Description of the SDC-9™ culture.

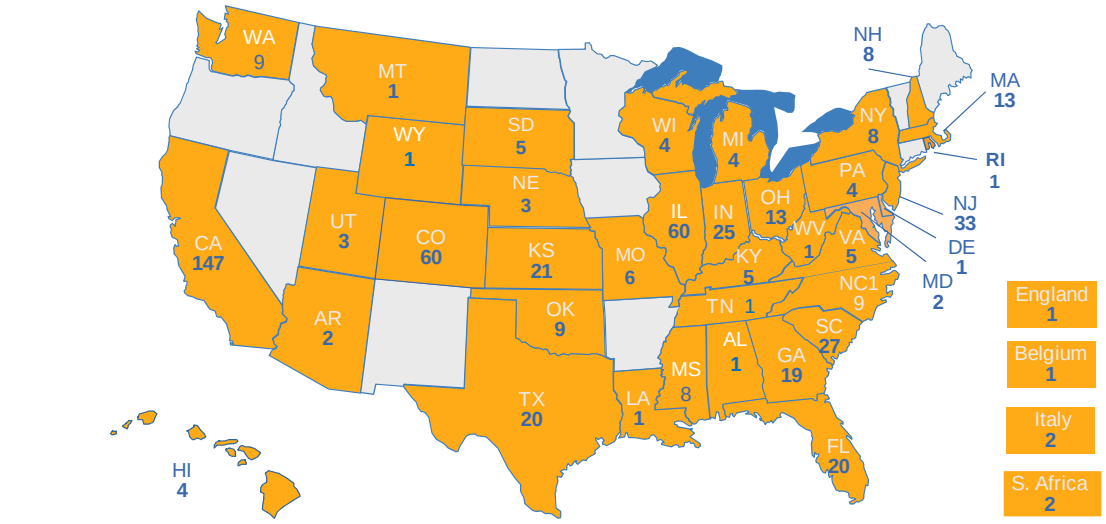
By, Robert J. Steffan, Ph.D.

The SDC-9™ culture produced by CB&I Federal Services, LLC (CFS; Formerly Shaw Environmental, Inc.) is a mixture of anaerobic bacteria that was selected by enrichment culturing of environmental samples with lactate as an electron donor and perchloroethylene (PCE) as an electron acceptor (Vainberg et al., 2009; attached). The culture rapidly degrades chlorinated ethenes via reductive dehalogenation; it converts highly chlorinated solvents (e.g., PCE and TCE) completely to ethene. It has been applied more than 600 times at sites throughout United States, Europe, and S. Africa (Figure 1), and described in many published scientific papers. The culture is sold under a variety of trade names by licensed distributors. Trade names for the SDC-9™ culture include: SDC-9™, BAC-9 ™, RCB-1 ™, BCIplus ™, and TSI-DC ™ .

Because of the extreme challenge of individually isolating anaerobic dechlorinating organisms in pure culture, the SDC-9™ culture is maintained as a consortium of many organisms, and the microbial population of the culture has been characterized by performing Denaturing Gradient Gel Electrophoresis (DGGE) analysis. This method is the most widely accepted method for characterizing such mixed cultures, and it relies on PCR amplification of the 16S rDNA sequences of the individual members of the consortium. The amplified DNA is separated by using denaturing gradient electrophoresis, and the individual DNA bands are sequences to determine the identity of the member organisms. The sequences obtained are compared to sequences in the GenBank DNA sequence database. The SDC-9™ culture contains multiple strains *Dehalococcoides* spp. organisms that are well known for their ability to dehalogenate a wide range of pollutants including PCE, TCE, DCE, and vinyl chloride. It also contains multiple *Desulfovibrio* spp. strains. These organisms are common sulfate reducing bacteria as are found widely distributed in nearly all anaerobic environments, and they are known to dehalogenate PCE and TCE. The culture also contains a *Desulfobacterium* sp. strain. These organisms also are common in anaerobic environments and are characterized as fermentative organisms. It is believed that these organisms play an important role in ensuring the dehalogenating activity of dechlorinating microbes in the consortium by producing fermentative H_2 that is used as an electron donor by the dechlorinating organisms, and by producing important co-factors needed by the dechlorinators.

The efficacy of using *Dehalococcoides sp.*-containing cultures for remediating chlorinated solvent contaminated groundwater is now rarely questioned, provided site conditions are appropriate for the survival and distribution of the organisms. Success of bioaugmentation with the SDC-9™ culture has been documented in large number of published studies and abstracts at national and international remediation conferences. A partial list of SDC-9 papers and a copy a hallmark study on the field application of the culture are below and attached (Attachment 2), respectively. The culture also has been widely applied in California (Attachment 3)

Figure 1. Application of the SDC-9™ dechlorination culture up to June 16, 2013.



Total Applications:596

Includes SDC-9™, PJKS™, and Hawaii-05™

Total Volume Delivered: ~103,470.8 L

* Data represent culture deliveries as of 6/19/13 and include licensed culture distributors

Partial list of papers describing the successful application of the SDC-9™ culture.

Steffan, R. J. and S. Vainberg. 2013. Production and handling of *Dehalococcoides* bioaugmentation cultures. pp. 89-113 in, H.F. Stroo, A. Leeson, and C.H. Ward (eds) *Bioaugmentation for Groundwater Remediation*. Springer Science+Business Media, New York.

Stroo, H .F., D. W. Major, R. J. Steffan, S. S. Koenigsberg, C. H. Ward. 2013. Bioaugmentation with *Dehalococcoides*: A decision guide. pp. 117-140 in, H.F. Stroo, A. Leeson, and C.H. Ward (eds) *Bioaugmentation for Groundwater Remediation*. Springer Science+Business Media, New York.

Aziz, C., R.Wymore, and R. Steffan. 2013. Bioaugmentation considerations. pp. 141-169 in, H.F. Stroo, A. Leeson, and C.H. Ward (eds) *Bioaugmentation for Groundwater Remediation*. Springer Science+Business Media, New York.

Schaefer, C.E., D. R. Lippincott, and R. J. Steffan. 2010. Field-scale evaluation of bioaugmentation dosage for treating Chlorinated ethenes. Ground Water Monitor. Remediat. 30:113-124.

Schaefer, C.E., R.M. Towne, S. Vainberg, J.E. McCray, and R.J. Steffan. 2010. Bioaugmentation for treatment of dense non-aqueous phase liquid in fractured sandstone blocks. Environ. Sci. Technol. 44:4958-4964.

Schaefer, C. E., S. Vainberg, C. Condee, R.J. Steffan. 2009. Bioaugmentation for chlorinated ethenes using *Dehalococcoides* sp.: Comparison between batch and column experiments. Chemosphere 75:141-148.

Vainberg, S., C.W. Condee, R.J. Steffan. 2009. Large scale production of *Dehalococcoides sp.*-containing cultures for bioaugmentation. J. Indust. Microbiol. Biotechnol. 36:1189-1197.

Ladaa, T. S. Scarborough, S. Crouch, R. Mayer, D. Leigh, B. Porter, R. Steffan, S. Vainberg, and S. Anderson, 2004, "Bioaugmentation Using SDC-9 to Treat Chlorinated Solvents," *Poster Presentation at the Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compounds,* Monterey, California, May 24-27.

Ladaa, T. and R. Steffan, 2004, "Bioaugmentation Approaches for Treating Chlorinated Solvent-Contaminated Aquifers," *Presented at the Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compounds,* Monterey, California, May 24-27.

Leigh, D. B. Porter, J. Baur, S. Clarke, T. Ladaa, and S. Anderson, 2004, "In Situ Bioremediation of a Tetrachloroethene Source Area at Naval Station Treasure Island,"

Presented at the Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, California, May 24-27.

Steffan, R. J., and T. Ladaa. 2004. Bioaugmentation approaches for treating chlorinated solvent-contaminated aquifers. *AEHS 14th Annual West Coast Conference on Soils, Sediments, and Water.* March 15-18. San Diego, CA.

Vainberg, S. , R. J. Steffan, R. Rogers, T. Ladaa, D. Pohlmann, and D. Leigh. 2006. Production and application of large-scale cultures for bioaugmentation. *The Fifth International Conference on Remediation of Chlorinated and Recalcitrant Compounds.* May 22-25. Monterey, CA.

Pohlmann, D., L. Combass, and A. Kolodziejski. 2006. Comparison of full-scale passive and active TCE remediation using SDC-9 at Moody AFB, Valdosta, GA. *The Fifth International Conference on Remediation of Chlorinated and Recalcitrant Compounds.* May 22-25. Monterey, CA.

Steffan, R. J., S. Vainberg, and K. McClay. 2005 Bioaugmentation for groundwater remediation: Culture production. *Partners in Environmental Technology: Meeting DoD's Environmental Challenges.* Nov. 30-Dec. 2, 2005. Washington, D.C.

Cacciatore, D., D. P. Leigh, and S. Anderson. 2006. Design and Implementation of an Expanded In Situ Bioremediation System**.** *Proceedings of the Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compounds,* Monterey, CA, May 22-25, 2006

Clarke, S., D. P. Leigh, and S. Anderson, 2006, Accelerated Anaerobic In Situ Bioremediation Coupled with an EHC[tm] Permeable Reactive Barrier. *Proceedings of the Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compounds,* Monterey, CA, May 22-25, 2006

Thomas, M. J., D. P. Leigh, and N. Laurente, 2006, In Situ Anaerobic Bioremediation of a Mixed TCE and Perchlorate Plume at Vandenberg Air Force Base . *Proceedings of the Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compounds*, Monterey, CA, May 22-25, 2006

Leigh, D. P., T. Ladaa, R. Steffan, and S. Anderson, 2005, Comparison of Biostimulation and Bioaugmentation for In Situ Bioremediation of Chlorinated Ethenes. *Proceedings of the Seventh International In Situ and On-Site Bioremediation Symposium*, Baltimore MA, June 6-9, 2005

Porter, B., D. Leigh, D. Cacciatore, and W, Schaal, 2005, Design and Operations of an In Situ Anaerobic Bioremediation System**.** *Proceedings of the Seventh International In Situ and On-Site Bioremediation Symposium,* Baltimore MA, June 6-9, 2005

Porter, B., D. Leigh, S. Clarke, J. Baur, D. Cacciatore, and S. Anderson, 2005, Design and Implementation of an Expanded In Situ Anaerobic Bioremediation System. *Proceedings of the Seventh International In Situ and On-Site Bioremediation Symposium,* Baltimore MA, June 6-9, 2005

Leigh, D. P. J. Baur, S. Anderson, and G. Christensen, 2005, Application of In Situ Bioremediation of Various Chlorinated Organics, *Proceedings of the Shaw Environmental & Infrastructure Technical Symposium,* Orlando Florida, April 14 - 16, 2005.

Leigh, D. P., T. Ladaa, R. J. Steffan, and S. Anderson, 2005 Field Comparison of Biostimulation and Bioaugmentation for In Situ Bioremediation of Chlorinated Ethenes. *The 1st International Conference on Environmental Science and Technology, America Academy of Science* – New Orleans, Louisiana, January 23-26, 2005.

Leigh, D. P., T. Ladaa, and R. J. Steffan, 2004, Field Demonstration of Three Techniques for Bioremediation of Chlorinated Ethenes at Naval Station Treasure Island. *The 20th Annual International Conference on Soils, Sediments & Water, University of Massachusetts,* Amherst, MA., October 18-21, 2004

Steffan, R. J., S. Vainberg, T. Ladaa, and D. Leigh, 2004 Anaerobic Bioaugmentation Approaches For Treating Chlorinated Solvent *Contaminated Aquifers. The 20th Annual International Conference on Soils, Sediments & Water, University of Massachusetts,* Amherst, MA., October 18-21, 2004

Leigh, D. P., 2005, In Situ Anaerobic Bioremediation of a Former Dry Cleaning Facility at Naval Station Treasure Island, Lessons Learned – *Invited Speaker, Site Assessment and Mitigation Program Technical Forum,* San Diego, CA September 22, 2004.

J Ind Microbiol Biotechnol (2009) 36:1189–1197
DOI 10.1007/s10295-009-0600-5

ORIGINAL PAPER

Large-scale production of bacterial consortia for remediation of chlorinated solvent-contaminated groundwater

Simon Vainberg · Charles W. Condee · Robert J. Steffan

Received: 22 October 2008 / Accepted: 21 May 2009 / Published online: 12 June 2009

Abstract Chlorinated solvents such as perchloroethylene (PCE) and trichloroethylene (TCE) continue to be significant groundwater contaminants throughout the USA. In many cases efficient bioremediation of aquifers contaminated with these chemicals requires the addition of exogenous microorganisms, specifically members of the genus *Dehalococcoides* (DHC). This process is referred to as bioaugmentation. In this study a fed-batch fermentation process was developed for producing large volumes (to 3,200 L) of DHC-containing consortia suitable for treating contaminated aquifers. Three consortia enriched from three different sites were grown anaerobically with sodium lactate as an electron donor and PCE or TCE as an electron acceptor. DHC titers in excess of 10^{11} DHC/L could be reproducibly obtained at all scales tested and with all three of the enrichment cultures. The mean specific DHC growth rate for culture SDC-9™ was 0.036 ± 0.005 (standard error, SE)/h with a calculated mean doubling time of 19.3 ± 2.7 (SE) h. Finished cultures could be concentrated approximately tenfold by membrane filtration and stored refrigerated (4°C) for more that 40 days without measurable loss of activity. Dehalogenation of PCE by the fermented cultures was affected by pH with no measurable activity at pH <5.0.

S. Vainberg · C. W. Condee · R. J. Steffan (✉)
Shaw Environmental, Inc., 17 Princess Road,
Lawrenceville, NJ 08648, USA
e-mail: rob.steffan@shawgrp.com

S. Vainberg
e-mail: simon.vainberg@shawgrp.com

C. W. Condee
e-mail: charles.condee@shawgrp.com

Keywords Bioremediation · Bioaugmentation · PCE · TCE · Fermentation · *Dehalococcoides* · Dechlorination · SDC-9 · Groundwater

Introduction

Chlorinated ethenes have been used extensively as industrial solvents and cleaning agents, and their widespread use and improper disposal practices have led to them becoming common groundwater contaminants throughout the USA and the world [25, 33]. Because of the widespread occurrence of chlorinated solvent contamination, a number of treatment technologies have emerged and evolved. Currently, the most common treatment alternative involves biological degradation of the solvents.

The predominant biodegradation pathway for chlorinated ethenes under anaerobic conditions is reductive dechlorination. During reductive dechlorination, chlorinated ethenes are used as electron acceptors by specialized microorganisms, and during the process a chlorine atom is removed and replaced with a hydrogen atom [12, 13, 16, 30]. Sequential dechlorination of perchloroethylene (PCE) most commonly proceeds to trichloroethene (TCE), *cis*-1,2-dichloroethene (*c*DCE), vinyl chloride (VC), and finally the desired end product, ethene. In some cultures *trans*-1,2-DCE and 1,1-DCE also can be produced through the reductive dechlorination of TCE [6, 35]. In situ biodegradation of chlorinated ethenes can be performed by indigenous microorganisms at contaminated sites that use endogenous resources to support contaminant degradation (i.e., intrinsic bioremediation), or nutrients that are purposefully added to support their activity (i.e., biostimulation). The lack of an adequate microbial population capable of completely dechlorinating PCE and TCE to ethene at some sites,

however, may lead to the accumulation of *cis*-DCE and VC [11]. Consequently, the addition of exogenous organisms (i.e., bioaugmentation) is sometimes used to supplement the indigenous microbial population [5, 15, 21].

While many dechlorinating microorganisms have been identified [30], bacteria of only one microbial genus, *Dehalococcoides* (DHC), have been shown to completely reduce *c*DCE and VC to ethene [7, 8, 22, 23, 26, 31]. These organisms use molecular hydrogen as an obligate electron donor and halogenated compounds as obligate respiratory electron acceptors. Acetate (e.g., from lactate fermentation) is used as a carbon source. Studies of field sites have strongly correlated the presence of DHC strains with complete dehalogenation of chlorinated ethenes in situ [11]. Therefore, microbial cultures used to remediate chlorinated solvent-contaminated groundwaters contain at least one strain of *Dehalococcoides* sp. Because of the difficulty of growing DHC-type organisms in pure culture [7, 8, 10, 23], however, cultures used for bioaugmentation applications are consortia that contain DHC as well as fermentative and other microbes that support the growth and activity of the DHC strains [4, 5, 15, 21]. The consortia, and the DHC therein, can be grown on a wide range of carbon sources provided the substrate is fermented to hydrogen.

