Dehalococcoides-containing microbial consortium (SDC-9^{TM}) for anaerobic bioremediation

1. Dr. Robert J. Steffan, CB&I Federal Services, LLC. (formerly Shaw Environmental, Inc.)
2. Composed of anaerobic bacteria including *Dehalococcoides mccartii* in an aqueous medium.
3. MSDS and Technical Data Sheet
4. Number of Field-scale Applications to Date: 650+ applications
5. Case Studies – Attached.
6. Technical Summary. The SDC-9^{TM} culture is a pathogen-free, non-genetically altered microbial consortium capable of biologically degrading halogenated aliphatic pollutants including , 1,1,1-TCA, 1,1,2-TCA, 1,1-DCA, 1,2-DCA, 1,2-DBE, TeCA, CT, CF, PCE, TCE, DCE, VC, and Freon 113 (1,2-dichloro-1,2-difluoroethane), and also mixtures thereof. Molecular biological analyses of the SDC-9 culture has demonstrated that the culture has at least three closely-related strains of *Dehalococcoides* sp. bacteria which is the only bacterial genus known to degrade chlorinated ethenes completely to ethene. In addition, the culture contains other known pollutant degrading bacteria including *Desufitobacterium* and *Desulfovibrio* strains. It has now been successfully applied motre than 600 times, and 100,000 L have been delivered to sites throughout the United States. The culture has been applied commercially since 2003, and it is sold by licensed distributors under several trade names including RTB-1^{TM}, BAC-9^{TM}, TSI-DC^{TM}, and BDIplus^{TM}.

Material Safety Data Sheet

SECTION 1 – CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

Product Name: DHC microbial consortium (SDC-9)

Manufacturer CB&I 17 Princess Road, Lawrenceville,
NJ 08648. Phone (609) 895-5340

CAS #: N/A (Not Applicable)

Product Use: For remediation of contaminated groundwater (environmental applications).

Material Description: Non-toxic, naturally occurring, non-pathogenic, non-genetically altered anaerobic microbes in a water-based medium.

IN CASE OF EMERGENCY CALL CHEMTREC 24 HOUR EMERGENCY RESPONSE PHONE NUMBER (800) 424-9300

SECTION 2 – COMPOSITIONS AND INFORMATION ON INGREDIENTS

Components	%	OSHA PEL	ACGIH TLV	OTHER LIMITS
Non-Hazardous Ingredients	100	N/A	N/A	N/A

DHC microbial consortium (SDC-9) comprised of microorganism of the genus *Dehalococcoides, Desulfovibrio, and Desulfitobacterium,* and methanogenic archebacteria.

SECTION 3 – HAZARDS IDENTIFICATION

The available data indicates no known hazards associated with exposure to this product. Nevertheless, individuals who are allergic to enzymes or other related proteins should avoid exposure and handling. Health effects associated with exposure to similar organisms are listed below.

Ingestion: Ingestion of large quantities may result in abdominal discomfort including nausea, vomiting, cramps, diarrhea, and fever.

Inhalation: Hypersensitive individuals may experience breathing difficulties after inhalation of aerosols.

Skin Absorption: May cause irritation upon prolonged contact. Hypersensitive individuals may experience allergic reactions..

Eye contact: May cause irritation unless immediately rinsed.

SECTION 4 – FIRST AID MEASURES

Ingestion: Thoroughly rinse mouth with water. Do not induce vomiting unless directed to do so by medical personnel. Get immediate medical attention. Never give anything by mouth to an unconscious or convulsing person.

Inhalation: Get medical attention if allergic symptoms develop.

Skin Absorption: N/A

Skin Contact: Wash affected area with soap and water. Get medical attention if allergic symptoms develop.

Eye Contact: Flush eyes with plenty of water for at least 15 minutes using an eyewash fountain, if available. Get medical attention if irritation occurs.

NOTE TO PHYSICIANS: All treatments should be based on observed signs and symptoms of distress in the patient. Consideration should be given to the possibility that overexposure to materials other than this material may have occurred.

SECTION 5 – FIRE AND EXPLOSION DATA

Flammability of the Product: Non-flammable

Flash Point: N/A

Flammable Limits: N/A

Fire Hazard in Presence of Various Substances: N/A

Explosion Hazard in Presence of Various Substances: N/A

Extinguishing Media: Foam, carbon dioxide, water

Special Fire Fighting Procedures: None

Unusual Fire and Explosion Hazards: None

SECTION 6 – ACCIDENTAL RELEASE MEASURES

Reportable quantities (in lbs of EPA Hazardous Substances): N/A

No emergency results from spillage. However, spills should be cleaned up promptly. Absorb with an inert material and put the spilled material in an appropriate waste disposal container. All personnel involved in the cleanup must wear protective clothing and avoid skin contact. After clean-up, disinfect all cleaning materials and storage containers that come in contact with the spilled liquid.

SECTION 7 – HANDLING AND STORAGE

Avoid breathing breathe aerosol. Avoid contact with skin. Use personal protective equipment recommended in Section 8.
Keep containers tightly closed in a cool, well-ventilated area. The DHC microbial consortium (SDC-9) can be supplied in stainless steel kegs designed for maximum working pressure of 130 psi and equipped with pressure relief valves. The kegs are pressurized with Nitrogen up to the pressure of 15 psi. Do not exceed pressure of 15 psi during transfer of DHC microbial consortium (SDC-9) from kegs. Don't open keg if content of the keg is under pressure.

DHC microbial consortium (SDC-9) may be stored for up to 3 weeks at temperature 2-4°C without aeration. Avoid freezing.

SECTION 8 – EXPOSURE CONTROLS/PERSONAL PROTECTION

Hand Protection: Rubber, nitrile, or vinyl gloves.

Eye Protection: Safety goggles or glasses with side splash shields.

Protective Clothing: Use adequate clothing to prevent skin contact.

Respiratory Protection: N95 respirator if aerosols might be generated.

Ventilation: Provide adequate ventilation to remove odors.

Other Precautions: An eyewash station in the work area is recommended.

SECTION 9 – PHYSICAL/CHEMICAL CHARACTERISTICS

Physical state and appearance: Light greenish murky liquid. Musty odor.

Boiling Point: 100°C (water)	Specific Gravity (H_2O = 1): 0.9 - 1.1
Vapor Pressure @ 25°C: 24 mm Hg (water)	Melting Point: 0°C (water)
Vapor Density: N/A	Evaporation Rate (H_2O = 1): 0.9 - 1.1
Solubility in Water: Soluble	Water Reactive: No
pH: 6.0 - 8.0	

SECTION 10 – STABILITY AND REACTIVITY DATA

Stability: Stable

Conditions to Avoid: None

Incompatibility (Materials to Avoid): Water-reactive materials

Hazardous Decomposition Byproducts: None

SECTION 11 – TOXICOLOGICAL INFORMATION

This product contains no toxic ingredients.
SDC-9 consortium has tested negative for pathogenic microorganisms such as Bacillus cereus, Listeria monocytogens, Salmonella sp., Fecal Coliform, Total Coliform, Yeast and Mold and Pseudomonas sp.

SECTION 12 – ECOLOGICAL INFORMATION

Ecotoxicity: this material will degrade in the environment.

SECTION 13 – DISPOSAL CONSIDERATIONS

Waste Disposal Method: No special disposal methods are required. The material is compatible with all known biological treatment methods. To reduce odors and permanently inactivate microorganisms, mix 100 parts (by volume) of SDC-9 consortium with 1 part (by volume) of bleach. Dispose of in accordance with local, state and federal regulations.

SECTION 14 – TRANSPORT INFORMATION

DOT Classification:	N/A
Labeling:	NA
Shipping Name:	Not regulated

SECTION 15 – REGULATORY INFORMATION

Federal and State Regulations: N/A

SECTION 16 – OTHER INFORMATION

MSDS Code: ENV 1033
MSDS Creation Date: 10/06/2003
Last Revised: April 30, 2013.

While the information and recommendations set forth herein are believed to be accurate as of the date hereof, CB&I MAKES NO WARRANTY WITH RESPECT HERETO AND DISCLAIMS ALL LIABILITY FROM RELIANCE THEREON.

SDC-9 Technical Data

Robert J. Steffan, Ph.D.

CB&I Federal Services, LLC

(Formerly, Shaw Environmental, Inc.)

About SDC-9

Isolated in 2002

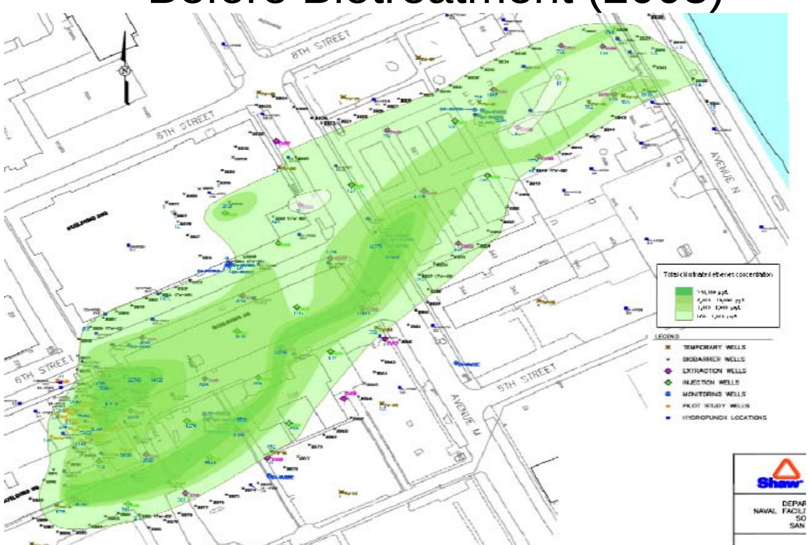
Enrichment culturing with samples from North Island Naval Station, CA Site 9

Grown exclusively on Lactate plus PCE with trace amounts of Yeast Extract

Grown under strict anaerobic conditions

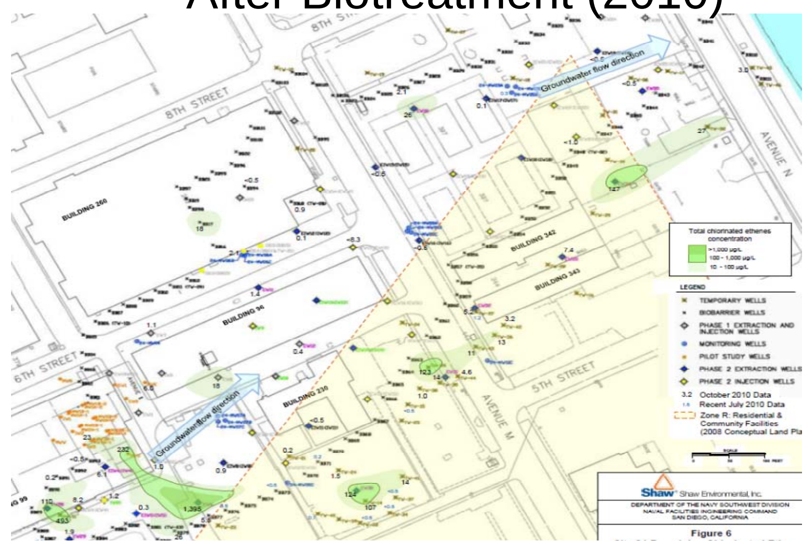
First commercial-scale application – Treasure Island, CA Site 24– October 6, 2003

Before Biotreatment (2003)

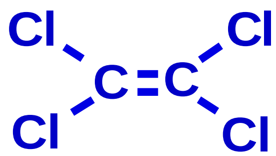


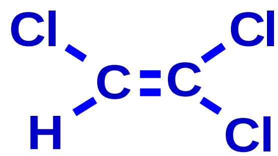
Dark Green >10 mg/L TCE

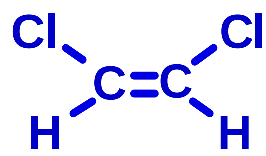
After Biotreatment (2010)

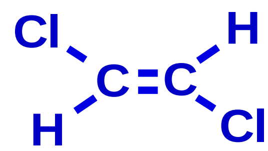


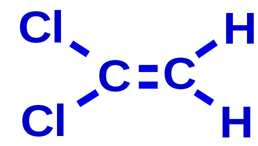
Pollutants Degraded by SDC-9^{TM}

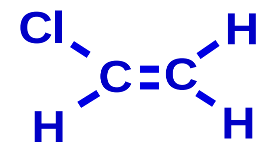

PCE


TCE


*c*DCE


*t*DCE


1,1-DCE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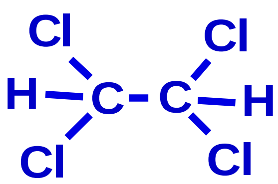
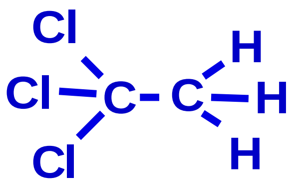
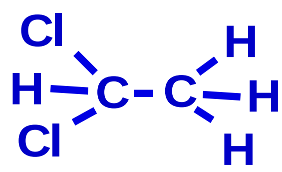
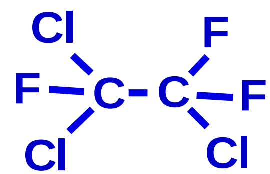
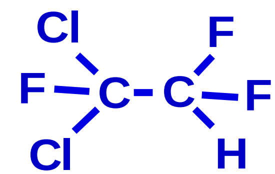
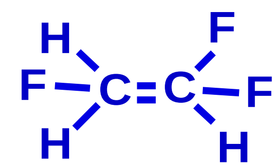

1,1,2,2-TeCA

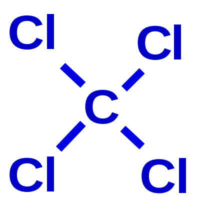

1,1,1-TCA

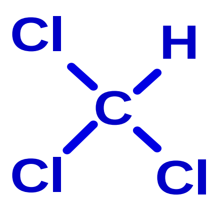

1,1-DCA


Freon 113


HCFC


TFE

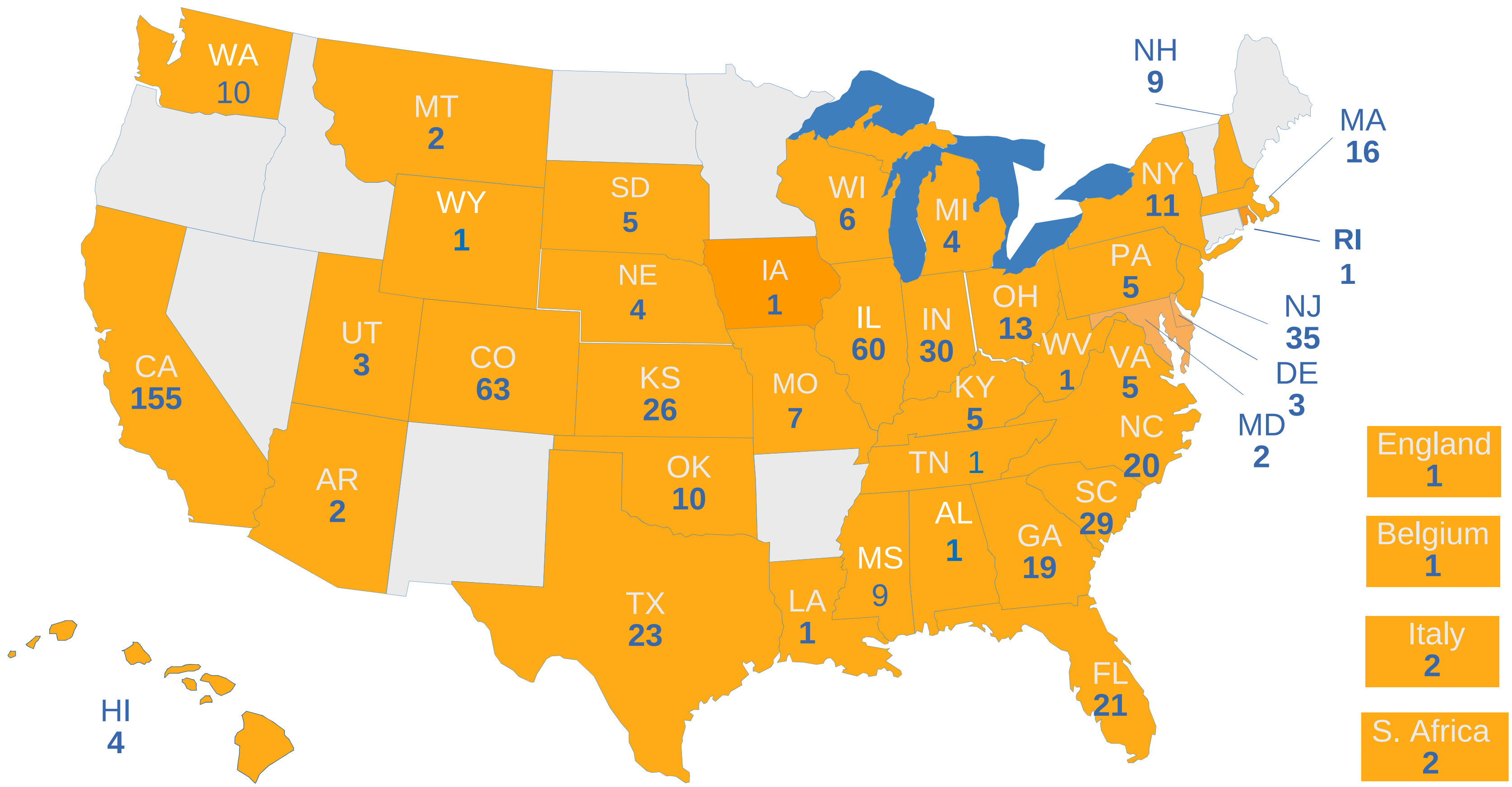

CT


CF

SDC-9 Vendors and Trade Names

CB&I	SDC-9™
FMC	SDC-9™
EOS Remediation, Inc.	Bac-9™
REDOX TECH, LLC	RTB-1™
Terra Systems	TSI-DC™
REGENESIS Advanced Technologies for Groundwater Resources	BDIplus™
JRW BIOREMEDIATION LLC	SDC-9™

Bioaugmentation Culture Applications*



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

Total Applications:641

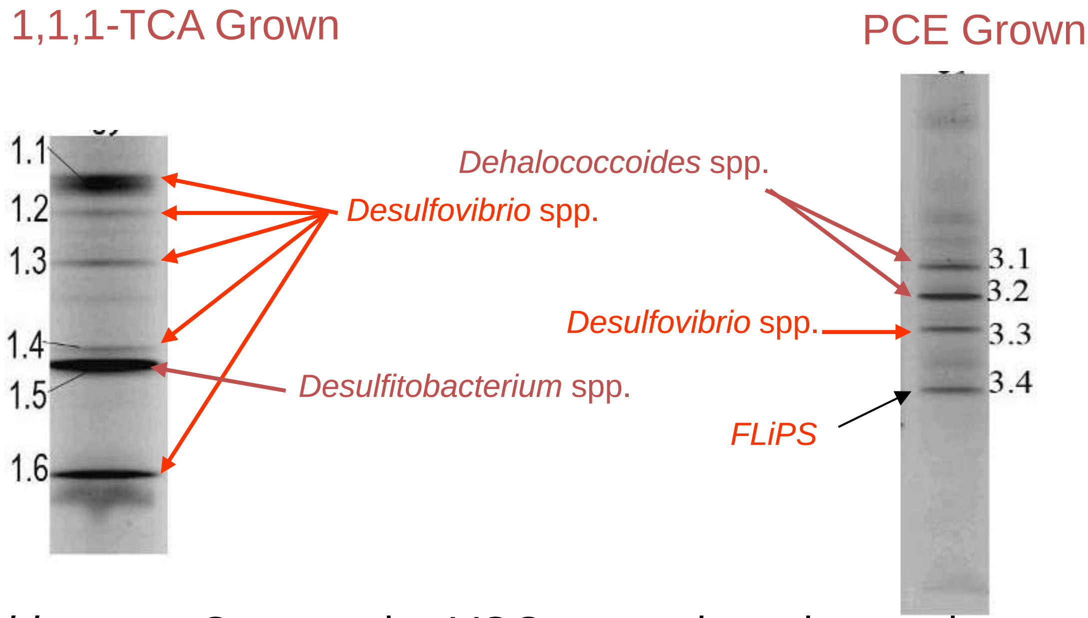
Total Volume Delivered: ~106,206 L

* Data represent culture deliveries as of 9/24/13 and include licensed culture distributors

Gene Library Analysis by CDM et al.

- 4 *Dehalococcoides* strains
- *vcrA* present
  - Most closely related to Strain VS *vcrA*
  - >99% sequence similarity
- *bvc*A not present

DGEE Analysis of SDC-9



Dehalococcoides sp. – Common in cVOC-contaminated groundwater – dechlorinate DCE and VC to ethene

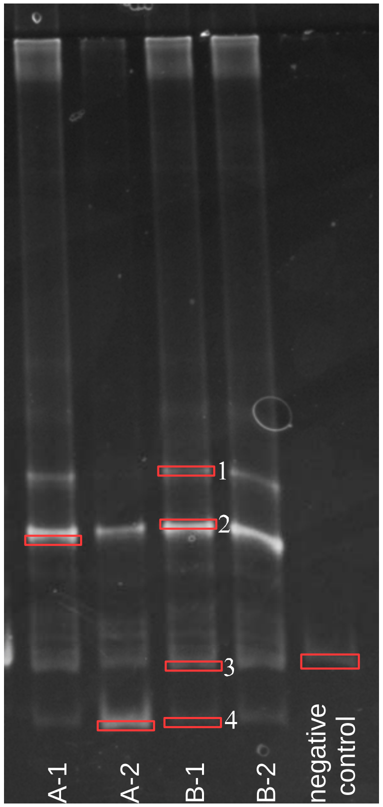
Desulfovibrio spp. – Common groundwater microbes – reduce sulfate, may dechlorinate PCE and TCE

Desulfitobacterium spp. – Common groundwater microbes – ferment, may dechlorinate PCE and TCE

FLiPS – Common in DHC consortia - free living polymorphic spirochaetes- believed to ferment. Not recently detected in SDC-9

Also contains Methanogens

DGGE Analysis of Carbon Tet-Grown SDC-9



Band	ID	E value	Identities	
A-1	Uncultured bacterium	3.00E-75	188/202 (93%)	Community Uranium Reduction and Reoxidation
A-2	Uncultured bacterium	3.00E-84	172/172 (100%)	Anaerobic Polychlorinated Biphenyl Dechlorinating Consortia
B-1, band 1	*Bacteroidales bacterium*	1.00E-93	189/189 (100%)	Dehalococcoides Population Dechlorinating PCB Mixture Aroclor 1260
B-1, band 2	Same as A-1			
B-1, band 3	Uncultured bacterium	2.00E-97	196/196 (100%)	Polychlorinated-dioxin-dechlorinating microbial community
B-1, band 4	Same as A-2			
negative control	*Shigella boydii, E. coli*	5.00E-98	197/197 (100%)	

SDC-9 Pathogen Analysis

Microbac Laboratories, Inc.

Baltimore Division

2101 Van Deman Street • Baltimore, MD 21224

Phone: 410-633-1800
Fax: 410-633-6553
www.microbac.com

CERTIFICATE OF ANALYSIS

SHAW ENVIRONMENTAL & INFRA.	Project: CONSORTIUM SAMPLES	Report: 12C0784
17 PRINCESS ROAD	Project Number: CONSORTIUM SAMPLES	Reported: 03/20/2012 13:37
LAWRENCEVILLE, NJ 08648	Project Manager: SIMON VAINBERG	

SDC-9

12C0784-01 (Water) Sampled: 03/13/2012 00:00; Type: Not Specified

Analyte	Result	Reporting Limit	Units	Prepared	Analyzed	Analyst	Method	Notes
Microbac Laboratories, Inc., Baltimore Division								
Microbiology								
Bacillus cereus	ND	3.0	CFU/g	031312 1103	031712 1600	JAT	AOAC 980.31	
Coliform, Total	ND	3.0	MPN/g	031312 1114	031512 0945	DML	FDA-BAM	
E. Coli	ND	3.0	MPN/g	031312 1114	031512 0945	DML	FDA-BAM	
Fecal Coliform	ND	3.0	MPN/g	031312 1130	031512 0945	DML	FDA BAM	
Listeria monocytogens	**NEGATIVE**		per 25g	031312 1120	031612 0935	JAT	AOAC 2003.12	
Salmonella	**NEGATIVE**		per 25g	031312 1122	031512 0630	DML	AOAC 2003.09	
Yeast and Mold	ND	10	CFU/g	031312 1123	031812 1140	JAT	FDA-BAM	
Microbac Laboratories, Inc., Central Pennsylvania								
MICROBIOLOGY								
Pseudomonas	ND	10	CFU/g	031412 1845	031612 1600	GLF	ISO 13720	

Testing performed at least annually; Data available from 2005 8

Batch QA/QC

Shaw® Shaw Environmental, Inc.	17 Princess Road Lawrenceville, NJ 08691 (609) 895-5340 Fax (609) 895-1858

CERTIFICATE OF QUALITY

Batch # JS70730-1 (04/04/2011)

Test	Results	Date	Method
DHC content of Pre-concentrated culture, copies/L	3.90E+11	4/6/2011	qPCR
DHC content of Concentrated culture, copies/L	6.00E+12	4/6/2011	qPCR
PCE dechlorination activity, mg/h per gram of dry weight	240	4/4/2011	Bottle Assay
cDCE dechlorination activity, mg/h per gram of dry weight	201	4/4/2011	Bottle Assay

This certificate has been reviewed and is signed by:

Robert J. Steffan, Ph.D.
Director,
Biotechnology Development and Applications Group
Telephone: (609) 895-5350

Bioaugmentation/SDC-9 Literature

•Stedtfeld, R.D., T.M. Stedfeld, M. Kronlein, G. Seyrig, **R.J. Steffan**, A.M. Cupples, and S.A Hashsham. DNA-extraction free quantification of *Dehalococcoides* spp. in groundwater using a hand-held device. In press.

