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Aerobic biodegradation of vinyl chloride by a highly enriched mixed culture

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Abstract

Lower chlorinated compounds such as *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC) often accumulate in chloroethene-contaminated aquifers due to incomplete reductive dechlorination of higher chlorinated compounds. A highly enriched aerobic culture that degrades VC as a growth substrate was obtained from a chloroethene-contaminated aquifer material. The culture rapidly degraded 50–250 μM aqueous VC to below GC detection limit with a first-order rate constant of 0.2 day^{-1}. Besides VC, the culture also degraded ethene as the sole carbon source. In addition, the culture degraded *cis*-DCE, but only in the presence of VC. However, no degradation of *trans*-DCE or TCE occurred either in the presence or absence of VC. The ability of the TRW culture to degrade *cis*-DCE is significant for natural attenuation since both VC and *cis*-DCE are often found in chloroethene-contaminated groundwater. Experiments examining the effect of oxygen threshold on VC degradation showed that the culture was able to metabolize VC efficiently at extremely low concentrations of dissolved oxygen (DO). Complete removal of 150 μmoles of VC occurred in the presence of only 0.2 mmol of oxygen (1.8 mg/L DO). This is important since most groundwater environments contain low DO (1–2 mg/L). Studies showed that the culture was able to withstand long periods of VC starvation. For example, the culture was able to assimilate VC with minimal lag time even after 5 months of starvation. This is impressive from the point of its sustenance under field conditions. Overall the culture is robust and degrades VC to below the detection limit rendering this culture suitable for field application.

Introduction

Considerable research has focused on the anaerobic transformation of tetrachloroethene (PCE) and trichloroethene (TCE), two of the most common chlorinated solvents found in groundwater. The dechlorination of PCE to *cis*-dichloroethene (*cis*-DCE) is a relatively fast process and can be easily stimulated by a diverse group of bacteria. However, further reduction of *cis*-DCE to vinyl chloride (VC) and then to ethene is a significantly slower process, often resulting in the accumulation of *cis*-DCE and VC as dead-end products at most sites (Hirata et al. 1992; 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). Both *cis*-DCE and VC are US EPA priority pollutants and VC is also a known human carcinogen (Kielhorn 2000).

Numerous studies have demonstrated that VC can be readily degraded under aerobic conditions by many pure and mixed cultures expressing mono and di-oxygenase enzymes when grown on a variety of 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 VC was apparently a fortuitous occurrence with no clear advantage to the organism, and consequently requires a sufficient concentration of a primary substrate to support growth. In addition, cometabolic processes are inherently slow due to substrate competition, enzyme inactivation, and toxicity to the organism (Alvarez-Cohen & McCarty 1991; Arp et al. 2001; Dolan & McCarty 1995; Newman & Wackett 1997; Oldenhuis et al. 1991; Rasche et 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).

Although VC is readily degraded under aerobic conditions and yields a large amount of free-energy ($\Delta G^{0'} = -1137$ kJ/mol), organisms belonging to only two genera capable of assimilating VC have been isolated and characterized (Coleman et al. 2002a; Hartmans et al. 1985; Hartmans & de Bont 1992; Hartmans et al. 1992; Verce et al. 2000). Similarly, only one organism capable of utilizing *cis*-DCE has been reported (Coleman et al. 2002b). Although isolation of new VC-assimilating organisms is important for understanding diversity, ecology, and mechanisms of degradation, studies using mixed cultures can provide important information about VC oxidation under competing environments. Additionally, the data from mixed culture studies can be used to compare rates and stability of the processes with pure cultures. Such information is important for field application. In the present study we describe VC metabolism by a highly enriched mixed culture obtained from a chloroethene-contaminated site (TRW site, Minerva, OH)

Materials and methods

Aquifer material and chemicals

A VC degrading mixed culture was enriched from an aquifer material obtained from a chloroethene plume at the TRW facility in Minerva, OH. The aquifer material was retrieved using a Geoprobe from 10 feet below ground surface at a location downstream from a TCE source area. Groundwater at the sample location mainly contained *cis*-DCE and VC and <1 mg/L dissolve oxygen (DO).