One of the significant challenges of performing bioaugmentation at a commercial scale is the large size of contaminant plumes and the large amount of culture needed to facilitate timely and successful remediation. Contaminant plumes can range from less than an acre (0.4 ha) in size to several kilometers long and hundreds of meters wide. Recent studies of in situ chlorinated ethene degradation have suggested that DHC concentrations in the range of 10^7 DHC/L of groundwater are needed to support acceptable degradation rates [19, 28]. To illustrate the challenge of applying bioaugmentation in the field, a 0.4-ha (one-acre) aquifer with a saturated zone 3 m (10 ft) thick and porosity of 25% would contain $\sim 3 \times 10^6$ L of groundwater and require 3×10^{13} DHC based on the findings of Lu et al. At the reported DHC concentrations of early bioaugmentation cultures (10^9 DHC/L; [21]), as much as 10^4 L of culture could be required to treat a one-acre site. Of course other factors affect the amount of culture applied at a site [14, 28], but it is clear that large-scale production of high-density cultures is necessary to apply bioaugmentation economically, especially at large sites.

The objective of this study is to evaluated large-scale production of a DHC-containing consortium, SDC-9™, for full-scale remedial applications. The culture was grown in small (3-L) to large (4,000-L) fermentors by using sodium lactate as a carbon and electron donor source and PCE as an electron acceptor. DHC concentrations of $>10^{11}$/L could be achieved, and the culture could be concentrated and stored prior to field application. The fermentation procedure produced similar results with two other DHC cultures enriched from different sites.

Materials and methods

Chemicals

Sodium-(L)-lactate (60% solution) was purchased from Purac America (Lincolnshire, IL), yeast extract (bacteriological grade) was purchased from Marcor Development Corp. (Carlstadt, NJ), and PCE (99.9%) was from Sigma/Aldrich (Milwaukee, WI). Unless otherwise stated, all other chemicals were of the highest purity available and purchased from either Aldrich Chemical Co. (Milwaukee, WI), Mallinckrodt Specialty Chemical Co. (Paris, KY), J.T. Baker Inc. (Phillipsburg, NJ.), Spectrum Chemical Manufacturing Corp. (Garden, CA) or Sigma Chemical Co. (St. Louis, MO).

Bacterial cultures

An anaerobic dechlorinating consortium designated SDC-9™ was isolated by enrichment culturing of samples from a chlorinated solvent-contaminated aquifer in southern California with lactate as an electron donor and PCE as an electron acceptor. The culture has been maintained on sodium lactate and PCE in reduced anaerobic mineral medium (RAMM) [29], but without sodium sulfide and rezasurin, for more than 4 years. Hawaii-05™ was enriched in 2005 by enrichment culturing of aquifer samples from Hickam Air Force Base, Hawaii on sodium lactate and TCE, and PJKS™ was enriched in 2005 from aquifer samples from Air Force Plant PJKS in Colorado on sodium lactate and TCE. The latter cultures are maintained as described for SDC-9™. All three cultures are marketed commercially by Shaw Environmental, Inc. (Lawrenceville, NJ).

Fermentation equipment

Bench-scale fermentation experiments and seed culture production were performed in a 3-L or 7-L Applicon fermentor (Cole Palmer, Vernon Hills, IL.) equipped with pH and mixer controls. Substrate and NaOH feeds were controlled by using syringe pumps (Harvard Apparatus, Holliston, MA). Larger seed cultures were produced in a similarly equipped 20-L Biolafitte fermentor (Pierre Guerin, Inc., Spring Lake Park, MN). Larger cultures were produced in a 750-L ABEC fermentor (Bethlehem, PA) or a custom-built 4,000-L stainless-steel fermentor. In each case anaerobic conditions were maintained by pressurizing the vessels with nitrogen. Cells in the fermentation broth were concentrated by passing the broth over a custom-built concentrator

constructed with six Kerasep™ tubular ceramic membranes (Novasep, Inc., Boothwyn, PA). Concentrated cells were stored at 4°C in 18.5-L stainless-steel soda kegs that were pressurized with nitrogen.

Fermentation protocol

For seed culture production RAMM medium [29] without $NaHCO_3$ and Na_2S was added to the 20-L fermentor and steam sterilized at 121°C and 15 psi pressure for 45 min. After sterilization the fermentor was connected to a nitrogen tank to maintain a positive pressure of nitrogen in the fermentor during cooling to 30°C. After the temperature in the fermentor reached the set-point temperature of fermentation (28–30°C) and anaerobic condition were achieved [measured dissolved oxygen (DO) = 0 mg/L], nitrogen flow was stopped and $NaHCO_3$ solution was added aseptically to the medium. The fermentor was then inoculated with 2 L of SDC-9™, PJKS™ or Hawaii-05™. The final volume of medium in the fermentor was 16–18 L.

After inoculation of the fermentor, sterile 10% yeast extract (YE) solution was added to a final concentration of 0.1% YE (w/v) and PCE or TCE was added to a final concentration of 10 mg/L. SDC-9™ was grown on PCE, but PJKS™ and Hawaii-05™ were grown on either PCE or TCE. The fermentor was operated at 28–30°C with an agitator speed of 100 rpm. pH was maintained at 6.4–7.2 by addition NaOH (2 N). Alternatively, to increase pH during fermentation, the fermentor was sparged with nitrogen to remove dissolved CO_2. To control foam in the fermentor Antifoam 289 or 204 (Sigma) was applied automatically. After 1 day of fermentation, sodium lactate (60% solution) was added continuously to the fermentor at flow rate of 0.02–0.04 mL/h × liter of media. Subsequent additions of PCE or TCE (10 mg/L) were made to the fermentor only after complete dechlorination of PCE/TCE but before complete dechlorination of *c*DCE. Typically, PCE/TCE was added to the medium when the concentration of *c*DCE in the medium was reduced to 1–3 mg/L. When the culture reached an optical density (OD) at 550 nm (OD_{550}) of approximately 1.0 it was transferred anaerobically to the 750-L fermentor.

The 750-L fermentor was prepared with 550 L RAMM medium and sampled and monitored essentially as described above. The fermentor was connected to a nitrogen tank to maintain anoxic conditions, and it was operated under the same conditions as described for the 20-L fermentor except the agitator speed was set at 60 rpm. The automatic pH control system on the fermentor was inactivated to avoid addition of excess sodium. After 1 day of fermentation a continuous feed of sodium lactate (60% solution) was initiated with flow rate of 0.02–0.04 mL/h × L. When the specific PCE and *c*DCE dechlorination activity reached 1.3–1.7 mg/h × gram of dry weight, a continuous feed of neat PCE/TCE was initiated at rate of 0.18–0.25 μL/h × L. This rate was increased to 0.9–1.2 μL/h × L as the culture cell density and dechlorination activity increased. The culture was grown for 13–15 days until an $OD_{550} \approx 0.7–1.1$ or 10^{10}-10^{11} DHC/L was achieved. Higher DHC concentrations could be obtained by extending the fermentation for up to 35 days.

Growth of the cultures in the 4,000-L fermentor (working volume 3,200 L) was performed essentially as described for the 750-L fermentor, but because the 4,000-L fermentor did not have an impeller, cells were continuously suspended by using a centrifugal pump that circulated the culture medium. The 4,000-L fermentor was chemically sterilized by using NaOH and a clean-in-place system. The culture medium in the 4,000-L fermentor was not sterilized. Substrate feeding and other parameters were as described for the 750-L fermentor. The fermentor was inoculated with either culture from the 750-L fermentor or refrigerated concentrated cell stocks.

Degradation assays and analytical procedures

Whenever possible, analytical methods performed during this project followed US Environmental Protection Agency (USEPA) SW-846 methods [32] that are available online at http://www.epa.gov/epawaste/hazard/testmethods/sw846/index.htm. Biodegradation assays were incubated at 28 ± 1°C in the dark in serum vials essentially as described by Schaefer et al. [28]. Chlorinated ethene analyses were performed by gas chromatography using USEPA method 8260 [gas chromatography/mass spectrometry (GC/MS) with purge and trap injection]. Methane and ethene were monitored by GC/flame ionization detection (FID) according to USEPA SW846 method 8015b. Lactate and volatile fatty acids (VFAs) were measured by ion chromatography using USEPA method 300.0-modified on a Dionex DX600 ion chromatograph (Dionex Corp., Bannockburn, IL). Hydrogen concentration in the fermentors was measured by analyzing the headspace of 100-mL samples in 120-mL vials on a Varian 3800 gas chromatograph (Varian, Inc., Walnut Creek, CA) equipped with a Valco pulsed discharge helium ionization detector (PDHID), a helium gas purifier to achieve helium carrier and makeup gas of 99.999% purity, and Varian Pora Bond Q (10 m, 0.32 inner diameter, 5 uM df) and Varian Molsieve 5A (10 m, 0.32 inner diameter, 5 μM df) columns operated in series. Concentration of hydrogen was determined by comparison to a standard curve. Dry weight (Dwt) was determined by concentrating 15–30 mL culture in a RC5C centrifuge (10,000×*g*; Sorval Instruments, Newtown, CT), removing the supernatant, suspending the pellet in deionized (DI) water, and repeating the procedure twice. The washed cell pellet was suspended

in DI water, transferred to an aluminum weighing dish, and dried at 105°C.

DHC quantification

DHC-like organisms were quantified by using real-time quantitative polymerase chain reaction (qPCR). Following collection of fermentor samples, the OD_{550} of the sample was measured and the cells were either concentrated by centrifugation or diluted with water to an OD_{550} of approximately 0.5. OD was then remeasured for verification. One milliliter of the OD_{550} = 0.5 cells were then concentrated by centrifugation (16,000×*g* for 2 min) and resuspended in 100 µL distilled water. The cells were then processed using an Idaho Technologies 1-2-3 RAPID DNA purification kit (Idaho Technology Inc. Salt Lake City, UT) as per manufacturer instructions and using a Bead Beater (BioSpec Products Inc., Tulsa, OK). DNA was eluted from columns in a final volume of 100 µL buffer rather than the prescribed 400 µL.

Quantitative real-time PCR was performed with a RAPID PCR machine (Idaho Technologies Inc.) and a Lightcycler FastStart DNA Master Hybprobe probe kit (Roche Diagnostics GmbH, Manheim, Germany) and primers developed by us with the assistance of Idaho Technologies, Inc. to amplify and quantify 16 s ribosomal RNA (rRNA) gene DNA. DNA amplification used a forward primer (5′-GAAGTAGTGAACCGAAAGG-3′) and a reverse primer (5′-TCTGTCCATTGTAGCGTG-3′), and the amplified DNA was quantified using a fluorescence resonance energy transfer (FRET) probe system that employed a Light Cycler Red 640 fluorophore (5′-AGCGAGAC TGCCCC-3′) and an fluorescein isothiocyanate (FITC)-labeled probe (5′-CCCACCTTCCTCCCCGTTTC-3′). The amplification conditions were as follows: denaturation at 95°C for 10 min, followed by 40 cycles of melting at 94°C for 20 s, annealing at 53°C for 10 s, and extension at 72°C for 20 s. *Dehalococcoides* sp. chromosomal DNA was quantified by comparison to a standard curve generated by amplifying serial dilutions of a known concentration of plasmid (pSC-A vector; Stratagene Inc. La Jolla, CA) containing a cloned 16S rRNA gene from the SDC-9™ culture.

Results and discussion

Culture growth

A typical growth curve of large-scale (3,200-L) production of SDC-9™ is shown in Fig. 1a. Monod kinetics parameters for SDC-9™ are reported elsewhere [28]. The cells were grown with lactate as an electron donor and PCE as an electron acceptor, and yeast extract was added periodically as indicated. Although the OD_{550} of the culture increased rapidly in the fermentor, DHC concentrations remained constant for 5 days before the initiation of the exponential growth phase. This DHC lag phase, however, did not occur in all fermentation runs and it could be the result of variability in the qPCR quantification method. During the exponential growth phase when both *c*DCE and VC were present in excess the specific growth rate (*m*) reached 0.032/h with a cell doubling time of 21.5 h. During multiple fermentation runs at both the 550-L and 3,200-L scale (n = 5) (data not shown), specific DHC growth rates ranged from 0.027 to 0.043/h with mean rate of 0.036/h (19.3 ± 2.7 h doubling time).

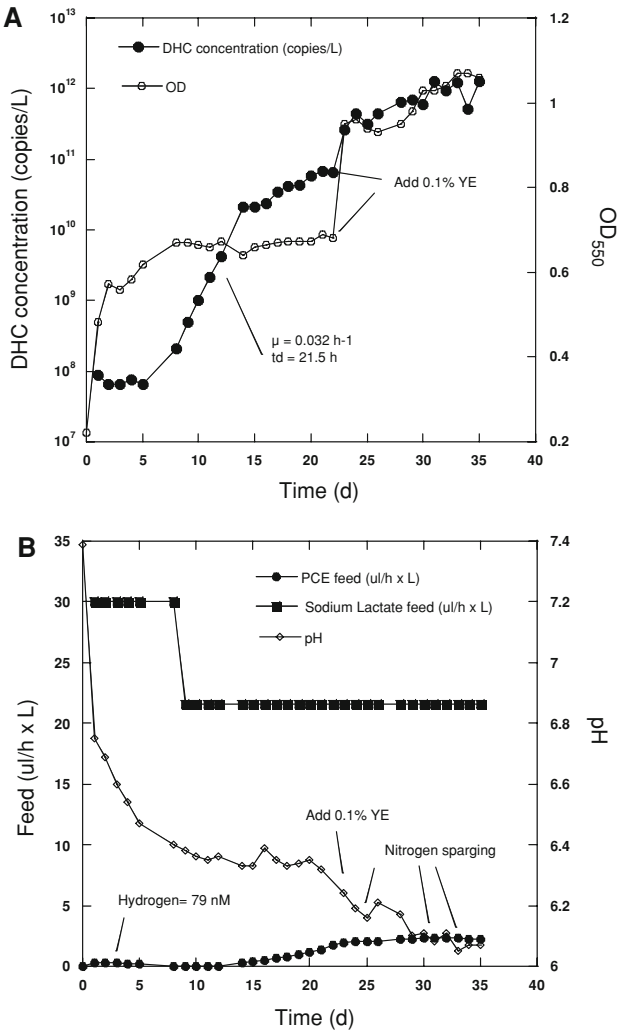


Fig. 1 Growth of SDC-9™ in a 4,000-L fermentor. **a** Concentration of DHC as measured by qPCR (*filled circle*) and total cell concentration as estimated by OD at 550 nm (*open circle*). DHC first-order growth rate (*μ*) and doubling time (td) are indicated on the graph. **b** Feed rate of neat PCE (*filled circle*) and 60% sodium lactate (*filled square*), and the pH of the culture medium (*open diamond*) are indicated. Yeast extract (YE) solution was added at the beginning of the fermentation and as indicated. The fermentor was sparged with N_2 as indicated to control pH

Although the OD of the culture stabilized after approximately 10 days, exponential growth of DHC continued until approximately day 24. These results suggest that non-DHC microorganisms in the consortium initially grew much faster than DHC. During this early fermentation period, DHC represented a relatively low proportion of the total bacterial population of the culture, but during extended growth the relative abundance of DHC in the culture increased. The results also demonstrate that, at least during the early stages of fermentation, OD measurements are not a good indicator of DHC concentration in the culture, and more advanced measurements such as qPCR are needed to estimate DHC numbers in the culture effectively [17, 27].

During the initial stages of 3,200-L fermentation (to day 25) a maximum DHC concentration of $\sim 10^{11}$ DHC/L was achieved in the fermentor, even though growth substrates were still present in the culture broth (Fig. 1a). DHC concentrations in the fermentor, however, could be increased approximately tenfold by the addition of YE as a nutrient source. The exact role of the YE is not known, but its addition also revived the growth of non-DHC organisms in the consortium (Fig. 1a). Because the RAMM medium used in this study did not contain sodium sulfide or other sulfur-containing salts, it is possible that the yeast extract provided a needed source of sulfur for the cultures. Based on our analysis (data not shown) 1 g/L YE was estimated to provide 5 mg/L sulfur and 0.48 mg/L iron. YE also could provide a needed source of amino acids and/or precursors for the production of corrinoid cofactors that are necessary for dehalogenation by DHC strains [23]. During this extended growth of the culture there was a correlation between culture OD_{550} and DHC concentrations, suggesting that during this period of the fermentation process measurements of OD may be useful for estimating DHC levels in the fermentor and to automate the control of the fermentation process.