•**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.

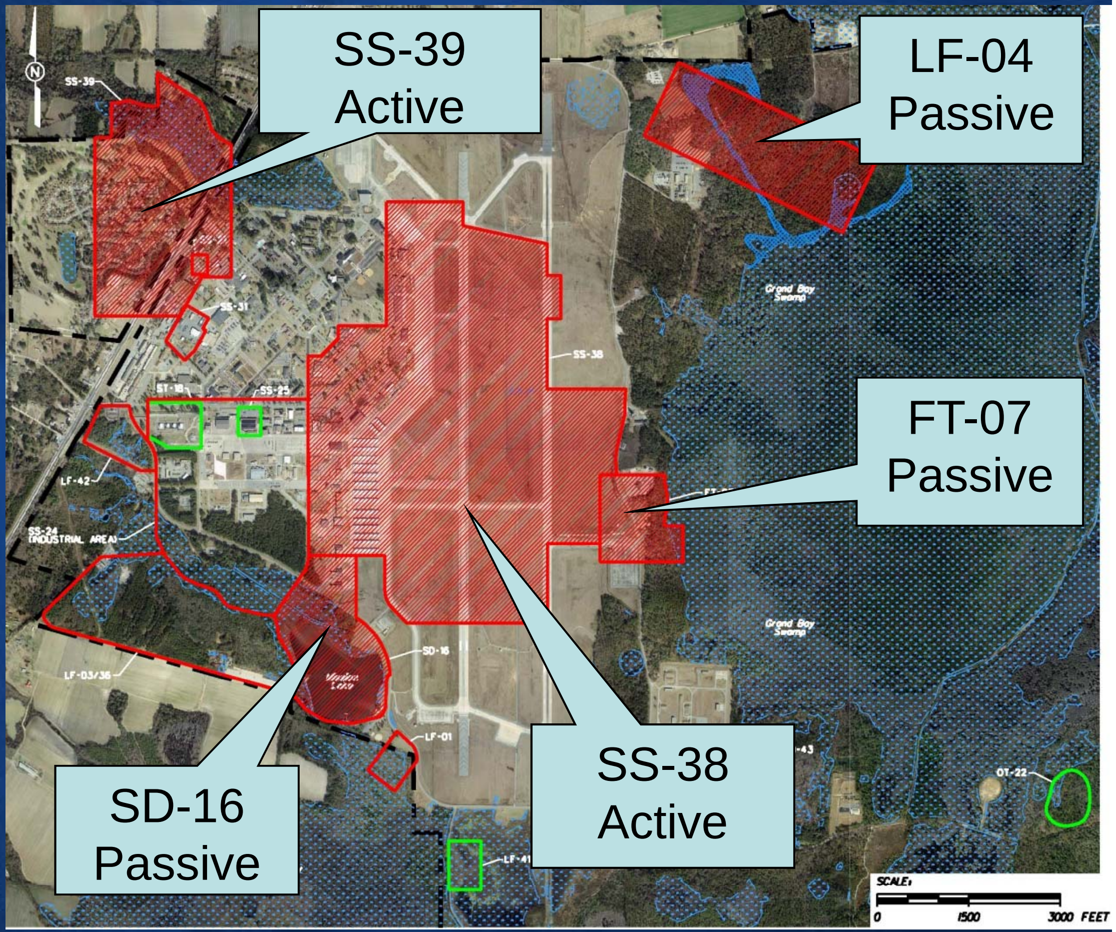
SDC-9^{TM} Case Studies

Track #

Moody AFB Bioremediation Site Locations

Site Characteristics

- **Chlorinated Ethenes**
  - TCE from 100 – 10,000 µg/L
  - Little or no cis-1,2-DCE, VC
- **Aquifer Conditions**
  - DO >1 mg/L
  - ORP >200 mV
  - pH between ~4.5 – 6.5
  - GW velocity ~ 150ft/yr
- **Active Remediation Performance Standards**
  - **TCE/DCE 50 – 1,000 µg/L**



Track #

Shaw® a world of Solutions™

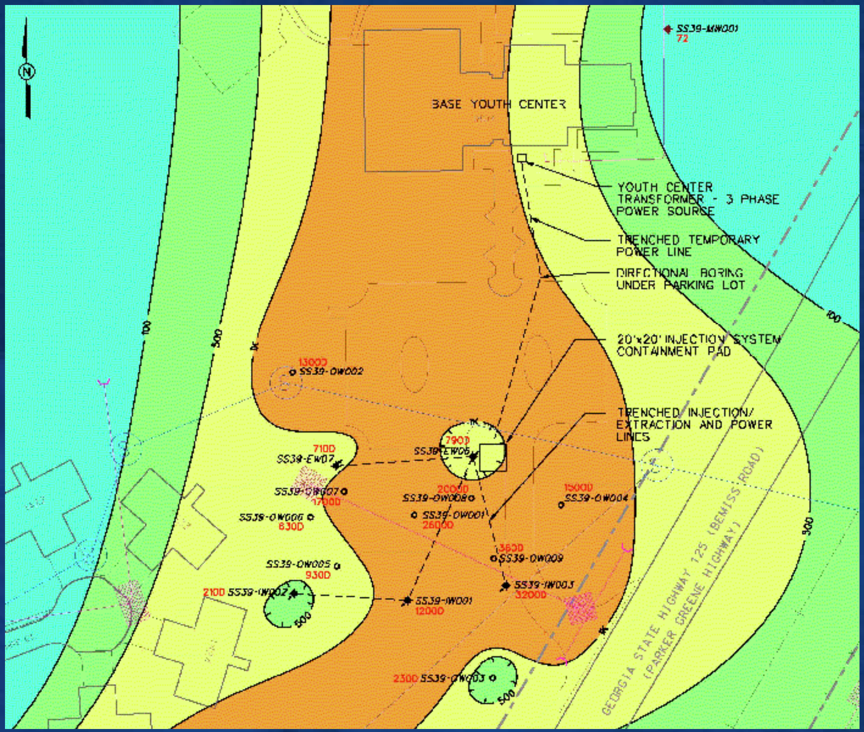
Golf Course Area

- TCE >500 ppb
- Recirculation
- Lactate
- Bioaugmentation with SDC-9

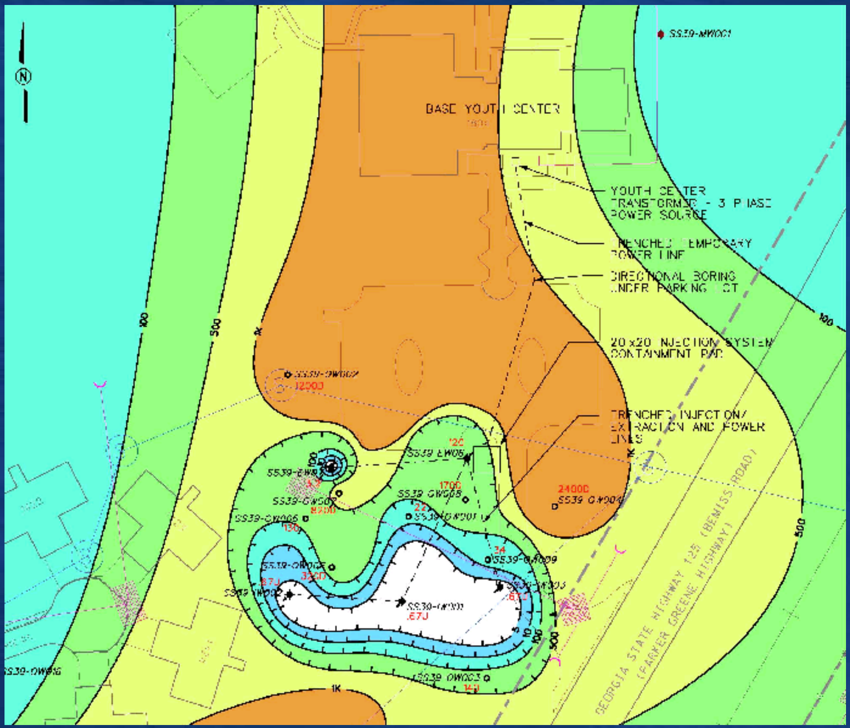
Track #

Shaw® a world of Solutions™

Golf Course Area (SS-39)
Pilot Study Results



TCE concentrations in DEC 03 (prior to system start-up)



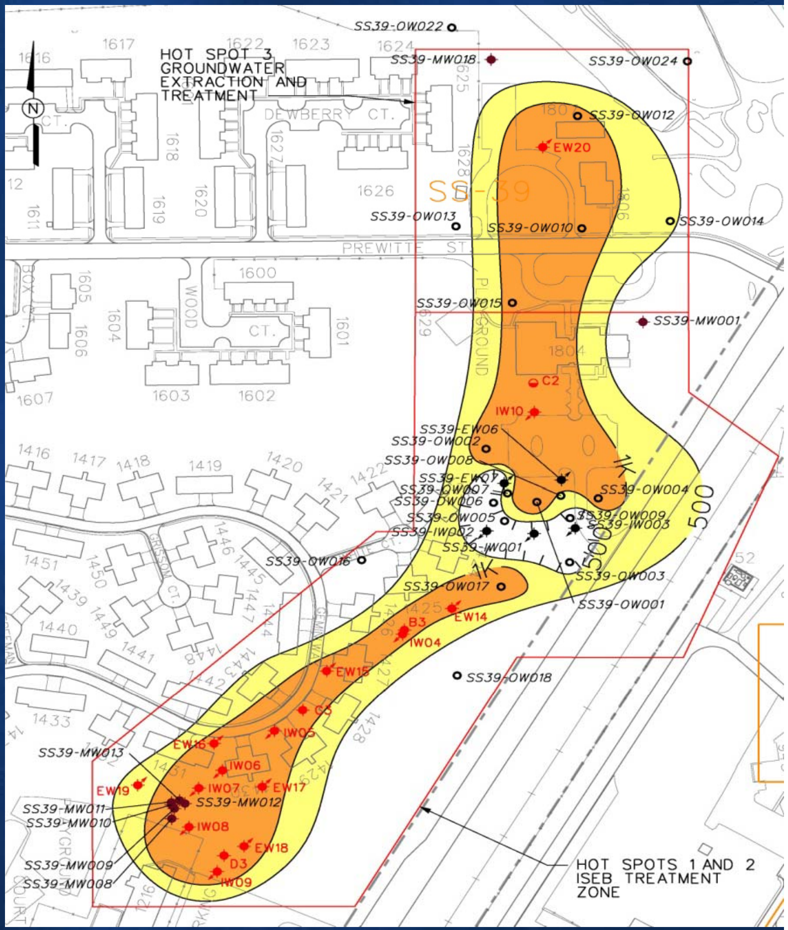
TCE concentrations in JAN 05 (two years post system start-up)

Shaw® a world of Solutions™

Golf Course Area (SS-39) Expanded Groundwater Recirculation System

Expanded System

- TCE > 500 µg/L
- 10 Injection Wells
- 8 Extraction Wells
- Carbon Source
  Sodium Lactate
- Bioaugmentation
  SDC-9



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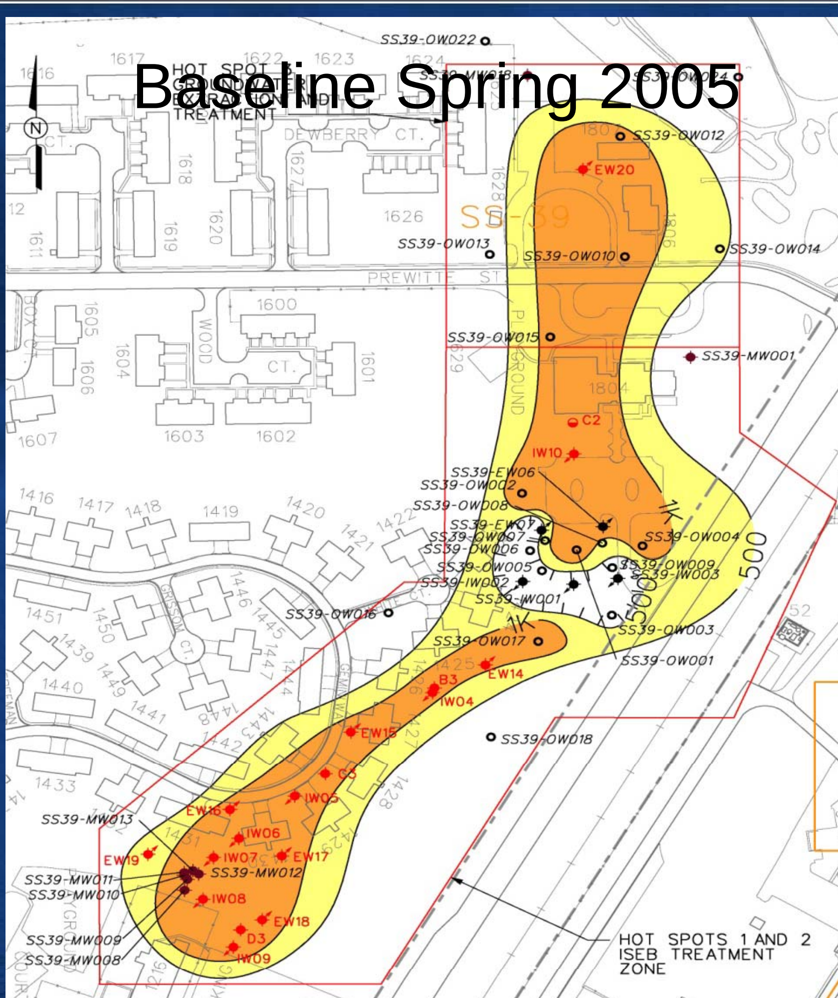
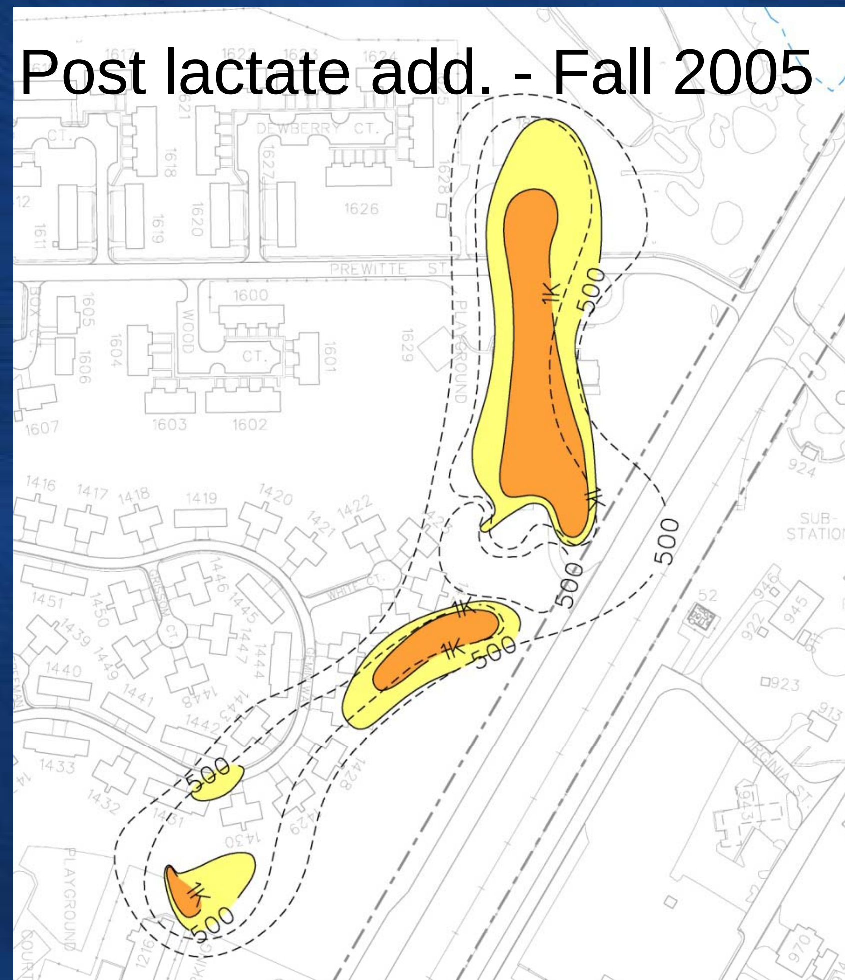
Golf Course Area (SS-39) Results





System currently shut down – No further action required

Shaw® a world of Solutions™

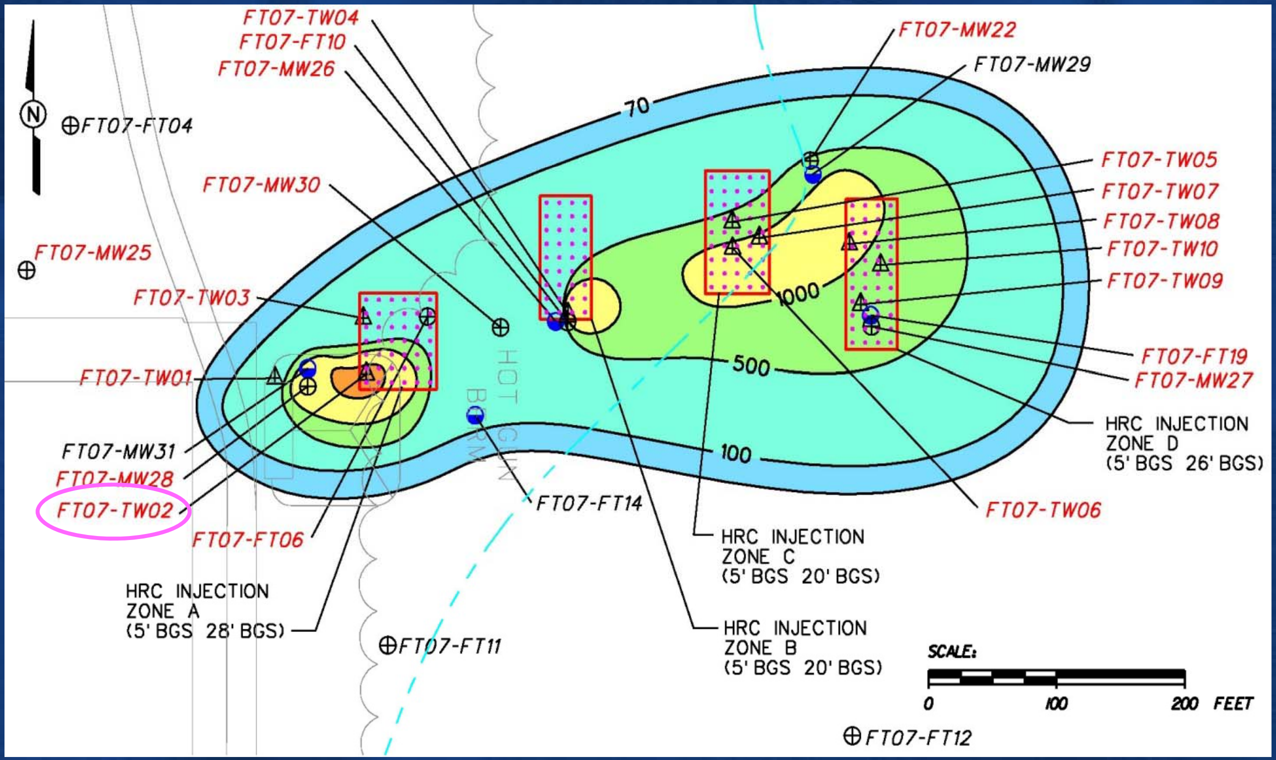
Fire Training Area

- Passive Treatment
- Lactate
- Bioaugmentation with SDC-9

Track #

Former Fire Training Area (FT-07)
Anaerobic Bioremediation Pilot Study

A pilot study was initiated in November 2002 to evaluate direct injection and distribution via ambient groundwater flow.

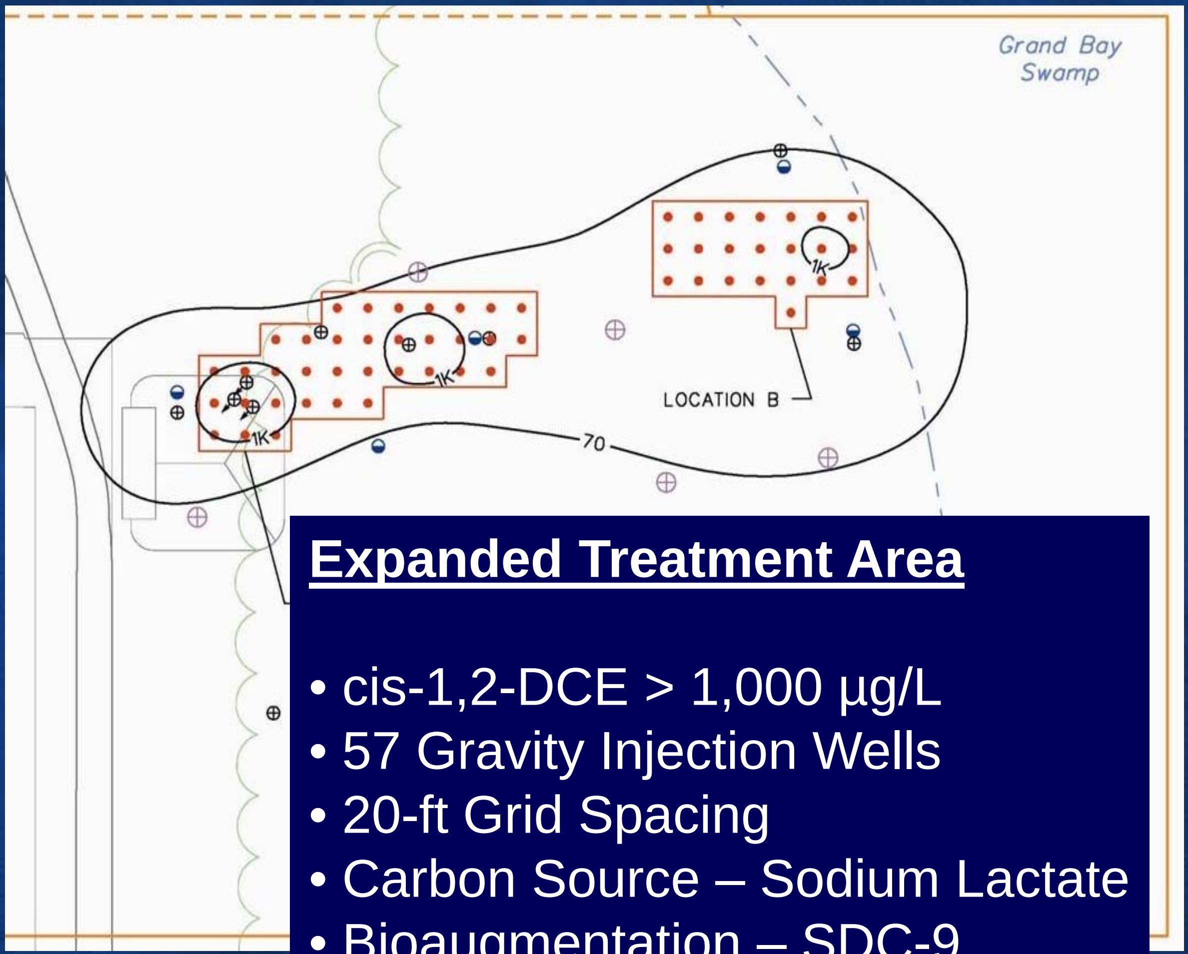


- HRC injection November 2002
- HRC injection December 2003
- Bioaugmentation June 2004