TCE, *cis*-DCE, *trans*-DCE, VC, ethene, ethane, and methane were >98% pure and obtained from Aldrich Chemical Co., Milwaukee, WI. All other chemicals used were of reagent grade. The following mineral salts medium (MSM) was used in this study and it contained (g/l): NaCl, 1.0; NH_4Cl, 1.0; KCl, 0.1; KH_2PO_4, 0.45; K_2HPO_4, 0.9; $MgSO_4.7H_2O$, 0.3 and $CaCl_2.2H_2O$, 0.04. The pH was adjusted to 7.0.

Microcosm setup

Microcosms were constructed using 160-ml serum bottles (Wheaton) closed with 20-mm Teflon-lined septa and aluminum caps (The West Co). Initial slurry microcosms study was done using 10 g (wet weight) aquifer material from the TRW site and 90-ml MSM. All experiments using the TRW culture were performed in 160-ml serum bottles with 50-ml MSM. The bottles were injected with VC, methane, ethane, and/or ethene using 1 or 5-ml disposable syringes. Addition of TCE, *cis*-DCE, or *trans*-DCE to microcosms was done with dedicated 10-μl glass syringes (Hamilton Co., Reno, NV). The amount of VC and other substrates added to microcosms were calculated using a Henry's constant (Gossett 1987). Henry's constant (concentrations in gas phase/concentrations in aqueous phase) 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, ethane, methane, *cis*-DCE, *trans*-DCE, and/or TCE was tested in serum bottles containing 50-ml MSM inoculated with 2.5-ml active TRW culture. The effect of oxygen levels on the degradation of VC was evaluated by purging microcosms with nitrogen for 25 min to remove oxygen from the bottles. The bottles were crimp sealed and inoculated with active TRW culture. Different amounts of oxygen in the headspace were achieved by replacing the known volume of headspace with pure oxygen. The volume of oxygen needed to achieve desired concentrations of oxygen in liquid was calculated using the Henry's constant (concentrations in gas phase/concentrations in aqueous phase) of 31.5 for oxygen at 25 °C. The potential of TRW culture

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. When all the added VC in the headspace was removed, the bottles were incubated in the absence of VC for various periods of time. At the end of each starvation period, the bottles were purged with air, spiked with VC and degradation was monitored by GC.

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). TCE, *cis*-DCE, and *trans*-DCE were separated on a DB-624 capillary column of 30 m $\times$ 0.323 mm $\times$ 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 $\times$ 0.53 mm $\times$ 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 were 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 spiltless mode.

For quantification, 20 or 30 μl of headspace gases from microcosms were withdrawn using a 50-μl capacity gas-tight glass syringe and analyzed by the GC. Total mass of each chlorinated and non-chlorinated compound in bottles was determined by comparing the headspace measurements to standards. Separate standards were prepared for each compound tested by adding a range of mass of the test compound to 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 was plotted and aqueous concentrations were calculated using Henry's constant. The GC detection limit for aqueous VC by our method was <0.1 μM (much lower concentrations of VC could be detected when larger volumes were injected).

Development of enrichment cultures and analysis of community structure

Slurry microcosms that consistently degraded VC in the absence of added co-substrates were selected for further enrichment. Five percent (vol/vol) of the sediment-free slurry was transferred to a new set of serum bottles containing fresh MSM spiked with 250 to 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 culture to fresh MSM several times. After 12 months of enrichment process, the culture consistently degraded added VC within 2–3 days. All experiments were initiated by transferring 2.5-ml of the enriched mixed culture from the 1-L bottles to 160-ml serum bottles containing 50-ml MSM.

The analysis of microbial diversity of the TRW culture was done by the Microbial Insights, Inc., (Rockford, Tenn.). Approximately, 10-ml of the VC-grown culture was used for analyses of both community structure and phylogenetic identification of the dominant members of the culture using denaturing gradient gel electrophoresis (DGGE) (Nicholson & Fathepure 2004).