Similar fermentation results were obtained with two other chloroethene dechlorinating bacterial consortia, PJKS™ and Hawaii-05™, at both the 550-L and 3,200-L scale (Table 1), by using the described procedures. Both cultures could be grown to high DHC concentration ($>10^{11}$ cells/L), and both the final OD_{550} and total cell mass obtained were similar to the results obtained with SDC-9™.

No other studies have evaluated or reported large-scale production of DHC-containing consortia, but the DHC cell concentration achieved in our studies were similar to those obtained by others in small-scale laboratory tests. For example, Couples et al. [1] calculated final DHC concentrations of up to 4×10^{11}/L during growth of the VS culture in TCE-fed 60-mL batch cultures, and He et al. [9], achieved up to 1.8×10^{11} copies/L of the *tce*A gene in 100-mL batch cultures of *D. ethenogenes* strain 195 containing a coculture of a sulfate-reducing bacterium. Similarly, whereas we observed DHC doubling times of 19.3 h during large-scale fermentation, DHC doubling times from small laboratory studies of 19.5 h to 2 days have been reported [2, 9, 10, 22].

The results of this study demonstrate that culture volumes and DHC cell densities sufficient to treat even relatively large contaminated aquifers can be obtained. Assuming that 10^7 DHC/L of contaminated groundwater are needed to obtain effective and timely remediation [19], 3,200 L of culture with 10^{11} DHC/L could potentially support remediation of 3.2×10^7 L of groundwater, even without further in situ growth of the organisms.

Factors affecting fermentation

Several factors could affect the results obtained during growth of the test cultures, including substrate type and feed rates, pH, and VFA accumulation. Growth of DHC requires the presence of a chlorinated substrate as an electron acceptor, H_2 as an electron donor, and a carbon growth source such as acetate [8, 16, 23]. In consortia such as those used in this study, the primary growth substrate (i.e., lactate) is fermented by non-DHC members of the consortia to H_2 and acetate that can be utilized by DHC. The presence of excess H_2, however, can lead to substrate competition with methanogenic bacteria in the consortia that also can use H_2, albeit at a higher substrate threshold than DHC [18, 20, 34]. Therefore, in developing a fermentation protocol for the described cultures, attempts were made to maintain

Table 1 Results of multiple fermentation runs with the tested chlorinated solvent-dechlorinating consortia

Culture	Date (M/Y)	Volume (L)	Final OD_{550}	Final DHC (cells/L)[a]	Dwt (mg/L)	PCE activity (mg/h/g Dwt)	*c*DCE activity (mg/h/g Dwt)
SDC-9	01/2006	550	1.3	1.4 E11	0.51	16	13
SDC-9	02/2008	550	1.7	2.8 E11	0.66	22	14
SDC-9	03/2008	3,200	1.6	1.4 E11	0.65	41	37
SDC-9	05/2008	2,500	1.6	2.4 E12	0.59	42	39
SDC-9	08/2008	2,000	1.4	1.0 E12	0.51	80	69
PJKS	01/2008	2,500	1.1	9.4 E11	0.41	32	14
PJKS	02/2008	1,700	1.3	1.0 E11	0.50	64	45
Hawaii-05	11/2007	550	1.2	1.5 E11	0.50	23	16

[a] Based on qPCR assuming 1 16S rRNA gene copy/cell

consistent low H_2 concentrations within the reactor. The sodium lactate feed rate used during the fermentation process resulted in sustained dissolved hydrogen concentration in the reactor of <20 nM. During utilization of the initial batch feeding of lactate and YE added prior to inoculation, H_2 concentrations sometimes exceeded 100 nM, but during the extended fermentation process H_2 concentrations were typically 3–5 nM, which was similar to the half-velocity coefficient for hydrogen calculated for the VS culture (7 ± 2 nM; [3]).

Fermentation of lactate also led to an accumulation of VFAs (e.g., propionate and acetate) that could potentially inhibit dechlorinating organisms in the consortia. Studies with SDC-9™ demonstrated that dehalogenation of chlorinated ethenes by the culture was not inhibited by propionate and acetate concentrations to 6,000 mg/L (data not shown). Figure 2a, b shows the formation of VFAs during growth of SDC-9™ and PJKS™, respectively. In both cases, the VFA concentrations did not reach inhibitory levels with the fermentation protocol described here. Notably, the SDC-9™ culture accumulated much less propionate and acetate than the PJKS™ culture grown under the same conditions. Although the reason for this lower accumulation of VFAs is not certain, it is likely due to evolution of the SDC-9™ consortium during several years of maintenance on lactate as a primary growth substrate, either in activity or member composition, to utilize VFAs more efficiently.

To optimize the growth of the SDC-9™ consortium it was necessary to determine a relationship between PCE feed rate and DHC cell concentration. We were most concerned about maintaining the VC-reducing population(s) in the consortia because VC reduction is less energetically favorable than the other dehalogenating reactions, so it was possible that PCE and TCE dehalogenating populations could outcompete the VC reducers if the higher chlorinated substrates were maintained in excess. Furthermore, Cupples et al. [3] observed that net decay in dechlorinating microorganisms could occur in the VS culture if DCE plus VC concentrations were below 0.7 μM. In addition, with SDC-9™, based on many biodegradation assays, the VC dechlorination rate is 28–35% of the PCE dechlorination rate. Therefore, there was a tendency for VC to accumulate in the fermentor during high-rate PCE feeding. Consequently, PCE feed rates were adjusted to prevent accumulation of PCE, TCE or *cis*-DCE while maintaining a residual VC concentration in the medium of ~1 mg/L (16 μM). Evaluating the PCE feed rates during multiple fermentation runs, the results of the biodegradation assays, and the analyses of PCE, TCE *c*DCE, and VC concentrations during fermentation allowed us to optimize PCE feed rates for the growth of SDC-9™ consortium. The relationship between DHC yield and PCE feed rate could be described by the following equation: DHC concentration (cells/L) = $-6.77 \times 10^{11} + [8.40 \times 10^{11} \times$ PCE feed rate (mg/h × L)] ($R = 0.999$).

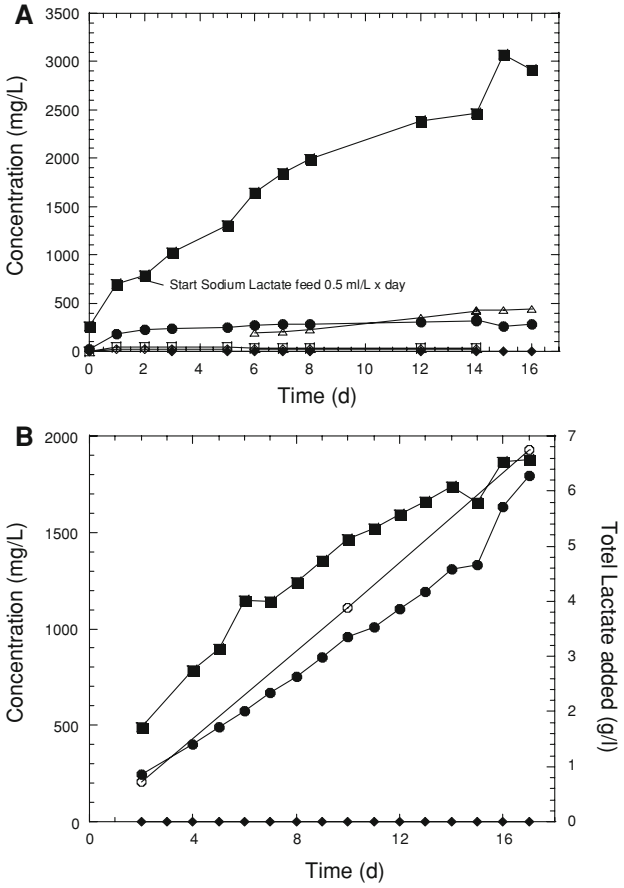


Fig. 2 Accumulation of VFAs during growth of SDC-9™ (**a**) or PJKS™ (**b**) in a 750-L fermentor. Symbols indicate lactic acid (*filled diamond*), propionic acid (*filled circle*), formic acid (*open diamond*), pyruvic acid (*open square*), butyric acid (*open triangle*), and acetic acid (*filled square*), or the total amount of sodium lactate added to the fermentor (*open circle*; **b**)

Dehalogenation of chloroethenes by SDC-9™ also was affected by culture pH, with little or no dehalogenation below pH 5.0 (Fig. 3). Both reductive dehalogenation and fermentation of the growth substrates used to grow the cells consumes considerable amounts of alkalinity [24]. The pH of the medium in the 4,000-L fermentor decreased from an initial pH of 7.4 to approximately 6.1 during the first 30 days of cell growth (Fig. 1b). Because the culture was fed sodium lactate, however, the addition of NaOH to control pH could have led to an excess of sodium ions in the reactor that could affect cell growth. Therefore, instead of adding NaOH, the fermentors were sparged periodically with N_2 to remove dissolved CO_2 from the culture medium. This approach sufficiently regulated the medium pH to allow completion of the culture production (Fig. 1b), even though this may have been below the optimum pH for dehalogenation by the cultures.

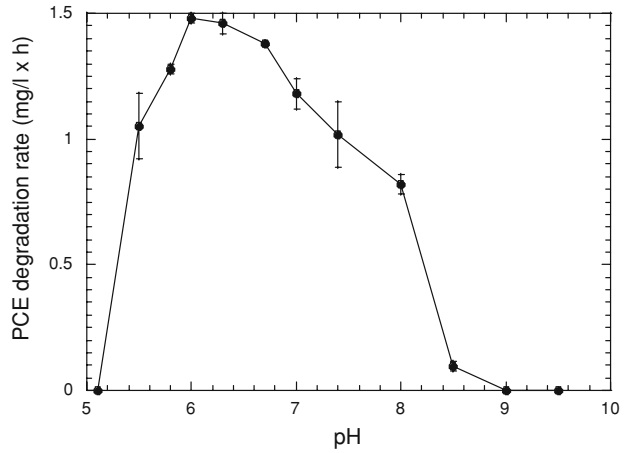


Fig. 3 Effect of culture pH on PCE dehalogenation by SDC-9™. Values represent the mean of triplicate samples, and *error bars* represent one standard error of the mean

Culture activity

The relative degradative activity of the grown dehalogenating cultures was evaluated by performing serum bottle biodegradation assays with the grown culture. The biodegradation assays evaluated the ability of the grown cultures to dehalogenate PCE and *c*DCE by incubating the cells in individual serum vials with either PCE or *c*DCE. An example of a PCE degradation activity assay is presented in Fig. 4. A summary of results from several assays with the three test cultures evaluated here is presented in Table 1. In each case, the specific activities of the resulting cultures were of a similar order of magnitude, but some variation was observed. Several factors could cause the observed differences, including variability in the concentration of nondehalogenating organisms produced. That is, because the cultures were mixtures of dehalogenating and nondehalogenating microbes, even relatively small differences in the total concentration of nondehalogenating microbes could greatly affect the measured specific, dry-weight-based, activity measurements. Repetitive fermentation of SDC-9™ culture over the last 4 years has resulted in development of the current fermentation protocol that has resulted in a general increase in the specific activity of the produced cultures.

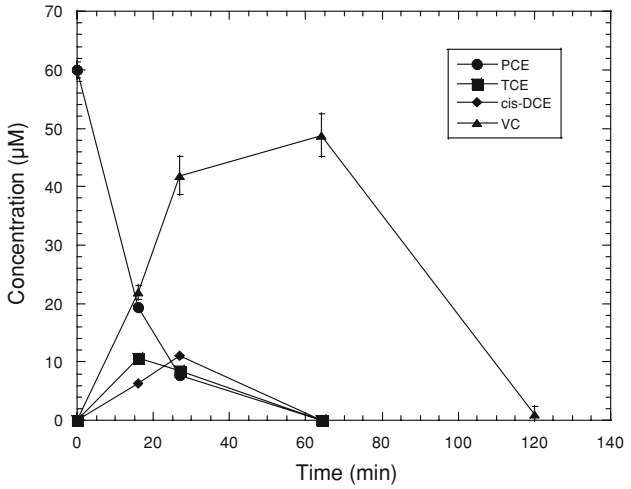


Fig. 4 Results of a PCE degradation assay with samples from a 550-L fermentation batch of the SDC-9™ culture. The assay was performed in 60-mL serum vials containing 60 mL SDC-9™ culture (0.52 g/L Dwt), 6 mM sodium lactate, and 10 mg/L PCE. Values represent means of triplicate samples and *error bars* represent one standard error of the mean

Related issues

The use of bioaugmentation to remediate chlorinated solvent-contaminated sites requires the shipment of cultures throughout the USA and elsewhere. Shipping a large volume of culture is costly, and ground transportation can require that the culture spend several days in shipping, which could affect culture activity. An alternate approach is to concentrate the culture to allow overnight shipping of a reduced culture volume. We used a tubular ceramic membrane system to concentrate consortia. The cell culture was chilled during concentration to ensure maintenance of cell viability. Analysis of the specific activity of the cells before and after concentration demonstrated only slight changes in activity during concentration. For example, specific activity of two cultures tested were 24.5 and 16.5 mg PCE/h × g Dwt before concentration and 22.6 and 15.1 mg PCE/h × g Dwt after concentration, respectively. Concentration resulted in approximately 90% reduction in culture volume, and it also removed ~90% of any fermentation byproducts remaining in the culture broth. It also allowed us to standardize the DHC concentration and activity of culture batches, thereby allowing users to more accurately estimate the volume of culture needed for field applications.

Storage of bacterial cultures also is critical for allowing timely delivery of cultures to contaminated sites to coordinate culture injection with the availability of field personnel and equipment (e.g., drilling rigs). To evaluate storage longevity, tenfold-concentrated SDC-9™ cultures were incubated for up to 90 days at either 4°C, 13°C, 22°C, or 28°C in stainless-steel containers. Periodically, samples of the stored cultures were removed and assayed for their ability to degrade PCE and *c*DCE. Activity of the culture decreased rapidly if stored at 13°C or 28°C, but SDC-9™ could be stored at 4°C for >40 day without measurable loss of activity (Fig. 5).

Conclusions

A fermentation protocol was developed for large-scale production of DHC-containing cultures for in situ

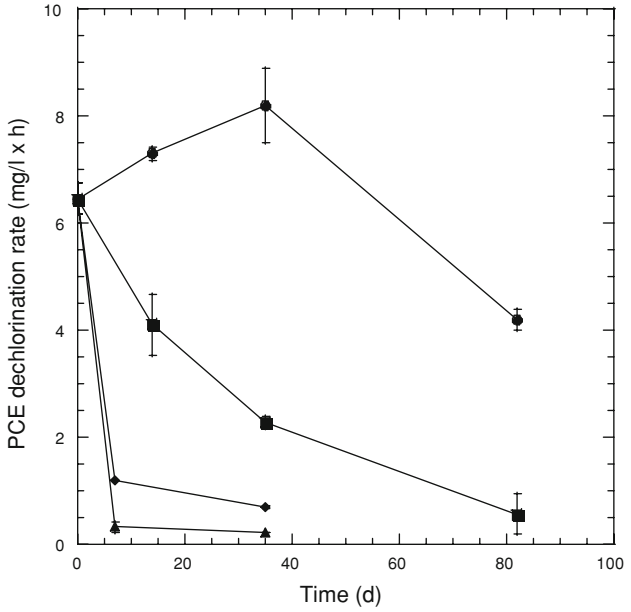


Fig. 5 Effect of storage conditions on the activity of concentrated SDC-9™ culture. Tenfold-concentrated SDC-9™ culture was stored anaerobically and without substrate at either 4°C (*filled circle*), 13°C (*filled square*), 22°C (*filled diamond*) or 28°C (*filled triangle*). Values represent means of triplicate samples, and *error bars* are one standard error of the mean

bioaugmentation of chlorinated ethene-contaminated aquifers. The performance of the SDC-9™ culture in contaminated aquifer material is described elsewhere [28]. Success of the fermentation process was dependant on electron donor (i.e., lactate) and acceptor (PCE) feed rate, and the addition of YE greatly improved cell yield. The initial stages of fermentation were characterized by a rapid growth of non-DHC organisms in the culture, while the growth rate of DHC within the consortia tested exhibited a short lag and then was relatively constant to final DHC concentrations of $>10^{11}$/L. The fermentation protocol was scalable to 550 L and 3,200 L and produced comparable results for consortia enriched from three different sites.

