Bio-Dechlor INOCULUM® for Bio Augmentation:

1. Daniel Nunez, Regenesis
2. Soil Born Bacteria Extract Solution (Dehalococcoides ethenogenes, Bio-Dechlor INOCULUM® Plus BDI+ and SDC-9)
3. MSDS & Technical Data Sheet - Attached
4. Number of Field-scale Applications to Date: 1,000+ sites.
5. Case Studies – Attached
6. Bio-Dechlor INOCULUM® PLUS is an enriched natural microbial consortium containing species of *Dehalococcoides* sp. (DHC). This product has been used successfully over the last 10-15 years. This microbial consortium has since been enriched to increase its ability to rapidly dechlorinate contaminants during in situ bioremediation processes. Bio-Dechlor INOCULUM has been shown to stimulate the rapid and complete dechlorination of compounds such as tetrachloroethene (PCE), trichloroethene (TCE), dichloroethene (DCE), and vinyl chloride (VC). The most current culture of Bio Dechlor INOCULUM PLUS(+) now contains microbes capable of dehalogenating halomethanes (e.g. carbon tetrachloride and chloroform) and
   haloethanes (e.g. 1,1,1 TCA and 1,1, DCA) as well as mixtures of these halogenated contaminants. Bio-Dechlor INOCULUM PLUS(+) is provided in a liquid form and is designed to be injected directly into the contaminated subsurface. Once in place, this microbial consortium works to accelerate the extant rate of chlorinated ethane degradation. When faced with an insufficient quantity of critical dechlorinating microbes, Bio-Dechlor INOCULUM PLUS(+) supplies many beneficial chlorinated solvent degraders including the all-important DHC required to achieve complete and rapid dechlorination. This microbial consortium is compatible with most electron donors however it is often optimized with the addition of any of Regenesis' Hydrogen Release Compound (HRC®) products. There are no know health and safety effects on this product.

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.

Bio-Dechlor INOCULUM™ +
MATERIAL SAFETY DATA SHEET (MSDS)

Last Revised: January 5, 2010

Section 1 - Material Identification

Supplier:

REGENESIS

1011 Calle Sombra
San Clemente, CA 92673

Phone: **949.366.8000**

Fax: **949.366.8090**

E-mail: **info@regenesis.com**

Chemical Name:	**Soil Born Bacteria Extract Solution**
Chemical Family:	**Organic Chemical**
Trade Name:	**Bio-Dechlor INOCULUM™ +, BDI+, (SDC-9)**
Product Use:	**Used in the remediation of contaminated groundwater (environmental applications)**

Section 2 – Chemical Information

CAS#	Chemical	
Not Available (NA)	**Soil Bacteria**	**Non Hazardous Ingredients**

Section 3 - Physical Data

Physcial State:	**Liquid**
Boiling Point:	**100ºC**
Flash Point:	**ND**
Density:	**0.9-1.1 g/cc**
Solubility:	**Water**
Appearance:	**Murky Yellow Liquid**
Odor:	**Musty Odor**

Section 4 - Fire and Explosion Hazard Data

Extinguishing Media: **Carbon Dioxide, Water, Foam.**

Water may be used to keep exposed containers cool.

For large quantities involved in a fire, one should wear full protective clothing and a NIOSH approved self contained breathing apparatus with full face piece operated in the pressure demand or positive pressure mode as for a situation where lack of oxygen and excess heat are present.

Section 5 - Health Hazard Data

Handling: **Avoid contact with skin. Avoid contact with eyes.**

In any case of any exposure which elicits a response, a physician should be consulted immediately.

First Aid Procedures

Inhalation:	**Remove to fresh air. If not breathing give artificial respiration. In case of labored breathing give oxygen. Call a physician.**

Section 5 - Health Hazard Data (cont)

Ingestion:	**No effects expected. Do not give anything to an unconscious person. Call a physician immediately.**
Skin Contact:	**Flush with plenty of water. Contaminated clothing may be washed or dry cleaned normally.**
Eye contact:	**Wash eyes with plenty of water for at least 15 minutes, lifting both upper and lower lids. Call a physician.**

Section 6 - Toxicological Information

Acute Effects:	**May be harmful by inhalation, ingestion, or skin absorption. May cause irritation. To the best of our knowledge, the chemical, physical, and toxicological properties of Bio-Dechlor INOCULUM + have not been investigated.**

Section 7 - Reactivity Data

Conditions to Avoid:	**Strong oxidizing agents, bases and acids**
Hazardous Polymerization:	**None known**

Section 8 - Spill, Leak or Accident Procedures

After Spillage or Leakage:	**Neutralization is not required. The area should be disinfected with a 5% bleach solution**
Disposal:	**Laws and regulations for disposal vary widely by locality. Observe all applicable regulations and laws. This material, may be disposed of in a solid waste landfill. Material is readily degradable and hydrolyses in several hours.**

No requirement for a reportable quantity (CERCLA) of a spill is known.

Section 9 - Special Protection or Handling

Should be stored in plastic lined steel, plastic, glass, aluminum, stainless steel, or reinforced fiberglass containers.

Protective Gloves:	**Vinyl or Rubber**
Eyes:	**Splash Goggles or Full Face Shield. Area should have approved means of washing eyes.**
Ventilation:	**General exhaust.**
Storage:	**Store in cool, dry, 4-5 ºC area. Protect from incompatible materials.**

Section 10 - Shipping Information

D.O.T Shipping Name	**No limitations on shipping this material.**

Section 11 - Other Information

This material will degrade in the environment. Materials containing reactive chemicals should be used only by personnel with appropriate chemical training.

The information contained in this document is the best available to the supplier as of the time of writing. Some possible hazards have been determined by analogy to similar classes of material. No separate tests have been performed on the toxicity of this material. The items in this document are subject to change and clarification as more information becomes available.

Bioaugmentation After a Stalled Biostimulation Application

Pawan Sharma
(CDM, Walnut Creek, California)
Al Bourquin
(CDM, Denver, Colorado)

Hypothesis

After a stalled biostimulation test for enhanced reductive dechlorination (ERD) of trichloroethene (TCE), a one-time direct injection event of a bacterial consortium of naturally occurring species of Dehalococcoides can successfully increase the rate of complete ERD of cis-1,2-dichloroethene (cDCE).

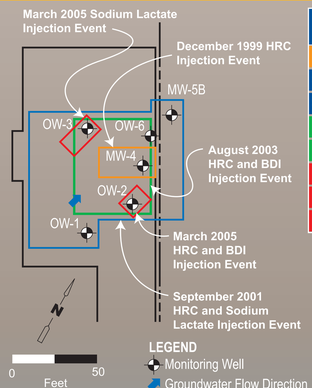
Abstract

Bioaugmentation was tested as an alternative to continued biostimulation of groundwater containing trichloroethene (TCE), cis-1,2-dichloroethene (cDCE), and other chlorinated organic compounds to promote enhanced reductive dechlorination (ERD). Specifically, direct injection of a proprietary bacterial consortium of naturally occurring species of Dehalococcoides: Bio-Dechlor INOCULUM™ (BDI), manufactured and provided by Regenesis, was evaluated. Quantitative real-time polymerase chain reaction (PCR) analysis of groundwater and biotrap samples was used to evaluate effectiveness of the injection procedure and Dehalococcoides activity in the aquifer system.

Background

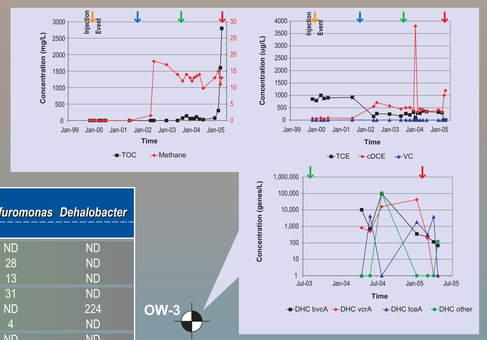
Based on results from a natural attenuation study, an in situ biostimulation test for enhanced reductive dechlorination (ERD) of trichloroethene (TCE) was initiated. The biostimulation test included injection of HRC® and sodium lactate and induced strongly reducing methanogenic conditions. Decreases of TCE concentrations in the test area accompanied increases in concentrations of cis-1,2-dichloroethene (cDCE) and to a lesser degree vinyl chloride (VC). Continued HRC and sodium lactate injections maintained methanogenic conditions, but did not lead to complete reductive dechlorination to ethene.