Results

Biodegradation of VC by the TRW culture

Complete removal of VC occurred within 33 days with 2-week of 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). VC-assimilating populations were enriched from these slurry microcosms that degraded 250–300 μM aqueous VC within <3

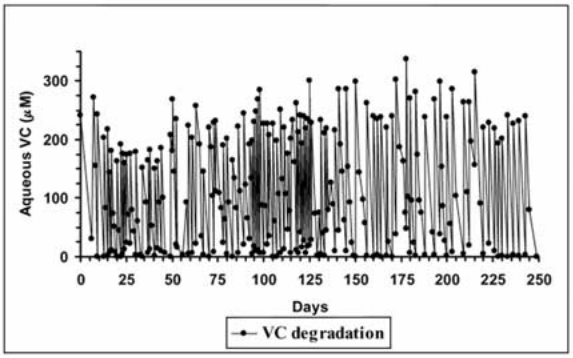

Figure 1. Sustained use of VC as a growth substrate by the TRW culture, indicated by the repeated use of VC. The data are shown for the last 250 days of the culture maintenance on VC as the sole carbon source.

days after a 12-months of enrichment process. Microscopic observations as well as colony morphology on agar plates suggested that the culture was mainly composed of 3 or 4 morphologically distinct organisms. The results in Figure 1 demonstrate the performance of the TRW culture for the next 250 days after the initial 12 months of enrichment process. The partial identity of the dominant members of the culture was obtained by DGGE profiles of 16S rDNA amplified PCR products (Figure 2). DGGE analysis showed 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* (>97% sequence similarity).

Experiments were carried out to evaluate degradation potential of the TRW culture at varied concentrations of VC ranging from 50 to 2,500 μM. The initial rate of VC degradation increased linearly with increasing aqueous VC ranging from 50 to 250 μM yielding a first-order rate constant of 0.214 day^{-1}. The rates further increased with increasing initial aqueous VC from 500 to 1,500 μM, but this removal rate was not linear and the removal could not be adequately described by Michaelis-Menten kinetics (data not shown). It is conceivable that the culture may have harbored more than one species of VC-degraders and also factors such as diffusional barrier or accumulating intermediates at higher VC concentrations may have affected the rates. At >1,000 μM aqueous VC, removal occurred following 2 days of lag time.

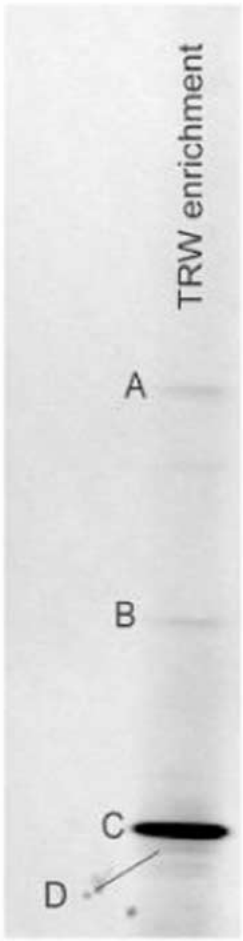

Figure 2. DGGE gel image of PCR products amplified from bacterial 16S rDNA gene extracted from the TRW culture grown on VC as the sole carbon source. Prominent bands were excised and sequenced. Results from sequences were compared with the DNA sequences in databases, RDP and GenBank. Bands A and D failed to produce usable sequences, while bands B and C most closely matched (>97%) with *Rhodococcus* and *Mycobacterium* sequences in GenBank database, respectively. The GenBank access numbers for band B is AY 114109 and for band C is AY 114109 and AJ 429044.

