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26 July 2013

Alan Seech, Ph.D
Director - Soil & Groundwater Remediation
FMC Environmental Solutions

via email: Alan.Seech@fmc.com

Reference: Use and Safety Information for KB-1® Bioaugmentation Culture

Dear Dr. Seech:

Further to your request SiREM is pleased to the following information related to product use and safety of SiREM's bioaugmentation culture KB-1®.

This document also contains the following attachments for your reference:

Attachment A: Information Regarding the Initial Isolation of KB-1®
Attachment B: Locations of KB-1® Bioaugmentation
Attachment C: Selected Peer Reviewed KB-1® Publications
Attachment D: Pathogen Screening Methods and Data for KB-1®
Attachment E: Health Canada Biosafety Safety Level 1 Designation Letter for KB-1®
Attachment F: Data from Ecotoxicity Testing of KB-1® on Frogs
Attachment G: Photographs of KB-1® Vessels and Shipping Overpacks

1. Introduction to KB-1®

KB-1® is a natural, non-pathogenic, anaerobic microbial consortium that has been proven to rapidly and completely degrade chlorinated solvents such as tetrachloroethene (PCE), trichloroethene (TCE), cis-1,2-dichloroethene (cDCE), 1,1-dichloroethene (1,1-DCE) and vinyl chloride (VC) and 1,2-dichloroethane (1,2-DCA) to non-toxic, environmentally-acceptable end products such as ethene. The KB-1® culture has not been genetically modified in any manner and was derived from naturally occurring bacterial populations found in soil and groundwater impacted by TCE. Details regarding the isolation and enrichment of KB-1® are included in Attachment A. Laboratory and fieldwork with KB-1® for more than 10 years has demonstrated it to be a robust, stable and predictable consortium. Microbial analyses have been conducted to characterize the major components of the KB-1® microbial bioaugmentation at a site in consortium including molecular cloning, microbial plate counts and denaturing gradient gel electrophoresis (DGGE). DGGE, a molecular genetic technique that is used to characterize mixed microbial communities, is performed on a quarterly basis to ensure a stable microbial structure, and to identify (through 16S rRNA gene sequences) the predominant organisms in the microbial consortium. The organisms detected in KB-1® are predominantly dechlorinating *Dehalococcoides* organisms, a member of the iron reducing group *Geobacter* and methanogens

belonging to the to the genera *Methanomethylovorans*. Together these three microbial groups comprise approximately 98% of the microbes found in KB-1®. *Dehalococcoides* species have been detected at numerous sites across the United States and Europe, indicating that these bacteria are widespread but not ubiquitous. *Dehalococcoides, Geobacter, Methanomethylovorans* or the trace microorganisms in KB-1® are not expected to represent human health hazards.

To date, more than 300 sites in 38 US States as well as in the countries of Denmark, Germany, Hungary, Sweden, Canada (Ontario, British Columbia) and the United Kingdom and Malaysia have received regulatory approvals and have been injected with KB-1®. US States where KB-1® has been applied include: Alaska, California, Colorado, Connecticut, , Delaware, Florida, Georgia, Kansas, Kentucky, Indiana, Illinois, Maine, Massachusetts, Maryland, Michigan, New Jersey, New York, North Carolina, Oregon, Ohio, Pennsylvania, South Carolina, South Dakota, Texas, Washington, West Virginia, Wisconsin, Wyoming, Vermont and Virginia. A map providing an overview of KB-1® applications is provided in Attachment B. A selection of peer reviewed technical papers documenting the use and characterization of KB-1® is provided in Attachment C.

2. KB-1® Production Methods and Quality Control

KB-1® is produced in SiREM's laboratory facility under sterile conditions following stringent quality assurance/quality control (QA/QC) procedures including steam in place sterilization of fermenters. These procedures ensure that each lot of KB-1® shipped to an application site is consistent in terms of microbial content, titer of *Dehalococcoides* and degradation performance. The KB-1® culture is screened annually for pathogens. A copy of pathogen test results (March 2013) indicating negative results for all tested pathogens and methods are included in Attachment D. Since the commencement of large scale production of KB-1® in 2002, pathogen test results have consistently been negative for *Salmonella* sp., *Listeria monocytogenes*, *Vibrio* sp., *Campylobacter* sp., *Clostridium* sp., *Bacillus anthracis*, *Pseudomonas aeruginosa*, *Yersinia* sp., Pathogenic Yeast and Mold (includes *Candida albicans*, *Aspergillus fumigatus*), fecal Streptococci, fecal coliforms and Enterococci. The absence of fecal related organisms (fecal coliforms, fecal streptococci) makes it unlikely that other fecal related diseases (viruses, etc.) are present in the culture.

3. Environment Canada/Health Canada Assessments of KB-1®

As part of Canadian regulatory compliance requirements, Health Canada conducted a Biosafety Assessment of KB-1® to define the biosafety level (i.e., pathogencity level) of the culture. All organisms identified in KB-1® were designated by Health Canada as risk group 1 (the safest rating in a four level scale) which is defined as non-pathogenic to healthy humans. In a letter dated 14 January 2004 (Attachment E) Health Canada states that:

"All of the microbial components of the KB-1® preparation are considered risk group 1 organisms (non-pathogenic to healthy humans) and you have tested the preparation to confirm the absence of various pathogenic bacteria, yeast and molds…. "

In a further Canadian regulatory assessment, KB-1® was added to Environment Canada's Domestic Substances List (DSL) for use in groundwater remediation in Canada on August 20, 2008 (http://www.ec.gc.ca/subsnouvelles-newsubs/default.asp?lang=En&n=C4E09AE7-1).

The regulatory approval for use of KB-1® in Canada was received as a result of a very detailed multi-year effort by SiREM and the University of Toronto to demonstrate the stability, safety and effectiveness of KB-1® under the Federal governments New Substances Notification program The information required by Environmental Canada/ Health Canada for review included:

- Its identification of microorganisms in the KB-1® consortium and the information substantiating their identification including analysis of stability using DGGE, clone reference ladders and quantitative polymerase chain reaction (qPCR) analysis.
- The history of the KB-1® consortium including microcosm construction and incubation, and initial enrichment of KB-1®.
- A description of the methods used to distinguish and detect the KB-1® consortium.
- A description of the biological and ecological characteristics of the KB-1® consortium including its life cycle, infectivity, pathogenicity to non-human species, toxicity and toxigenicity, evidence for absence of ecotoxicity, its involvement in biogeochemical cycling, the conditions required for, and conditions that limit, its survival, growth and replication, and the mechanisms of its dispersal and the modes of interaction with any dispersal agents.
- A description of the mode of action in relation to the intended use.
- The dispersal by gene transfer of traits of pathogenicity to non-human species, toxigenicity and resistance to antibiotics, including a description the genetic basis for pathogenicity to non-human species, toxigenicity and resistance to antibiotics, capability to transfer genes, and the conditions that might select for dispersal of traits of pathogenicity to non-human species, toxigenicity and resistance to antibiotics, and whether the conditions are likely to exist at the locations of introduction or within the range of dispersal of the microorganisms.

4. Ecotoxicity Testing of KB-1®

KB-1® has been subject to testing by Environment Canada for potential ecotoxicity effects on frogs in a model aquifer system (McDaniel et al., 2006; provided in Attachment F). The study compared the ecotoxicological and pathogenic effects of effluents from KB-1® bioaugmented zones to non-bioaugmented zones. The study concluded:

"The results presented in this paper suggest that bioremediation using the addition of a bacterial consortium known to degrade chlorinated solvents, specifically KB-1®, has little or no impact on amphibian growth, development, or survivorship and was not pathogenic".

5. KB-1® Handling

While KB-1® is considered to be non-pathogenic, SiREM has developed specific safe-handling procedures for the application of KB-1® that limit the potential for exposure of personnel to KB-1®. Specifically, the culture is transported to application sites in stainless steel vessels that are contained in over-packs that act as secondary containment and protects the vessels. The injection process is performed by trained personnel to oversee the delivery of the vessels to the site, perform the injection and oversee the return of the vessels to SiREM. Photographs of the delivery vessel, the over-pack and the fittings used to pressurize the vessel with argon gas for groundwater injection are included in Attachment G. Injection under argon gas is necessary to prevent exposure of the organisms in KB-1® to oxygen, as exposure to oxygen kills the vast majority of organisms in KB-1®.

6. Summary

KB-1® has a history of safe use having been approved for use and applied safely at more than 300 Sites in the United States (38 States) and Canada, Europe (England, Denmark Hungary, Germany and Sweden) Malaysia. KB-1® was recently approved for import into Australia.

Phylogenetic analysis of KB-1® indicates that the microorganisms in the consortium consists predominantly of microorganisms commonly found inhabiting subsurface environments, including many drinking water aquifers. The KB-1® microbial consortium has not been genetically modified in any manner; it is an enrichment derived from naturally occurring bacteria found in soil and groundwater. Ongoing microbial screening has confirmed that KB-1® does not contain a number of common human pathogens and the culture has been proven to have no adverse effect on frogs.

Introduction of KB-1® to subsurface environments is not expected to significantly alter subsurface microbial conditions, but to supplement the natural microbiota with bacteria, particularly *Dehalococcoides,* that promote the rapid and complete dechlorination of PCE and TCE to ethene. Following degradation of the chlorinated solvents, these *Dehalococcoides* bacteria (which respire only using chlorinated solvents) are expected to become inactive. Furthermore, the sensitivity of KB-1® to oxygen generally limits its spread within aerobic systems.

For field demonstrations/applications, the fate of the KB-1® can be tracked using 16S rRNA-based and/or vinyl chloride reductase (*vcrA*) based PCR detection methods (e.g., Gene-Trac® Tests) to confirm and monitor its distribution, transport, survival and attenuation at the subject site.

If you require additional details regarding the use and safety of KB-1®, please contact me at SiREM, toll free at 519-515-0836 or by email at pdennis@siremlab.com.

Sincerely,

Phil Dennis, M.A.Sc.
Senior Manager

\attach.

Attachment A:

Information Regarding the Initial Isolation of KB-1®

HISTORY OF THE KB-1® CONSORTIUM

KB-1® was enriched from soil and groundwater collected at a former manufacturing facility in southwestern Ontario (the Site) where historical releases of TCE and other industrial chemicals have been degraded and high levels of ethene were produced naturally (Edwards et al., 1995).

i) Sample Collection and Handling

Soil

Soil used for KB-1® enrichment was collected aseptically from a borehole drilled in the shallow aquifer. No drilling fluids, greases, or petroleum-based solvents were used during drilling or soil collection. All down hole drilling equipment (back of drill rig, auger flights/casing, drilling rods, and other down-hole equipment) and sampling equipment were decontaminated (steam-cleaned) prior to use.

Representative aquifer material (i.e., material not contaminated by surface microorganisms) was collected from the borehole using an ethanol-sterilized core barrel sampler. Samples retrieved from the aquifer were aseptically transferred to sterile plastic liners. Liners were capped with sterile end caps, cut into 1.5-foot lengths, and delivered to the treatability laboratory. All equipment coming into contact with the soil samples was sterilized with ethanol prior to sample collection and between each sample location.

Groundwater

Groundwater was collected for the microcosms from a monitoring well. The monitoring well was purged (three casing volumes) prior to sampling. Groundwater samples were collected in sterile glass containers.

ii) Microcosm Construction and Incubation

Aquifer material was removed from the liners and mixed in a sterile plastic bag. Microcosms were constructed by adding approximately 60 grams (g) of homogenized, saturated soil to 100 milliliter (mL) (nominal volume) sterile glass serum bottles. Serum bottles were filled with 70 mL of groundwater, allowing a 40 mL headspace for sampling of dissolved hydrocarbon gases, and to allow gas (methane) production. All manipulations (including the initial processing) of the microcosms were conducted in an anaerobic chamber. Resazurin was added to microcosms to monitor groundwater redox conditions (resazurin is clear under anaerobic conditions but turns pink if exposed to oxygen). All bottles were sealed with Mininert™ caps and incubated in an inverted position at room temperature in the anaerobic chamber.

iii) Initial enrichment of KB-1®

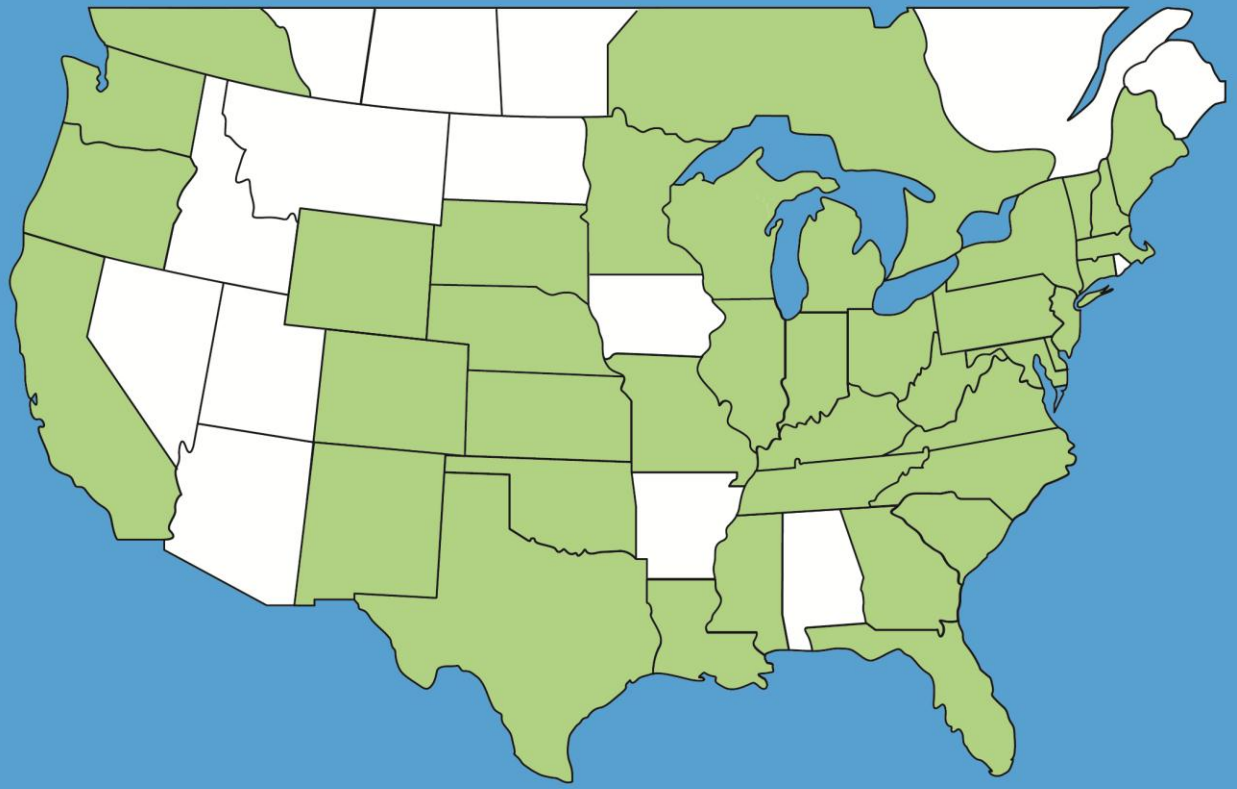
The microcosms were initially amended with an electron donor mixture of acetate, methanol, ethanol and lactate (approximately 1 millimolar [mM] each) and incubated anaerobically at room temperature (18-22°C). The TCE (15 micromoles [µmoles]) in the groundwater was dechlorinated to ethene within a few weeks. After several repeated amendments of TCE and methanol to the microcosms, the supernatant (30% volume/volume [v/v]) from shaken microcosms was transferred into sterile anaerobic defined mineral medium. Complete dechlorination of progressively higher concentrations of TCE to ethene was sustained through repeated transfers (10% v/v) into sterile defined mineral medium (Edwards and Grbic-Galic, 1994). After one year, the TCE-dechlorinating enrichment culture (referred to as KB-1) was routinely fed TCE (150 µM aqueous concentration) and methanol (1.5 mM) every two weeks in the laboratory of Dr. Elizabeth Edwards at the University of Toronto (Duhamel et al., 2002). The consortium was transferred (50% v/v) every 6-9 months into fresh sterile mineral medium to maintain dechlorination rates. The KB-1® consortium has been further reinnoculated by SiREM using culture that originated from the University of Toronto's KB-1/TCE culture in 2003.

Attachment B:

Locations of KB-1® Bioaugmentation

SiREM KB-1®/KB-1® Plus Bioaugmentation Locations

North America/Europe/Asia



November 2012

Europe

Alaska

Malaysia

*Insets not to scale

Attachment C:

Selected Peer Reviewed KB-1® Publications

PERGAMON

Water Research 36 (2002) 4193–4202

WATER RESEARCH

www.elsevier.com/locate/watres

Comparison of anaerobic dechlorinating enrichment cultures maintained on tetrachloroethene, trichloroethene, *cis*-dichloroethene and vinyl chloride

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Abstract

An anaerobic mixed microbial culture was enriched from soil and groundwater taken from a site contaminated with trichloroethene (TCE). This enrichment culture was divided into four subcultures amended separately with either perchloroethene (PCE), TCE, *cis*-dichloroethene (cDCE) or vinyl chloride (VC). In each of the four subcultures, the chlorinated ethenes were rapidly, consistently, and completely converted to ethene at rates of 30–50 μmol/l of culture per day, or an average 160 μ-electron equivalents/l of culture per day. These cultures were capable of sustained and rapid dechlorination of VC, and could not dechlorinate 1,2-dichloroethane, differentiating them from *Dehalococcoides ethenogenes*, the only known isolate capable of complete dechlorination of PCE to ethene. Chloroform (CF) and 1,1,1-trichloroethane, frequent groundwater co-contaminants with TCE and PCE, inhibited chlorinated ethene dechlorination. Most strongly inhibited was the final conversion of VC to ethene, with complete inhibition occurring at an aqueous CF concentration of 2.5 μM. Differences in rates and community composition developed between the different subcultures, including the loss of the VC enrichment culture's ability to dechlorinate PCE. Denaturing gradient gel electrophoresis of amplified bacterial 16S rRNA gene fragments identified three different DNA sequences in the enrichment cultures, all phylogenetically related to *D. ethenogenes*. Based on the PCR-DGGE results and substrate utilization patterns, it is apparent that significant mechanistic differences exist between each step of dechlorination from TCE to ethene, especially for the last important dechlorination step from VC to ethene.

Keywords: Anaerobic reductive dechlorination; DGGE; Microorganisms; Bioaugmentation

1. Introduction

The microbially mediated dechlorination of chlorinated ethenes to non-toxic ethene has been observed in the laboratory since 1989 [1] and in the field since 1991 [2]. Given the widespread and serious nature of chlorinated solvent groundwater contamination, much attention has been focused on understanding and optimizing in situ biological remediation of these contaminants. Biological reductive dechlorination of perchloroethene (PCE) or trichloroethene (TCE) occurs readily at many field sites and in many laboratory cultures. However, only partial dechlorination to *cis*-dichloroethene (cDCE) is often observed. A significant challenge is to understand the factors that prevent complete dechlorination beyond cDCE and vinyl chloride (VC) to ethene. While many PCE-dechlorinating organisms have been isolated [3–9], only one, *Dehalococcoides ethenogenes* Strain 195, is capable of

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completely reducing PCE to ethene [10]. Moreover, there is now evidence from laboratory and field investigations that organisms closely related to *D. ethenogenes* are present where complete dechlorination is observed, and are absent where dechlorination is incomplete [11–13].

In 1996, we constructed microcosms with soil and groundwater samples obtained from a TCE-contaminated site in Southern Ontario. This particular site was chosen because a geochemical investigation revealed high concentrations of TCE and daughter products including high levels of ethene (270 μM) in the groundwater [14], suggesting that organisms capable of complete dechlorination to ethene were active in situ. Indeed, TCE was rapidly converted to ethene in microcosms constructed with freshly collected site soil and groundwater (collected aseptically and anaerobically) and incubated in the laboratory under anaerobic conditions. Here, we describe the characteristics of stable enrichment cultures derived from these original TCE-dechlorinating microcosms, and demonstrate that significant differences develop between enrichment cultures established on each individual chlorinated ethene.

The motivation for this study is the growing understanding and appreciation of the function and distribution of dechlorinating organisms in the subsurface [12,13,15–17]. With a focus on managing the innumerable chlorinated ethene-contaminated sites worldwide, new insight into the mechanisms and organisms involved could significantly impact the optimization of biological remediation strategies. We have conducted a number of laboratory and field bioaugmentation studies using the TCE enrichment culture described herein as inoculum [18–20]. These studies and those of others have indicated that the reason for stalled or incomplete dechlorination in the field may not be the lack of appropriate conditions in situ, but rather the lack of appropriate organisms to carry the dechlorination to completion [13]. Since, many contaminated sites do not exhibit dechlorination past cDCE, and only one isolate is known to perform this function, the discovery of new organisms capable of dechlorinating cDCE and VC to ethene is clearly needed. Ultimately, one could envisage a strategy where bioaugmentation with specific dechlorinating enrichment cultures could be used to stimulate slow dechlorination steps in situ.

2. Materials and methods

2.1. Chemicals

Chlorinated alkanes and alkenes (>98% purity, Sigma-Aldrich) were used to amend cultures and for analytical standards. Methanol (HPLC Grade), ethanol, sodium lactate, and a hydrogen gas mix (80% H_2/20% CO_2, Praxair Specialty Gases and Equipment, Danbury, CT) were used as electron donors. An 80% N_2/20% CO_2 gas mix (Praxair) was used as an anaerobic purge gas. A 1% gas mix of methane, ethene and ethane in nitrogen (Scotty II mix 216, Alltech Associates, Deerfield, IL) was employed as an analytical standard.

2.2. Enrichment of dechlorinating cultures

The dechlorinating culture under study was originally enriched anaerobically from soil and groundwater obtained from a Southern Ontario TCE-contaminated site. TCE (15 μM) present in the sample groundwater was dechlorinated to ethene within a few weeks. After several repeated amendments of TCE and methanol to the microcosms, supernatant (30% v/v) from shaken microcosms was transferred into sterile anaerobic defined mineral medium [21]. Complete dechlorination of progressively higher concentrations of TCE to ethene was sustained through repeated transfers (10% v/v) into sterile defined mineral medium. After 1 year, this TCE-dechlorinating enrichment culture (referred to as KB-1) was routinely fed TCE (150 μM aqueous concentration) and methanol (1.5 mM) every 2 weeks.

In 1998, four separate sets of cultures of KB-1 were prepared that were amended with either PCE, TCE, cDCE, or VC (referred to as KB-1/PCE, KB-1/TCE, KB-1/cDCE and KB-1/VC, respectively). These cultures were amended every 2 weeks with 100–300 μM (aqueous) of one of the four chlorinated ethenes as electron acceptor and about 1–3 mM methanol as electron donor. PCE, TCE, and cDCE were added from solutions of chlorinated ethenes in methanol. VC was added as a neat gas using a gas-tight syringe. In the VC-amended cultures, methanol was added from a separate neat stock. These cultures have since been repeatedly transferred to select for organisms specifically required for each dechlorination step. Incubation of all enrichment cultures was carried out statically in an anaerobic chamber (Coy Lab. Products Inc., Glasslake, MI) at room temperature. For all culture transfers and for sampling, either sterile disposable syringes and needles were used or needles were sterilized with an incandescent loop in the anaerobic chamber immediately prior to use. Culture stoppers were wiped with isopropyl alcohol immediately before sampling to maintain sterility and prevent cross-contamination.

2.3. Electron donors and chlorinated electron acceptors used by KB-1

A variety of substrates were tested to determine the range of electron donors and acceptors used by KB-1 culture amended with TCE (called KB-1/TCE). For each substrate tested, a set of vials sealed with Mininert

screw caps (VICI Precision Sampling, Inc., Baton Rouge, LA) was filled with anaerobic medium. The vials were amended with equal electron-mole equivalents of each donor or acceptor being tested and inoculated with 5% (v/v) KB-1/TCE culture. Each condition was tested in duplicate or triplicate. Vials were sampled every 2–3 days for chlorinated ethenes, ethene and methane concentrations.

2.4. Effect of chloroform (CF) and 1,1,1-trichloroethane (1,1,1-TCA) on TCE dechlorination

In a preliminary experiment, the effects of CF and 1,1,1-TCA on the dechlorination process were evaluated at concentrations ranging from 4 to 20 μM (aqueous). CF was found to have a much stronger inhibitory effect on TCE dechlorination than 1,1,1-TCA, therefore, a second experiment was conducted to look at the effects of CF more closely. Twenty-four 40-ml vials sealed with Mininert caps were prepared as follows. The vials were amended with excess methanol (1 mM) and equal electron equivalents of one of either TCE, cDCE or VC (corresponding to aqueous concentrations of 14 μM TCE, 21 μM cDCE and 42 μM VC) along with either zero, 0.4, 2.5 or 6.7 μM (aqueous) CF. Each condition was tested in duplicate, and all treatments received an identical 10% (v/v) inoculum from the same bottle of KB-1/TCE culture.

2.5. Cross-acclimation study

This experiment was designed to determine if the cultures KB-1/TCE, KB-1/cDCE, and KB-1/VC could still dechlorinate chlorinated ethenes more chlorinated than their respective enrichment substrates. These cultures had not been exposed to more chlorinated ethenes for over 2 years. A series of glass bottles sealed with Teflon-coated butyl rubber stoppers were amended with the equal electron equivalents of either PCE, TCE, cDCE or VC and neat methanol (in excess) as electron donor.