Based on 16S RNA gene sequencing the SDC-9™ culture contains multiple DHC strains (data not shown), and it is possible that growth of the individual dehalogenating strains within the culture might be different during the fermentation process. Although this could not be monitored during this study, our results demonstrated that both PCE and *c*DCE dehalogenation activities were high in the final cultures, and the culture degraded VC well, albeit at a lower rate than PCE and *c*DCE dehalogenation. This suggests that the described procedure supports the growth of DHC that are able to completely dehalogenate chlorinated ethenes, including vinyl chloride. Our results also demonstrate that DHC-containing cultures designed for bioaugmentation can be concentrated by cross-flow filtration to reduce shipping volumes, and that the concentrated cultures can be stored under refrigeration for >40 days to allow for injection schedule flexibility.

With the increased use of bioaugmentation to treat challenging chlorinated ethene-contaminated sites, the ability to produce large volumes of high-density cultures is becoming increasingly important. This study provides useful information to aid in the production of cultures for bioaugmentation, even at scales suitable for treating large contaminant plumes.

Acknowledgments The authors thank Randi Rothmel, Antonio Soto, Kevin McClay, and Paul Hedman for excellent analytical support. This project was supported by the Environmental Security Technology Certification Program (ESTCP) project number CU-0515. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect those of the US Army Corp. of Engineers, Humphreys Engineer Center Support Activity.

References

1. Cupples AM, Spormann AM, McCarty PL (2003) Growth of a *Dehalococcoides*-like microorganism on vinyl chloride and *cis*-dichloroethene as electron acceptors as determined by competitive PCR. Appl Environ Microbiol 69:953–959. doi:10.1128/AEM.69.2.953-959.2003
2. Cupples AM, Spormann AM, McCarty PL (2004) Comparative evaluation of chloroethene dechlorination to ethane by *Dehalococcoides*-like microorganisms. Environ Sci Technol 38:4768–4774. doi:10.1021/es049965z
3. Cupples AM, Spormann AM, McCarty PL (2004) Vinyl chloride and *cis*-dichloroethene dechlorination kinetics and microorganism growth under substrate limiting conditions. Environ Sci Technol 38:1102–1107. doi:10.1021/es0348647
4. Duhamel M, Mo K, Edwards EA (2004) Characterization of a highly enriched *Dehalococcoides*-containing culture that grows on vinyl chloride and trichloroethene. Appl Environ Microbiol 70:5538–5545. doi:10.1128/AEM.70.9.5538-5545.2004
5. Ellis DE, Lutz EJ, Odom JM, Ronald J, Buchanan J, Bartlett C, Lee MD, Harkness MR, Deweerd KA (2000) Bioaugmentation for accelerated in situ anaerobic bioremediation. Environ Sci Technol 34:2254–2260. doi:10.1021/es990638e
6. Griffin BM, Tiedje JM, Löffler FE (2004) Anaerobic microbial reductive dechlorination of tetrachloroethene (PCE) to predominately trans-1, 2 dichloroethene. Environ Sci Technol 38:4300–4303. doi:10.102/es035439g
7. He J, Ritalahti KM, Aiello MR, Löffler FE (2003) Complete detoxification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as *Dehalococcoides* species. Appl Environ Microbiol 69:996–1003. doi:10.1128/AEM.69.2.996-1003.2003
8. He J, Ritalahti KM, Yang KL, Koenigsberg SS, Löffler FE (2003) Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature 424:62–65. doi:10.1038/nature01717
9. He J, Holmes V, Lee PKH, Alvarez-Cohen L (2007) Influence of vitamin B_{12} and co-cultures on the growth of *Dehalococcoides* isolates in defined medium. Appl Environ Microbiol 73:2847–2853. doi:10.1128/AEM.02574-06
10. He J, Sung Y, Krajmalnik-Brown R, Ritalahti KM, Löffler FE (2005) Isolation and characterization of *Dehalococcoides* sp.

Strain FL2, a trichloroethene (TCE) and 1,2-dichloroethene-respiring anaerobe. Environ Microbiol 7:1442–1450. doi:10.1111/j.1462-2920.2005.00830.x
11. Hendrickson ER, Payne JA, Young RM, Starr MG, Perry MP, Fahnestock S, Ellis DE, Ebersole RC (2002) Molecular analysis of *Dehalococcoides* 16S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe. Appl Microbiol 68:485–495. doi:10.1128/AEM.68.2.485-495.2002
12. Holliger C, Wohlfarth G, Diekert G (1999) Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol Rev 22:383–398. doi:10.1111/j.1574-6976.1998.tb00377.x
13. Holliger C, Schumacher W (1994) Reductive dehalogenation as a respiratory process. Antonie Van Leeuwenhoek 66:239–246. doi:10.1007/BF00871642
14. Lee MD, Odom JM, Buchanan RJ Jr (1998) New perspectives on microbial dehalogenation of chlorinated solvents: insights from the field. Annu Rev Microbiol 52:423–452. doi:10.1146/annurev.micro.52.1.423
15. Lendvay JM, Löffler FE, Dollhopf M, Aiello MR, Daniels G, Fathepure BZ, Gebhard M, Heine R, Helton R, Shi J, Krajmalnik-Brown R, Major CL Jr, Barcelona MJ, Petrovskis E, Tiedje JM, Adriaens P (2002) Bioreactive barriers: bioaugmentation and biostimulation for chlorinated solvent remediation. Environ Sci Technol 37:1422–1431. doi:10.102/es025985u
16. Löffler FE, Cole JR, Ritalahti KM, Tiedje JM (2003) Diversity of dechlorinating bacteria. In: Häggblom MM, Bossert ID (eds) Dehalogenation: microbial processes and environmental applications. Kluwer Academic Press, New York, pp 53–87. doi:10.1007/0-306-48011-5_3
17. Löffler FE, Sun Q, Li J, Tiedje JM (2000) 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. Appl Environ Microbiol 66:1369–1374. doi:10.1128/AEM.66.4.1369-1374.2000
18. Löffler FE, Tiedje JM, Sanford RA (1999) Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology. Appl Environ Microbiol 65:4049–4056
19. Lu X, Wilson JT, Kampbell DH (2006) Relationship between *Dehalococcoides* DNA in ground water and rates of reductive dechlorination at field scale. Water Res 40:3131–3140. doi:10.1016/j.watres.2006.05.030
20. Lu X-X, Tao S, Bosma T, Gerritse J (2001) Characteristic hydrogen concentrations for various redox processes in batch study. J Environ Sci Health A 36:1725–1734. doi:10.1081/ESE-100106254
21. Major DW, McMaster ML, Cox EE, Edwards EA, Dworatzek SM, Hendrickson ER, Starr MG, Payne JA, Buonamici LW (2002) Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. Environ Sci Technol 36:5106–5116. doi:10.1021/es0255711
22. Maymó-Gatell X, Anguish T, Zinder SH (1999) Reductive dechlorination of chlorinated ethenes and 1, 2-dichloroethane by "*Dehalococcoides ethenogenes*" 195. Appl Environ Microbiol 65:3108–3113
23. Maymó-Gatell X, Chien YT, Gossett JM, Zinder SH (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 276:1568–1571. doi:10.1126/science.276.5318.1568
24. McCarty PL, Chu M-Y, Kitanidis PK (2006) Electron donor and pH relationships for biologically enhanced dissolution of chlorinated solvent DNAPL in groundwater. Eur J Soil Biol 43:276–282. doi:10.1016/j.ejsobi.2007.03.004
25. Moran MJ, Zogorski S (2007) Chlorinated solvents in groundwater of the United States. Environ Sci Technol 41:74–81. doi:10.1021/es061553y
26. Müller JA, Rosner BM, von Abendroth G, Meshulam-Simon G, McCarty PL, Spormann AM (2004) Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution. Appl Environ Microbiol 70:4880–4888. doi:10.1128/AEM.70.8.4880-4888.2004
27. Ritalahti KM, Amos BK, Sung Y, Wu Q, Koenigsberg SS, Löffler FE (2006) Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple *Dehalococcoides* strains. Appl Environ Microbiol 72:2765–2774. doi:10.1128/AEM.72.4.2765-2774.2006
28. Schaefer CE, Condee CW, Vainberg S, Steffan RJ (2009) Bioaugmentation for chlorinated ethenes using *Dehalococcoides* sp.: comparison between batch and column experiments. Chemosphere 75:141–148. doi:10.1016/j.chemosphere.2008.12.041
29. Shelton DR, Tiedje JM (1984) General method for determining anaerobic biodegradation potential. Appl Environ Microbiol 47:850–857
30. Smidt H, de Vos WM (2004) Anaerobic microbiol dehalogenation. Annu Rev Microbiol 58:43–73. doi:10.1146/annurev.micro.58.030603.123600
31. Sung Y, Ritalahti KM, Apkarian RP, Löffler (2006) Quantitative PCR confirms purity of strain GT, a novel trichloroethene-to-ethene-respiring *Dehalococcoides* isolate. Appl Environ Microbiol 72:1980–1987. doi:10.1128/AEM.72.3.1980-1987.2006
32. U.S. EPA (1998) U.S. EPA test methods for evaluating solid waste, physical/chemical methods SW846, 3rd edn. Revision 5, 1998
33. Westrick JJ, Mello JW, Thomas RF (2004) The groundwater supply survey. J Am Water Works Assoc 76:52–59
34. Yang Y, McCarty PL (1998) Competition for hydrogen within a chlorinated solvent dehalogenating anaerobic mixed culture. Environ Sci Technol 32:3591–3597. doi:10.1021/es991410u
35. Zhang J, Andrew AP, Chiu PC (2006) 1,1-Dichloroethene as a predominant intermediate of microbial trichloroethene reduction. Environ Sci Technol 40:1830–1836

Ground Water Monitoring&Remediation

Field-Scale Evaluation of Bioaugmentation Dosage for Treating Chlorinated Ethenes

by Charles E. Schaefer, David R. Lippincott, and Robert J. Steffan

Abstract

A field demonstration was performed to evaluate the impacts of bioaugmentation dosage for treatment of chlorinated ethenes in a sandy-to-silty shallow aquifer. Specifically, bioaugmentation using a commercially available *Dehalococcoides* (DHC)-containing culture was performed in three separate groundwater recirculation loops, with one loop bioaugmented with 3.9×10^{11} DHC, the second loop bioaugmented with 3.9×10^{12} DHC, and the third loop bioaugmented with 3.9×10^{13} DHC. Groundwater monitoring was performed to evaluate DHC growth and migration, dechlorination rates, and aquifer geochemistry. The loop inoculated with 3.9×10^{12} DHC showed slower dechlorination rates and DHC migration/growth compared with the other loops. This relatively poor performance was attributed to low pH conditions. Results for the loops inoculated with 3.9×10^{11} and 3.9×10^{13} DHC showed similar timeframes for dechlorination, as evaluated at a monitoring well approximately 10 feet downgradient of the DHC injection well. Application of a recently developed one-dimensional bioaugmentation fate and transport screening model provided a reasonable prediction of the data in these two loops. Overall, these results suggest that increasing bioaugmentation dosage does not necessarily result in decreased dechlorination timeframes in the field. The ability to predict results suggests that modeling potentially can serve as an effective tool for determining bioaugmentation dosage and predicting overall remedial timeframes.

Introduction

Chlorinated ethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE), have been used extensively as industrial solvents and cleaning agents at several government and private sector facilities. This widespread use, in addition to improper disposal practices and the stability of chlorinated ethenes, has led to them becoming common groundwater contaminants. One in situ technology that has proven to be effective at treating chlorinated ethenes is bioaugmentation (United States Environmental Protection Agency [USEPA] 2004; Interstate Technology & Regulatory Council 2005, 2007). Bioaugmentation for chlorinated ethenes involves delivery of electron donor, bacteria, and (if needed) nutrients to the subsurface for the purpose of facilitating microbially enhanced reductive dechlorination. The most accepted form of bioaugmentation for chlorinated ethenes involves the use of mixed anaerobic cultures that contain *Dehalococcoides* (DHC) sp., or closely related strains, that can reductively dechlorinate the chlorinated ethenes; DHC are the only bacteria known to completely dechlorinate PCE and TCE (Maymó-Gatell et al. 1997).

Several studies have been performed using model or real aquifers to evaluate bioaugmentation for treating chlorinated ethenes and for evaluating the relationship between measured DHC concentration and observed dechlorination rates. Using laboratory silica sand columns, Amos et al. (2009) showed that bioaugmented DHC responsible for dechlorination were primarily associated with the solid phase. In contrast, Schaefer et al. (2009) showed that the bioaugmented DHC were primarily associated with the aqueous phase (with the exception of a localized region near the column influent), and Lu et al. (2006) showed that there was a relationship between DHC in groundwater and observed dechlorination rates.

Although the studies referenced earlier have provided substantial insight into the processes that control DHC growth, distribution, and dechlorination kinetics during bioaugmentation, there currently exists considerable uncertainty when designing and implementing bioaugmentation at the field scale. These uncertainties can have substantial ramifications on the technical and economic success of in situ bioaugmentation. Key unknowns include uncertainty related to the inoculated DHC dosage needed to treat a contaminated site, the transport and distribution of DHC in the aquifer, and DHC activity with respect to growth and dechlorination rates (Environmental Security Technology Certification Program 2005). In particular, the relationship

doi: 10.1111/j1745-6592.2010.01297.x

between DHC injection dosage and aquifer response with respect to DHC distribution and observed dechlorination rates is poorly understood. No generally accepted conceptual model exists and (to the best of our knowledge) no published field studies exist that can sufficiently address these uncertainties.

The purpose of this study was to quantitatively evaluate bioaugmentation performance at the field scale by measuring DHC distribution and growth and dechlorination of TCE, *cis*-1,2-dichloroethene (DCE), and vinyl chloride as a function of bioaugmentation dosage. Field results were evaluated using a previously developed bioaugmentation model. The model was used to provide additional insights into the mechanisms controlling the observed behavior.

Methods

Generalized Approach

The bioaugmentation evaluation was performed by delivering DHC to three groundwater recirculation loops for treating TCE and DCE; each groundwater recirculation loop was inoculated with a different DHC dosage. A fourth groundwater recirculation loop, which received no DHC inoculation, served as a control. Groundwater was monitored within each recirculation loop to evaluate the extent of TCE and DCE dechlorination over time and to determine DHC growth and migration. Results among the recirculation loops were compared to assess the impact of bioaugmentation dosage on observed treatment timeframes and overall effectiveness.

Demonstration Location and Description

The bioaugmentation demonstration was performed at Fort Dix, which is located in Burlington and Ocean counties, New Jersey, approximately 25 miles southeast of Trenton. The actual demonstration plot was located within the MAG-1 Area, which is located in the northern part of the Cantonment Area at Fort Dix. The geology underlying the field demonstration site consisted of unconsolidated materials from the Kirkwood and Manasquan formations. Results of the predemonstration testing to evaluate the hydrogeology and contaminant distribution in the test area are summarized in Figure 1. Soils from the targeted bioaugmentation zone (approximately 104 to 90 feet mean sea level [MSL]) consisted of saturated, light gray silty fine sands (Kirkwood Formation). A 4- to 8-inch-thick interface zone, consisting of fine-to-coarse sands and fine gravel, is present at the base of this unit. The interface zone appears to exhibit significantly higher permeability than the formations above and below. Dissolved contaminants consisted primarily of TCE and DCE at concentrations up to 2900 µg/L, as measured via discrete Geoprobe® sampling points. Baseline sampling events showed that no vinyl chloride or ethene was present in the test area groundwater. Hydraulic conductivities estimated using slug test data ranged from 0.6 to 1.8 m/day in the targeted zone of the Kirkwood Formation. Ambient groundwater velocity through the demonstration zone was approximately 0.0018 m/day. Measurement of TCE and DCE concentrations in soil samples collected adjacent to the Geoprobe groundwater sampling points allowed for estimation of a linear adsorption coefficient; the estimated values for TCE and DCE were 2.1 and 1.1 L/kg, respectively.