Injection Events



Date	Material	Injection Area (sq. ft.)	Number of Injections	Injection Interval	Amount per Injection Foot
December 1999	HRC	1200	15	8 to 20 feet	6 pounds
September 2001	HRC	7000	20	8 to 20 feet	6 pounds
September 2001	60% NaLac	7000	20	8 to 20 feet	9 pounds
August 2003	HRC	3800	25	8 to 20 feet	4 pounds
August 2003	BDI	3800	25	8 to 20 feet	0.25 liter
March 2005	HRC	200	5	8 to 20 feet	6 pounds
March 2005	BDI	200	3	8 to 20 feet	0.6 liter
March 2005	60% NaLac	300	6	8 to 20 feet	12 pounds

Test Layout and Monitoring Results



Quantitative PCR Analysis of Biotraps

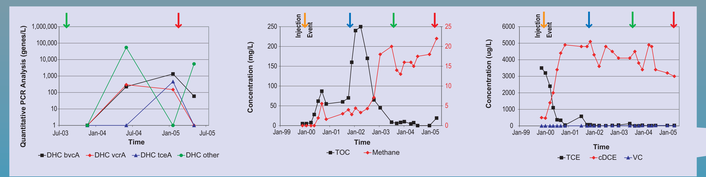
Location	Date	Methanogens	Sulfate & Iron Reducing	*Dehalococcoides*	*Desulfuromonas*	*Dehalobacter*
		Gene Copies Per Bead				
OW-2	Mar-04	434,000	2,880	ND	ND	ND
	Apr-04	3,400,000	27	ND	28	ND
	May-04	608,000	ND	ND	13	ND
OW-3	Mar-04	851,000	ND	ND	31	ND
	Apr-04	2,500,000	160	ND	ND	224
	May-04	427,000	8,450	ND	4	ND
OW-6	Mar-04	211,000	816	ND	ND	ND
	Apr-04	1,420,000	124	ND	19	ND
	May-04	170,000	ND	ND	2	ND

OW-3

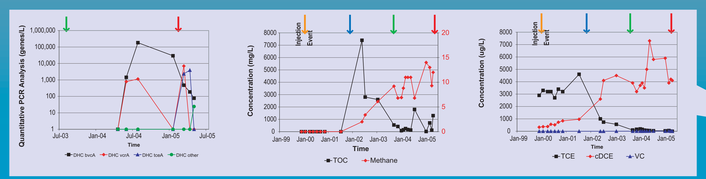
Biotraps were suspended in the wells in Feb 2004 and retreived after 30, 60, and 90 days.

OW-6

Converted BDI injection location.



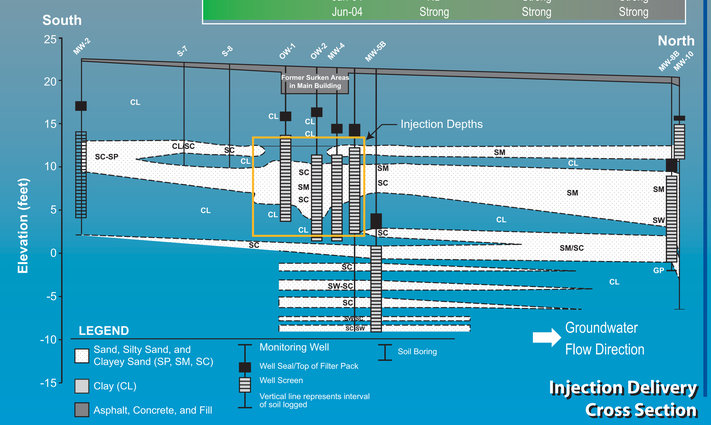
MW-4



OW-2

Qualitative PCR Analysis

Location	Matrix	Date	*Dehalococcoides*	*Desulfuromonas*	*Dehalobacter*
		Before Bioaugmentation			
MW-4	GW	Jul-03	ND	Strong	ND
OW-1	GW	Jul-03	ND	Strong	ND
BDI-6	Soil	Aug-03	ND	Strong	Strong
		After Bioaugmentation			
MW-4	GW	Sep-03	ND	Strong	Strong
		Nov-03	ND	Strong	Strong
		Jan-04	Weak	Strong	Strong
OW-2	GW	Feb-04	Strong	Strong	Strong
		Jun-04	Strong	Strong	Strong
		Jul-04	Strong	Strong	Strong
OW-3	GW	Feb-04	Strong	Strong	Strong
		Jun-04	Strong	Strong	ND
		Jul-04	Strong	Weak	Weak
OW-6	GW	Sep-03	Strong	Strong	Strong
		Nov-03	ND	Strong	Strong
		Jan-04	ND	Strong	Strong
		Jun-04	Strong	Strong	Strong



Injection Delivery Cross Section

Results and Findings

The introduction of *Dehalococcoides* type microbes (those present in the BDI culture) was successful as evidenced by the results from quantitative and qualitative PCR analyses. However, subsequent growth of these microbes to sufficient populations in the aquifer system appears to have been insufficient to accelerate the complete ERD of TCE and cDCE. While the introduction of low populations of dehalorespiring microbes at low concentrations appears to be successful, the bioaugmentation test has resulted in little change in contaminant concentrations.

The causes for this hindered growth were primarily uneven distribution of electron donor, evidenced by varying total organic carbon (TOC) levels, and potentially competition from non-dehalorespiring microbes. The groundwater concentrations of indigenous microbes, at the time of the initial BDI injections, were 1 to 3 orders of magnitude higher than those of *Dehalococcoides* type microbes injected into the groundwater.

If population competition is a factor in the lack of complete ERD, it is not attributed to microbe characteristics or their suitability to the geochemical conditions in the aquifer, but perhaps simply a function of initial population sizes. The injected *Dehalococcoides* mass was insufficient to gain substantial footing in the aquifer's microbial ecosystem in the time period of the test.

Based on the pressence of the *bvcA* gene of *Dehalococcoides*, associated with complete metabolic reductive dechlorination of cDCE and VC to ethene, it is possible that complete dechlorination is currently occurring, but not measurable.

Dehalorespiring microbes can out-compete many anaerobic microorganisms due to a greater enzyme affinity for hydrogen, the electron donor. Therefore, it is expected that complete reductive dechlorination will be evidenced once the *Dehalococcoides* type microbes grow to a suitable population size. This will likely require the injection of additional electron donor.

Two contrasting approaches to DNA sample collection were utilized at the site. One approach was the use of Biotraps that were suspended in monitoring wells in order to allow attachment of indigenous bacteria over a period of 30, 60, and 90 days. The Biotraps were then removed from the wells and DNA was extracted from the attached bacteria. This method is purported to provide a more representative sample of the attached microbial community in the subsurface. The second approach was a DNA extraction directly from groundwater samples using standard methods. Somewhat surprisingly, analysis of samples from the Biotraps never detected *Dehalococcoides*, and frequently did not detect *Desulfuromonas* or *Dehalobacter*. All of these were detected through direct analysis of groundwater. This suggests that while the Biotraps do sample a different portion of the microbial community than groundwater samples, they may be ineffective for detecting the populations of interest.

CDM
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CVOC Remediation Using Bio-Dechlor INOCULUM™ and Diagnostics in a Methanogenic Aquifer

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Pueblo, CO 81006-9330, USA)

ABSTRACT: Chlorinated volatile organic compounds (CVOCs) are common contaminants found across the United States. Enhanced reductive dechlorination is a viable technology for the remediation of CVOCs in groundwater. An appropriate suite of groundwater analyses must be performed to effectively design a remedy for groundwater treatment. Biological testing using molecular biological diagnostics is rapidly becoming part of these protocols. In addition, the use of microorganisms (bioaugmentation) can be part of these advanced operations designed to affect the accelerated degradation of CVOCs.