Track #

Shaw® a world of Solutions™

Former Fire Training Area (FT-07)
Expanded Passive Delivery System

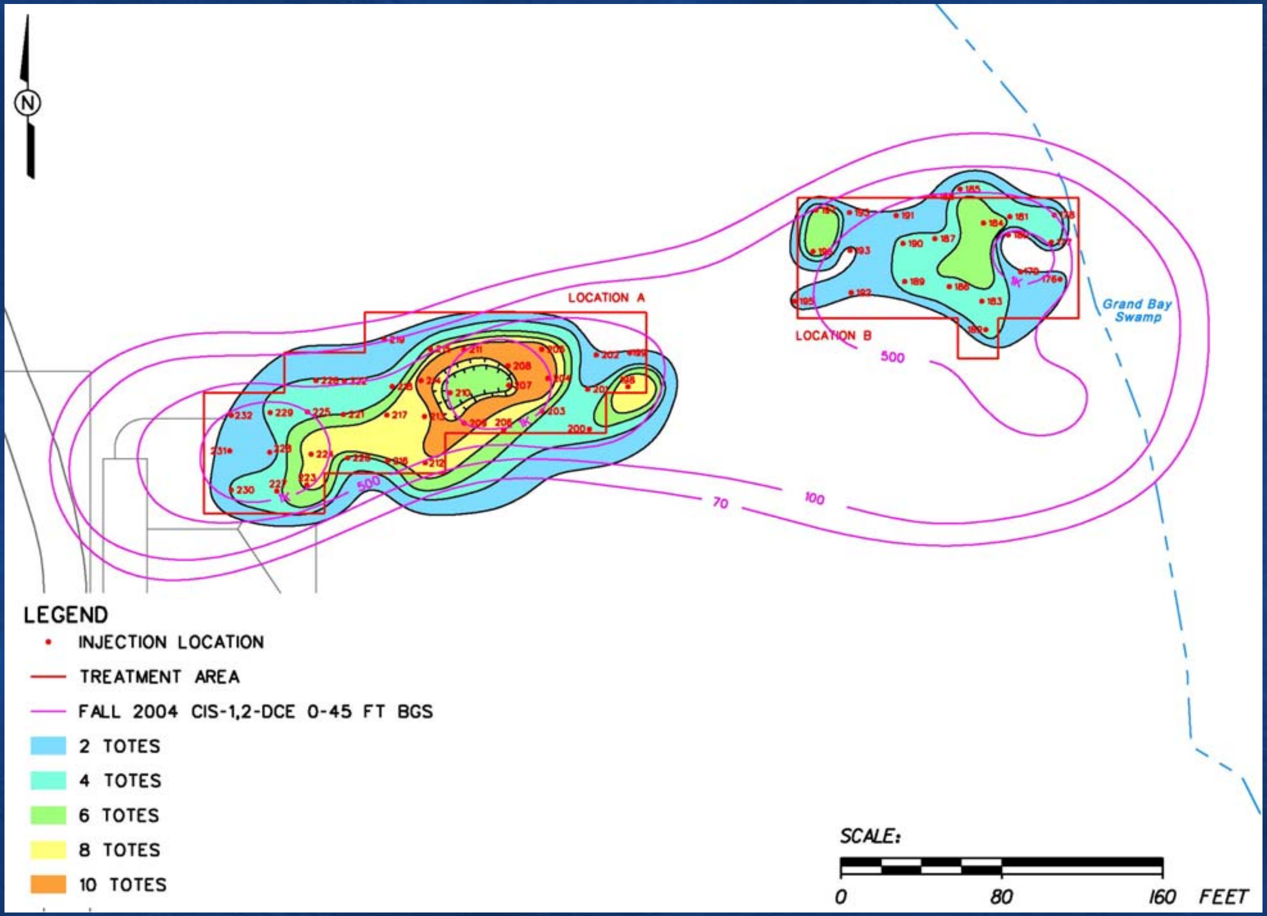


Expanded Treatment Area

- cis-1,2-DCE > 1,000 µg/L
- 57 Gravity Injection Wells
- 20-ft Grid Spacing
- Carbon Source – Sodium Lactate
- Bioaugmentation – SDC-9

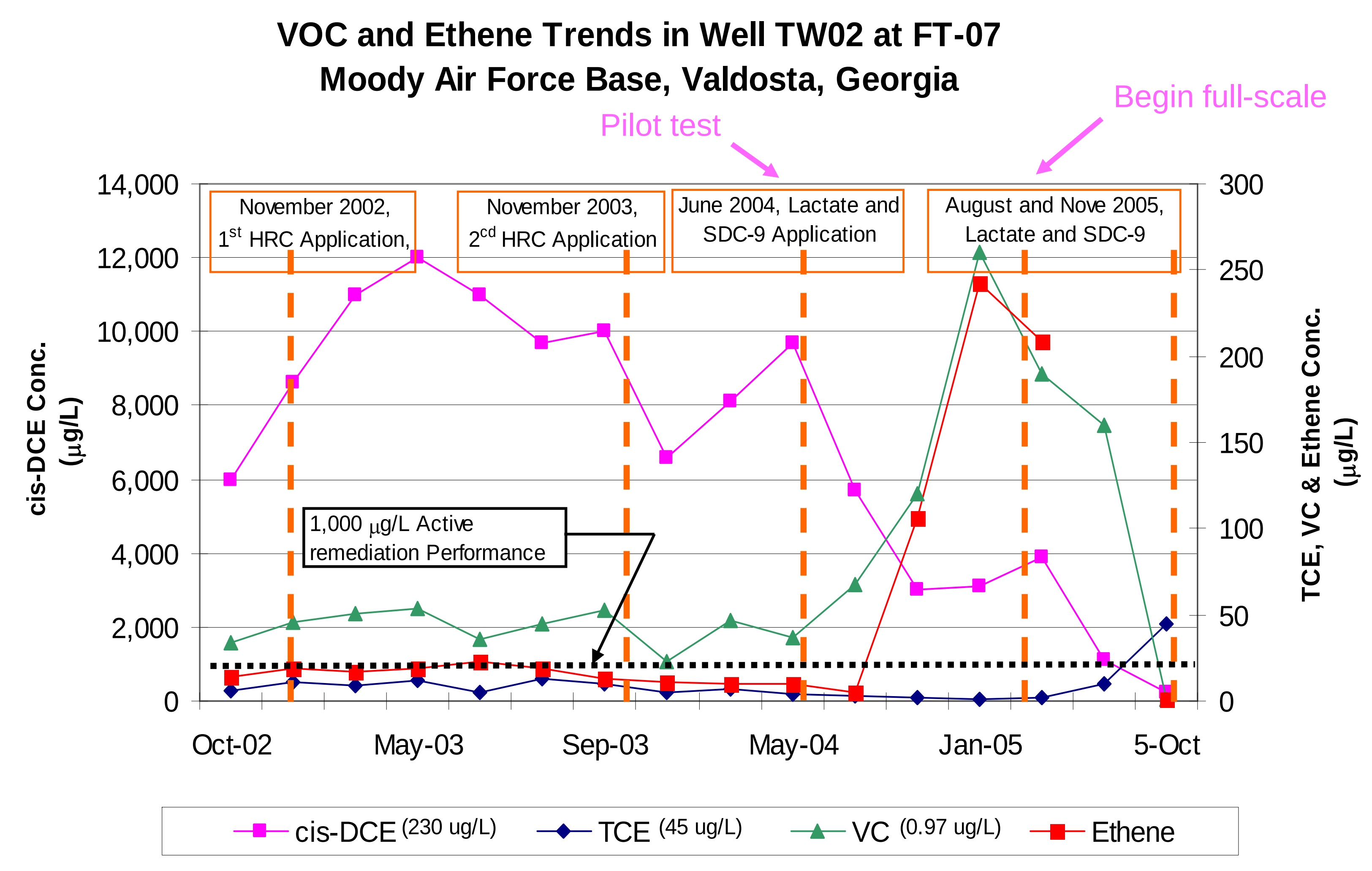
Shaw® a world of Solutions™

Former Fire Training Area (FT-07) Lactate Distribution via Gravity Feed



Shaw® a world of Solutions™

Former Fire Training Area (FT-07) Pilot Study Results



Shaw® a world of Solutions™

Flight Line Storm Drain Area

- Passive Treatment
- Veg. Oil
- Bioaugmentation with SDC-9

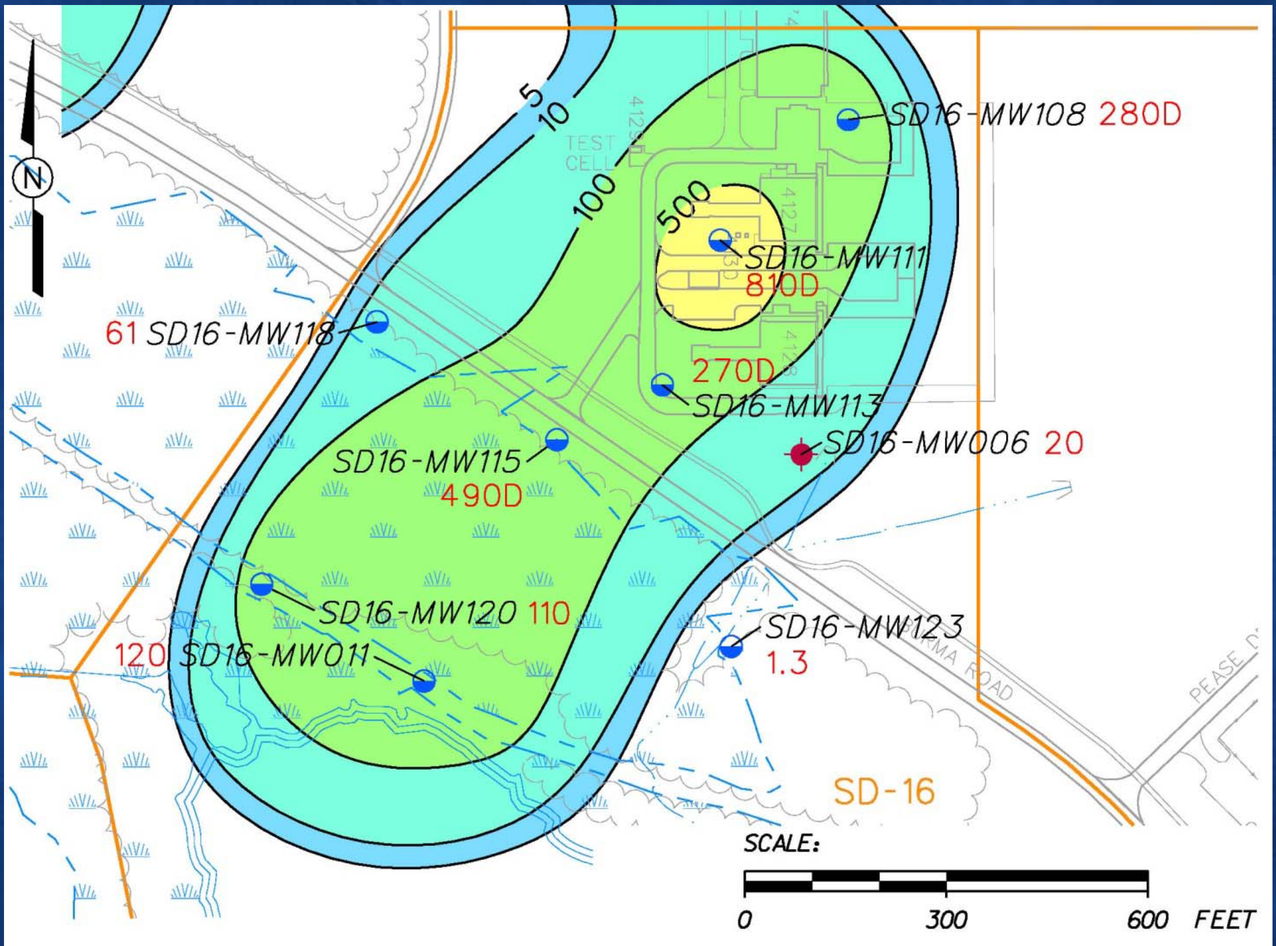
Track #

Shaw® a world of Solutions™

Flightline Storm Drain Outfall & Mission Lake (SD-16)
Full-Scale Anaerobic Bioremediation

Treatment Area

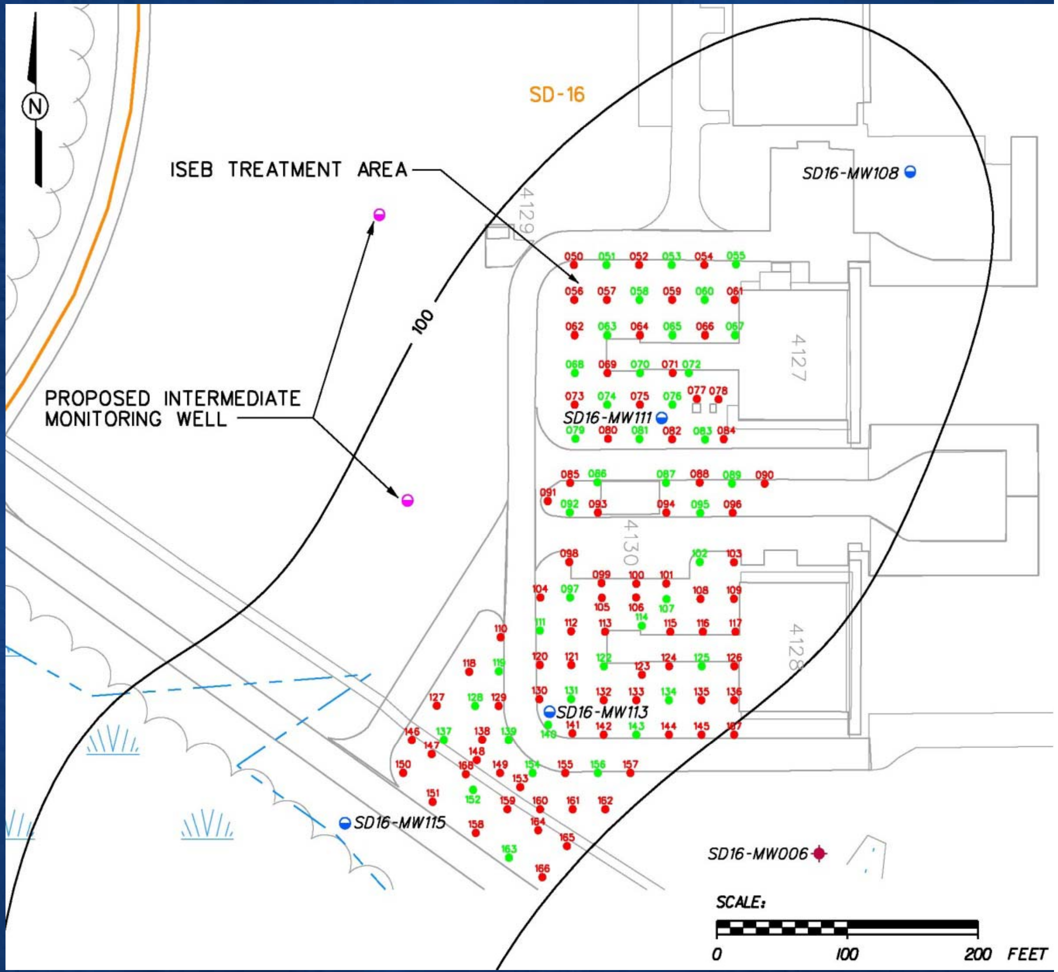
- TCE > 100 µg/L
- 118 Injection Wells
- 25-ft Grid Spacing
- Carbon Source
  Emulsified Oil
- pH Buffer
  Sodium Bicarbonate
- Bioaugmentation
  SDC-9
  ~2900 L



Track #

Shaw® a world of Solutions™

Flightline Storm Drain Outfall & Mission Lake (SD-16)
Grid Application of Emulsified Oil and Bioaugmentation



Track #

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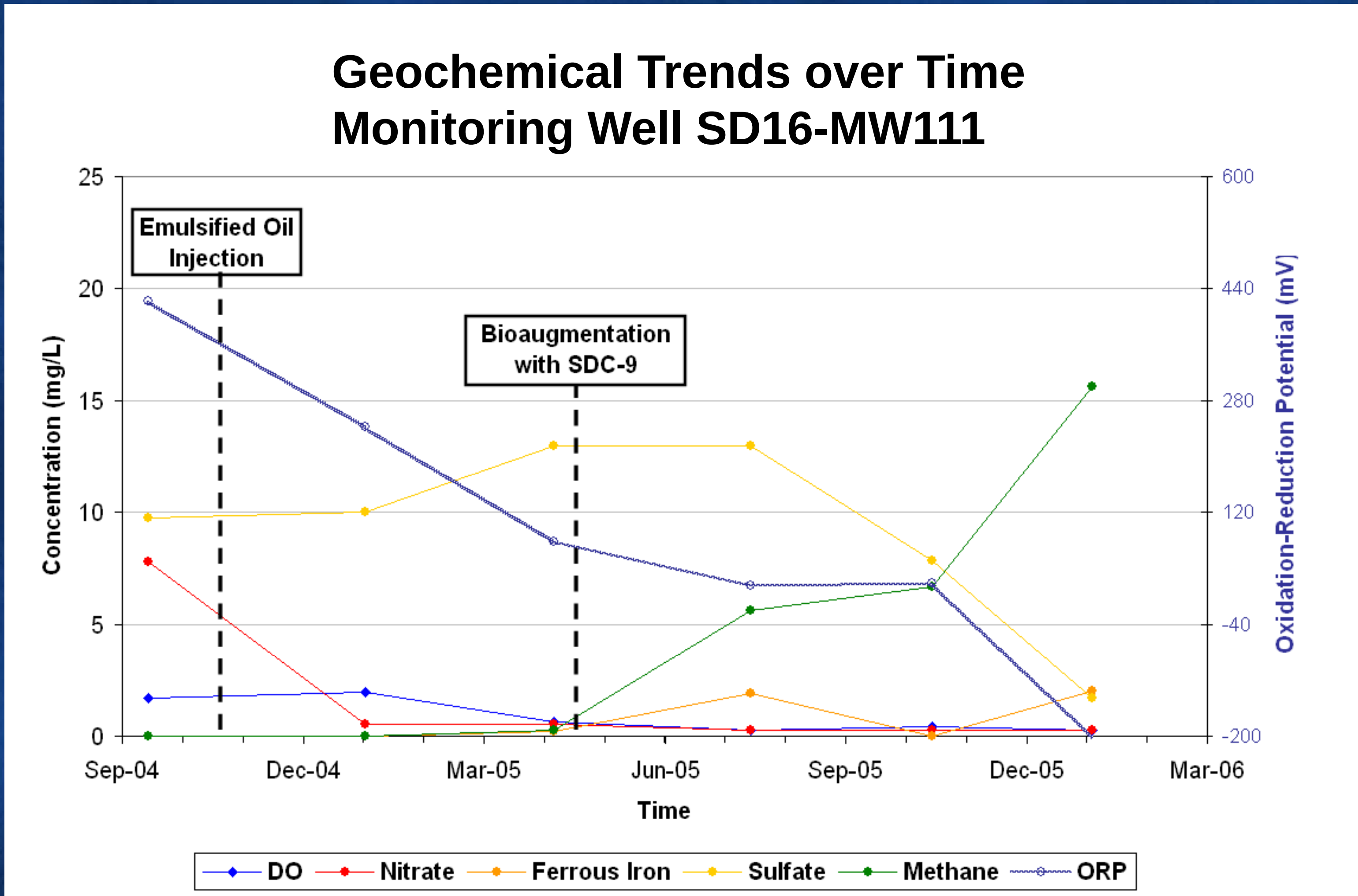
Flightline Storm Drain Outfall & Mission Lake (SD-16) Performance Monitoring Results



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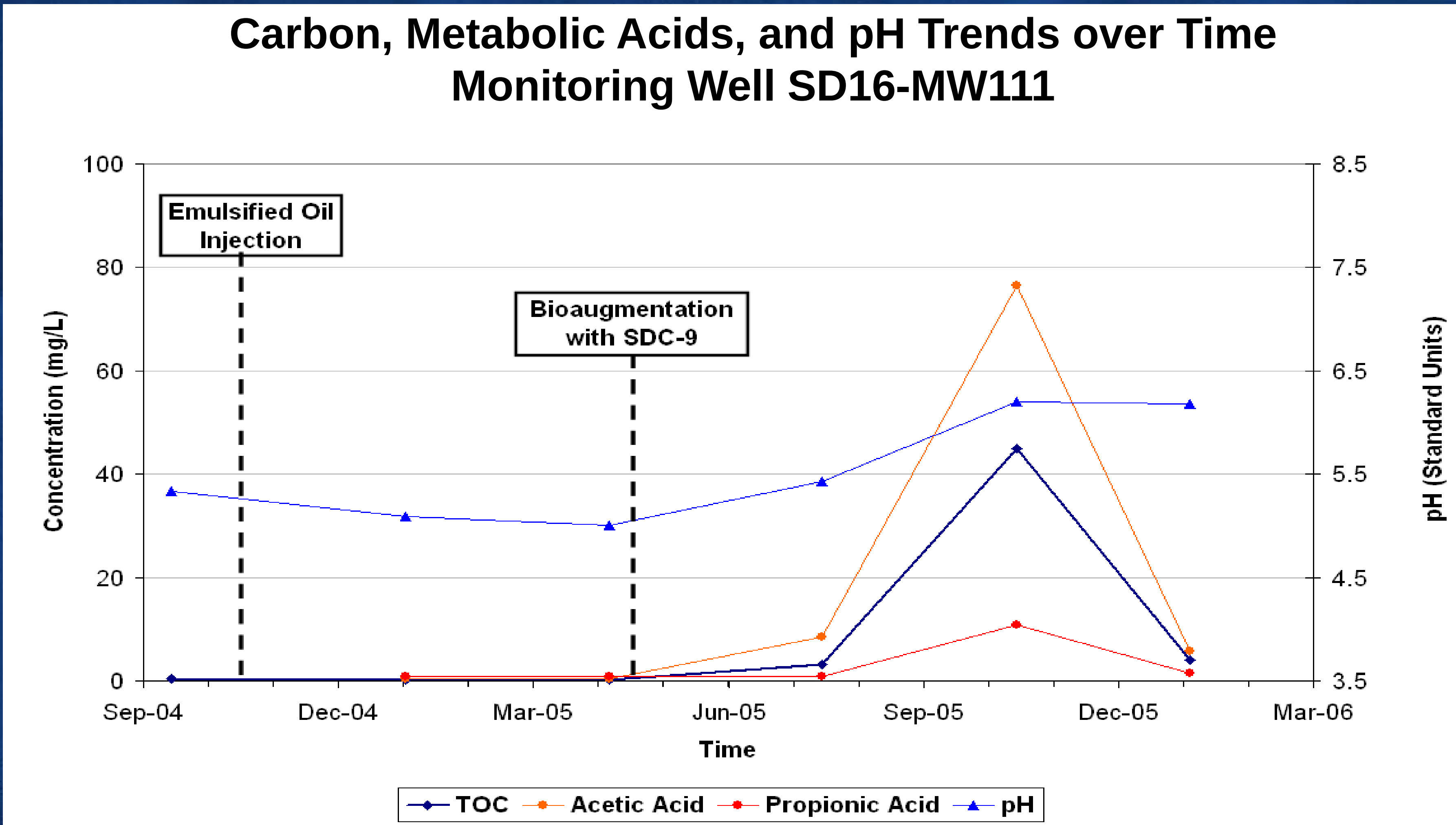
Shaw® a world of Solutions™

Flightline Storm Drain Outfall & Mission Lake (SD-16) Performance Monitoring Results



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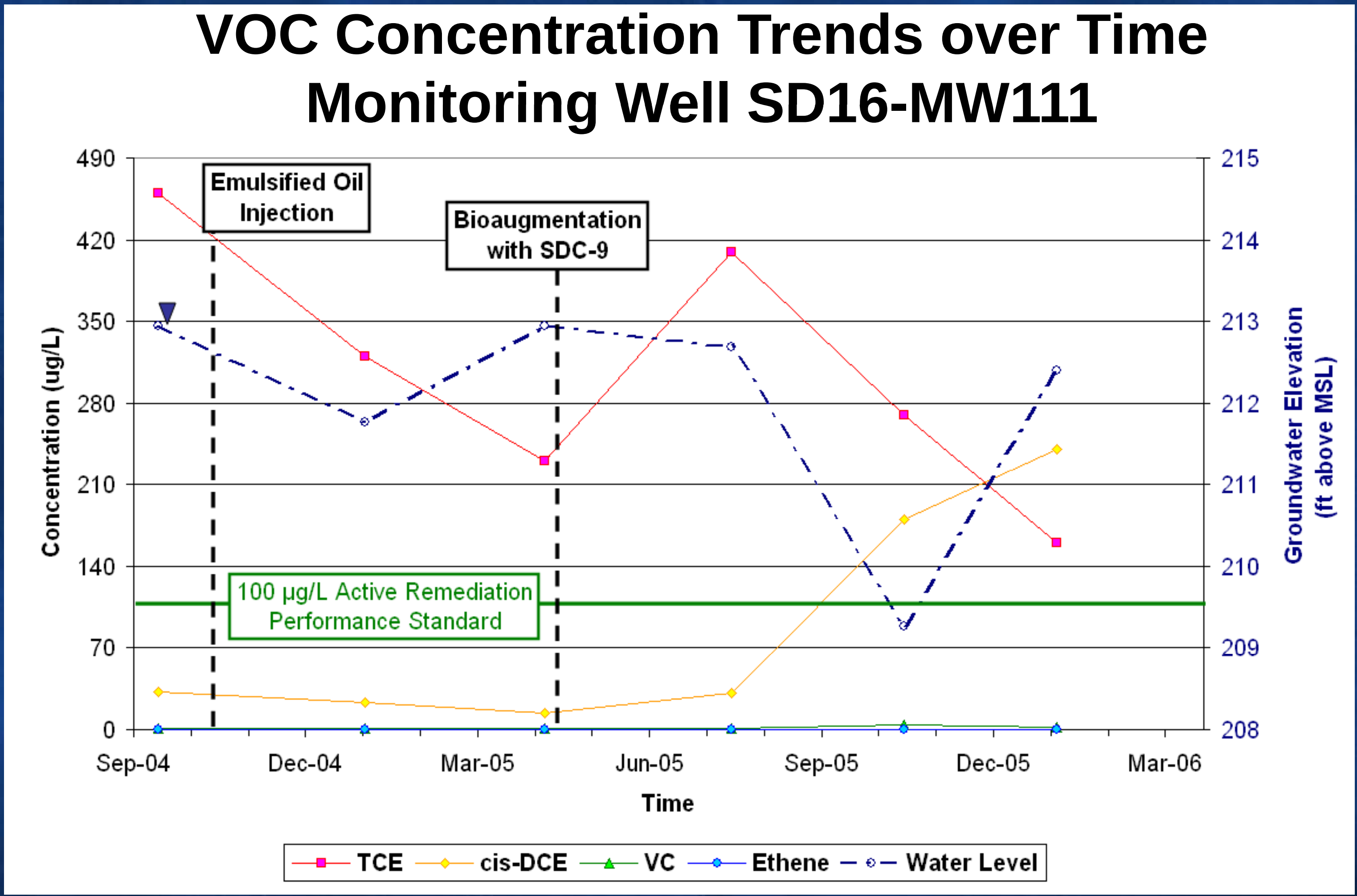
Flightline Storm Drain Outfall & Mission Lake (SD-16) Performance Monitoring Results