Recirculation System Design and Amendment Addition

A groundwater recirculation system was installed and implemented for the bioaugmentation demonstration. The system design consisted of four pairs of injection/extraction wells (IW-1 through IW-4 and EX-1 through EX-4) operating at approximately 1.9 L/min/pair; this system was located in the center of the TCE/DCE groundwater plume. The actual surveyed system layout, including performance monitoring wells (BMW-1 through BMW-8) within each recirculation loop, is shown in Figure 2. These monitoring wells were spaced approximately 10 and 20 feet downgradient of the groundwater injection well. Three additional performance-monitoring wells (BMW-9 through BMW-11) were located between or sidegradient of select loops. Loop 4 was used as a control loop. Well construction details are summarized in Table 1.

Amendment metering pumps for delivery of electron donor (sodium lactate), tracer (sodium bromide), and buffer (sodium bicarbonate and/or sodium carbonate) solutions were installed within a Conex box. A 836-L polyethylene tank containing a 50:50 volume mix of 60% liquid sodium lactate solution and deionized water was used to deliver electron donor to each of the recirculation loops. The lactate solution was metered into each of the four injection wells (operating at approximately 1.9 L/min) at 0.0025 L/min, thereby attaining a final sodium lactate injection concentration of 400 mg/L. An additional eight 836-L polyethylene tanks were used to deliver buffer and nutrients (diammonium phosphate and yeast extract). The solution was metered into each of the injection wells between 0.048 and 0.12 L/min, thereby attaining a final buffer injection concentration of between approximately 1700 and 4300 mg/L. Sodium bicarbonate buffer was used from start-up (November 16, 2007) until December 11, 2007, at which time the buffer used was changed to sodium carbonate to more effectively increase pH within the aquifer. Additionally, diammonium phosphate was mixed into the buffer solution tanks, attaining a final injection concentration of approximately 75 mg/L. The final injection concentration for the yeast extract was approximately 50 mg/L. Individual feed lines were run from the tanks to the corresponding metering pump and from the metering pump to injection racks installed within a second Conex box. The injection racks contained filter housings, flow meters, pressure gauges, and injection ports for the amendments.

Bulk injections of sodium carbonate were performed on December 27, 2007 (45 kg/well) and January 15, 2008 (68 kg/well) at each of the four groundwater injection wells. Sodium carbonate powder was mixed in drums with groundwater extracted from each of the injections wells, then reinjected into the wells. These bulk injections were performed to further elevate groundwater pH values that still largely remained below 5.5 standard units after several weeks of system operation.

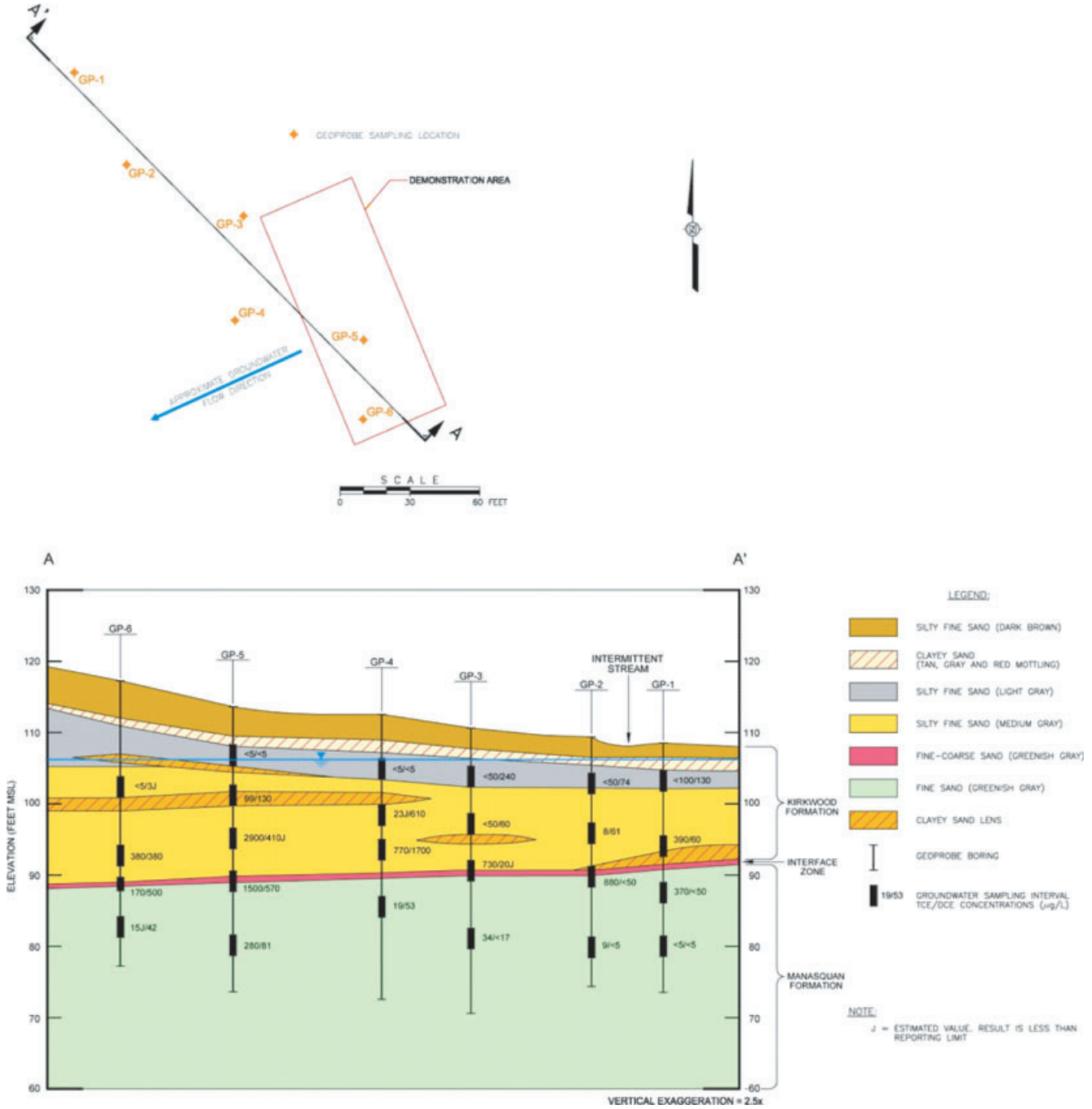


Figure 1. Demonstration area's geologic cross section and contaminant distribution.

Tracer Testing

Amendment delivery and recirculation, as described in the previous section, were performed for a 10-week start-up period. During this start-up period, a tracer test was performed concurrently using sodium bromide in loops 1 and 3. Forty-five kilograms of sodium bromide was mixed into the buffer tanks with site groundwater. A total of 1938 L of solution (three 646-L batches), with an average bromide concentration of approximately 9100 mg/L, was prepared in the buffer tanks for loops 1 and 3. Tracer injections began on November 16, 2006, and were completed on December 14, 2007. The buffer metering pumps were used to inject the tracer solution continuously into the injection wells during active groundwater recirculation periods. The bromide solution was metered into the injection wells at 0.048 L/min at an average injection well concentration of approximately 225 mg/L.

Groundwater sampling was performed at select monitoring locations within the demonstration area to monitor migration of tracer, lactate, and carbonate, to determine the appropriate changes in aquifer geochemical conditions (i.e., decreases in dissolved oxygen and other electron acceptors and decreases in oxidation-reduction potential [ORP]), to evaluate changes in dissolved chlorinated ethene concentrations due to system mixing, and to determine baseline conditions prior to bioaugmentation.

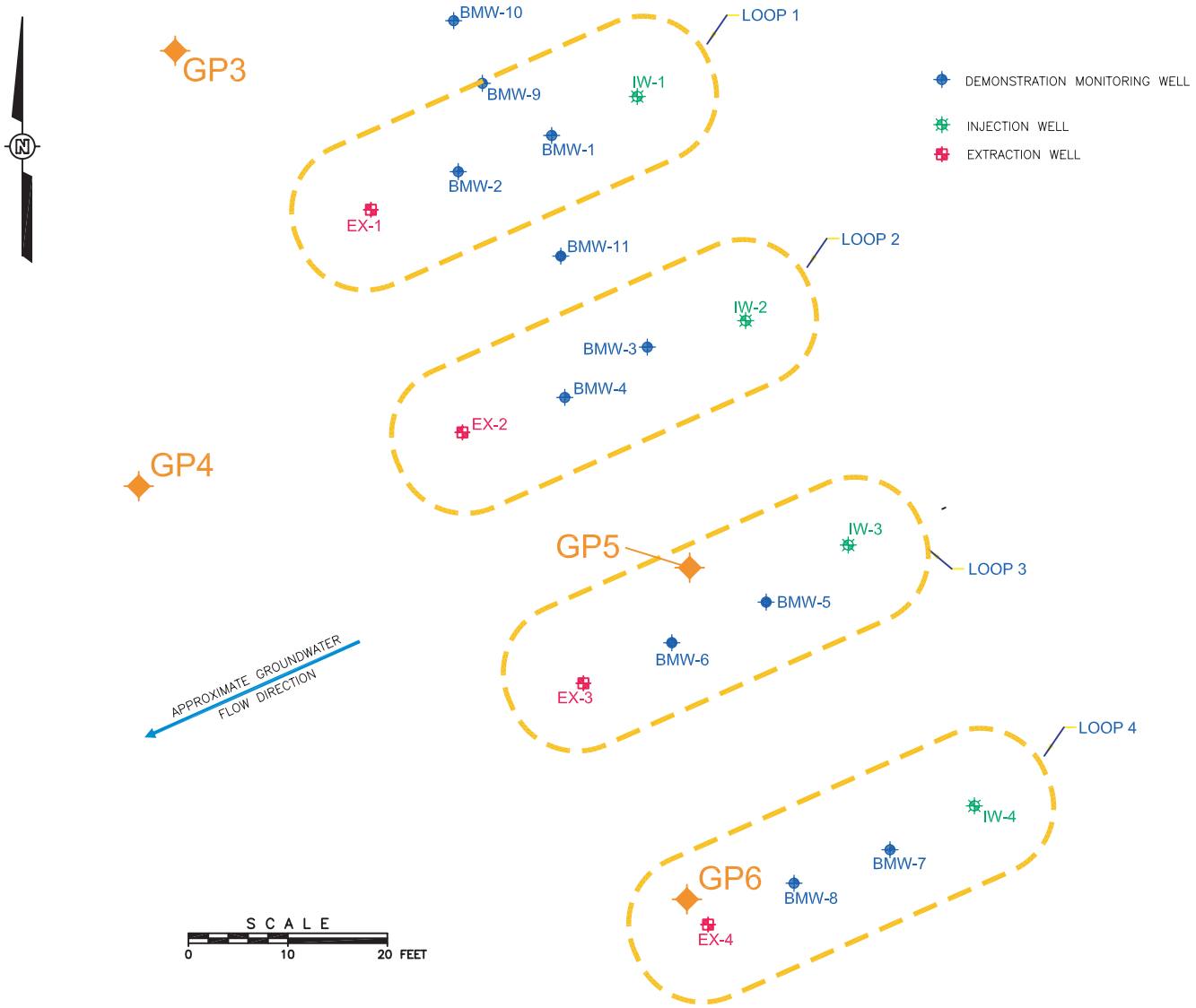


Figure 2. Demonstration layout.

Bioaugmentation

Bioaugmentation was performed on May 1, 2008 (approximately 150 days after recirculating amendments) using the commercially available SDC-9 culture (Shaw Environmental Inc., Lawrenceville, New Jersey). The dechlorination and growth kinetics of this DHC-containing culture have been described previously (Schaefer et al. 2009). Bioaugmentation implementation consisted of first pumping approximately 190 L of groundwater from wells BMW-1, BMW-3, and BMW-5 into individual 55-gallon drums. Drums were amended with lactate, diammonium phosphate, and yeast extract for final concentrations of 16,000, 1000, and 1000 mg/L, respectively. The SDC-9 culture, which was delivered to the site under nitrogen pressure in three individual soda kegs, was injected into wells BMW-1, BMW-3, and BMW-5 through Tygon tubing that was lowered into the water column within each well to the approximate middle of the screened interval. The groundwater injection wells (IW1 through IW4) were not used for delivery of the SDC-9 culture because of locally elevated pH (~10) measured in these wells. The concentration of DHC in the soda kegs, as measured via quantitative polymerase chain reaction (qPCR), was 3.9×10^{11} DHC/L. The tubing was connected to a valve on the outlet port of each soda keg containing the bacteria. A nitrogen cylinder was connected to the inlet port of the soda keg. The soda keg was pressurized to approximately 10 psi using the nitrogen, and the outlet valve was opened allowing the culture to be injected into each well.

A total of 100 L (10 L of culture concentrated 10 times; 3.9×10^{13} DHC), 10 L (3.9×10^{12} DHC), and 1 L (3.9×10^{11} DHC) of culture was injected into wells BMW-1, BMW-3, and BMW-5, respectively. Bioaugmentation was not performed at well BMW-7 in recirculation loop 4, as this was used as the control loop. Each bioaugmentation injection took approximately 20 min to perform. Once the injection of the culture was complete, the 190 L of groundwater extracted from each of the injection wells was pumped back into the respective wells to further distribute the culture within the surrounding formation.

Table 1
Well Construction Details

Well ID	Ground Surface Elevation (feet MSL)	Top of Casing Elevation (feet MSL)	Well Diameter (inch)	Depth to Top of Screen (feet bgs)	Depth to Bottom of Screen (feet bgs)	Screen Length (feet)	Top of Screen Elevation (feet MSL)	Bottom of Screen Elevation (feet MSL)
Injection wells								
IW-1	109.27	111.44	6.0	8.0	18.0	10.0	101.3	91.3
IW-2	110.93	113.54	6.0	9.5	19.5	10.0	101.4	91.4
IW-3	112.38	115.28	6.0	11.5	21.5	10.0	100.9	90.9
IW-4	114.87	118.70	6.0	13.5	23.5	10.0	101.4	91.4
Extraction wells								
EX-1	110.15	113.85	6.0	8.5	18.5	10.0	101.7	91.7
EX-2	111.90	115.06	6.0	10.5	20.5	10.0	101.4	91.4
EX-3	113.46	116.54	6.0	12.0	22.0	10.0	101.5	91.5
EX-4	116.25	118.91	6.0	15.0	25.0	10.0	101.3	91.3
Monitoring wells								
BMW-1	109.76	112.10	2.0	8.0	18.0	10.0	101.8	91.8
BMW-2	110.10	112.44	2.0	8.5	18.5	10.0	101.6	91.6
BMW-3	111.43	111.14	2.0	10.0	20.0	10.0	101.4	91.4
BMW-4	110.70	111.28	2.0	10.5	20.5	10.0	100.2	90.2
BMW-5	112.98	115.38	2.0	11.5	21.5	10.0	101.5	91.5
BMW-6	113.25	112.88	2.0	11.5	21.5	10.0	101.8	91.8
BMW-7	115.50	117.77	2.0	14.0	24.0	10.0	101.5	91.5
BMW-8	116.31	118.31	2.0	14.5	24.5	10.0	101.8	91.8
BMW-9	109.66	111.96	2.0	8.0	18.0	10.0	101.7	91.7
BMW-10	109.24	111.72	2.0	8.0	18.0	10.0	101.2	91.2
BMW-11	110.27	109.92	2.0	9.0	19.0	10.0	101.3	91.3

System Operation and Monitoring

After bioaugmentation was performed, the recirculation system was operated in an intermittent mode (approximately 10 days "on" and 10 days "off"). In addition, groundwater recirculation flow rates were decreased to approximately 0.57 L/min due to increasing pressures at the injection wells and to limit cross flow between the loops.

Groundwater samples were collected by utilizing low-flow purging in accordance with New Jersey Department of Environmental Protection Low Flow Purging and Sampling Guidance, with the exception of purge times being limited to 60 min at each well before samples are collected. Samples were obtained using dedicated submersible bladder pumps and Teflon® tubing. A YSI field meter (YSI, Inc.) with a flow-through cell was used to collect measurement of field geochemical parameters (pH, ORP, temperature, specific conductivity, and dissolved oxygen). Analyses of groundwater collected during the performance monitoring sampling events included volatile organic compounds, reduced gases, volatile fatty acids (VFAs), anions, and qPCR to measure DHC concentrations in groundwater.