A treatability study using in-situ reductive dechlorination with Bio-Dechlor INOCULUM™ (BDI), Hydrogen Release Compound® (HRC), and HRC-primer to dechlorinate CVOCs in heterogeneous soil was conducted at a study area, Solid Waste Management Unit (SWMU) 58, at the Pueblo Chemical Depot in Colorado. The treatability study for SWMU 58 was initiated in June 2004, and post injection groundwater monitoring for CVOCs, geochemistry, and microbiological activity was conducted from July 2004 to March 2005.

Cis-1,2-dichloroethene (cDCE) is the only contaminant of concern that exceeds the Colorado Groundwater Quality Standard of 70 micrograms per liter (µg/L) in SWMU 58. cDCE concentrations in groundwater samples, prior to injection, ranged from 22 to 100 µg/L. The HRC, HRC-primer, and BDI pilot study grid design consists of 75 injection points installed in a series of nine rows perpendicular to the groundwater flow direction. HRC-primer was injected into three of the rows while HRC and BDI were injected into separate injection points in the remaining six rows. The direct push subsurface injection probes were advanced to the alluvium/bedrock (aquitard) interface approximately 15 ft below ground surface. HRC, HRC-primer, and BDI were injected into approximately 8 ft of the saturated alluvium.

Based on the last round of sampling, the CVOC concentrations at the site are on a downward trend in areas where methanogens and/or methane levels have lessened. The *Dehalococcoides* (DHC) population, the microbes responsible for complete cDCE and vinyl chloride (VC) degradation, has begun to increase in these areas. Reductive dechlorination continues to stall at VC in areas where methanogenesis is occurring. Methanogens seem to be the dominating group of bacteria during the pilot study due to the existing, strongly methanogenic conditions. DHC and other bacteria, such as sulfate-reducers, increased in population only as the methanogen population decreased, which is likely a result of diminishing hydrogen levels.

INTRODUCTION

Anaerobic reductive dechlorination is a naturally occurring mechanism by which chlorinated hydrocarbons are degraded under anaerobic conditions [without oxygen (O_2)]. Reductive dechlorination rates are highest for more chlorinated compounds such as tetrachloroethene (PCE) and trichloroethene (TCE) and lower for less chlorinated compounds such as cis-1,2-dichloroethene (cDCE), trans-1,2-DCE (tDCE), and vinyl chloride (VC). The effectiveness of reductive dechlorination is dependent on the selection of the most appropriate electron donor to stimulate biodegradation and the presence or addition of the correct consortium of bacteria needed to degrade the chlorinated compounds. The effectiveness is also dependent upon sufficient delivery of the electron donor and any necessary bacteria and obtaining adequate coverage in the contaminated zone. The electron donors used in this pilot study were Hydrogen Release Compound® (HRC) and HRC-primer. Bioaugmentation with Bio-Dechlor INOCULUM™ (BDI) was performed because of low baseline detections of *Dehalococcoides* (DHC) discussed below in the Results section.

Methanogenesis is the production of methane (CH_4) by the fermentation of simple organic carbon compounds or oxidation of hydrogen (H_2) under anaerobic conditions with simultaneous production of carbon dioxide. This process is carried out by methanogens in anaerobic and reductive aquifers. Methanogens are single celled microorganisms, which are members of the superkingdom *Archaea* and are within the phylum Euryarchaeota. *Archaea* are unique because, unlike most life on Earth that relies on oxygen and complex organic compounds for energy, *Archaea* rely on simple organic compounds (e.g., acetate) and H_2 for energy. Methanogenic conditions prevail in many contaminant plumes after all other electron acceptors (O_2, nitrate [NO_3], ferrous iron [Fe^{+3}], and sulfate [SO_4]) have been used up by other members of the subsurface microbial community.

HRC is a commercially available, patented compound developed by Regenesis which releases lactate gradually upon hydration in groundwater. Indigenous anaerobic microorganisms metabolize the lactate, and H_2 is produced as a result. The H_2 is then available for reductive dechlorination processes.

BDI is a highly specialized microbial consortium containing species of DHC, a group of organisms that has been shown to be responsible for the rapid and complete degradation of chlorinated ethenes. BDI includes a strain of DHC identified as BAV1, which was discovered at a contaminated site in Oscoda, Michigan. After researchers at Georgia Institute of Technology observed its ability to rapidly and completely dechlorinate cDCE and VC metabolically instead of cometabolically, they isolated it and further enriched the culture to increase its ability to dechlorinate cDCE and VC. (He, et al., 2003). The resulting culture was then used in BDI.

Solid Waste Management Unit (SWMU) 58, within the South Central Terrace (SCT) of the Pueblo Chemical Depot, consists of alluvial and colluvial deposits that unconformably overlie the Pierre Shale bedrock. Much of the alluvium in SWMU 58 is described as clay, clayey sand, and silt. Unnamed Creek, which flows north to south, bisects the pilot study area. Historic sampling has shown that contamination does not extend past the creek bed and is contained to the east and west by areas of unsaturated alluvium that flank both sides of the plume. Contamination at SWMU 58 consists of a cDCE plume that remains as a result of the incomplete natural degradation of TCE in the

previously excavated source area. Hydraulic conductivities in SWMU 58 range from 7.2-60 ft/day. Historic dissolved oxygen (DO) and oxidation-reduction potential (ORP) measurements indicate a natural anaerobic tendency in the aquifer. The depth to groundwater varies seasonally and can fluctuate up to 5 feet. The groundwater within the SCT flows in a general southwesterly direction and has been historically high in sulfate and nitrate.

The HRC, HRC-primer, and BDI pilot study consists of 75 injection points installed in a series of nine rows perpendicular to the groundwater flow direction (Figure 1). HRC-primer (red) was injected into three of the rows. HRC (blue) and BDI (green) were injected into the remaining six rows in alternating points. The gray dots denote points that were not injected due to obstructions. The direct push subsurface injection probes were advanced to the alluvium/bedrock (aquitard) interface approximately 15 feet (ft) below ground surface. Prior to injection activities, the saturated alluvium thickness was measured at 8 ft. BDI was injected into the saturated alluvium at 0.19 liters/foot (L/ft), HRC was injected at 20 pounds/foot (lbs/ft), and HRC-primer was injected at 8 lbs/ft. The HRC injection depth was decreased on the western portion of the pilot study area due to the decrease in thickness of saturated alluvium. The decrease in saturated alluvium is associated with shallow Pierre Shale bedrock along the upper bank of a paleochannel.

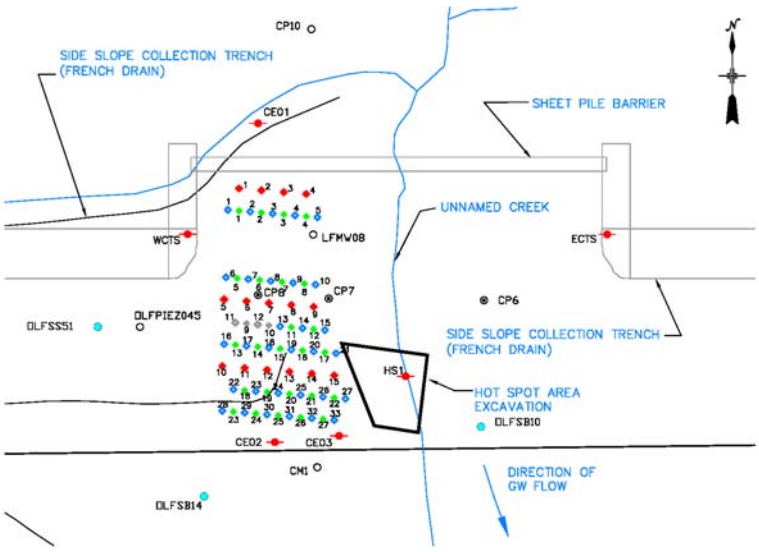

Figure 1: Biostimulation and Bioaugmentation Pilot Study Barrier Design

MATERIALS AND METHODS

Six monitoring wells were sampled at SWMU 58 to assess the pilot study. CE01, an upgradient well, received microbial analysis only during a one-time event to determine unaugmented levels of naturally occurring bacteria. Groundwater was collected from the site using bailer methods during the initial, 1-, and 3-month sampling events. Low-flow micropurge methods were used during the 6-month and 9-month sampling events. The sampling method was changed to increase sampling sensitivity of dissolved gases and chlorinated volatile organic compounds (CVOCs). Bio-traps, or small sampling devices suspended in monitoring wells to collect representative sample of the microbial

community over time, were used in wells CM1, CP8, and LFMW08. Groundwater grab samples were collected from wells CE02 and CE01 to collect microbiological samples. Grab samples were collected in these wells because they are extraction wells. The bio-traps would not be able to be placed down the well because pumps are in the well.