Biodegradation of VC at different oxygen concentrations

Degradation of VC occurred over a wide range of oxygen in the headspace (Figure 3). Addition of as low as 0.06–0.08 mmol of O_2 per bottle (0.5–0.7 mg/L DO) resulted in the degradation of all the added 42 μmol of VC. However, degradation was relatively rapid in microcosms containing 0.2 mmol of O_2/bottle (1.8 mg/L DO). Repeated additions of VC (total 150 μmol) to these bottles (no additional O_2

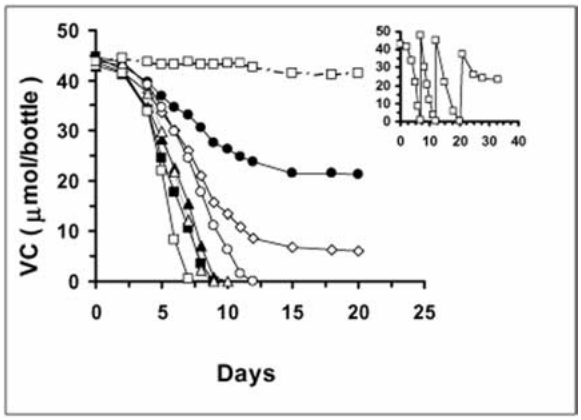

Figure 3. Biodegradation of VC as a function of mass of oxygen ranging from 0.02 to 0.2 mmol/bottle (0.18 to 1.8 mg/L DO). Symbol: (●), 0.02 mmol O_2/bottle; (◇), 0.04 mmol O_2/bottle; (▲), 0.06 mmol O_2/bottle; (○), 0.08 mmol O_2/bottle; (■), 0.1 mmol O_2/bottle; (△), 0.14 mmol O_2/bottle; (□), 0.2 mmol O_2/bottle; (- - □ - -), 0.2 mmol O_2/sterile bottle. Inset shows repeated removal of VC (total 150 μmoles) in microcosms amended with 0.2 mmoles of oxygen (1.8 mg/L DO). The bottles were purged with N_2 for 20 to 25 min. prior to the addition of a known volume of pure oxygen. Similar experiment was done using higher amount of oxygen ranging from 0.2 to 4.47 mmol/bottle (1.8 to 35 mg/L DO, data not shown). Results are means of three active and two control bottles.

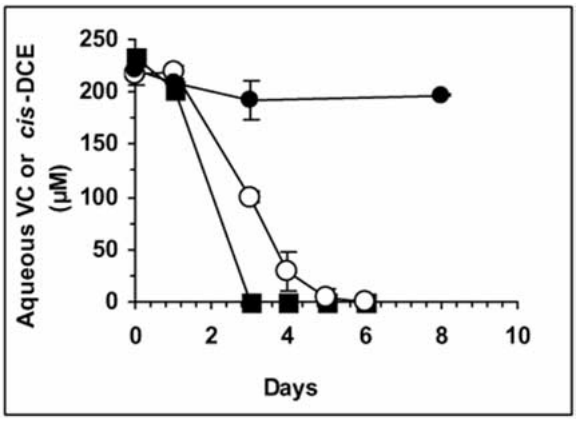

Figure 4. Cometabolic degradation of *cis*-DCE in the presence of VC added as a primary carbon source. Symbol: (■), VC degradation (*cis*-DCE present); (○), *cis*-DCE degradation (VC present); (●), *cis*-DCE degradation (no other substrate present). Results are means of three active bottles. Error bars represent standard deviations.

added) showed continued removal of VC suggesting that the TRW culture can degrade VC at low levels of DO (see inset, Figure 3). Experiments involving the addition of higher amounts of oxygen ranging from 0.2 to 4.47 mmol/bottle did not show an enhanced rate of VC degradation (data not shown).