Analytical Methods

Analysis of chloride, bromide, nitrate, nitrite, and sulfate by EPA Method 300.0, VFAs by EPA Method 300m, chlorinated ethenes by EPA Method 8260, and reduced gases by EPA Method 8015 were performed at Shaw's certified analytical laboratory in Lawrenceville, New Jersey. DHC concentrations in the groundwater samples were determined by quantitative real-time PCR with primers (5′-gaagtagtgaaccgaaagg and 5′-tctgtccattgtagcgtc) that amplified a 235-bp fragment of the 16s rRNA gene of DHC-type organisms.

Results and Discussion

Tracer and Amendment Distribution

The bromide tracer was distributed through loops 1 and 3 quickly, with detectable concentrations of bromide observed at extraction wells EX-1 and EX-3 within 10 and 18 days, respectively. Analysis of the tracer test data indicated that the estimated travel time of the bromide tracer through loops 1 and 3 (from the injection to the extraction

well) was approximately 30 to 40 days, with an average groundwater velocity of 0.23 to 0.30 m/day. These estimates were based on groundwater extraction/reinjection rates of 1.9 L/min/loop. However, because groundwater extraction rates were reduced to 0.57 L/min and were operated in an intermittent mode after bioaugmentation was performed, the average groundwater velocity was significantly decreased (to approximately 0.025 m/day) during the bioaugmentation portion of the demonstration. Tracer results for BMW-1 are provided in the Supporting Information.

Limited cross flow occurred between loops 1 and 2 and loops 3 and 4 during the tracer test. Bromide concentrations observed within loops 2 and 4 were generally 1 to 2 orders of magnitude below those observed in loops 1 and 3. As previously discussed, groundwater extraction rate was 1.9 L/min for each of the four extraction wells during the tracer testing. This pumping rate was reduced after the tracer test was completed, which resulted in a decrease in bromide concentration in loops 2 and 4 to approximately 1 mg/L (bromide concentrations remained above 20 mg/L in loops 1 and 3 throughout the demonstration). Additionally, as discussed in subsequent sections, vinyl chloride, ethene, and elevated DHC concentrations were not observed in the control loop (loop 4), indicating that significant cross flow between loops 3 and 4 likely was not occurring at the reduced (0.57 L/min) flow rates during the bioaugmentation portion of the demonstration.

Sidegradient monitoring well BMW-9 showed elevated VFA and bromide concentrations throughout the demonstration. However, sidegradient monitoring wells BMW-10 and BMW-11 did not show any impacts of the recirculation system (i.e., no measureable bromide or VFAs). Based on these data, amendment distribution in each loop subsequent to bioaugmentation was estimated at 15 to 25 feet perpendicular to recirculation flow (as indicated by the dashed outline for each loop in Figure 2).

During amendment delivery, but prior to bioaugmentation, several changes in aquifer geochemical and contaminant conditions were observed. Monitoring wells BMW-1 through BMW-8 showed that addition of the buffer solutions resulted in a gradual increase in aquifer pH from approximately 4.5 to 6.5. Distribution of lactate was evidenced by VFA concentrations (predominantly lactate fermentation products acetate and propionate) ranging from 50 to 2000 mg/L at the monitoring wells. ORP values decreased from baseline levels of approximately +100 mV to approximately −200 mV in the monitoring wells in each of the four loops, and sulfate concentrations decreased from approximately 50 to 3 mg/L.

Prebioaugmentation amendment delivery also resulted in substantial decreases in TCE at BMW-5 and small-to-moderate decreases in TCE at BMW-7 and BMW-8 (Figures 3 through 6). Results of preliminary laboratory column experiments using site soil and groundwater showed that addition of electron donor without bioaugmentation resulted in dechlorination of TCE but no subsequent dechlorination of DCE and vinyl chloride. The observed decreases in TCE concentrations in the field results are consistent with this laboratory result. However, as shown in Figures 3 through 6, a stochiometric increase in DCE (or any other ethene) was not observed in the field prior to bioaugmentation. This is particularly evident at BMW-5. Thus, the decreases in TCE observed prior to bioaugmentation may be partially due to in situ mixing effects rather than reductive dechlorination.

No generation of vinyl chloride or ethene occurred prior to bioaugmentation in any of the monitoring locations. Measured DHC concentrations at monitoring wells in all four loops increased from baseline concentrations of approximately 10^3 (prior to amendment addition) to 10^4 to 10^5 DHC/L (after approximately 140 days of amendment addition and just prior to bioaugmentation) (Figures 3 through 6). The lack of measureable DCE dechlorination despite these increasing DHC levels likely is the result of slow dechlorination kinetics and/or the inability of native DHC to dechlorinate DCE.

Bioaugmentation

As shown in Figures 3 through 5, bioaugmentation at BMW-1, BMW-3, and BMW-5 resulted in a substantial increase in DHC concentrations; DHC concentrations in these wells measured 18 days after bioaugmentation showed increases that were approximately proportional to the DHC injection dosage. Bioaugmentation also resulted in dechlorination of TCE and DCE, as evidenced by vinyl chloride and ethene generation measured in the bioaugmentation injection locations. With the exception of BMW-1, DHC concentrations increased in the monitoring wells following the initial bioaugmentation (the reason for this lack of observed growth in BMW-1 is discussed in the modeling Results section). DHC concentrations in the control loop show a gradual increase to 10^6 DHC/L over the course of the demonstration. This increase could be due to a slow migration of DHC from loop 3 and/or the slow growth of indigenous DHC. However, no measureable DCE dechlorination (as evidenced by vinyl chloride or ethene generation) was observed in the control loop during the duration of the demonstration (Figure 6).

Comparison among BMW-1, BMW-3, and BMW-5 shows that DHC dosage affects the timeframe for DCE dechlorination. DCE conversion to ethene was most rapid in BMW-1 (highest DHC dosage, with conversion occurring within 14 days) and slowest in BMW-5 (lowest DHC dosage, with substantial conversion occurring in 50 to 100 days). These data also suggest that DHC groundwater concentrations were (approximately) proportional to the observed dechlorination timeframes.

Results at the downgradient monitoring well in each treatment loop (i.e., BMW-2, BMW-4, and BMW-6) also were compared. Evidence of DCE dechlorination and increases in DHC concentration were delayed in BMW-2 and BMW-6 by several weeks (relative to the bioaugmentation injection wells). This delay is presumably due to the travel time required for DHC and treated groundwater to migrate downgradient. Interestingly, both BMW-2 and BMW-6 show removal of DCE in approximately 250 days, despite a 100-fold difference in DHC dosage in the treatment loop.

In contrast, results at BMW-4 show limited DCE dechlorination, and DHC concentrations remained below

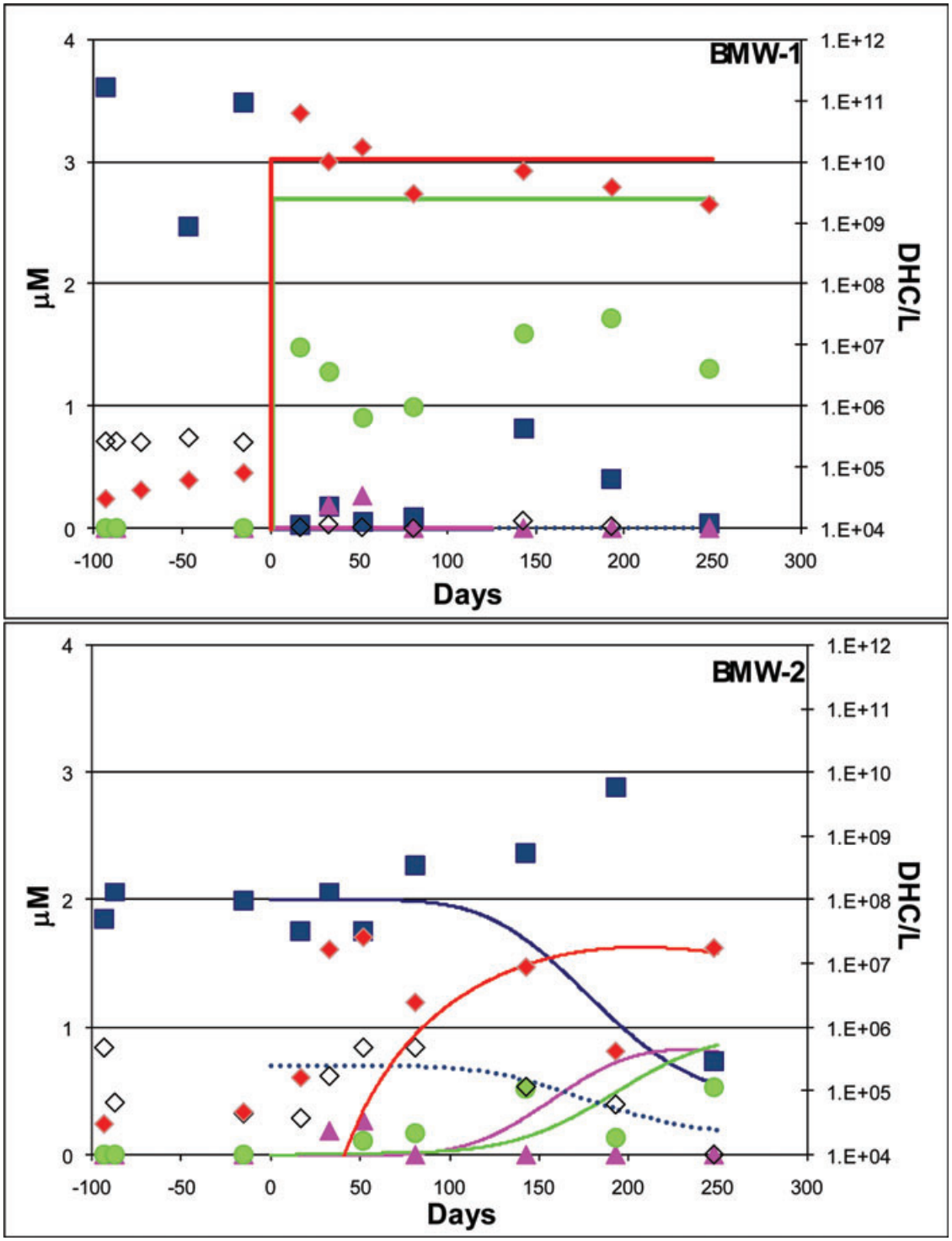


Figure 3. Ethenes and DHC concentrations plotted as a function of time for loop 1. Bioaugmentation was performed at 0 days. ◊, TCE; ■, DCE; ▲, vinyl chloride; ●, ethene; ◆, DHC. Solid and dotted lines represent corresponding model simulations. Simulated DHC concentrations in the bioaugmentation injection well (BMW-1) include the total (mobile and immobile) DHC.

10^7 DHC/L. One explanation for the relatively poor treatment at this monitoring location is that pH levels ranged from 4.9 to 5.8 during at least a 64-day period (days 116 to 180) in this well. At these pH levels, DHC dechlorination of DCE is severely inhibited (Vainberg et al. 2009). Increasing the buffer concentration ultimately resulted in an increase in pH within this loop. The decrease in DCE, accompanied by the increase in DHC and vinyl chloride, at day 150 suggests that treatment was beginning to occur in this well by the end of the demonstration period.

Increases in DHC levels (~10^7 DHC/L) were measured in EX-1 by day 193. Increases in DHC levels at EX-2 and EX3 (10^7 and 10^8 DHC/L, respectively) were measured by day 248. Ethene concentrations at EX-1 through EX-3 by

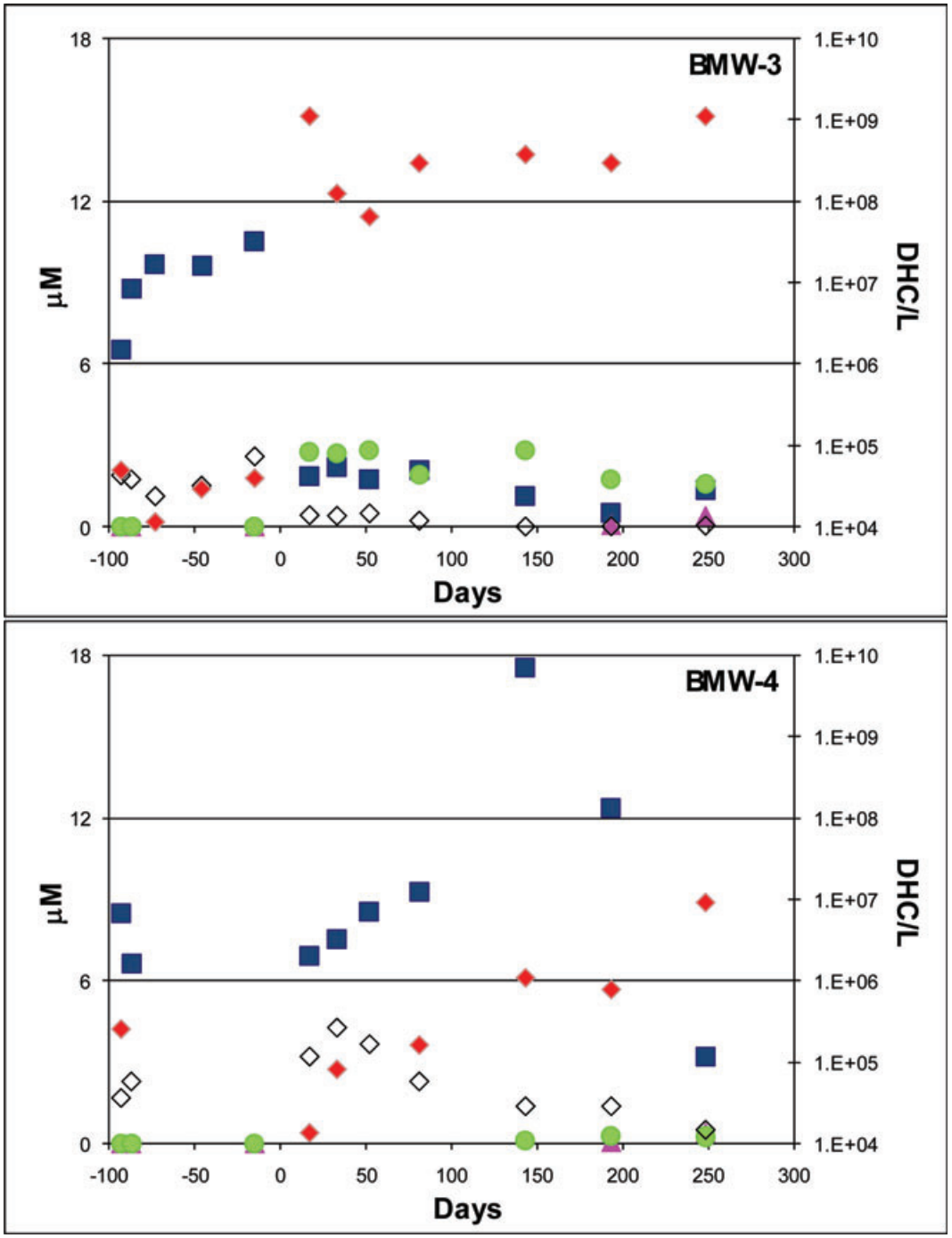


Figure 4. Ethenes and DHC concentrations plotted as a function of time for loop 2. Bioaugmentation was performed at 0 days. ◊, TCE; ■, DCE; ▲, vinyl chloride; ●, ethene; ◆, DHC.

day 248 were 0.5, 0.06, and 1.3 µM, respectively. These data suggest that DHC and treated groundwater were migrating toward the extraction wells. However, no measureable decrease in DCE concentrations was measured at the extraction wells, suggesting that the extraction wells were still capturing untreated groundwater from the sidegradient and/or downgradient aquifer.

