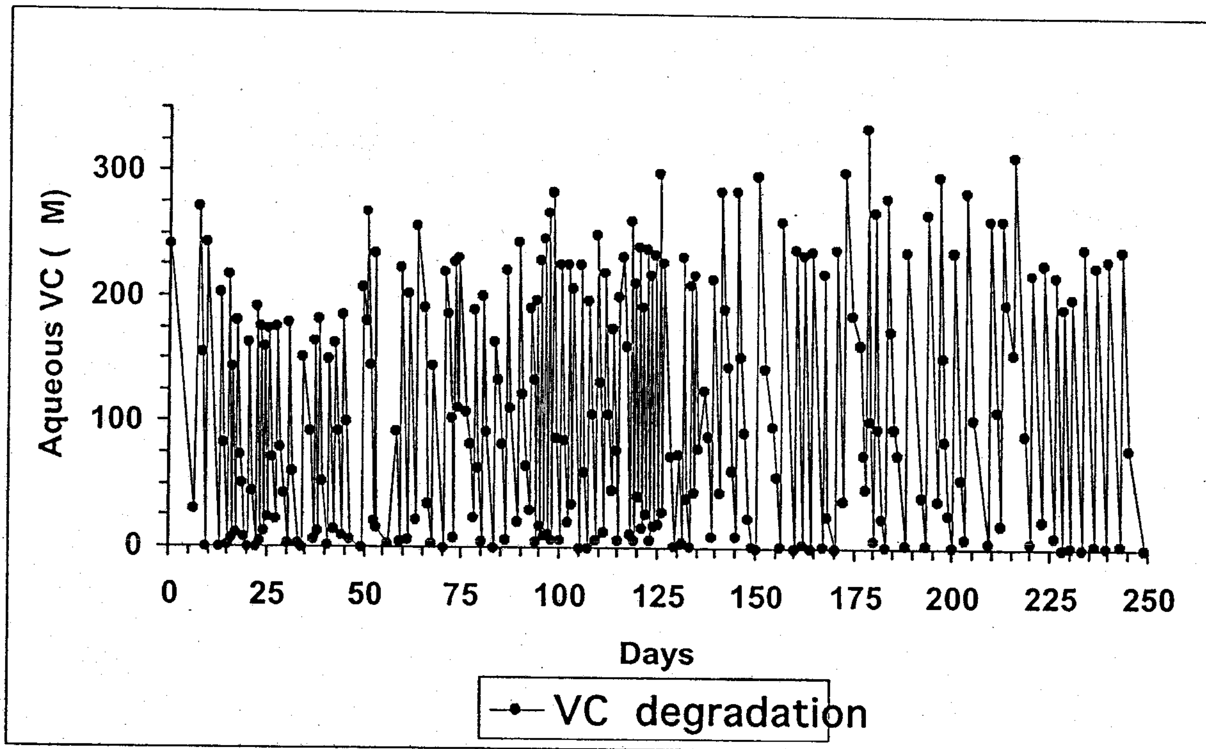


**FIGURE 2**

DGGE gel image of PCR amplified bacterial 16S rRNA genes from the TRW enrichment grown on VC as the sole carbon source. The banding pattern and relative intensity of the recovered bands provide a measure of community complexity. Dominant species must contribute at least 1-2 % of the total bacterial 16S rRNA genes to form a visible band. Labeled bands were excised and sequenced. Results from sequences were compared with the DNA sequences in databases, RDP and GenBank. Bands A and C did not yield sequence data, and bands B and C closely matched *Mycobacterium* and *Rhodococcus* sequences in the GenBank database (>94% identity). The GenBank access numbers for band B is AY 114109 and for band C is AY 114109 and AJ 429044.

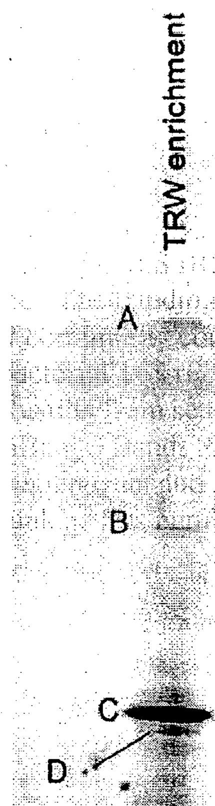


FIGURE 3

Biodegradation of VC as a function of concentration of aqueous VC. Data were averaged from three replicate microcosms. Inset: VC removal rate at varied initial dissolved concentrations of VC ranging from 50 μM to 250 μM. The rates are calculated from the depletion curves for each concentration tested.

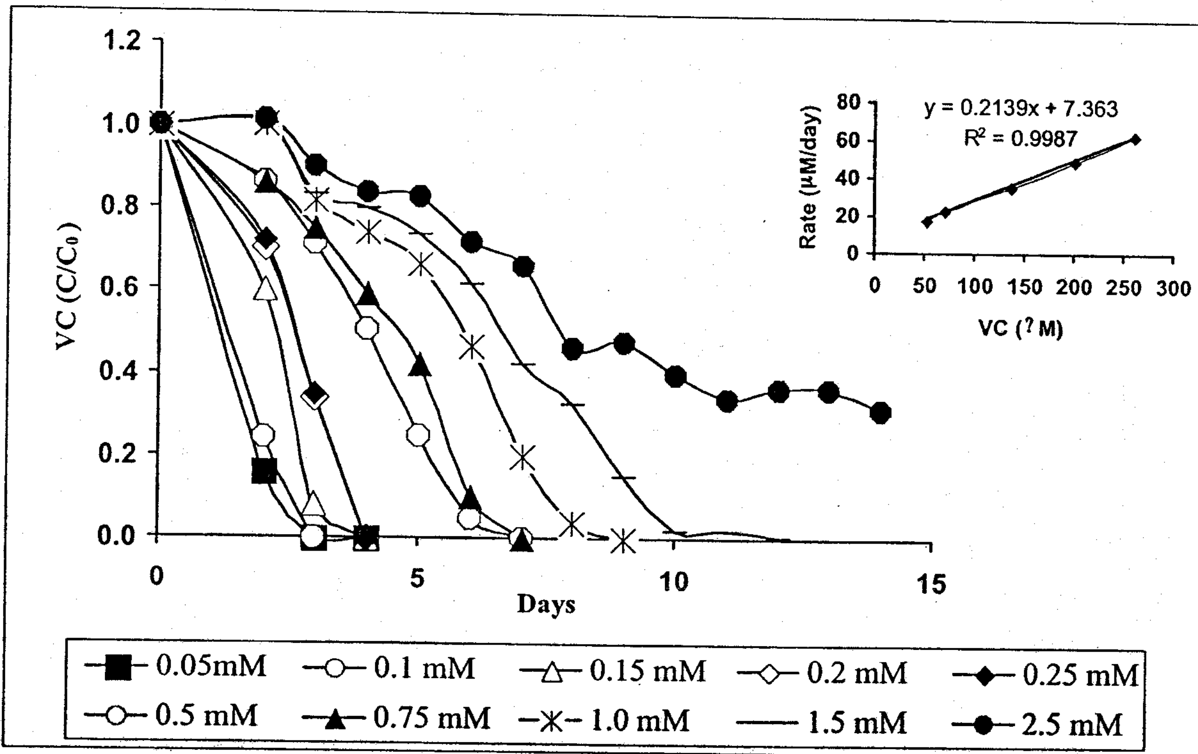


FIGURE 4

Biodegradation of VC as a function of mass of oxygen ranging from 0.02 to 0.2 mmol/bottle (0.18 to 1.8 ppm DO). Inset shows removal of VC when fed repeatedly to microcosms amended with 0.2 mmol of oxygen (1.8 ppm DO). The bottles were purged with $N_2$ for 20 to 25 min., prior to the addition of a known volume of pure oxygen.