Biodegradation of ethene, ethane, methane, cis-DCE, trans-DCE and TCE

Experiments were initiated to evaluate the metabolic versatility of the TRW culture. The culture readily degraded 125 μM aqueous ethene both in the presence or absence of added VC. Further, the culture could be maintained on ethene alone as a growth substrate for an extended period of time (data not shown). The culture also degraded *cis*-DCE, but only in the presence of VC as the primary carbon. No degradation of *cis*-DCE occurred when added alone even after incubating for longer periods (Figure 4). No degradation of *trans*-DCE or TCE occurred either in the presence or absence of added VC. Also, the culture did not degrade methane or ethane when added alone or in conjunction with VC. Biodegradation of VC was tested in the presence of easily assimilable compounds including acetate, glucose, pyruvate, yeast-extract, or trypticase soy (Table 1). VC degradation occurred in

Table 1. Biodegradation of VC in the presence of an alternate substrate by the TRW culture[1]

Alternate substrate	Aqueous concentrations	Degradation of alternate substrate[2]	VC assimilation[3]
VC	250 μM	none added	+
Ethene	125 μM	+	+
Ethane	220 μM	–	+
Methane	120 μM	–	+
Cis-DCE	210 μM	+	+
Trans-DCE	225 μM	–	+
TCE	192 μM	–	+
Acetate	5 mM	ND[4]	+
Pyruvate	5 mM	ND	–
Glucose	5 mM	ND	–
Trypticase soy	0.1%	ND	+
Yeast-extract	0.1%	ND	+

[1]Results are averages of three bottles.
[2]Degradation of altenate substrate was evaluated in the presence of VC.
[3]Degradation of VC was done in the presence of altenate substrate.
[4]ND = not determined.

the presence of all the tested substrates except glucose or pyruvate. However, when a 5% culture from these bottles was transferred to fresh MSM devoid of added 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 5). The data showed that VC degradation resumed

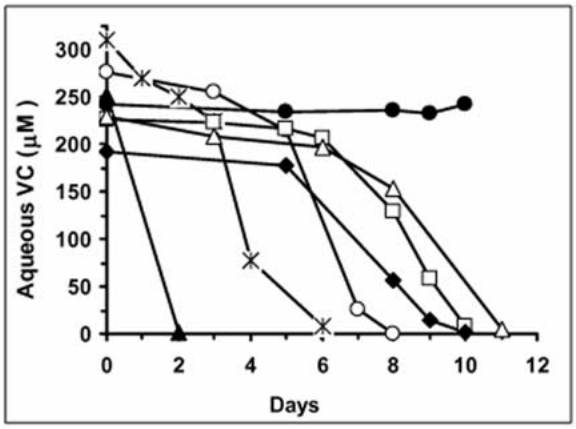

Figure 5. Biodegradation of VC at the end of various starvation periods by the TRW culture. Bottles containing 50-ml of MSM and inoculated with 2.5-ml of TRW culture were spiked with 250 μM aqueous VC three times. After third spike when all the added VC was degraded, bottles were incubated at 30 °C in the absence added VC for various time periods. Symbol: (▲), 0 day starvation; (⋇), 15 days of starvation; (○), 30 days of starvation; (□), 60 days of starvation; (△), 90 days of starvation; (⧫), 150 days of starvation; (●), 150 days of starvation in control bottles. At the end of each starvation period, the bottles were purged with air and amended with VC and degradation monitored periodically. The autoclaved control bottles were purged with sterile air and VC was injected with the help of a 1-ml sterile disposable syringe and a 0.2-micron sterile filter. Each data point represents average value of triplicate active and duplicate control bottles.

with a minor lag after each starvation period. For example, a lag of roughly 3–6 days was seen when the culture was incubated at 30 °C without VC for a period ranging from 15 to 150 days. Interestingly, the lag time did not increase proportionally with increased starvation times.

Discussion

A stable microbial culture capable of using VC as the sole source of carbon was developed using the TRW site material. The TRW culture differs in many respects from previously reported VC-degrading enrichments or pure cultures (Coleman et al. 2002a; Hartmans 1995; Hartmans et al. 1985, 1992; Hartmans & de Bont 1992; Verce et al. 2000, 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 repeatedly degraded roughly 250 μM aqueous VC to below detection limit within 1–3 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 was 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 indicated the production of HCl due to mineralization of added VC (Hartmans et al. 1985). DGGE analysis revealed that *Mycobacterium* spp and *Rhodococcus* spp were the dominant members of the TRW enrichment. These results are consistent with the recent observation that VC-assimilating *Mycobacterium* spp are widespread and commonly found at most chloroethene-contaminated aquifers (Coleman et al. 2002a). However, no report exists in the literature showing the ability of *Rhodococcus* spp to assimilate VC as the growth substrate. 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 environments and degrade a variety of compounds including haloalkanes (Parales et al. 2002).

