

A 1,1,1-Trichloroethane-Degrading Anaerobic Mixed Microbial Culture Enhances Biotransformation of Mixtures of Chlorinated Ethenes and Ethanes[∇]

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1,1,1-Trichloroethane (1,1,1-TCA) is a common groundwater pollutant as a result of improper disposal and accidental spills. It is often found as a cocontaminant with trichloroethene (TCE) and inhibits some TCE-degrading microorganisms. 1,1,1-TCA removal is therefore required for effective bioremediation of sites contaminated with mixed chlorinated organics. This study characterized MS, a 1,1,1-TCA-degrading, anaerobic, mixed microbial culture derived from a 1,1,1-TCA-contaminated site in the northeastern United States. MS reductively dechlorinated 1,1,1-TCA to 1,1-dichloroethane (1,1-DCA) and then to monochloroethane (CA) but not further. Cloning of bacterial 16S rRNA genes revealed among other organisms the presence of a *Dehalobacter* sp. and a *Desulfovibrio* sp., which are both phylogenetically related to known dehalorespiring strains. Monitoring of these populations with species-specific quantitative PCR during degradation of 1,1,1-TCA and 1,1-DCA showed that *Dehalobacter* proliferated during dechlorination. *Dehalobacter* growth was dechlorination dependent, whereas *Desulfovibrio* growth was dechlorination independent. Experiments were also performed to test whether MS could enhance TCE degradation in the presence of inhibiting levels of 1,1,1-TCA. Dechlorination of *cis*-dichloroethene (cDCE) and vinyl chloride (VC) in KB-1, a chloroethene-degrading culture used for bioaugmentation, was inhibited with 1,1,1-TCA present. When KB-1 and MS were coinoculated, degradation of cDCE and VC to ethene proceeded as soon as the 1,1,1-TCA was dechlorinated to 1,1-DCA by MS. This demonstrated the potential application of the MS and KB-1 cultures for cobioaugmentation of sites cocontaminated with 1,1,1-TCA and TCE.

The heavy industrial use of trichloroethene (TCE) as a chlorinated solvent peaked in the early 1970s, when the compound was shown to have both adverse human and adverse environmental effects (12). Consequentially, a seemingly safer alternative, 1,1,1-trichloroethane (1,1,1-TCA), was widely adopted. While acute exposure to large doses of 1,1,1-TCA is known to depress the central nervous system, no link to cancer through chronic exposure has been demonstrated (4). However, 1,1,1-TCA is an ozone-depleting substance, so its manufacture and use have been phased out as of 1996 in accordance with the Montreal Protocol on Substances that Deplete the Ozone Layer (48). Nonetheless, due to a legacy of historical accidental spillage and improper disposal, 1,1,1-TCA is a common groundwater contaminant. In the United States, 1,1,1-TCA has been found as a contaminant in at least 459 sites (30% of total) designated to the National Priorities List (NPL) by the U.S. Environmental Protection Agency (data from a search of the NPL database in January 2006).

1,1,1-TCA undergoes both abiotic and biotic degradation under natural conditions. Abiotically, the compound is dehydrohalogenated to 1,1-dichloroethene (1,1-DCE) and acetic acid, with a half-life of >2.8 years (24, 50). Biological degradation by aerobic microorganisms can occur cometabolically, with the production of 2,2,2-trichloroethanol, trichloroacetic

acid, and dichloroacetic acid (26). However, because impacted groundwater is often anoxic, anaerobic degradation of 1,1,1-TCA has greater relevance for site remediation. The high density and low solubility of 1,1,1-TCA allow it to form dense non-aqueous-phase layers deep in the subsurface, making anaerobic conditions particularly important. Fortunately, several anaerobic microorganisms, including methanogens (e.g., references 1 and 10) and sulfate reducers (10), can reductively dechlorinate 1,1,1-TCA cometabolically, usually resulting in the partially dechlorinated daughter products 1,1-dichloroethane (1,1-DCA) and monochloroethane (CA). Several years ago, an organism (strain TCA1) phylogenetically related to the tetrachloroethene (PCE)- and TCE-respiring *Dehalobacter restrictus* that coupled 1,1,1-TCA and 1,1-DCA degradation to growth was isolated (45). It is not known how widespread 1,1,1-TCA-dehalorespiring organisms are, but these studies clearly illustrate the potential for using biological processes to remediate sites contaminated with 1,1,1-TCA.

While 1,1,1-TCA can be biologically degraded, it has also been observed to inhibit some anaerobic biological processes, including methanogenesis (2, 11, 43) and reductive dechlorination (17). Inhibition of reductive dechlorination is significant because many sites are contaminated by multiple chlorinated constituents, and it is often the more toxic compounds, such as PCE and TCE, that drive remedial efforts; bioremediation of chlorinated, ethene-contaminated sites has proven to be a successful technology (19), but its success can be limited by cocontaminants (32). The most prevalent inhibitory cocontaminants are chloroform and 1,1,1-TCA, both of which interfere

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with the complete detoxification of chlorinated ethenes (17). Given that 1,1,1-TCA and TCE are found as cocontaminants in at least 310 (20%) USEPA NPL sites (data from a search of the NPL database in May 2006), the inhibitory effects of 1,1,1-TCA are a real obstacle to bioremediation efforts.

In this study, a mixed anaerobic microbial culture that reductively dechlorinates 1,1,1-TCA to 1,1-DCA and CA is described. This culture, known as MS, was enriched from sediment derived from a 1,1,1-TCA-contaminated site in the northeastern United States. 16S rRNA gene cloning and quantitative PCR (qPCR) were used to identify the dechlorinating organism and to demonstrate that 1,1,1-TCA degradation occurs metabolically. The potential to overcome 1,1,1-TCA-mediated inhibition of reductive dechlorination of chlorinated ethenes by simultaneous bioaugmentation with the MS and KB-1 cultures was also investigated.