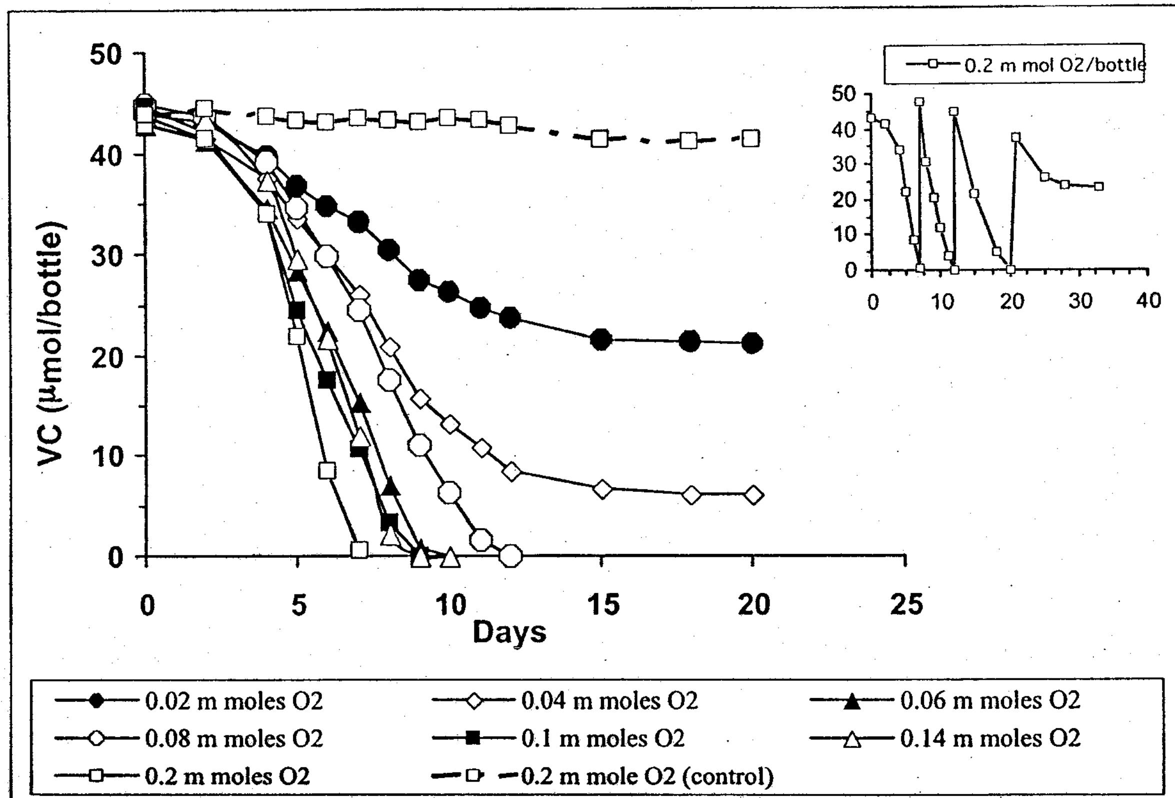


FIGURE 5

Biodegradation of ethene in the presence or the absence of VC (225 µM). Results are averages of three active and two control bottles. The error bars represent standard deviations.

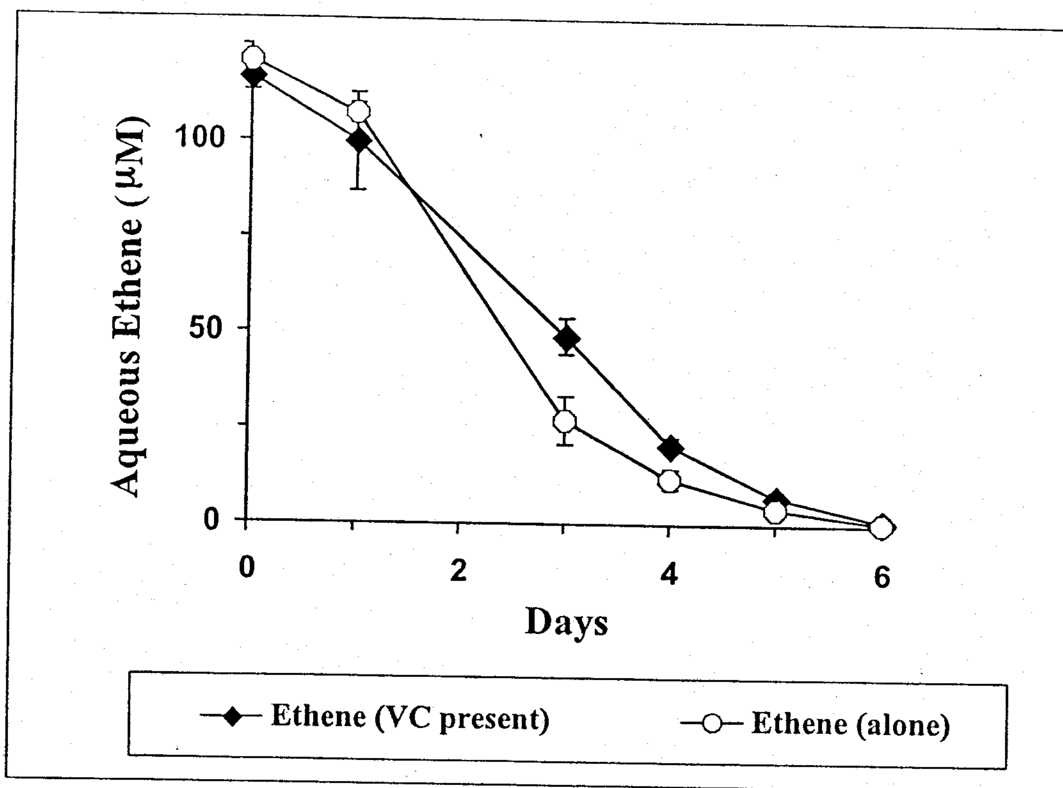


FIGURE 6

Cometabolic degradation of *cis*-DCE in the presence of VC as a primary carbon source. Results are means of three active and two control bottles.