Screening-Level Model

To provide a first-level evaluation of in situ dechlorination rates and DHC growth, and to further evaluate the mechanisms responsible for the observed microbial growth and dechlorination rates, the one-dimensional screening-level bioaugmentation model developed by Schaefer et al. (2009) for the SDC-9 culture was applied to demonstration loops 1 and 3. This model uses Monod kinetics to describe DHC growth and dechlorination rates (determined for the SDC-9 culture in batch kinetic studies) and applies an attachment-detachment–type mechanism to describe DHC migration through soil. The model assumes that both immobile and mobile DHC near the bioaugmentation injection well, and mobile DHC migrating downgradient

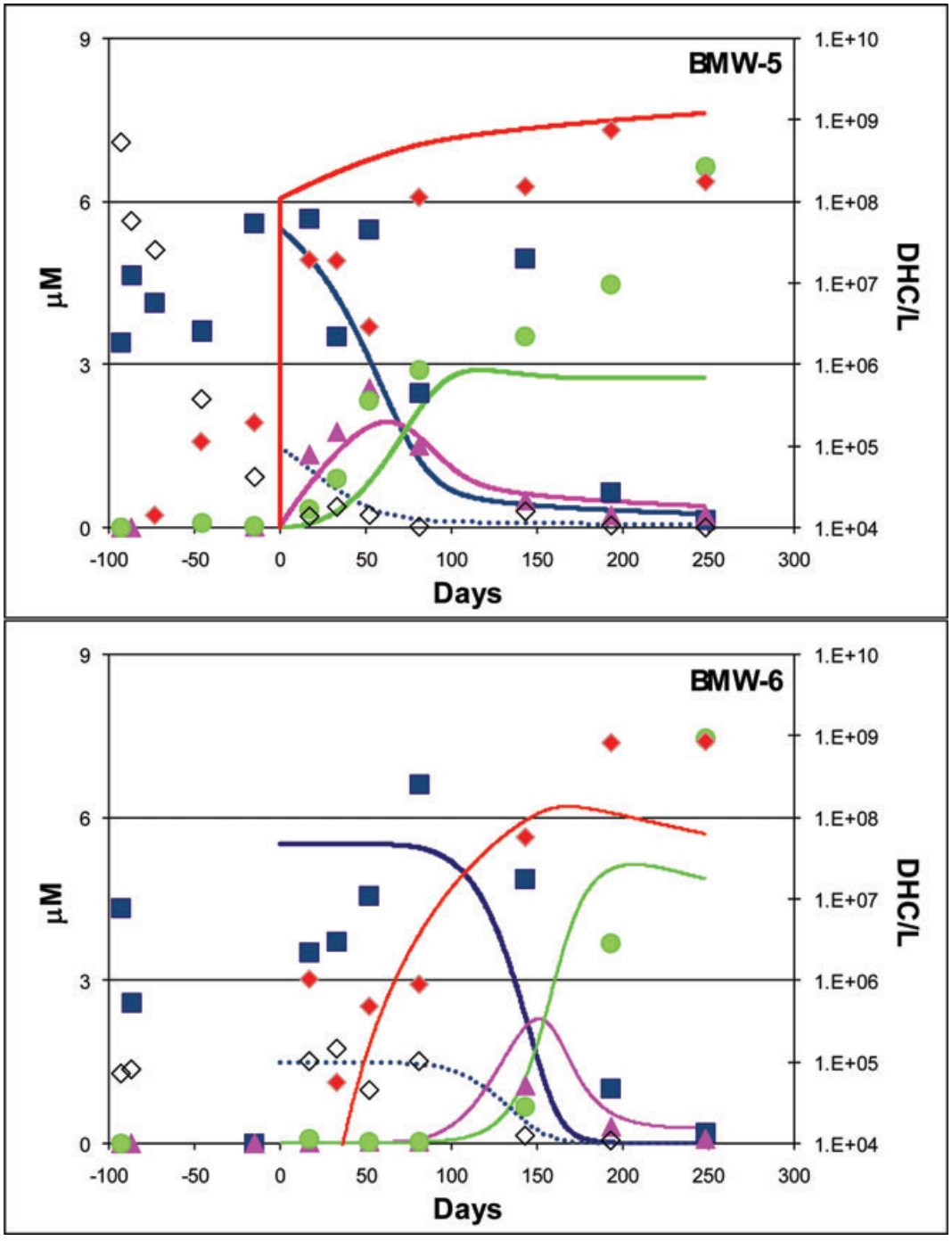


Figure 5. Ethenes and DHC concentrations plotted as a function of time for loop 3. Bioaugmentation was performed at 0 days. ◊, TCE; ■, DCE; ▲, vinyl chloride; ●, ethene; ◆, DHC. Solid and dotted lines represent corresponding model simulations. Simulated DHC concentrations in the bioaugmentation injection well (BMW-5) include the total (mobile and immobile) DHC.

from the bioaugmentation injection well, contribute to contaminant dechlorination. This finite difference model (Δx = 1 foot, Δt = 0.4 days) was applied to describe DHC growth and dechlorination from BMW-1 to BMW-2 and from BMW-5 to BMW-6. Because of the low pH issue at BMW-4, which likely resulted in inhibition of DCE dechlorination, the model was not applied to loop 2. The simulated porosity was assumed to be 0.35, and the superficial velocity for loops 1 and 3 was estimated (based on the bromide tracer data and adjusted based on the reduction in recirculation flow rate after bioaugmenting in each loop) at 0.021 and 0.029 m/day, respectively. The dispersivity was estimated based on the bromide tracer data at 0.15 m. The linear sorption coefficient for vinyl chloride was estimated at 0.58 L/kg, which was calculated based on the DCE sorption coefficient and the organic carbon partition coefficient of vinyl chloride relative to that of DCE (USEPA 1996). The linear sorption coefficient for ethene was assumed equal to that of vinyl chloride. The lone fitting parameter in the model was the attachment-

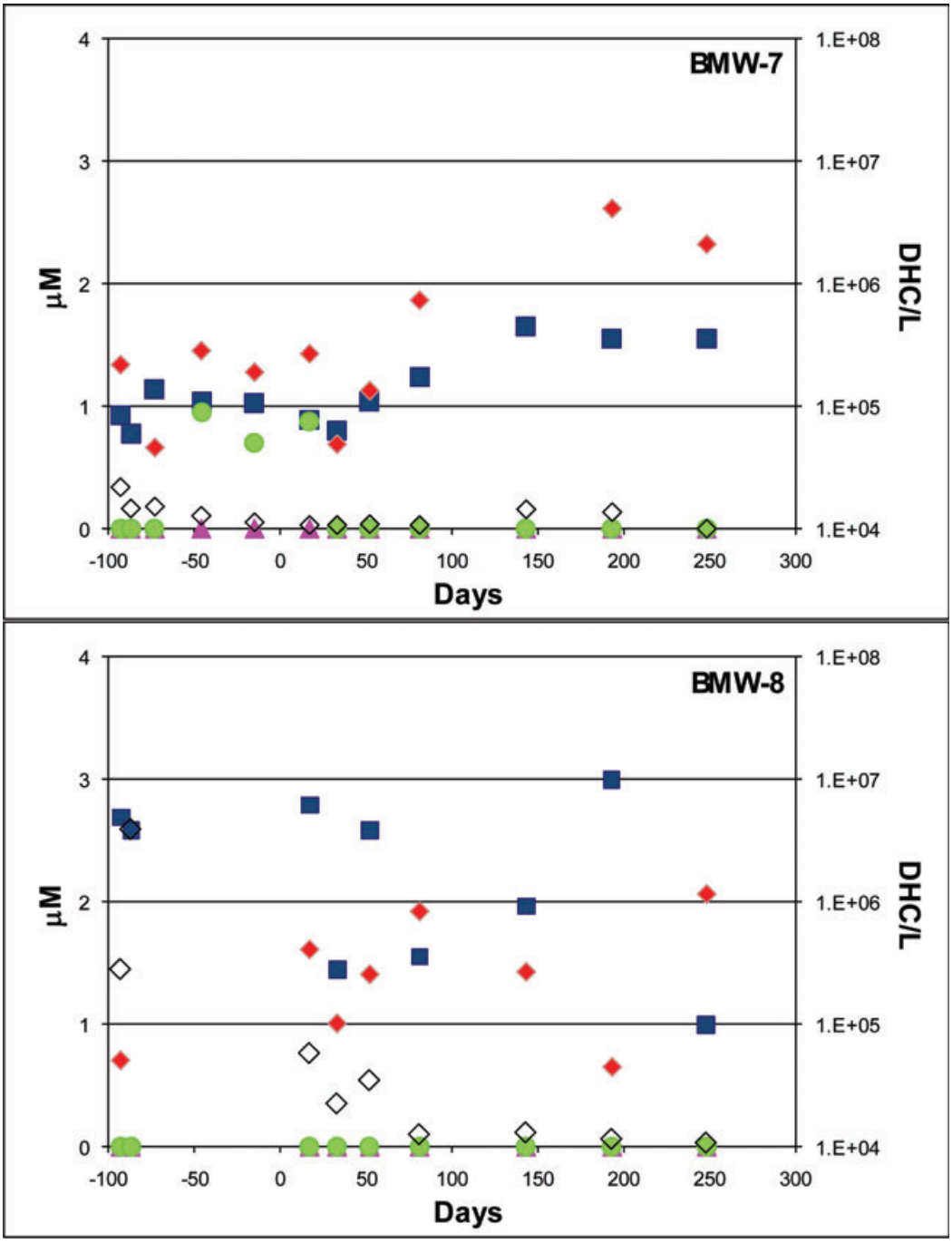


Figure 6. Ethenes and DHC concentrations plotted as a function of time for loop 4 (control loop). Bioaugmentation was performed at 0 days. ◊, TCE; ■, DCE; ▲, vinyl chloride; ●, ethene; ◆, DHC. No detection of vinyl chloride or ethene were observed.

detachment ratio of growing DHC in the soil. The best fit of this parameter (*f*) was approximately 0.9, indicating that 10% of the DHC growing in the soil detach and subsequently migrate through the aquifer. Model details are provided in the Supporting Information.

Model predictions for loops 1 and 3 are shown in Figures 3 and 5. Although intended to serve as only a semiquantitative tool, the model provided a reasonable prediction of the timeframe for DCE treatment at each of the monitoring wells in these treatment loops. In addition, the model provided a reasonable prediction of the DHC concentrations in groundwater, although the elevated DHC levels at BMW-2 at 40 to 50 days after bioaugmentation are not readily explained. Most importantly, the model showed that treatment timeframes at BMW-2 and BMW-6 were similar despite a 100-fold difference in DHC bioaugmentation dosage at BMW-1 and BMW-5. The model also showed that in situ DHC growth in loop 3 was greater than the DHC growth in loop 1. The rapid decrease in chlorinated ethene concentrations in BMW-1, which resulted from the large DHC inoculation dosage in this well, limits the subsequent rate of DHC growth within this treatment loop. Thus, in situ growth in loop 3 acted to compensate for the decreased DHC inoculation

dosage, and this explains why results for these two treatment loops are similar despite the 100-fold difference in bioaugmentation dosage. Thus, the model provides a reasonable explanation for the observed similarity between loops 1 and 3. Simulation of the loop 3 bioaugmentation dosage using the flow rate and chlorinated ethene concentrations in loop 1 did not substantially affect the simulated remedial timeframe or DHC levels obtained for loop 3. Thus, the similarity in the observed experimental results between loops 1 and 3 was not due to any artifacts caused by differences in chlorinated ethene or groundwater velocity between the recirculation loops.

Both the experimental data and model simulations show that DHC concentrations at BMW-5 and BMW-6 are similar (within about an order of magnitude). This level of agreement is reasonable considering the variability associated with aqueous phase DHC sampling (Schaefer et al. 2009). The agreement between model simulations and the experimental data confirm our qualitative and quantitative interpretation of the processes controlling DHC migration and DCE dechlorination at both high and low bioaugmentation dosages.

The question then arises as to whether continuing to decrease the bioaugmentation dosage would result in any substantial increases in remedial timeframe. Performance of a simulation using a DHC inoculation of 0.1-times which was used in loop 3 resulted in an additional 50 days of treatment required for DCE removal at the downgradient well (BMW-6). Thus, based on the combination of field and simulation results, the dosage used in loop 3 appears to be near optimal for the conditions of this study, balancing the benefits of high dosage and rapid treatment near the injection well to sustained growth and detachment of DHC to facilitate treatment downgradient.

Conclusions

Results of this field demonstration were used to evaluate the impacts of DHC dosage on effectiveness and rates of bioaugmentation. For the conditions of this demonstration, a 100-fold difference in bioaugmentation dosage using a commercially available DHC-containing culture did not result in an apparent difference in bioaugmentation performance, as measured at a monitoring well 10 feet downgradient of the bioaugmentation injection well. A one-dimensional screening-level model provided a reasonable prediction of the dechlorination rates and was able to predict the impacts of DHC dosage on bioaugmentation performance. Thus, this type of model potentially can serve as a tool for estimating DHC dosage in some field applications. The successful application of the model to the field results also verifies that the dechlorination and microbial processes observed at the bench scale (Schaefer et al. 2009) are applicable at the field scale, at least for the conditions of our study. Low pH conditions likely were responsible for inhibition of DCE dechlorination and DHC growth and migration in loop 2.

Results of this demonstration and others show that many factors including groundwater flow velocity, contaminant concentration, groundwater chemistry, and heterogeneity of the subsurface can affect the amount of culture needed to effectively treat chlorinated solvent-contaminated aquifers. As a result, precisely determining the amount of culture needed for a given site still requires a site-by-site evaluation. Importantly, the one-dimensional model used to predict and evaluate growth of DHC and treatment effectiveness (Schaefer et al. 2009) reasonably described the results of the demonstration. Consequently, the model appears suitable for evaluating the affect of different DHC dosages on treatment times and effectiveness and may serve a useful design tool for planning bioaugmentation applications. Validation of the model under a wider range of bioaugmentation field conditions would be useful in more fully demonstrating the robustness of this model. A significant component of its use, however, is the need to determine the attachment-detachment factor (f) that may vary based on aquifer geochemistry and soil texture. Work is continuing to allow up-front estimates of this factor based on analysis of site samples, and efforts are in progress to incorporate the one-dimensional model into existing groundwater flow and bioremediation models to make them more accessible to remediation practitioners.

Acknowledgment

Funding for this project was attained through the U.S. DoD Environmental Security Technology Certification Program (ESTCP) project ER-0515.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

Amos, B.K., E.J. Suchomel, K.D. Pennell, and F.E. Löffler. 2009. Spatial and temporal distributions of *Geobacter lovleyi* and *Dehalococcoides* spp. during bioenhanced PCE-DNAPL dissolution. *Environmental Science & Technology*, 43, 1977–1985.

Environmental Security Technology Certification Program (ESTCP). 2005. *Bioaugmentation for remediation of chlorinated solvents: technology development, status, and research needs*. http://docs.serdp-estcp.org/viewfile.cfm?Doc=BioaugmentationWhitePaper.pdf (accessed May 11, 2010).

Interstate Technology & Regulatory Council (ITRC). 2005. *Overview of In Situ Bioremediation of Chlorinated Ethene DNAPL Source Zones*. BioDNAPL-1. ITRC Bio DNAPL Team, Washington, DC.

Interstate Technology & Regulatory Council (ITRC). 2007. *In Situ Bioremediation of Chlorinated Ethene DNAPL Source Zones: Case Studies*. BioDNAPL-2. ITRC Bio DNAPL Team, Washington, DC.

Lu, X., J.T. Wilson, and D.H. Campbell. 2006. Relationship between *Dehalococcoides* DNA in groundwater and rates of reductive dechlorination at field scale. *Water Research*, 40, 3131–3140.

Maymó-Gatell, X., Y. Chien, J.M. Gossett, and S.H. Zinder. 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science*, 276, 1568–1571.

Schaefer, C.E., C.W. Condee, S. Vainberg, and R.J. Steffan. 2009. Bioaugmentation for chlorinated ethenes using *Dehalococcoides* sp.: Comparison between batch and column experiments. *Chemosphere*, 75, 141–148.

United States Environmental Protection Agency (USEPA). 2004. *Demonstration of Bioaugmentation of DNAPL Through Biostimulation and Bioaugmentation at Launch Complex 34 Cape Canaveral Air Force Station, Florida*. EPA/540/R-07/007. Battelle, Columbus, Ohio.

United States Environmental Protection Agency (USEPA). 1996. *Soil Screening Guidance: User's Guide*, 4–23. Publication 9355. Office of Solid Waste and Emergency Response, Washington, DC.

Vainberg, S., C.W. Condee, and R.J. Steffan. 2009. Large-scale production of bacterial consortia for remediation of chlorinated solvent-contaminated groundwater. *Journal of Industrial Microbiology and Biotechnology*, 36, 1189–1197.