Groundwater samples from six monitoring wells, CM1, CP7, CP8, CE02, CE03, and LFMW08, were analyzed for CVOCs in all sampling events. Four of the seven wells, CM1, LFMW08, CP7, and CP8, were sampled for total organic carbon (TOC), metabolic acid, ethene, ethane, methane, nitrate, nitrite, sulfide, sulfate, dissolved manganese and iron, and CVOCs during the 6- and 9-month sampling events. Three of the seven wells, CM1, LFMW08, and CP8, received microbial analyses. Real-time quantitative Polymerase Chain Reaction (qPCR) tests were performed to quantify the presence of "Universal Bacteria" (eBAC), methanogens (MGN), DHC, sulfate and iron reducing bacteria (SRB/IRB), and the *bvcA* gene. Microbial analysis was performed on samples from CE02 during the 6- and 9-month sampling events and on CE01 during the 9-month sampling event. Additional test methods and wells were included in the later sampling events to aid in understanding the aquifer conditions during the pilot study. Microbiological, geochemical, and organic acid samples were preserved by chilling to 4°C. All samples were shipped overnight by courier under chain-of-custody.

The bio-traps and grab samples were submitted to Microbial Insights for microbial analysis. Dissolved manganese and iron were analyzed using field test kits. All other samples were submitted to Kemron Laboratories for analysis. DO, ORP, pH, conductivity, and temperature were measured with field meters.

RESULTS AND DISCUSSION

Initial sampling done in February 2004 indicated non-detect (ND) DHC levels at CP8 and low DHC counts at CM1 (Table 1). The original bio-traps were later analyzed for MGN and eBAC after high methane concentrations were discovered during the pilot study. Results showed that MGN levels were extremely high in CP8 and CM1 (see Table 1) prior to the injection of HRC, indicating that aquifer conditions were strongly methanogenic prior to the HRC and BDI application.

The December 2004 microbial analysis of CE01, an upgradient monitoring well, showed high levels of eBAC, SRB/IRB, and MGN. The *bvcA* gene was ND and DHC levels were very low. This is to be expected, as the site is naturally low in DHC, and CE01 should not have been affected by the BDI and HRC application. The microbial community in CE01 appears to be similar to the community present in CP8 prior to the injection of HRC and BDI.

Real-time qPCR testing also revealed notable changes in the microbial communities during this study. eBAC results indicated that biomass remained fairly stable over the study period at ~10^{6-7}cells/bead for all locations, which is typical for most sites. Notable exceptions to this were observed in samples LFMW08 and CP8 where eBAC increased ~1 order of magnitude between the initial February 2004 event and the August 2004 sampling event. Marked decreases (~3 orders of magnitude) in eBAC were seen in LFMW08 and CE02 between February and August. SRBs/IRBs increased over time at all locations. The abundance of MGNs and of DHC was more variable over time. It did appear, however, that there may be an inverse relationship between MGNs and DHC at the site as some of the highest levels of DHC observed in the wells correlate to some of

the lowest levels of MGNs at the site, and DHC levels tended to increase when MGN levels went down (although not in all sampling events). qPCR results are presented in Table 1, and are briefly summarized below.

Decreases in levels of eBAC, BVC, MGN, and DHC and increases in SRB/IRB were observed in LFMW08 (located within treatment area) during the 3- to 6-month monitoring periods. CP8, also located within the treatment area, displayed an increase in eBAC, SRB/IRB, and MGN levels and a decrease in levels of *bvcA* and DHC throughout the 9-month pilot study. Decreases in eBAC, *bvcA*, and MGN levels and increases in SRB/IRB and DHC levels were observed in CM1 (downgradient of treatment area) during the pilot study. CE02 showed a decrease in all bacteria except SRB/IRB from 6 months to 9 months. The *bvcA* gene was ND at CE02, located downgradient of the treatment area, during this time period. Although movement of DHC has been observed on some sites, BAV1 is a sessile organism that lives on the surface of soil particles. Therefore it is not surprising that increases in the BAV1 population were not observed in CM1 and CE02, which are both downgradient monitoring wells. Although an increase in total DHC were observed in CM1, this could be a result of biostimulation and not the bioaugmentation application.

Table 1: qPCR Analysis Results During Pilot Study

Well	Units	Month After Injection	Universal Bacteria (eBAC)	Sulfate and Iron Reducing Bacteria (SRB/IRB)	*bvcA* gene (BVC)	Methanogens (MGN)	*Dehalococcoides* spp. (DHC)
LFMW08	gen/bd	3	7.03E+07	NS	NS	4.57E+06	ND
	gen/bd	6	5.85E+07	3.79E+04	9.58	5.73E+05	6.11E+02
	gen/bd	9	3.24E+04	1.14E+06	ND	ND	ND
CP8	gen/bd	initial	4.71E+06	NS	NS	1.91E+05	ND
	gen/bd	3	7.03E+07	NS	NS	2.19E+06	ND
	gen/bd	6	3.94E+07	4.51E+04	4.37	8.96E+05	3.48E+02
	gen/bd	9	8.40E+07	7.00E+05	ND	5.14E+06	3.49E+01
CM1	gen/bd	initial	1.27E+07	NS	NS	1.03E+05	1.00E+02
	gen/bd	3	1.11E+07	NS	NS	2.03E+03	ND
	gen/bd	6	3.03E+07	1.62E+03	6.44	1.84E+06	4.17E+02
	gen/bd	9	1.91E+07	2.89E+04	ND	2.21E+04	2.19E+03
CE02	gen/ml	6	8.23E+06	1.81	ND	6.92E+03	1.12E+01
	gen/ml	9	2.43E+03	3.76E+03	ND	ND	9.98E-01
CE01	gen/ml	9	1.27E+07	5.64E+08	ND	2.57E+06	2.38E+01

Six groundwater monitoring wells were sampled and analyzed for CVOCs, including TCE, cDCE, tDCE, and VC, prior to injection activities and 1 month, 3 months, 6 months, and 9 months after injection activities (Table 2). Most of the trends discussed below are based upon the more recent sampling events. Additional sampling events are expected to confirm these trends.

In CE02, all CVOC concentrations increased during the pilot study. CE03 has shown a decrease in TCE and cDCE and a corresponding increase in VC throughout the pilot study. A slight decline in tDCE is shown in the 9-month data after concentrations steadily increased during the 6-month sampling event. TCE concentrations in CM1 are generally stable, although cDCE concentrations have dropped an order of magnitude during the

pilot study. tDCE and VC show the beginning of a downward trend during the 9-month sampling event after peaking during the 6-month sampling event. CP7 displayed decreasing trends in all CVOCs during the pilot study. TCE concentrations for CP8 lowered to ND, cDCE concentrations steadily increased until after the 6th month when downward trends began, and tDCE and VC concentrations increased throughout the pilot study. LFMW08 has shown a recent decrease in TCE after the 6th month and in cDCE, tDCE, and VC after the 3rd month. It is possible there was an influx of mass prior to the 6-month sampling event, as chlorinated solvent concentrations increased in several wells during this time. It is also possible that these increases could have been due to mobilization of sorbed-phase mass during the physical injection, although these increases are typically observed right after the injection and are usually gone within a few months after the injection. Rising groundwater levels may have also contributed to the mobilization of sorbed-phase mass into the groundwater. Extraction wells, CE02 and CE03, were turned off prior to injection activities, which may also be contributing to mass increases.