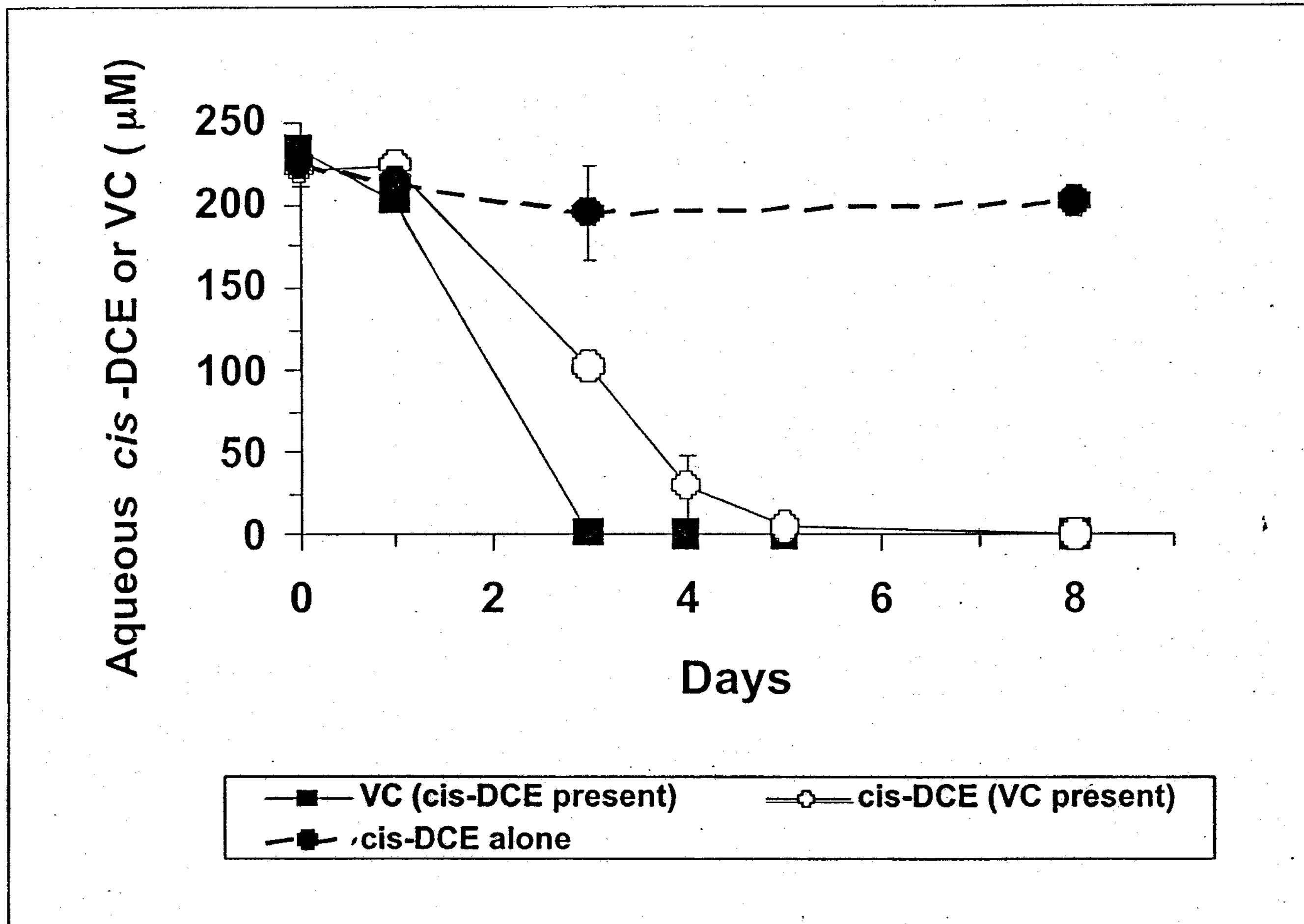


FIGURE 7

Biodegradation of VC at the end of various starvation periods by the TRW culture. The bottles were incubated static in the dark at $30^0$ C in the absence of VC for various time periods. At end of each starvation regimen, the bottles were purged with air, amended with VC, and degradation was monitored on daily basis. Each data point represents the average value of triplicate active and duplicate control bottles.

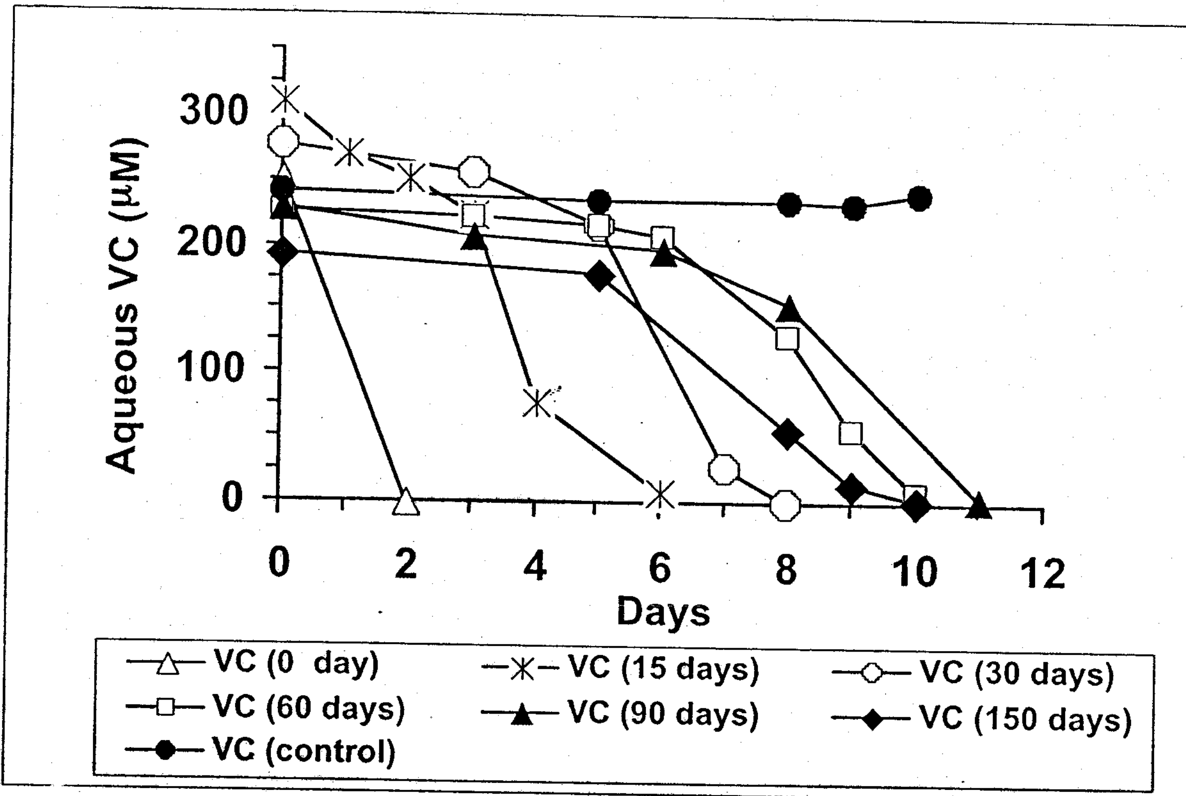


Figure 8a. Biodegradation of VC or Ethene by the *Ochrobacterium* isolate.

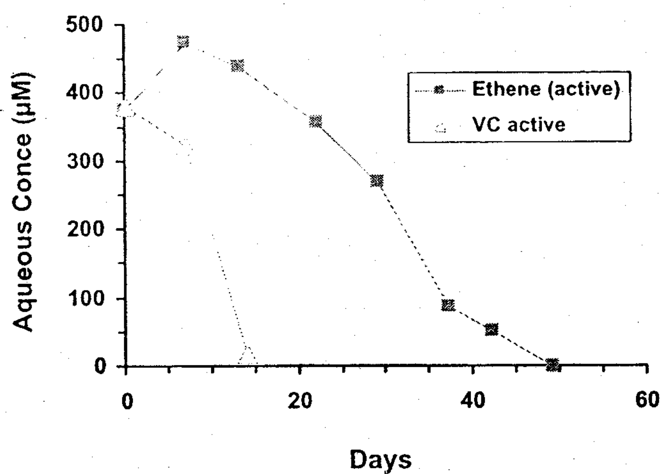



Figure 8b. Biodegradation of VC or Ethene by the *Mycobacterium* isolate.