MATERIALS AND METHODS

Establishment of enrichment cultures and subcultures. Anaerobic microcosms were constructed in 2001 with groundwater and solids from a northeastern United States industrial area contaminated with high concentrations of 1,1,1-TCA (38 μ M) and TCE (8 μ M). Subsurface cores were collected from two locations in the saturated anoxic zone at a depth of approximately 30 feet. The cores consisted of fine and silty sand and were combined and homogenized prior to being dispensed into microcosm bottles. Microcosm preparation was conducted in a disposable glove bag under an N_2 headspace. Homogenized subsurface material (30 g) and site groundwater (100 ml) were anaerobically transferred to sterile bottles (250 ml), purged with N_2 - CO_2 (80%/20%) to remove volatile organic compounds, and then amended with a carbon source mixture of methanol, ethanol, acetate, and lactate (a mixture of equal electron equivalents of each compound, referred to as "MEAL") as the electron donor and 1,1,1-TCA (0.04 mM) as the electron acceptor. The original microcosms and subsequent enrichment cultures were maintained statically in an anaerobic chamber (Coy Laboratory Products, Madison, WI) filled with a CO_2 - H_2 - N_2 (10%/10%/80%) gas mixture. After dechlorination activity was observed (5 months), transfer cultures were prepared in defined anaerobic mineral medium containing vitamins (18). These transfer cultures were amended with 1,1,1-TCA at initial aqueous concentrations ranging from 0.08 to 0.20 mM. MEAL was provided to these cultures as an electron donor at concentrations ranging from 0.27 to 1.33 mM, each compound representing approximately 10 times the number of electron equivalents required for dechlorination. These amendments were calculated on the assumption that two electron equivalents of donor are required per dechlorination step and that methanol, ethanol, acetate, and lactate provide 6, 12, 8, and 12 electron equivalents per mol, respectively. When dechlorinating activity ceased, 20 to 50% of the culture was replaced with fresh mineral medium (12 times since 2001). This enrichment culture is referred to as MS.

To test whether hydrogen could act as a sole electron donor and to initiate the isolation of the 1,1,1-TCA-degrading microorganism, a subculture of MS, with hydrogen as the electron donor and 1,1,1-TCA (0.07 mM) as the electron acceptor, was established in 160-ml bottles containing 100 ml of mineral medium amended with 5 mM acetate and 6 ml of H_2 - CO_2 (80%/20%). Upon the degradation of two amendments of 1,1,1-TCA, a 5% transfer into a new bottle was made. Five transfers have been performed to date in this manner. This subculture is referred to as MS/ H_2 .

In some experiments, KB-1, a TCE-dechlorinating enrichment culture used commercially for bioaugmentation (SIREM, Guelph, ON, Canada), was also used. This culture has been maintained on TCE and methanol as an electron donor for 8 years (17) and is dominated by *Dehalococcoides* (16).

Substrate range study. The ability of the MS culture to degrade 1,2-dichloroethane (1,2-DCA), 1,1,2-trichloroethane (1,1,2-TCA), PCE, TCE, *cis*-dichloroethene (cDCE), and vinyl chloride (VC) over a 6-week period was tested. Duplicate screw-top vials (45 ml) with Mininert septa (VICI Precision Sampling, Baton Rouge, LA) were filled with 10 ml of mineral medium and 10 ml of MS culture. These were amended with each potential chlorinated electron acceptor at an aqueous concentration of 0.06 to 0.10 mM (10 mg/liter) and MEAL as an electron donor, with each MEAL component at a concentration representing approximately 10 times the number of electron equivalents required for dechlorination. Uninoculated controls were prepared for each electron acceptor.

The maximum concentration of 1,1,1-TCA that could be degraded by MS was determined by inoculating 5 ml mineral medium with 5 ml of MS culture in 17-ml screw-top vials with Mininert septa. Duplicate vials were amended with increasing volumes of a saturated aqueous 1,1,1-TCA solution (1,400 mg/liter), and MEAL was added as an electron donor, with each component at a concentration representing approximately 10 times the number of electron equivalents required for dechlorination.

16S rRNA gene cloning. To identify the bacterial populations present, bacterial 16S rRNA genes were cloned from MS following extraction of total genomic DNA, as previously described (25). Thirty-seven 16S rRNA gene clones were sequenced by the University Health Network Research DNA Sequencing Facility (Toronto, ON, Canada) with the primer 8f (38), and the closest sequence match was identified with a BLASTN search of GenBank (www.ncbi.nlm.nih.gov/blast).

Time course experiment. The growth of putative dechlorinating microorganisms during degradation of 1,1,1-TCA and 1,1-DCA was monitored. Screw-top bottles (250 ml) with Mininert septa were filled with either 160 ml (for 1,1,1-TCA treatments and no-electron-acceptor controls) or 120 ml (for 1,1-DCA treatments) mineral medium. Three bottles were amended with either neat 1,1,1-TCA (initial aqueous concentration of 0.19 mM) or neat 1,1-DCA (0.26 mM) and MEAL (each component at a concentration representing approximately 5 times the number of electron equivalents required for dechlorination, for a total of 20 times the number of electron equivalents). Other bottles were amended with MEAL only, as a no-electron-acceptor control. A 1.5% (vol/vol) inoculum of MS was added. Immediately after inoculation, 40 ml of culture was removed for DNA extraction (time point 0). For the 1,1,1-TCA treatments, DNA was subsequently extracted from 40-ml samples from all bottles when 1,1,1-TCA was completely degraded to 1,1-DCA (time point 1), when 1,1-DCA was 60% degraded to CA (time point 2), and when 1,1-DCA was fully degraded to CA (time point 3). Samples from the no-electron-acceptor bottles were taken at the same times as those from the 1,1,1-TCA treatment bottles. For the 1,1-DCA treatments, DNA was extracted when the 1,1-DCA was 60% degraded to CA (time point 1) and when 1,1-DCA was fully degraded to CA (time point 2). All bottles were reamended with MEAL (with a total of 5 times the number of electron equivalents) after the DNA extractions at time point 1 to ensure that the electron donor was not limiting.