TABLE 2: TCE, cDCE, tDCE, and VC Concentrations in µg/L

Well ID	Month After Injection	TCE	cDCE	tDCE	VC
CE02	initial	1.5	100	47	1.3
	1	1.0	120	49	3.1
	3	3.5	250	103	6.8
	6	5.9	400	150	14
	9	12	510	168	35
CE03	initial	1.4	22	8.0	0.25
	1	2.1	64	20	0.93
	3	2.0	52	33	32
	6	0.58	8.8	24	65
	9	0.48	5.7	21	81
CM1	initial	0.32	41	15	0.44
	1	ND	41	12	ND
	3	0.8	44	18	10
	6	0.33	4.9	22	84
	9	0.46	2.3	17	46

Well ID	Month After Injection	TCE	cDCE	tDCE	VC
CP7	initial	2	56	14	2.1
	1	3.8	74	21	1.7
	3	1.6	98	25	12
	5	2.1	21	6.8	0.94
	9	1.6	14	4.0	0.35
CP8	initial	15	57	32	0.52
	1	8.6	120	67	0.99
	3	7.5	52	27	0.47
	6	1.1	200	77	2.0
	9	ND	140	97	43
LFMW08	initial	2.1	63	17	4.1
	1	3.5	35	7.6	0.40
	3	4.2	91	23	15
	6	4.5	58	14	6.3
	9	3.7	3	7.3	3.5

Table 3 summarizes the results for dissolved gases, anions, and TOC concentrations found during the pilot study. None of the wells had significant nitrate, nitrite, or sulfide concentrations throughout the pilot study. TOC levels decreased in the most recent sampling event in each of the monitoring wells sampled, and like the decrease in fermenting organisms, this can likely be attributed to the depletion of HRC-primer, which is a quickly-fermentable carbon source designed to remove large amounts of competing electron acceptors from the subsurface to allow the H_2 released slowly by HRC to be used by reductive dechlorinators for chlorinated solvent degradation. The typical longevity of the HRC-primer applied at the site is estimated to be approximately 3-4

months, at which time the amount of carbon delivered to the system should have declined along with the concentration of TOC.

Sulfate concentrations have generally increased in CM1, LFMW08, and CP7. CP8 was the only well sampled for sulfate that displayed a decrease in concentration. Low levels of ethane and ethene were detected in all wells except CM1. Although concentrations of ethane and ethene in CM1 decreased from 6th month to the 9th month, it is interesting to note that CM1 also has the highest levels of DHC observed at the site. Methane concentrations in CP7 decreased during the pilot study. Methane concentrations in CM1 and LFMW08 peaked after 6 months and decreased by 9 months. This data correlates with the qPCR data, as methanogen levels decreased in each of these wells by several orders of magnitude from the 6-month to the 9-month sampling event. CP8 displayed an increase in methane concentration from the 6-month sampling event to the 9-month sampling event. CP7 methane concentrations decreased from the 6th month to the 9th month.

Table 3: Dissolved Gases, Anions, and TOC Concentrations

Well ID	Month After Injection	Methane (µg/L)	Ethane (ug/L)	Ethane (ug/L)	Nitrates (mg/L)	Nitrite (mg/L)	Sulfates (mg/L)	Sulfides (mg/L)	TOC (mg/L)
CM1	Initial	3.3	ND	ND	NS	NS	NS	NS	NS
	1	13	ND	ND	NS	NS	NS	NS	NS
	3	280	ND	ND	NS	NS	NS	NS	NS
	6	5860	4.7	4.3	ND	ND	16	17	160
	9	3150	2.0	1.2	ND	ND	270	8.2	49
LFMW08	Initial	24	ND	ND	NS	NS	NS	NS	NS
	1	2	ND	ND	NS	NS	NS	NS	NS
	3	52	0.63	ND	NS	NS	NS	NS	NS
	6	160	0.34	ND	0.11	ND	570	5.1	160
	9	140	ND	ND	0.15	ND	840	2.8	53
CP8	6	280	ND	ND	ND	ND	270	0.9	160
	9	570	0.85	0.70	ND	ND	120	ND	75
CP7	6	120	ND	ND	ND	ND	560	0.9	26
	9	3.0	ND	ND	ND	ND	840	ND	10

CONCLUSIONS

The high loading rate of HRC-primer and HRC, injected into the saturated alluvium, increased methanogenic activity in some of the wells in the existing methanogenic aquifer. It has been shown that methanogenic activity increases in proportion to higher H_2 concentrations (Hemond and Fechnew, 1994). After H_2 levels are reduced to a "threshold" concentration, methanogenesis becomes less favorable (Wright and Cox, 2004). Most of the wells have shown reduction of TCE and DCE to VC under methanogenic conditions. In wells where methanogens and/or methane concentrations decreased (CM1, LFMW08, and CP7) VC has begun, per the last sampling round, to decrease and the DHC population has begun to increase. This observation may demonstrate the ability of DHC to compete with methanogens at lower H_2 levels in areas where existing methanogenic conditions exist.

VC levels remain relatively constant in areas where methanogens still dominate and where methane levels continue to increase. DHC populations in these areas have decreased with increasing methanogen populations. Methanogens easily dominate DHC when H_2 levels are high and disrupt the reductive dechlorination process (Kean et. al., 2003).

Sulfate-reducing bacteria have not yet shown to hinder the remediation of VC in this study, despite previous studies that have resulted in the inhibition of VC remediation (Weidemeier et al., 1998). In fact, a lot of the 'conventional wisdom" that dictates sulfate inhibits reductive dechlorination is being challenged. The problem is more related to the production of toxic sulfide as the by-product of sulfate reduction. This can be "neutralized" by several factors including the fugacity of sulfide gases and the formation of metal sulfides. The concept that sulfate reduction "captures" all available electrons as a means of inhibition is spurious. As methane and sulfate concentrations continue to dwindle, VC concentrations are expected to decrease.

Bioaugmentation has served as an important supplementary technology on this project and the use of the diagnostic tools as described was important in site management.

ACKNOWLEDGEMENTS

The authors would like to thank the Regenesis staff, especially Mr. Todd Herrington and Dr. Stephen Koenigsberg, for reviewing and commenting on this paper and for providing additional groundwater monitoring analysis for the pilot study. We would also like to thank Mr. Greg Davis with Microbial Insights for his review.

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Bioaugmentation After a Stalled Biostimulation Application

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Hypothesis

After a stalled biostimulation test for enhanced reductive dechlorination (ERD) of trichloroethene (TCE), a one-time direct injection event of a bacterial consortium of naturally occurring species of Dehalococcoides can successfully increase the rate of complete ERD of cis-1,2-dichloroethene (cDCE).

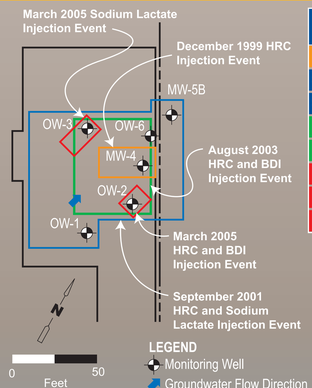
Abstract

Bioaugmentation was tested as an alternative to continued biostimulation of groundwater containing trichloroethene (TCE), cis-1,2-dichloroethene (cDCE), and other chlorinated organic compounds to promote enhanced reductive dechlorination (ERD). Specifically, direct injection of a proprietary bacterial consortium of naturally occurring species of Dehalococcoides: Bio-Dechlor INOCULUM™ (BDI), manufactured and provided by Regenesis, was evaluated. Quantitative real-time polymerase chain reaction (PCR) analysis of groundwater and biotrap samples was used to evaluate effectiveness of the injection procedure and Dehalococcoides activity in the aquifer system.