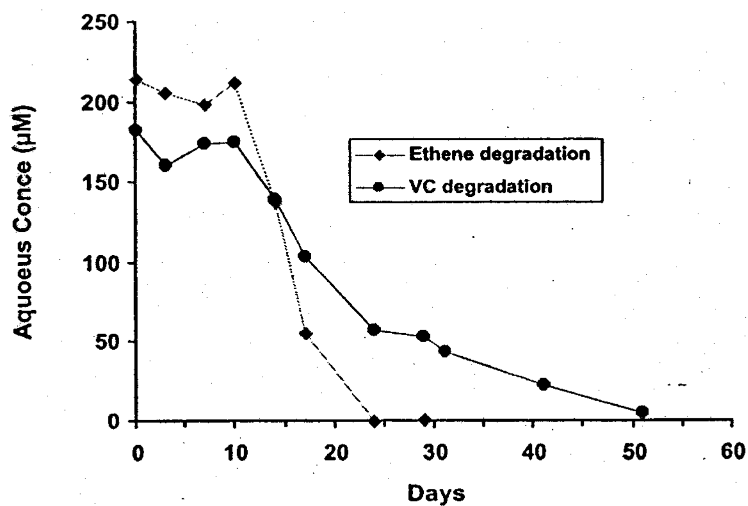


Figure 9. Microcosm studies to assess the bioaugmentation potential of *Mycobacterium* sp. in the (a) absence or (b) presence of native populations associated with Wichita site aquifer material.

**Figure 9a.**

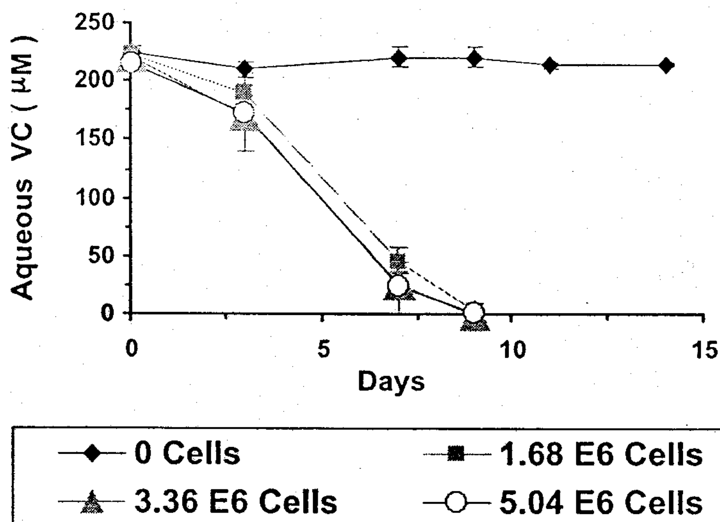



**Figure 9b**

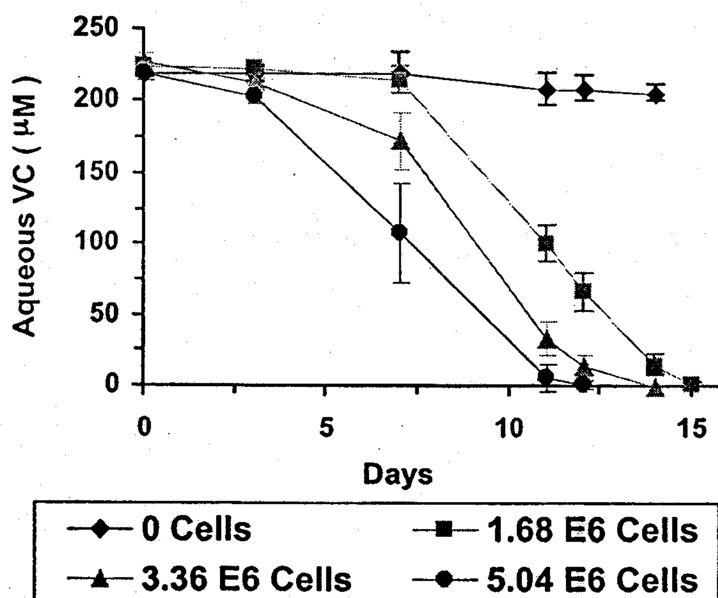


**Figure 10. Microcosm studies to assess the bioaugmentation potential of *Ochrobacterium* sp. in the (a) absence or (b) presence of native populations associated with Wichita site aquifer material.**

Figure 10a

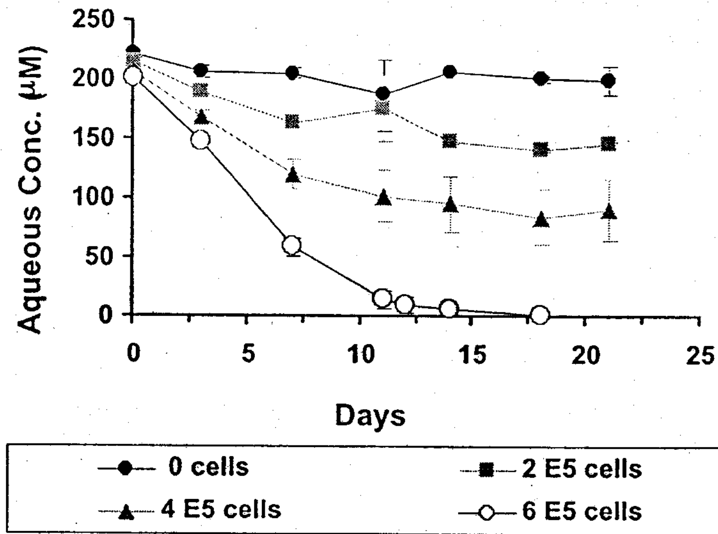
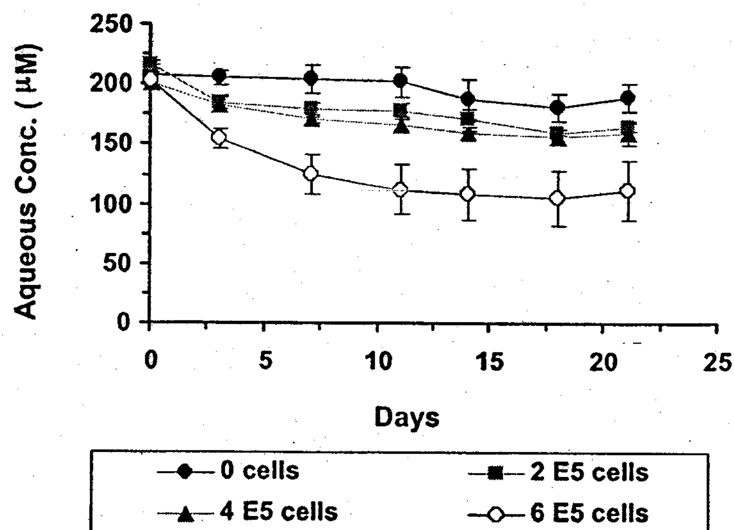