Track #

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Flight Line Full Scale

- Recirculation – Horizontal Wells
- Lactate
- Bioaugmentation with SDC-9

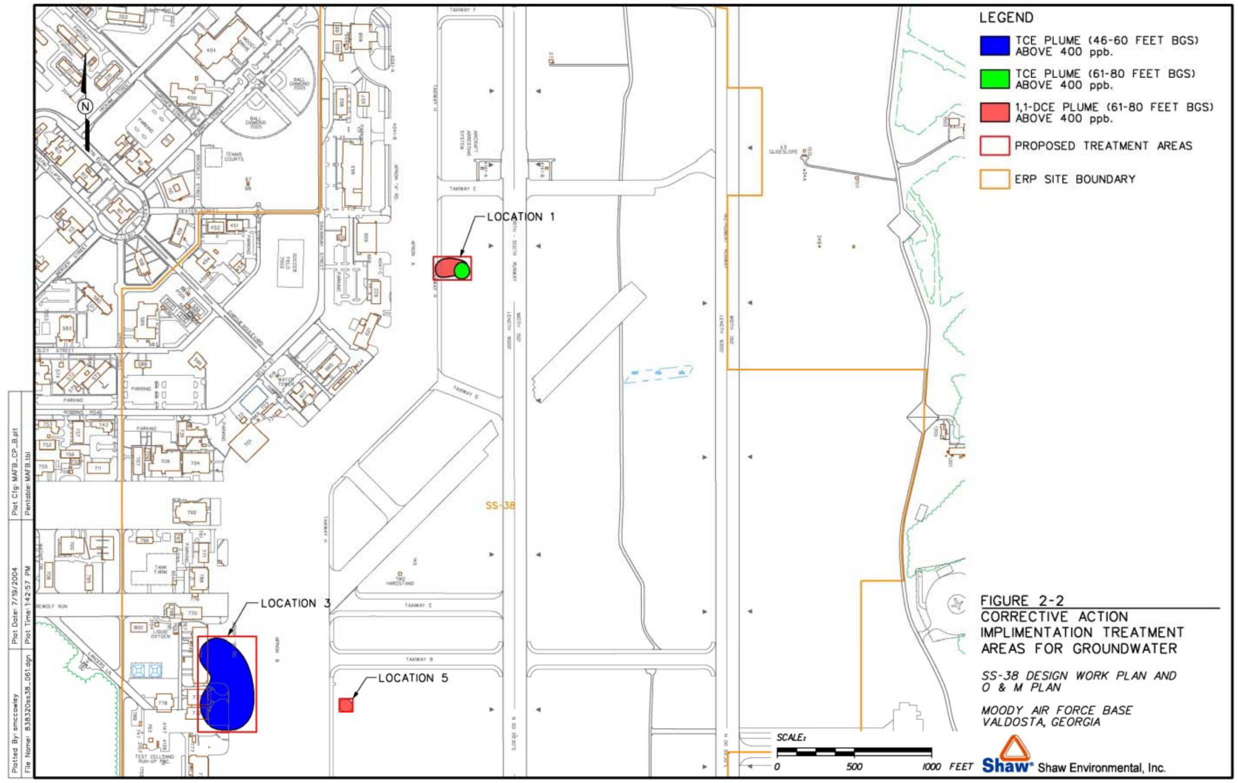
Flightline Area (SS-38)
Full-Scale Anaerobic Bioremediation

Treatment Areas

- TCE and 1,1-DCE > 400 µg/L
- Locations 1 and 5 Passive Distribution
- Location 3 Ground-water Recirculation

•Lactate

•Bioaug. with SDC-9

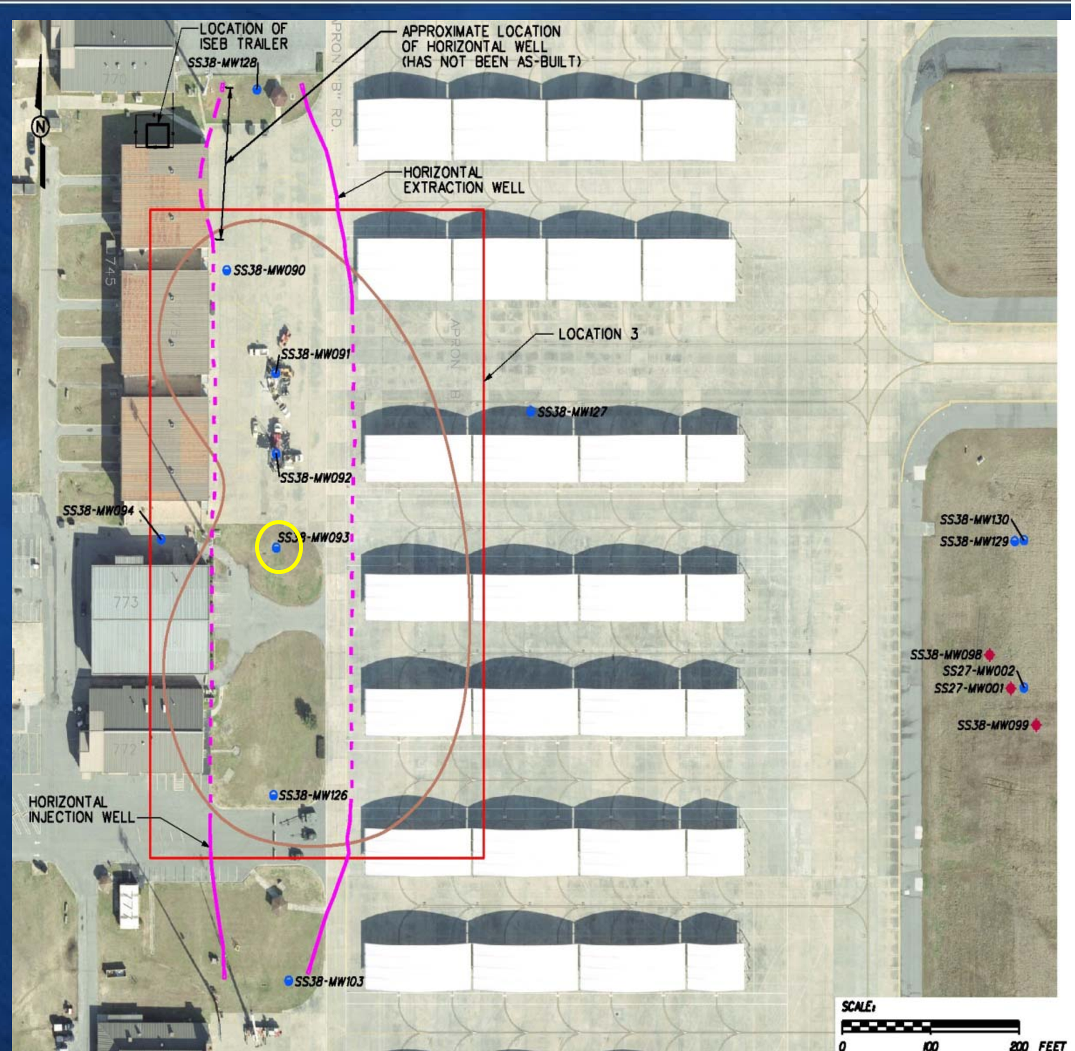


Flightline Area (SS-38)
Anaerobic Bioremediation w/ Groundwater Recirculation

Location 3 Treatment Area

- 1 Horizontal Injection Well
- 1 Horizontal Extraction Well
    - 700 ft, 500 ft. screened
- Carbon Source – Lactate
- Bioaugmentation – SDC-9
    - 925 L



Track #

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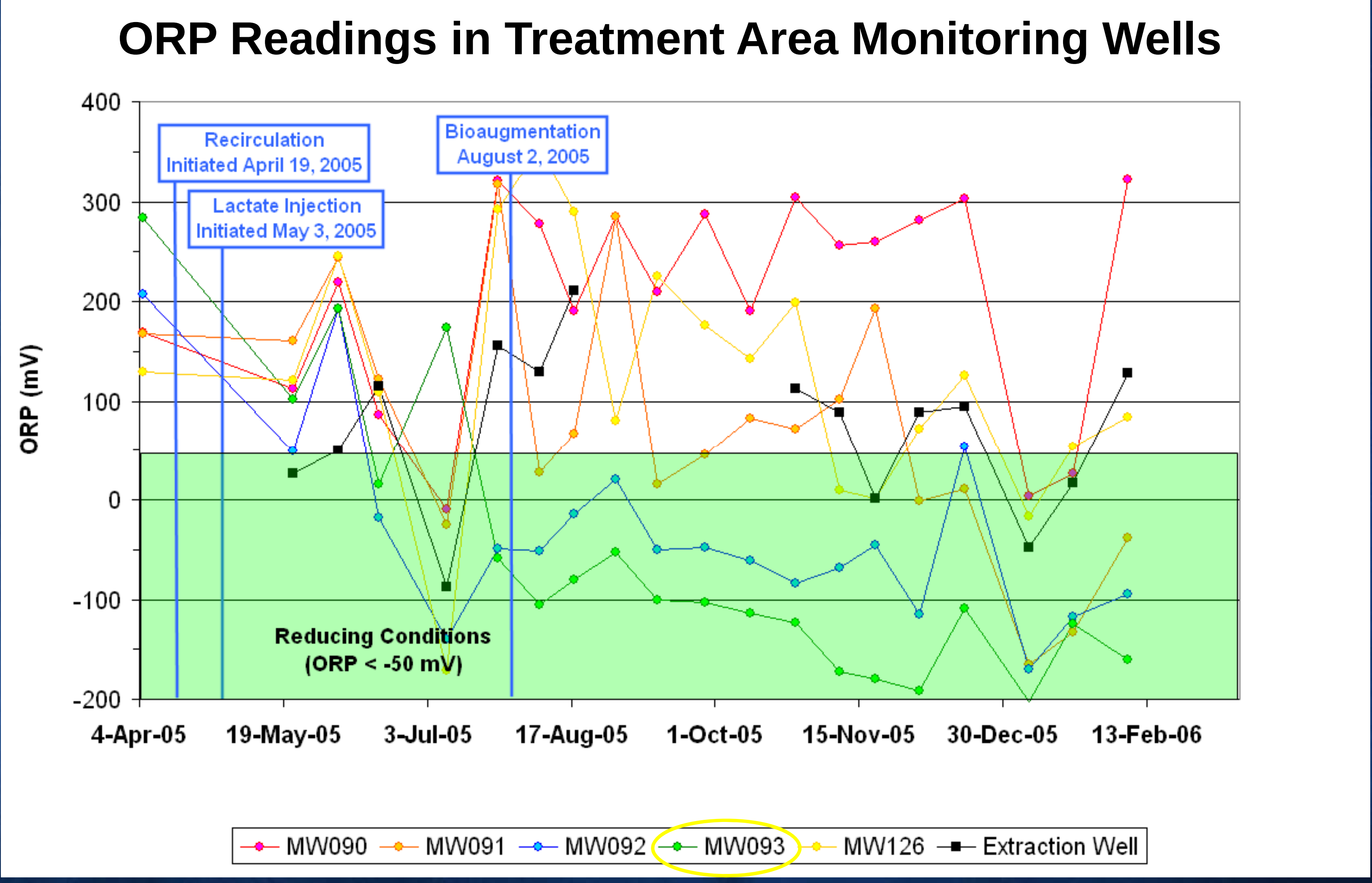
Flightline Area (SS-38)
Performance Monitoring Results



Track #

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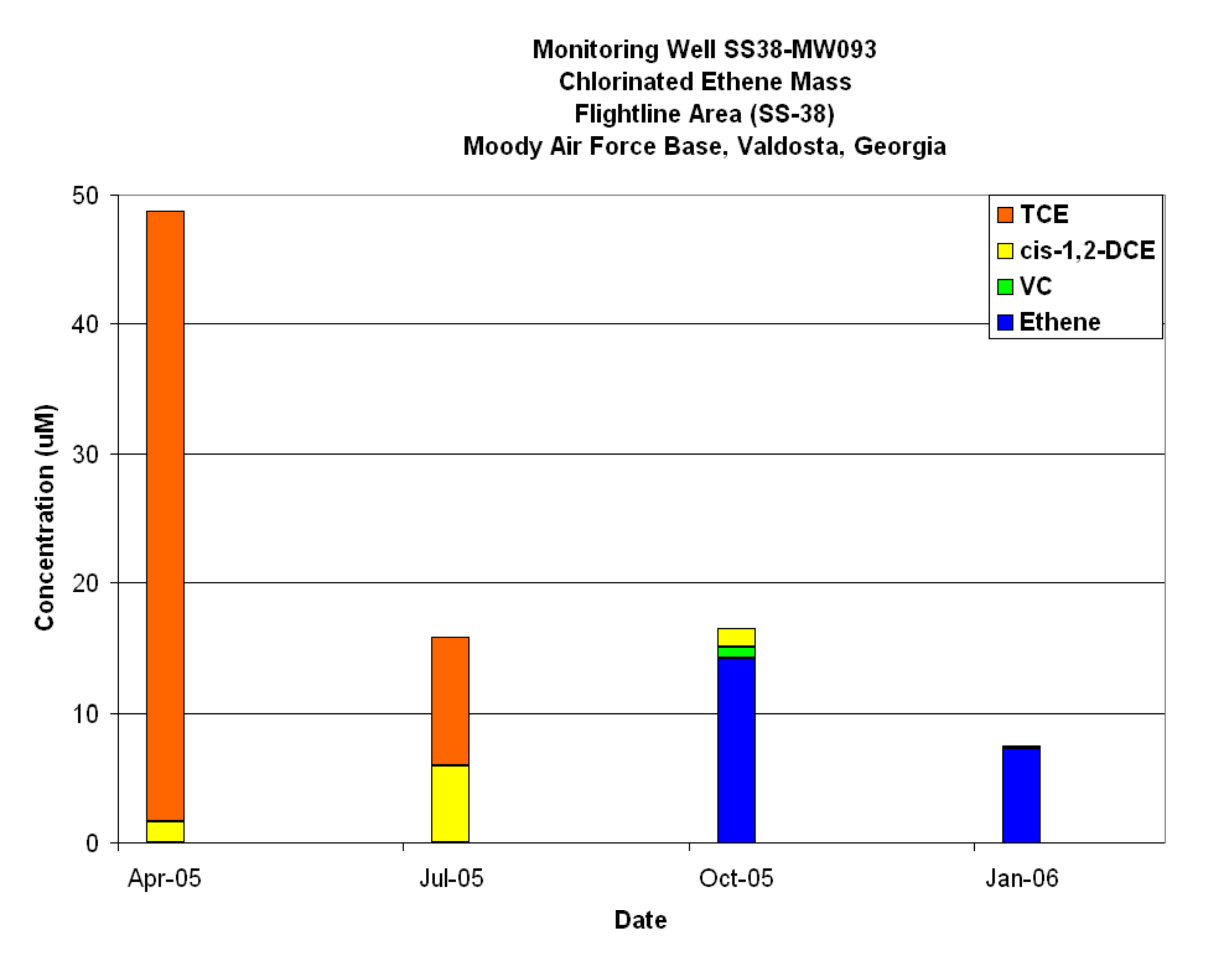
Flightline Area (SS-38)
Monitoring Well SS38-MW093 Results



Track #

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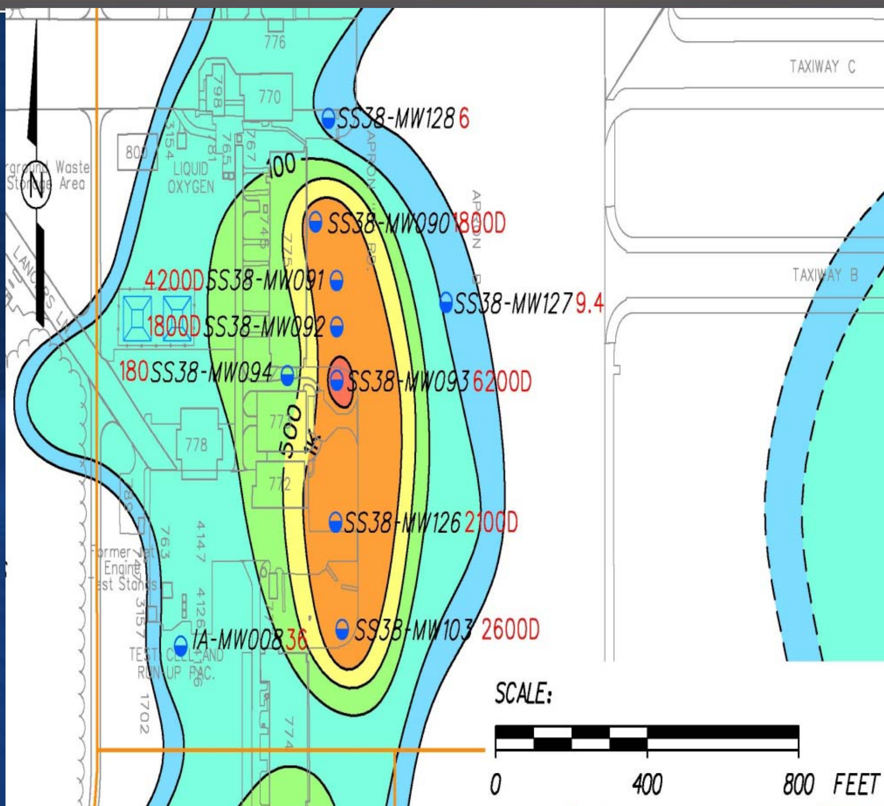
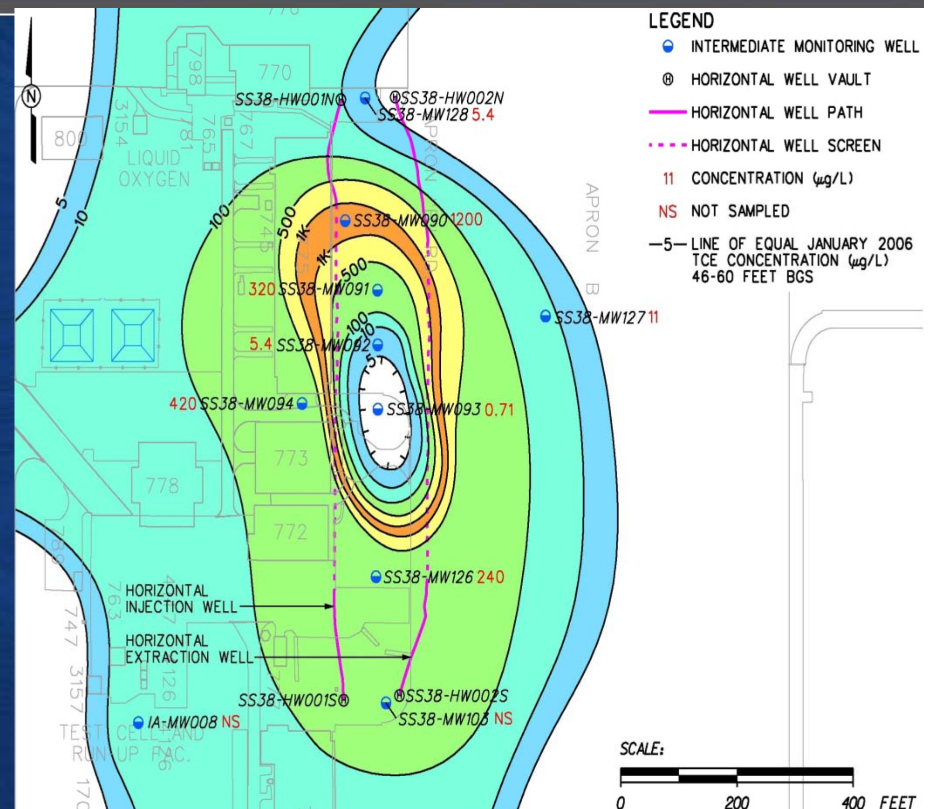
Flightline Area (SS-38)
Performance TCE Monitoring Results



Base line
April 2005



January 2006
9-months post system start-up

System currently shut down – No further action required

Track #

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Active versus Passive Distribution

Site	Anaerobic Conditions	Daughter Products	Adequate Distribution
SS-39 (active)	< 3 months	< 3 months	Yes
FT-07 (passive)	NA	3-6+ months	OK
SD-16 (passive)	3-6 months	9-12 months	Yes
SS-38 (active)	< 3 months	< 3 months	Yes
SS-38 (passive)	3-6 months	9-12 months	Yes
LF-04 (passive)	Performance Monitoring Data Not Yet Available		

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).

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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 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.

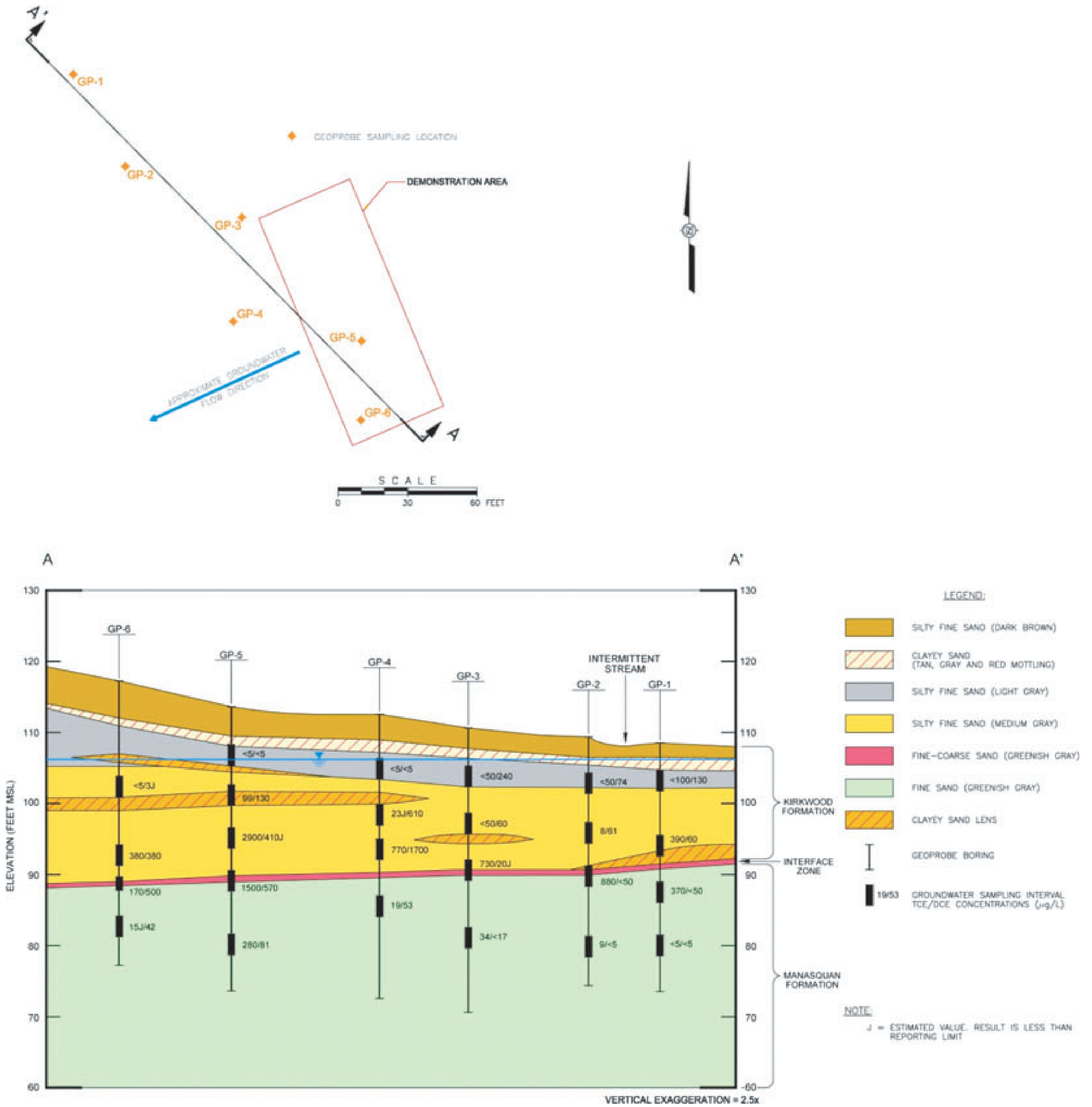

Figure 1. Demonstration area's geologic cross section and contaminant distribution.

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.