For DNA extractions, culture was transferred to anaerobic conical centrifuge tubes (50 ml) (Fisher Scientific, Toronto, ON, Canada) and centrifuged at $2,300 \times g$ for 50 min at 4°C. The pellet was collected and DNA extracted with a MoBio UltraClean soil DNA kit according to the manufacturer's alternative protocol, except that the DNA was finally eluted with 5 mM Tris-HCl, pH 8.0. The copies of *Dehalobacter* and *Desulfovibrio* 16S rRNA genes in the extracted DNA were analyzed by quantitative PCR (see below).

The growth yield of each organism was determined by assuming 100% DNA extraction efficiency (16). Yield was calculated by first determining how many moles of the chlorinated compound (1,1,1-TCA or 1,1-DCA) were degraded between two time points, considering both liquid and headspace in the bottle and taking into account mass removed with DNA extraction. Changes in 16S rRNA gene copy number were calculated for the same time periods. A yield in units of 16S rRNA gene copies per mole of chlorinated compound degraded during a single reductive dechlorination step was then determined.

Coaugmentation studies. Coaugmentation studies were conducted to evaluate interactions between 1,1,1-TCA and TCE dechlorination because 1,1,1-TCA can inhibit methanogenesis (2, 11, 43) and TCE dechlorination (17). A comprehensive treatment matrix was prepared to compare the dechlorination of 1,1,1-TCA alone, TCE alone, or 1,1,1-TCA and TCE together and was inoculated with either MS, KB-1, or both MS and KB-1. Duplicate screw-top vials (45 ml) with Mininert septa were filled with 15 ml (for single-culture inoculation treatments) or 10 ml (for coinoculation treatments) of mineral medium and 5 ml each of MS and/or KB-1. Vials were amended with either 1,1,1-TCA (initial aqueous concentration of 0.30 mM), TCE (0.30 mM), or both (each at 0.30 mM). The MEAL electron donor mixture was added to provide approximately 20 times the number of electron equivalents required for dechlorination. Uninoculated controls were prepared for each electron acceptor. The vials were reamended with MEAL when dechlorination stalled.

This experiment was repeated using lower concentrations of 1,1,1-TCA (0.03 mM) and TCE (0.03 mM) and less inoculum (10% of each culture). Electron donor mixture concentrations were decreased correspondingly. Each treatment was prepared in triplicate.

Quantitative PCR. qPCR for enumerating *Dehalobacter* 16S rRNA gene copies was conducted as described previously (25), except that 20- μ l reaction mixtures were used, with each reaction mixture containing 10 μ l of SYBR green JumpStart *Taq* ReadyMix, 7.2 μ l of sterile water, 2 μ l of a DNA template, and 0.5 μ M each of the forward and reverse primers. *Desulfovibrio*-specific qPCR was

performed in the same manner by using primers DSB1180F (5'-CCTAGGGCTACACACGTAATAA-3') (22) and DSB1405R (5'-CCGGCTTCGGGTAATAA-3') with an annealing temperature of 61°C. Calibration was performed with serial dilutions of a known quantity of M13r-/T7f-amplified fragments of *Dehalobacter* or *Desulfovibrio* 16S rRNA gene-containing plasmids generated in the cloning study described above. The dynamic range for qPCR for both targeted 16S rRNA genes was 2×10^3 to 4×10^8 16S rRNA gene copies/reaction. DNA concentrations were determined by UV absorbance measurement (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE).

Analytical procedures. For culture maintenance and the time course experiment, chlorinated ethanes, ethenes, methane, and ethene were measured by injecting a 300- μ l-headspace sample onto a Hewlett-Packard 5890 Series II gas chromatograph (GC) fitted with a GSQ column (J&W Scientific), as previously described by Grostern et al. (25) for chlorinated ethanes and Duhamel et al. (17) for chlorinated ethenes. In coaugmentation experiments, the former GC method was used to analyze vials amended with 1,1,1-TCA only, while the latter GC method was used to analyze vials amended with TCE only. To analyze headspace samples for vials amended simultaneously with 1,1,1-TCA and TCE, the following method was used: the carrier gas pressure was initially 70 kPa, and the oven temperature was programmed to hold at 50°C for 90 s and then increase to 155°C at 30°C/min and then increase to 190°C at 4°C/min and hold for 2 min.

For substrate range experiments, chlorinated ethanes, ethenes, methane, and ethene were analyzed either in a headspace sample as described above or in a 1-ml liquid sample that was mixed with 5 ml of acidified water in a headspace vial (10 ml), using an HP 7694 headspace sampler (Hewlett Packard, Mississauga, ON, Canada) connected to an HP 5890A gas chromatograph (Hewlett Packard) fitted with a GSQ column and a flame ionization detector, as described previously (25).

Nucleotide sequence accession numbers. The cloned *Dehalobacter* and *Desulfovibrio* sequences identified in these cultures were deposited in GenBank with the following accession numbers: for the *Dehalobacter* sp. in MS, DQ663785; for the *Desulfovibrio* sp. in MS, DQ663786.

RESULTS

Culture development and characterization. Microcosms established with aquifer material and groundwater and amended with MEAL demonstrated dechlorination of 1,1,1-TCA to 1,1-DCA after 70 days and further dechlorination to CA after 130 days incubation. No degradation of CA was observed. Methanogenesis was absent in the presence of 1,1,1-TCA but commenced once 1,1,1-TCA was depleted and only 1,1-DCA remained. Active microcosms were successively transferred (12 transfers to date) into defined mineral medium, and the rate of dechlorination progressively increased from about 0.6 to over 13 μ mol/liter/day.