2.6. Denaturing gradient gel electrophoresis (DGGE) of amplified bacterial 16S rRNA gene fragments

DNA was extracted from each enrichment culture using a method adapted from Miller et al. [22]. Briefly, 50 ml of culture was centrifuged to pellet cells, and resuspended in TENS buffer (50 mM Tris-HCl; 20 mM EDTA pH 8; 100 mM NaCl; 1% w/v SDS). Glass beads (106 μm) were then added to the mixture. After vortexing, the samples were incubated at 70°C for 20 min, and then homogenized in a Mini-Beadbeater™ Model 3110 BX (Biospec Products, Bartlesville, OK) on medium speed for 190 s. The samples were then centrifuged, and the supernatant was transferred to fresh (1.5 ml) Eppendorf tubes. DNA was precipitated using sodium acetate and 100% ethanol, centrifuged, and then washed with 70% ethanol to remove salts. The final DNA pellet was resuspended in TE buffer (10 mM Tris-HCl pH 8; 1 mM EDTA pH 8; in autoclaved water) and frozen at −20°C. DGGE analysis, including PCR amplification of 16S rRNA gene fragments, DGGE, and sequencing of bands was conducted by Microbial Insights Inc. (Rockford, TN). 16S rRNA gene fragments were amplified from genomic DNA using the general bacterial primers 5′-*CGCCCGCCGCGCGCGGCGGGC-GGGGCGGGGGCACGGGGGG*CCTACGGGAGGCA-GCAG-3′ and 5′-ATTACCGCGGCTGCTGG-3’ as described in Muyzer et al. [23], except that 35 thermocycles were carried out per amplification. These primers amplify a region of the 16S rRNA gene corresponding to positions 341–533 in *E. coli*. DGGE was performed using a 16 × 16 cm gel system (BioRad, Hercules, CA) maintained at a constant temperature in TAE buffer (40 mM Tris-HCl; 40 mM glacial acetic acid; 1 mM EDTA pH 8). A gradient ranging from 20% to 55% denaturant (7 M urea with 40% v/v deionized formamide) as denaturant was employed. After electrophoresis, the gel was stained with ethidium bromide and gel images were captured using an Alpha Imager® system. The central portions of bands of interest were excised and soaked in elution buffer (0.3 M NaCl, 0.3 mM EDTA, 30 mM Tris, pH = 7.6) overnight at 37°C. The DNA was purified using a Gene-Clean® kit and reamplified with the same primers as for the initial PCR reaction. The reamplified products were again purified and sequenced (each sample sequenced only once) with an ABI-Prism automatic sequencer using the reverse primer listed above, resulting in a sequence of about 145 base pairs long. Sequence comparisons 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 (RDPII, [24]) (http://www.cme. msu.edu/RDP/analyses.html).

2.7. Analytical procedures

Chlorinated ethenes and ethanes, methane, and ethene were analyzed by injecting a 300 μl headspace sample onto a Hewlett-Packard 5890 Series II Gas Chromatograph (GC) fitted with a GSQ 30 m × 0.53 mm I.D. PLOT column (J&W Scientific, Folsom, CA) and a flame ionization detector. The oven temperature was programmed to hold at 35°C for 2 min to elute methane and ethene, then to increase to 180°C at 60°C/min, and finally hold for 4 min at 180°C. Calibration was with external standards. Chlorinated ethene aqueous standard solutions were prepared gravimetrically from concentrated methanolic stock solutions (also prepared gravimetrically). GC peak areas were correlated to the

total mass of chlorinated ethene added to a given calibration bottle. Calibration bottles were prepared with the same volumes of headspace and liquid as in experimental bottles. The dimensionless Henry's law constants used to estimate actual aqueous phase concentrations at 25°C were 0.93 for PCE, 0.48 for TCE, 0.31 for cDCE and 0.95 for VC [25]. For methane and ethene, a purchased gas standard containing a mix of 1% methane, ethene and ethane in nitrogen was used for calibration. Because of the significant volatility of the chlorinated ethenes, ethene, and methane, mole balances and absolute rates of dechlorination were calculated using total moles or electron-mole equivalents per bottle (in both the gas and liquid phases). However, for the sake of comparison with other studies, aqueous concentrations are often reported. Protein concentration in cultures was measured by the method of Bradford [26] using a microassay kit (Bio-Rad) and bovine serum albumin as a standard. The detection limit for protein was 3 μg/ml of sample. Lower detection limits were achieved by first concentrating a culture sample by centrifugation.

3. Results and discussion

3.1. Enrichment of KB-1 and development of subcultures

A stable TCE-dechlorinating culture, called KB-1/TCE, was enriched from initial microcosms. Subcultures maintained with either PCE, cDCE, or VC for over 2 years (referred to as KB-1/PCE, KB-1/cDCE and KB-1/VC, respectively) were generated from the original TCE-fed culture. In all cultures, dechlorination rates have steadily increased with time. Of particular interest was the sustained dechlorination in the KB-1/VC enrichment cultures. These cultures, when closely monitored, sustained dechlorination rates of up to 100 μmol VC/l/day with either methanol or hydrogen as electron donor. Flynn et al. [27] also described an enrichment culture capable of sustained VC dechlorination in a completely defined medium. Sustained dechlorination of VC in the absence of other chlorinated ethenes is significant, since dechlorination of VC with *D. ethenogenes* Strain 195 is cometabolic and cannot be sustained without exposure to a higher chlorinated ethene, as well as anaerobic digestor sludge supernatant [28]. The long-term viability (>3 years) and high dechlorination rates in enrichment cultures sustained on weekly additions of PCE, TCE, cDCE, and VC suggested that dechlorination was associated with growth. Moreover, protein concentration increased more in cultures amended with chlorinated ethenes compared to cultures to which donor, but no chlorinated ethenes, had been added (data not shown). Specific rates of dechlorination normalized to total protein concentration were calculated for 14 different enrichment cultures dechlorinating different chlorinated ethenes. Overall, these dechlorination rates were similar and averaged about 1.0 (± 0.4) μmol/h/mg total protein. Protein concentrations were typically around 5 mg/l, but values as high as 20 mg/l were obtained in some cultures. However, in a mixed culture, total protein concentration may not be a particularly good quantity for normalization, because it includes not only the protein from active dechlorinating organisms, but also the protein from non-dechlorinating organisms such as acetogens and methanogens, and possibly inactive organisms as well. Therefore, when comparing our own enrichment cultures in this report, rates are reported per unit volume of culture medium. In any case, care should be taken in comparing dechlorination rates from different studies, as they are frequently normalized to different quantities, such as total biomass, total protein, or culture volume.

3.2. Electron donors used by KB-1

Methanol, ethanol, hydrogen, lactate, and propionate (but not acetate) could all serve as electron donors for sustaining dechlorination. Moreover, when a stable toluene-degrading, non-dechlorinating methanogenic culture [29] was mixed with KB-1, toluene could also serve as an effective electron donor. Although methanol is not thought to be a good substrate for dechlorination because it is a substrate for methanogenic bacteria and does not yield a large amount of hydrogen on fermentation, we used methanol for long-term culture maintenance since it worked well. Varying the initial methanol concentration did not significantly affect the rate or extent of dechlorination, but did have a profound effect on the amount of methane produced. By feeding lower concentrations of methanol, we could minimize methane production. Dechlorination rates with methanol were always equal to or better than rates with other donors. It is possible that the methanol-degrading organisms, known to synthesize high concentrations of cobalt-containing cofactors [30], may be providing key trace nutrients to the dechlorinators.

3.3. Maximum concentrations of chlorinated ethenes dechlorinated by specific KB-1 cultures

Complete dechlorination readily occurred with initial aqueous concentrations of TCE up to a maximum of 1.5 mM, VC up to 1.4 mM, cDCE up to 800 μM, and PCE up to 800 μM. It is possible that the lower concentration maximum for cDCE was due to the presence of CF or other contaminants in the cDCE, as reported by others [17]. With PCE, dechlorination stopped abruptly, even when H_2 was added as a direct electron donor, when concentrations approached the solubility of PCE (about 800 μM). This inhibition may

have been related to the development of a separate, non-aqueous PCE phase.

3.4. Compounds dechlorinated by KB-1/TCE

The ability of KB-1/TCE to dechlorinate a variety of other chlorinated substrates, including 1,1-DCE and *trans*-1,2-DCE, 1,2-dicloroethane, 1,1,1-trichloroethane and chloroform, at concentrations of approximately 30–50 μM was evaluated. KB-1/TCE rapidly and sustainably dechlorinated 1,1-DCE at a rate comparable to that of cDCE. In contrast, KB-1/TCE dechlorinated *trans*-DCE at a rate that was approximately five times slower than that with cDCE, and an increase in rate with time was not observed. No dechlorination of 1,2-dichloroethane (1,2-DCA) was observed over several months of incubation. This is in contrast to other TCE-dechlorinating pure and mixed cultures that have been shown to also readily dechlorinate 1,2-DCA [3,28,31]. CF and 1,1,1-TCA (3–30 μM) were not significantly dechlorinated by KB-1/TCE over a 1-month period in incubations with and without chlorinated ethenes present. This was somewhat unexpected given the possibility for cometabolic degradation of these compounds by methanogens [32,33].

3.5. Effect of CF and 1,1,1-TCA on TCE dechlorination

Since many sites are contaminated with mixtures of chlorinated solvents, a better understanding of co-contaminant effects is needed for the selection of appropriate remediation strategies. 1,1,1-TCA [34] and especially CF [35] are known to inhibit methanogenesis, and may also interfere with reductive dechlorination [36,17]. A preliminary experiment with KB-1/TCE demonstrated that both CF and 1,1,1-TCA slowed dechlorination. Most notably, ethene production from VC was completely inhibited at a CF aqueous concentration of 3.8 μM (450 μg/l) and at a 1,1,1-TCA concentration of between 5.2 and 22 μM (700 and 3000 μg/l). In a second experiment, the effect of CF at 0.4, 2.5 and 6.7 μM (50, 300 and 800 μg/l) on each step in the dechlorination of TCE was examined. In this experiment, it is important to note that a single sample from a KB-1/TCE culture was divided into aliquots, which were then used to inoculate all treatments (TCE-, cDCE- and VC-amended). This was done to ensure that the initial culture concentration and composition were as uniform as possible in all treatments. The effect of CF on the rate of each dechlorination step differed significantly (Fig. 1). TCE dechlorination was completely inhibited when the CF concentration reached 6.7 μM. The effect of CF on cDCE dechlorination was less pronounced; only minor inhibition was initially observed, even at 6.7 μM. In contrast, VC dechlorination rates were significantly affected by CF, even at the lowest concentration tested (0.4 μM), and complete inhibition of dechlorination was observed at 2.5 μM CF. Bagley et al. [36] observed complete inhibition of PCE dechlorination at 4 μM CF. Maymó-Gatell et al. [17] found that 1.6 μM CF completely inhibited cDCE dechlorination by *D. ethenogenes* Strain 195. Increased sensitivity of the VC to ethene step in the presence of another common groundwater co-contaminant, carbon tetrachloride, has also been observed [37]. In this study, as CF concentrations increased, methanogenesis also decreased (data not shown); at the highest CF concentration tested, no methanogenesis was observed at all. CF may have directly affected the dechlorinating organisms or may have indirectly affected dechlorination by inhibiting other microorganisms, for example,

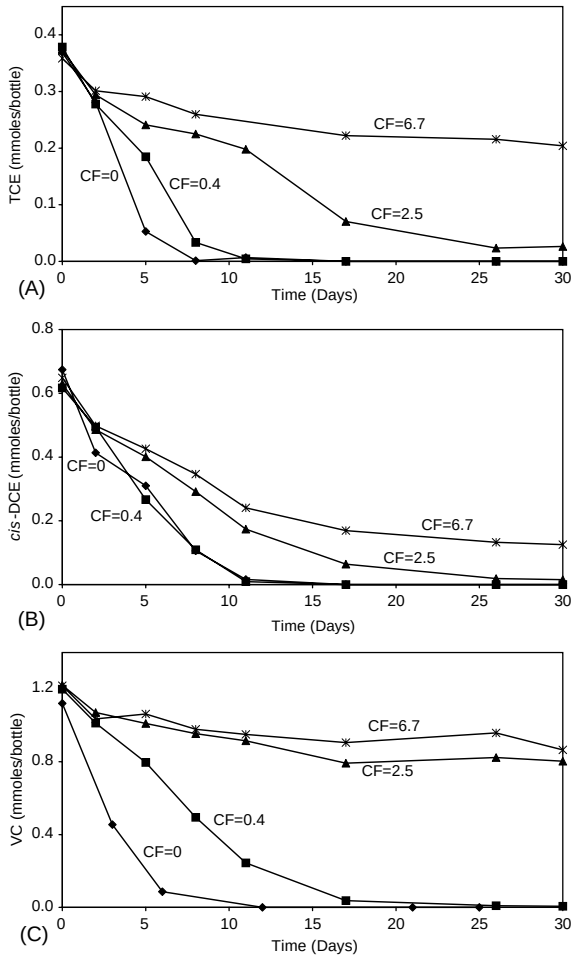

Fig. 1. Effect of chloroform on the rate of dechlorination of (A) TCE, (B) cDCE, and (C) VC by KB-1/TCE. Chloroform concentrations are in μM (aqueous). Each experimental vial was inoculated with the same sample of culture from a TCE-fed enrichment culture. Data are the average of duplicates; duplicates differed by $<15\%$.

methanogenic bacteria, on which the dechlorinating organisms depended.

3.6. Direct comparison of the four enrichment cultures

To better understand differences between the microbial populations responsible for each dechlorination step, the four enrichment cultures were directly compared in two experiments: (1) a cross-acclimation study to evaluate any changes in substrate range and (2) a molecular study to contrast bacterial community composition.

3.7. Cross-acclimation study

This study was performed to determine if, over the 2 years since their creation in 1998, the KB-1 enrichment cultures sustained on less chlorinated ethenes had lost the ability to transform any of the higher chlorinated ethenes. The most significant finding was that PCE was no longer dechlorinated by the KB-1/VC enrichment culture (Table 1). This result has also been observed for another VC enrichment culture [27]. In addition, while both TCE and cDCE were dechlorinated by the KB-1/VC enrichment culture, dechlorination rates decreased as the extent of chlorination increased. Results for both the KB-1/cDCE and the KB-1/TCE enrichment cultures revealed a similar trend, although PCE was still dechlorinated by these cultures (Table 1); a similar result was observed by Maymó-Gatell et al. [17] for *D. ethenogenes* Strain 195.

Table 1
Relative dechlorination rates for three enrichment cultures tested with substrates more chlorinated than the enrichment substrate

Culture enrichment substrate	Chlorinated substrate tested	Dechlorination rate[a] (μmol/l/day)
VC	VC	22±1.9
	cDCE	17±0.5
	TCE	7.0±1.5
	PCE	Not dechlorinated
cDCE	cDCE	30±7.5
	TCE	12±0.3
	PCE	3.6±2.4
TCE	TCE	14±3.0
	PCE	3.2±0.2

[a] Data are mean±range from duplicate experiments.

3.8. Microbial community composition

To determine if the microbial community composition shifted when enriched on a single chlorinated ethene for over 2 years, the cultures were compared using PCR-DGGE of bacteria 16S rRNA gene fragments (Fig. 2). In DGGE, each band in a lane theoretically corresponds to a different organism in the culture, although one organism may have multiple 16S rRNA genes with slightly different sequences, which may cause multiple bands for one organism. Another artifact, heteroduplex formation, occurs when two very similar PCR products are in the same sample [38]. In our study, PCR-DGGE revealed firstly that all of the cultures were of low complexity, since there were relatively few bands per lane. Secondly, the four cultures were not identical. The separation patterns corresponding to the KB-1/PCE and

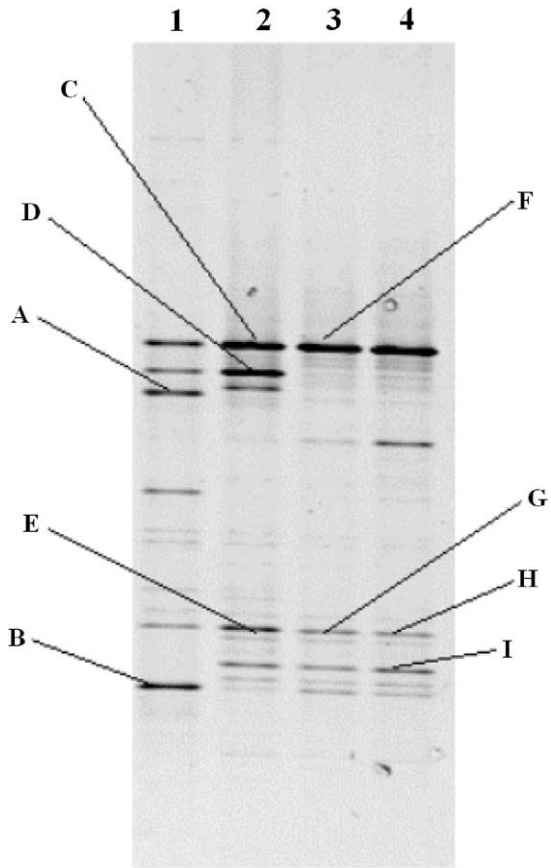

Fig. 2. Image of PCR-DGGE of 16S rDNA gene fragments amplified from four enrichment cultures. (Lane 1) KB-1/VC, (Lane 2) KB-1/cDCE, (Lane 3) KB-1/TCE, and (Lane 4) KB-1/PCE. Letters correspond to bands that were excised and the DNA sequenced, as listed in Table 2. Unlabelled bands could not be sequenced.

KB-1/TCE cultures were similar. There was a clear shift in the population composition between the KB-1/TCE and KB-1/cDCE cultures, and another substantial shift between the KB-1/cDCE and KB-1/VC cultures. These data provide evidence for different microbial communities in each culture, consistent with the cross-acclimation study results.

The brightest bands in the denaturing gradient gel (labeled with letters in Fig. 2) were excised and the reamplified DNA fragments were partially sequenced and compared to known sequences using BLASTN and the RDP (Table 2). Certain bands in the gel corresponded to organisms closely related to *D. ethenogenes* Strain 195 or another very similar organism based on 16S rRNA sequence; called CBDB1 [40]. The separation of KB-1 DNA by DGGE yielded three different bands with sequences very similar to that of *Dehalococcoides ethenogenes*. These data suggested that perhaps three different microorganisms related to *D. ethenogenes* were present in the cultures. The six sequences retrieved from these three band positions (C, E, F, G, H and I) were aligned with the sequence of *D. ethenogenes* Strain 195. Unfortunately, only a limited amount of sequence data (145 bp) was provided by Microbial Insights, corresponding to positions 303–449 of the *D. ethenogenes* 16S rRNA sequence. Overall, there was a maximum of 3 differences between the sequences obtained from DNA fragments in the gel and *D. ethenogenes* Strain 195 in this region. All of the sequences had a G deletion at position 432 of the *D. ethenogenes* sequence. Sequences for bands E, G, H and I had a T deletion at position 441 and band I had an A to C substitution at position 394. These slight differences in sequences were consistent with the observation of three distinct bands on a denaturing gradient gel. It is unlikely that these differences were due to PCR, sequencing errors, or chimeras since the same three bands were observed in sequences derived from three separate DNA extractions (i.e. the three lanes corresponding to the KB-1/cDCE, KB-1/TCE, and KB-1/PCE cultures). Recently, Hendrickson et al. [13] found two distinct *Dehalococcoides* sequences in a KB-1/TCE enrichment culture sample that we sent to them (GenBank Accession numbers AF388540 and AF388539). However, the differences we found in this present study using DGGE do not correspond to the same differences reported by Hendrickson et al. Further cloning work is underway to identify all of the *Dehalococcoides* 16S rRNA sequences in KB-1 enrichment cultures.

The sequences of the other bands in the DGGE gel indicated the presence of common soil organisms. Band D, whose 145 bp sequence was 100% match to several *Acetobacterium* species, was intense in the KB-1/cDCE separation pattern and to a lesser degree in the KB-1/VC separation pattern, but not in the KB-1/TCE and KB-1/PCE patterns. Although this may seem to illustrate that *Acetobacterium* species are present in greater number in KB-1/VC and KB-1/cDCE, biases in the DNA extraction steps and PCR amplification make quantitative assessment unreliable. *Acetobacterium* species can grow on CO_2 plus H_2 and other organic substrates such as methanol [41].

Of the four gel patterns, the one corresponding to KB-1/VC was the most distinct, containing two bright bands (A and B) not seen in other patterns. Band A may correspond to a distant relative of *Sulfurospirillum deleyianum*, an organism that has been shown to use H_2 and sulfide as electron donors and acetate as a carbon source with a variety of electron acceptors [42]. Band B shared up to 99% similarity with many uncultured soil organisms, although its closest isolated relative (89% similarity) was *Hippea maritima*, a

Table 2
DGGE[a] band sequencing results

Band	Best matches	Accession numbers	Similarity[b] (%)
A	*Sulfurospirillum deleyianum*	Y13671	83
B	Uncultured soil bacterium PBS-111-32a	AJ390460	99
	Hippea maritima	Y18292	89
D	*Acetobacterium paludosum*	X96958	100
	Acetobacterium psammolithicum	AF132739	100
	Acetobacterium carbinolicum	X96956	100
C and F	*Dehalococcides ethenogenes*	AF004928	98
	CBDB1	AF230641	99 (F); 100 (C)
E, G, H	*Dehalococcides ethenogenes*	AF004928	96
	CBDB1	AF230641	98
I	*Dehalococcides ethenogenes*	AF004928	98
	CBDB1	AF230641	97

[a] DGGE shown in Fig. 2.
[b] Based on BLASTn search of ca.145 bp fragments [39].

thermophilic sulfate-reducer found in submarine hot vents [43]. No potential role for the population associated with this band has yet been identified. Another distinguishing feature of the KB-1/VC gel pattern is that one of the bands for *D. ethenogenes*-like sequences (corresponding to band I) was absent. Perhaps this band corresponds to a strain similar to *D. ethenogenes* Strain 195 that cannot grow on VC alone. Efforts are underway to isolate dechlorinating organisms from KB-1/VC.

The observed differences in degradative abilities and community composition between the various KB-1 cultures are consistent with the results of carbon fractionation studies done with KB-1 and other dechlorinating cultures [44,45]. These studies showed that isotope fractionation during each dechlorination step was reproducible, was different for each step in the dechlorination pathway, and fit to a Raleigh model [44,45]. The fractionation factor, the parameter of the Raleigh model, is a characteristic of the reaction. Significantly different fractionation factors were observed for each dechlorination step, implying that a different reaction mechanism is used for each dechlorination step.

4. Conclusions

- A set of highly enriched and effective dechlorinating enrichment cultures have been developed that use methanol, hydrogen and other electron donors and dechlorinate PCE, TCE, cDCE or VC completely to ethene.
- Cultures enriched on cDCE, TCE and PCE may harbor three distinct close relatives of *D. ethenogenes* Strain 195, an organism that completely dechlorinates chlorinated ethenes and 1,2-dichloroethane to ethene in pure culture.
- The KB-1/TCE enrichment culture does not dechlorinate 1,2-dichloroethane (in contrast to *D. ethenogenes* Strain 195 and other dechlorinating enrichment cultures).
- The KB-1/VC enrichment culture can be maintained on VC indefinitely and, after a prolonged period of time, lost the ability to dechlorinate PCE.
- CF and 1,1,1-TCA, frequent groundwater co-contaminants with chlorinated ethenes, are potent inhibitors of chlorinated ethene dechlorination by KB-1/TCE.

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Field Demonstration of Successful Bioaugmentation To Achieve Dechlorination of Tetrachloroethene To Ethene

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A laboratory microcosm study and a pilot scale field test were conducted to evaluate biostimulation and bioaugmentation to dechlorinate tetrachloroethene (PCE) to ethene at Kelly Air Force Base. The site groundwater contained about 1 mg/L of PCE and lower amounts of trichloroethene (TCE) and *cis*-1,2-dichloroethene (cDCE). Laboratory microcosms inoculated with soil and groundwater from the site exhibited partial dechlorination of TCE to cDCE when amended with lactate or methanol. Following the addition of a dechlorinating enrichment culture, KB-1, the chlorinated ethenes in the microcosms were completely converted to ethene. The KB-1 culture is a natural dechlorinating microbial consortium that contains phylogenetic relatives of *Dehalococcoides ethenogenes.* The ability of KB-1 to stimulate biodegradation of chlorinated ethenes in situ was explored using a closed loop recirculation cell with a pore volume of approximately 64 000 L. The pilot test area (PTA) groundwater was first amended with methanol and acetate to establish reducing conditions. Under these conditions, dechlorination of PCE to cDCE was observed. Thirteen liters of the KB-1 culture were then injected into the subsurface. Within 200 days, the concentrations of PCE, TCE, and *cis*-1,2-DCE within the PTA were all below 5 μg/L, and ethene production accounted for the observed mass loss. The maximum rates of dechlorination estimated from field data were rapid (half-lives of a few hours). Throughout the pilot test period, groundwater samples were assayed for the presence of *Dehalococcoides* using both a *Dehalococcoides*-specific PCR assay and 16S rDNA sequence information. The sequences detected in the PTA after bioaugmentation were specific to the *Dehalococcoides* species in the KB-1 culture. These sequences were observed to progressively increase in abundance and spread downgradient within the PTA. These results confirm that organisms in the KB-1 culture populated the PTA aquifer and contributed to the stimulation of dechlorination beyond cDCE to ethene.

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Introduction

Chlorinated ethenes such as tetrachloroethene (PCE) and trichloroethene (TCE) are some of the most pervasive groundwater contaminants. Biodegradation is a promising remedial alternative in many cases. The principle biodegradation mechanism for chlorinated ethenes is reductive dechlorination, which involves the sequential replacement of chlorine atoms on the alkene molecule by hydrogen atoms (*1*−*3*). Some microorganisms, termed dehalorespiring microorganisms, including *Dehalospirillium multivorans* (*4*), *Dehalobacter restrictus* (*5*), and *Dehalococcoides ethenogenes* (*1*), use chlorinated ethenes as their terminal electron acceptors in metabolism and gain energy from reductive dechlorination (*6*−*8*). Hydrogen produced by fermentation is often the electron donor. Of these microorganisms, *D. ethenogenes* is the only one able to completely dechlorinate chlorinated ethenes to ethene but does not appear to be present at all sites. At some sites, dechlorination of PCE and TCE stalls at *cis*-1,2-dichloroethene (cDCE) resulting in an accumulation of partially dechlorinated products. In a recent survey of 24 chlorinated ethene-contaminated sites, *Dehalococcoides* spp. (*Dhc*) were not detected at "partially dechlorinating" sites (*9*).