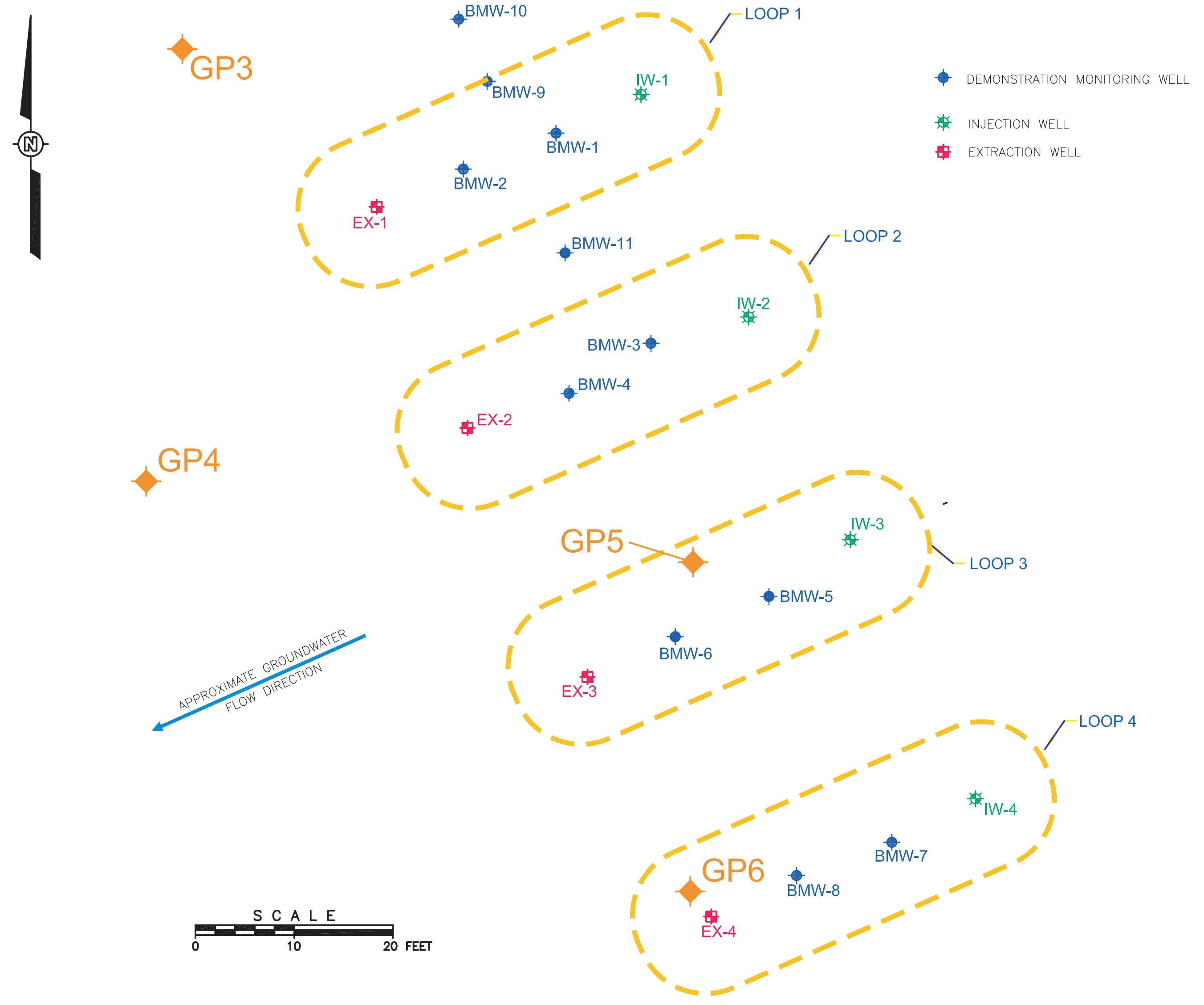

Figure 2. Demonstration layout.

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

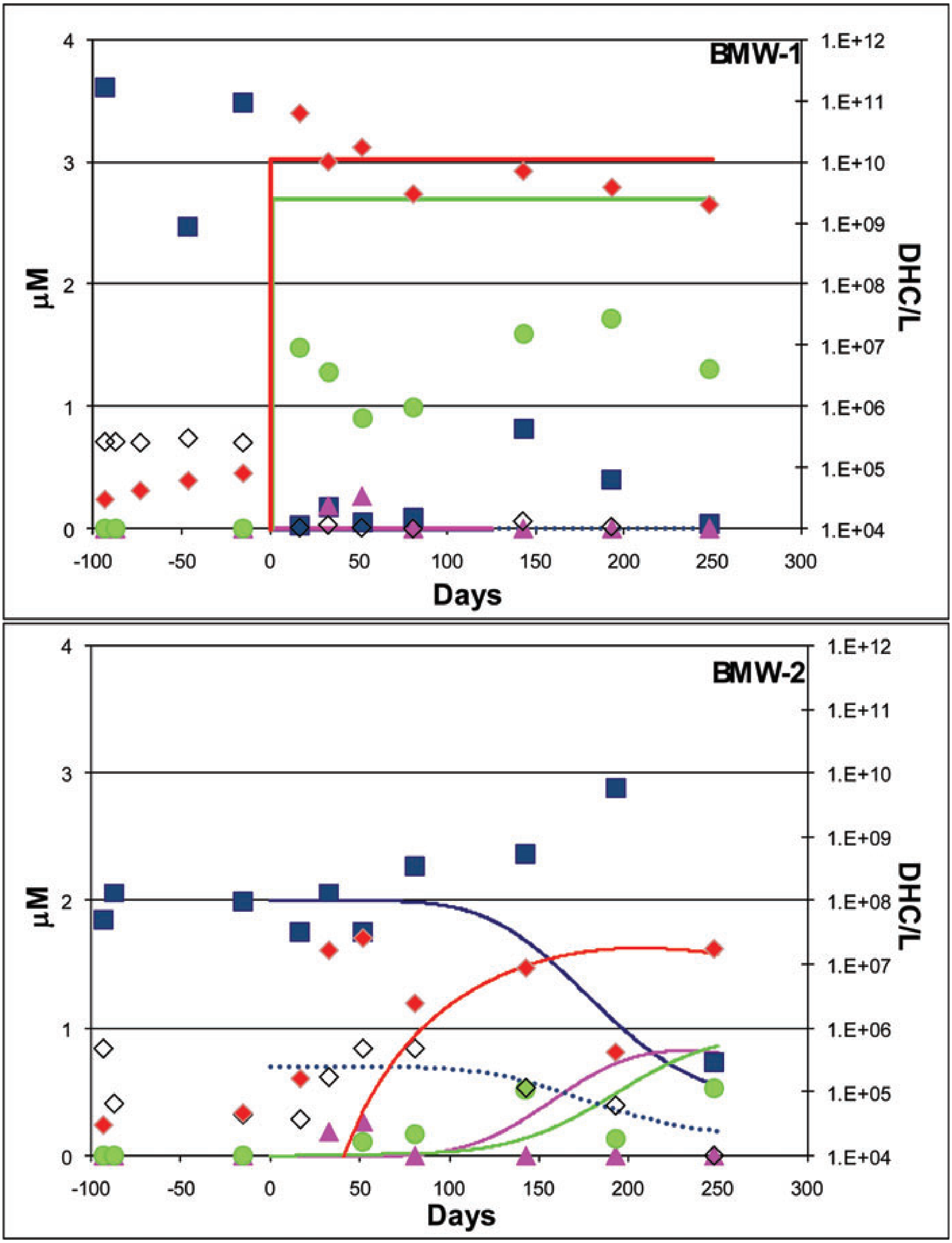

Figure 3. Ethenes and DHC concentrations plotted as a function of time for loop 1. Bioaugmentation was performed at 0 days. ◊, TCE; ■, DCE; ▲, vinyl chloride; ●, ethene; ◆, DHC. Solid and dotted lines represent corresponding model simulations. Simulated DHC concentrations in the bioaugmentation injection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

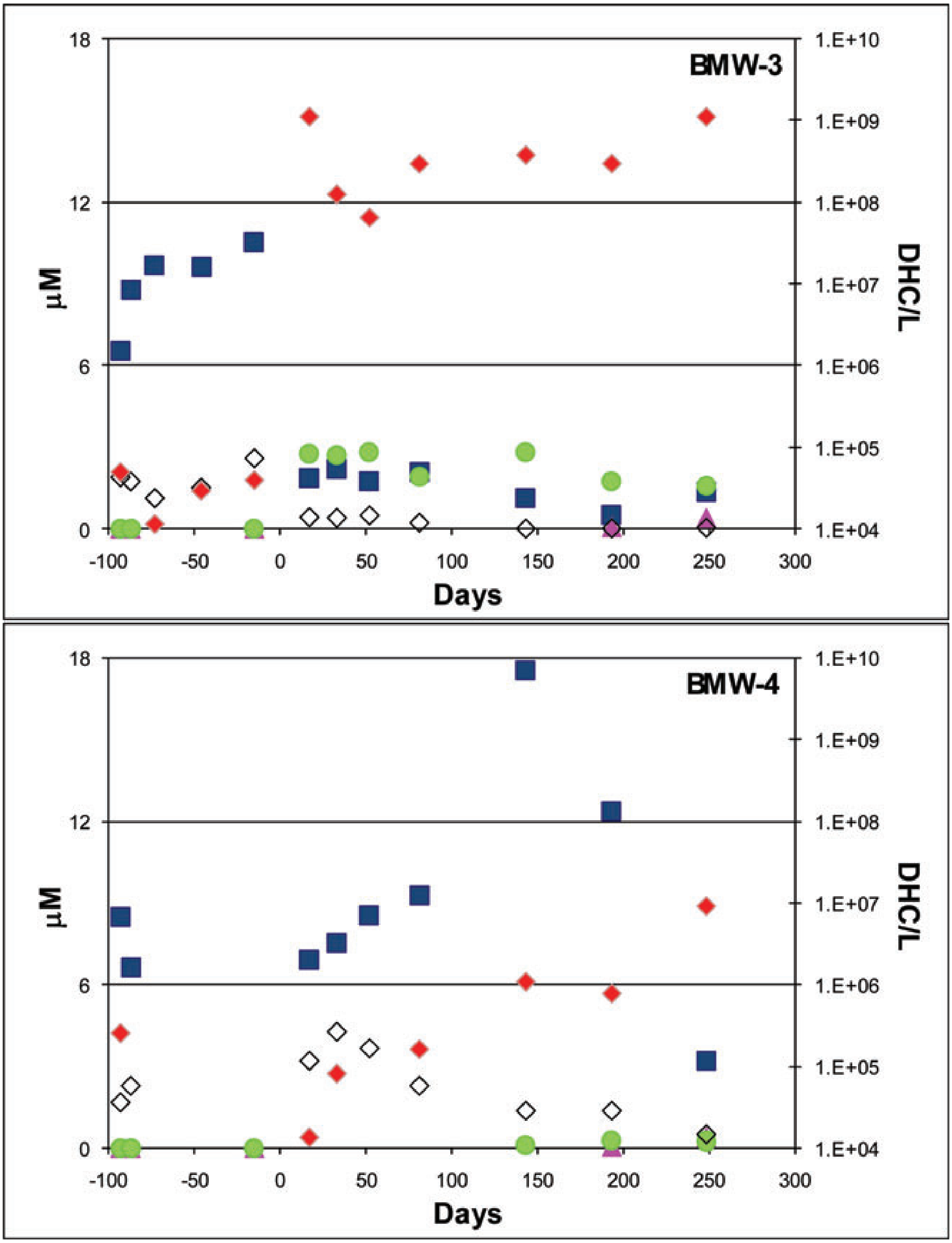

Figure 4. Ethenes and DHC concentrations plotted as a function of time for loop 2. Bioaugmentation was performed at 0 days. ◊, TCE; ■, DCE; ▲, vinyl chloride; ●, ethene; ◆, DHC.

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

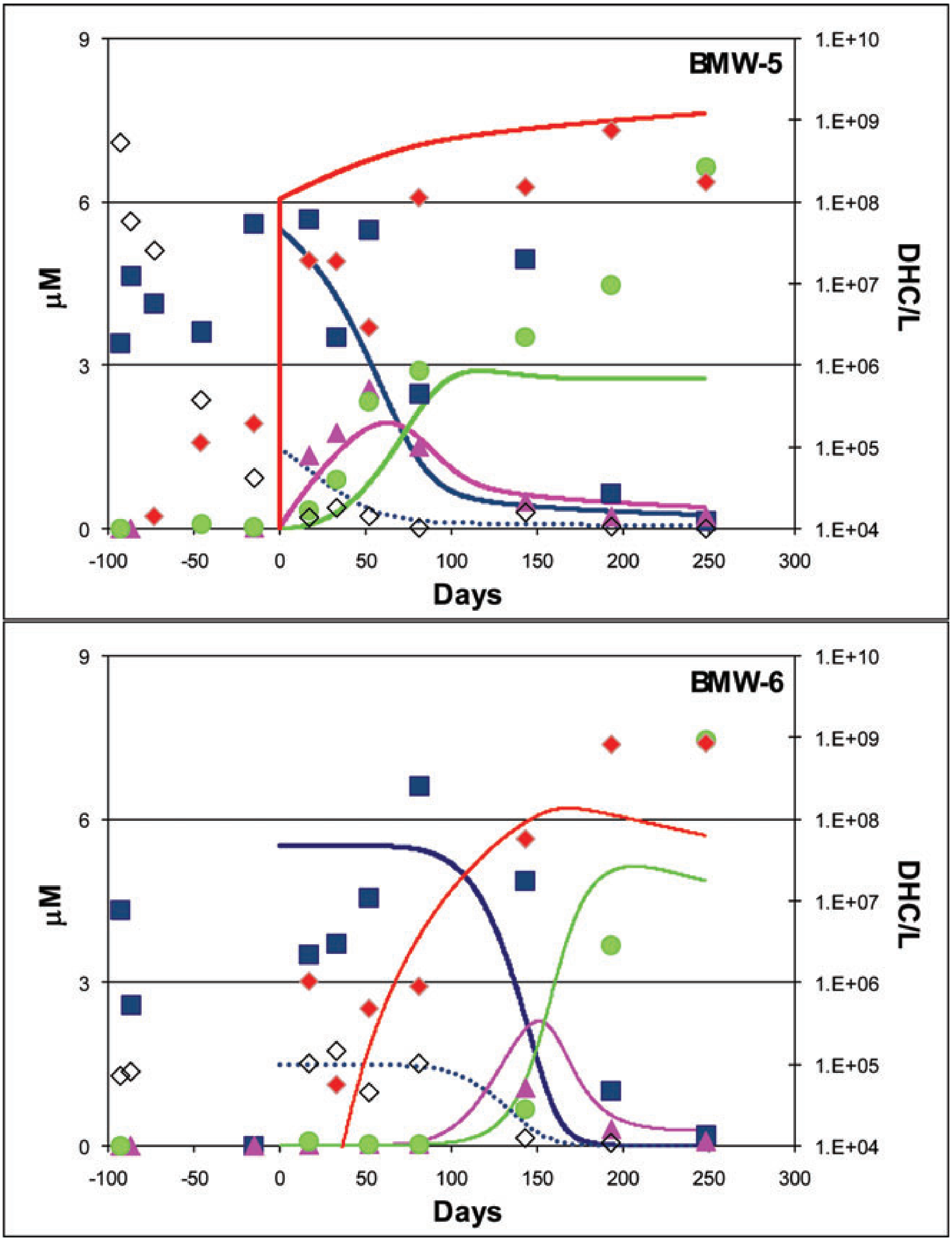

Figure 5. Ethenes and DHC concentrations plotted as a function of time for loop 3. Bioaugmentation was performed at 0 days. ◊, TCE; ■, DCE; ▲, vinyl chloride; ●, ethene; ◆, DHC. Solid and dotted lines represent corresponding model simulations. Simulated DHC concentrations in the bioaugmentation injection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

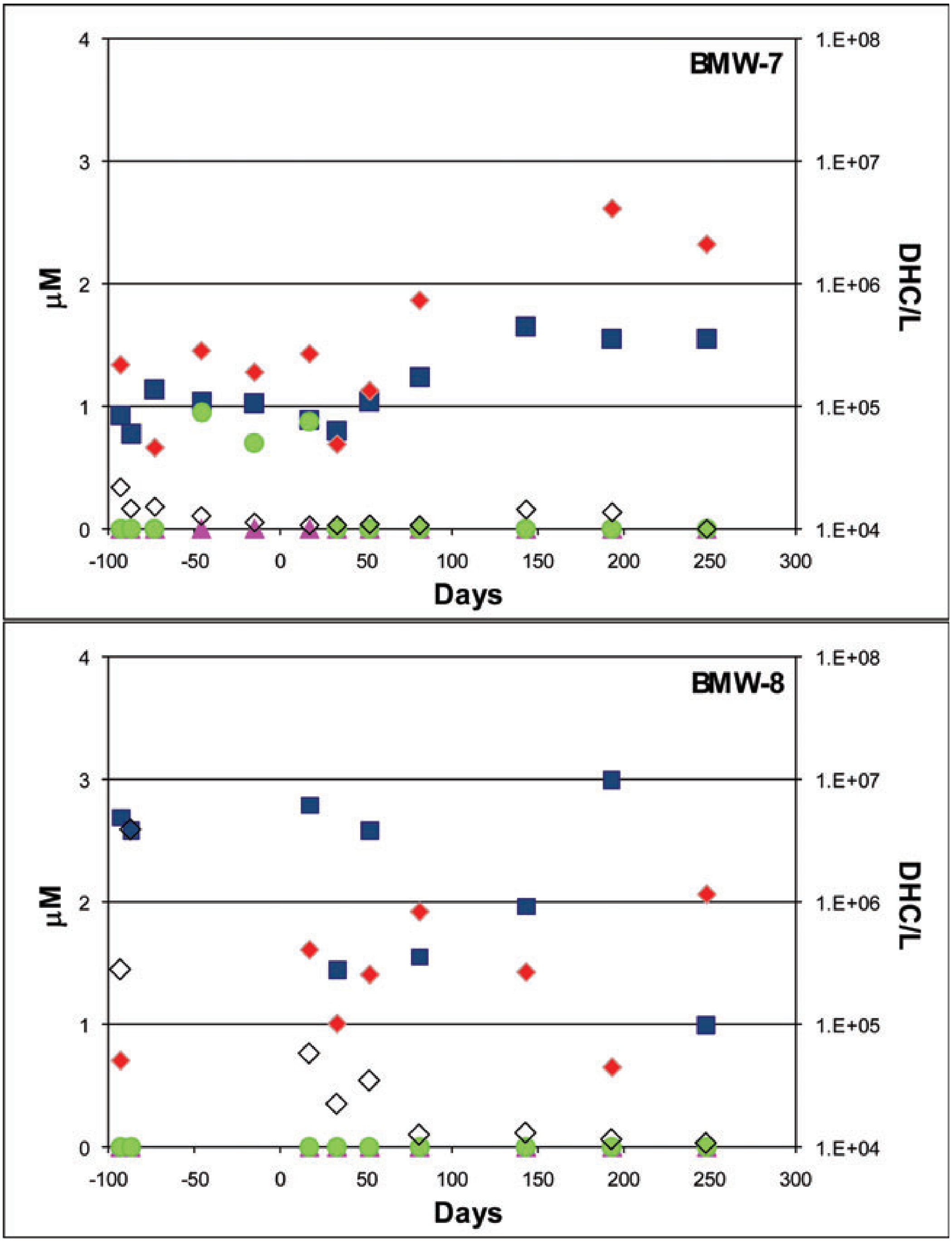

Figure 6. Ethenes and DHC concentrations plotted as a function of time for loop 4 (control loop). Bioaugmentation was performed at 0 days. ◊, TCE; ■, DCE; ▲, vinyl chloride; ●, ethene; ◆, DHC. No detection of vinyl chloride or ethene were observed.

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

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Supporting Information

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

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Bioaugmentation for Treatment of Dense Non-Aqueous Phase Liquid in Fractured Sandstone Blocks

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Laboratory experiments were performed in discretely fractured sandstone blocks to evaluate the use of bioaugmentation to treat residual dense non-aqueous phase liquid (DNAPL) tetrachloroethene (PCE). Significant dechlorination of PCE and growth of *Dehalococcoides* spp. (DHC) occurred within the fractures. DNAPL dissolution was enhanced during bioaugmentation by up to a factor of approximately 3.5, with dissolved PCE concentrations at or near aqueous solubility. The extent of dechlorination and DNAPL dissolution enhancement were dependent upon the fracture characteristics, residence time in the fractures, and dissolved concentration of PCE. No relationship was observed between planktonic DHC concentrations exiting the fracture and the observed extents of PCE dechlorination and DNAPL dissolution. Measured planktonic DHC concentrations exiting the fracture increased with increasing flow rate and bioaugmentation dosage, suggesting that these parameters may be important for distribution of DHC to treat dissolved chlorinated ethenes migrating downgradient of the DNAPL source. Bioaugmentation dosage, for the DHC dosages and conditions studied, did not have a measurable impact on DNAPL dissolution or dechlorination within the fractures themselves. Overall, these results indicate that bioaugmentation may be a viable remedial option for treating DNAPL sources in bedrock.

Introduction

Bioaugmentation for treatment of tetrachloroethene (PCE) and trichloroethene (TCE) in groundwater has been successfully applied at numerous sites. Bioaugmentation for chlorinated ethenes typically involves the subsurface delivery of mixed anaerobic cultures that contain *Dehalococcoides* spp. (DHC) that can reductively dechlorinate the chlorinated ethenes; DHC are the only bacteria known to completely dechlorinate PCE and TCE (*1*).

While several laboratory and field studies have demonstrated the effectiveness of bioaugmenting with DHC for treating dissolved phase PCE and TCE (*2*−*5*), the use of this approach for treating PCE or TCE when present as a dense non-aqueous phase liquid (DNAPL) has received far less attention. Treatment of DNAPL source areas has increasingly become a focus at many contaminated sites (*6*−*8*). Batch and column studies have indicated that the presence of PCE DNAPL can have an inhibitory effect on the reductive dechlorination of PCE during bioaugmentation (*9*−*11*). Adamson et al. (*10*) noted the accumulation of TCE and *cis*-1,2-dichloroethene (DCE) in the DNAPL source zone, without further dechlorination to vinyl chloride (VC) or ethene until PCE concentrations decreased to approximately 10 μM.

Despite this apparent inhibitory effect of DNAPL on the reductive dechlorination of PCE, bioaugmentation has been shown to enhance the rate of PCE DNAPL dissolution in sand columns and flow cells by factors ranging from approximately 1.1 to 21 (*11*−*13*); enhancement rates generally were on the high end of this range when the dissolved concentration of PCE was less than approximately 300 μM (*11, 13*). Other factors shown to impact DNAPL dissolution enhancement and overall PCE dechlorination during bioaugmentation include microbial dechlorination kinetics, water velocity (which impacts residence time and shear stress), electron donor supply, bioclogging, pH, and DNAPL saturation and architecture (*13*−*17*). Becker and Seagren (*15*) also show that non-DHC partial dechlorinators can play an important role in DNAPL dissolution.

While prior studies have provided insight into DNAPL dissolution processes during bioaugmentation in unconsolidated media, published studies that evaluate similar mechanistic processes of PCE DNAPL dissolution in fractured bedrock systems currently are lacking. Several recent experimental and theoretical studies have focused on evaluating abiotic DNAPL dissolution in bedrock fracture systems (*18*−*20*), and DNAPL dissolution in fractures during implementation of chemical oxidation (*21*). Similar studies evaluating DNAPL dissolution in bedrock fractures during bioaugmentation are needed.

The objective of this study was to quantify the extent to which bioaugmentation could enhance PCE DNAPL dissolution in discretely fractured sandstone blocks. PCE DNAPL dissolution enhancement, dechlorination kinetics, and microbial growth and transport were evaluated in four fracture systems, and bioaugmentation effectiveness was evaluated for various DNAPL saturations, water velocities, and DHC inoculation dosages. Results of this study provided insight into the mechanisms controlling DNAPL dissolution in bedrock fractures during bioaugmentation.

Experimental Section

Materials. Sandstone blocks were used to create bench-scale fracture systems (two Colorado Red (C1 and C2) and two Arizona Buff (A1 and A2) sandstones). Construction and characterization of these systems, which were used for the bioaugmentation experiments presented herein, have been previously described (*20*). Briefly, a discrete fracture was created in each block (29 cm ×29 cm ×5 cm) along naturally occurring bedding planes. The outside edges of the fracture were sealed with epoxy. Twelve holes (0.20 cm diam.) were drilled approximately 5 mm into the rock along the influent edge; 28 holes of similar size and depth were drilled along the effluent fracture edge. Stainless steel needles (16G) were inserted into each hole and connected to an influent and effluent manifold. The fracture apparatus is presented in the Supporting Information (Figure S1). Fracture properties for each experimental fracture system are shown in Table 1.

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TABLE 1. Fracture Properties for Each Rock, As Calculated by Schaefer et al. (*20*)

rock fracture	fracture volume (cm^3)	fracture aperture (cm)	DNAPL residual saturation (cm^3/cm^3)	DNAPL-water interfacial area (cm^2/cm^3)
C1	31	0.038	0.18[a]	21
C2	32	0.039	0.19[a]	48
A1	45	0.054	0.39	56
A2	82	0.098	0.43	20

[a] Previously measured at 0.24 and 0.21 for C1 and C2, respectively, by Schaefer et al. (*20*)

Artificial groundwater was prepared using deionized water amended with the following reagent grade chemicals purchased from Sigma-Aldrich (St. Louis, MO): 180 mg/L $NaSO_4$, 113 mg/L NaCl, 50 mg/L $NaHCO_3$, 1.0 mg/L $MnSO_4 \cdot H_2O$, and HCl for a final pH of approximately 6.5. PCE was purchased from Sigma Aldrich (St. Louis, MO). Sodium-(L)-lactate (60% solution) was purchased from Purac America (Lincolnshire, IL). Yeast extract (bacteriological grade) was purchased from Marcor Development Corp. (Carlsdtadt, NJ). The DHC-containing consortium used in this study for bioaugmentation was the commercially available SDC-9 ((*22*); Shaw Environmental, Inc., Lawrenceville, NJ). Additional details on the SDC-9 culture are provided in the Supporting Information.

Batch Experiments. Batch reactor experiments were performed to evaluate PCE dechlorination kinetics by DHC in the presence of PCE DNAPL. Experiments were performed by adding 6 L of artificial groundwater to each of two nitrogen sparged 6.5 L autoclavable bioreactors (Applicon, Inc., Foster City, CA). Approximately 10 mL of PCE was added to each reactor. Each reactor also was amended with sodium lactate (final concentration of 1,000 mg/L), yeast extract (final concentration of 300 mg/L), and DHC (10^9 cell/L). The reactors were continuously mixed (250 rpm) and maintained at room temperature (~21 °C). The reactors were operated for 50 days, with periodic sampling for chlorinated ethenes, reduced gases, anions, and volatile fatty acids. For anions and volatile fatty acids, 1 mL aqueous samples were collected and filtered (0.25 μm) prior to analysis. For chlorinated ethenes and reduced gases, 5 mL aqueous samples were collected, preserved with acid, and equilibrated in a 14 mL glass serum bottle (with septa) prior to headspace analysis.