To identify the most abundant species as well as possible dechlorinating organisms in the enriched cultures, bacterial 16S rRNA gene fragments were cloned with general bacterial primers 4 years after the initial microcosms were established. Only bacterial rRNA genes were targeted because our goal was to investigate organisms that degrade the chlorinated compounds through dehalorespiration, which is energy yielding and growth supporting. To date, no archaeal species has been shown to respire with chlorinated compounds, although cometabolic dechlorination has been observed. The closest GenBank matches for the partially sequenced clones included *Dehalobacter* (10 clones), *Clostridium* (10 clones), *Desulfovibrio* (6 clones), *Sedimentibacter* (1 clone), *Spirochaete* (1 clone), and clones distantly related to *Aminomonas* (1 clone), *Oscillospira* (2 clones), and *Streptomyces* (1 clone). One operational taxonomic unit (five clones) could not be related to any cultured microorganisms. The *Dehalobacter* and *Desulfovibrio* sequences, in addition to being phylogenetically related to known dechlorinators, were among the most abundant in the clone library. The *Dehalobacter* sequence had 1,374/1,384 bp identity

(99.3%) to *Dehalobacter restrictus* (27) and 1,353/1,384 bp identity (97.8%) to *Dehalobacter* strain TCA1 (45), while the *Desulfovibrio* sequence had 1,163/1,303 bp identity (89.3%) to *Desulfovibrio* strain 2BP-48 (21). Subsequent analysis focused on *Dehalobacter* as the putative dechlorinating organism and *Desulfovibrio* as a control for nondechlorinating activity.

Substrate range studies were performed on the culture to test its ability to degrade select commercial chlorinated ethanes and ethenes. Under the conditions tested, dechlorination of PCE, TCE, cDCE, VC, and 1,2-DCA was not observed in 40 days. However, 1,1,2-TCA was stoichiometrically transformed to VC via dihaloelimination in 8 days (data not shown). Concentrations of up to 1.5 mM 1,1,1-TCA (200 mg/liter) were completely dechlorinated to CA. Between 1.5 mM and 2.2 mM (300 mg/liter) 1,1,1-TCA, dechlorination proceeded only as far as 1,1-DCA during the 2-month observation period. Dechlorination was completely inhibited above 2.2 mM 1,1,1-TCA.

A common characteristic of many dehalorespiring bacteria is their ability to use dihydrogen as an electron donor. Therefore, the ability of H₂ to support reductive dechlorination in an MS subculture was tested. Transfer cultures sustained dechlorination of 1,1,1-TCA upon successive transfers when amended with only acetate as a carbon source and H₂ as an electron donor. Methanogenic activity was no longer observed after two transfers. However, after three transfers, the ability to reductively dechlorinate 1,1-DCA to CA was lost, despite the addition of more electron donors and prolonged incubation (data not shown).

1,1,1-TCA and 1,1-DCA degradation time course experiments. The potential for growth-linked dechlorinating activity by the *Dehalobacter* and *Desulfovibrio* populations identified in the clone library was investigated with a degradation time course experiment. Vials amended with MEAL as an electron donor and either 1,1,1-TCA or 1,1-DCA as an electron acceptor were inoculated with MS culture; no-electron-acceptor controls were also prepared. The *Dehalobacter* and *Desulfovibrio* populations were monitored during dechlorination by analyzing them for the presence of each organism with species-specific qPCR following DNA extraction. No degradation of the tested chlorinated compounds was observed in uninoculated controls.

In the 1,1,1-TCA-amended bottles, degradation of 1,1,1-TCA occurred sequentially. First, 1,1,1-TCA was reductively dechlorinated with no lag to 1,1-DCA in 10 days (Fig. 1A). At this point, DNA was extracted from an aliquot of the 1,1,1-TCA and no-electron-acceptor treatment bottles. 1,1-DCA was reductively dechlorinated to CA in a further 14 days; DNA was extracted during mid-1,1-DCA degradation and when 1,1-DCA degradation was completed. Mass decreases immediately following sampling time points (Fig. 1) were due to sample removal for DNA extraction. Methanogenesis did not occur while 1,1,1-TCA was present, although it commenced during 1,1-DCA degradation. Dechlorination in the 1,1-DCA treatment bottles started with no lag and was complete in 12 days (Fig. 1B). Methanogenesis occurred throughout 1,1-DCA degradation.

qPCR monitoring of the *Dehalobacter* population during dechlorination revealed that growth occurred, as shown by a 10-fold increase in 16S rRNA gene copies, during both 1,1,1-TCA and 1,1-DCA degradation in the 1,1,1-TCA amendment