Several stable enrichment cultures that contain organisms phylogenetically closely related to *D. ethenogenes* are capable of mediating complete dechlorination of TCE to ethene. Examples include the Cornell Culture (*1*), the Victoria Culture (*9*), and the Pinellas culture (*10*−*13*). A field demonstration by the Remediation Technologies Development Forum (RTDF) at Dover Air Force Base in Delaware demonstrated that dechlorination of cDCE to ethene occurred after the pilot test area (PTA) was bioaugmented with the Pinellas culture, a culture enriched from soil and groundwater samples from a U.S. Department of Energy site in Largo, FL (*11*, *12*). After complete dechlorination of TCE to ethene had occurred, samples taken from the Dover PTA were shown to contain, via 16S rDNA analysis, at least three different dechlorinating species similar to *D. restrictus*, *D. multivorans*, and *D. ethenogenes* (*9*, *10*, *13*). Furthermore, the 16S rDNA sequences obtained from the Dover pilot site were found to have signature sequences that were the same as those found in the Pinellas bioaugmentation culture. These findings suggest, although did not prove, that the inoculum abetted the dechlorination and that some of the culture had indeed survived and populated the pilot subsurface (*10*, *13*). To date, all cultures that dechlorinate chlorinated ethenes completely to ethene have been found to contain a phylogenetically close relative of *Dehalococcoides* (*1*, *9*, *14)*. While *Dehalococcoides* may not be the only group of organisms capable of complete dechlorination (others have yet to be discovered), they have been shown to be an important indicator for dechlorination beyond cDCE (*9*, *14*).

In this study, we report the results of a second successful pilot scale bioaugmentation demonstration. The goals of this study were to correlate the dechlorination to ethene with the presence of dechlorinating organisms, in particular *Dehalococcoides*, whether they were indigenous to the Kelly Air

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Force Base (AFB) pilot plot or whether they were introduced during bioaugmentation. The program had two stages. The first stage, biostimulation, consisted of the addition of electron donors to stimulate the formation of an anaerobic, reductive subsurface environment suitable for reductive dechlorination and to try to stimulate the activity of indigenous dehalorespiring organisms. The addition of electron donors over 173 days failed to stimulate dechlorination beyond cDCE. Therefore a second stage was initiated with the inoculation (bioaugmentation) of the pilot test area with an enrichment culture referred to as KB-1 (*15*). The formation of chlorinated ethene degradation products was monitored to determine when cDCE transformation to ethene began. Over the period of electron donor addition, both prior to and after bioaugmentation, the distribution of *Dehalococcoides* organisms in and around the pilot test area was assessed using a *Dehalococcoides*-specific polymerase chain reaction (PCR) assay combined with sequence analysis of the amplified sequences (*9*). From the sequence of amplified DNA, it was possible to determine that the specific *Dehalococcoides* organisms detected in the pilot test area were not native to the site but were introduced with the KB-1 culture.

Methods

Site Description. Kelly AFB is located in south central Texas, approximately eleven kilometers Southwest of downtown San Antonio. The geology in the vicinity of the PTA consisted of unconsolidated alluvial deposits overlying an undulatory erosional surface of Cretaceous alluvium and Navarro Clay. The alluvial deposits consisted of gravel, sand, silt, and clay, ranging in thickness from 6 to 12 m. From the surface downward, the geology typically consisted of the following: 0.3 to 1.2 m of black organic clay; 1.8 to 4.9 m of tan silty, calcareous clay; and 1.2 to 6.1 m of clayey limestone and chert gravel. The groundwater flow velocity is about 0.9 m/day. Volatile organic compounds in site groundwater consisted primarily of PCE, with lesser amounts of TCE, and cDCE. Sulfate, nitrate, dissolved iron, and oxygen concentrations and reduction−oxidation potentials indicated that the site groundwater was predominantly aerobic and oxidizing. Methane, ethene, and ethane were not detected in site groundwater (*16*, *17*).

A second chlorinated ethene-contaminated site existed on the Kelly AFB. This site consisted of several landfill trenches located 2.4 km west of the PTA. The subsurface below the landfill trenches was anaerobic and reduced, and PCE, TCE, cDCE, VC and ethene were detected in soil and groundwater samples from this area (*16*).

KB-1 Enrichment Culture. The dechlorinating culture used for bioaugmentation was anaerobically enriched from soil and groundwater obtained from a Southern Ontario TCE-contaminated site over 6 years ago. Dechlorination of progressively higher concentrations of TCE or PCE to ethene was sustained through repeated transfers (10% v/v) into sterile defined prereduced mineral medium (*18*). This TCE-dechlorinating enrichment culture (referred to as KB-1) was routinely fed TCE (300 μM aqueous concentration) and methanol (1.5 mM) every two weeks. The culture was routinely grown under strictly anaerobic conditions in 1 or 2 L glass bottles sealed with screw caps modified with a small hole to fit a black butyl rubber stopper. The rubber stoppers facilitated sampling and feeding without opening the cap. The bottles were regularly amended with TCE and methanol as soon the previous TCE dose was converted to ethene. Pressure buildup from methanogenesis was relieved weekly with a syringe. Approximately every two to three months, about 20% of the culture volume was removed and replaced with fresh medium and the pH was readjusted to 7 with NaOH if required. The culture would grow to a maximum cell density of about 10^8 cells per mL, corresponding to a maximum protein concentration of about 40 mg/L. No pathogenic bacteria were found in a 16S rDNA clone library of KB-1 (*19*). In addition, quadruplicate samples obtained from various batches of KB-1 were sent to GAP Enviro-Microbial Services (London, Ontario) for routine pathogenicity testing using standard plate count assays for the most common pathogens. None of the following microorganisms were detected in KB-1: *Salmonella* sp., *Listeria monocytogenes*, *Vibrio* sp., *Campylobacter* sp., hemolytic *Clostridia* sp., *Pseudomonas aeruginosa*, *Yersinia* sp., *Candida albicans*, *Aspergillus fumigatus*, fecal coliforms and Enterococci. Additionally, culture-based tests by GAP Environmental Services, and PCR-based tests by Aerobiology Laboratory Associates, Inc. (Reston, Virginia) were negative for *Bacillus anthracis*.

Two 8 L stainless steel pressure vessels were used to transport the culture to the site. These vessels were inoculated each with about 1 L of culture in 6 L of medium several months prior to use and amended with TCE (300 μM) and methanol three times to confirm that the culture was active before inoculating the site. The two stainless steel vessels were purged with N_2/CO_2 (80:20) and then analyzed to ensure that there were no chlorinated ethenes or ethene in the inoculum prior to shipping.

Procedure for Collecting Soil Samples for Microcosm Studies. Soil samples for microcosm studies were obtained in 15.2 cm long by 8 cm diameter stainless steel core liners. The samples were collected in the PTA prior to the initiation of the study at depths of 7 to 8.2 m below ground surface using split spoon sampling methods. The cores were shipped on ice to the Edwards' laboratory at the University of Toronto. Upon arrival all samples were stored in an anaerobic chamber (Coy Laboratory Products Inc., Glasslake, MI) filled from a gas cylinder containing 10% hydrogen and 10% carbon dioxide in nitrogen.

Microcosm Study. A microcosm study was conducted prior to the field study. Microcosms consisted of 250 mL (nominal volume) sterile glass bottles filled with 60 g of site soil and 150 mL of site groundwater, leaving 70 mL of headspace. The microcosms were prepared inside a disposable glovebag purged with Nitrogen. The microcosms were sealed with Mininert screw caps and then transferred to a Coy anaerobic chamber for incubation and sampling. Seven different treatments (sterile control, intrinsic control, lactate-amended, methanol-amended, and 3 methanol-amended and bioaugmented), prepared in triplicate, were evaluated. Sterile control microcosms were amended with 1.0 mL of 5% mercuric chloride and 1.0 mL of 5% sodium azide to inhibit microbial activity. The intrinsic control microcosms were not amended with any exogenous electron donor. Methanol- or sodium lactate-amended microcosms contained target initial concentrations of about 3 mM for each electron donor and 6 μM (aqueous) TCE. KB-1 bioaugmented microcosms were prepared with three different initial concentrations of TCE: 6, 60, and 600 μM (0.8, 8, and 80 mg/L). These microcosms were each amended with 3 mL of the same culture that was used to inoculate the stainless steel vessels that were shipped to the field site. Methanol (3 mM) was added as electron donor from a neat stock. Electron donors were added to donor-amended and bioaugmented microcosms initially and periodically throughout the incubation period (days 16, 42, and 56). One replicate from each treatment was amended with resazurin to monitor redox conditions.

Analytical Procedures for the Microcosm Study. Chlorinated ethenes, ethene, methane, and methanol concentrations were obtained by removing up to 1 mL sample of liquid from each microcosm and injecting the sample into a 10-mL headspace vial containing 5 mL of acidified (pH 2) water and then sealing the vial with a Teflon-coated septum and aluminum crimp cap. The samples were acidified to inhibit

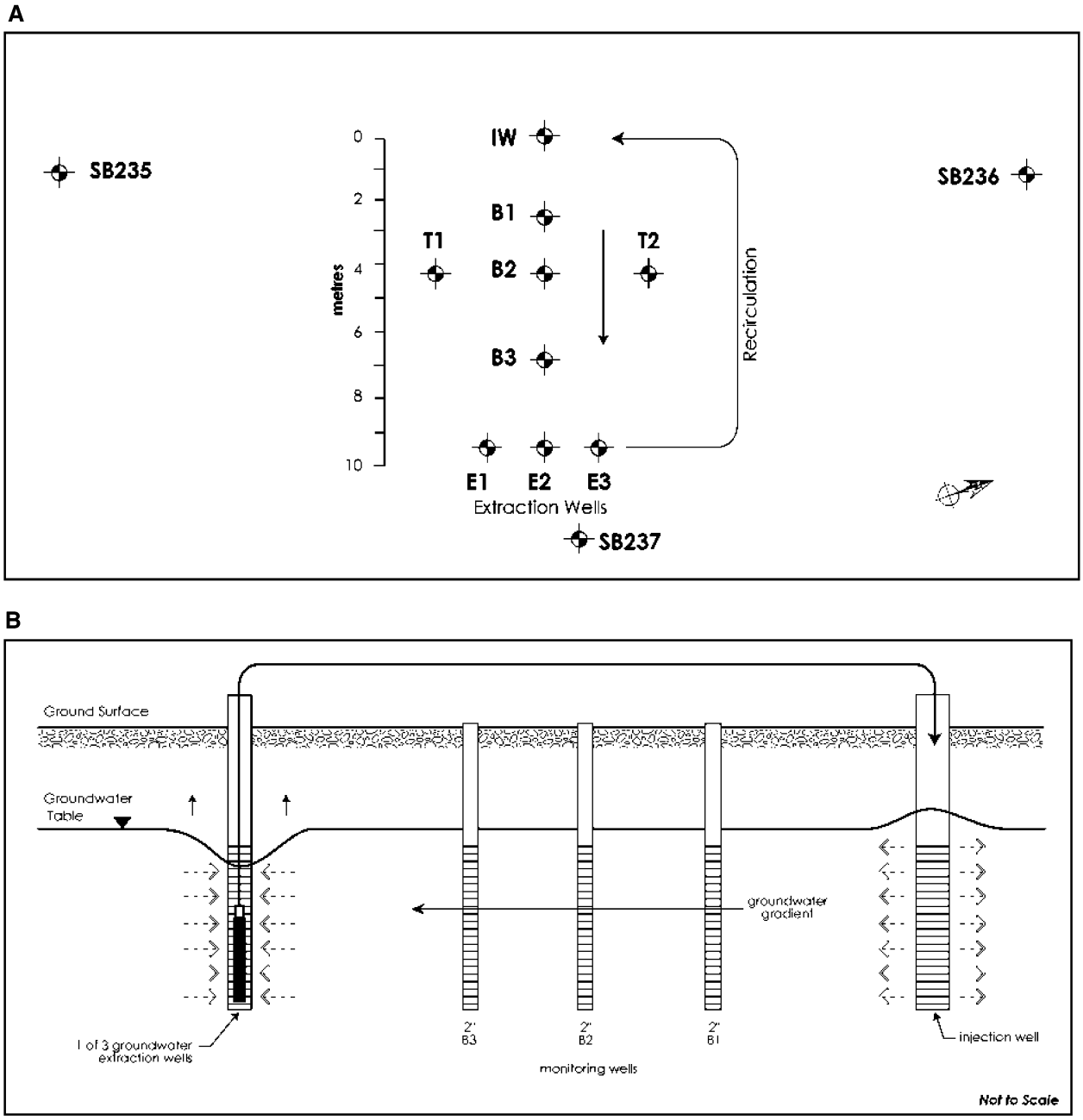

FIGURE 1. Diagram of pilot test area (PTA). Plan view (A) and cross-section (B). The distance between the each of the extraction wells was approximately 0.9 m and the distance between the injection well (IW) and middle extraction well (E2) was approximately 9.1 m. The monitoring wells B1, B2, and B3 were positioned along the groundwater flowpath centerline and spaced approximately 2.4, 4, and 7 m from the IW. Transgradient wells (T1, T2) were spaced approximately 3.1 m from the groundwater flowpath centerline. Monitoring and extraction wells were installed in September 1999, with the exception of the IW, which was a preexisting well. Wells were developed after installation following standard protocols. All wells were screened across the aquifer thickness (approximately 3.1 m).

degradation. Analysis was conducted using a HP 7694 headspace autosampler and a HP5890 series II gas chromatograph (GC) (Hewlett-Packard, Wilmington, DE) equipped with a GSQ plot column (0.53 mm × 30 m, J&W, Agilent, Palto Alto, CA) and a flame ionization detector (FID). The injector temperature was set at 200 °C, and the detector temperature was set at 250 °C. The oven temperature was programmed as follows: 35 °C for 2 min, increase to 100 °C at 10 °C/min, then increase to 180 °C at 6 °C/min, and held at 180 °C for 1.34 min. The carrier gas was helium at a flow rate of 11 mL/min. The autosampler was programmed to heat each sample to 75 °C for 40 min prior to injection. The detection limits for a 1 mL aqueous sample were 5 μg/L for chlorinated ethenes, ethene, and methane and 1 mg/L for methanol.

Pilot Test Area Layout and Operation. The PTA consisted of a closed loop recirculation system, including 3 extraction wells (only two used for the test), one injection well, and 5 monitoring wells. Figure 1 presents schematics of the system in plan view (panel A) and cross section (panel B). Groundwater was extracted at a combined flow of 5.7 L.min^{-1} using two SQ/SQE pumps (Grundfos Pumps Corp., Oakville, ON) installed in extraction wells 1 and 2 (E1, E2) powered by single-phase power and a master control module. Extraction well E3 was not used, as it did not increase capture efficiency. The extracted groundwater from each well was combined through a common header and injected via the injection well (IW). The system was equipped with an inline paddle wheel flow sensor installed downstream of the common header to continuously measure the flow rate of the

recirculating groundwater. Output from the flow sensor controlled the speed of an Ismatec multichannel cartridge pump (MIDI-MS/CA 4−6, Ismatec SA, Glattbrugg, Switzerland) delivering tracer or electron donor into the feed groundwater to maintain the desired concentration of these compounds in the amended groundwater.

Two bromide conservative tracer tests were conducted at the beginning to optimize groundwater capture and to estimate flow velocities. In the first test, bromide was added to the PTA groundwater as a short duration pulse (9 h interval). Background bromide, as determined using a bromide ion selective electrode (model 27502, Cole-Parmer, Vernon Hills, IL), was less than 1 mg/L. A total of 5 kg of sodium bromide was added to the PTA groundwater and was monitored at each well in the PTA using a bromide-specific electrode. Data were collected hourly for the period prior to and as the tracer pulse passed through each well. Visual MODFLOW Pro, V 2.8 (Waterloo Hydrogeologic, Waterloo, ON), was used to numerically model groundwater flow and solute transport through the PTA. The numerical model was calibrated using the bromide tracer data and was then used to evaluate how to modify extraction rates to achieve greater groundwater capture. The second bromide test confirmed the MODFLOW predictions that decreasing the total flow rate from 11.4 to 7.6 L min^{-1}, with extraction wells E1 and E2 at 5.7 and 1.9 L min^{-1}, respectively, increased the capture efficiency of the PTA.

Electron Donor Addition and Bioaugmentation. Groundwater was recirculated without electron donor addition for the first 89 days to equilibrate the system and to conduct a bromide conservative tracer test. The tracer test was used to verify system hydraulics, to estimate groundwater capture efficiency, and to estimate the pore volume of recirculating water. After day 89, methanol and acetate were added as electron donors at an approximate time-weighted average concentration of 3.6 mM each, until the termination of the test (day 319). Methanol was chosen because it was the enrichment substrate for KB-1. Acetate was added to promote anaerobic conditions more rapidly. Bioaugmentation began on day 176 with the addition of 13 L of KB-1 culture. The culture was shipped to the site in two 8-L stainless steel vessels under a N_2/CO_2 atmosphere. To inoculate the PTA with the KB-1 culture, groundwater recirculation was stopped, and the water column and air above the water column in the IW were purged with argon to reduce the amount of oxygen prior to addition of the KB-1 culture. The culture was injected into the PTA using an argon-flushed delivery line that was submerged in the injection well (IW). The culture was pushed through the delivery line and into the well by pressurizing the tank (5 psig) with argon delivered from a gas cylinder connected to the vessel. After injection of the KB-1 culture, recirculation was restarted briefly to allow injection of three well-casing volumes of reduced groundwater containing electron donors. Recirculation was stopped once again for 24-hours to provide time for the bacteria in the culture to adhere or otherwise become established near the IW.

Design and Operation of the Control Plots. Two biostimulation control plots were constructed in the same manner as the PTA. Both plots were amended with the same concentrations of electron donors as used in the PTA but were never amended with KB-1. Control plot 1 was located approximately 80 m upgradient and north of the PTA and control plot 2 was located approximately 41 m downgradient and south of the PTA. Control plot 1 was sampled at day 0 and day 216. Control plot 2 was operated for 117 days and was sampled five times.

Collection of Groundwater Samples for Chemical Analysis. Groundwater samples from monitoring wells were collected using dedicated tubing (Waterra Pumps, Ltd., Mississauga, ON) using standard sampling protocols (i.e., the well was purged a minimum of three casing volumes and field parameters (dissolved oxygen, redox parameters, and pH) were monitored prior to collecting analytical samples). Samples were express-shipped, on ice, to Pioneer Analytical Laboratory (Mississauga, ON).

Analytical Procedures for Field Samples. Groundwater samples were analyzed for chlorinated ethenes and methanol using EPA Method 8260 (purge and traps GC/MS). Dissolved hydrocarbon gases were analyzed by headspace GC using a method provided by Dow Chemical Co. (Midland, MI) (*9*). Anions and volatile fatty acids (e.g., acetate, lactate, propionate, and butyrate) were analyzed using ion chromatography. Metals were determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). Field duplicate samples and trip blanks were incorporated into the groundwater sample collection and analysis program.

Collection of Soil Samples for DNA Analysis. Soil samples were collected at depths ranging from 7 to 8.2 m below ground surface using Hollow Stem Auger and split spoon samplers. Soil samples were collected in 6 in. brass liners, capped, and shipped to the laboratory for *Dhc* analysis. Soil samples were collected 3 days prior to bioaugmentation (day 173) and at completion (day 304). The prebioaugmentation samples were collected from a single core taken between the injection well and B1, slightly off the direct flow path. On day 304 samples were taken from a soil boring that was 2 m downgradient of the extraction wells (SB 237, outside of the capture zone) and from borings in the center of each of the two control plots (SB 235 and SB 236).

Collection of Groundwater Samples for DNA Analysis. Groundwater samples were collected from the injection well, the monitoring wells, the extraction wells, and the transgradient wells into 1-L sterile plastic sampling bottles, filled-up to the top, sealed, doubled bagged, and shipped to the laboratory on ice. Upon arrival, the samples were either stored overnight at 4 °C or immediately centrifuged at 9000 × g at 10 °C for 30 min using a GSA or a G-3 rotor in a RC5B Sorvall Superspeed centrifuge (Kendro Laboratory Products, Newtown, CT). The resulting pellets were resuspended in 2 mL of 1× PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.6) and either stored at −20 °C or processed immediately for DNA extraction.

DNA Extraction from Groundwater and Soil Samples. DNA was extracted from pelleted cells from one liter of groundwater or from 10 mL of KB-1 cultures using a bead mill homogenization procedure as previously described (*9*), with the FastDNA Spin Kit (Bio 101, Vista, CA). The purified DNA was eluted from the kit matrix by adding 80 μL of sterile, deionized water, mixing with a pipet tip, and centrifuging for 1 min at 14 000 × g. The flow through product, 50 μL, was transferred to a screw capped 1.5 mL tube and stored at −20 °C until it is needed for the PCR assay. DNA was extracted from 10 g of soil samples using the Mo Bio Soil DNA Kit (Mega Prep, Mo Bio, and Carlsbad, CA) as previously described (*9*). The extracted DNA was ethanol precipitated and resuspended in a final volume of 200 μL of sterile, deionized water.

PCR Amplification of Extracted DNA. DNA was amplified using one of the following *Dehalococcoides*-specific 16S rDNA PCR primer sets: DHC 1F (5′-GATGAACGCTAGCGGCG-3′) with DHC 1377R (5′-GGTTGGCACATCGACTTCAA-3′) or DHC 385F (5′-GGGTTGTAAACCTCTTTTCAC-3′) with DHC 692R (5′-TCAGTGACAACCTAGAAAAC-3′) producing 1377 and 307 bp fragments, respectively (*9*). In some cases, general Bacterial primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used (*9*). PCR amplifications were performed using a Perkin-Elmer 9600 GeneAmp PCR thermal cycler (Applied Biosystems, Norwalk, CT). The PCR reactions contained 1 × PCR Buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl), 1.5 mM $MgCl_2$, 10 μM of each deoxynucleoside triphosphate, 20 pmol of each primer, 2.5

U of Taq polymerase, and 1 μL of extracted DNA in a final reaction volume of 50 μL. The following PCR thermocycling program was used: 2 min of denaturation at 95 °C, followed by using 40 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and finally cooled to 4°C. The PCR products were visualized by agarose gel electrophoresis and stained with ethidium bromide. Product yield was measured by electronic imaging the ethidium bromide stained PCR products, using the Eagle Eye II Still Video System (Stratagene, La Jolla, CA). The electronic images were analyzed for DNA band intensities using NIH Image 1.62 (written by Wayne Rasband at NIH and available from the Internet http://rsb.info.nih.gov/nih-image/download.html). As demonstrated previously, we observed a dose response in the PCR assay over several orders of magnitudes of target copy number (*9*). Using the DHC 385F/DHC 692R primer set with a cloned *Dhc* 16S rDNA sequence (*9*), the detection limit was 10−100 copies of the *Dehalococcoides* sequence per PCR reaction.

Cloning and Sequencing of Amplified *Dhc* Sequences. The PCR fragments produced from the *Dehalococcoides*-specific amplification reactions were cloned into vector pCR4-TOPO using the TOPO TA Cloning system (Invitrogen, Inc., San Diego, CA) as previously described (*9*). Plasmid DNA was extracted and purified using the QIAvac system (Qiagen, Inc., Valencia, CA) as previously described (*9*). The cloned amplicons were sequenced using the fluorescent Dye-Deoxy method and the Model 377 DNA Sequencer system, (Applied Biosystems, Perkin-Elmer, Foster City, CA). Both strands of the cloned DNA were completely sequenced. The sequences were assembled and edited using the Seqman II program (ver. 4.0) (*20*) (DNAstar, Inc., Madison, WI).

Phylogenetic Analysis of 16S rRNA. Using Blast (*21*) or FastA sequence analysis programs (*22*), 16S rDNA sequences were analyzed to verify their identity. The sequences were also verified using the Ribosomal Database Project II (RDP) database and its online programs at http://www.cme.msu.edu/RDP/cgis/probe_match.cgi?su=SSU, RDPII (Michigan State University, East Lansing, MI) (*23*). Default values of the software were used unless otherwise specified. The 16S rDNA *Dehalococcoides* sequences found in the KB-1 culture were compared to one another and to *Dehalococcoides* sequences from the databases (*1*, *9*, *24*, *25*) to determine their sequence relationship using either the Pileup and Neighbor-joining tree GCG programs in the Wisconsin Package Version 10.0 (Genetics Computer Group (GCG), Madison, WI) or the Clustal alignment and phylogenetic tree functions of the MegAlign program in the DNAStar package (DNAStar, Inc., Madison, WI).

Results

Characterization of the KB-1 Enrichment Culture. Many of the features of KB-1 have been reported elsewhere (*18*). KB-1 was found to contain as many as three different 16S rDNA sequences, all closely related to *D. ethenogenes* Strain 195 (*18*). Two of these have been sequenced (*9*) and were designated as *Dhc-kb1P* and *Dhc-kb1C* (GenBank accession numbers AF388540 and AF388530, respectively). The *Dhc-kb1P* sequence was much more abundant, representing 99% (190 of 192) of the16S rDNA clones tested. This sequence has unique signatures that provided a specific method to track the fate of the introduced culture and distinguish it from other *Dehalococcoides* (Table 3). The titer of *Dehalococcoides* within the culture was approximately 10^6 cells/mL at the time of this study as determined by DHC PCR of serially diluted genomic DNA.

Microcosm Study with Samples from the PTA. Prior to the pilot test, a microcosm treatability study was conducted using water and soil samples taken in the PTA before the recirculation system was constructed. The objective of the microcosm study was to determine if the native microorganisms could be stimulated to effect dechlorination beyond cDCE. The microcosms also provided a method to test the efficacy of bioaugmentation. TCE rather than PCE was amended to the microcosms because there was sufficient field data indicating that PCE was being transformed to TCE. Furthermore, dechlorination past cDCE was the focus of the microcosm test; therefore, TCE was selected as the starting compound. There was no significant loss of TCE in sterile or intrinsic control microcosms and no formation of degradation products after 120 days of incubation (data not shown). Stoichiometric conversion of TCE (6 μM) to cDCE was observed in the lactate or methanol-amended microcosms (Figure 2A,B). Dechlorination of TCE to cDCE was completed sooner in the lactate-amended microcosms; however, neither treatment resulted in further conversion of cDCE to VC or ethene, even after over 120 days of incubation (Figure 2A,B). In all of the microcosms inoculated with KB-1 and amended with TCE at 6 μM (Figure 2C), 60 μM (data not shown), and 600 μM (Figure 2D) and methanol, all of the TCE was converted stoichiometrically to ethene.