An additional batch experiment was performed similar to those described by Schaefer et al. (*5*) to evaluate microbial PCE dechlorination at elevated dissolved concentrations, but in the absence of DNAPL. Glass serum bottles (13 mL) with Teflon-lined rubber septa were prepared in duplicate with 13 mL artificial groundwater and dissolved PCE (final concentration of approximately 0.95 mM). The bottles were inoculated with bacteria to attain a final concentration of approximately 5×10^{11} DHC/L. Sodium lactate was added for a final concentration of 1,000 mg/L. A parallel set of controls were prepared without bacteria or lactate. Periodic sampling for chlorinated ethenes and reduced gases was performed.

Bioaugmentation Experiments. Bioaugmentation experiments on the fractured sandstones were performed after residual PCE DNAPL saturation was attained in the rocks. Procedures for attaining residual DNAPL saturation in the rocks have been previously described (*20*). Briefly, residual PCE DNAPL saturation was obtained by flooding the water saturated rock with PCE, followed by water flushing the fracture at a high capillary number until no further PCE DNAPL was displaced. The residual DNAPL saturation (DNAPL volume divided by the fracture volume) for each rock is provided in Table 1. The seven sandstone bioaugmentation experiments are summarized in Table 2.

After attaining residual DNAPL saturation, artificial groundwater was amended with 500 mg/L sodium lactate, 300 mg/L yeast extract, and 200 mg/L diammonium phosphate. The artificial groundwater solution used in the bioaugmentation experiments was prepared in an anaerobic chamber (Coy Laboratory Products, Inc., Model AALC) and transferred to Tedlar bags with single polypropylene fittings (SKC, Inc.) to serve as influent reservoirs for the fractures. The Tedlar bags were connected to a Whatman filter device (Polycap 36 HD disposable filter capsule, MAPP filter media with polypropylene housing, 10 μm pore size) with Viton tubing and delivered to the fractured rocks at the selected flow rates (0.02 to 0.2 cm^3/min) using piston driven high performance metering pumps. Resultant groundwater velocities in the fractures (Table 2) were within ranges observed under natural conditions in conductive bedrock fractures (*23*, *24*).

After flushing the fracture with this anaerobic solution for a minimum of 2 days, bioaugmentation with DHC was performed. Five milliliters of the desired inoculation dosage was delivered to the fractures using a syringe pump at a flow rate of 0.1 mL/min. After the DHC inoculation, delivery of the lactate/yeast extract/diammonium phosphate solution from the Tedlar bags recommenced.

The effluent artificial groundwater was drip-collected in a 10 mL plastic tube for analysis of pH, anions, DHC, and volatile fatty acids. This collection process limited any further reductive dechlorination by exposing the DHC to atmospheric air (*22*). Lack of additional dechlorination during sample collection and storage was verified by analyzing for chloride at 1, 2, and 4 days and observing no increasing trend in chloride concentration.

For analysis of chlorinated ethenes and reduced gases in the effluent, effluent lines from the fracture effluent manifold were connected to a 3 mL sample vial that was crimp sealed with Teflon-lined butyl rubber septa. The sample vials were configured so the effluent exited approximately 1 cm above the fracture to prevent gravity drainage of liquid from the fractures. Effluent flowed through the trap and exited via a 20 gauge needle (Supporting Information, Figure S1). The sample vial was amended with 15 μL of 18% hydrochloric acid approximately 30 min prior to collection to maintain the pH less than 2, thereby inhibiting the dechlorination activity of the DHC (*22*). Contents of the vials were then transferred to 5 mL glass vials with Teflon-lined septum screw caps (containing an additional 15 μL of 18% hydrochloric acid) for headspace analysis. Parallel testing was performed to ensure that this sample collection method did not result in any appreciable volatilization losses. Fractures were flushed sequentially with ethanol, base (0.1 N NaOH), acid (0.1 N H_2SO_4), and artificial groundwater after each experiment to remove remaining DNAPL and biomass.

Experiment 7 (C1) was performed to evaluate the impacts of flow rate on effluent DHC concentrations, and to verify that injected DHC were not rapidly eluting through the fracture prior to collecting effluent samples. All fracture effluent was continuously containerized in glass beakers over a 10 day period following inoculation. The flow rate was increased after 29 days to assess the impact of velocity on DHC elution. Effluent monitoring of chlorinated ethenes, reduced gases, anions, or volatile fatty acids was not performed for this experiment.

Analytical Procedures. Chlorinated ethenes and reduced gases concentrations were determined via headspace analysis using a Varian 3900 gas chromatograph equipped with a Flame Ionizing Detector (FID) and Aluminum RT column. Aqueous concentrations were determined by applying Henry's Law. Hydrogen headspace analysis was performed on a Varian 3800 GC equipped with a Valco Pulsed Discharge Helium Ionized Detector (PDHID) and tandem Pora Bond

TABLE 2. Experimental Design for the Bedrock Fracture Bioaugmentation Experiments[a]

experiment	rock	DHC iInoculation (cells DHC)	flow velocity (cm/min)	dissolution enhancement factor (eq 1)
1	A1	3×10^6 (day 0) 8×10^8 (day 91)	0.10	1.1
2	A2	3×10^6 (day 0) 8×10^8 (day 91)	0.062	5.0
3	A1	4×10^8	0.10 (decreased to 0.025 on day 13)	3.5
4	A2	4×10^8	0.062 (decreased to 0.015 on day 13)	3.4
5	C1	2×10^9	0.11 (decreased to 0.022 on day 37)	1.4
6	C2	2×10^9 2×10^{11} (day 58)	0.11 (decreased to 0.022 on day 37)	1.3
7	C1	4×10^8	0.044 (increased to 0.22 on day 29)	not calculated

[a] Flow velocity is calculated by dividing the influent volumetric flow rate by the fracture aperture (calculated by bromide tracers (*20*)), the fracture width (29 cm), and the water-filled porosity. Dissolution enhancement factors were calculated at the end of the experiment, or the end of active bioaugmentation.

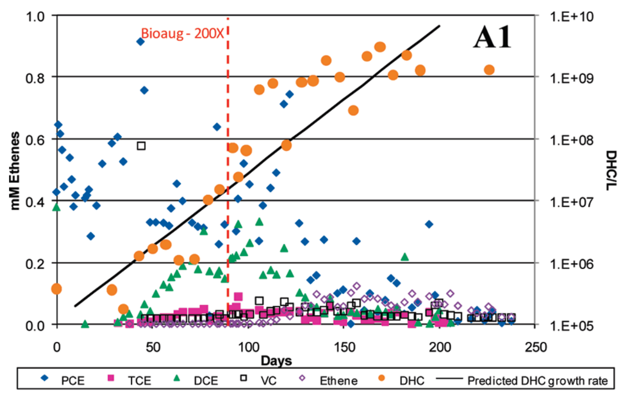
Q and Molesieve columns. Volatile fatty acid analysis was performed on a Dionex AS 50 Chromatograph equipped with a Dionex IonPac AS11-HC column and CD 25 Conductivity Detector. Anions were analyzed via ion chromatography (Dionex DX-120, Sunnyvale, CA). The effluent pH was monitored periodically with pH test strips. Aqueous DHC concentrations were determined by quantitative real-time PCR with primers (5′- gaagtagtgaaccgaaagg and 5′- tctgtc-cattgtagcgtc) that amplified a 235bp fragment of the 16s rRNA gene of DHC-type organisms. Biomass was concentrated via centrifugation at 3700 rpm for a minimum of 1 h. Our previous work (*5*) demonstrated that measurement of DHC for the culture used in this study was sufficient to assess overall dechlorination activity and kinetics.

Results

Batch Experiments. Results of the batch testing showed that no dechlorination daughter products (including chloride) were generated over the duration of the experiment in any experiments that contained PCE DNAPL. In addition, no fermentation of the lactate was observed, and no sulfate reduction occurred in experiments where DNAPL was present. Thus, the presence of the PCE DNAPL exhibited an apparent inhibitory effect not only on the DHC, but on the fermentative and sulfate reducing bacteria present in the consortium. The addition of hydrogen to the batch reactor systems (900 nM final concentration) did not result in any PCE dechlorination. For the batch experiments containing only dissolved PCE, PCE dechlorination to VC occurred within 20 h, with continued transformation to ethene over the next several days.

Results for these batch studies indicated that the presence of PCE DNAPL was inhibitory to PCE dechlorination, which is consistent with the results of others (*9*−*11*). The lack of PCE dechlorination in the presence of DNAPL could be due to toxicity from the sustained elevated dissolved PCE concentration from the dissolution of the DNAPL, and/or solvation of the DHC into the non aqueous phase.

Fracture Experiments - General Observations. Prior to bioaugmentation and during delivery of the lactate and yeast extract amended artificial groundwater, no dechlorination was observed, and DHC levels were below the detection limit ($<10^5$ DHC/L). Dissolved PCE concentrations measured prior to bioaugmentation (or within a few weeks after bioaugmenting, but prior to observation of measurable dechlorination) generally were in reasonable agreement (±20%) to the abiotic PCE DNAPL dissolution concentrations previously measured in these same bedrock fractures at the initial flow velocities listed in Table 2 (*20*). The two exceptions were Experiments 1 and 5, where the effluent PCE concentrations varied by up to 40% from those previously measured; this discrepancy may have been due to slight differences in flow



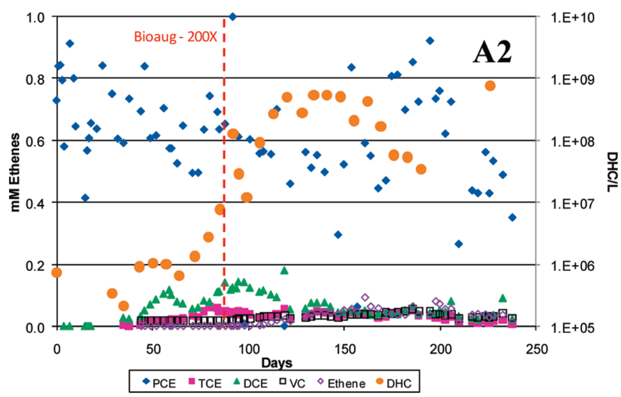


FIGURE 1. Effluent concentrations of ethenes and DHC from rock A1 (top) and A2 (bottom) in Experiments 1 and 2, respectively. Fractures were bioaugmented on Day 0. The vertical dashed lines indicate when inoculation with additional culture was performed. The solid line for A1 represents the predicted rate of DHC growth (*5*). For A2, some interruptions in flow occurred between days 150 and 190. Chloride concentrations were not monitored in Experiments 1 and 2.

paths within the fracture because of introduction of the microbial culture. During all bioaugmentation experiments, effluent pH levels remained at approximately 6.5, and lactate and/or intermediate fermentation products were consistently observed in the fracture effluent indicating that electron donor was present in excess.

Arizona Rocks - Low Flow Rate (Experiments 1 and 2). After inoculating with DHC, monitoring showed that dechlorination of PCE occurred in the presence of DNAPL, with generation of measurable quantities of ethene, despite relatively short fracture residence times (270 and 450 min for A1 and A2, respectively) (Figure 1). DCE initially was the primary dechlorination product in both rocks, but ethene became the predominant dechlorination product at later times. VC and ethene concentrations between days 125 and 195 generally ranged between 0.01 and 0.03 mM. The shift from DCE accumulation to ethene was most apparent in A1, where the DCE to ethene transition correlated with decreases in dissolved PCE concentration to approximately 0.3 mM. These observations suggest that elevated dissolved PCE concentrations limited the further dechlorination of DCE. Similar DCE dechlorination inhibition near PCE DNAPL sources has been observed (*10*).

DNAPL mass removal was estimated by calculating the mass of PCE and daughter products exiting the fracture effluent. Approximately 16% of the residual DNAPL was removed in A1 during the experiment (15% as PCE, 1% as reductive dechlorination products). Approximately 8% of the residual DNAPL was removed in A2 during the experiment, with <1% as dechlorination products (as shown in Table 1, A2 contained approximately twice the DNAPL residual volume as A1). While no observable decrease in effluent PCE concentration was observed in A2, effluent PCE concentrations decreased to approximately 0.01 mM in A1, which represents approximately a 98% decrease in effluent PCE concentration with only a 16% decrease in residual PCE DNAPL. This result suggests that most of the residual PCE residing in A1 was in low permeability regions that were not well contacted by migrating water. After day 195, as PCE concentrations continued to decrease in A1, ethene concentrations increased to approximately twice that of PCE, and VC concentrations increased to approximately equal that of PCE. This observation may suggest that the extent of dechlorination within a PCE DNAPL source zone may be dependent upon the DNAPL source zone strength function (*25*). DNAPL sources in bedrock that have dissolved concentrations that decrease rapidly in response to a relatively small fraction of DNAPL source removal may exhibit greater conversion to VC and ethene in response to bioaugmentation treatment than DNAPL sources that maintain elevated dissolved concentrations until nearly all the DNAPL mass is depleted.

An increasing trend in eluted DHC concentrations in both A1 and A2 was observed, as effluent DHC levels increased by nearly 4 orders of magnitude. The rate of DHC growth observed in the fracture effluent is well predicted by our previously developed DHC Monod kinetic model (*5*), as indicated by the solid line for Experiment 1. This model (*5*) is based on DHC that have been proven capable of complete dechlorination to ethene. These data suggest that the dechlorination and DHC growth in this study are likely because of DHC capable of complete dechlorination. Thus, for the SDC-9 culture and the conditions of this study, the role of any partial dechlorinators likely was limited. It is unclear if adding additional culture had a substantial impact on effluent DHC levels, as effluent DHC levels may have been increasing prior to reinoculation. DHC concentrations in A1 were slightly greater than in A2, possibly because of the lower dissolved PCE concentration in A1 that may have resulted in decreased inhibition/toxicity on DHC growth and subsequent detachment. Integrating the DHC elution curve and multiplying by the flow rate showed that the amount of DHC eluted from A1 and A2 were 28- and 7-times greater (respectively) than the total amount of DHC inoculated, verifying that DHC growth occurred in the fractures. Some flow interruptions occurred in A2 between 150 and 190 days, which might explain the decrease in DHC and increase in PCE within this interval.

The influent solution for both A1 and A2 was switched to aerobic artificial groundwater (without lactate) on day 197; 500 ppm hypochlorite was added to the influent solution between days 210 and 212 to remove biomass that may have been sustaining (but at a substantially decreased rate) dechlorination (*26, 27*). Despite the absence of any substantial

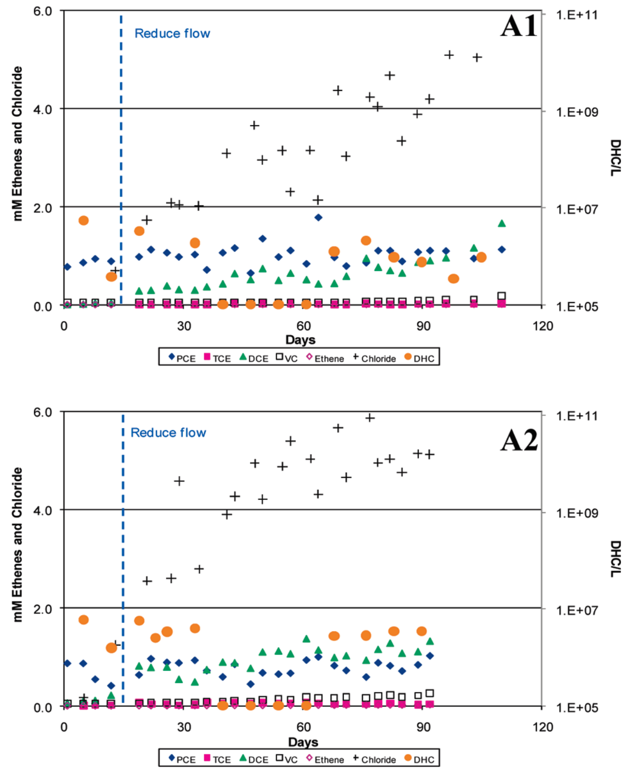


FIGURE 2. Effluent concentrations of ethenes, chloride, and DHC from rock A1 (top) and A2 (bottom) in Experiments 3 and 4, respectively. Fractures were bioaugmented on Day 0. The vertical lines indicate when the fractures flow was decreased from approximately 0.10 cm^3/min to 0.025 cm^3/min. Experiment 4 was terminated prior to Experiment 3.

dechlorination by the end of the experiment (after 212 days), PCE concentrations remained low in Experiment 1, and did not rebound, indicating that the decreased PCE concentrations were the result of a decrease in DNAPL mass, and were not suppressed because of biological reaction.

Arizona Rocks - High Flow rate (Experiments 3 and 4). Results of bioaugmentation in the Arizona rocks for Experiments 3 and 4 (Figure 2) showed that DCE was the primary dechlorination daughter product, and that DCE concentrations were elevated (~1 mM) compared to DCE concentrations measured in Experiments 1 and 2 (0.1 to 0.3 mM). VC concentrations in Experiments 3 and 4 were 10-times greater than in Experiments 1 and 2. The increased DCE and VC concentrations in Experiments 3 and 4 were likely due to the increased residence time, which allowed for a greater extent of PCE dechlorination within the fractures. Conversely, despite the increased residence time, ethene concentration in Experiments 3 and 4 were 3-times less than in Experiments 1 and 2. One explanation for this is that VC dechlorination in Experiments 3 and 4 may have been inhibited by the elevated dissolved PCE concentrations, which remained near solubility (approximately 1 mM) throughout the experiment. Alternately, the elevated DCE concentrations in Experiments 3 and 4 may have inhibited VC dechlorination (*12*).

Despite the generation of DCE to levels near that of the initial PCE effluent concentration, no decreases in effluent PCE concentration were observed during the duration of the experiment. This indicates that biotic dechlorination of the PCE increases the concentration gradient for DNAPL dissolution, thereby enhancing the overall rate of DNAPL dissolution. The quantity of chloride generated was approximately two-times greater than the expected quantity based on the measured chlorinated ethene (and ethene) daughter products. This discrepancy likely is due to back-

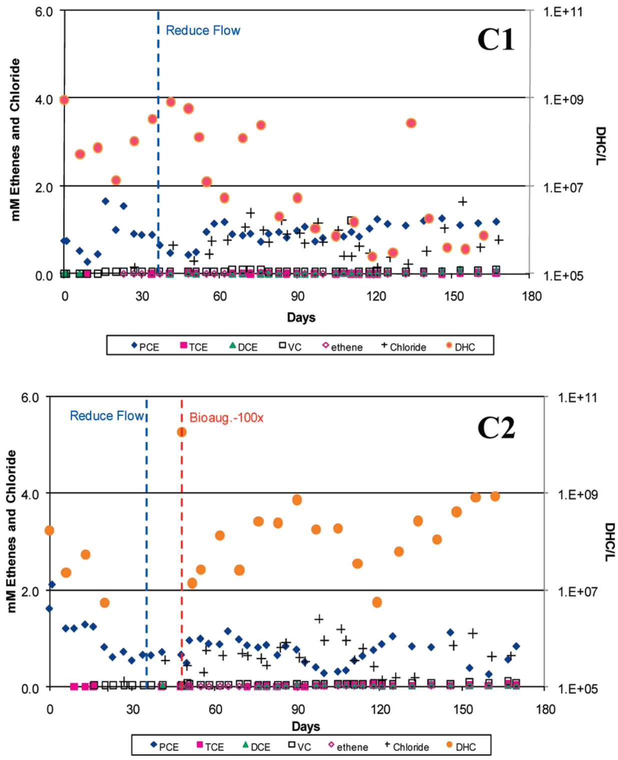


FIGURE 3. Effluent concentrations of ethenes, chloride, and DHC from rock C1 (top) and C2 (bottom) in Experiments 5 and 6, respectively. Fractures were bioaugmented on Day 0. The vertical lines indicate when the fractures flow was decreased or when inoculation with additional culture was performed.

partitioning of chlorinated ethene daughter products into the DNAPL (*15*).

The effluent DHC concentrations in Experiments 3 and 4 were 3 orders of magnitude less than in Experiments 1 and 2, despite similar total bioaugmentation dosages. In addition, eluted DHC in Experiments 3 and 4 account for only 10% of the bioaugmented dosage. These findings suggest that most of the DHC likely became retained within the fractures. The increased DCE generation was proportional to the increase in residence time, suggesting that DHC were still controlling dechlorination. However, a contribution from non-DHC partial dechlorinators cannot be completely ruled out.

Less than 6% of the initial residual PCE DNAPL was removed from the fractures during the duration of Experiments 3 and 4. PCE mass balance analysis performed on A1 showed that PCE mass removed during bioaugmentation and the post-treatment ethanol flushing accounted for 90% of the initially emplaced DNAPL mass; similar analysis performed for A2 showed 86% PCE mass recovery.

Colorado Rocks - High Flow Rate (Experiments 5 and 6). Results for rock C1 (Figure 3) were similar to Experiments 3 and 4. Effluent DHC levels decreased from 10^9 DHC/L to 10^6 DHC/L after reducing the flow rate, consistent with shear-controlled detachment process and consistent with comparison of the low and high flow rate experiments for the Arizona rocks. No increasing trend in effluent DHC was observed throughout the experiment, which is consistent with the low flow rate experiments in A1 and A2. Most of the injected DHC was retained within C1, as eluted DHC accounted for approximately 30% of the total bioaugmented cells.

Approximately 7% of the residual PCE DNAPL mass was removed (as PCE and reductive dechlorination daughter products) during the C1 experiment (Experiment 5). PCE mass balance analysis performed on C1 showed that PCE mass removed during bioaugmentation and the post-treatment ethanol flushing accounted for 96% of the initially emplaced DNAPL mass.

For C2 (Experiment 6), adding additional culture at a 100-fold greater DHC dosage resulted in elevated DHC concentrations in the effluent, but no increase in dechlorination. However, less than 1% of the total DHC injected eluted through the fracture. DHC effluent concentrations increased after adding the additional culture, but then remained constant (within an order of magnitude) throughout the remainder of the experiment. Approximately 8% of the residual PCE DNAPL mass was removed (as PCE and reductive dechlorination daughter products). PCE mass balance analysis performed on C2 showed that PCE mass removed during bioaugmentation and the post-treatment ethanol flushing accounted for 59% of the initially emplaced DNAPL mass; this relatively low mass recovery may have been due to losses (leakage, spillage) during the ethanol flushing.

Impact of Increased Flow (Experiment 7). qPCR results indicated that no rapid DHC elution or "spike" was observed (greater than 90% of the injected DHC were retained within the fracture), and effluent DHC concentrations were consistent with those obtained in Experiment 5 (data not shown). Increasing the flow rate resulted in an increase in DHC effluent concentration of approximately a factor of 5. Combined with the observed decrease in effluent DHC concentrations observed in Experiments 3 and 4 (which were operated at a lower flow rate than in Experiments 1 and 2), these data suggest that DHC detachment and downgradient migration is proportional to the shear.

Discussion

DNAPL Dissolution. Similar to the approach employed by others (*28*), a maximum biotic dissolution enhancement factor (*E*) to describe the increased rate of DNAPL dissolution during bioaugmentation was calculated as follows:

$$E = \frac{[PCE] + ([TCE] + [DCE] + [VC] + [ethene])\dfrac{[Cl^-]_{measured}}{[Cl^-]_{theoretical}}}{[PCE]} \qquad (1)$$

where the bracketed terms represent measured molar concentration (mM), and the theoretical chloride concentration is the expected chloride concentration based on the measured chlorinated ethene (and ethene) daughter products. The difference between $[Cl^-]_{measured}$ and $[Cl^-]_{theoretical}$ is assumed to be due to back-partitioning of the organic daughter products into the DNAPL. For [PCE] in eq 1, the PCE solubility was used for Experiments 3 through 6, since effluent PCE concentrations remained at solubility throughout the duration of the experiment. In Experiments 1 and 2, the PCE concentrations measured at 197 days were used because these concentrations were unchanged after terminating active bioaugmentation.

For the high-flow Experiments 1 and 2, the values of *E* at the end of bioaugmentation (197 days) were approximately 5 and 1.1, respectively. The reason for the difference in these two experiments likely is due to the decreased PCE concentrations in Experiment 1 that resulted in less toxicity, which is consistent with observed PCE DNAPL inhibition on dechlorination observed by others (*10*). It is noted that chloride concentrations were not measured in Experiments 1 and 2, so the actual dissolution enhancement may have been greater than that calculated based on measurement of ethenes alone. For Experiments 3 and 4, the values of *E* at the end of the experiments were 3.5 and 3.4, respectively. Despite having elevated dissolved PCE concentrations, the dissolution enhancement factor is greater than in Experiment

2 because of the decreased flow rate (longer residence time) in the fracture that provided more time for reductive dechlorination. Daughter product concentrations were gradually increasing at the end of the experiment, so a longer duration experiment would likely have yielded increased values of E.

The calculated values of *E* for Experiments 1 through 4 generally are within the general range observed by others working with unconsolidated materials (*11*−*13*). However, on the basis of the results of our batch testing and the results of others (*11*), negligible PCE dechlorination would have been expected, because of the presence of DNAPL and dissolved PCE concentrations near 1 mM. The presence of regions within the fracture where the dissolved PCE concentration was reduced (because of flow heterogeneity), thereby facilitating DHC growth and dechlorination activity, is one possible explanation for the observed enhancement factors.