Background

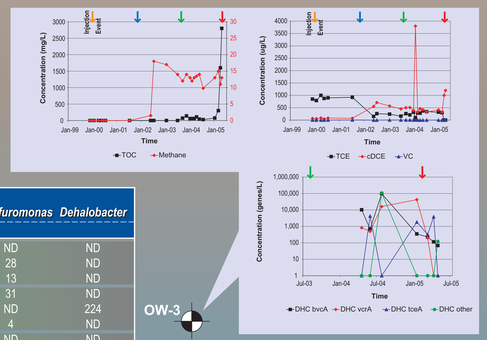
Based on results from a natural attenuation study, an in situ biostimulation test for enhanced reductive dechlorination (ERD) of trichloroethene (TCE) was initiated. The biostimulation test included injection of HRC® and sodium lactate and induced strongly reducing methanogenic conditions. Decreases of TCE concentrations in the test area accompanied increases in concentrations of cis-1,2-dichloroethene (cDCE) and to a lesser degree vinyl chloride (VC). Continued HRC and sodium lactate injections maintained methanogenic conditions, but did not lead to complete reductive dechlorination to ethene.

Injection Events



Date	Material	Injection Area (sq. ft.)	Number of Injections	Injection Interval	Amount per Injection Foot
December 1999	HRC	1200	15	8 to 20 feet	6 pounds
September 2001	HRC	7000	20	8 to 20 feet	6 pounds
September 2001	60% NaLac	7000	20	8 to 20 feet	9 pounds
August 2003	HRC	3800	25	8 to 20 feet	4 pounds
August 2003	BDI	3800	25	8 to 20 feet	0.25 liter
March 2005	HRC	200	5	8 to 20 feet	6 pounds
March 2005	BDI	200	3	8 to 20 feet	0.6 liter
March 2005	60% NaLac	300	6	8 to 20 feet	12 pounds

Test Layout and Monitoring Results



Quantitative PCR Analysis of Biotraps

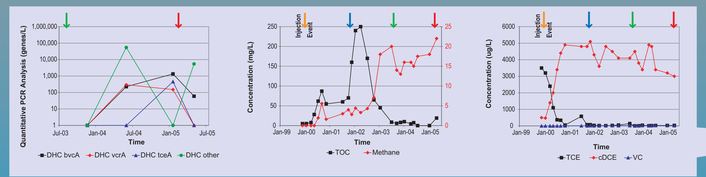
Location	Date	Methanogens	Sulfate & Iron Reducing	*Dehalococcoides*	*Desulfuromonas*	*Dehalobacter*
		Gene Copies Per Bead				
OW-2	Mar-04	434,000	2,880	ND	ND	ND
	Apr-04	3,400,000	27	ND	28	ND
	May-04	608,000	ND	ND	13	ND
OW-3	Mar-04	851,000	ND	ND	31	ND
	Apr-04	2,500,000	160	ND	ND	224
	May-04	427,000	8,450	ND	4	ND
OW-6	Mar-04	211,000	816	ND	ND	ND
	Apr-04	1,420,000	124	ND	19	ND
	May-04	170,000	ND	ND	2	ND

OW-3

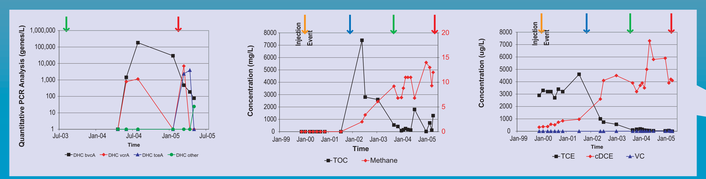
Biotraps were suspended in the wells in Feb 2004 and retreived after 30, 60, and 90 days.

OW-6

Converted BDI injection location.



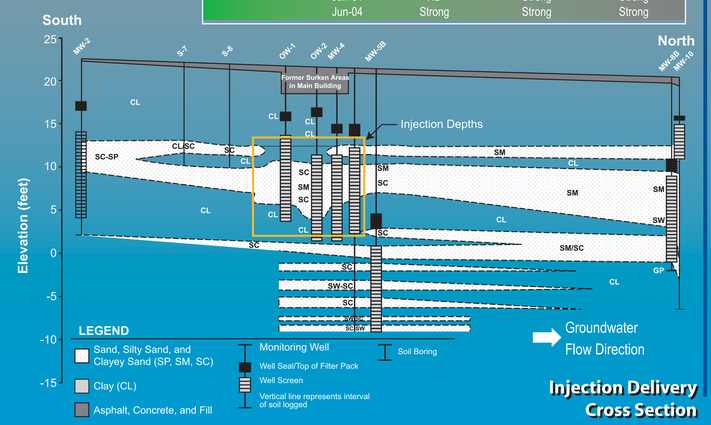
MW-4



OW-2

Qualitative PCR Analysis

Location	Matrix	Date	*Dehalococcoides*	*Desulfuromonas*	*Dehalobacter*
		Before Bioaugmentation			
MW-4	GW	Jul-03	ND	Strong	ND
OW-1	GW	Jul-03	ND	Strong	ND
BDI-6	Soil	Aug-03	ND	Strong	Strong
		After Bioaugmentation			
MW-4	GW	Sep-03	ND	Strong	Strong
		Nov-03	ND	Strong	Strong
		Jan-04	Weak	Strong	Strong
OW-2	GW	Feb-04	Strong	Strong	Strong
		Jun-04	Strong	Strong	Strong
		Jul-04	Strong	Strong	Strong
OW-3	GW	Feb-04	Strong	Strong	Strong
		Jun-04	Strong	Strong	ND
		Jul-04	Strong	Weak	Weak
OW-6	GW	Sep-03	Strong	Strong	Strong
		Nov-03	ND	Strong	Strong
		Jan-04	ND	Strong	Strong
		Jun-04	Strong	Strong	Strong



Injection Delivery Cross Section

Results and Findings

The introduction of *Dehalococcoides* type microbes (those present in the BDI culture) was successful as evidenced by the results from quantitative and qualitative PCR analyses. However, subsequent growth of these microbes to sufficient populations in the aquifer system appears to have been insufficient to accelerate the complete ERD of TCE and cDCE. While the introduction of low populations of dehalorespiring microbes at low concentrations appears to be successful, the bioaugmentation test has resulted in little change in contaminant concentrations.

The causes for this hindered growth were primarily uneven distribution of electron donor, evidenced by varying total organic carbon (TOC) levels, and potentially competition from non-dehalorespiring microbes. The groundwater concentrations of indigenous microbes, at the time of the initial BDI injections, were 1 to 3 orders of magnitude higher than those of *Dehalococcoides* type microbes injected into the groundwater.

If population competition is a factor in the lack of complete ERD, it is not attributed to microbe characteristics or their suitability to the geochemical conditions in the aquifer, but perhaps simply a function of initial population sizes. The injected *Dehalococcoides* mass was insufficient to gain substantial footing in the aquifer's microbial ecosystem in the time period of the test.

Based on the pressence of the *bvcA* gene of *Dehalococcoides*, associated with complete metabolic reductive dechlorination of cDCE and VC to ethene, it is possible that complete dechlorination is currently occurring, but not measurable.

Dehalorespiring microbes can out-compete many anaerobic microorganisms due to a greater enzyme affinity for hydrogen, the electron donor. Therefore, it is expected that complete reductive dechlorination will be evidenced once the *Dehalococcoides* type microbes grow to a suitable population size. This will likely require the injection of additional electron donor.

Two contrasting approaches to DNA sample collection were utilized at the site. One approach was the use of Biotraps that were suspended in monitoring wells in order to allow attachment of indigenous bacteria over a period of 30, 60, and 90 days. The Biotraps were then removed from the wells and DNA was extracted from the attached bacteria. This method is purported to provide a more representative sample of the attached microbial community in the subsurface. The second approach was a DNA extraction directly from groundwater samples using standard methods. Somewhat surprisingly, analysis of samples from the Biotraps never detected *Dehalococcoides*, and frequently did not detect *Desulfuromonas* or *Dehalobacter*. All of these were detected through direct analysis of groundwater. This suggests that while the Biotraps do sample a different portion of the microbial community than groundwater samples, they may be ineffective for detecting the populations of interest.