Characterization of Kelly AFB *Dehalococcoides* 16S rDNA Sequences Detected in and around the PTA before Bioaugmentation. Soil and groundwater from the PTA were tested for *Dehalococcoides* organisms using the DHC PCR assay. *Dehalococcoides* sequences were not detected in any of the six samples tested. These results are consistent with the microcosm studies, where reductive dechlorination stalled at cDCE. Together, the results of the DHC PCR assay and of the microcosm study indicated that *Dehalococcoides* organisms capable of dechlorinating beyond cDCE to VC and ethene were not present in the PTA. In contrast, *Dehalococcoides* sequences were detected in soil and groundwater samples from the landfill trenches 2.4 km away where complete dechlorination was being observed. The same *Dehalococcoides* sequence was found in both soil and groundwater samples taken from the landfill trenches. This sequence, designated as *Dhc-klf* (GenBank Accession no. AF388541) belongs to the *Dehalococcoides* Cornell sequences group (*9*) and is closely related to the *D. ethenogenes* Strain 195 16S rDNA sequence. *Dhc-klf* is significantly different from the sequences in KB-1.

PTA Hydraulics. The size of the capture zone in the PTA was approximately 10 m long, 7.6 m wide, and 3.1 m deep. This volume was estimated using an iterative process of comparing the flow and transport model output to the tracer breakthrough data. Using a porosity of 0.3, the approximate volume of water in the PTA was approximately 64 m^3. Under pumping conditions, it was determined that the average bromide breakthrough time at wells B1, B2, and B3 was 4.1, 7.8, and 21.7 h, respectively, with distances from the IW to B1, B2, and B3 of 2.4, 4, and 7 m, respectively. The estimated average linear groundwater flow velocity was 14.3 $m.day^{-1}$. Based on the time to recover the mass of bromide injected, the time to capture and recirculate one pore volume was approximately 7.8 days. From day 89 (electron donor addition started) to day 318 (last sampling event) a total of 39 pore volumes were recirculated through the PTA. Approximately 24 pore volumes were recirculated during the bioaugmentation period (from day 176 to day 318). A bromide mass balance indicated that the capture efficiency of the PTA was approximately 90%.

Chlorinated Ethenes and Ethene Concentration Trends in the PTA. The concentrations of PCE, TCE, cDCE, VC, ethene, and methane were measured in all the wells at specific time intervals. The concentrations in samples taken from each well were plotted as a function of time (Figure 3). During 173 days of biostimulation, which involved the addition of only electron donors (no inoculum), PCE concentrations in the PTA declined by more than 90%, and the dominant degradation product was cDCE. VC and ethene were not detected. On day 176, the PTA subsurface was inoculated with KB-1. After 52 days (day 228) trace amounts of VC were

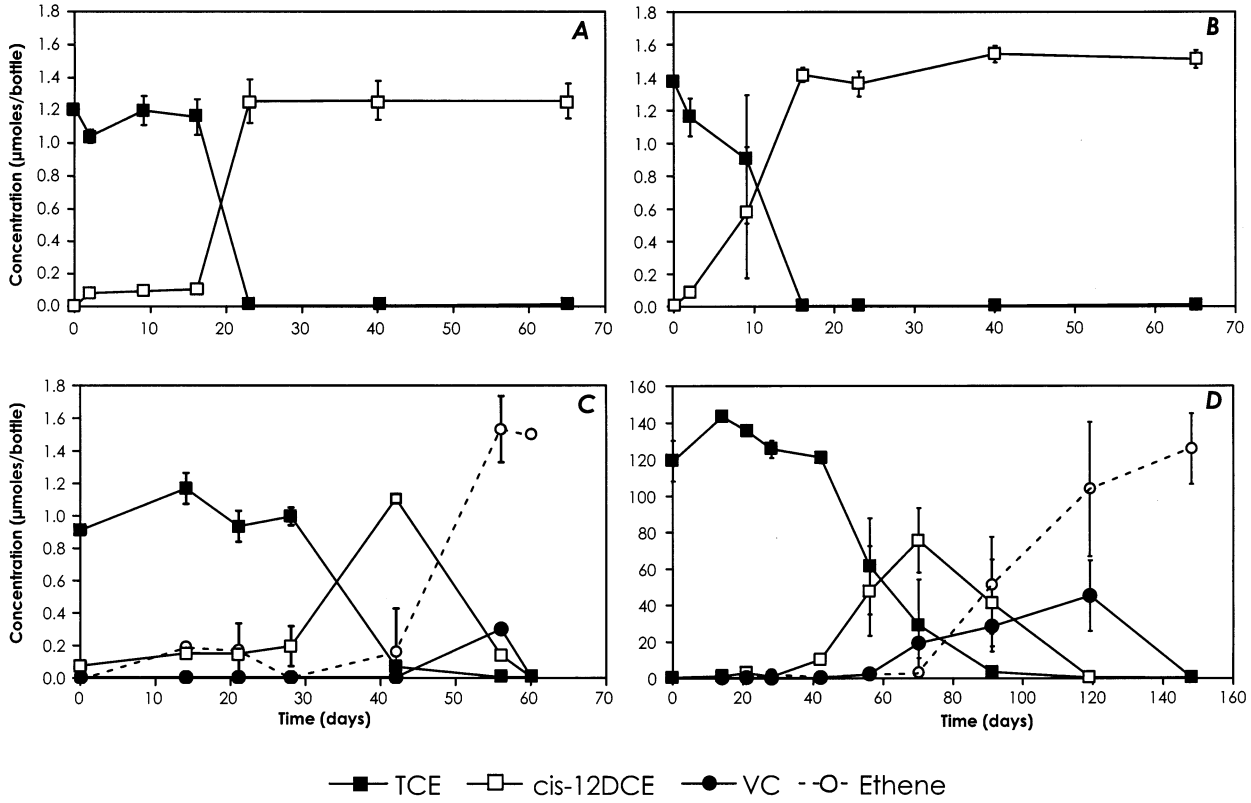

FIGURE 2. TCE dechlorination in microcosms. Methanol-amended microcosms (A), Lactate-amended microcosms (B); KB-1 bioaugmented microcosms (C and D). The initial aqueous TCE concentration was 6 μM (0.8 mg/L) in graphs A, B, and C and was 600 μM in graph D. The data are presented in units of μmoles per bottle to account for both liquid and gas-phase concentrations. TCE − closed squares; cDCE − open squares; VC − closed circles; ethene − open circles and dashed line. Data represent average of 3 microcosms. Error bars represent one standard deviation from the mean.

detected. Ethene was detected 21 days later on day 249. By day 318, ethene was the predominant compound within the PTA (Figure 3).

The change in concentration of chlorinated ethenes and ethene between the injection well (IW) and first monitoring well (B1) from day 248 to 318 (72 to 142 days after bioaugmentation) was used to estimate maximum field biodegradation rates. This location and time period represented the area where maximum biodegradation rates would be observed because of the continuous presence of electron donors, highest concentrations of chlorinated ethenes, and probably greatest biomass density. The short travel time of approximately 4.1 h between the IW and B1 also minimized concentration changes due to advection and dispersion relative to biodegradation. The following equations were used to estimate a first-order reaction rate constant (h^{-1}) based on the sequential decay and production of the daughter products.

$$r_{PCE} = -\lambda_1 C_{PCE}$$

$$r_{TCE} = \lambda_1 C_{PCE} - \lambda_2 C_{TCE}$$

$$r_{DCE} = \lambda_2 C_{TCE} - \lambda_3 C_{DCE}$$

$$r_{VC} = \lambda_3 C_{DCE} - \lambda_4 C_{VC}$$

$$r_{ETH} = \lambda_4 C_{VC}$$

λ_1, λ_2, λ_3, and λ_4 are first-order biodegradation rate constants and C_{PCE}, C_{TCE}, C_{DCE}, C_{VC}, and C_{ETH} are the aqueous molar concentrations of PCE, TCE, DCE, VC, and ethene, respectively. First-order reaction rates were assumed to be adequate given the low concentrations of chlorinated ethenes in the PTA. Ethene was assumed not to undergo further degradation under these conditions. The actual micromolar concentrations of chlorinated ethenes and ethenes on specific sampling days in the IW and first monitoring well B1 are presented in Figure 4. These data demonstrate that good mass recovery was achieved between these two wells. Rates were estimated assuming a travel time from IW to B1 of 4.1 h. Using an Excel (Microsoft, Redmond, WA) spreadsheet and the equations listed above, we could estimate the rates of degradation that best reproduced the observed concentrations of chlorinated ethenes and ethene in B1 (see predicted concentrations in Figure 4). This rough exercise yielded approximate reaction rate constants for the less chlorinated ethenes (cDCE and VC) of the order of 0.1−0.9 h^{-1} and for PCE and TCE of 1−3 h^{-1} (Table 1). Moreover, the data also suggest that the rates of cDCE and VC dechlorination increased over time. The same rate constants also predicted the concentrations observed at B2 reasonably well (data not shown).

In the laboratory microcosms with the lowest initial TCE concentrations (similar to field conditions), the maximum rate of ethene production corresponded to first order rate constants of about 0.01 per hour. This rate was about 10 times slower than the maximum rate of ethene production observed between the injection well (IW) and first monitoring well (B1). We attributed this difference to local donor limitations in the batch microcosms, because the microcosms were intermittently amended with donor and were not shaken. Moreover, the calculations in the field represent maximum field rates where the highest concentrations of organisms, acceptor (chlorinated ethenes) and donor were present. In other parts of the PTA, the rates of ethene production were considerably slower, especially further downgradient where electron donor became depleted.

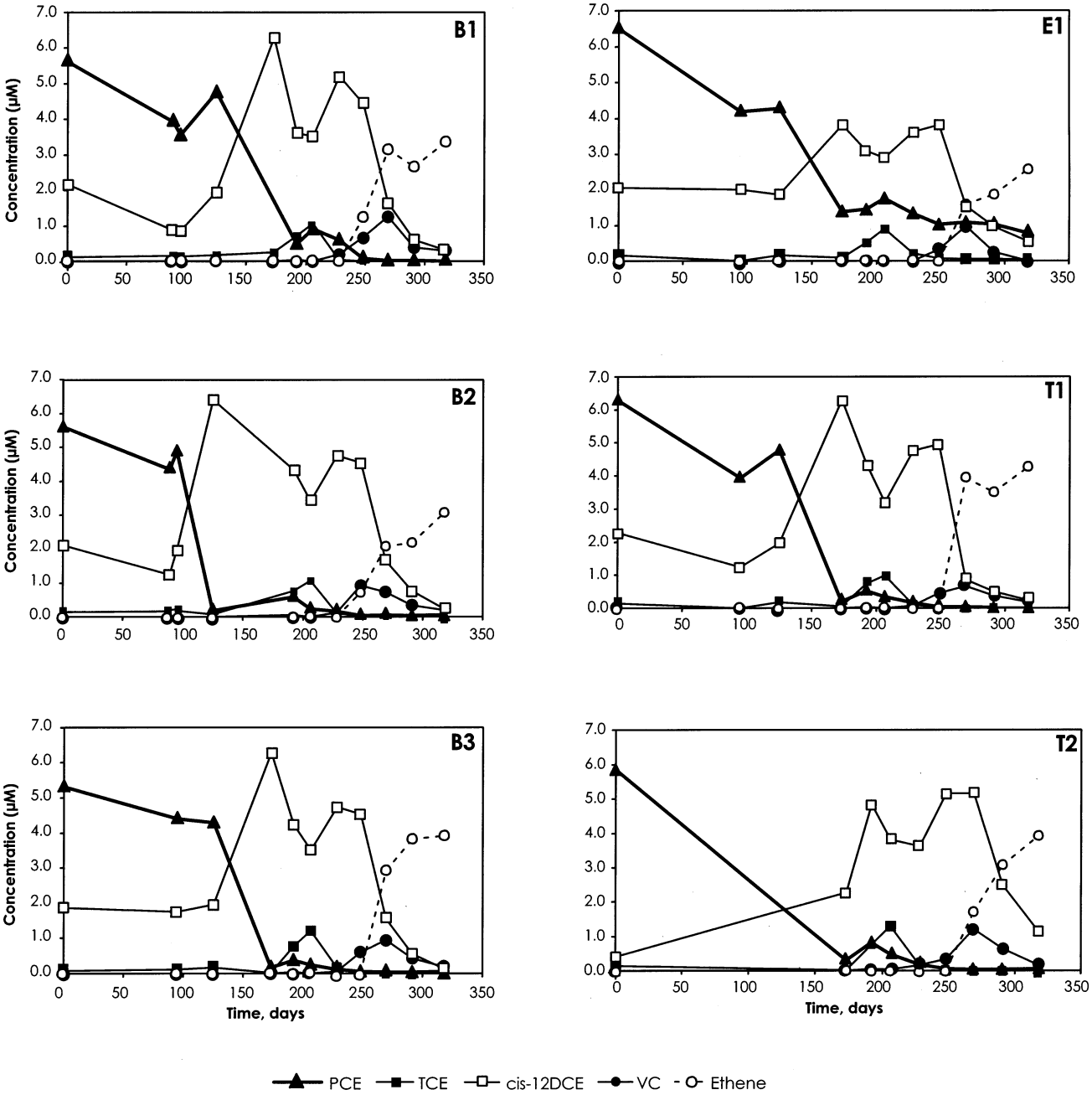

FIGURE 3. Chlorinated ethenes and ethene concentrations in each well (B1, B2, B3, E1, T1, and T2, see Figure 1 for location of wells) over time in the Pilot Test Area. Bioaugmentation with KB-1 occurred on day 176.

Geochemical Trends in the PTA. Prior to biostimulation, the groundwater was generally aerobic and oxidizing as indicated by the average concentrations of major geochemical parameters (21 mg/L nitrate, 16 mg/L sulfate, 0.1 mg/L dissolved iron, 3 mg/L dissolved oxygen and nondetectable concentrations of methane). Prior to bioaugmentation, the biostimulated groundwater was anaerobic and reduced, as indicated by the average concentration of major geochemical and redox parameters (<0.5 mg/L nitrate, 7 mg/L sulfate, 3 mg/L dissolved iron, 6 mg/L methane, less than 1 mg/L of dissolved oxygen, and about −200 mV oxidation−reduction potential). Anaerobic and reducing conditions were maintained throughout the remainder of the pilot test period.

Concentration Trends in the Two Control Plots. Dechlorination beyond cDCE was not observed in either control plot. After 216 days of donor amendment, VC and ethene were still not detected (<3 ug/L) in control plot 1. Initially, the mass distribution of PCE, TCE, and cDCE was 60%, 2%, and 38%, respectively. On day 216, the distribution was 37%, 23%, and 40%, respectively. Methane concentrations in Control plot 1 increased from nondetect at day 0 to an average of 1.3 mg/L by day 216 indicating the establishment of strongly reducing conditions. Similarly, in control plot 2, the addition of electron donors led to the stoichiometric formation of cDCE from PCE between days 50 and 76, with no trace of VC or ethene after continued donor amendment through 117 days. These results were similar to what was observed in the donor-amended microcosms.

Detection of *Dehalococcoides* 16S rDNA Sequences during Biostimulation and Bioaugmentation. *Dehalococcoides* 16S rDNA sequences were not detected in groundwater samples from the PTA taken during the biostimulation phase of the pilot test on day 1, day 126, or day 173 (the latter only 3 days before bioaugmentation) (Table 2; Figure 5). After bioaugmentation, *Dehalococcoides* sequences were detected initially only in the injection well (IW) but were later detected

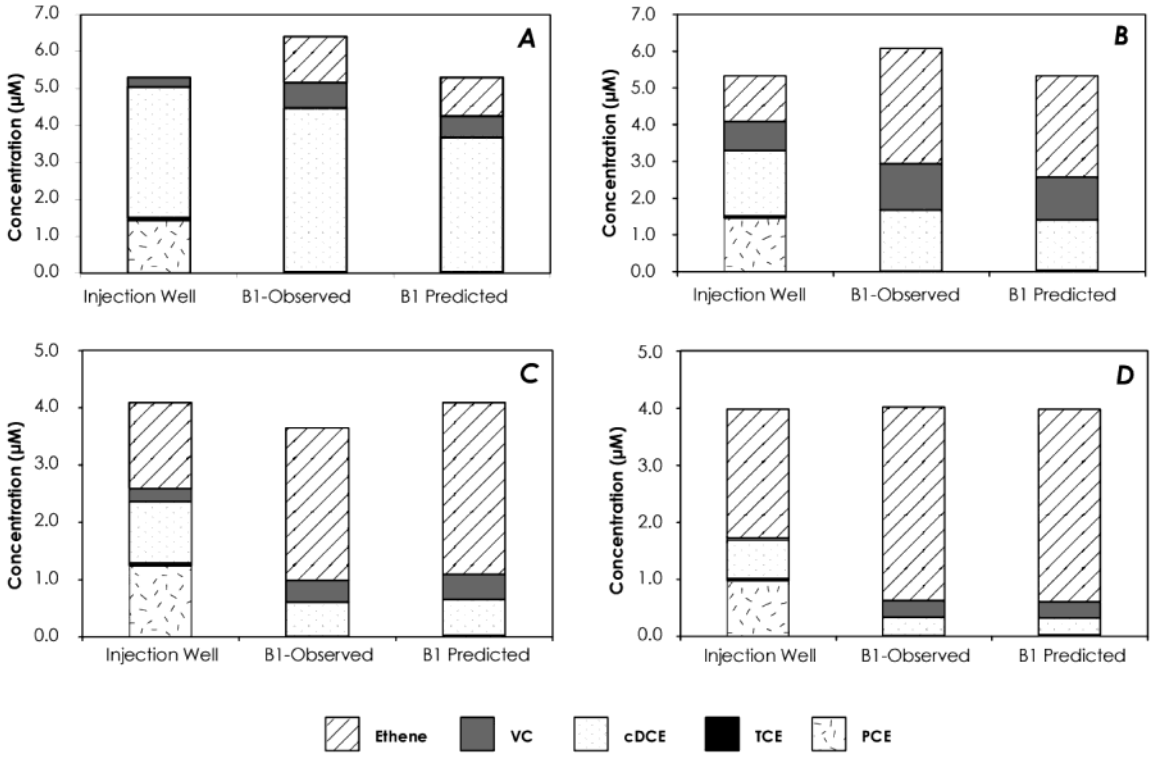

FIGURE 4. Figure 4. Distribution of observed and modeled chlorinated ethenes and ethene concentration after bioaugmentation. A. 72 days after bioaugmentation; B. 93 days after bioaugmentation; C. 115 days after bioaugmentation. D. 142 days after bioaugmentation. The travel time between IW and B1 was estimated at 4.1 h. The predicted concentrations were determined using the sequential first-order decay and production equations presented in the text. The reaction rate constants were modified for PCE, TCE, cDCE, and VC to achieve the best match to the observed concentrations of each compound in B1 given the starting concentration in the IW for each date. The best match reaction rate constants are presented in Table 1.

TABLE 1. Estimated First-Order Degradation Rate Constants for Dechlorination of Each Chlorinated Ethenes in the Most Active Zone between the Injection Well (IW) and the First Monitoring Well (B1)[a]

days post bioaugmentation	modeled dechlorination first-order rate constants (h^{-1})			
	PCE	TCE	cDCE	VC
72	1.0	3.0	0.1	0.6
93	1.0	2.5	0.3	0.4
115	1.0	2.5	0.4	0.8
142	1.0	2.5	0.6	0.9

[a] An Excel spreadsheet using the equations presented in the text was used to model the sequential first-order decay and production of daughter products. The first-order decay rate for PCE, TCE, cDCE, and VC were adjusted up or down by 0.1 $hour^{-1}$ increments until observed and predicted values matched as closely as possible. These values result in a good match between the observed and predicted molar ratios of each chlorinated ethene and ethene as shown in Figure 4.

TABLE 2. Detection of *Dehalococcoides* Sequences in PTA Groundwater after Bioaugmentation

sample date	day[a]	monitoring well number							
		IW	B1	B2	B3	T1	T2	E1	E2
3-May-00	−3	0	0	0	NT	NT	NT	0	NT
6-May-00	1	1	0	NT	NT	NT	NT	0	NT
22-May-00	17	3	0	NT	NT	NT	NT	0	NT
5-Jun-00	30	3	1	NT	NT	NT	NT	0	NT
27-Jun-00	51	2	2	NT	NT	NT	NT	0	NT
17-Jul-00	72	1	3	2	1	NT	NT	0	NT
7-Aug-00	93	1	3	3	1	NT	NT	1	NT
29-Aug-00	115	1	3	3	2	2	2	2	1
24-Sep-00	142	1	3	3	2	3	2	2	1

[a] Day − number of days after bioaugmentation. NT- Not tested. Relative Pixel PCR pixel density as measured by NIH Image 1.62. 3 = highest pixel density of *Dehalococcoides* sequence (> 10^5 *Dhc* copies per reaction). 2 = Midrange pixel density of DHC PCR product (10^3–10^5 *Dhc* copies per reaction). 1 = Low range pixel density of DHC PCR product (10–10^3 *Dhc* copies per reaction). 0 = No DHC PCR product detected (<100 *Dhc* copies per reaction).

downgradient and throughout the PTA (Table 2; Figure 5). Furthermore, there appeared to be an increase in the abundance of the *Dehalococcoides* sequences in the samples over time based on the intensity of the signal as well as movement of the organisms through the PTA (Table 2; Figure 5). At the completion of the study, *Dehalococcoides* sequences were detected well above the detection limit in all monitoring and extraction wells (Table 2). In all samples analyzed from day 173 (just prior to bioaugmentation) and after bioaugmentation, a strong signal was obtained using the Bacterial PCR primer set, confirming that the DNA extraction efficiency was satisfactory and relatively consistent from sample to sample (data not shown). From the DHC PCR assay results, we can estimate the relative cell counts for *Dehalococcoides* in the PTA over time. If we assume that the DNA extraction efficiency was similar for each analysis, then we can compare the PCR results. This assumption is reasonable since the same DNA extraction protocol was used for all aqueous samples, the matrix (i.e. groundwater) was the same for all samples, and the PCR signal using bacterial primers was relatively consistent. The initial 13 L inoculum contained at least 10^6 *Dehalococcoides* cells/mL. If those cells were simply completely mixed into the PTA, with an estimated volume of 64 m^3, then the average concentration of *Dhc sequences* in the PTA would be 200 cells/mL. This is well above the detection limit of the assay, even assuming a really poor DNA extraction efficiency. The results in Table 2 show that 17 days after inoculation, or after over 2 pore volumes had been recirculated, *Dehalococcoides* was still only detected in the injection well and not detected in the first monitoring well

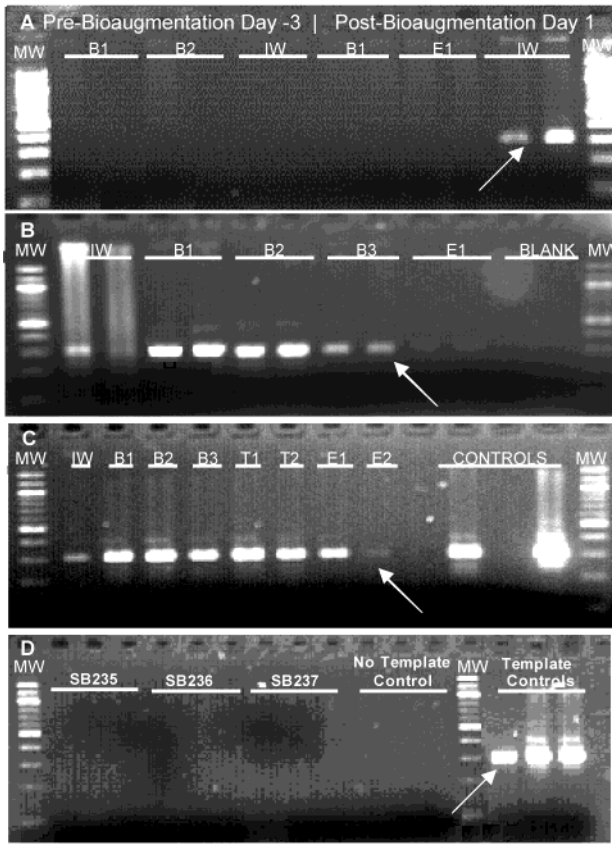

FIGURE 5. Detection of *Dehalococcoides* sequences using DHC PCR assay with primers DHC 385F and DHC 692R in groundwater and soil samples from the wells in the pilot test area and borings in the two control plots. The white arrows indicate the 307 bp DHC PCR product. MW: Molecular weight marker (100 bp ladder, New England BioLabs, Beverly, MA). The PCR template control was a cloned *Dehalococcoides*-related 16S rDNA, *Dhc-vic* (*9*). See Figure 1 for names and location of wells. (A) Groundwater samples collected day 173, 3 days prior to bioaugmentation with KB-1 and from day 177, 1 day after bioaugmentation. (B) Groundwater samples collected 73 days after bioaugmentation on day 269. (C) Groundwater samples collected 142 days after bioaugmentation on day 318. (D) Soil samples taken on day 304, 128 days after bioaugmentation, from the Control Plots (SB 235, SB 236) and from 2 m downgradient of E1 (SB 237).

or in the extraction well. These data indicate that the organisms remained attached or trapped in the aquifer near the injection well and were not significantly transported with groundwater flow. However, as time progressed, so did the number of wells downgradient where *Dehalococcoides* was detected, suggesting transport of a small number of cells (initially undetected) and subsequent growth. At the end of the test, 142 days after bioaugmentation, all of the wells had detectable concentrations of *Dehalococcoides* DNA, even the transgradient wells, and most had strong signals indicating relatively high concentrations. Overall, the signal intensity in all samples was far above background, suggesting that all samples contained greater than 10^4 copies of *Dhc* DNA per PCR reaction. Assuming 100% DNA extraction efficiency (which is unrealistic, but conservative in this calculation), this PCR results indicated that there was at least an average of 10^3 cells per mL of groundwater in the PTA on day 318. This is an order of magnitude higher than the original inoculum. The true cell concentration is likely higher, because DNA extraction is not 100%, and because we did not account for cells attached to aquifer solids. In addition, the concentration of *Dehalococcoides* at the end of the study was clearly much higher than the initial concentration in several downgradient wells (signal range 3 or greater than 10^4 cells/mL), and these relatively high concentrations are consistent with cell growth occurring in the vicinity of these wells.