Despite the reduced flow velocities, the dissolution enhancement factors for C1 and C2 (Experiments 5 and 6) were 1.4 and 1.3, respectively. The reason for these relatively low values of *E* compared to Experiments 3 and 4 (which also were performed at reduced flow velocities) may be due to differences in the effective aperture distribution in each set of rocks. The Colorado rocks have an effective fracture distribution that is substantially more uniform than the Arizona rocks (*20*). The Arizona rocks contain a comparatively greater number of small aperture regions along the flow path; these small aperture regions likely are devoid of DNAPL, and may serve as optimal locations for DHC biofilm growth and dechlorination activity. Thus, comparison of the Colorado and Arizona bioaugmentation data suggest that aperture distribution (and the distribution of DNAPL within the fractures) may control dechlorination kinetics and overall bioaugmentation effectiveness in these systems.

When considering the measured dissolution enhancement factors in this bench scale study, it is important to consider the length scale of the experiment. If the length scale of the fracture was 10-times greater, the bioaugmentation residence time correspondingly would be 10-times greater, resulting in approximately a 10-fold increase in dechlorination daughter products. This would result in a calculated value of *E* that was approximately 10-times greater than those calculated in this study. Thus, results from all six dissolution experiments suggest that bioaugmentation has the potential to substantially increase the rate of DNAPL dissolution at larger scales.

DHC Migration. As observed by comparing Experiments 1 and 2 to Experiments 3 and 4, effluent DHC concentrations were determined to not be a useful metric for assessing DNAPL dechlorination kinetics. These observations differ from our previous studies that evaluated dissolved phase DCE dechlorination in sand columns (*5*), where effluent DHC concentrations were correlated to the dechlorination kinetics observed in the column. The presence of the PCE DNAPL, and its inhibitory effects on planktonic DHC at elevated aqueous PCE concentrations, is one possible reason for this discrepancy. DHC present in biofilms, which might be more resistant to toxicity than planktonic cells (*29, 30*), could have played a dominant role in the observed PCE dechlorination. Additional study is needed to further evaluate the role of DHC in biofilms in bedrock fractures when PCE DNAPL is present.

While increasing the DHC inoculation dosage did not have a measurable impact on dechlorination (as observed by comparing Experiments 5 and 6), the flux of DHC leaving the column was approximately 100-times greater in C2 than in C1. This increased concentration of DHC migrating downgradient of the DNAPL source zone could provide additional remedial benefit for the downgradient plume (*12*).

This study has shown that bioaugmentation using DHC can substantially enhance PCE DNAPL dissolution rates in bedrock fractures. Planktonic DHC in batch systems were unable to dechlorinate PCE when DNAPL was present, but substantial dechlorination was observed in the DNAPL-impacted bedrock fractures even when dissolved PCE concentrations were at or near solubility. Biofilms and/or the presence of regions with lower dissolved PCE concentrations may have mitigated the inhibitory effects of sustained and elevated PCE levels, resulting in the observed dechlorination. Effluent DHC concentrations did not correlate to observed dechlorination activity, but rather were a function of the flow rate and shear. For the experimental fracture systems and DHC dosages examined, DNAPL dechlorination kinetics were more sensitive to fracture properties than to bioaugmentation dosage, as demonstrated by the differences in the Arizona and Colorado rocks, and the negligible impact on dechlorination rates after increasing the bioaugmentation dosage. However, downgradient migration of DHC, and potential treatment of the dissolved downgradient plume, was impacted by the DHC dosage. Although additional studies are needed to further examine these processes under a wider range of conditions, these results suggest that bioaugmentation may be a viable option for treatment of DNAPL in bedrock, and that bench scale batch testing may not be an appropriate indicator of in situ dechlorination kinetics when DNAPL is present.

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Supporting Information Available

Additional details are available regarding the SDC-9 culture and the experimental fracture apparatus constructed from sandstone blocks. This material is available free of charge via the Internet at http://pubs.acs.org.

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ORIGINAL PAPER

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

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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.

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,

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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

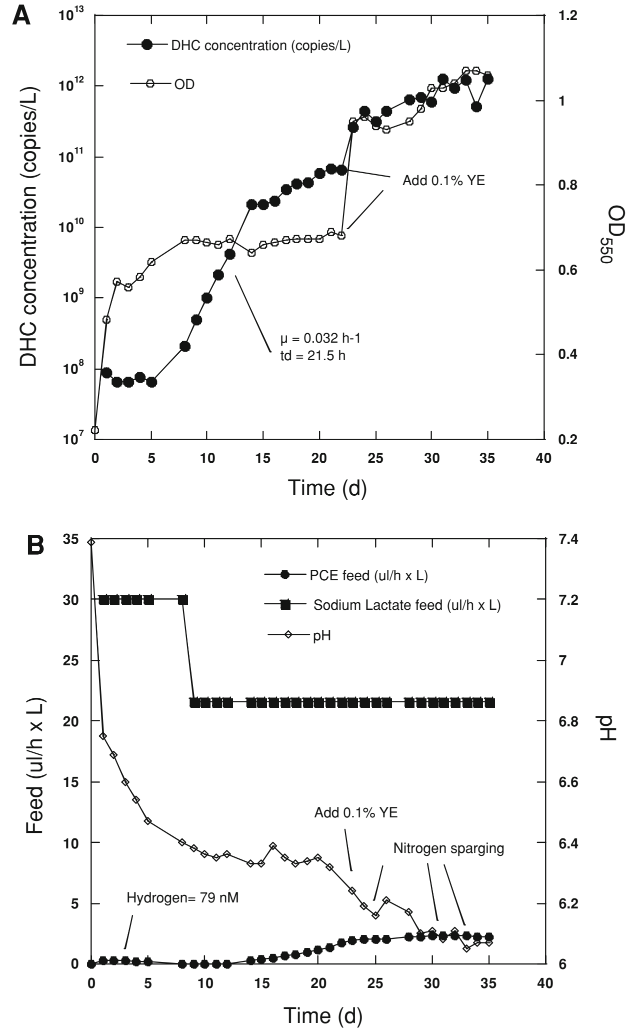

Fig. 1 Growth of SDC-9™ in a 4,000-L fermentor. **a** Concentration of DHC as measured by qPCR (*filled circle*) and total cell concentration as estimated by OD at 550 nm (*open circle*). DHC first-order growth rate (μ) and doubling time (td) are indicated on the graph. **b** Feed rate of neat PCE (*filled 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

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).

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)] ($R = 0.999$).

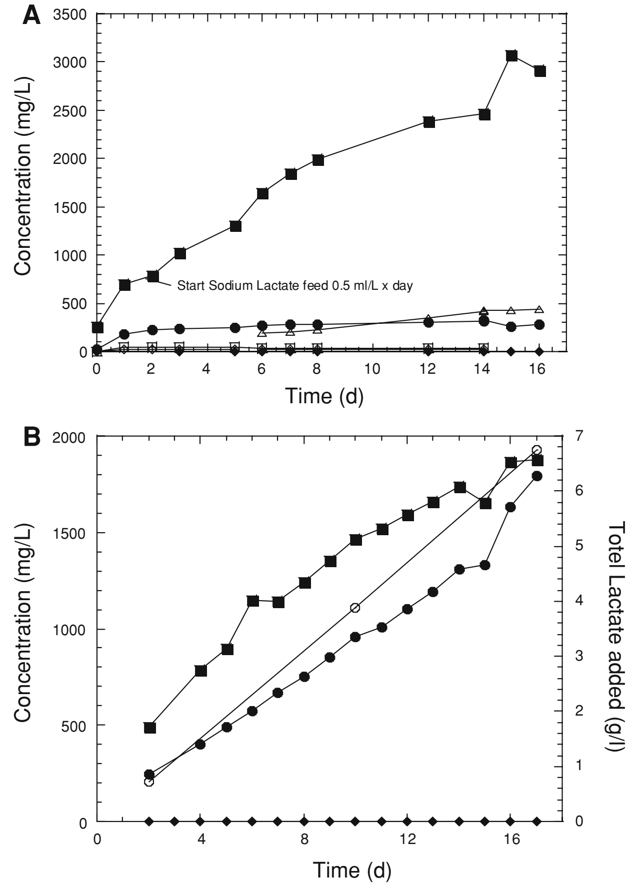

Fig. 2 Accumulation of VFAs during growth of SDC-9™ (**a**) or PJKS™ (**b**) in a 750-L fermentor. Symbols indicate lactic acid (*filled diamond*), propionic acid (*filled circle*), formic acid (*open diamond*), pyruvic acid (*open square*), butyric acid (*open triangle*), and acetic acid (*filled square*),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

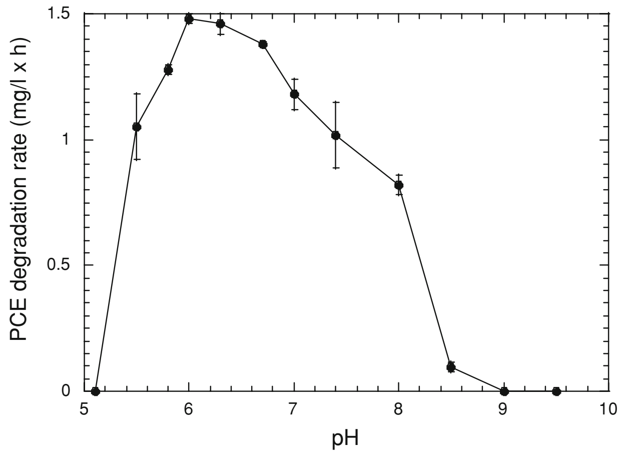

Fig. 3 Effect of culture pH on PCE dehalogenation by SDC-9™. Values represent the mean of triplicate samples, and *error bars* represent one standard error of the mean

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.

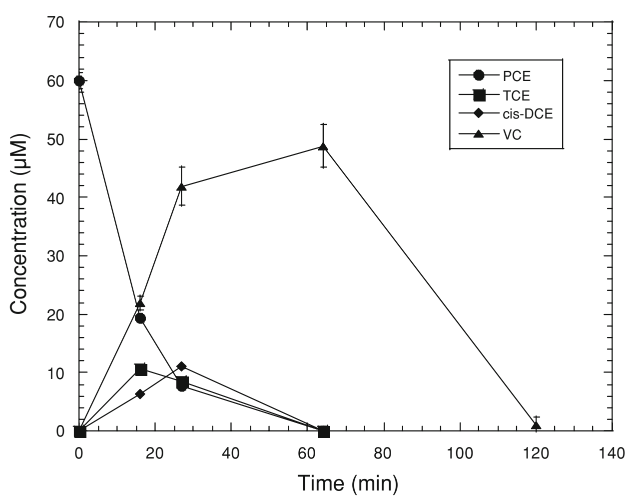

Fig. 4 Results of a PCE degradation assay with samples from a 550-L fermentation batch of the SDC-9™ culture. The assay was performed in 60-mL serum vials containing 60 mL SDC-9™ culture (0.52 g/L Dwt), 6 mM sodium lactate, and 10 mg/L PCE. Values represent means of triplicate samples and *error bars* 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 $\times$ g Dwt before concentration and 22.6 and 15.1 mg PCE/h $\times$ 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

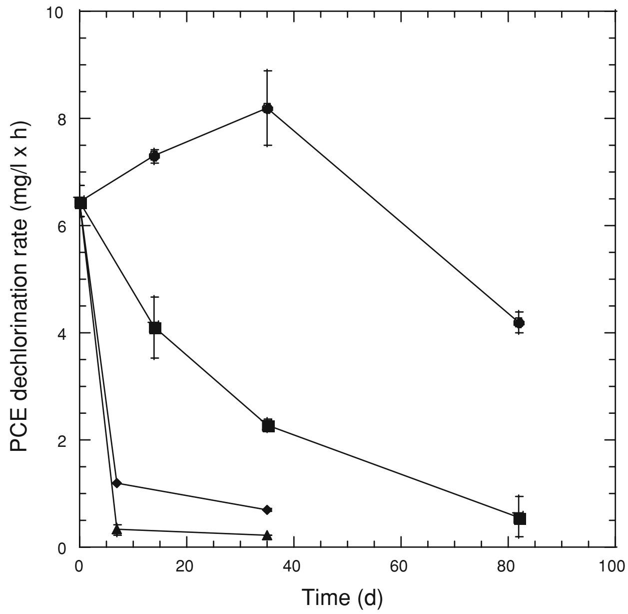

Fig. 5 Effect of storage conditions on the activity of concentrated SDC-9™ culture. Tenfold-concentrated SDC-9™ culture was stored anaerobically and without substrate at either 4°C (*filled circle*), 13°C (*filled square*), 22°C (*filled diamond*) or 28°C (*filled triangle*). Values represent means of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

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Field-Scale Evaluation of a Biobarrier for the Treatment of a Trichloroethene Plume

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In the 1960s, trichloroethene (TCE) was used at what is now designated as Installation Restoration Program Site 32 Cluster at Vandenberg Air Force Base to flush missile engines prior to launch and perhaps for other degreasing activities, resulting in releases of TCE to groundwater. The TCE plume extends approximately 1 kilometer from the previous launch facilities beyond the southwestern end of the site. To limit further migration of TCE and chlorinated degradation by-products, an in situ, *permeable, reactive bioremediation barrier (biobarrier) was designed as a cost-effective treatment technology to address the TCE plume emanating from the source area. The biobarrier treatment would involve injecting carbon-based substrate and microbes to achieve reductive dechlorination of volatile organic compounds, such as TCE. Under reducing conditions and in the presence of certain dechlorinating microorganisms, TCE degrades to nontoxic ethene in groundwater.*

To support the design of the full-scale biobarrier, a pilot test was conducted to evaluate site conditions and collect pertinent design data. The pilot test results indicated possible substrate delivery difficulties and a smaller radius of influence than had been estimated, which would be used to determine the final biobarrier well spacing. Based on these results, the full-scale biobarrier design was modified. In January 2010, the biobarrier was implemented at the toe of the source area by adding a fermentable substrate and a dechlorinating microbial culture to the subsurface via an injection well array that spanned the width of the TCE plume.

After the injections, the groundwater pH in the injection wells continued to decrease to a level that could be detrimental to the population of Dehalococcoides in the SDC-9™ culture. In addition, 7 months postinjection, the injection wells could not be sampled due to fouling. Cleaning was required to restore their functions. Bioassay and polymerase chain reaction analyses were conducted, as well as titration tests, to assess the need for biobarrier amendments in response to the fouling issues and low pH. Additionally, slug tests were performed on three wells to evaluate possible localized differences in hydraulic conductivity within the biobarrier. Based on the test results, the biobarrier was amended with sodium carbonate and inoculated a second time with SDC-9™. The aquifer pH was restored, and reductive dechlorination resumed in the treatment zone, evidenced by the reduction in TCE and the increase in degradation products, including ethene. © 2011 Wiley Periodicals, Inc.

INTRODUCTION

Located along the central California coast in Santa Barbara County, between San Luis Obispo and the city of Santa Barbara, Vandenberg Air Force Base (VAFB) occupies

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approximately 98,000 acres and 35 miles of coastline. Currently, VAFB is an active U.S. military installation and supports major launch operations for both military and commercial satellites. The project site, Installation Restoration Program Site 32C, is a site "cluster" and refers to two sites, IRP Sites 32 and 35, which share similar hydrogeologic settings and operational history. IRP Site 32C, located in the northern portion of VAFB, is a former missile launch facility. During its use as a launch site in the 1960s, missile engines were flushed with TCE for degreasing purposes; these past operations likely resulted in TCE releases to soil and groundwater. An investigation conducted in 2009 assessed the vertical and lateral extent of soil and groundwater contamination in the source area, located approximately 300 feet (ft) upgradient of the project location. The investigation results concluded that the groundwater plume emanated from a location southeast of Building 1930 (Exhibit 1) and continued southwest following groundwater flow. Groundwater is unconfined and is approximately 25 ft below ground surface (bgs) at the project location. Previous investigations at IRP Site 32C assessed the TCE plume as relatively narrow, approximately 200 ft in width, following bedrock topography within a paleochannel and extending almost 1 kilometer to the southwest.

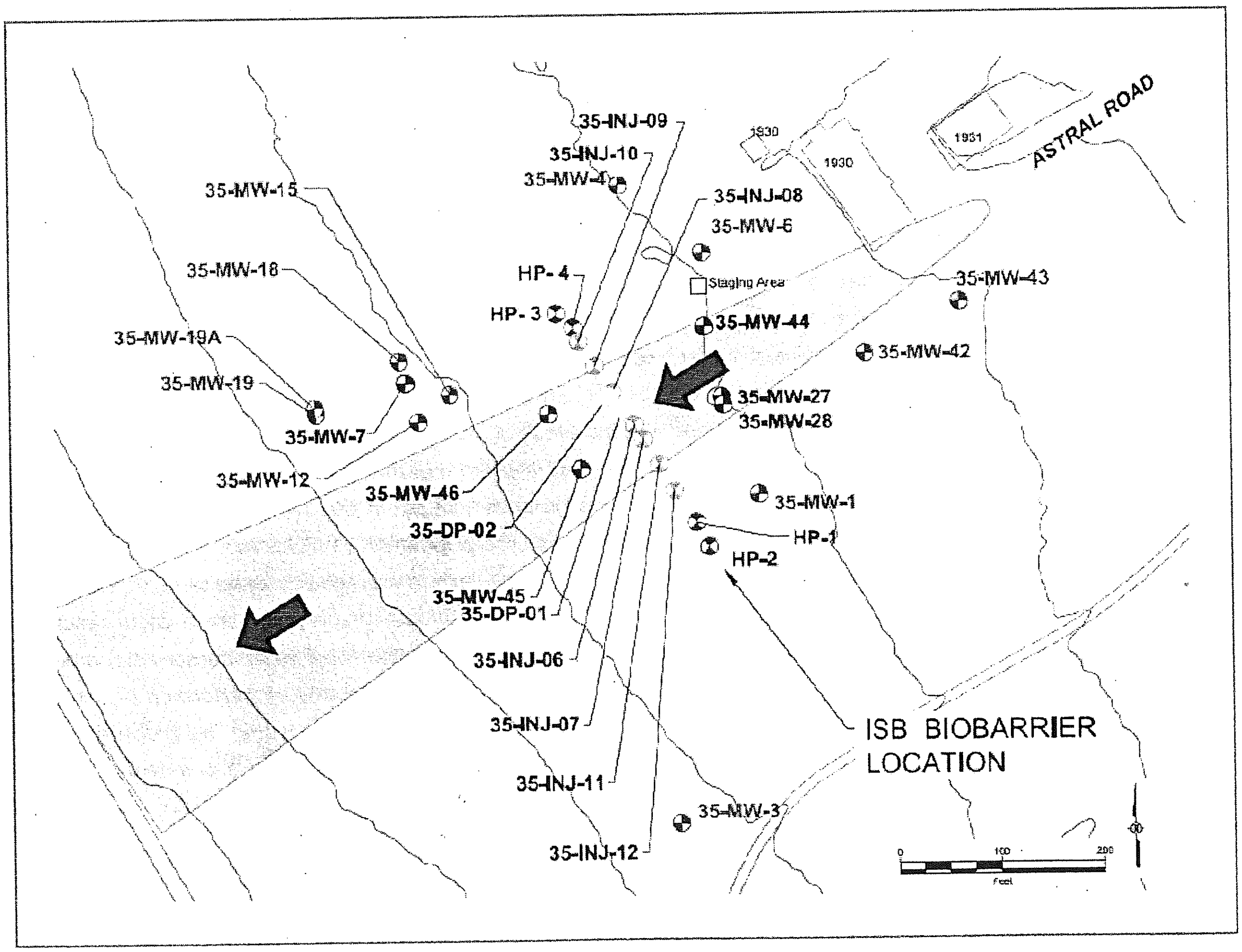

Exhibit 1. The arrows indicate the approximate direction of groundwater flow and TCE plume migration. The plume contour represents the approximate 50 micrograms per liter TCE in groundwater prior to the biobarrier installation

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Engineered *in situ* bioremediation in the form of a permeable, reactive biobarrier was selected as the remedy for this site based on the narrow groundwater plume configuration and long-term treatment goals. Permeable reactive barriers are intended to allow groundwater to pass through a reactive zone, in this case a biologically active zone, which treats the contaminants while not impeding groundwater flow (U.S. Environmental Protection Agency, 1998). To reduce the concentrations of TCE and its chlorinated degradation by-products, such as *cis*-1,2-dichloroethene (*cis*-1,2-DCE) and vinyl chloride, in the groundwater plume and to limit further migration of these VOCs, a biobarrier consisting of seven 4-inch-diameter injection wells (35-INJ-6–35-INJ-12) was constructed in August 2009 (Exhibit 1). These seven wells were used as conduits for substrate delivery to promote the formation of a biologically active zone. This zone was created to encourage reductive dechlorination via biostimulation with a carbon substrate, such as emulsified soybean oil, and bioaugmention with a dechlorinating microbial culture, such as SDC-9™. The SDC-9™ culture is a consortium comprised of a variety of bacteria including *Clostridium* (fermenters that convert complex carbon compounds into hydrogen), *Desulfitobacterium* (facultative dehalogenators), and at least three different strains of *Dehalococcoides* (Dhc) (R. J. Steffan, personal communication, May 31, 2011). Certain strains of Dhc are capable of anaerobically degrading DCE and vinyl chloride (Maymó Gatell, Chien, Gossett, & Zinder, 1997; Maymó Gatell, Anguish, & Zinder, 1999).

Refined soybean oil is the most widely used food-grade substrate for enhanced *in situ* bioremediation (Air Force Center for Environmental Excellence, 2007). Due to its low solubility, this carbon source acts as a slow-release electron donor in the aquifer matrix. Microbes use the carbon source and produce hydrogen, which in turn is used by other dechlorinating bacteria, such as Dhc, as an electron donor. The electrons are accepted by the chlorinated compound, and a chloride ion is released into solution. This process of reductive dechlorination (or reductive dehalogenation) reduces toxic TCE to the nontoxic, nonchlorinated ethene (Vogel, Criddle, & McCarty, 1987). A slow-release, persistent substrate, such as LactOil™ (JRW Bioremediation LLC, Lenexa, Kansas), was selected as the injectate in order to maintain the reactive zone over an extended time period without the need for substrate reinjection or buffering. LactOil™ is buffered to pH 6.5 (JRW Bioremediation LLC, 2011).

At the time of the biobarrier installation, the source area was scheduled for soil removal and groundwater treatment by others. In April 2010, following the targeted soil excavation of 200 cubic yards in the source area, confirmation soil samples collected along the side walls had TCE concentrations ranging from 15 to 2,700 micrograms (mcg) per kilogram. No further source area removal action had been completed as of July 2011 and, thus, the TCE loading on the biobarrier continues.

DESIGN AND CONSTRUCTION

Although extensive remedial investigations had been conducted at the site at the time the remedy was under consideration, very little soil or groundwater sampling in the immediate vicinity of the proposed biobarrier had been completed. Therefore, a pilot test was performed with three biobarrier wells spaced approximately 20 ft and 40 ft apart. The initial spacing was based on previous biobarrier projects at another VAFB site. Tracer tests were

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conducted on these three wells prior to the installation of the remaining four wells to determine the site-specific radius of influence (ROI) and evaluate the final spacing of the wells.

The biobarrier injection wells and two downgradient monitoring wells were installed to bedrock, approximately 40 ft bgs using the hollow stem auger drilling method; split spoon samples were collected and logged by the project geologist every 5 ft to determine the soil lithology. Subsurface soils were identified as predominantly poorly graded, fine-grained sand with some interbedded lenses of clayey sand. Several borings were also identified with fine-grained silty sands in the upper 10 to 20 ft, and a gravel layer overlaying the bedrock.

With the additional soil characterization information and the limited results of the tracer test, a groundwater simulation using Visual MODFLOW was run to estimate the ROI.

PILOT TEST

During the installation of the first three biobarrier wells (35-INJ-6–35-INJ-8), four direct-push borings (HP-1–HP-4) were advanced for grab groundwater sampling to assess the longitudinal ends of the proposed biobarrier width (Exhibit 1). The proposed target depth of the four grab sampling locations was between 33 and 37 ft bgs. The formation failed to yield groundwater at 37 ft within a reasonable amount of time, so groundwater samples were collected at either 30 or 33 ft bgs. The northernmost boring, HP-4, failed to yield any water during sampling, indicating preferential migration of groundwater around this area. Based on the analytical results of the grab samples and samples collected from existing wells both up- and downgradient of the proposed biobarrier location, the width of the plume was estimated at approximately 150 ft.

Tracer tests using fluorescein dye and bromide were conducted on two of the initial three injection wells, 35-INJ-6 and 35-INJ-7; tracer was injected into 35-INJ-6, while 35-INJ-7 was pumped and monitored. The bromide levels rose only slightly in 35-INJ-7, and red dye was not observed during the test. The tracer test results were deemed inconclusive; thus, an additional soil investigation was performed to further investigate subsurface soil characteristics near the injection wells 35-INJ-6 and 35-INJ-8 as these wells yielded little to no water during well development.

Two additional borings, 35-DP-01 and 35-DP-02, were advanced via direct-push technology and continuously logged from 0 to 20 ft bgs and 24 to 36 ft bgs, respectively. The intention had been to continuously log from 0 to approximately 40 ft bgs (surface to bedrock); however, the dense, dry sands created substantial friction causing the acetate liners to "melt" in the drill rods. The soils were classified as primarily sand with clay and sand with silt.

With the additional soil characterization information and the limited results of the tracer test, a groundwater simulation using Visual MODFLOW was run to estimate the ROI. Based on the model results, the original well spacing of 40 ft was decreased. The remaining biobarrier wells were installed approximately 30 ft apart, spanning the width of the estimated 150-ft TCE plume and oriented in a northwest–southeast direction.