Biographical Sketches

***Charles E. Schaefer**, Ph.D., corresponding author, may be reached at Shaw Environmental Inc., Lawrenceville, NJ 08648; charles.schaefer@shawgrp.com.*

***David R. Lippincott**, P.G., may be reached at Shaw Environmental Inc., Lawrenceville, NJ 08648.*

***Robert J. Steffan**, Ph.D., may be reached at Shaw Environmental Inc., Lawrenceville, NJ 08648.*

Attachment 3. Shipments of the SDC-9 Culture for Remediation of California Sites

Order Date	Initial shipment date	Actual shipment date	Shipping Address	City	State	Zip Code
11/20/2007	11/28/2007	11/28/2007	1370 W. San Marcos Blvd	San Marcos	CA	92078
3/4/2008	4/7/2008	4/7/2008	200 20 th Street	Vandenberg AFB	CA	93437
4/23/2008	4/23/2008	4/23/2008	Bourns Hall room A242 Univ of Calfornia 3401 Watkins	Riverside	CA	92521
10/13/2008	10/28/2008	10/28/2008	M. Peterson301 Mentor Drive Suite A	Santa Barbara	CA	93111
10/14/2008	10/20/2008	10/16/2008	Pick up here			
10/16/2008	10/20/2008	10/20/2008	1305 North H street Ste A Attn. Scott Potter	Lompoc	CA	93436
12/8/2008	1/26/2009	2/2/2009	Derek Hiser 7725 W Reno Avenue	Oklahoma City	OK	73127
12/8/2008	12/16/2008	12/16/2008	4005 Port Chicago Highway	Concord	CA	94520
12/11/2008	12/15/2008	12/15/2008	Nick Trimble 10461 Old Placerville Rd	Scaramento	CA	95827
12/15/2008	1/5/2009	1/6/2009	David Friese 3330 Vincent Road Pleasant Hill CA 94523, phone 925-977-1811	Pleasant Hill	CA	94523
12/16/2008	1/28/2009	1/28/2009	Building 570 Ave M	Treasure Island	CA	94130
1/6/2009	1/12/2009	1/12/2009	Sibel Tekce 111 Academy Suite 150	Irvine	CA	92617
1/27/2008	2/19/2009	2/19, 2/23, 2/24	1565 Macarthur BLVD.	Coata Mesa	CA	92626
2/12/2009	3/16/2009	3/16/2009	200 20street Vandenberg AFB, CA 93437	Vandenberg AFB	CA	93437
2/23/2009	3/5/2009	3/5/2009	Sibel Tekce 111 Academy Suite 150	Irvine	CA	92617
3/16/2008	3/30/2009	3/30/2009	Toddd Hana 1225E. McFadden Ave.	Santa Ana	CA	92705
3/17/2009	3/19/2009	3/19/2007	Bourns Hall A242 University of California	Riverside	CA	92521
3/25/2009	4/6/2009	4/6/2009	301 Mentor Drive St. A	Santa Barbara	CA	93111
3/30/2009	3/30/2009	3/30/2009	Michael Building 570 Ave M	Treasure Island	CA	94130
4/20/2009	5/11/2009-5/18/09	5/19/2009	Pick up here			
4/30/2009	5/11/2009	5/11/2009	Michael Building 570 Ave M	Treasure Island	CA	94130
6/1/2009	6/8/2009	6/8/2009	/IBM C/O Hitachi Global Storage Technol	San Jose	CA	95193
6/17/2009	6/24/2009	6/24/2009	200 20 Street	Vandenberg AFB	CA	93437
7/21/2009	7/22/2008	7/22/2008	Bourns Hall A242 University of California,	Riverside	CA	92521
8/3/2009	11/9/2009	11/9/2009	Attn: Jason Stormo . 200 20th Street	Vandenberg AFB,	CA	93437
8/28/2009	9/2/2009	9/2/2009	Cindy G. Schreier President 5070 Robert J. Mathews Parkway, Suite 300 ,	El Dorado Hills	CA	95762
9/8/2009	9/10/2009	9/10/2009	5601 Great Oaks Parkway Attn. David Walter	San Jose	CA	95119
9/8/2009	9/17/2009	9/17/2009	5601 Great Oaks Parkway Attn. David Walter	San Jose	CA	95119
10/21/2009	11/9/2009,	11/9-1keg (18.5L, conc)	Eric Jones , Inc. 200 20th Street	Vandenberg AFB	CA	93437
11/19/2009	11/24/-3k, 12/2-4k, 12/10-3k	11/24-3k, 12/2-4k, 12/10-3K	Todd Hanna 1225 E. McFadden Avenue phone 714-647-6290	Santa Ana	CA	92705
11/23/2009	12/8/2009-1 keg, 1 k-12/29, 1k-1/5	12/11/09-1 keg, 1 k-12/29, 1k-1/5/10	Attn: Jason Stormo 200 20th Street	Vandenberg AFB	CA	93437
11/24/2009	11/30/2009	11/30/2009	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
1/7/2009	1/13/2010	1/13/2010	Attn: Jason Stormo 200 20th Street	Vandenberg AFB	CA	93437
1/7/2010	1/12/2010	1/12/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
1/7/2010	2/1/2010	2/1/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
2/11/2010	2/15/2010	2/15/2010	Eric Jones . 200 20th Street	Vandenberg AFB	CA	93437
2/24/2010	3/1/2010	3/1/2010	Eric Jones 200 20th Street ,	Vandenberg AFB	CA	93437
3/10/2010	3/11/2010	3/11/2010	Eric Jones 200 20th Street ,	Vandenberg AFB	CA	93437
3/17/2010	3/17/2010	3/17/2010	200 20th Street	Vandenberg AFB	CA	93437
3/25/2010	3/25/2010	3/25/2010	200 20th Street	Vandenberg AFB	CA	93437
3/31/2010	3/31/2010	3/31/2010	200 20th Street	Vandenberg AFB	CA	93437
3/30/2010	4/12/2010	4/12/2010	Attn. Kamran Saber 400 North Tustin Avenue suite 230	Santa Ana	CA	92705
4/7/2010	4/7/2010	4/7/2010	200 20th Street	Vandenberg AFB	CA	93437
4/14/2010	4/14/2010	4/14/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
4/20/2010	4/26/2010	4/26/2010	Ivan Vargas 2245 Craeber St. MWH c/o Bldg 300 , (phone cell 818-391-4246	Match ARB	CA	92518
4/23/2010	8/9/2010	8/9/2010	Ivan Vargas 2245 Graeber St. MWH c/o Bldg 300, (phone cell 818-391-4246	March ARB	CA	92518
4/20/2010	5/18/2010	5/18/2010	OTIE-Oneida 1777 N. California Blvd Suite 310 , J.D. Lensing, phone 925-906-9698	Walnut Creek	CA	94596
4/21/2010	4/22/2010	4/22/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
4/30/2010	5/3/2010	5/3/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
4/30/2010	5/3/2010	5/3/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
5/18/2010	June -July		Vandenberg AFB	Vandenberg AFB	CA	
7/2/2010	7/21/2010	7/21/2010	Paula Jewell 760-274-3458	Tustin	CA	92780
7/5/2010	8/4/2010	8/4/2010	pick up			
7/8/2010	07/15/10 -3 (60L)	7/15/2010	Attn: Richard Orens 15375 Barranca Parkway, Suite J-101 , 714.612.7491 cell	Irvine	CA	92618
7/9/2010	07/26/10-3 (64L)	7/26/2010	Attn: Richard Orens 15375 Barranca Parkway, Suite J-101 , 714.612.7491 cell	Irvine	CA	92618
7/9/2010	7/26/2010	7/26/2010	Mark Vennemeyer Building 107 Moffett Field phone 925-383-6502	Moffett Field	CA	94035
7/9/2010	8/2/2010	8/2/2010	Mark Vennemeyer Building 107 Moffett Field phone 925-383-6502	Moffett Field	CA	94035
7/9/2010	8/9/2010	8/9/2010	Mark Vennemeyer Building 107 Moffett Field phone 925-383-6502	Moffett Field	CA	94035
7/16/2010	7/29/2010	7/29/2010	Jason Stormo 200 20th Street phone805.276.5806	Vandenberg AFB	CA	93437
8/4/2010	8/11/2010	8/11/2010	Jason Stormo 200 20th Street phone 805.276.5806	Vandenberg AFB	CA	93437
8/18/2010	8/18/2010	8/18/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
8/25/2010	8/26/2010	8/26/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
8/31/2010	9/1/10 Replacement for 8/26/10	9/1/2010	Eric Jones, 200 20th Street	Vandenberg AFB	CA	93437
9/2/2010	9/7/2010	9/7/2010	Jason Stormo 200 20th Stree phone 805.276.5806	Vandenberg AFB	CA	93437
9/16/2010	9/20/2010	9/20/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
9/8/2010	9/20/2010	9/20/2010	Mary C. Holland-Ford, 600 Grand Avenue Suite 300 phone 510-628-3221	Oakland	CA	94610
9/28/2010	9/29/2010	9/29/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
9/30/2010	10/6/2010	10/6/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
9/28/2010	10/13/2010	10/13/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
10/21/2010	10/21/2010	10/21/2010	Lloyd Guss 6330 Getaway Dr. Suite B phone 714-484-8600	Cypress	CA	90630
10/25/2010	11/1/2010	11/1/2010	Derek Payne399 W Seaplane Lagoon	Alameda	CA	94501
10/26/2010	10/27/2010	10/27/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
11/4/2010	11/4/2010	11/14/2010	Derek Payne 399 W Seaplane Lagoon	Alameda	CA	94501
11/8/2010	12/1/2010	12/1/2010	attention Sydney Geels 4005 Port Chicago Highway phone 925-288-9898	Concord	CA	94520
11/8/2010	12/8/2010	12/8/2010	attention Sydney Geels 4005 Port Chicago Highway phone 925-288-9898	Concord	CA	94520
9/12/2010	11/16/2010	11/16/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
11/30/2010	12/13/2010	12/13/2010	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
1/4/2011	1/10/2011	1/10/2011	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
1/28/2010	1/31/2010	1/31/2011	Eric Jones 200 20th Street	Vandenberg AFB	CA	93437
9/24/2010	2/14/2011	2/14/2011	Jason Stormo (cell: 805-276-5806) 200 20th Street 93437 805-605-0509 - landline	Vandenberg AFB,	CA	
2/25/2011	3/8/2011	3/8/2011	Don Winglewich 220 N. East Street (530) 668 2424 / (415) 235-69	Woodland	CA	95776
3/11/2011	3/14/2011	3/14/2011	Jason Stormo (cell: 805-276-5806) 200 20th Street , 805-605-0509 - landline	Vandenberg AFB	CA	93437
4/2/2011	6/2/2011	6/2/2011	Dan Leigh	Concord	CA	
4/20/2011	4/25/2011	4/25/2011	Attn: John McAssey 1641 Chalenge Drive	Concord	CA	94520
5/5/2011	5/10/2011	5/10/2011	Jason Stormo (cell: 805-276-5806) 200 20th Street , 805-605-0509 - landline	Vandenberg AFB	CA	93437
6/2/2011	6/2/2011	6/2/2011	Ted Lizee 299 West Hillcrest Dr, Suite 220, Office: (805) 373-9	Thousand Oaks	CA	91360
	6/6/2011	6/6/2011	Ted Lizee 2335 W. Rosecrans Avenue , 290 Field Cell Phone#: 805.	Gardena	CA	90249
	6/8/2011	6/8/2011	Ted Lizee 2335 W. Rosecrans Avenue , 290 Field Cell Phone#: 805.	Gardena	CA	90249
7/7/2011	7/18/2011	7/18/2011	Apgar 530 Showers Dr. Suite 7 1457 616-340-0878	Mountainview	CA	94040
7/12/2011	7/18/2011	7/18/2011	Jason Stormo (cell: 805-276-5806) 200 20th Street , 805-605-0509 - landline	Vandenberg AFB	CA	93437
8/12/2011	8/15/2011	8/15/2011	Jason Stormo (cell: 805-276-5806) 200 20th Street , 805-605-0509 - landline	Vandenberg AFB	CA	93437
11/4/2011	11/7/2011	11/7/2011	c/o Kent Deacon 200 20th Street phone 8055039698	Vandenberg AFB	CA	93437
11/15/2011	11/15/2011	11/15/2011	c/o Kent Deacon 200 20th Street phone 8055039698	Vandenberg AFB	CA	93437
11/15/2011	11/16/2011	11/16/2011	c/o Kent Deacon 200 20th Street phone 8055039698	Vandenberg AFB	CA	93437
11/16/2011	11/21/2011	11/21/2011	Pick up			
11/23/2011	12/1/2011	12/1/2011	Andrew Schmidt 400 N Tustin Ave #230 Phone 714-973-2230	Santa Ana	CA	92705
12/5/2011	12/6/2011	12/6/2011	c/o Kent Deacon 200 20th Street phone 8055039698	Vandenberg AFB	CA	93437
12/22/2011	1/9/2012	1/9/2012	UPS Store Attn; Mike Apgar 530 Showers Dr. STE 7 phone 616-340-0878	Mountain View	CA	94040-1457
1/20/2012	2/14/2012 delay	2/29/2012	75 Coromar Drive Buiding 5, MS 96	Goleta	CA	93117
1/25/2012	2/1/2012	2/1/2012	950 Avenue M (Bldg 570) Treasure Island	San Francisco	CA	94130
3/7/2012	3/13/2012	3/13/2012	950 Avenue M (Bldg 570) Treasure Island	San Francisco	CA	94130
2/6/2012	2/13/2012	2/13/2012	711 Grand Ave, Suite 220	San Rafael	CA	94901
2/7/2012	2/8/2012	2/8/2012	Vironex, Inc. 1225 E McFadden Avenue	Santa Ana	CA	92705
3/5/2012	3/6/2012	3/6/2012	URS corrporation One Montgomery St Suite 900 San Francisco CA 94104	San Francisco	CA	94104
4/3/2012	4/4/2012	4/4/2012	TRC 2300 Clayton Rd., Suite 610	Concord	CA	94520
4/25/2012	5/7/2012	5/7/2012	1225 E. McFadden Avenue Santa Ana , CA 92705	Santa Ana	CA	92705
5/7/2012	5/8/2012	5/8/2012	Jason Stormo (cell: 805-276-5806) 200 20th Street , 805-605-0509 - landline	Vandenberg AFB	CA	93437
6/26/2012	7/2/2012	7/2/2012	Jason Stormo (cell: 805-276-5806) 200 20th Street , 805-605-0509 - landline	Vandenberg AFB	CA	93437
7/10/2012	8/13/2012	8/13/2012	399 West Seaplane Lagoon Alameda CA 94501	Alameda	CA	94501
7/20/2012	7/26/2012	7/26/2012	GHD Inc. 400 N. Tustin Ave, Suite 230 Santa Ana CA 92705	Santa Ana	CA	92705
8/14/2012	8/15/2012	8/15/2012	PES Environmental, Inc. 1682 Novato BLVD. Suite 100 Novato, CA 94947	Novato	CA	94947
9/11/2012	9/17/2012	9/17/2012	399 West Seaplane Lagoon Alameda CA 94501	Alameda	CA	94501
9/18/2012	9/20/2012	9/20/2012	PES Environmental, Inc. 1682 Novato BLVD. Suite 100 Novato, CA 94947	Novato	CA	94947
10/5/2012	10/9/2012	10/9/2012	Shaw E&I 200 20th Street Vandenberg AFB, CA 93437	Vandenberg AFB	CA	93437
11/9/2012	11/12/2012	11/12/2012	Shaw E&I 200 20th Street Vandenberg AFB, CA 93437	Vandenberg AFB	CA	93437
11/15/2012	11/29/2012	11/29/2012	OTIE Raytheon B-2 Construction Trailer 75 Coromar Drive Building 5, MS 96	Goleta	CA	93117
12/14/2012	12/17/2012	12/17/2012	Kent Deacon (cell: 805-605-0509) 200 20th Street , 805-605-0509 - landline	Vandenberg AFB	CA	93437
1/3/2013	1/10/2013 and 1/24/2013	1/10/2013	OTIE 317 East Main Street Ventura CA 93001	Ventura	CA	93001
1/4/2013	1/7/2013	1/7/2013	15991 Armstrong Ave Tustin CA, 92606	Tustin	CA	92606
1/22/2013	2/11/2013	2/11/2013	Vironex, Inc. 1225 E McFadden Avenue Santa Ana CA 92705	Santa Ana	CA	92705
2/4/2013	2/11/2013	2/11/2013	2640 El Camino Real Santa Clara, CA 95051	Santa Clara	CA	95051