TABLE 3. 16S rDNA Sequence Differences Relative to *Dehalococcoides* Strain 195[a]

strain 195 base no.	strain 195 AF004928B	*Dhc-klf* AF388541	*Dhc-kb1P or Dhc-kafb* AF388540 or AF388537
66	A	A	G
144	A	A	G
150	T	T	C
157	A	A	T
160	C	C	T
167	T	T	C
176	C	C	T
181	T	T	C
265	A	G	A
294	G	A	G
295	G	C	C
405	C	C	T
480	C	−	−
570	T	T	A
919+	−	C	C
942	A	A	T
953	C	C	T
961	A	A	G
982	G	G	A
983	T	T	C
1031	G	T	T
1057+	−	C	C
1112	G	A	G
1195	T	T	C
1291	A	A	T

[a] Base no.: base position in the 16S rDNA sequence for *Dehalococcoides ethenogenes* Strain 195. Positions not listed were identical to Strain 195 in the other three sequences. "+": insertion in sequence relative to Strain 195 (after specified base no.). "−": deletion in sequence relative to the other sequences.

At five different times after bioaugmentation, PCR-amplified DNA was cloned and sequenced. In all, 192 clones containing the 1377 base PCR product were sequenced. All of the amplified sequences from within the PTA were found to be identical and were designated *Dhc-kafb* (GenBank Accession no, AF388537) (*9*). The 1377 base sequences of *D. ethenogenes* Strain 195 (Accession No. AF004928), *Dhc-klf* (*Dhc* sequence from landfill trenches), and *Dhc-kb1P* (*Dhc* sequence from KB-1) and *Dhc-kafb* (detected at the site after bioaugmentation) were aligned and compared (Table 3). The sequence repeatedly found in the PTA samples after bioaugmentation, *Dhc-kafb*, was an exact match to the KB-1 sequence *Dhc-kb1P*. Therefore, based on both the PCR monitoring data and sequence comparisons, we can conclude that not only did *Dehalococcoides* organisms grow in the PTA during the bioaugmentation phase but also that these organisms originated from KB-1. If a native *Dehalococcoides* organism had been stimulated, for example with a sequence similar to that found in the landfill trenches 2.4 km away, then we would have detected different sequences in the genomic DNA extracted from PTA samples.

On day 304, 128 days after bioaugmentation, background control soil samples were taken and assayed for *Dhc*. SB236 and SB235 were taken from the center of control plots 1 and 2, respectively, and a third soil sample, SB237, was taken 2 m downgradient from the extraction wells. The DHC PCR assay for each of these background samples was below detection limit (Figure 5D). Samples were also taken from the Kelly PTA, one year after the pilot pump and feeding system had been turned off. The *Dhc-kafb* sequences still could be detected in IW, B2, and E1, indicating that the organisms were still present in the PTA (data not shown).

Discussion

The presence of organisms in the *Dehalococcoides* group appears to correlate with the complete dechlorination of

chlorinated ethenes to ethene (*9*, *10*, *14*, *26*). At present it is the only group of organisms known to transform cDCE and VC to ethene. Moreover, these microorganisms are generally not found at locations where only partial dechlorination of PCE and TCE to cDCE is observed (*9*). The explanation for the specific localization of *Dehalococcoides* in the environment is unclear but may be mediated by geochemical factors, presence of electron donors, microbial populations, and other historical practices. Amending contaminated subsurfaces with electron donor, biostimulation, was not successful at stimulating complete dechlorination at both at the Dover site (*12*) and in this study at the Kelly site, despite achieving the appropriate reducing conditions.

This study has demonstrated that bioaugmentation with a dechlorinating enrichment culture stimulated complete dechlorination at Kelly AFB. The changes in concentration and location of *Dehalococcoides* sequences with time in the PTA correlated with the transformation of PCE, TCE, and cDCE to vinyl chloride and finally to ethene. Most importantly, the final concentration and distribution of *Dehalococcoides* sequences in the PTA could only be explained if growth had occurred. Several observations support this conclusion: 1) the organisms did not travel through the PTA as rapidly as groundwater (i.e., they were not simply recirculated with the pore water); 2) the concentration of *Dehalococcoides* sequences in downgradient wells increased with time; 3) the total number of *Dehalococcoides* in the PTA at the end of the test was significantly greater than the number originally introduced with the inoculum; and 4) the KB-1 *Dehalococcoides* sequences were detected in the PTA one year after the end of the test.

Concentration and flow data from test and control plots confirmed that PCE was converted completely to ethene in the Kelly AFB pilot test only after bioaugmentation. The role of the inoculum (KB-1) in promoting complete transformation to ethene was firmly established by the following observations: 1) incomplete dechlorination over 160 days in donor-amended microcosms, 2) incomplete conversion in 117 or 216 days in biostimulated control plots, 3) absence of *Dehalococcoides* DNA sequences in the control plots and in the PTA before bioaugmentation, 4) stoichiometric conversion of PCE through to ethene in the PTA after bioaugmentation with KB-1, 5) detection of specific sequences unique to KB-1 in the PTA after inoculation, and 6) increase in abundance of KB-1 *Dhc* sequences over time. The estimated rates of dechlorination were fastest in the zone between the injection well and the first monitoring well, where the highest concentrations of donor, acceptor, and biomass were found. Moreover, the rates of cDCE and VC dechlorination were found to increase with time, further supporting that growth of the organisms occurred in the PTA.

Bioaugmentation may offer a solution for contaminated sites where dechlorination is not occurring naturally (where the appropriate organisms are lacking) or where it is too slow to be practical (very low numbers of dechlorinating organisms). Bioaugmentation may therefore increase the number of sites where anaerobic bioremediation could be applied. The prospects for scale-up of the technology are encouraging, as only a small inoculum volume of KB-1 (0.02%) was required to effectively inoculate the PTA. Observed half-lives of chlorinated ethenes to ethene in the most active zone were in the order of hours, meaning that this technology could be competitive with other remediation technologies provided that donor concentrations and organism populations can be maintained in situ over the long term.

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Multiple Reductive-Dehalogenase-Homologous Genes Are Simultaneously Transcribed during Dechlorination by *Dehalococcoides*-Containing Cultures

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Degenerate primers were used to amplify 14 distinct reductive-dehalogenase-homologous (RDH) genes from the *Dehalococcoides*-containing mixed culture KB1. Most of the corresponding predicted proteins were highly similar (97 to >99% amino acid identity) to previously reported *Dehalococcoides* reductive dehalogenases. To examine the differential transcription of these RDH genes, KB1 was split into five subcultures amended with either trichloroethene, *cis*-1,2-dichloroethene, vinyl chloride, 1,2-dichlorethane, or no chlorinated electron acceptor. Total RNA was extracted following the onset of reductive dechlorination, and RDH transcripts were reverse transcribed and amplified using degenerate primers. The results indicate that the transcription of RDH genes requires the presence of a chlorinated electron acceptor, and for all treatments, multiple RDH genes were simultaneously transcribed, with transcripts of two of the genes being present under all four electron-accepting conditions. Two of the transcribed sequences were highly similar to reported vinyl chloride reductase genes, namely, *vcrA* from *Dehalococcoides* sp. strain VS and *bvcA* from *Dehalococcoides* sp. strain BAV1. These findings suggest that multiple RDH genes are induced by a single chlorinated substrate and that multiple reductive dehalogenases contribute to chloroethene degradation in KB1.

The industrial solvents trichloroethene (TCE) and tetrachloroethene (PCE) are pervasive and persistent groundwater contaminants. TCE- and PCE-contaminated sites frequently contain substantial quantities of the degradation products *cis*-1,2-dichloroethene (cDCE) and vinyl chloride (VC), which accumulate due to biotic and abiotic processes (4, 10, 23). Because of the acute and chronic toxicity of these chlorinated compounds, they pose risks to human and environmental health (13). In addition, these recalcitrant compounds tend to persist in anoxic subsurface environments (32). Fortunately, anaerobic dechlorinating microorganisms have been identified in a variety of genera. Among these, several isolates of the genus *Dehalococcoides* can completely reductively dechlorinate chlorinated ethenes to the nontoxic end product ethene (31). KB1 is a microbial consortium dominated by *Dehalococcoides* organisms (5). The successful stimulation of complete dechlorination of chlorinated ethenes in situ through bioaugmentation has been demonstrated in the field (16, 20). Molecular tools such as PCR detection of dehalogenase genes and transcripts have the potential to aid in monitoring and diagnosing the dechlorinating abilities at a site.

To date, 15 different reductive dehalogenases (RDases) have been at least partially purified from a variety of microbial genera (3, 11, 17, 19, 22–27, 30, 34, 38). These include the first RDase purified from a *Dehalococcoides* organism, TceA of *Dehalococcoides ethenogenes* strain 195 (18, 19). More recently, a VC RDase was partially purified from *Dehalococcoides* sp. strain VS (23). All but one of the RDases have been copurified with a corrinoid cofactor, and all contained iron-sulfur clusters. Physiological studies of whole cells and cell extracts have suggested the expression of different dechlorinating enzymes depending on the type and concentration of the initial dechlorinating substrate (8, 12, 37).

Biochemical purification has proved laborious due to the low growth yields of these organisms as well as the hydrophobic nature and oxygen sensitivity of their membrane-associated proteins. However, molecular investigations of the reductive dehalogenase genes have provided a wealth of sequence information. An analysis of the genome of *Dehalococcoides* strain 195 revealed 17 intact genes that appeared homologous to genes encoding biochemically purified reductive dehalogenases (31, 39). Based on these sequences, Krajmalnik-Brown et al. (15) designed degenerate primers to amplify *Dehalococcoides*-specific reductive-dehalogenase-homologous (RDH) genes. This allowed for the amplification of many RDH genes from different strains of *Dehalococcoides*, including strain BAV1 (7 homologues), strain FL2 (14 homologues), and strain CBDB1 (14 homologues). Although some strain-specific genes were identified, Hölscher et al. (14) identified 10 subclusters of genes with orthologues in two or more different *Dehalococcoides* strains.

The fact that each strain possesses multiple RDHs has prompted investigations of what controls the expression of these genes. Studies with the *cpr* gene cluster of *Desulfitobacterium dehalogenans* have identified two regulatory proteins, *cprC* and *cprK*, whose gene products belong to the NirI/NosR and CRP/FNR families, respectively (33). The transcription of *cprA* and *cprB* was shown to be dependent on the chlorinated substrate 3-chloro-4-hydroxyphenylacetate. A recent examina-

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tion of the *Dehalococcoides ethenogenes* strain 195 genome revealed homologues of *cprC* and *cprK*; however, the majority of the transcriptional regulators were two-component signal transduction systems (31). What remains to be investigated is if these regulatory genes respond to specific substrates and thus cause the transcription of different dehalogenase genes based on the presence of specific chlorinated compounds used as electron acceptors.

The aims of these studies were to first identify the *Dehalococcoides* RDH genes in KB1 and then to understand which of these RDH genes have an active role in reductive dechlorination of TCE, cDCE, VC, and 1,2-dichloroethane (1,2-DCA). To address the latter issue, we identified RDH genes that were transcribed in KB1 during the dechlorination of these four different electron acceptors. Our results indicated that two genes were transcribed in response to all four of the chlorinated electron acceptors and that multiple genes were simultaneously transcribed during the degradation of each of the compounds. This work adds to the RDH sequence data set and further extends it by investigating the differential transcription of these genes under different electron-accepting conditions. This information will be useful in creating molecular tools to aid in the design and monitoring of bioremediation schemes. Accumulating research indicates that the 16S rRNA gene is not sufficient to distinguish the dechlorinating abilities of different strains of *Dehalococcoides* (5, 9, 28). However, the detection of specific RDH genes may allow for the prediction of a site's degradation potential, and the detection of RDH gene transcripts may allow estimates of in situ dechlorination activity.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma-Aldrich, unless noted otherwise. Chlorinated ethenes were of >97% purity (Sigma-Aldrich). A 1% gas mixture of ethene and methane (Scotty II; Alltech Associates, Inc.) was used for gas chromatograph calibration.

Cultures and growth conditions. All KB1 subcultures grown with different chloroaliphatic compounds were derived from a single, TCE-dechlorinating parent culture (6). Subcultures were maintained with different chlorinated electron acceptors in 200- to 2,000-ml volumes in glass bottles (with approximately 10% of the total volume being headspace) and amended with methanol or hydrogen plus acetate as previously described (5). The cultures were maintained in defined mineral medium (7). All KB1 cultures were replenished with 20 to 50% (vol/vol) fresh medium if degradation rates decreased, approximately every 3 to 6 months. A KB1 enrichment culture that was maintained with VC and methanol for 5 years (KB1/VC-MeOH) was the source of inoculum for the transcription experiment described herein. This ethene-producing culture was grown with weekly amendments of 445 μM (liquid concentration) VC from a 100% gas stock and of 2.2 mM methanol. The KB1/VC-MeOH culture contains two identified *Dehalococcoides* 16S rRNA gene sequences, KB1/VC (AY146779) and KB1/PCE (AY146780), and *Dehalococcoides* accounts for about 50% of the biomass, with acetogens and methanogens comprising the remainder. A second subculture (KB1/VC-H_2) was also used to assay specific RDH genes. This subculture contains only one *Dehalococcoides* 16S rRNA gene sequence, KB1/VC, and in this culture, *Dehalococcoides* accounts for >90% of the biomass (5).

Transcription experiment. The KB1/VC-MeOH subculture (2 liters) was purged with a sterile N_2-CO_2 (80:20 [vol/vol]) gas stream that was passed over heated copper filings until the methane, ethene, and VC concentrations were below the detection limits. Following a 70-hour incubation, 400-ml portions of the culture were dispensed into five identical 1-liter bottles (Pyrex; VWR, Mississauga, Ontario, Canada) in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI). The bottles were sealed with plastic screw-cap tops in which a hole had been drilled in order to insert a black butyl rubber septum (Geo-Microbial Technologies, Ochelata, OK) for repeated sampling. Individual chlorinated electron acceptors were added to each bottle by syringe as undiluted compounds in the following amounts: TCE, 150 μmol; cDCE, 300 μmol; VC, 150 μmol; and 1,2-DCA, 200 μmol. These amounts correspond to the following initial aqueous concentrations: TCE, 200 μM; cDCE, 475 μM; VC, 135 μM; and 1,2-DCA, 465 μM. One bottle was not amended with a chlorinated electron acceptor. Headspace samples (300 μl) were then taken (after the compounds had equilibrated) for compositional analysis, and an initial well-mixed liquid sample (50 ml) was taken from the unamended treatment for RNA analysis. Subsequently, methanol was added as the electron donor (495 μmol) to all five bottles to initiate dechlorination. In an effort to detect genes responding to the chlorinated substrate that was added to the culture and not to subsequent dechlorination products, samples for RNA extraction were taken soon after the onset of dechlorination.

Analytical procedures. Chlorinated ethenes and ethanes, methane, and ethene were analyzed by gas chromatography of headspace samples as described previously (5).

Nucleic acid extraction. Genomic DNAs were extracted from 15 ml of liquid culture using an UltraClean soil DNA kit (Mo Bio Laboratories Inc., Solana Beach, CA) as previously described (5). In total, DNA was extracted from six different cultures to capture the breadth of KB1 cultures. For RNA extraction, 50-ml samples were withdrawn from the culture bottles inside an anaerobic chamber (Coy Laboratory Products). To avoid creating excessive vacuum, 60 ml of N_2-CO_2 was injected into each culture bottle with a sterile 60-ml syringe prior to withdrawing 50 ml of culture in the same syringe. The culture sample was then dispensed into an anaerobic centrifuge tube on ice. Cells were collected by centrifugation at 2,000 × *g* for 40 min at 4°C. After discarding the supernatant, the pellet was resuspended in 300 μl of ice-cold lysis solution (1.4 M NaCl, 22 mM EDTA, 35 mM sodium dodecyl sulfate) and transferred to a 1.5-ml screw-cap microcentrifuge tube. Subsequently, 900 μl of ice-cold acid–phenol-chloroform-isoamyl alcohol (125:24:1, pH 4.5) (Ambion, Austin, TX) and 100 μl of zirconia/silica beads (0.5 mm; BioSpec Products Inc., Bartlesville, OK) were added to the microcentrifuge tube. The tube was then agitated horizontally on a vortex machine (VELP Scientifica, Plainview, NY) for 4 min at maximum speed followed by centrifugation at 14,000 × *g* for 3 min at 4°C. The aqueous supernatant was removed and transferred to a new 1.5-ml microcentrifuge tube. Ammonium acetate (0.1 volume of a 5 M solution) and isopropanol (1.1 volumes) were added, and the RNA was precipitated overnight at −20°C. The RNA solution was then purified using an RNeasy spin column (QIAGEN, Valencia, Calif.). Contaminating DNAs were removed using two successive treatments of a DNA-free kit (Ambion, Austin, TX).

Reverse transcription. Each RNA sample was divided into two, with one half serving as a control to which no reverse transcriptase (RT) was added. Total RNA was quantified using the Ribogreen method according to the manufacturer's recommendations (Molecular Probes, Burlington, Ontario, Canada). First-strand cDNA synthesis was performed using 6 to 12 μg of RNA, 250 ng of random hexamers (Invitrogen, Carlsbad, CA), and 10 nmol of deoxynucleoside triphosphates (Invitrogen). The final volume was adjusted to 15 μl using RNase-free water. This mixture was then heated to 65°C for 5 min in a PTC-200 DNA engine thermocycler (MJ Research). The sample was cooled in an ice bath before 2 μl of a 0.1 M dithiothreitol solution (Invitrogen) and 4 μl of 5× first-strand buffer (Invitrogen) were added. The sample was then incubated at 25°C for 5 min. Finally, 1 μl of SuperScript II (Invitrogen) was added, and the thermocycler program was continued at 25°C for 10 min, 42°C for 1 h, and then 70°C for 15 min.

PCR amplification of RDH genes from DNA or cDNA. Degenerate primers B1R and RRF2 (15) were used to amplify RDH genes from genomic DNA (for KB1 RDH gene identification) and cDNA templates (for transcription experiments). PCR mixtures (50 μl) contained 1 to 10 μl of cDNA or 50 ng of genomic DNA, a 0.5 μM concentration of each primer, 2.5 mM $MgCl_2$, a 0.25 mM concentration of each deoxynucleotide (MBI Fermentas, Burlington, Ontario, Canada), 0.13 mg/ml of bovine serum albumin, and 1.25 U of *Taq* DNA polymerase (New England Biolabs, Beverly, MA) in 1× PCR buffer (New England Biolabs). PCRs were carried out with the following parameters: 130 s at 94°C; 30 cycles of 30 s at 94°C, 45 s at 48°C, and 130 s at 72°C; and a final extension of 6 min at 72°C. Agarose gels (1%) were run to verify amplification and amplicon sizes. To detect contamination of the cDNA with genomic DNA, the PCR products from cDNAs generated without RT were also analyzed in agarose gels.

RDH-specific primers. Specific PCR primers were designed for each of the RDH genes found in the KB1 subcultures (Table 1). For specific RDH genes, PCRs were carried out as described above, except that the annealing and extension steps were performed for only 30 s, and the annealing temperature was 60°C. The specificity of these primers was assessed by attempting PCR amplification of nontarget DNA from *Escherichia coli*, a mixed toluene-degrading consortium, and clones containing nontarget RDH genes. In addition, PCRs were performed

TABLE 1. Specific primer sequences designed for KB1 RDH genes

KB1 gene target	Primer name[a]	Sequence (5′-3′)
rdhA1	rdhA1_246f	ATCGGAGCTGCACAAGTAGG
	rdhA1_336r	TCTTGTGAGCGGTGTCTTTG
rdhA2	rdhA2_720f	CAAAGGAGATGTTCCGGTGT
	rdhA2_985f	CAGGTGGAAAAGACCGGTTA
rdhA3	rdhA3_1149f	CATTCTCCGGGAAGAAAACA
	rdhA3_1379r	CCAGGCTTCCTTGTCTTCAG
rdhA4	rdhA4_754f	TTGTTATGCCGCCAATATGA
	rdhA4_925r	TCTATCCATTTCGCCCAGAC
rdhA5	rdhA5_1017f	GATGCAGGCATTTACCGTTT
	rdhA5_1137r	GTCTCTTTGCCTTCGGTCAG
rdhA6	rdhA6_318f	ATTTAGCGTGGGCAAAACAG
	rdhA6_555r	CCTTCCCACCTTGGGTATTT
rdhA7	rdhA7_1391f	GCTAAAGAGCCGTCATCCTG
	rdhA7_1539r	GCAGTAACAACAGCCCCAAT
rdhA8	rdhA8_845f	CCCAAGGTAGGTGTGCAGAT
	rdhA8_1016r	CCCGGTTAGTTACCCCGTAT
rdhA9	rdhA9_251f	CTGACCTTGAAACCCCTGAA
	rdhA9_425r	TTGCCACCCATTTCCATATT
rdhA10	rdhA10_710f	GCTGAAACACCCACCAAACT
	rdhA10_860r	CGACAAAGGGGAATCTTTGA
rdhA11	rdhA11_429f	TAATGGCAACCGGAGGTAAG
	rdhA11_609r	TCTACCGGTATGGCCTGAAC
rdhA12	rdhA12_864f	AGGAGTTCCTGTGGGGACTT
	rdhA12_994r	TTTGGGGGTCATAACTGCTC
rdhA13	rdhA13_1356f	CAGGGTACCTGTCCCTTCAA
	rdhA13_1493r	AGGGTTCTTCCGTCCGTACT
rdhA14	rdhA14_642f	GAAAGCTCAGCCGATGACTC
	rdhA14_846r	TGGTTGAGGTAGGGTGAAGG

[a] The position numbers are shown within the names with respect to the first nucleotide of each gene following the RRF2 primer binding site. f, forward; r, reverse.

with genomic DNA from three different KB1 subcultures at three different annealing temperatures. The resulting amplicons were sequenced, the chromatograms were visually inspected, and the sequences were aligned.

Cloning. For each cloning reaction, amplicons from three to five PCRs were pooled, ligated into the pCR2.1-TOPO vector, and used to transform One Shot TOP10 competent *E. coli* cells (Invitrogen, Carlsbad, California). The success of the cloning reaction was determined by using an overnight *E. coli* culture as the template for PCR amplification of the insert with T7f and M13r primers and subsequent product visualization on a 1% agarose gel. For cultures in which the plasmids had appropriate insert sizes (1.4 to 1.7 kb), plasmid DNA was extracted using a GeneElute plasmid miniprep kit (Sigma-Aldrich, Oakville, Ontario, Canada).

Sequence determination. Sequencing of the plasmid inserts was carried out by the Ontario Cancer Institute's sequencing facility (Princess Margaret Hospital, Toronto, Ontario, Canada), using a Beckman Coulter CEQ 2000 automatic sequencer. Inserts were sequenced using vector primers T7 forward and M13 reverse (Invitrogen).

Sequence assembly and analysis. A total of 87 inserts, derived from both DNA and cDNA templates, were sequenced with both the T7f and M13r primers. For each unique insert, 3 to 10 times sequence coverage was obtained over the entire length. Internal sequencing primers were designed using Primer3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The sequences were assembled into contigs using the EditSeq and SeqMan programs of the DNAStar software package (DNASTAR Inc., Madison, WI). Conceptual translations were made using the translate tool of Expasy (http://us.expasy.org/tools/dna.html). These amino acid sequences were aligned and compared to known sequences using ClustalX (36). Manual editing of the alignment was performed using Genedoc (http://www.psc.edu/biomed/genedoc). Additionally, an identity/similarity matrix was constructed using MatGAT, a program that creates pairwise alignments (2). Phylogenetic trees were constructed using PAUP* (Sinauer Associates, Inc., Sunderland, MA).

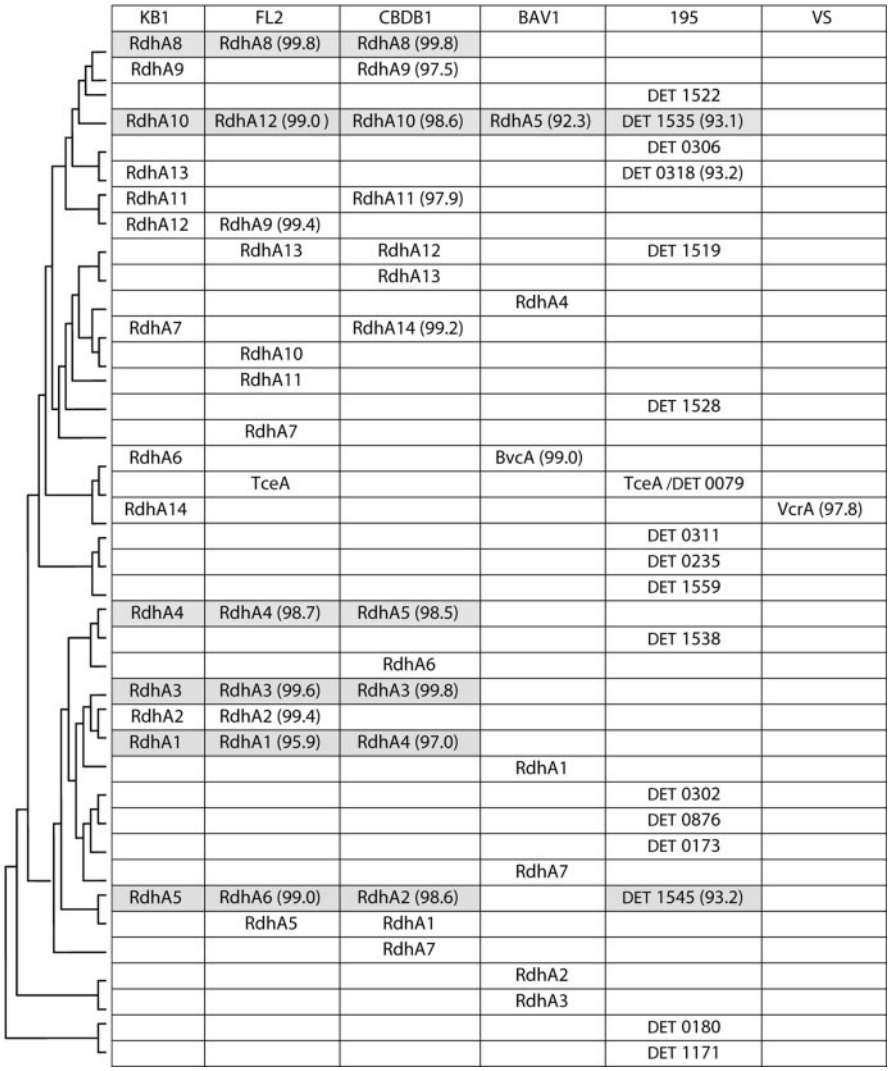
Nucleotide sequence accession numbers. The sequences of the RDH genes and their associated B gene fragments have been deposited in GenBank under the following accession numbers: DQ177506 to DQ177519 (KB1 *rdhA1* to *rdhA14*) and DQ115513 and DQ115514 (FL2 *rdhA12* and *rdhA13*).