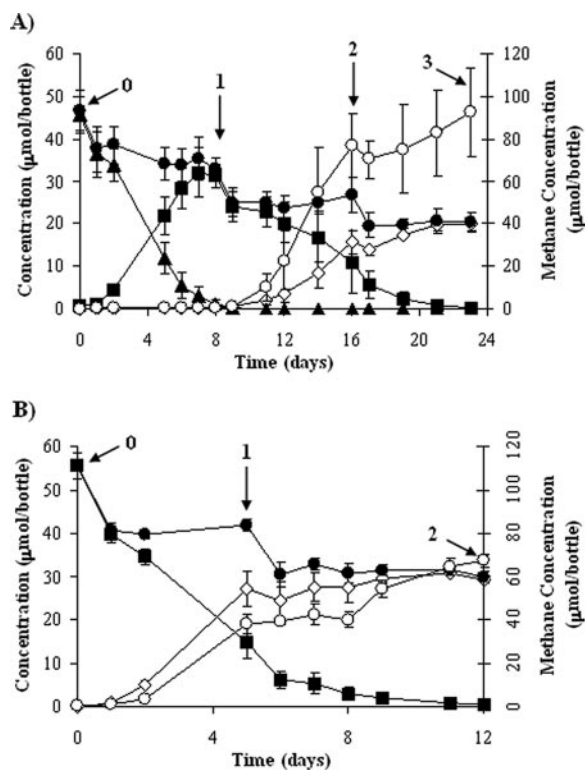


FIG. 1. Degradation profiles for 1,1,1-TCA and 1,1-DCA time course experiments. (A) Degradation in 1,1,1-TCA-amended cultures. (B) Degradation in 1,1-DCA-amended cultures. Closed triangles, 1,1,1-TCA; squares, 1,1-DCA; open diamonds, CA; closed circles, total chlorinated ethanes; open circles, methane. Numbers indicate the approximate time points for DNA extraction in the respective experiments (see the text). Each curve shows the mean value for three bottles. Error bars show the standard deviations for three bottles.

treatment bottles (Fig. 2). Growth during 1,1-DCA degradation was confirmed for the 1,1-DCA amendment treatments (Fig. 2). No proliferation of *Dehalobacter* was seen in the no-electron-acceptor treatment bottles, indicating that chlorinated compounds can be used as electron acceptors for growth by this population. These results agree with previous findings for *Dehalobacter* strains (27, 45, 49, 53).

qPCR monitoring of the *Desulfovibrio* population revealed growth in all treatment bottles (Fig. 2), including the no-electron-acceptor control. Hence, this population is not strictly dependent on a chlorinated compound as an electron acceptor for growth. However, in the 1,1,1-TCA and 1,1-DCA treatment bottles, overall increases in 16S rRNA gene copies for *Desulfovibrio* were considerably less ($\sim 1,600$ -fold) than those for *Dehalobacter*. Additionally, the absolute concentration of *Desulfovibrio* was 3 orders of magnitude lower than that for *Dehalobacter* in all treatment bottles. Therefore, the *Desulfovibrio* population's direct involvement in dechlorination, if any, was likely minor compared to the energy-yielding dehalorespiration performed by *Dehalobacter* in this experiment.

Assuming that most of the dechlorination was performed by *Dehalobacter*, growth yields for 1,1,1-TCA and 1,1-DCA could be determined by calculating the moles of the electron acceptor dechlorinated between the extraction time points, taking into account mass lost due to DNA sampling. The yields for

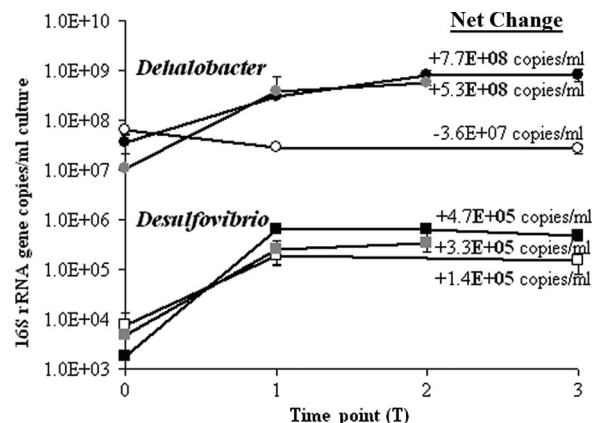


FIG. 2. *Dehalobacter* and *Desulfovibrio* growth during 1,1,1-TCA and 1,1-DCA degradation. Circles represent *Dehalobacter*; squares represent *Desulfovibrio*. Open symbols represent controls (amended with 1,1,1-TCA); black symbols represent bottles amended with 1,1-DCA. 16S rRNA gene copy values are mean values for three bottles and are expressed as numbers of copies per ml of culture. Numbers on the right represent net increases (+) or decreases (–) in 16S rRNA gene copies/ml from the start to the end of the experiment for each target organism in each treatment bottle. Error bars show the standard deviations for three bottles.

Dehalobacter during dechlorination were $(9.4 \pm 1.7) \times 10^8$ 16S rRNA gene copies/ μmol 1,1,1-TCA degraded to 1,1-DCA ($n = 3$) and $(2.4 \pm 1.2) \times 10^9$ 16S rRNA gene copies/ μmol 1,1-DCA degraded to CA ($n = 6$). Sun et al. (45) determined a yield of 5.60 g of cells (dry weight) per mol of Cl^- evolved for *Dehalobacter* strain TCA1. Given the cell dimensions of strain TCA1 (a short rod with a diameter of 0.4 to 0.6 μm and a length of 1.0 to 2.0 μm) reported by Sun et al. (45), approximating the cell shape to that of a cylinder, and assuming that cell density is equal to that of water and that a cell is 80% water, a yield of 8.10×10^7 cells/ μmol of Cl^- evolved was calculated. For the MS culture, an average yield of $(1.4 \pm 0.3) \times 10^9$ cells/ μmol of Cl^- evolved was calculated for the 1,1,1-TCA-to-CA experiment, assuming that the number of 16S rRNA gene copies in the *Dehalobacter* genome is one. These yields can also be compared to results from our previous study (25) on a *Dehalobacter*-containing culture that degraded 1,1,2-TCA (Table 1). The yield for the *Dehalobacter* described herein is highest on a comparable per-electron-equivalent-reduced basis and is similar to the yields obtained for a mixed culture dechlorinating TCE at a high rate.