Figure 10b



# Appendix III

**Background**

The chlorinated solvents tetrachloroethene (PCE), trichloroethene (TCE), and their anaerobic degradation products *cis*-1,2-dichloroethene (*cis*-DCE) and vinyl chloride (VC) are common ground water pollutants. PCE is resistant to degradation under aerobic conditions but can be reductively dechlorinated to less chlorinated ethenes under anaerobic conditions. TCE, *cis*-DCE, and VC can be completely dechlorinated to ethene (ETH), and sometimes to ethane, by anaerobic bacteria, or they can be mineralized to carbon dioxide and hydrochloric acid under both anaerobic and aerobic conditions. Recent research has significantly improved our understanding of the microbial strategies leading to the reductive dechlorination of PCE to TCE and *cis*-DCE under anaerobic conditions. Also, some progress has been made towards a better understanding of the aerobic oxidation of TCE and VC. Despite these efforts, the accumulation of *cis*-DCE and VC at many PCE/TCE contaminated sites remains a pressing problem, even after injection of substrates to stimulate biodegradation. For the successful remediation of a contaminated site, the complete reduction to the environmentally benign products ETH and ethane, or complete mineralization must be achieved. The different possible processes involved in the dechlorination/degradation of chloroethenes are summarized in Table 1. Anaerobic halorespiration (Process 2) and aerobic energy-yielding oxidation (Process 5) are the most promising processes involved in the degradation of chlorinated solvents.

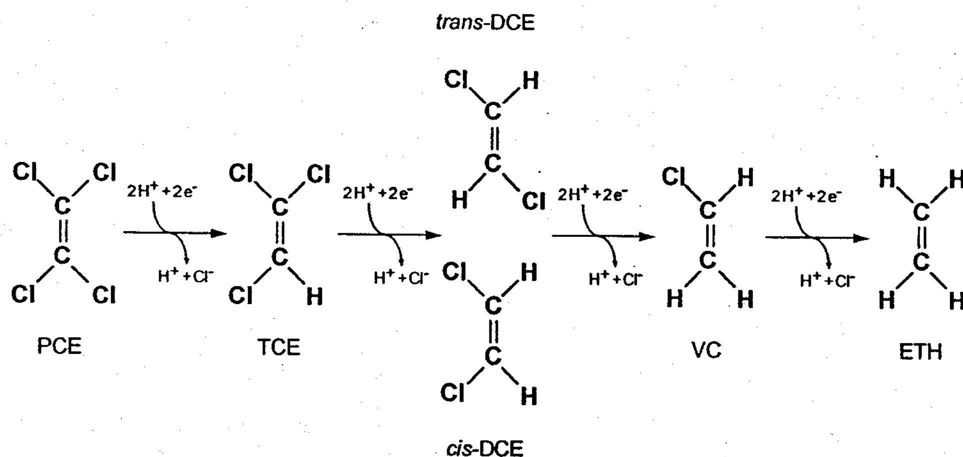
**Table 1:** Summary of different anaerobic and aerobic processes that can lead to the dechlorination/degradation of chlorinated ethenes

| | Biodegradation Process | | | | |
|---|---|---|---|---|---|
| | Anaerobic | | | Aerobic | |
| Characteristics | **Process 1** Cometabolic reductions | **Process 2** Halorespiration (energy-yielding) | **Process 3** Oxidation (energy-yielding) | **Process 4** Cometabolic oxidations | **Process 5** Oxygen-dependent oxidation (energy-yielding) |
| Metabolic group(s) | Sulfidogens Methanogens Acetogens | Halorespirers | Denitrifiers? Fe(III)-reducers Mn(IV)-reducers Humic acid-reduc. | Organisms with broad range oxygenases | *Mycobacterium* sp. and others? |
| Relative dechlorination/degradation rates | + | ++++ | ? | ++ | +/? |
| Frequency of active organisms in nature | High | High in anaerobic environments | ? | High in aerobic environments | Unknown |
| Favorable site conditions | Anaerobic, not $e^-$-donor and $e^-$-acceptor limited | Anaerobic, appropriate $e^-$-donor present no interfering TEAPs | Denitrifying conditions? Fe(III) reducing Mn(IV)-reducing | Aerobic, Inducer-compound present | Aerobic |

***Process 2 (Metabolic reductive dechlorination, halorespiration)***

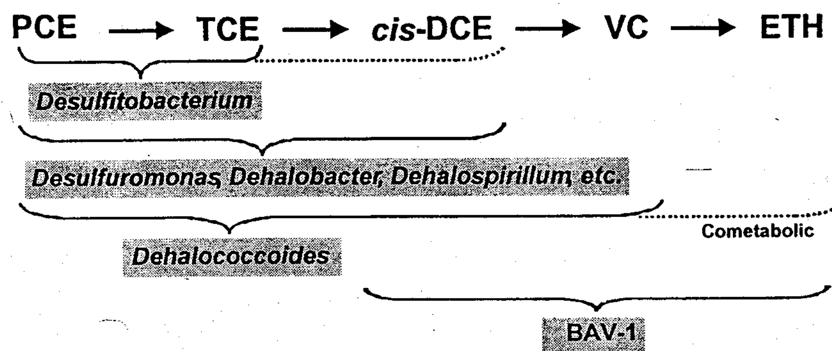
In laboratory studies with anaerobic microcosms and soil columns, as well as in field demonstrations, the stepwise reductive dechlorination of PCE to TCE, *cis*-DCE, VC, ETH, and sometimes ethane has been observed. The sequence of reductive dechlorination steps leading to ETH is depicted in Figure 1.

**Fig. 1: Sequential reduction of PCE to ETH observed under anaerobic conditions**




The energy available from reductive dechlorination of chloroethenes is substantial with Gibbs free energy changes ranging from -139 kJ to -173 kJ per chlorine released (under standard conditions with $H_2$ as the electron donor). The common characteristic of halorespiring bacteria is their ability to couple the energy released during the reductive dechlorination of chlorinated compounds to growth. Several organisms capable of using PCE, TCE, *cis*-DCE and VC as terminal electron acceptors in their energy metabolism (and hence for growth) have been isolated. Halorespiriers exhibit dechlorination rates several hundred times faster than previously known for organisms that reduce chlorinated ethene in cometabolic processes, and halorespirers are likely to be major contributors to microbially mediated dechlorination reactions in anaerobic environments. The complete dechlorination of PCE to ETH is a multi-step process and is most effectively carried out by more than one population. Figure 2 summarizes the current knowledge of microbial populations involved in the reductive dechlorination of PCE to ETH.

**Fig. 2: Populations involved in steps of complete reductive dechlorination of PCE to ETH**




Several members of the genus *Desulfitobacterium* have been identified to dechlorinate PCE to TCE in a respiratory process. One strain, *Desulfitobacterium* PCE-S, was shown to dechlorinate PCE to *cis*-DCE. Several organisms belonging to four different genera (*Desulfuromonas*, *Dehalobacter*, *Dehalospirillum*, *Enterobacter*) have been isolated on their ability to couple growth to the reductive dechlorination of PCE to *cis*-DCE. *Dehalococcoides ethenogenes* was shown to carry out the complete dechlorination sequence leading to the complete reduction of PCE to ETH (Maymó-Gatell, et. al., 1997). The final dechlorination step, the reduction of VC to ETH, however, was cometabolic and did not support a terminal electron accepting process (TEAP). *Dehalococcoides ethenogenes* requires unidentified growth factors, which limit the use of this organism for bioremediation, and the identification of the growth factors is a high priority goal. BAV-1 has been shown to couple growth to utilization of *cis*-DCE and VC (He et al., 2003).