RESULTS

RDH genes in KB1 culture. Fourteen distinct RDH gene sequences were identified in DNA samples from different KB1 subcultures (Fig. 1). All of these genes were also amplified from DNAs from the culture used in the transcription study (KB1/VC-MeOH). Furthermore, fragments of seven of these RDH genes (KB1 *rdhA1*, *rdhA2*, *rdhA3*, *rdhA8*, *rdhA10*, *rdhA12*, and *rdhA14*) were identified in a random genomic fragment library of KB1/VC-MeOH (E. A. Edwards, unpublished data). Each of the KB1 RDH genes identified is highly similar (93 to >99% identity at the protein level) to a previously identified RDH gene in one or more *Dehalococcoides* strains. Sequences with >99% identity differ by only one or two amino acids. KB1 shares nine highly similar RDH genes with strain CBDB1, eight with strain FL2, three with strain 195, and two with strain BAV1. Of the three *Dehalococcoides* RDH genes with ascribed functions, KB1 contains genes that are highly similar to both reported VC RDase genes (VS *vcrA* and BAV1 *bvcA*) but no genes similar to *Dehalococcoides* TCE RDase genes (195 *tce*A and FL2 *tceA*) (11). Although the primer set RRF2/B1R does not amplify *tceA*-like genes, even specific *tceA*-targeted primers (18) failed to amplify *tceA* from different KB1 subcultures (data not shown). Phylogenetic analyses of the KB1 and other *Dehalococcoides* RDH genes using both parsimony and neighbor-joining methods produced trees that were similar in topology to the tree presented by Hölscher et al. (14).

A similarity analysis performed with conceptual protein translations of the KB1 RDH genes indicated that the majority share about 30% amino acid identity with each other (Table 2). The two most similar proteins are KB1 RdhA8 and RdhA9, which share 91.4% amino acid identity. Although the KB1 RDH protein sequences are not similar (<25% amino acid identity) to those of RDHs in other dechlorinating organisms, such as *Sulfurospirillum*, *Dehalobacter*, and *Desulfitobacterium* (data not shown), they still share common conserved motifs such as the two iron-sulfur cluster binding motifs and the twin arginine leader peptide.

Identification of specific RDH genes in KB1 *Dehalococcoides* strains. Two strains of *Dehalococcoides* have been identified in KB1, based on differences in 16S rRNA gene sequence and substrate range (5). The corresponding 16S rRNA gene sequences have been designated KB1/PCE and KB1/VC, because cultures that harbor only the latter do not dechlorinate PCE (5). Genomic DNA from the mixed culture KB1/VC-MeOH (which contains both KB1/PCE and KB1/VC sequences) and genomic DNA from the KB1/VC-H_2 culture (which contains only the KB1/VC sequence) were assayed for RDH genes, using the 14 primer pairs for each specific KB1 RDH gene. All 14 KB1 RDH genes were detected in culture KB1/VC-MeOH, while all except the sequence corresponding to KB1 *rdhA6* (*bvcA*-like gene) were detected in culture KB1/VC-H_2 (Fig. 2). These data suggest that the BAV1 *bvcA*-like gene originated from the *Dehalococcoides* strain with the KB1/PCE sequence and not from the strain containing the KB1/VC sequence. These data reinforce that these two 16S rRNA gene sequences (which differ by only 1 bp) do indeed correspond to two distinct strains of *Dehalococcoides*.



KB1	FL2	CBDB1	BAV1	195	VS
RdhA8	RdhA8 (99.8)	RdhA8 (99.8)			
RdhA9		RdhA9 (97.5)			
				DET 1522	
RdhA10	RdhA12 (99.0)	RdhA10 (98.6)	RdhA5 (92.3)	DET 1535 (93.1)	
				DET 0306	
RdhA13				DET 0318 (93.2)	
RdhA11		RdhA11 (97.9)			
RdhA12	RdhA9 (99.4)				
	RdhA13	RdhA12		DET 1519	
		RdhA13			
			RdhA4		
RdhA7		RdhA14 (99.2)			
	RdhA10				
	RdhA11				
				DET 1528	
	RdhA7				
RdhA6			BvcA (99.0)		
	TceA			TceA /DET 0079	
RdhA14					VcrA (97.8)
				DET 0311	
				DET 0235	
				DET 1559	
RdhA4	RdhA4 (98.7)	RdhA5 (98.5)			
				DET 1538	
		RdhA6			
RdhA3	RdhA3 (99.6)	RdhA3 (99.8)			
RdhA2	RdhA2 (99.4)				
RdhA1	RdhA1 (95.9)	RdhA4 (97.0)			
			RdhA1		
				DET 0302	
				DET 0876	
				DET 0173	
			RdhA7		
RdhA5	RdhA6 (99.0)	RdhA2 (98.6)		DET 1545 (93.2)	
	RdhA5	RdhA1			
		RdhA7			
			RdhA2		
			RdhA3		
				DET 0180	
				DET 1171	

FIG. 1. Comparison of translated sequences of RDH genes detected in cultures KB1 and VS and strains FL2, CBDB1, and 195. Highly similar sequences (~93 to 100% amino acid identity) in different strains are grouped in the same row. The percent identity compared to the sequence in KB1 is shown in parentheses. The phylogenetic relationship among the proteins is indicated by the most parsimonious cladogram to the left of the table. The KB1 sequences that were detected in cDNA are listed in Table 3. Highly similar homologues found in three or more cultures are shaded. No extensive RDH gene survey has been conducted with culture VS, and *vcrA* is the only reported sequence.

Transcription experiment. Complete dechlorination to ethene was observed in all cultures amended with chlorinated electron acceptors (Fig. 3). RNA extraction and cDNA synthesis were successful for each sample taken. Furthermore, the RNA preparations were not contaminated with genomic DNA because no visible amplicons were generated from PCRs of samples processed without RT (Fig. 4).

RDH genes identified in transcription experiments. In total, seven different RDH sequences were identified in cDNA samples from cultures amended with different substrates (Table 3). In each cDNA sample, multiple distinct RDH transcripts were detected, with up to six being simultaneously detected in the cDCE treatment. Two genes, i.e., KB1 *rdhA6* and KB1 *rdhA5*, those most similar to BAV1 *bvcA* and FL2 *rdhA6*, were transcribed regardless of which chlorinated electron acceptor was provided to the culture. No RDH sequences were amplified from cDNAs generated from the unamended culture.

DISCUSSION

KB1/VC contains RDH genes that are highly similar to genes in strains CBDB1, FL2, BAV1, 195, and VS. In combination with previously reported RDH gene sequences, these results suggest that a pool of *Dehalococcoides* RDH genes exists and that each strain possesses a different complement of these genes. Phylogenetic analyses by Hölscher et al. (14) delineated 10 subclusters of highly similar genes in strains CBDB1, FL2, BAV1, and 195. With new sequence information from the KB1 culture, six additional subclusters based on sequence similarity are evident (Fig. 1, with each row being a

TABLE 2. Identity/similarity matrix of RDH gene translations in KB1

Protein	% Identity or similarity with KB1 protein[a]													
	RdhA8	RdhA9	RdhA10	RdhA13	RdhA11	RdhA12	RdhA7	RdhA6	RdhA14	RdhA4	RdhA3	RdhA2	RdhA1	RdhA5
RdhA8		91.4	59.3	46.1	35.8	37.3	31.7	34.2	34.6	35.0	33.4	32.1	31.8	32.5
RdhA9	95.6		59.3	47.4	36.6	37.3	31.6	34.7	35.2	34.8	34.5	32.1	33.2	32.9
RdhA10	75.8	75.6		46.5	35.5	35.0	34.3	33.3	32.4	32.4	32.5	32.4	32.5	30.2
RdhA13	63.5	64.9	64.3		39.5	40.6	34.5	33.0	35.1	32.3	31.0	32.0	31.2	30.8
RdhA11	53.0	54.1	53.9	58.2		61.0	31.2	31.2	31.1	31.8	28.9	30.7	29.2	29.8
RdhA12	54.5	55.7	55.0	59.9	74.9		29.9	31.8	30.9	30.6	30.5	32.2	30.4	30.1
RdhA7	51.3	52.1	50.7	53.8	49.1	50.1		31.0	31.5	28.5	30.9	30.4	33.2	31.5
RdhA6	49.8	50.0	52.6	52.4	47.6	49.6	51.6		39.0	31.1	29.8	29.7	30.2	28.4
RdhA14	51.9	50.9	50.3	50.7	48.9	49.9	49.9	55.5		30.3	29.2	31.6	31.1	28.2
RdhA4	53.2	52.4	51.9	49.7	49.9	50.2	48.9	47.4	47.3		38.0	38.5	38.7	35.6
RdhA3	51.0	52.7	51.7	52.1	47.3	49.0	53.2	48.6	49.1	56.2		55.4	54.1	36.5
RdhA2	50.1	49.1	48.7	49.1	49.3	49.5	50.3	50.0	48.5	54.0	69.6		58.3	40.0
RdhA1	50.3	51.6	51.5	49.9	48.0	48.1	53.2	49.4	46.3	56.4	67.4	72.7		39.9
RdhA5	51.9	52.7	49.0	51.9	47.3	48.8	50.3	47.4	43.3	53.1	56.4	56.9	57.7	

[a] The upper triangle of data represents the percent identity between two amino acid sequences in a pairwise alignment, and the lower triangle of data represents percent similarity. Similarity was calculated using the BLOSUM50 scoring matrix and a modified Smith-Waterman algorithm (2). Nearly complete translations of the *rdhA* genes starting after the RRF2 primer proteins were used to generate the identity and similarity matrixes.

subcluster), as genes that seemed to be unique now have highly similar genes in KB1. *Dehalococcoides* strains 195 and BAV1 still have the largest proportions of unique RDH genes, with 12 out of 17 (71%) and 5 out of 7 (71%), respectively. Without more information on the evolutionary relationship between these genes, it is not possible to tell if these subclusters of highly similar homologues represent true orthologues or not; however, their close identity suggests that they share function, perhaps even to the level of substrate specificity. For example, the KB1 mixed culture contains highly similar homologues of both reported VC RDase genes, *bvcA* (15) and *vcrA* (23), consistent with the observation that this culture dechlorinates VC efficiently.

A comparison of the dechlorination conditions under which particular RDH genes were transcribed in KB1 and the substrate range of *Dehalococcoides* strains that possess highly similar homologues to those genes suggests that, in contrast to expectations, these highly similar genes do not necessarily encode RDases that share substrate specificity. For example, KB1 *rdhA5* was transcribed under all four electron-accepting conditions. This gene has highly similar homologues in three other *Dehalococcoides* strains, namely, FL2 (*rdhA6*), CBDB1 (*rdhA2*), and 195 (DET1545). No chlorinated substrate has been reported to be common among these three organisms and KB1, suggesting a broad substrate range, an unidentified common substrate, different regulatory mechanisms in different strains, or a nonspecific regulatory mechanism for this putative dehalogenase. KB1 *rdhA6*, which is highly similar to BAV1 *bvcA*, was transcribed during the degradation of all four compounds (TCE, cDCE, VC, and 1,2-DCA); however, TCE is not a growth substrate for *Dehalococcoides* strain BAV1 (10). It could be that transcription of KB1 *rdhA6* was induced by the presence of small amounts of cDCE that were formed from TCE dechlorination. KB1 *rdhA14*, which is highly similar to VS *vcrA*, was transcribed during TCE degradation, in contrast to the tested substrate range of partially purified protein fractions containing VcrA. These partially purified fractions dechlorinated all three DCE isomers at rates similar to the VC dechlorination rate; however, TCE was dechlorinated at 5% of this rate (23). Although *vcrA* of strain VS is closely related to KB1 *rdh14*, there are still significant sequence differences between these genes. There are 18 nucleotide differences in the first 72 base pairs, corresponding to six amino acid substitutions at the N terminus of the protein, which could explain differences in the substrate specificity of the enzyme. The identification of a transcript's function is complicated in sequential reactions,

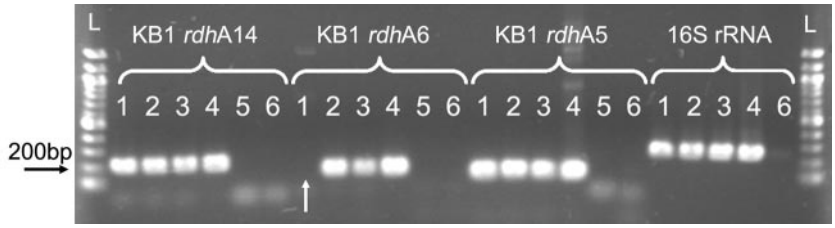


FIG. 2. PCR amplification of DNA from different KB1 subcultures using primers specific to frequently encountered RDH genes and the *Dehalococcoides* 16S rRNA gene. Four different specific primer sets (*rdhA14*, *rdhA6*, *rdhA5*, and 1f/259r [5]) were tested on six different DNA templates. Template 1, genomic DNA from KB1/VC-H_2 (only contains *Dehalococcoides* sequence KB1/VC); template 2, genomic DNA from a KB1/TCE-MeOH culture (contains both KB1/VC and KB1/PCE sequences); template 3, genomic DNA from KB1/VC-MeOH (contains both KB1/VC and KB1/PCE sequences); template 4, positive control (plasmid DNA containing the corresponding RDH gene or 16S rRNA gene); template 5, negative control (plasmid DNA containing a nontarget RDH gene); and template 6, negative control (no template). L, 100-bp ladder. The white arrow points to the result showing that KB1 *rdhA6* (*bvcA*-like gene) was not detected in KB1/VC-H_2.

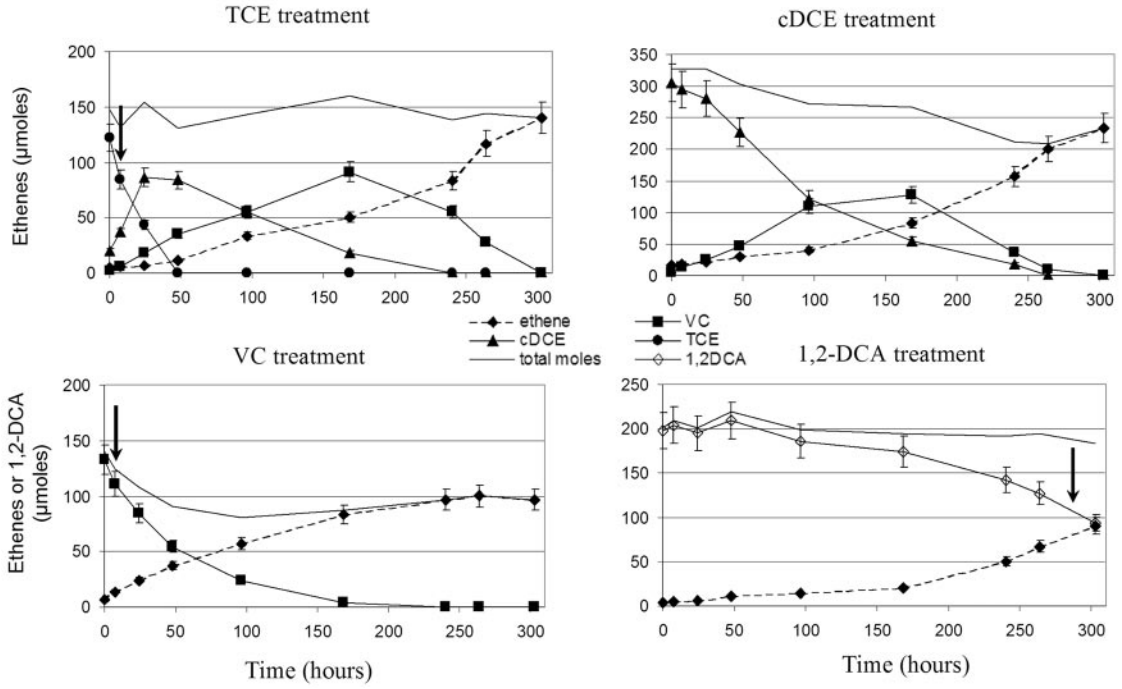


FIG. 3. Dechlorination profiles of four treatments amended with chlorinated substrates. The arrows indicate when RNAs were extracted. The TCE-amended culture was sampled 7 h after electron donor addition; at this point, 30 μmol of TCE had been dechlorinated to cDCE, and only traces of VC were present. The cDCE-amended culture was sampled after 24 h, during the transformation of cDCE to VC before ethene production began. The VC-amended culture was also sampled after 24 h. The 1,2-DCA-amended culture was sampled after 300 h (12.5 days) owing to a long lag time of 7 days before appreciable degradation took place.

where dechlorination of parent and intermediate products often occurs simultaneously. However, even during VC and 1,2-DCA dechlorination reactions, each of which only involves a single dechlorination step, multiple genes were transcribed. Sequencing of *Dehalococcoides* genomes will provide additional insight into regulatory networks and allow more detailed experimentation to explore RDase gene transcription.

A recent analysis of the *Dehalococcoides* strain 195 genome described a close association between most of the RDH genes and genes coding for transcription regulators (31). Furthermore, many of these regulatory genes are homologous to those belonging to two-component signal transduction systems, whose associated histidine kinase sensors appear to reside in the cytoplasm rather than the typical membrane location (29, 31, 35). Because of the presence of PAS/PAC motifs implicated in sensing cytosolic changes in redox potential, oxygen, and light, it has been suggested that the histidine kinase sensors respond to intracellular stimuli to modulate the energy level of the cell (31, 35). In addition, an analysis of the genome of strain 195 also revealed that one RDase gene, *tceA*, is not situated near a transcriptional regulator. This is interesting because the corresponding RDase, TceA, was partially purified from a mixed TCE-dechlorinating culture containing strain 195. It may be that within one *Dehalococcoides* organism, individual RDase genes are regulated by distinct transcriptional control mechanisms, and that these controls respond to multiple stimuli.

An alternative explanation for the different substrate ranges of the *Dehalococcoides* strains that transcribe highly similar RDH genes is that although similar apoenzymes may be produced from these genes, perhaps different corrinoid cofactors modulate substrate specificity. All but one of the purified RDases are dependent on the corrinoid cofactors that are associated with the enzyme. The genome sequence of *Dehalo-*

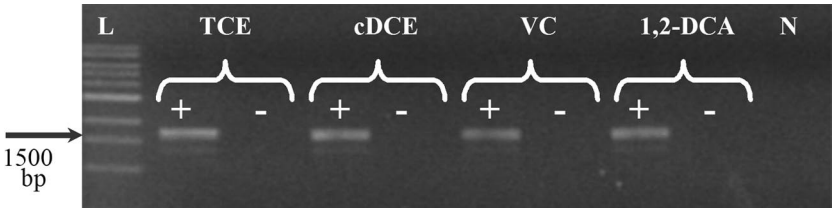


FIG. 4. PCR amplification of cDNAs with degenerate primers targeting RDH genes (primers RRF2 and BR1). For cDNA samples from each treatment group (TCE, cDCE, VC, and 1,2-DCA), two PCRs were performed: "+" indicates that RT was used for cDNA synthesis, and "−" indicates that no RT was used. L, 1,000-bp ladder (New England Biolabs); N, PCR product from RNA extracted from the unamended culture.

TABLE 3. Comparison of the different RDH genes identified in RNA samples

KB1 RDH gene	Most similar RDH gene	No. of clones containing each RDH gene transcript per treatment[a]			
		TCE	cDCE	VC	1,2-DCA
rdhA6	BAV1 *bvcA*	5	3	4	3
rdhA14	VS *vcrA*	2	0	5	0
rdhA5	FL2 *rdhA6*	3	7	1	12
rdhA3	CBDB1 *rdhA3*	0	1	0	0
rdhA7	CBDB1 *rdhA14*	0	1	0	0
rdhA1	CBDB1 *rdhA4*	0	1	1	0
rdhA8	FL2 *rdhA8*	0	1	0	0
Total		10	14	11	15

[a] For each chlorinated substrate, RNA was extracted at one time point soon after dechlorination began; RDH genes were amplified from cDNA samples, cloned, and sequenced. The number of occurrences of each RDH gene transcript from the different treatments is reported.

coccoides strain 195 suggests that these organisms cannot synthesize the corrinoid ring structure and must salvage them from the environment (31), which in the case of pure cultures must be the medium, but in the case of a mixed culture or the environment, might also be other organisms. Therefore, different cofactors may be associated with the RDases when strains are grown in mixed or pure cultures. Strain CBDB1 was able to degrade pentachlorobenzene and hexachlorobenzene when grown in a mixed culture, but not when grown in isolation (1). This was attributed to the state in which the compound was administered (i.e., in crystal form, as opposed to a hexadecane phase); however, this different substrate range could be due to the availability of different cofactors associated with the RDase(s). *Dehalococcoides* strain 195 exhibited different rates and extents of dechlorination depending on what amendments were made to the medium. For example, when mixed-culture supernatant was added to the pure culture, TCE was degraded to ethene, but when yeast extract or *E. coli* extract was added, no degradation beyond cDCE occurred (21). The possibility that the corrinoid cofactors affect the substrate range and specificity of the holoenzyme would explain observations made with mixed and pure cultures, and this hypothesis should be explored further.

Understanding the transcriptional controls of reductive dechlorination is relevant for bioremediation applications. Mixed consortia like KB1 have been used successfully in bioaugmentation field studies (20). The sequence information described in this paper will be useful in designing probes or primers to detect the presence of these genes or transcripts in situ. Dehalogenase genes offer more resolution than 16S rRNA gene-based probes, which cannot distinguish between *Dehalococcoides* strains with differing dechlorinating activities. The detection of a specific complement of RDH genes may provide more reliable strain identification. Furthermore, the detection of transcription of certain RDH genes may provide quantitative information about the metabolic processes occurring in situ. However, more research is required to determine if the identification of certain transcripts at a site correlates with a particular compound being degraded or if site geochemical parameters such as available corrinoid cofactors modulate substrate degradation. These approaches will lead to the development of refined molecular tools to predict and monitor in situ dechlorination processes.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Since the acceptance of this paper, the publication of a study analyzing the genome sequence of *Dehalococcoides* strain CBDB1 revealed the presence of 32 RDH genes (M. Kube, A. Beck, S. H. Zinder, H. Kuhl, R. Reinhardt, and L. Adrian, Nat. Biotechnol. **23:**1269–1273, 2005). This is significantly more than the 14 RDH genes detected by Hölscher et al. using the same degenerate primers that were used to amplify the RDH genes in KB1. However, this does not change the topology of the tree in Fig. 1, nor does it change the conclusions of this paper.

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Attachment D:

Pathogen Screening Methods and Data for KB-1®

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METHOD FOR DETECTION OF MICROBIAL SPECIES.

Our laboratory is accredited by the Canadian Association for Environmental Analytical Laboratories (CAEAL), and all of the proper quality control procedures were conducted and documented when performing these methods.

***Vibrio* sp.**
Based on Standard Methods for the Examination of Water and Wastewater (20th Ed.) method 9260H and Health Canada method MFLP-72.
Sample was enriched in 90 mL of alkaline peptone water (APW), and incubated stationary at 35°C. At 6-8 h and 24 h, 0.1 mL of APW enrichment broth was added to Thiosulfate Citrate Bile Sucrose (TBCS) agar, and incubated at 35°C for 18 to 24 h. Confirmation using TSI slant, catalase, oxidase, Gram-strain, API 20E.

***Campylobacter* sp.**
Based on Madden *et al.* (2000) J. Microbiol. Methods 42:115-119.
Sample was added to 90 mL of modified Cefoperazone Charcoal Desoxycholate broth (mCCD) broth, and enriched under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 42°C for 24 and 48 h. A 0.1 mL sample of mCCD broth was then added to modified Cefoperazone Charcoal Desoxycholate agar (m CCDA), and incubated under microaerophilic conditions at 42°C for 48 h. Confirmation using microscopic examination, Gram-stain, blood agar, catalase test, oxidase test, API Campy strip.

Pseudomonas aeruginosa
Based on Standard Methods for the Examination of Water and Wastewater (20th Ed.) method 9213 E.
Sample was processed by membrane filtration, and the filter was transferred onto mPAE agar. mPAE agar is prepared as described for mPA agar, with the following additions (g/L): $MgSO_4 \cdot 7H_2O$, 5.0; sodium deoxycholate, 0.1. Plates were incubated at 41.5 °C for 46 to 50 h. Confirmation using Gram-stain, oxidase test, Milk agar and Acetamide agar (Atlas, Handbook of Microbiological Media, 1993).

***Salmonella* sp.**
Based on Standard Methods for the Examination of Water and Wastewater (20th Ed.) method 9260B.
Sample was added to 90 mL of Tetrathionate broth, and incubated at 35°C. After 24 and 48 h, a 0.1 mL sample of enrichment broth was added to Brilliant Green agar, and incubated for 24 hours at 35°C. Suspect colonies were inoculated into a Lysine Iron agar slant, and incubated at 35°C for 24 h. Further confirmation by Gram-stain, oxidase test, catalase test and API 20E.

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Listeria monocytogenes
Sample was added to 90mL of UVM Listeria Enrichment broth (Difco), and incubated 30°C for 48 h. A 0.1 mL sample of enrichment broth was streaked onto Listeria Selective agar (Difco), and incubated at 30°C for 24 h. Confirmation using blood agar, Gram-stain, catalase test, oxidase test, TSI agar and API Listeria.

Yersinia enterocolitica
Method based on that of Bhaduri *et al.* (1997) Appl. Environ. Microbiol. 63:1657-1660.
Sample was processed by membrane filtration onto Yersinia selective (CIN) agar (Oxoid), and incubated at 28°C for 24 h. Confirmation using Gram-stain, oxidase test and API 20E.

***Clostridium* sp.**
Sample was added to 90 mL of Thioglycollate medium (BBL), and incubated anaerobically at 35°C for 24 h. A 0.1 mL sample of enrichment broth was added to Clostrisel agar (BBL), and incubated anaerobically at 35°C for 48 hours. Confirmation by Gram-stain, blood agar, skim mild broth and species identification (16S rDNA analysis).

Total yeast and mold
Based on Standard Methods for the Examination of Water and Wastewater (20th Ed.) method 9610 D.
Sample was processed by membrane filtration, and the filter was transferred onto Rose Bengal agar (Difco). Plates were incubated at 26°C for 7 days.