FULL-SCALE DESIGN

The remaining four injection wells (35-INJ-9–35-INJ-12) and the two downgradient monitoring wells (35-MW-45 and 35-MW-46) were screened from bedrock at approximately 40 ft bgs to the top of the water table at approximately 16 ft bgs, targeting the saturated zone. Prior to the injections, baseline sampling was conducted with stacked, passive diffusion bag samplers in select wells to assess the vertical stratification of

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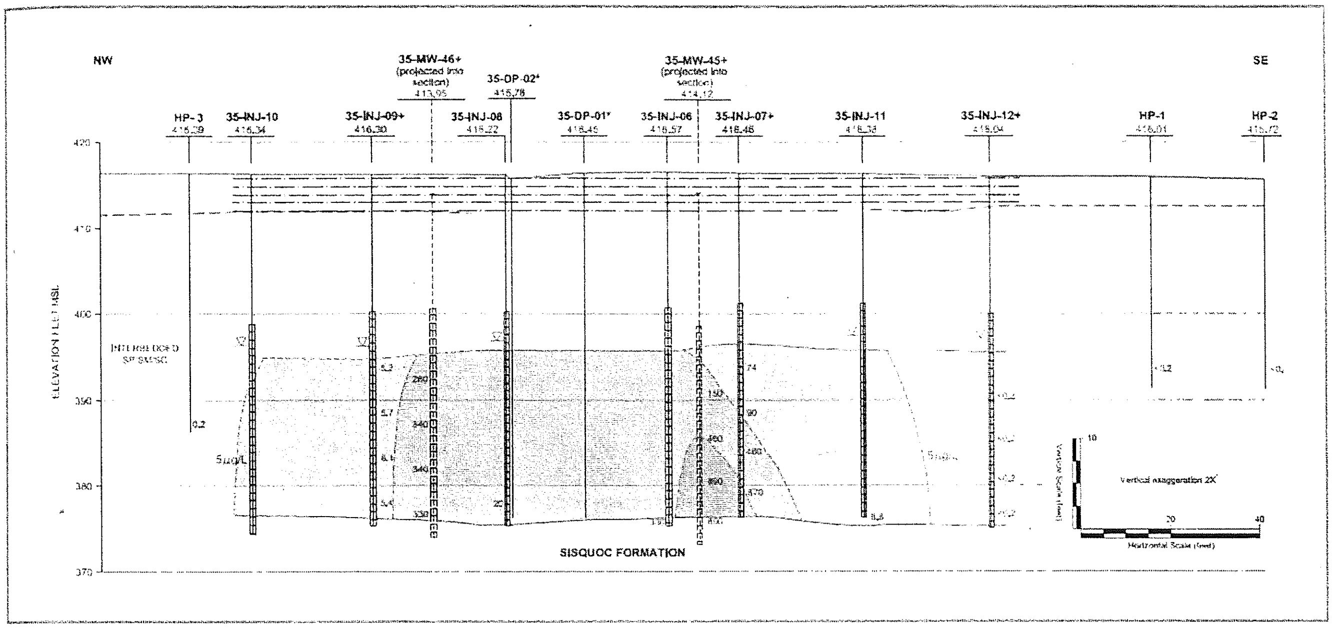

Exhibit 2. Biobarrier hydrogeologic cross section with baseline TCE concentrations in micrograms per liter; select wells sampled with diffusion bags

contamination across the treatment zone. Diffusion bag sampling delays groundwater quality parameter monitoring (i.e., dissolved oxygen, oxidation-reduction potential [ORP], and pH); thus, some wells were sampled without diffusion bags. The baseline sampling results indicated the presence of higher TCE concentrations toward the bottom of the water-bearing zone (Exhibit 2); hence, all future groundwater samples would be collected from this interval.

In preparation for the substrate injections, water was extracted from two biobarrier wells, 35-INJ-7 and 35-INJ-12, at approximately 8 gallons per minute (gal/m), and stored in two 21,000-gal tanks on-site to be used during the substrate and microbe injections. The stored groundwater was amended with very low concentrations of sodium lactate (less than 0.3 percent solution) to create anaerobic conditions. An injection manifold was constructed connecting the Baker tanks and a smaller, portable tank to each wellhead.

In November 2009, LactOil™ and SDC-9™ injections commenced. The SDC-9™ microbial culture requires low dissolved oxygen, preferably less than 0.5 milligram per liter (mg/L), an ORP less than −100 millivolts (mV), and a relatively neutral pH for optimal performance (Vainberg, Condee, & Steffan, 2009). The LactOil™ was diluted to less than the manufacturer's recommended 10 percent by volume; however, a higher-concentration solution was added to each biobarrier well to prime the groundwater prior to the addition of the SDC-9™. At the request of the state regulatory agencies to minimize aquifer displacement, the total injectate volume did not exceed 10 percent of the total groundwater treatment zone volume. Each injection well received approximately 20 L of SDC-9™ via a delivery system that displaces the culture from the shipping canisters with nitrogen rather than air to maintain an anaerobic environment for the microbes (Exhibit 3). As expected following the pilot test, the site lithology limited injection rates (between 0.5 and 2.7 gal/m) at some biobarrier well locations. As a result,

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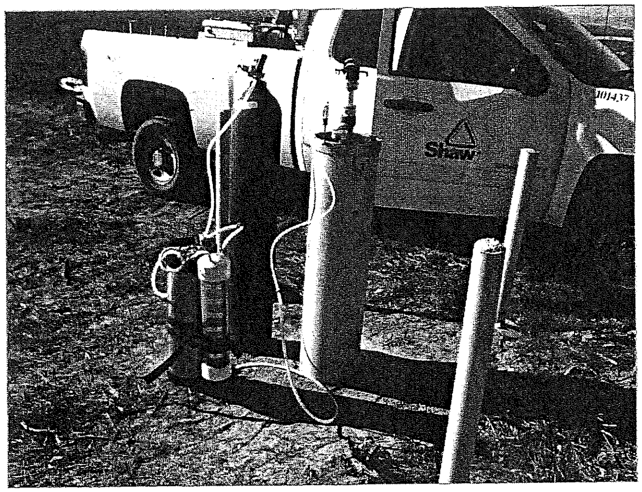

Exhibit 3. Nitrogen delivery system for the SDC-9™ bacteria injections

injections were not completed until the end of January 2010. Subsurface heterogeneity encountered in the groundwater plume contributed to significant variation in injectate distribution observed in the biobarrier treatment zone, resulting in varied ROI among the injection wells. This information was important for monitoring purposes; because the southern biobarrier wells (35-INJ-7– 35-INJ-12) accepted the injectate better than the northern biobarrier wells, it was expected that the substrate effects would appear sooner in 35-MW-45, the more southern of the two wells downgradient of the biobarrier.

SAMPLING AND MONITORING

The seven injection wells, two downgradient wells (35-MW-45 and 35-MW-46), and existing upgradient well (35-MW-27) were sampled for VOCs and groundwater quality parameters on the last day of injections and then approximately 30, 60, and 90 days postinjection, followed by quarterly sampling, which is expected to continue for the next 2 years. Only the well 35-MW-45, located downgradient of the southern portion of the biobarrier, showed a significant reduction in TCE and an increase of *cis*-1,2-DCE by the day-60 sampling event. The TCE concentrations at 35-MW-45 decreased from 910 μg/L to 7.5 μg/L by day 60; the *cis*-1,2-DCE concentrations during this same time period increased from 22 μg/L to 520 μg/L, with trace amounts of vinyl chloride and ethene. The well immediately upgradient of the biobarrier, 35-MW-27, has consistently had TCE concentrations exceeding 2,000 μg/L since the construction of the biobarrier.

Decreasing pH levels were recorded in all the biobarrier wells from the time of the injections until the day-90 sampling event.

Decreasing pH levels were recorded in all the biobarrier wells from the time of the injections until the day-90 sampling event. The pH levels recorded in the biobarrier wells prior to injections ranged between 6.61 and 6.81; by day 90, the pH levels in these same wells ranged between 4.82 and 5.01. The pH range for the growth of dechlorinating microbes is between 5.5 and 8 (Vainberg et al., 2009).

Based on previous aquifer tests conducted prior to the biobarrier installation, the estimated hydraulic conductivity for the site is between 1.1 and 5.8 ft per day (ft/day). During groundwater level gauging conducted in March 2010, the average hydraulic gradient was estimated at 0.064 ft/1 ft to the southwest. Assuming an effective porosity

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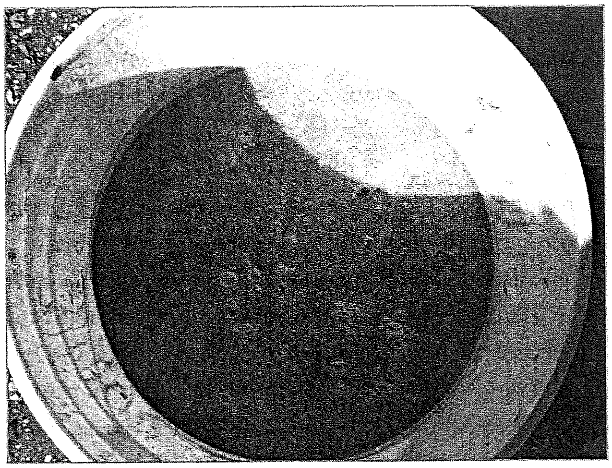

Exhibit 4. Gray substance discovered in the biobarrier wells approximately 7 months after injections

of 0.3, the seepage velocity was estimated between 0.23 and 1.2 ft/day. Monitoring wells 35-MW-45 and 35-MW-46 are approximately 60 to 70 ft downgradient of the biobarrier; thus, hydrogeologic modeling suggests that water traveling from the biobarrier would reach the downgradient wells between 50 and 300 days under ambient conditions. This time frame does not account for the injection delivery process, which would decrease the travel time of the substrate to the monitoring wells. The downgradient well 35-MW-45, which is located closest to injection well 35-INJ-7, began showing the full effect of the substrate injections within 60 days. Although the substrate effects had reached the downgradient well 35-MW-46, the percent reduction was less than that occurring at 35-MW-45; the moderate reduction is likely due to its location downgradient of the northern portion of the biobarrier, where hydraulic conductivity is suspected to be much lower based on well development records and injection rates.

By September 2010, almost 7 months postinjection, the biobarrier wells were so obstructed with a gray substance that the wells could not be sampled (Exhibit 4). The obstruction was possibly due to biofouling and/or the presence of emulsion breakdown product. Fouling was not noted by the field team during the May 2010 sampling event. The gray substance was subsequently removed from five of the injection wells, and groundwater conditions were examined in December 2010. Two injection wells had more persistent, crusted layers of the gray substance and were not cleaned successfully until January 2011. Injection wells that could be sampled had an average groundwater pH of 5, a level that could be detrimental to the population of Dhc.

TESTS

In response to the low pH and the possible biofouling or formation of breakdown products in the injection wells, several tests were conducted to assess the biobarrier condition and the need for amendments to restore the biobarrier to optimum performance conditions. These tests included microbial analyses, a titration test, and well slug tests. Microbial analyses were performed to determine the presence of Dhc. A titration test was conducted to establish the buffering requirements for the groundwater

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within the treatment zone, and slug tests were conducted to evaluate the hydraulic conductivities across the biobarrier.

Microbial (qPCR) Analysis

The laboratory concluded that, although both samples had a sufficient amount of hydrogen, the initial pH of the samples was below the range required for dehalogenation activity.

In November 2010, groundwater samples were collected from injection well 35-INJ-7 and downgradient monitoring well 35-MW-45 and were sent to Shaw's biotechnology laboratory in Knoxville, Tennessee, for quantitative polymerase chain reaction (qPCR) analysis. The number of Dhc in the sample was determined based on the number of 16s rRNA gene sequences detected via qPCR using a Roche Real-Time LightCycler PCR instrument. The sample from 35-INJ-7 contained a large amount of solid gray material, causing matrix interference, and could not be filtered for analysis. The microbial communities from the groundwater sample collected at 35-MW-45 were screened for Dhc presence; Dhc were not observed above the detection limit of 40 cells per milliliter (mL). Although this information assisted in determining the extent of Dhc presence, it was essential to assess the viability of the microbes within the biobarrier wells. Thus, once the wells were cleared of the interfering gray material, a sample was collected again from 35-INJ-7 and from 35-INJ-8. The samples were sent again for qPCR analysis, but this time to Shaw's biotechnology laboratory in Lawrenceville, New Jersey, for verification. The laboratory reported that 813 cells/mL were detected in 35-INJ-7 and no Dhc were detected in 35-INJ-8. To evaluate the dechlorination activity in the groundwater samples, the laboratory also performed a degradation assay. Each sample was amended with sodium carbonate to increase the pH to the optimum level. The pH in the sample from 35-INJ-7 was raised from 5.33 to 6.34 and that from 35-INJ-8 was increased from 5.07 to 6.42. TCE was added to the vials at a final concentration of 1 mg/L. The vials were incubated for 20 days at 23°C and then 5 days at 30°C. The samples were submitted for VOC analysis after 20 and 25 days of incubation and for Dhc quantification by qPCR analysis after 25 days. The laboratory concluded that, although both samples had a sufficient amount of hydrogen, the initial pH of the samples was below the range required for dehalogenation activity. Additionally, even after buffering and incubating the samples, the growth of Dhc bacteria was insignificant.

Titration Test

On December 17, 2010, a titration test was performed using groundwater from 35-INJ-7 and 35-INJ-8 to determine buffering requirements needed to restore the aquifer pH. Titration was performed on two different groundwater samples of 250 mL each using sodium carbonate solutions at two different concentrations: 4 grams (g) per L and 50 g/L.

Prior to buffering, the pH at 35-INJ-7 had decreased to 5.3. The low-concentration sodium carbonate buffering solution was added to a sample of the water collected from 35-INJ-7 in increments until the pH reached 7.31. The test was repeated with a water sample from 35-INJ-7 using the high-concentration, sodium carbonate solution. The same titrations were performed with a groundwater sample from 35-INJ-8. The results were graphed and a best-fit linear regression analysis was used to determine the buffering requirements for both wells. Approximately 5.1 g sodium carbonate would be required to adjust the pH of 1 gallon of groundwater at 35-INJ-7 to a pH of 8.0. This amounts to 11 pounds (lb) per 1,000 gallons (gal) of groundwater, which was determined to be the

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amount required to adjust the pH in the column of water within and immediately around 35-INJ-7. Similarly, approximately 34 lb/1,000 gal were required for pH adjustment in and around 35-INJ-8. Background alkalinity concentrations in the vicinity of the biobarrier are on average 100 mg/L; thus, the buffering capacity of the aquifer is limited.

Slug Tests

Slug tests were performed on 35-INJ-7, 35-INJ-8, and 35-INJ-10 to assess the possibility of variable hydraulic conductivity across the biobarrier, and potential changes to the subsurface conditions, such as reduced hydraulic conductivity as a result of the injections. These three wells were selected based on their spacing of approximately 50 ft apart, representing different portions of the biobarrier. The addition of substrate likely reduced hydraulic conductivity adjacent to the injection wells, across the biobarrier; however, it was suspected that the groundwater moved faster through the southern portion of the biobarrier than through the northern portion, even after injections. Based on the slug test results, the mean hydraulic conductivities ranged between 0.045 ft/day at 35-INJ-7, 0.008 ft/day at 35-INJ-10, and 0.003 ft/day at 35-INJ-8, signifying large lateral differences in the groundwater flow near the biobarrier wells, across a 100-ft span. These hydraulic conductivity values were lower by 2 to 3 orders of magnitude from the hydraulic conductivity range previously estimated for the site of 1.1 to 5.8 ft/day.

BIOBARRIER AMENDMENTS

Based on the qPCR analyses and the field tests previously described, the conclusion was that the biobarrier would require amendments in order to restore optimum performance. These amendments included buffering the groundwater at the injection wells and introducing additional Dhc.

The conclusion was that the biobarrier would require amendments in order to restore optimum performance.

Buffering

To restore the aquifer pH, the groundwater at the biobarrier wells was buffered with sodium carbonate in February 2011. Due to the low yield of the biobarrier wells, the original plan to buffer using extracted site water, as had been done during substrate injections, was abandoned. Instead, hydrant water, pretreated through a granular activated carbon system to remove disinfectant by-products, was transported to the site, and the buffering requirements were recalculated based on the hydrant water pH.

Bioaugmentation

Once the pH stabilized between 6 and 8 and reducing conditions were established (low dissolved oxygen ranging between 0.22 mg/L and 0.80 mg/L, and low ORP ranging between –135 mV and –227 mV) the biobarrier wells were inoculated again in March 2011. Approximately 5 L SDC-9™ were injected into each injection well via a polyvinyl chloride drop pipe and the nitrogen delivery system. Several hundred gallons of site water were added to each injection well as a chaser to push the microbes into the formation and away from the well. Since the total organic carbon levels were still high, ranging between 3,100 mg/L and 10,300 mg/L as of the last sampling event in May 2010, only a minimal

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amount of sodium lactate was added to the chaser water with the SDC-9™ to sustain anaerobic conditions.

RESULTS AND DISCUSSION OF THE BIOBARRIER AMENDMENTS

Prior to the substrate injections completed in January 2010, groundwater was extracted from two biobarrier wells, 35-INJ-7 and 35-INJ-12, at approximately 8 gal/m per well. During the buffering preparations, these same two wells were pumped dry with very slow recharge rates; injection well 35-INJ-7 ran dry after 4 minutes of pumping at 5 gal/m, and 35-INJ-12 had similar results. The injectate likely temporarily reduced the hydraulic conductivity, which would increase the residence time of groundwater in the treatment zone and the travel time to the downgradient monitoring wells. Based on quarterly sampling results from wells to the north and south of the biobarrier, it does not appear as if the groundwater is moving around the treatment zone at this time. It is possible that the reduced hydraulic conductivity is localized to the injection wells and may not fully extend into the treatment zone.

Although substrate degradation results in organic fatty acid production, which can lower the groundwater pH, the LactOil™ product is buffered to pH 6.5; therefore, the significant pH depression in the groundwater within the treatment zone was unexpected. As such, all future LactOil™ injections at the site will require additional buffering using sodium carbonate. To mitigate the possibility of biofouling and/or emulsion breakdown product formation within the injection wells, the wells will also be flushed with unamended site water after any subsequent substrate injections.

Since the sodium carbonate buffering and second inoculation of SDC-9™ in March 2011, groundwater quality parameters were reexamined from the biobarrier wells and the two downgradient monitoring wells. The groundwater quality parameters collected from the injection wells indicated that the buffering had restored the pH levels to baseline conditions, ranging between 6.32 and 6.74, in the treatment zone. Additionally, the dissolved oxygen concentrations ranged between 0.13 mg/L and 0.45 mg/L, and ORP levels ranged between −106 mV and −160 mV. The field crew noted that the groundwater samples were gray to black and produced a very strong odor indicative of ongoing biological reactions.

Groundwater sampling was performed in June 2011 on the biobarrier wells, the two downgradient monitoring wells, and existing vicinity wells. Based on the sampling results, reductive dechlorination is occurring, as indicated by the decrease in TCE in the injection wells and downgradient monitoring wells and the simultaneous increase in *cis*-1,2-DCE and vinyl chloride in the downgradient monitoring wells, 35-MW-45 and 35-MW-46 (Exhibit 5). Because the upgradient well, 35-MW-27, has consistently had TCE concentrations exceeding 2,000 μg/L, the decreases in TCE downgradient of the biobarrier are fully attributed to the effectiveness of the biobarrier. The increase in the ethene concentration at downgradient monitoring well 35-MW-46 confirms that complete reductive dechlorination has also occurred. The other downgradient well, 35-MW-45, had ethene detections 30, 60, and 90 days after the first biobarrier treatment, but since the second inoculation in March 2011, this well has yet to show any significant increases in ethene.

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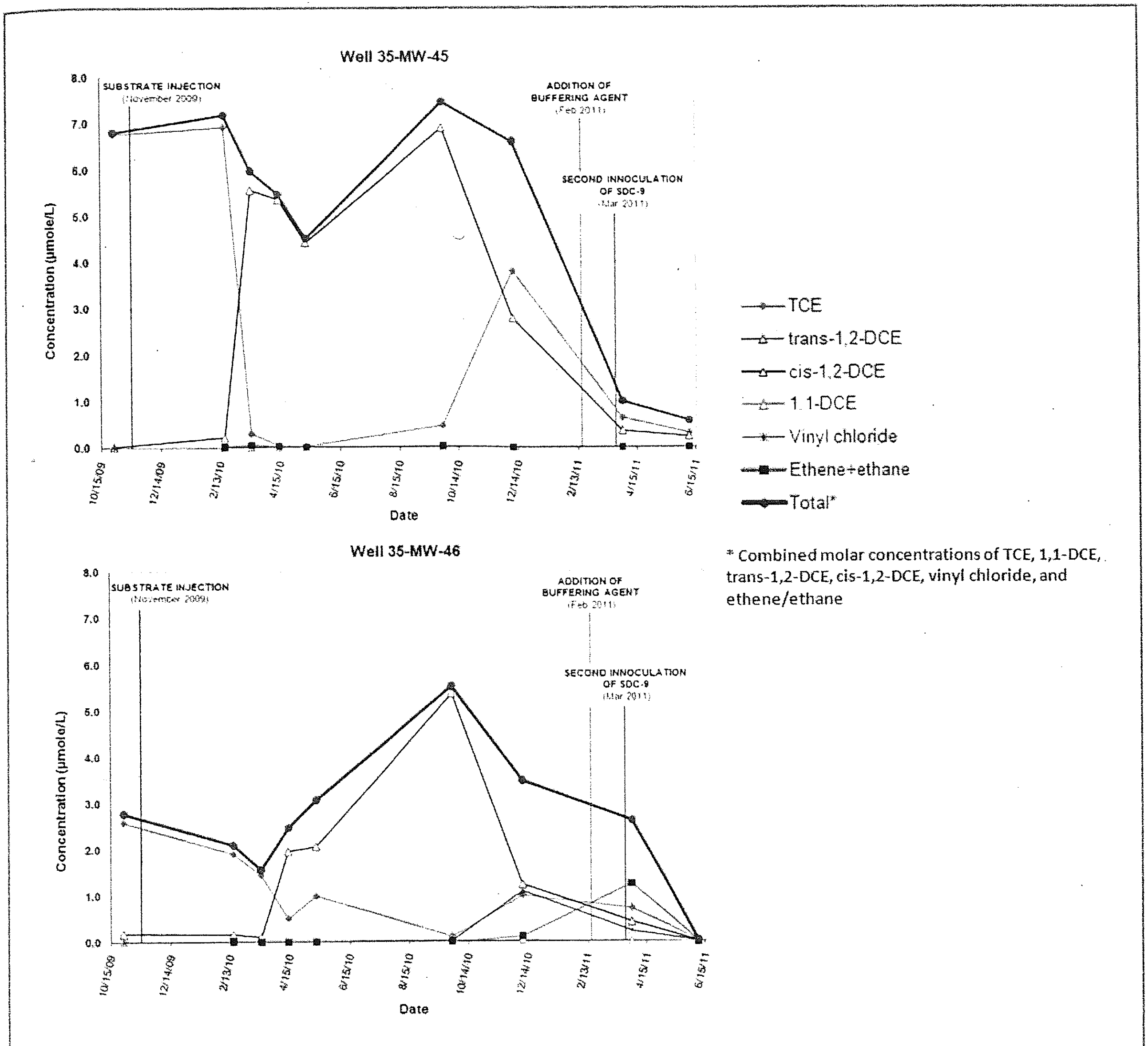

Exhibit 5. Molar concentrations of VOCs at the downgradient monitoring wells from the time of the biobarrier installation until June 2011

As of June 2011, dissolved oxygen concentrations in the biobarrier wells ranged between 0.49 mg/L and 2.36 mg/L, pH ranged between 6.60 and 7.27, and ORP levels ranged between −121 mV and −152 mV. The dissolved oxygen level increases (greater than 0.5 mg/L) in several of the injection wells since the May 2011 sampling event could be the result of using different sampling devices; a portable bladder pump was used during the May sampling and a 2-inch (in.) variable-speed Grundfos® Redi-Flo pump was used during the June sampling. The record rainfall that occurred in California between the May and June sampling events was also considered as contributing to the increased dissolved oxygen levels. According to the County of Santa Barbara Public Works (2011), the area received 1 in. precipitation between the sampling events, possibly recharging the aquifer. The groundwater elevation from May to June rose approximately 1.2 in.

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Although the greater than 0.5 mg/L dissolved oxygen concentrations are not conducive to reductive dechlorination, other factors, such as degradation product increases, methane increases, low ORP, and neutral pH, indicate that the treatment zone conditions are still reducing and the biobarrier performance has improved.

The biobarrier pilot test provided useful design data that were implemented during the full-scale design and construction, such as the final well spacing; however, regular monitoring of the biobarrier prevented complete biobarrier failure. The fouling in the injection wells and the very low pH levels were remedied with well cleaning and biobarrier amendments. Since the biobarrier was amended, it continues to operate as intended by limiting VOC migration.

PATH FORWARD

As of June 2011, the pH levels at all biobarrier wells and both downgradient monitoring wells were within the range that sustains optimal microbial activity. TCE and *cis*-1,2-DCE concentrations continue to decrease in the downgradient monitoring wells despite the continual TCE loading from the source area onto the biobarrier. Ethene has been detected at both downgradient wells since the initial biobarrier treatment, and higher concentrations of ethene appeared in one downgradient well since the second inoculation. Future sampling results may differ since the travel time through the treatment zone has likely increased due to the decrease in hydraulic conductivity as demonstrated during the slug tests.

The total organic carbon concentrations remain relatively high in the injection wells; thus, there are no future plans to inject additional substrate. As of July 2011, the source area removal was still incomplete. If TCE concentrations upgradient of the biobarrier do not decrease, the biobarrier will likely require additional substrate and amendments in the next two years. The next groundwater sampling is scheduled for September 2011.

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