Coaggregation studies. The potential for the MS culture to relieve 1,1,1-TCA inhibition of TCE degradation by the KB-1 culture was investigated. Replicate vials were amended with 1,1,1-TCA, TCE, or 1,1,1-TCA and TCE and were inoculated with MS, TCE-grown KB-1, or MS and KB-1, with MEAL as the electron donor mixture. This experiment was performed twice, with different starting concentrations for the chlorinated compounds and different strengths of inoculum. In the first experiment, a 25% inoculum was used, and the targeted concentrations were 0.30 mM (40 mg/liter) for each of the chlorinated compounds. No degradation was observed in the uninoculated controls. While complete degradation of 1,1,1-TCA to 1,1-DCA and then to CA was observed in 8 days

TABLE 1. Comparison of yields for selected *Dehalobacter* and *Dehalococcoides* spp. during dehalorespiration

Organism group (culture) ^a	Process ^b	$\Delta G^{\circ c}$	Yield ^d	Source or reference
<i>Dehalobacter</i> sp. (MS)	1,1,1-TCA→DCA→CA	-115.8	$(7.0 \pm 1.5) \times 10^8$	This study
<i>Dehalococcoides</i> sp. (KB-1/VC)	VC→ethene	-75.0	$(2.8 \pm 0.7) \times 10^8$	16
<i>Dehalobacter</i> sp. (WL)	1,1,2-TCA→VC	-103.1	$(1.5 \pm 1.4) \times 10^8$	25
<i>Dehalococcoides</i> sp. (WL)	VC→ethene	-75.0	$(4.4 \pm 1.3) \times 10^7$	25
<i>Dehalobacter</i> strain TCA1	1,1,1-TCA→DCA→CA	-115.8	4.0×10^{7e}	45

^a Except for *Dehalobacter* strain TCA1, all organisms were in mixed cultures.

^b Each dechlorination step (indicated by →) requires two electron equivalents. Except for 1,1,2-TCA→VC, all indicated processes result in release of one Cl⁻ ion per dechlorination step.

^c Gibbs free energies of formation based on the work of Dolfig and Janssen (13). Values are kJ/electron equivalents for overall reactions, with H₂ used as an electron donor.

^d Number of 16S rRNA gene copies/ μ mol electron equivalents reduced.

^e Assuming that the genome of *Dehalobacter* strain TCA1 has one copy of the 16S rRNA gene.

in the MS-inoculated vials, no degradation by-products were detected in 1,1,1-TCA-amended vials that were inoculated with KB-1, confirming this culture's inability to degrade this compound (data not shown). In TCE-amended vials inocu-

lated with KB-1, complete dechlorination of TCE to ethene occurred in 20 days, whereas in the MS-inoculated vials, TCE was not degraded.

Vials amended simultaneously with 1,1,1-TCA and TCE showed more-complex degradation patterns. In vials inoculated with MS only, 1,1,1-TCA was slowly dechlorinated to 1,1-DCA (93% in 90 days) in the presence of TCE, and no further dechlorination to CA was observed (Fig. 3A). This indicates that 1,1,1-TCA degradation and, in particular, 1,1-DCA degradation by the MS culture were inhibited by TCE. Conversely, in vials inoculated with KB-1 only, no degradation of 1,1,1-TCA was observed, while TCE dechlorination stalled at cDCE and VC; no ethene was produced, even when an electron donor was provided (Fig. 3B). These results were consistent with the previous finding for KB-1 (17) that 1,1,1-TCA inhibits the degradation of TCE daughter products rather than the reductive dechlorination of TCE itself.

In vials amended with both MS and KB-1, dechlorination of TCE past VC did not occur until 1,1,1-TCA was fully converted to 1,1-DCA (Fig. 3C). With the 1,1,1-TCA converted, degradation of the chlorinated ethenes to ethene proceeded quickly, but degradation of 1,1-DCA stalled. After the chlorinated ethenes were fully converted to ethene, dechlorination of 1,1-DCA to CA eventually proceeded. The benefit of coaugmentation is most vividly illustrated in Fig. 4, which pre-

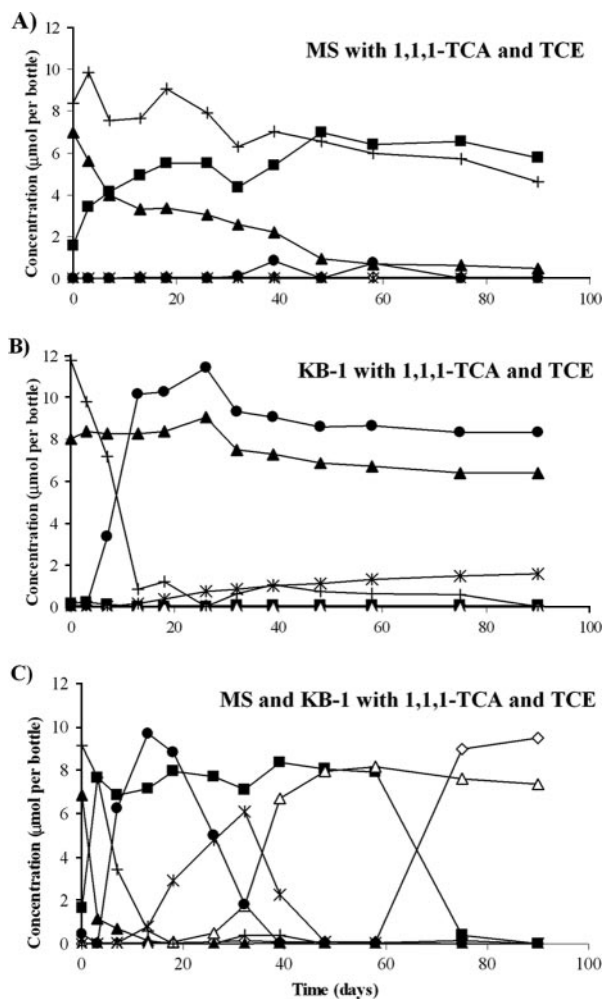


FIG. 3. Representative degradation profiles of 1,1,1-TCA- and TCE-amended vials in the coaugmentation study. (A) Vials inoculated with MS only. (B) Vials inoculated with KB-1 only. (C) Vials inoculated with both MS and KB-1. Closed triangles, 1,1,1-TCA; squares, 1,1-DCA; open diamonds, CA; crosses, TCE; circles, cDCE; asterisks, VC; open triangles, ethene.