Fecal Streptococci (Enterococci)
Sample was processed by membrane filtration, and the filter was transferred onto mEnterococcus agar (BBL). Plates were incubated at 35°C for 48 h. Confirmation using Ethyl Violet Azide (EVA) broth and incubation at 35°C for 24 h, and Gram-stain.

Fecal coliforms
Sample was processed by membrane filtration, and the filter was transferred onto mFC agar (BBL). Plates were incubated at 44.5°C for 20 h.

Aerobic Plate Count
Sample dilutions were prepared in Phosphate Buffered Water, and spread plated onto R2A agar (Difco). Plates were incubated at 28°C for 4 days.

Anaerobic Plate Count
Sample dilutions were prepared in Phosphate Buffered Water, and spread plated onto R2A agar (Difco). Plates were incubated in anaerobic jars, using the BBL GasPak system (H_2 + CO_2 generator envelops) at 28°C for 4 days. Anaerobic conditions were monitored using a BBL GasPak disposable anaerobic indicator strip.

CALA FINAL RESULTS FORM

GAP EnviroMicrobial Services Ltd.
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Tel: (519) 681-0571 Fax: (519) 681-7150

GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab	
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb	
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2	
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2	
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151
PAGE: 1 of 6	**ANALYSIS FINISH DATE:** 07-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	MF Results (CFU per 1 mL)							Benchsheet Page Reference	Quality Control Comments
		Total Coliforms (TCMF-0001)	Background (TCMF-0001)	Detection Limit	Fecal Coliforms (FCMF-0001)	Detection Limit	*E. coli* (ECMF-0001)	Detection Limit		
939		<1	<1	1	<1	1	<1	1	Page 63	QC Passed

CALCULATED BY: J. Patterson **POSITION:** Lab Manager **MANAGER APPROVAL:** S. Verhoeven (Technical Manager)

SIGNATURE: J Patterson **SIGNATURE:** S Verh **DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

CALA Testing Accreditation No. A 2914

FINAL RESULTS FORM

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GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab	
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb	
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2	
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2	
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151
PAGE: 2 of 6	**ANALYSIS FINISH DATE:** 08-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	Heterotrophic Plate Count		Fecal Streptococci		Benchsheet Page Reference	Quality Control Comments
		CFU per mL HPCMF-0001	Detection Limit CFU per mL	CFU per mL MF, mENTER, 35°C, 48h	Detection Limit CFU per mL		
939		3	1	<1	1	Page 63	QC Passed

CALCULATED BY: J. Patterson **POSITION:** Lab Manager

SIGNATURE: J Patterson

MANAGER APPROVAL: S. Verhoeven (Technical Manager)

SIGNATURE: S Verh **DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

FINAL RESULTS FORM

GAP EnviroMicrobial Services Ltd.
1020 Hargrieve Road. Unit 14
London, ON N6E 1P5
Tel: (519) 681-0571 Fax: (519) 681-7150

GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab	
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb	
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2	
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2	
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151
PAGE: 3 of 6	**ANALYSIS FINISH DATE:** 08-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	*Clostridium perfringens*		*Pseudomonas aeruginosa*		Benchsheet Page Reference	Quality Control Comments
		CFU per mL CLOSP-0001	Detection Limit CFU per mL	CFU per mL SOP #46	Detection Limit CFU per mL		
939		<1	1	<1	1	Page 63 & Reverse	QC Passed

CALCULATED BY: J. Patterson **POSITION:** Lab Manager

MANAGER APPROVAL: S. Verhoeven (Technical Manager)

SIGNATURE: J Patterson **SIGNATURE:** S Verh **DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

FINAL RESULTS FORM

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London, ON N6E 1P5
Tel: (519) 681-0571 Fax: (519) 681-7150

GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab	
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb	
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2	
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2	
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151
PAGE: 4 of 6	**ANALYSIS FINISH DATE:** 11-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	Yeast		Mold		Benchsheet Page Reference	Quality Control Comments
		CFU per mL FUNGIID-0001	Detection Limit CFU per mL	CFU per mL FUNGIID-0001	Detection Limit CFU per mL		
939		<1	1	<1	1	Page 63 Reverse	QC Passed

CALCULATED BY: J. Patterson **POSITION:** Lab Manager	**MANAGER APPROVAL:** S. Verhoeven (Technical Manager)
SIGNATURE: J Patterson	**SIGNATURE:** S Verh **DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

FINAL RESULTS FORM

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London, ON N6E 1P5
Tel: (519) 681-0571 Fax: (519) 681-7150

GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab			
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb			
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2			
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2			
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151		
PAGE: 5 of 6	**ANALYSIS FINISH DATE:** 11-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**		

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	*Salmonella* sp. P/A per 10 mL	*Listeria monocytogenes* P/A per 10 mL	*Vibrio* sp. P/A per 10 mL	*Yersinia* sp. P/A per 10 mL	Benchsheet Page Reference	Quality Control Comments
939		Absent	Absent	Absent	Absent	Benchsheets	QC Passed

CALCULATED BY: J. Patterson	**POSITION:** Lab Manager	**MANAGER APPROVAL:** S. Verhoeven (Technical Manager)	
SIGNATURE: JPatterson		**SIGNATURE:** SL Verh	**DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

FINAL RESULTS FORM

GAP EnviroMicrobial Services Ltd.
1020 Hargrieve Road. Unit 14
London, ON N6E 1P5
Tel: (519) 681-0571 Fax: (519) 681-7150

GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab			
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb			
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2			
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2			
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151		
PAGE: 6 of 6	**ANALYSIS FINISH DATE:** 12-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**		

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	*Campylobacter* sp. P/A per 10 mL	*Bacillus anthracis* P/A per 10 mL			Benchsheet Page Reference	Quality Control Comments
939		Absent	Absent			Benchsheets	QC Passed

CALCULATED BY: J. Patterson **POSITION:** Lab Manager

MANAGER APPROVAL: S. Verhoeven (Technical Manager)

SIGNATURE: J Patterson **SIGNATURE:** S Verh **DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

5 Mar 2013

GAP EnviroMicrobial Services Ltd.

GAP EnviroMicrobial Services Ltd.
1020 Hargrieve Road, Unit 14; London, Ontario; Canada N6E 1P5
Tel: (519) 681-0571 Fax: (519) 681-7150 www.gapenviromic.com

CHAIN OF CUSTODY

Client: Geosyntec / SIREM Lab	ANALYSES REQUESTED
Client Contact: Jennifer Webb	GAP Project # A 10284
Address: 130 Research Lane, Suite #2, Guelph, Ontario, N1G 5G2	Invoice to:
Tel: 519-822-2230 x307 Fax: 519-822-3151	
Email: jwebb@siremlab.com	
Client Project # P.O. # TL500534	
GAP Contact: Water Works #	

Fecal streptococci; Pseudomonas aeruginosa; bacillus anthrasis; fecal coliforms

SAMPLE IDENTIFICATION	# of containers	Matrix	Please check analyses requested	Date/Time Collected	Volume Sampled	Lab Sample No.
KB-1 / WBC-2 / ACT-3	1	aqueous	✓ ✓ ✓ ✓	5 mar 13		939

Turnaround Time:

☒ Normal (5 to 10 business days)

☐ *Rush: Date _____

* Double Rates Apply for Rush Analysis

Report Options:

☐ Fax

☒ Email

☐ Mail

Special Instructions / Comments:

Sampler (Print & Sign Name)

Collected by: Jon Webb

Signature: J-Webb

Laboratory Information

Rental Equipment (Completed by Sampler)

Equipment Standard Operating Procedure Read and Understood Initial ☐

Laboratory Use Only

Sample Information

Sample Temperature 8.1 °C Temp

Condition Acceptable Upon Receipt (Y/N) _____

Received at Lab by: JPatt

Date: 6 mar 13 Time: 10:30 am

Please read the reverse side of this form for sampling and shipping instructions.

5 Mar 2013

GAP EnviroMicrobial Services Ltd.

1020 Hargrieve Road, Unit 14; London, Ontario; Canada N6E 1P5
Tel: (519) 681-0571 Fax: (519) 681-7150 www.gapenviromic.com

CHAIN OF CUSTODY

Client: Geosyntec / SIREM Lab
Client Contact: Jennifer Webb
Address: 130 Research Lane, Suite #2, Guelph, Ontario, N1G 5G2.
Tel: 519-822-2230 x307 | Fax: 519-822-3151
Email: jwebb@siremlab.com
Client Project # | P.O. #: TL500534
GAP Contact: | Water Works #

ANALYSES REQUESTED

GAP Project # A 10284

Invoice to:

SAMPLE IDENTIFICATION	# of containers	Matrix	Total Coliforms	Escherichia coli	heterotrophic plate count	Yeast & Mold	Salmonella sp.	Listeria monocytogenes	Vibrio spp.	Yersinia spp.	Campylobacter spp.	Clostridium perfringens	Date/Time Collected	Volume Sampled	Lab Sample No.
			Please check analyses requested												
KB-1 / WBC-2 / AO-3	1	aqueous	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	5 Mar 13		939

Turnaround Time:
☒ Normal (5 to 10 business days)
☐ *Rush: Date _____
* Double Rates Apply for Rush Analysis

Report Options:
☐ Fax
☒ Email
☐ Mail

Special Instructions / Comments:

Sampler (Print & Sign Name)
Collected by: Jen Webb
Signature: J. Webb

Laboratory Information
Rental Equipment (Completed by Sampler)
Equipment Standard Operating Procedure Read and Understood Initial ☐

Laboratory Use Only
Sample Information
Sample Temperature 8.1 C Temp
Condition Acceptable Upon Receipt (Y/N) _____
Received at Lab by: JPatt
Date: Mar 6/13 Time: 1030 am

Please read the reverse side of this form for sampling and shipping instructions.

Attachment E:

Health Canada Biosafety Level 1 Designation Letter for KB-1®

Health Canada
Santé Canada

Population and Public Health Branch
Direction générale de la santé de la population et de la santé publique

January 14, 2004

Your file Votre référence

Your file Votre référence

Phil Dennis, M.A.Sc.
SiREM
130 Research Lane, Suite 2
Guelph, ON N1G 5G3
pdennis@siremlab.com

Re: Biosafety Level Assessment for KB-1™ Dechlorinator

Dear Mr. Dennis,

We have reviewed the updated list of organisms detected in the KB-1 culture, submitted December 17 for biosafety level determination. Please note that our biosafety level assessment applies only to the status of your preparation as a human pathogen; our assignment of a biosafety level does not involve assessment of the preparation for potential as an animal and/or plant pathogen.

The submitted sequence information indicates the presence of organisms belonging to the genus *Dehalococcoides*, family *Geobacteriaceae* and family *Desulfovibrionaceae,* in the KB-1 preparation.

The final conclusion on the risk group of the KB-1 preparation, assuming microbial stability with respect to the information submitted, is that the material is *reasonably believed not to contain human pathogens* and, therefore, is classified as risk group 1. Please note the use of "reasonably believed not to contain human pathogens"; the rationale for use of this statement is outlined below.

Sequences 0687A and 0687B

There is no evidence to suggest that members of the genus *Dehalococcoides* are human pathogens and all members of the *Geobacteriaceae* family, previously assessed by our office, have been classified as risk group 1.

Sequences 0774A, 0854B and 0854C

The *Desulfovibrionaceae* family includes the genera *Desulfovibrio, Biophila* and *Lawsonia. B. wadsworthia* has been classified as a risk group 2 human pathogen. Species of the *Desulfovibrio* genus infrequently cause disease in humans (predominately in immunocompromised or elderly persons).

The sequence data only indicate the nearest relative for the sequence source, and the organisms themselves are relatively new and not well characterized, therefore, some assumptions were made for the risk group assessment.

- Sequence 0774A: The nearest relative identified for this sequence source is an uncultured and uncharacterized bacterium, isolated from sediment of an underground aquifer (Alfreider et al., 2002). Due to the environmental source and lack of evidence that the nearest relative may cause disease in humans, the source of this sequence is presently classified as risk group 1.
- Sequence 0854B: The nearest relative identified for this sequence source is itself most closely related to *D. desulfuricans* (Zengler et al., 1999) which is classified as risk group 1, therefore, the source of this sequence is presently classified as risk group 1. Please note that *D. desulfuricans* is considered an opportunistic human pathogen which may cause disease in immunocompromised or elderly individuals.
- Sequence 0854C: The nearest relative identified for this sequence source is *D. africanus* (Devereux et al., 1990) which is classified as risk group 1, therefore, the source of this sequence is presently classified as risk group 1.

All of the microbial components of the KB-1 preparation are considered risk group 1 organisms (non-pathogenic to healthy humans) and you have tested the preparation to confirm the absence of various pathogenic bacteria, yeast and moulds, therefore, the overall preparation is classified as risk group 1.

Please note, however, that the preparation may contain opportunistic pathogens that can cause illness in immunocompromised individuals or elderly persons. If these risk factors apply to members of your staff who will be working directly with the preparation, please take appropriate precautions to ensure their safety.

We thank you for your efforts to characterize the KB-1 preparation, as they have facilitated the biosafety level assessment. Please do not hesitate to contact our office if you require clarification or wish to discuss this matter in more detail.

Best regards,

Stacey Mantha
Head, Importation and Biosafety Programs
Office of Laboratory Security
CEPR, PPHB, Health Canada
100 Colonnade Road, AL 6201A
Ottawa, ON K1A 0L2
613.957.1779
Stacey_Mantha@hc-sc.gc.ca

Attachment F:

Data from Ecotoxicity Testing of KB-1® on Frogs
(McDaniel et. al, 2006)

Arch. Environ. Contam. Toxicol. 52, 410–417 (2007)
DOI: 10.1007/s00244-005-8218-8

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and Toxicology

Bioremediation of Tetrachloroethylene-Contaminated Groundwater in a Model Aquifer: Effects on Green Frogs (*Rana clamitans*) and *Xenopus laevis* as Potential Wetland Receptors

Tana V. McDaniel,[1] Nathalie Ross,[2] Pamela A. Martin,[1] Helena Steer,[2] Ann-Marie Irwin Abbey,[2] Suzanne Lesage[2]

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Received: 28 August 2005 /Accepted: 16 September 2006

Abstract. Recent regulations require that the ecological effects of microorganisms introduced into the environment, such as for groundwater bioremediation, be assessed prior to their utilization. A native anuran (*Rana clamitans*) and a model anuran (*Xenopus laevis*) were used as potential wetland receptors of tetrachloroethylene (PCE)-contaminated groundwater, undergoing three bioremediation treatments: natural attenuation (NA), biostimulation (ST), and bioaugmentation (AU). Eggs of both species were exposed acutely (96 h) to remediated effluents. *Xenopus* tadpoles were chronically exposed to the effluents for 100 days and were screened for the presence of bacterial pathogens. There was no impact on the survivorship of the frogs exposed either acutely or chronically to the NA, ST, or AU effluents; nor was there any evidence of bacterial infection found, with the exception of control individuals. The results of these exposures suggest that bioremediation with KB-1™ culture poses a minimal threat to anuran development and survivorship.

Chemical contamination of groundwater is a serious issue affecting the availability and quality of drinking water in North America. Efficient technologies to deal with these concerns include *in situ* enhancement of microbial degradation of contaminants to less harmful substances. While these technologies have proven effective in the breakdown of contaminants, concern regarding the environmental impacts of introduced microorganisms into contaminated aquifers has prompted the regulation of their use (Environment Canada 1997; US EPA 1997). In the current paper, we present the results of an exposure study of effluents from a model aquifer undergoing bioremediation of chloroethylene contaminants on anuran larvae.

Correspondence to: Pamela A. Martin; *email:* pamela.martin@ec.gc.ca

To comply with recent federal regulations, more research is needed on the environmental impact of introducing novel microorganisms into the environment for groundwater bioaugmentation (AU). Groundwater AU consists of injecting microorganisms, isolated or cultured, that can degrade specific contaminants in an aquifer (Devinny and Chang 2000). Pure cultures of organisms or mixed consortia may be used to provide missing species, avoid lag phases, provide organisms in large numbers, ensure dispersal, remove contaminants at low concentrations, or provide secondary factors such as surfactant-producers. Although AU has been compared to other bioremediation approaches for its efficacy to biotransform chemical products (Lendvay *et al*. 2003), little information exists on its ecological effects.

Other bioremediation approaches include biostimulation (ST) and natural attenuation (NA). The ST approach promotes the biodegradative activity of groundwater indigenous species after ensuring adequate nutrients and electron acceptors are provided (Devinny and Chang 2000), and the NA approach refers to all naturally occurring processes that reduce contaminant concentrations or toxicity (Lorah and Olsen 1999; Sun *et al*. 2000). Although these approaches are exempted from notification regulations since no microbial additions are involved (Environment Canada 1997), it has been shown that changing environmental conditions, such as injected nutrients, may cause ecological effects (Ross *et al*. 1998; Iwamoto *et al*. 2000). All of these bioremediation approaches, applied to groundwater, might impact wetlands through groundwater resurgence (Tobias *et al*. 2001, Négrel *et al*. 2003).

Tetrachloroethylene (PCE), a volatile chlorinated solvent used extensively in the dry-cleaning and degreasing industries, is a common contaminant of groundwater. In a US EPA national survey of volatile chemicals in groundwater supplies used for drinking water, PCE and its major metabolites were among the ten most commonly encountered chemicals (Westrick *et al*. 1984). Concentrations of PCE as high as 75 mg/L have been measured in Canadian aquifers (Environment Canada 1994). Although concentrations of PCE as high as 190 μg/L have been recorded in surface waters, concentrations are typically in the high ng/L to low μg/L range. The bacterial culture KB-1™ Dechlorinator has been introduced in several

sites successfully promoting PCE dechlorination to ethene (Duhamel *et al*. 2002; Major *et al*. 2002). PCE can be degraded *in situ* via anaerobic microorganisms in a series of reductive dechlorinations to produce trichloroethylene (TCE), dichloroethylene (DCE), vinyl chloride (VC), ethene, and ethane. The propensity of the chloroethylenes for microbial degradation has provided the impetus for the assessment of fully utilizing this process for bioremediation of contaminated sites. The KB-1™ culture is an enriched anaerobic bacterial consortium isolated from soil and groundwater impacted by TCE. The two main species in KB-1 responsible for the dechlorination of PCE are Geobacter and Dehalococcoides (DHC), which use dehalorespiration. Other species include *Sulfurospirillum deleyianum, Sporomusa* sp., *Spirochaeta* sp., *Methanosarcina* sp., *Methanomethylovarans* sp., and *Acetobacterium* (Duhamel *et al*. 2004). It is believed that KB-1™ consists predominantly of microorganisms that inhabit subsurface environments and is exempt from known human pathogens. Additional testing is required for assessing its potential ecological effects.

One primary concern to environmental regulators is the potential toxicity and pathogenicity of introduced microorganisms towards non-target organisms (Environment Canada 1997). Amphibians are an ideal model species because of their use in standardized toxicity assays, their susceptibility to pathogenic bacteria, and their potential for exposure. As a key component of wetland ecosystems, amphibians may be exposed to remediated effluents through groundwater recharges of wetlands (Todd 1980). Standardized toxicity assays, such as FETAX (ASTM 1998), allow for the testing of complex effluents for both acute toxicity and teratogenicity. Bacterial pathogens of amphibians are relatively well documented. Amphibians are susceptible to infection from a wide taxonomic range of bacterial pathogens (Anver and Pond 1984).

The goal of the present study was to assess the safety of three approaches of bioremediation of PCE using amphibian embryos and larvae as model wetland receptor species. Of primary interest was the introduction of a bacterial consortium, KB-1™, to enhance the degradation of chloroethylenes and its potential impact on amphibian survivorship and development. To assess the short-term impact on hatching success and early life stage development, green frog (*Rana clamitans*) and African clawed frog (*Xenopus laevis*) embryos were exposed acutely to effluent from each of the three bioremediation treatments, AU, ST, and NA, produced in a model aquifer. *Xenopus* larvae were exposed chronically to aquifer effluents for 100 days to assess long-term impacts on growth, development, and survivorship. To specifically address the potential for pathogenicity of the microbial consortium, *Xenopus* froglets from the chronic exposure were screened for incidences of bacterial infection.

Materials and Methods

Experimental Setup and Effluent Composition

A model aquifer was constructed at the Canada Centre for Inland Waters (CCIW, Burlington, ON) to compare the biosafety of three bioremediation approaches; NA, ST, and AU. The design and the characterization of the model aquifer are described in detail by Ross *et al*. (2002), but will be briefly outlined below. The model aquifer consisted of a stainless-steel tank (6.0 m long, 2.4 m wide, and 1.8 m deep) divided into three 0.8-m-wide parallel lanes and filled with clean, medium to fine grain sand. Water was pumped into the head tank of each lane, and the flow was maintained gravimetrically at 80 ml per min. The contamination source in each lane of the model aquifer consisted of PCE in silicone oil (10% w/w) mixed with coarse sand introduced in a 30-cm, 200-µm meshed sock inserted at 1 m depth. The chloroethylene concentration around the model aquifer injection point varied between 1 and 5 ppm and decreased to values between 0.89 to 0.10 ppm in the effluents (Figure 1).

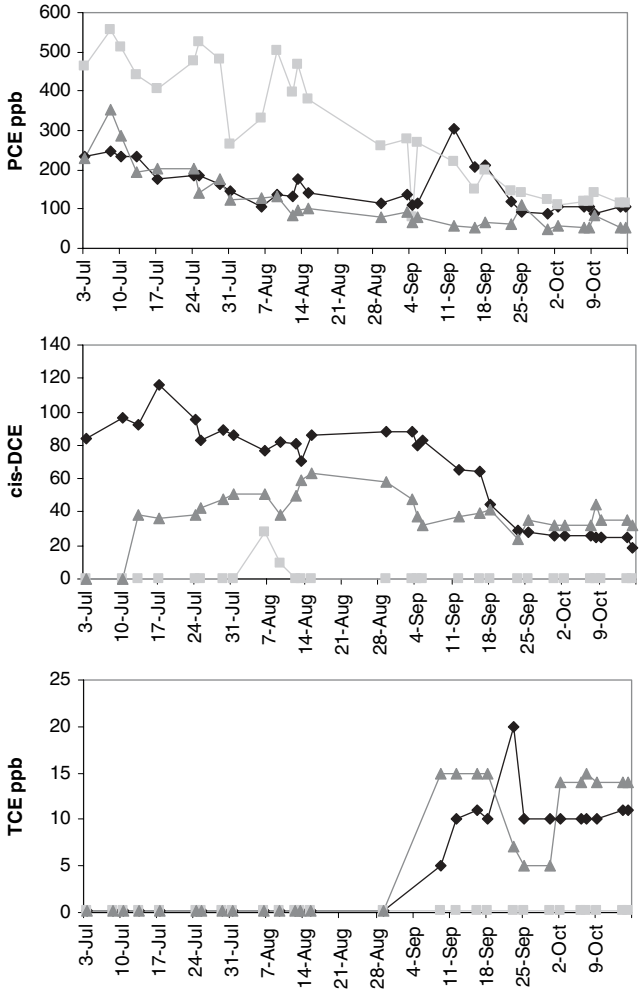

Fig. 1. Measured concentrations of perchloroethylene (PCE), trichloroethylene (TCE), and *cis*-dichloroethylene (*cis*-DCE) in the aquifer effluents from the three bioremediation lanes [(♦) Bioaugmentation (BA), (▲) Biostimulation (ST), and (■) Natural Attenuation (NA)] during the four-month period for which frogs were exposed

The NA lane was left unaltered. The ST lane received injections of nutrients (methanol and lactic acid) twice weekly, and the AU lane received the same regime of nutrient injection as the ST lane plus a single injection of KB-1™ culture (Major *et al*. 2002). The effluents were sampled at the exit of the model aquifer, located approximately 4.3 m from the contamination source and 4.0 m from the nutrient injection/AU injection zone.

The source of water for the model aquifer was groundwater from a research well located on the grounds of the CCIW. Carbon-filtered, dechlorinated tap water was used to dilute the aquifer effluent

during amphibian testing. Concentrations of chloroethylenes in the aquifer effluents (AU, ST, NA), as well as control groundwater and dechlorinated tap water, were measured biweekly using purge and trap (US EPA method 503 0B) GC/MS (US EPA method 82 60B; US EPA 1996) as described in Ross *et al*. (2002). The minimum detection limit for this technique was 0.1 ppb; however, the practical detection limit, or limit of quantification, was closer to 1.0 ppb for most samples. In addition to the chemical analyses, the effluents and KB-1 culture were tested for the presence of potential pathogens: *Cryptosporidium parvum, Campylobacter* sp., *Shigella* sp., *Staphylococcus aureus, Clostridium perfringins, Escherichia coli, Pseudomonas aeruginosa*, and *Yersinia* sp. (Bergey *et al*. 1984).

Effects of Acute Exposures to Effluents

Egg masses of green frogs *(R. clamitans)* and *X. laevis* were collected when less than 24 h old, before gastrulation was completed. Green frog egg masses were collected from a site in south central Ontario, Canada, where chloroethylenes were not detected in the surface water. *Xenopus* egg masses were obtained from a breeding colony at the University of Guelph (Guelph, ON). Three egg masses were used for each species with each egg mass representing a replicate. An egg mass represents the clutch of an individual female frog. Each egg mass was divided into lots of approximately 30 embryos and immersed in 300 ml of exposure solution contained in a sealed 1-L Mason® jar. The jars were held in a circulating water bath to maintain a constant temperature of 22°C ± 1°C. All exposures were conducted under an exhaust hood. A 14L/10D photoperiod, using full spectrum lamps (Interlectric Corporation F32T8 True Lite fluorescent tubes), was used to mimic natural light conditions.

Static renewal exposures (96 h) of aquifer effluent were conducted on green frogs and *Xenopus* using a modified FETAX protocol (ASTM 1998). Exposure solutions were composed of 12.5, 25.0, and 50.0% aquifer effluents. Controls consisted of groundwater from the aquifer head tank. The proportion of groundwater/effluent was made up to 50% consistently across all treatments by the addition of the appropriate amount of groundwater and all solutions were brought up to 100% with aerated tap water. Exposure solutions were renewed once every 24 h. Exposure solutions were not aerated during the 96-h exposure. During one 24-h renewal period, chloroethylene levels were measured at 0 and 24 h, to determine the range of chloroethylene levels in the jars.