***Process 5 (Aerobic energy-yielding oxidation)***

Because *cis*-DCE and VC are relatively less oxidized than PCE and TCE, their tendency to undergo further dechlorination is significantly reduced under anaerobic conditions. In contrast, under aerobic conditions, the potential for metabolic or cometabolic oxidation of *cis*-DCE and VC increases. The commonly observed reductive dechlorination of highly chlorinated ethenes under anaerobic conditions combined with the potential for rapid aerobic oxidation of the dechlorinated daughter products has led several investigators to suggest that a two-stage approach would be superior and cost-effective for treating chloroethene-contaminated aquifers

that are characterized by upgradient anaerobic and down gradient aerobic zones. Under these conditions, however, the potential for bioremediation depends on the ability of indigenous microorganisms to completely oxidize *cis*-DCE and VC metabolically or cometabolically over a range of contaminant concentrations.

Although a large body of information exists on the cometabolic biodegradation of *cis*-DCE and VC (Process 4), not much is known about the mechanisms and the enzymes catalyzing these reations. Also, nothing is known about the organisms that have the potential to use VC and *cis*-DCE as electron donors in aerobic metabolism.

In the presence of oxygen, VC or cis-DCE can be used as primary substrates for oxygen-dependent microbial growth and can be degraded completely to $CO_2$ and water. This results in a net energy benefit to the microbe involved. Thermodynamic calculations indicate that significantly more energy is available when VC is used as a carbon source by aerobic bacteria ($\Delta G^{o\prime}$ = -121 kJ/mole e-) than when it is reductively dechlorinated by anaerobic bacteria ($\Delta G^{o\prime}$ = -75 kJ/mole e-). The microorganisms that use cis-DCE and/or VC as a carbon and energy source under aerobic conditions are not well-studied.

**Site Assessment**

Site assessment criteria have been developed for monitored natural attenuation (Weidemeier, 1998), for the RABITT protocol (Morse, et. al. 1998), for use of soluble carbohydrates to enhance reductive dechlorination (Suthersan, et. al., 2002), and for enhanced anaerobic bioremediation (Henry et. al., 2003). The degradation of *cis*-DCE and VC is dependent on several site characteristics, including hydrogeology, chlorinated solvent degradation patterns, groundwater geochemistry and microbiology. Determining the redox (aerobic or anaerobic) state of the aquifer is one of the most important factors in determining the relevance of Process 2 or Process 5. Additional site assessment criteria that follow can be used to further evaluate the potential for Process 2 or Process 5.

***Hydrogeology***

Subsurface hydrogeology is, perhaps, the most critical site characteristic in evaluating remedial options at a site. The design of an MNA program or an electron donor/acceptor injection scheme is dependent on the ability to predict groundwater flow characteristics. Typical site assessment

includes determining the hydraulic conductivity, horizontal and vertical hydraulic gradients, flow direction, and groundwater velocity. The key hydrogeological characteristic influencing the redox state of the aquifer is groundwater recharge. However, recharge is typically estimated for development of a groundwater flow model, and direct measurements of dissolved oxygen or ORP are used.

*Chlorinated Solvent Degradation Patterns*

Two energy-yielding processes can degrade *cis*-DCE and VC to non-toxic end products: reductively dechlorination (halorespiration- Process 2) to ETH or aerobic oxidation (Process 5) to $CO_2$. Chlorinated solvent degradation patterns can support the likelihood of Process 2 or 5, as described in Table 2.

**Table 2: Process 2 Degradation Products**

| Chlorinated solvent | Process 2 degradation products | Process 5 degradation products |
|---|---|---|
| PCE/TCE/*cis*-DCE | TCE, cis-DCE, VC, ETH | $CO_2$ (for *cis*-DCE) |
| VC | ETH | $CO_2$ |

Evaluating chlorinated solvent degradation patterns is complicated by incomplete degradation or overlapping processes. The accumulation of *cis*-DCE at sites has been observed at many sites, and VC/ETH may not be observed, even when Process 2 is dominant. This may be due to a lack of halorespriring bacteria that dechlorinate cis-DCE. Alternatively, Process 5 may follow Process 2, and *cis*-DCE/VC/ETH may not be observed.

*Geochemistry*

The redox state of the aquifer is a crucial determinant of the potential for Process 2 or 5 in degrading chlorinated solvents. The redox state can be best evaluated by determining the dominant terminal electron acceptor process (TEAP) through a variety of field and laboratory measurements (Chapelle 1997). Based on their energy derived from TEAPs, microbes typically utilize the following electron acceptors in order: oxygen, nitrate, manganese, iron, sulfate, and carbon dioxide. Process 5 occurs under aerobic conditions. Process 2 usually occurs in a redox state representative of sulfate-reduction or lower. The following geochemical measurements are used to determine the redox state of the aquifer.

***Dissolved oxygen*** – Dissolved oxygen controls redox processes in an aquifer. Dissolved oxygen can be introduced to an aquifer primarily by recharge and can be depleted by microbial respiration. Dissolved oxygen concentrations less than 0.5 mg/L are required for Process 2.

***Oxygen-Reduction Potential*** **(ORP)** – ORP can provide an indication of the general redox condition of an aquifer. However, due to interferences and multiple reactions in groundwater, ORP cannot be used to determine the redox state. ORP is a useful measure in conjunction with other geochemical parameters. Process 2 may dominate if the ORP is less than -100 mV with an Ag/AgCl reference electrode.

***Nitrate*** – Microbes can utilize nitrate as a terminal electron acceptor, sometimes in the presence of low concentrations of oxygen. Nitrate is typically depleted for Process 2 to control chlorinated solvent degradation.

***Iron*** – Solid-phase ferric iron can be utilized as a terminal electron acceptor. Dissolved ferrous iron is often measured in the field to assess the significance of iron-reduction. Dissolved ferrous concentrations above background are indicative of iron-reducing conditions. Process 2 can take place in the presence of ferrous iron. However, high concentrations of bioavailable ferric iron may inhibit Process 2. Process 5 does not occur under iron-reducing conditions.

***Sulfate*** – Sulfate can be reduced, yielding sulfide, which can then precipitate as a ferrous mineral. Depletion of sulfate compared to background concentrations is indicative of a sulfate-reducing environment. Process 2 can occur under sulfate-reducing conditions and has been observed in high concentrations (over 500 mg/L) of sulfate. However, high concentrations of sulfide may inhibit process 2. Process 5 does not occur under these conditions.

***Methane*** – The presence of methane in a plume above background concentrations indicates a highly reduced condition, favorable for Process 2. Process 5 does not occur under these conditions.

***Hydrogen*** – Hydrogen is an important electron donor for Process 2. Hydrogen is produced by fermentation of various carbon sources and utilized by halorespiring bacteria in the degradation of chlorinated solvents. In general, hydrogen concentrations greater than 1 nM are required for Process 2. Process 5 is favored when hydrogen concentrations are less than 1 nM under aerobic conditions.

Other redox parameters, such as pH, temperature, specific conductance, alkalinity, total organic carbon, and chemical oxygen demand, are collected during site assessment activities and are used to evaluate biodegradation.

*Microbiology*

Genetic techniques and microcosm studies have been utilized at many sites to develop an understanding of the potential for biodegradation of chlorinated solvents. In conjunction with geochemical and chlorinated solvent data, these tools can be used to determine the dominant biodegradation process at a site.