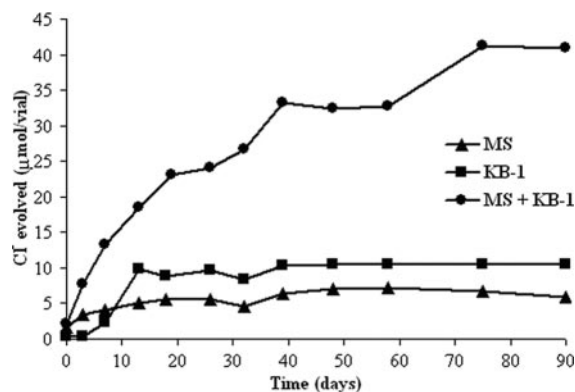


FIG. 4. Representative cumulative chloride production in 1,1,1-TCA- and TCE-amended vials from the coaugmentation study. Triangles represent vials inoculated with MS only. Squares represent vials inoculated with KB-1 only. Circles represent vials inoculated with both MS and KB-1.

sents the data for the cumulative chloride evolved (as calculated from the data for the disappearance of all chlorinated substrates) with the different inocula.

To investigate whether these results were concentration dependent, this experiment was repeated with a lower inoculum (10%) of each culture and significantly lower concentrations (0.03 mM) of 1,1,1-TCA and TCE (4 mg/liter and 5 mg/liter, respectively). Degradation and inhibition patterns were very similar to what was observed with the higher starting concentrations: in the 1,1,1-TCA- and TCE-amended vials inoculated with both MS and KB-1, chlorinated ethene degradation again stalled at cDCE and VC until the 1,1,1-TCA was fully converted to 1,1-DCA, and 1,1-DCA degradation did not proceed until after the chlorinated ethenes were fully converted to ethene. These results confirmed that the observed degradation and inhibition patterns were not concentration dependent.

DISCUSSION

Recent literature focusing on the biodegradation of 1,1,1-TCA is sparse relative to that devoted to the degradation of the chlorinated ethenes. Whereas at least 25 chloroethene-respiring anaerobic isolates (in the genera *Desulfomonile*, *Desulfuromonas*, *Dehalococcoides*, *Dehalobacter*, *Desulfitobacterium*, *Sulfospirillum*, and *Geobacter*) have been reported, only a single 1,1,1-TCA-respiring anaerobic bacterium has been isolated. Although this research bias may be due to the higher human toxicity of chlorinated ethenes, the widespread contamination of groundwater by 1,1,1-TCA warrants increased examination of the microorganisms and conditions required for its removal. This is especially important given that 1,1,1-TCA is a common cocontaminant with TCE and that 1,1,1-TCA can inhibit chlorinated ethene-degrading organisms, resulting in the undesirable accumulation of vinyl chloride.

The lack of reported 1,1,1-TCA-enriched anaerobic cultures makes it difficult to compare the degradation characteristics of the MS culture to those of other cultures. Adamson and Parkin (2) showed that a lactate- and PCE-enriched culture could transform 20 μM 1,1,1-TCA to 1,1-DCA and partially to CA in less than a day, despite no prior exposure; higher concentrations of 1,1,1-TCA were not tested. Similarly, a lactate-enriched methanogenic culture derived from digested sludge could degrade 2 μM 1,1,1-TCA cometabolically, but again, higher concentrations were not tested (23). *Dehalobacter* strain TCA1 could completely dechlorinate 450 μM (60 mg/liter) 1,1,1-TCA to CA in 5 weeks (45); no degradation characteristics have been reported for the mixed culture from which this isolate was obtained. Other studies (8, 42, 43, 54) have reported the degradation of significant amounts of 1,1,1-TCA, but these concentrations were less than those tested in the present study. Therefore, while the current study represents the highest reported 1,1,1-TCA concentration degraded, little data exist for comparison.

A culture-independent approach was used to identify the putative active dechlorinating organism(s) in MS. Organisms related to *Dehalobacter* and *Desulfovibrio* were identified by bacterial 16S rRNA gene cloning, and because of their phylogenetic relationship to known dehalorespiring organisms, their growth was tracked during dechlorination of 1,1,1-TCA and 1,1-DCA. Growth of the *Dehalobacter* sp. in MS was found to

be dependent on a chlorinated substrate, consistent with the growth requirements of other *Dehalobacter* strains (5, 25, 27, 45, 53). Several reported *Desulfovibrio* strains have the ability to reductively dehalogenate brominated or chlorinated compounds, and this occurs through either a cometabolic (5) or a dehalorespiratory (6, 21, 44) process. Given that the *Desulfovibrio* strain in MS grew in the absence of 1,1,1-TCA and 1,1-DCA, this strain is not dependent on a halogenated substrate for growth; rather, it was likely growing fermentatively on the ample electron donors provided. Growth in the presence of the chlorinated compounds was slightly higher than that in the absence of these compounds, presumably as a result of a syntrophic relationship between *Desulfovibrio* and *Dehalobacter*, mediated by interspecies H_2 transfer, similar to that seen between *Desulfitobacterium frappieri* TCE1 and *Desulfovibrio fructosivorans* (14).