Dissolved oxygen, pH, and temperature were monitored in all exposure jars daily to ensure that acceptable conditions for egg survival were maintained. At the end of the 96-h exposure period, all of the embryos had hatched and survivorship was assessed. Tadpoles were then euthanized using a 1% solution of tricaine methane sulfonate and were assessed for developmental malformations. Malformations were classified according to the *Atlas of Abnormalities* for *Xenopus* (Bantle *et al*. 1998).

Effects of Chronic Exposure to Effluents

Chronic exposures were initiated on *Xenopus* embryos less than 24 h old and continued for 100 days (d). Subsequent to this, tadpoles were raised in tap water until they completed metamorphosis, or until 156 d had passed. *Xenopus* tadpoles and embryos were exposed to effluents from each of three lanes mixed with aerated tap water to a dilution of 25% in order to simulate the mixing of groundwater into a freshwater wetland through groundwater recharge. Controls were exposed to clean groundwater at the same dilution rate. Three clutches from three individual females were used, and were divided up, each providing one replicate of each treatment. A total of 30 eggs were used in each replicate. *Xenopus* were raised in 1-L glass Mason® jars for 6 d until feeding began. At this point, tadpoles were transferred to 25-L glass aquaria containing 10 L of exposure solution. Tanks were suspended in a circulating water bath, with a constant temperature of 22°C ± 1°C. Tadpoles were fed Nasco Frog Brittle once per day; feces and leftover food were removed. The exposure solution was changed completely every 4 d; on the intervening days, 1.75 L of freshly collected aquifer effluent was added to each tank after removing an equal volume of water. For 4 h immediately following the addition of fresh effluent, tanks were not aerated. During one 24-h renewal period, chloroethylene levels were measured at 0, 4. and 24 h in the exposure tanks and at time 0 in the effluent and compared to expected nominal values to characterize the loss of chlorethylenes due to mixing and volatilization. Tanks were then aerated for 20 h; pretrials with the effluent indicated that aeration was necessary to maintain dissolved oxygen levels above 5 mg/L. When tadpoles reached metamorphosis, stage 66 (Nieuwkoop and Faber 1994), they were euthanized using a 1% solution of tricaine methane sulfonate, weighed, and the snout vent length (SVL) was measured. Frogs were assessed for malformations under a dissecting scope. Malformations were classified according to standard nomenclature (Meteyer *et al*. 2000; Ouellet 2000).

Bacterial Screening on Xenopus *Chronically Exposed to Effluents*

Upon euthanizing, frogs were initially examined macroscopically for any visible lesions. To determine if harmful bacterial infection would result from exposure to the elevated bacterial levels associated with the bioremediation treatments, swabs were taken from a subsample of frogs from each treatment group. Euthanized frogs were stored at −80°C and then thawed 20 min prior to swabbing. Swabs were conducted in sterile conditions under a laminar flow hood. Frogs were first swabbed externally with ethanol and their dorsal surface exposed to UV light for 20 min to sterilize the skin and prevent contamination while opening the abdominal cavity. Both aerobic and anaerobic swabs were taken from the abdominal cavity and from the lower intestinal tract to provide a fecal sample. BD BBL™ Port-A-Cul swabs were used in the collection and transport of aerobes and BD BBL™ Culture Swab Plus with charcoal were used for anaerobes. Three frogs from each treatment (one frog from each replicate tank) were swabbed; swabs were pooled so that each microbial assessment contained material from each of the three frogs. This may have led to higher than normal counts. Swabs were immediately transported to the Animal Health Laboratory at the University of Guelph for routine bacteriology including enterobacterial: *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Aeromonas* spp.; Gram-positive bacteria, and a subset of anaerobes including *Fusobacterium* spp. and bacteriodes. In addition, screening was conducted for the presence of specific pathogens: *Yersinia* spp. *Salmonella* spp., and *Campylobacter* spp. Anaerobic fecal samples were screened for *Clostridium perfringens*. If bacteria were detected in any of the abdominal samples, the analysis was redone with three new frogs to ensure results were replicable. The relative density of bacterial colonies was expressed as categories: 0 representing no colonies, 1+ occasional colonies, 2+ few colonies, 3+ moderate number of colonies, and 4+ representing a large number of colonies.

Statistics

All analyses were performed using STATISTICA 6.1 (StatSoft 2003). For the acute exposures, differences in survivorship and malformation rates among effluent concentrations were analyzed using a one-way

analysis of variance (ANOVA) with each jar considered a replicate ($n = 3$). Each effluent type was compared independently with the control group. For the chronic exposures, differences in survivorship, time to metamorphosis, and malformation rates among effluent groups (i.e., AU, ST, NA, Control) were analyzed using a one-way ANOVA with three replicate tanks per effluent type. Percentage data were normalized by using the arc-sign transformation (Sokal and Rohlf 1999). Clutch effects for survivorship, size, time to metamorphosis, and malformation rates were analyzed using an ANOVA with clutch as the categorical predictor variable across effluent type. An ANCOVA was conducted to test for differences in body size using cumulative tadpole density in each tank as a continuous predictor variable. Post hoc tests for differences among groups were Tukey's honestly significant difference test.

Results

Composition of Effluents

Neither PCE nor any of its breakdown products were detected in the groundwater used for control exposures. The parent contaminant, PCE, was the primary chloroethylene to be detected in effluent from the NA lane, with only low concentrations of *cis*-DCE occasionally detected (Figure 1). In the ST lane, biodegradation was promoted through reductive dechlorination; levels of PCE were lower than in the NA lane, and *cis*-DCE was measured at levels between 24 and 63 ppb. However, no further degradative products were observed in the ST lane throughout the period of the study. In the AU lane, PCE was dechlorinated to ethene (0 to 0.3 ppb) with intermediate degradation products, such as VC (5 to 9 ppb), remaining. At the beginning of the chronic exposures, concentrations of PCE in the aquifer effluents ranged from 440–550 ppb (NA), 200–350 ppb (ST), and 190–250 ppb (AU) with 70–100 ppb of *cis*-DCE in the AU lane.

Concentrations of chloroethylenes were measured in the effluent only, on a regular basis. After mixing and dilution of the effluent, concentrations of chloroethylenes in the tadpole exposure solutions were considerably lower. The nominal concentrations in the amphibian exposure tanks were expected to be 25% of the measured concentration of the effluent immediately after mixing with aerated water. In all likelihood, the actual concentrations were substantially less than the nominal concentration for most of the exposure period. Measurements of chloroethylenes in tank water 4 hours after mixing with the effluent indicated that PCE levels were 17% to 25% of nominal concentrations, while concentrations of *cis*-DCE were 27% to 40% of nominal concentrations. After aeration for 20 hours, concentrations of both compounds dropped to trace levels, at which point more effluent was added. This would bring the range of expected PCE concentrations in the tadpole tanks at between 0 and 100 ppb over the 4 months of exposure, and the range of *cis*-DCE concentrations between 0 and 8 ppb.

Other compounds present in the aquifer effluents include methane and ethane, and propionate. Levels of methane ranged from 0 to 52 ppb in the AU effluent, 0 to 67 ppb in the ST effluent, and 0 to 0.7 ppb in the NA effluent. Propionate was also found in the effluents in low concentrations around 20 ppb in all effluents. Within the aquifer, densities of viable bacteria ranged from $1.47 \times E05$ to $3.42 \times E05$ bacteria per ml in water samples taken from wells just prior to where the effluent exits the aquifer, suggesting that not all bacteria had been filtered out by the tank. Measured concentrations of total heterotrophic bacterial densities in the aquifer effluents ranged from 840 to 2300 cfu per 100 ml as quantified by membrane filtration; however, this method did not allow us to identify the bacteria. All three aquifer effluents tested negative for the pathogens *Cryptosporidium parvum, Campylobacter* sp., *Shigella* sp., *Staphylococcus aureus, Clostridium perfringins, Escherichia coli, Pseudomonas aeruginosa*, and *Yersinia* sp. However, the source water for the aquifer did contain large numbers of *Pseudomonas aeruginosa*, indicating that control individuals may have been exposed to this bacterium.

Effects of Acute Exposures of Effluents

Acute exposure to aquifer effluents (NA, ST, and AU) did not lead to a decrease in survivorship of either green frogs or *Xenopus* embryos (Table 1). Survivorship of green frog embryos did not fall below 95% in any of the treatment or control groups, whereas survivorship of *Xenopus* embryos was significantly lower in the control group than in the AU 25% effluent exposure group (ANOVA $F = 4.3$, $p = 0.044$). There was a slight nonsignificant increase in malformation rates in *Xenopus* embryos exposed to the highest concentration of the ST effluent and AU effluent relative to controls. The majority of these malformations involved dorsal flexure of the tail and notochord.

Effects of Chronic Exposure of Effluents

At 68 d prior to metamorphosis, the control group appeared to have higher survival than those exposed to the remediated effluents, although this was not significant (ANOVA $F = 2.0$, $p = 0.19$, Table 2). By day 100, any earlier differences were no longer apparent ($F = 0.35$ $p = 0.79$, Table 2). There was no significant clutch effect for survivorship. Of the surviving individuals, time to metamorphic climax did not vary among the groups. There was a significant clutch effect ($F = 3.1$ $p = 0.049$), with individuals from clutch three reaching metamorphic climax four to five days earlier than the other two clutches. There was no increase in the incidence of malformations in metamorphosed frogs exposed to any of the aquifer effluents as compared to the control group ($F = 1.44$, $p = 0.3$).

Size of frogs at metamorphic climax varied significantly among treatments (Table 2). There was a significant difference in weight, with frogs from the control and ST groups being 16% smaller on average than those from the AU and NA group. Snout-vent length was also significantly lower in control frogs as compared to the NA or AU group. However, size was significantly correlated with the number of frogs per tank (weight $R^2 = 0.25$, length $R^2 = 0.24$). When density was considered in the model as a covariate, there was no significant effect of treatment for either weight ($F = 0.9$, $p = 0.4$) or snout vent length ($F = 1.1$, $p = 0.35$).

Table 1. Survivorship and malformation rates in green frog and *Xenopus* embryos exposed to aquifer effluent from three bioremediation scenarios: Natural Attenuation (NA), Biostimulation (ST), and Bioaugmentation (AU) and control groundwater for 96 h

			Green Frogs				*Xenopus*			
			Survivorship (%)		Malformations (%)		Survivorship (%)		Malformations (%)	
Treatment	*cis*-DCE (ppb)	PCE (ppb)	mean	stdev	mean	stdev	mean	stdev	mean	stdev
Control	0	0	98.9	1.9	5.6	5.1	75.6*	8.4	7.8	5.1
NA 12.5	0	8 – 20	95.5	1.9	6.7	3.3	82.2	10.7	8.9	5.1
25	0	14 – 33	98.9	1.9	5.5	1.8	88.9	10.7	3.3	0
50	0	20 – 59	93.5	3.4	6.4	5.5	95.6	3.8	8.8	1.8
ST 12.5	0	5 – 8	95.7	1.7	2.2	1.9	88.9	7.7	6.7	3.3
25	0	7 – 13	95.6	3.8	2.3	4.0	93.3	6.7	5.6	3.9
50	0	16 – 28	97.8	3.9	5.3	4.7	82.2	16.4	14.4	1.9
AU 12.5	4 – 8	4 – 17	95.6	3.9	7.7	6.9	85.6	3.9	13.3	6.7
25	7 – 15	7 – 36	97.8	3.9	8.9	5.2	91.1	3.9	4.4	1.9
50	17 – 18	15 – 37	97.8	3.9	5.5	3.9	85.6	5.1	14.4	3.9

The range over the 24-h renewal period of measured concentrations of the two main contaminants in the effluent, *cis*-DCE and PCE., are given for each effluent type.
stdev = standard deviation.
* Significant at the $p < 0.05$ level.

Table 2. Survivorship (day 68 [stage 52–64] and day 100 [stage 60–66]), time to transformation, and size at metamorphosis of *Xenopus* tadpoles exposed to aquifer effluent from three bioremediation scenarios (Natural Attenuation (NA), Biostimulation (ST), and Bioaugmentation (AU)), and control for 100 days

	Survivorship day 68 (%)		Survivorship day 100 (%)		Time to transform (d)		Weight (g)		Snout-vent length (mm)	
Treatment	mean	stdev	mean	stdev	mean	SE	mean	SE	mean	SE
Control	88.3	1.4	70.2	11.4	104.3	2.2	0.36^{B}	0.01	15.3^{C}	0.2
NA	63.3	17.4	58.9	18.4	104.8	2.6	0.42^{A}	0.02	15.9^{AB}	0.2
ST	68.7	27.8	65.4	25.0	103.1	1.8	0.37^{B}	0.02	15.5^{BC}	0.2
AU	60.6	5.8	58.4	4.3	100.9	2.3	0.45^{A}	0.02	16.4^{A}	0.2

Letters indicate means that are significantly different at the $p < 0.05$ level. stdev = standard deviation; SE = standard error.

Bacterial Screening on Xenopus *Chronically Exposed to Effluents*

Only in the control frogs were any bacteria isolated from the abdominal cavity (Table 3). To ensure these large numbers were not due to contamination of the samples, the assay was repeated on three separate frogs from the control treatment with similar results. Frogs from NA, ST, and AU effluents, swabbed and cultured at the same time, did not show bacterial growth from abdominal swabs, eliminating the hypothesis of contamination of samples. No frogs, including controls, tested positive for *Yersinia*, *Salmonella*, or *Campylobacter*. Fecal swabs indicated a number of aerobic organisms in all treatment groups but contained no *Clostridium perfringens.*

Discussion

Few impacts on survivorship or malformation rates were seen as a result of acute embryonic exposure to effluents of an aquifer contaminated with low levels of PCE and treated with three bioremediation approaches. This suggests that bioremediation through the addition of a microbial consortia known to degrade chlorinated solvents (AU with KB-1™), or the enhancement of the native microbial community (ST), poses a minimal threat to early life stages of amphibians. There was no impact on the hatching success of either green frogs or *Xenopus* as compared to the control group. Hatching success was high with the exception of the control group for the *Xenopus* exposure. Mortality induced by chloroethylene toxicity was not expected given the low levels of contamination in the effluents. Concentrations of PCE and its metabolites were well below levels known to be toxic to fish by at least an order of magnitude (Call *et al*. 1983). Previous exposures indicated that levels as high as 20 mg/L PCE, 60 mg/L of TCE, and 100 mg/L of DCE did not induce significant mortality in American toad (*Bufo americanus*), green frog (*Rana clamitans*), wood frog (*Rana sylvatica*), or spotted salamander (*Ambystoma maculatum*) embryos (McDaniel *et al*. 2004).

Exposure to aquifer effluents did not result in an increase of malformation rates in green frog embryos or *Xenopus*. Levels of PCE, TCE, and *cis*- and *trans*-DCE in the aquifer effluents were considerably below those found to cause malformations in exposures to amphibian embryos (Fort *et al*. 1993). McDaniel *et al*. (2004) determined that the 96-h EC_{50} for malformations

Table 3. Counts of bacterial swabs of metamorphosed *Xenopus* froglet*s* raised in aquifer effluents from the three bioremediation scenarios and control groundwater

Treatment	Swab type	Bacteria	Count[a]
Control[b]	Abdominal aerobic	*Citrobacter freundii*	4 +
		Escherichia coli	4 +
		Pseudomonas sp.	4 +
		Klebsiella oxytoca	4 +
	Abdominal anaerobic	Mixed Anaerobes	3 +
	Fecal Aerobic	*Escherichia coli*	4 +
		Citrobacter sp.	4 +
		Klebsiella sp.	4 +
	Fecal anaerobic	*Clostridium pf*	0
NA	Abdominal aerobic	No bacterial growth	0
	Abdominal anaerobic	No bacterial growth	0
	Fecal aerobic	*Escherichia coli*	4 +
		Citrobacter sp.	NQ
	Fecal anaerobic	*Clostridium pf*	0
ST	Abdominal aerobic	No bacterial growth	0
	Abdominal anaerobic	No bacterial growth	0
	Fecal aerobic	*Escherichia coli*	4 +
		Klebsiella sp.	4 +
		Citrobacter freundii	4 +
	Fecal anaerobic	*Clostridium pf*	0
AU	Abdominal aerobic	No bacterial growth	0
	Abdominal anaerobic	No bacterial growth	0
	Fecal aerobic	*Citrobacter freundii*	3 +
		Aeromonas sp.	3 +
		Shewanella putrifaciens	3 +
		Pseudomonas sp.	1 +
	Fecal anaerobic	*Clostridium pf*	0

NQ is not quantified.
[a] The relative density of bacterial colonies given are categorized, with 0 being no colonies and 4 being a large number of colonies.
[b] Combined results of two separate tests.

caused by exposure to PCE for green frogs was 7.9 mg/L and the EC_{50} for TCE was 22 mg/L while no increase in the incidence of malformation was seen at the 2.5-mg/L level.

Concentrations of chloroethylenes in the aquifer lanes were well within the range of those measured in the groundwater of contaminated aquifers where concentrations of PCE as high as 75 ppm have been recorded (Environment Canada 1994). Concentrations of PCE and *cis*-DCE in tadpole exposure solutions, in contrast, were in the nd to 100-ppb range, and were reflective of levels in surface waters (Environment Canada 1994). We believe these concentrations accurately reflect a realistic scenario of surface water contamination from groundwater recharge of a wetland. These low chloroethylene levels allowed us to evaluate the effects of the remediation techniques on amphibian health without the confounding influence of chloroethylene toxicity.

Consistent differences were seen among clutches in *Xenopus* in terms of time to metamorphosis and growth. Such interclutch variability is thought to be the result of maternal effects arising from differences in egg size or maternal investment per egg (Parichy and Kaplan 1992). There is evidence to suggest that both maternal and genetic effects can lead to differences in tolerance to suboptimal water chemistry (Pierce and Sikand 1985). This variation in tolerance to water chemistry, growth rates, and time to metamorphosis due to maternal effect has important implications for both field exposure studies and lab toxicity testing. Importantly, it emphasizes the necessity of using multiple clutches in amphibian bioassays.

Chronic exposure to bioremediated effluents had a minimal impact on tadpole survivorship, growth, and development. Levels of chloroethylenes in the effluents were likely too low to have an inhibitory effect on growth and development. In a previous study, chronic exposures of American toad larvae for 30 days at much higher concentrations of PCE (4 ppm) and TCE (4 ppm) did not have an impact on survivorship and rate of development (McDaniel *et al*. 2004). At day 68 in the current study, survivorship was nonsignificantly lower in the aquifer treatment groups compared to controls. By the end of the 100-d exposure, however, these differences had disappeared as most control mortality occurred during metamorphosis; tadpoles exposed to effluents died throughout the entire exposure period. Because of these differing patterns in mortality, control tadpoles developed at higher densities and were, thus, significantly smaller than animals in the AU and NA treatments. This is consistent with tadpole husbandry literature, which suggests growth may be inhibited in crowded conditions (Richards 1958). When density was considered in the statistical model, all differences in body size between treatments disappeared, suggesting size differences were mainly due to differing tank densities. Early mortality in the treatments where tadpoles were exposed to effluents, may have resulted in larger individuals, which were more likely to survive metamorphosis. It is possible that undersized individ-

uals in the control groups may not have had sufficient energy reserves to survive the stress of metamorphosis. Increased body size at metamorphosis has been correlated with increased survivorship of transformed frogs (Alford 1999). In this way, there can be a compensatory effect of density in regard to pesticide-induced mortality and its impact on body size and later survivorship.

The survival of many of the bacterial species within the KB-1 culture would be expected to be limited in the aerobic conditions present in the tadpole exposure tanks since most of the dominant bacteria are thought to be obligate anaerobes (*Acetobacterium* sp. and *Dehalococcoides ethenogenes*). There is, however, some evidence to suggest that the effluent did contain live KB-1 bacteria. *Dehaloccocoides* was shown to survive the aerobic conditions present in the aquifer over part of the exposure period, indicating it can survive short periods of aerobic conditions. In addition, DNA from *Dehaloccocoides* was positively identified in the AU effluent (Sandra P. Toquica Diaz, unpublished data). Microfiltration of the effluent indicated numbers of unidentified bacteria in all effluents. However, neither the KB-1 culture nor the aquifer effluents tested positive for any of the pathogenic bacteria species for which they were screened. All the evidence suggests that KB-1 culture itself is not harmful to amphibians. In preliminary trials, American toad embryos, fathead minnows, and *Lemna minor* (duckweed) were exposed to groundwater containing KB-1 culture for between 4 (toads) and 7 (fathead minnow and *Lemna*) days. There were no significant effects on growth or survivorship from exposure to groundwater containing KB-1 culture (unpublished data). Therefore, any effects seen on the tadpoles were more likely a result of reduced water quality associated with either the by-products of bacterial metabolism, bacterial toxins, or with a change in the environmental conditions optimized to promote bacterial growth (Sun *et al*. 2000).

There is no indication that the addition of a microbial consortium initially isolated from a contaminated site (AU treatment) or the enhancement of the indigenous bacterial population (ST treatment) led to the increased risk of infection in *Xenopus*. Abdominal swabs of all *Xenopus* exposed to remediated aquifer effluents were clear of bacterial growth. This is promising in terms of the biosafety of these remediation techniques, and not unexpected. Previous screenings of KB-1 culture tested negative for a series pathogenic to humans, many of which are also pathogenic to amphibians: *Salmonella*, *Listeria monocytogenes*, *Vibrio* sp., *Campylobacter* sp., hemolytic *Clostridia* sp., *Bacillus anthracis*, *Pseudomonas aeruginosa*, *Yersinia* sp., pathogenic yeast and mold, fecal coliforms, and enterococci (Duhamel *et al*. 2002; Peter Dennis, SiREM, Guelph, ON, personal communication), nor did any of our pathogens screens of KB-1 or the effluents reveal any pathogenic bacteria species.

In contrast, abdominal swabs of control frogs tested positive for *Citrobacter freundii*, *Escherichia coli*, *Pseudomonas* sp., and *Klebsiella*, all of which are potential frog pathogens, but are also part of the normal intestinal flora. The presence of high numbers of bacteria in the abdominal cavity of control frogs may indicate a significant bacterial infection. *Citrobacter freundii* is a well-known anuran pathogen and has been found to opportunistically infect open wounds and lesions in farmed frogs, as has *E. coli* (Baldassi *et al*. 1995). While *Citrobacter freundii* was also isolated in the fecal samples of frogs from some of the other treatments, this was to be expected as it is known to be a normal component of the flora of the lower intestinal tract (Hird *et al*. 1983). *Klebsiella* is also both a normal component of the intestinal flora and a potential pathogen that has been related to septicemia (Hird *et al*. 1983). *Pseudomonas* sp. can lead to serious infection (Kaplan 1953). Bacterial infection may help to explain the smaller than average body size of control animals relative to those from other treatments if energy that would otherwise be allocated to growth is used to fight infection. Bacterial infection can lead to anorexia in amphibians (Crawshaw 2000).

It is not clear why bacterial infection may have been present in control individuals and absent in those raised in aquifer effluents. It is possible that populations of pathogenic bacteria were lower in aquifer effluent than they were in the control groundwater. This is supported by the fact that *Pseudomonus aeruginosa* was found in the source groundwater going into the tanks, and not in the aquifer effluents exiting the tanks. In fact, since the source groundwater was used for the control frog exposures, this is likely to have been the source of their *Pseudomonas* infections. All water from the aquifer effluent was passed through a large sand bed that may have filtered a significant proportion of the groundwater indigenous bacteria. Alternatively, the addition of chloroethylenes may have resulted in a changed bacterial community from that originally present in the groundwater. Indeed, it is expected that the addition of PCE at concentrations as high as 30 ppm at 1 m from the injection source had an adverse effect on the groundwater microbial community by reducing its structure and function including potential amphibian pathogens (Fuller *et al*. 1997; Baker *et al*. 2001). While we cannot say whether or not this was due to filtration or to biotic/abiotic conditions in the aquifer, filtration seems less likely as, overall, bacteria were still numerous in the effluent.

The results presented in this paper suggest that bioremediation using the addition of a bacterial consortium known to degrade chlorinated solvents, specifically KB-1™, has little or no impact on amphibian growth, development, or survivorship and was not pathogenic. This work was performed using a large-scale model aquifer to simulate conditions that may exist in field applications of this technique. Although amphibians represent one possible receptor, effects on additional potential receptor species are currently being tested.

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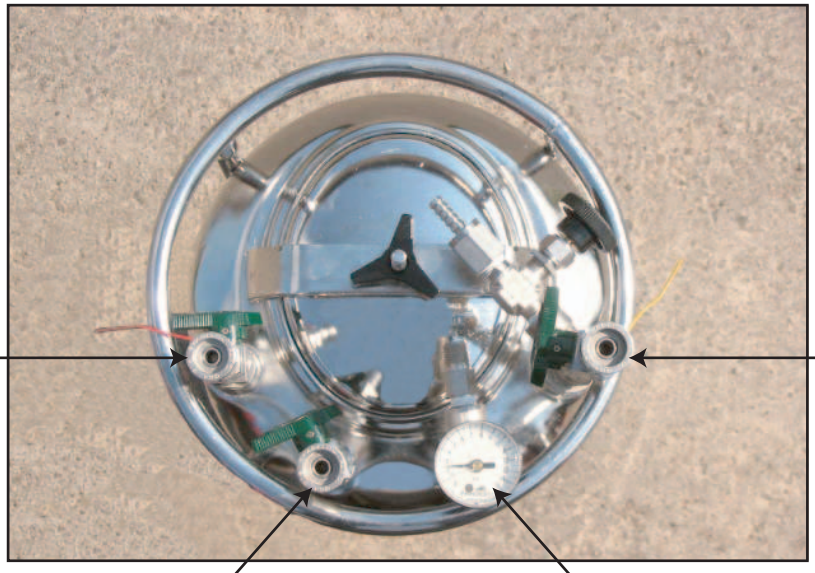
Attachment G:

Photographs of KB-1® Vessels and Shipping Overpacks

KB-1® Vessel
Empty Weight = 27 lbs
Full Weight = 77 lbs

KB-1® Vessel Shipping Case
Empty Weight = 28 lbs
Full Weight with KB-1 Vessel = 105 lbs



KB-1® Vessel and Shipping Case

Feb. 2007	Figure: **1**	SiREM Site Recovery & Management

KB-1 vessels.ai