*16S rRNA and Structural Gene Analysis* – A significant correlation has been developed between complete reductive dechlorination to ETH and the presence of at least one *Dehalococcoides* population (Ritalahti et al., 2002; Hendrickson et al., 2002). Other populations with dechlorinating activity (*Desulfitobacterium, Dehalobacter, Dehalospirillum,* and *Desulfuromonas*) have been identified at contaminated sites (Löffler et. al. 2000). DNA can be extracted from site soil or groundwater, amplified using Polymerase Chain Reaction (PCR) techniques, and analyzed for these populations to determine the potential for Process 2. PCR analyses are commercially available for approximately $300 per sample. However, one cannot rely solely on these techniques, as few specific 16S rRNA gene sequences have been linked to a particular dechlorinating activity. A high degree of sequence similarity has been identified among members of *Dehalococcoides* with varying dechlorination abilities. Furthermore, other yet unidentified microbial populations have halorespiring activity, which may result in false negative results in analyzing for *Dehalococcoides* populations. Real-Time PCR techniques allow for quantitative analyses of gene copies of halorespiring bacteria. As this tool becomes commercially available, correlations may be developed for threshold numbers of gene copies and dechlorinating activity. New techniques, such as terminal restriction fragment length polymorphism (T-RFLP) analysis have been developed by research laboratories to evaluate Process 2 activity (Richardson et. al., 2002), but have limited use to practitioners. T-RFLP analysis does not reliably distinguish *Dehalococcoides* species in diverse environmental samples, yielding false positives. Other techniques, such as phospholipids fatty acid (PFLA) analysis, are limited to providing information regarding known organisms with degradation activity.

At sites where reductive dechlorination (Process 2) is not active, aerobic energy-yielding oxidation (Process 5) may be the dominant biodegradation process. Relatively little is known about the microbial populations with the ability to degrade *cis*-DCE and VC as a sole source of

carbon and energy. As Process 5 microbial populations are defined, genetic tools will become available to assess a site.

***Microcosms*** – Site soil and groundwater samples can be incubated with combinations of chlorinated solvents and electron donors/acceptors to evaluate biostimulation of Process 2 or Process 5 in the laboratory. Microcosms can be bioaugmented with cultures of known activity to determine whether site-specific activity can be enhanced or inhibited. Although microcosm rate data cannot be directly extrapolated to the field, microcosms provide a good first approximation to the extent of activity that may be observed. Simple microcosms, which would include set-up with one or two chlorinated solvents and one electron donor, can be completed in a month for a reasonable cost to provide greater confidence in identification of Process 2 or 5 and in selection of a remedial option.

**Site Screening Matrix**

A site screening matrix was developed to develop correlations of geochemical and microbial data with contaminant degradation patterns. Although hydrogeological data is not necessary to evaluate the potential for Process 2, this information is critical in determining remedial options.

**Table 3: Key Indicators of Process 2**

| **Geochemical** | **Result** | **Conclusion** |
|---|---|---|
| Dissolved oxygen<br>Nitrate<br>Ferrous iron<br>Sulfate<br>Methane<br>Hydrogen<br>ORP | DO < 0.5 mg/L<br>$NO_3^-$ < 1 mg/L<br>$Fe^{2+}$ > background<br>$SO_4^{2-}$ < background<br>$CH_4$ > background<br>$H_2$ > 1 nM<br>ORP < -100 mV | Geochemical conditions are favorable for Process 2 |
| **Contaminant** | | |
| PCE, TCE, DCE, VC, ETH | Degradation products, such as cis-DCE, VC, and ETH are present | Reductive dechlorination is occurring |
| **Microbial** | | |
| 16S rRNA gene probe for<br>*Dehalococcoides* (*Dhc*)<br>*Desulfuromonas* (*Dsf*)<br>*Dehalobacter* (*Deb*)<br>TCE reductase gene (*tceA*) | Positive result compared to unimpacted area | Potential for Process 2 |

Modified from Wiedemeier MNA Technical Protocol (1998) and Suthersan (2002)

**Case Studies**

We have evaluated a number of sites for the potential for Process 2, the most significant biodegradation mechanism for *cis*-DCE and VC. The evaluation matrix, presented in Table 3, was used to evaluate site data. The results are presented below.

**Bachman Rd Site – Shallow transect**

| Key Indicator Results | Conclusion | Remedial Action |
|---|---|---|
| **Hydrogeological**<br>K – 20 ft/day | Suitable for nutrient delivery | MNA not sufficient. Bioaugmentation and biostimulation tested at pilot scale at site. |
| **Geochemical**<br>DO – 2.5 mg/L (avg)<br>Ferrous iron - up to 35 mg/L<br>Methane – up to 1.9 mg/L<br>Hydrogen – 0.8 nM<br>ORP - -125 mV | Anaerobic conditions suitable for Process 2 | |
| **Contaminant**<br>PCE (1,620 ug/L) to TCE (591 ug/L), cis-DCE (2,552 ug/L) and VC (29 mg/L)<br>No ethene production | Incomplete degradation with accumulation of cis-DCE | |
| **Microbial**<br>*Dhc*, *Dsf* and *Dhb* present. | Potential for Process 2. | |

**Hydrite Site – P19 (Layer 1)**

| Key Indicator Results | Conclusion | Remedial Action |
|---|---|---|
| **Hydrogeological**<br>K – $9 \times 10^{-4}$ cm/sec | Suitable for nutrient delivery | Continued monitoring. Hydraulic containment implemented for DNAPL source. |
| **Geochemical**<br>DO – 1.3 mg/L<br>Ferrous iron – 11 mg/L (> bkg)<br>Sulfate – 17 mg/L (< bkg) | Sulfate reduction active. Methanogenesis unknown. | |
| **Contaminant**<br>PCE (ND) and TCE (ND) to accumulation of cis-DCE (26 mg/L) and VC (4.2 mg/L) | Process 2 active, but incomplete. | |
| **Microbial**<br>*Dhc*, *Dhb* present.<br>*Dsf*, *tceA* absent. | Potential for Process 2. | |

**Hydrite Site – P95 (Layer 4)**

| Key Indicator Results | Conclusion | Remedial Action |
|---|---|---|
| **Hydrogeological**<br>K – 1.4 x $10^{-4}$ cm/sec | Suitable for nutrient delivery | Continued monitoring. Hydraulic containment implemented for DNAPL source. |
| **Geochemical**<br>DO – 5.1 mg/L<br>Ferrous iron - ND<br>Sulfate – 16 mg/L (< bkgd) | Aerobic conditions favoring Process 5 | |
| **Contaminant**<br>PCE (9 ug/L) and TCE (25 mg/L) present at low concentrations with cis-DCE (31 mg/L). No VC detected. | Process 2 inactive.<br>Potential for process 5. | |
| **Microbial**<br>Anaerobic microcosms inactive. | Process 2 inactive | |

**CA Site – PW-1**

| Key Indicator Results | Conclusion | Remedial Action |
|---|---|---|
| **Hydrogeological**<br>K – 3.3 ft/day | Suitable for nutrient delivery | Biostimulation with HRC implemented. Significant degradation. However, significant methane accumulation and stall at VC. Further monitoring recommended. |
| **Geochemical**<br>DO – 1.97 mg/L<br>Nitrate < 1 mg/L<br>Ferrous iron > background<br>Sulfate < background<br>Methane – 2,700 mg/L<br>Hydrogen – 7.1 nM<br>ORP - -107 mV | Methanogenic conditions favoring Process 2 | |
| **Contaminant**<br>TCE to cis-DCE to VC<br>Ethene accumulation | Significant degradation with stall at VC | |
| **Microbial**<br>*Dhc*, *Dsf* and *Dhb* present in impacted area. | Potential for Process 2 | |