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Biostimulation and Bioaugmentation of Recalcitrant VOCs in Groundwater using Hydrogen Release Compound (HRC®) and Bio-Dechlor Inoculum™ (BDI)

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ABSTRACT: In May, 2004, biostimulation/bioaugmentation using Hydrogen Release Compound (HRC®) and Bio-Dechlor Inoculum™ (BDI) was pilot tested to evaluate the effectiveness of the technology to degrade chlorinated volatile organic compounds (VOCs) in groundwater within a shallow perched sand and silty sand deposit at a commercial treatment, storage, disposal (TSD) facility in Ohio. Natural processes at the site have led to reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE), but have also resulted in a build up of cis-1,2-dichloroethene (cis-1,2-DCE) and vinyl chloride (VC). Pilot testing to accelerate the degradation of cis-1,2-DCE and VC involved injecting HRC/BDI into the perched unit in an area immediately upgradient of a monitor well containing cis-1,2-DCE, VC, and 1,1-dichloroethane (1,1-DCA), and monitoring groundwater at this location and downgradient of the injection area. The combined use of biostimulation and bioaugmentation during pilot testing was successful in accelerating the rate of cis-1,2-DCE and VC degradation in the vicinity of the injection area and downgradient of the injection area within the 133 day monitoring period. However, 1,1-DCA, a compound which the injected BDI microbial consortium has not been reported to metabolize, did not appear to be affected by the injection. These results indicate that the rapid biodegradation that took place at the site during the pilot test may be directly attributed to bioaugmentation and not just biostimulation alone.

INTRODUCTION

In-situ accelerated bioremediation of chlorinated VOCs in groundwater has evolved into a widely accepted remediation technology. These anaerobically mediated bioremediation efforts often focus on increasing the amount of electron donors present in the aquifer via biostimulation, and/or increasing the numbers of key microorganisms involved in certain steps of the degradation process through bioaugmentation. At many sites with PCE and/or TCE contamination in groundwater, natural processes have resulted in the reductive dechlorination of these compounds to dichloroethene (DCE) isomers dominated by cis-1,2-DCE. While biostimulation can be effective in accelerating the process past cis-1,2-DCE, biostimulation alone can sometimes lead to slow or incomplete degradation of both cis-1,2-DCE and VC. In such situations, bioaugmentation can be used to accelerate the rate of the final steps of the reductive dechlorination process through to ethene and reduce the time that it takes to reach remedial goals. This study documents the combined field application of biostimulation and bioaugmentation to effectively accelerate the complete dechlorination of these otherwise recalcitrant contaminants in groundwater at a commercial TSD facility in Ohio.

Site Description. The facility is located on the margin of a buried valley aquifer system consisting of unconsolidated deposits of coarse grained highly permeable glacial outwash separated by finer grained, low permeability glacial till. Beneath the facility, groundwater is present within two regional aquifers and a near surface, localized perched unit, termed the Upper Outwash. The Upper Outwash occurs within a channel feature in the glacial till (Figure 1), a portion of which is present beneath the operations area of the facility.

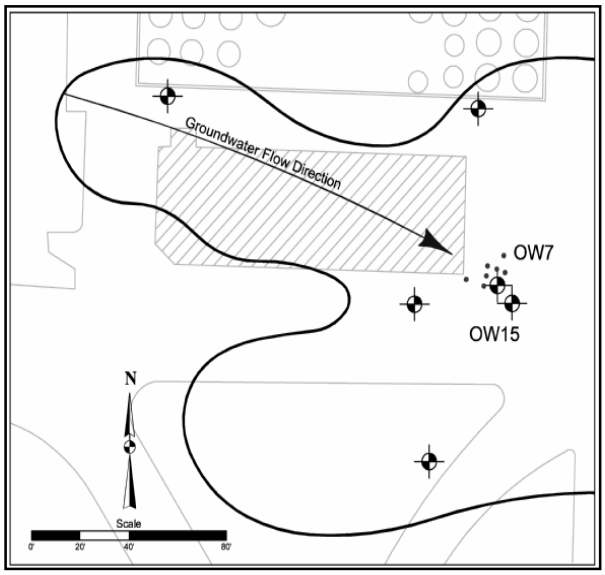

FIGURE 1. Location of injection points relative to monitor wells OW7 and OW15.

Lithologically, the Upper Outwash consists of a mixture of well graded medium sand and silty sand. Groundwater flow direction within the Upper Outwash is to the southeast. At its downgradient edge, groundwater infiltrates downward to the uppermost regional aquifer. Based on pneumatic slug testing, the average hydraulic conductivity within the Upper Outwash was calculated as 3.68×10^{-5}cm/sec. Using the calculated range of hydraulic conductivity values, the measure hydraulic gradient range, and estimated effective porosity values, groundwater flow velocity in the Upper Outwash has been calculated to range from 2.9×10^{-7}cm/sec to 5.7×10^{-5}cm/sec.

As the result of historical releases of both separate phase and dissolved phase chlorinated solvents, VOCs are present in the glacial till underlying the operations area of the facility. The VOCs in the glacial till act as a continuing source of VOC contamination to the underlying Upper Outwash, and to the uppermost regional aquifer through both the Upper Outwash and the base of the glacial till. Cis-1,2-DCE is the most commonly detected VOC in the Upper Outwash, followed by 1,1-DCA, VC, TCE, and chloroethane. Intrinsic degradation analysis conducted during an earlier phase of investigation indicated that reducing conditions existed in the Upper Outwash that might be favorable for reductive dechlorination. These conditions were evident in part based on oxidation-reduction potential (ORP) readings of near zero and slightly negative and the presence of cis-1,2-DCE and VC in groundwater.

Background. Investigation and remediation at the facility is being conducted under the RCRA Corrective Action program. Based on the detection of VOCs below maximum contaminant levels (MCLs) in groundwater in the uppermost regional aquifer, the facility voluntarily implemented interim measures under the Corrective Action Program to expeditiously address the source of VOC contamination and prevent further migration of VOCs into the regional aquifer. A secondary objective was to design and conduct interim measures capable of being the final Corrective Action remedy. Following the collection of additional data to further define the nature and extent of contamination and evaluate contaminant fate and transport, a streamlined Corrective Measures Study (CMS) was

conducted to select a remedial alternative capable of meeting the objectives of the interim measure. As part of the streamlined CMS, in-situ biostimulation/bioaugmentation using HRC and BDI was pilot tested as a potential technology to degrade VOCs in groundwater in the Upper Outwash prior to its discharge into the underlying regional aquifer.

MATERIALS AND METHODS

HRC is a polylactate ester, manufactured by Regenesis Bioremediation Products (Regenesis) that is specifically designed to slowly release lactic acid upon contact with water. The lactic acid is then metabolized by indigenous fermenting microbes to produce hydrogen, which is used in the reductive dechlorination process.

BDI is an enriched microbial consortium, also distributed by Regenesis, that contains species of *Dehalococcoides* (DHC). The consortium includes strain BAV1, an organism that can complete the final stages of chlorinated ethene degradation from the dichloroethene step through ethene metabolically and thus gain energy from each step of this process. This is considered to be a superior feature relative to other strains that can only degrade VC co-metabolically. BDI was developed in the lab specifically to address the more recalcitrant cis-1,2-DCE and VC compounds. Originally obtained from the Bachman Road Site in Oscoda, Michigan, the culture has since been optimized by Dr. Frank Löeffler at Georgia Tech to increase its dechlorinating ability. While bioaugmentation with BDI is often used to introduce DHC to sites where the dechlorinating organisms may be absent or present in very low levels, the addition of BDI also can be used to increase the numbers of native DHC within an aquifer, thereby increasing the rate at which degradation of chlorinated VOCs occurs (He, et al, 2003).