The culture efficiently degraded VC in the presence of extremely low DO. Results showed that 150 μmol of VC were completely degraded in the presence of only 0.2 mmol of O_2. Based on reaction stoichiometry, 150 μmol of VC would require 0.375 mmol of O_2, thus suggesting that incomplete mineralization and accumulation of partially oxidized metabolic intermediates had occurred. Continued degradation of VC at low DO apparently indicates the lack of toxicity expected by the accumulation of first metabolite, VC-epoxide (Hartmans 1995; Hartmans & de Bont 1992). Therefore, we speculate that perhaps VC may have been metabolized to intermediates past VC-epoxide in the metabolic pathway. However, additional research is needed to fully understand the pathways and specific intermediates that may accumulate under microaerophilic conditions. This ability of the 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 low Ks for oxygen during growth on VC indicating that these strains can metabolize VC under a low oxygen threshold. Our tests showed that the TRW culture did not degrade VC under denitrifying or sulfate-reducing electron accepting conditions (data not shown).

The enrichment's ability to metabolize ethene and *cis*-DCE is important for natural attenuation. Both ethene and *cis*-DCE are predominant products formed during reductive dechlorination of higher chloroethenes (e.g., PCE, TCE) and chloroethanes (e.g., 1,1,1-TCA) and can be commonly found in the leading fringes of most contaminated plumes (Lee et al. 1998). Interestingly, the culture did not degrade methane or ethane even when supplemented 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 & de Bont 1992). Alkene monooxygenases from *Xanthobacter* Py2 and *Rhodococcus corallinus* (*Nocardia corallina*) B-276 degraded alkenes and chloroalkenes but not alkanes and 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 the recently isolated VC-metabolizing *Pseudomonas* spp, strain MF1 (Verce et al. 2000, 2002). However, the strain MF1 did not begin to degrade *cis*-DCE until nearly all of the added VC was consumed (Verce et al. 2002). On the other hand, degradation of *cis*-DCE and VC occurred concurrently in the TRW culture. The culture's ability to degrade VC in the presence of methane, ethane, acetate, trypticase soy or yeast-extract is important for natural attenuation of VC in organic rich environments. The exact reason for the lack of VC degradation in the presence of glucose or pyruvate is not known.

The potential of the TRW culture to survive and initiate VC metabolism with a minimal lag even after 5 months of starvation is noteworthy and practical. The recently isolated VC degrading strain MF1 and the *Mycobacterium* spp strain JS60 also display similar capabilities. Strain MF1 degraded VC after 15 days of lag following a starvation period of 24 days (Verce et al. 2000), while strain JS60 readily degraded VC after 7 days of starvation (Coleman et al. 2002a). On the other hand, *Mycobacterium aurum* L1 and *Nocardioides* spp strain JS 614 completely lost their ability to degrade VC after a brief VC starvation. This loss of activity was attributed to the accumulation of VC-epoxide or chloroacetaldehyde (Coleman et al. 2002a; Hartmans 1995; Hartmans & de Bont 1992). The exact mechanism by which the TRW culture could survive the VC starvation is not known. It is highly probable that the dead cells might have served as a source of carbon and nutrients for the sustenance of growth. In addition, the absence of apparent toxicity in the TRW culture due to the accumulation of VC-epoxide that was evident in other organisms such as *M. aurum* strain L1 following a brief starvation is remarkable and warrants additional studies to identify the mechanism of VC breakdown in the TRW culture.

Overall, the TRW culture is robust and is capable of degrading VC below the GC detection limit when tested over a wide range of dissolved 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. In addition, its ability to survive extended starvations and also be able to metabolize VC at low DO is important for intrinsic remediation and the development of cost-effective treatment methods.

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