The narrowness of the substrate range observed for the MS culture is analogous to that observed for *Dehalobacter* isolates (27, 45, 53) and other reported *Dehalobacter*-containing enrichment cultures (25, 30, 40, 49, 51). For example, the only tested chlorinated compounds that *Dehalobacter* strain TCA1 can dehalorespire are 1,1,1-TCA and 1,1-DCA (45), while *Dehalobacter restrictus* strains PER-K23 and TEA can dehalorespire only PCE and TCE to cDCE (27, 53). Therefore, while it is known that *Dehalobacter* has a narrow electron donor range (hence the species name "*restrictus*"), it seems that a restricted chlorinated electron acceptor range is also a general property of this genus. A determining factor may be the presence of small numbers of narrow-substrate-range reductive dehalogenase (RDase) genes in the *Dehalobacter* genomes. A PCR-based study by Regard et al. (37) identified four putative or verified RDase genes in *Dehalobacter restrictus* PER-K23 genomic DNA. While the genome of this organism has not been sequenced, and thus the total number of putative RDase genes is unknown, this number of RDase genes can be compared to that found in recently sequenced genomes of *Dehalococcoides* and *Desulfitobacterium* species. *Dehalococcoides ethenogenes* strain 195 (41) and *Dehalococcoides* strain CBDB1 (31) have an astounding 17 and 32 putative RDase genes, respectively, and the organisms have been shown to degrade at least 25 (20, 33–35) and 16 (3, 7, 28, 29) chlorinated compounds, respectively. *Desulfitobacterium hafniense* strains Y51 (36) and DCB-2 (data from the *D. hafniense* DCB-2 whole-genome shotgun project; GenBank accession number AAW00000000) have two and nine putative RDase genes, respectively, and are known to degrade seven (47) and two (9) chlorinated compounds, respectively. Evidently, the *D. restrictus* PER-K23 putative RDase gene number is lower than those of many dechlorinating organisms studied, which correlates with its narrow chlorinated-substrate range.

The loss of 1,1-DCA-degrading ability in the MS/ H_2 transfers indicates that the dechlorination processes of 1,1,1-TCA and 1,1-DCA differ; this may be at either the organism or the enzyme level. While this study demonstrated growth of *Dehalobacter* during dechlorination of both 1,1-DCA and 1,1,1-TCA, it is possible that actually two or more distinct strains of *Dehalobacter* possessing near-identical 16S rRNA genes were present in the enrichment culture. Their growths would not be distinguished with the *Dehalobacter*-specific qPCR primers used here. Although the presence of multiple *Dehalobacter*

strains in mixed culture has not been reported, similar occurrences have been observed in chlorinated ethene-degrading cultures containing multiple strains of *Dehalococcoides* with identical or near-identical 16S rRNA genes (16, 39, 46, 52). In these cases, differentiation of the strains present came either by isolation or through identification of reductive dehalogenase genes whose population copy numbers were significantly different from the total *Dehalococcoides* 16S rRNA gene copy number. Alternatively, the loss of 1,1-DCA-dechlorinating ability may be due to different RDases acting on 1,1,1-TCA and 1,1-DCA, where a cofactor required for active 1,1-DCA RDase is not produced under the H₂-acetate conditions, either by the *Dehalobacter* sp. or by another organism in the mixed culture. A dependence on another organism for unknown factors influencing dechlorination has been previously reported by van Doesburg et al. (49), who observed that a *Dehalobacter* sp. could not degrade β-hexachlorocyclohexane in the absence of a *Sedimentibacter* sp.

The coaugmentation experiments described herein successfully demonstrated that if a microbial culture's ability to degrade a chlorinated compound is inhibited by the presence of a cocontaminant, that ability can be restored by removing the inhibiting cocontaminant via bioaugmentation with a second microbial culture. While the ideal situation for bioremediation involves the employment of a culture with a broad substrate range in order to degrade several cocontaminants simultaneously or sequentially, the reality is that a broad substrate range is a characteristic of highly complex microbial communities fed complex substrates (e.g., activated sludge), where many degrading populations are present. However, to obtain the large, consistent volumes of highly active culture required for the broad application of bioaugmentation for remediation of chloroorganic compounds, high-throughput culturing techniques that inevitably result in enrichment and consequently narrower substrate ranges must be employed. Therefore, the problem of degradation-inhibiting cocontaminants may necessitate bioaugmentation with two or more specialized microbial cultures. This study has shown that this strategy can, in principle, be successful.

These experiments helped to refine our understanding of the effects of complex mixtures of chlorinated compounds on the MS and KB-1 cultures. KB-1 is an ideal culture for these experiments not only because of its ability to fully degrade TCE but also because it can degrade 1,1-DCE (17), the abiotic breakdown product of 1,1,1-TCA. It was shown that the ability of KB-1 to reductively dechlorinate TCE to cDCE is not affected by 1,1,1-TCA but that subsequent degradation to VC and ethene is strongly, but reversibly, inhibited. KB-1 contains multiple dechlorinating organisms, including a *Geobacter* strain and at least two distinct *Dehalococcoides* strains (15, 17a). *Geobacter* grows during the reductive dechlorination of TCE to cDCE, but *Dehalococcoides* is likely responsible for subsequent degradation to ethene (15). Therefore, the stall at cDCE seen in the present study when KB-1 was exposed to both TCE and 1,1,1-TCA was due to direct or indirect inhibition of the *Dehalococcoides* populations in KB-1.

The inhibitory effect of TCE and its chlorinated degradation products on the degradation of 1,1,1-TCA and especially 1,1-DCA by the MS culture was an unexpected finding. These results revealed that a strategy consisting of first bioaugment-

ing a TCE-/1,1,1-TCA-cocontaminated site with MS to remove 1,1,1-TCA and then following with the application of KB-1 to remove the chlorinated ethenes could fail because these chlorinated ethenes may interfere with 1,1,1-TCA degradation. However, the inverse strategy, bioaugmenting the site with KB-1 to degrade TCE to cDCE, followed by bioaugmentation with MS to degrade 1,1,1-TCA, could result in remediation of the site. Additionally, the high chloroorganic concentrations treated in these experiments indicate that these cultures could be active in and near source zones, where high concentrations of the chlorinated compounds exist. Therefore, this study has contributed to a more informed application of these cultures for the bioremediation of chloroethane- and chloroethene-contaminated sites.

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