**FL Site – S9MW-15**

| Key Indicator Results | Conclusion | Remedial Action |
|---|---|---|
| **Geochemical**<br>DO – 0.2 mg/L<br>Sulfate < background<br>Hydrogen – 12.1 nM<br>Methane – 5,200 ug/L<br>ORP – -215 mV | Methanogenic conditions favoring Process 2 | Anaerobic conditions at baseline. Biostimulation with HRC resulted in increased methanogenesis. However, no degradation past cis-DCE.<br><br>Bioaugmentation recommended. |
| **Contaminant**<br>TCE (2 ug/L) to trans-1,2-DCE (740 ug/L) > cis-1,2-DCE (280 ug/L) | Process 2 stalled at DCE | |
| **Microbial** | Not available | |

**GA Site – MW-28/MW-7**

| Key Indicator Results | Conclusion | Remedial Action |
|---|---|---|
| **Hydrogeological**<br>K – 1.5 ft/day | Suitable for nutrient delivery | Anaerobic conditions at baseline. Bioaugmentation recommended to enhance DNAPL dissolution. |
| **Geochemical**<br>DO – 1.1 mg/L<br>Nitrate – < 0.5 mg/L<br>Sulfate < background<br>ORP – -21 mV | Limited evidence for conditions favorable for Process 2. | |
| **Contaminant**<br>TCE DNAPL. No significant degradation products. | Process 2 not active. | |
| **Microbial**<br>*Dhc* and *Dhb* only detected with nested PCR.<br><br>Microcosm study with lactate addition not active. Bioaugmentation successful for TCE and VC. | Process 2 not active. | |

**IN Site – PMW8 transect**

| Key Indicator Result | Conclusion | Remedial Action |
|---|---|---|
| **Hydrogeological**<br>K – 200 ft/day | Suitable for stimulation by nutrient/bacterial injection | Anoxic conditions at baseline. MNA not feasible. Bioaugmentation recommended. |
| **Geochemical**<br>DO – ND to 1.0 mg/L<br>Nitrate – 1.2 to 3.0 mg/L<br>Dissolved iron - ND<br>Sulfate - variable<br>Methane – 0.25 – 2.5 ug/L<br>ORP – 30 mV | Anoxic conditions with potential for process 2 | |
| **Contaminant**<br>TCE (4,100 ug/L) to low cis-DCE 160 ug/L). No VC | Limited activity of process 2 | |
| **Microbial**<br>Soil has weak signals for *Dhc*, *Dsf*, *Dhb*. Groundwater negative.<br>Microcosm study indicates cis-DCE stall with TCE and lactate.<br>No activity with VC and lactate.<br>Bioaugmented microcosms yield ethene. | Limited activity of process 2. | |

**WI Site – SVE 59/MW-53A**

| Key Indicator Result | Conclusion | Remedial Action |
|---|---|---|
| **Hydrogeological**<br>K – $1x10^{-3}$ cm/sec | Suitable for nutrient delivery | SVE 59: Anoxic conditions at baseline. MNA not feasible. Biostimulation/bioaugmentation recommended.<br><br>MW-53A: MNA feasible |
| **Geochemical**<br>DO – 0.8 mg/L/0.5 mg/L<br>Nitrate – not available<br>Dissolved iron – ND/1.5 mg/L<br>Sulfate – not available<br>Sulfide – ND/0.2 mg/L<br>Methane – 13 ug/L/720 ug/L<br>Ethene – 0.42 ng/L/930 mg/L<br>Hydrogen – 1.4 nM/1.5 nM<br>ORP – 114 mV/-11 mV | Anoxic conditions with potential for process 2 at SVE 59.<br><br>Anaerobic conditions suitable for process 2 | |
| **Contaminant**<br>SVE 59: TCE (4,800 ug/L) to cis-DCE (5%) and VC (2%).<br>MW-53A: TCE (ND) to cis-DCE (910 ug/L) and VC (9,100 ug/L) | SVE 59: Limited activity of process 2<br><br>MW-53A: Process 2 active | |
| **Microbial** | | |
| Not available | | |

**NJ Site – TWP K12**

| Key Indicator Result | Conclusion | Remedial Action |
| --- | --- | --- |
| **Hydrogeological**<br>K – 5 x $10^{-4}$ cm/sec (est) | Suitable for nutrient delivery | Source zone removal by excavation. MNA nbot feasible. Biostimulation recommended for dissolved groundwater plume. |
| **Geochemical**<br>DO – 0.5 mg/L<br>Nitrate – ND<br>Dissolved iron – 24.7 mg/L<br>Sulfate – 310 mg/L<br>Sulfide – 1.8 mg/L<br>Methane – 28 ug/L<br>Ethene – 33 ug/L | Anaerobic conditions suitable for Process 2 | |
| **Contaminant**<br>PCE (300 ug/L) to TCE (120 ug/L), cis-DCE (4500 ug/L) and VC (550 ug/L) | Process 2 active with cis-DCE accumulation | |
| **Microbial**<br>Nested PCR used to detect *Dhc* in all samples. Weak signals for *Dsf* and *Dhb*. 6,000-8,000 copies of *Dhc* per ml of groundwater. Microcosms with PCE produced ethene. Samples incubated with cis-DCE accumulated VC and some ethene. Microcosms with PCE DNAPL accumulated cis-DCE. | *Dhc* detected in all samples, indicating potential for Process 2. Microcosms indicate dissolved PCE can be transformed to ethene. | |

**MA Site 1 – LF-02A**

| Key Indicator Result | Conclusion | Remedial Action |
|---|---|---|
| **Hydrogeological**<br>K – 0.016 ft/day | Marginally suitable for nutrient delivery | Anaerobic conditions favorable for Process 2. Plume contained. Recommended biostimulation to accelerate Process 2. |
| **Geochemical**<br>DO – 0.1 mg/L<br>Nitrate – ND<br>Dissolved iron – 4.2 mg/L<br>Sulfate – 29 mg/L (< bkgd)<br>Methane – 2,900 ug/L<br>Ethene – ND<br>Hydrogen – not available<br>ORP – -75 mV | Methanogenic conditions favoring Process 2 | |
| **Contaminant**<br>1,1,-DCE (250 ug/L) to VC (180 ug/L)<br>Benzene - 28 ug/L | Process 2 active | |
| **Microbial**<br>Not available | | |

**MA Site 2 – Recovery well/G15**

| Key Indicator Result | Conclusion | Remedial Action |
|---|---|---|
| **Hydrogeological**<br>K – 0.04 ft/day | Marginally suitable for nutrient delivery | Anaerobic conditions favorable for Process 2. Plume contained. Recommended biostimulation to accelerate Process 2. |
| **Geochemical**<br>DO – not available<br>Nitrate – not available<br>Dissolved iron – G15S (19 mg/L) and G15D (5.3 mg/L)<br>Sulfate – not available<br>Methane – not available<br>ORP – not available | Anoxic conditions favoring Process 2 | |
| **Contaminant**<br>RW: PCE (22.3 ug/L) to TCE (323 ug/L), 1,2-DCE (484 ug/L), and VC (36 ug/L) | Process 2 active. | |
| **Microbial**<br>Not available | | |

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