Pilot testing involved injection of HRC/BDI into the Upper Outwash in an area immediately upgradient of a monitor well (OW7) containing cis-1,2-DCE, VC, and 1,1-DCA and monitoring the groundwater at this well and an additional newly installed well (OW15) 12ft (3.6m) downgradient of the area of injection (Figure 1). Monitor well OW15 was installed specifically to evaluate the downgradient affect of the pilot testing at a distance that would be traveled in a three to six month period at the higher calculated range of Upper Outwash flow velocities. To establish baseline (pre-injection) conditions, OW7 was sampled on April 16, 2004, twenty days prior to HRC/BDI injection. During this and subsequent sampling events, groundwater was analyzed for Appendix IX VOCs, nitrate, sulfate, dissolved gasses, total iron and manganese, organic acids, temperature, pH, dissolved oxygen, and ORP. Enumeration of DHC was accomplished by placing Bio-traps into the wells for a period of three weeks to a month. Bio-traps, developed by Microbial Insights in Rockford, Tennessee, are small plastic tubes containing colonizable Nomex beads which are designed to provide an integrated picture of the microbial community over time. Enumeration of native DHC was accomplished by placing a Bio-trap into OW7 20 days prior to injection. During the pilot test monitoring period, new Bio-traps were installed in OW7 and OW15 during each monitoring event. Microbial DNA was extracted from the Bio-trap and the amount of DHC present within the sample was measured using Bio-Dechlor CENSUS^{SM}, a unique and rapid assay for the quantitative detection of DHC. Bio-Dechlor CENSUS utilizes real-time Polymerase Chain Reaction (PCR) to quantify the number of target organisms present within a sample by measuring the amount of fluorescence produced during the DNA amplification step. The test can target the 16S rRNA gene sequence, a highly conserved region of DNA

common to all microorganisms. Targeting this sequence allows the total number of DHC present within a sample to be quantified but does not allow distinguishing among the different strains of DHC because the 16S rRNA gene sequence of each DHC strain is very similar. Because not all strains of DHC are involved in the reductive dechlorination process, "functional genes" also were targeted in order to determine if the organisms present were carrying out the desired dechlorination tasks. One of the genes targeted was the *bvcA* gene, a gene shown to be present in the BAV1 strain and directly involved in the VC to ethene step of the dechlorination process. This gene has also been shown to be absent in all strains of DHC that fail to metabolize VC (Krajmalnik-Brown, et al, 2004).

On May 6, 2004 (day 0), HRC and BDI were injected immediately upgradient of OW7 using Geoprobe direct-push equipment into side-by-side injection points at seven locations each. Figure 1 shows the locations of the injection points relative to OW7 and OW15. The application rate for HRC was 4lbs/ft (5.9kg/m) across a 5ft (1.5m) treatment thickness [3ft (0.9m) of saturated and 2ft (0.6m) of unsaturated thickness]. An application rate of 0.1L/ft (0.32L/m) of the undiluted culture across a similar treatment thickness was used for the BDI. Post-injection sampling of both OW7 and OW15 was conducted 32, 63, 84, 112 and 133 days after injection. In each event, the parameters analyzed were the same as those analyzed in the pre-injection sampling of OW7 (day -20). Pre-injection sampling of OW15 was not conducted, as this well was not installed at the time pre-injection sampling took place.

RESULTS AND DISCUSSION

Analytical results for select parameters from pre and post-injection samples from OW7 and OW15 are summarized on Tables 1 and 2, respectively.

Table 1. Summary of select analytical and field results during pilot testing at OW7.

Parameter	Days	-20	32	63	84	112	133
Cis-1,2-Dichloroethene	µg/l	2500	1600	2500	350	93	<1
Vinyl Chloride	µg/l	1700	630	2500	2000	1400	400
Ethene	µg/l	110	55	500	410	350	990
1,1-Dichloroethane	µg/l	140	94	220	220	170	200
Dehalococcoides spp.	g/b	120	51,900	9,490	17,300	153,000	192,000
bvcA gene	g/b	ND	ND	176	37.2	227	204
Organic Acids (total)	mg/l	8	13.6	24.3	751.2	450.4	421.8
Nitrate	mg/l	0.020	2.7	<0.10	<0.10	<0.10	<0.10
Sulfate	mg/l	17.0	45.4	1.4	0.88	0.72	0.35
ORP (field data)	mV	2	-181	-191	-146	-149	-181

Analytical results and interpretation of the results are discussed below for VOCs, Bio-Dechlor Census, and organic acids.

Table 2: Summary of select analytical and field results during pilot testing at OW15.

Parameter	Days	32	63	84	112	133
Cis-1,2-Dichloroethene	µg/l	840	83	17	2.8	<2.5
Vinyl Chloride	µg/l	<40	22	4.6	<5.7	<5
Ethene	µg/l	5.9	120	76	110	72
1,1-Dichloroethane	µg/l	140	150	150	180	110
Dehalococcoides spp.	g/b	1,330	25	994	1,200	555
*bvc*A gene	g/b	ND	ND	ND	46.8	ND
Organic Acids (total)	mg/l	< 1	511	463.7	691.2	526
Nitrate	mg/l	< 1	< 0.10	<0.10	<0.10	0.04
Sulfate	mg/l	5.5	4.4	0.99	0.58	0.47
ORP (field data)	mV	48	-139	-152	-152	-147

Volatile Organic Compounds. Concentrations of VOCs over time at monitoring wells OW7 and OW15 are shown graphically on Figures 2 and 3.

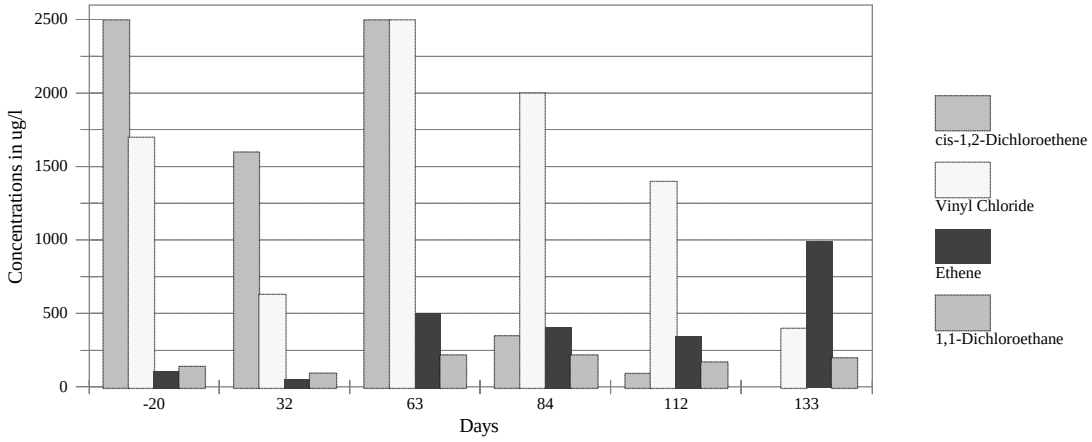

FIGURE 2. OW7 VOC concentrations.

At OW7, the concentration of cis-1,2-DCE dropped significantly in the first monitoring event following the injection (day 32), but then returned to baseline levels (2,500µg/l) in the second monitoring event (day 63). It is not clear if this decrease is due primarily to the injection or to the effect of a four-day long pilot test of high vacuum dual phase extraction (HVDPE) technology in the overlying glacial till. The HVDPE pilot test occurred from day 4 to day 8 of the biostimulation/bioaugmentation pilot test. Following the temporary increase to baseline levels, the concentrations of cis-1,2-DCE at OW7 decreased to below detection (<1µg/l) 133 days after injection. As with cis-1,2-DCE, the concentrations of VC at OW7 decreased significantly immediately following injection, but then increased during the second post-injection sampling event (day 63). Following this temporary increase, however, the concentrations of VC then decreased steadily throughout the remainder of the pilot test, although at a slower rate of decrease than cis-1,2-DCE. This is to be expected, as VC is produced during the degradation of cis-1,2-DCE. Ethene concentrations at OW7 increased by nearly an order of magnitude from 110µg/l during the baseline event to 990µg/l at day 133 (Figure 2). This significant

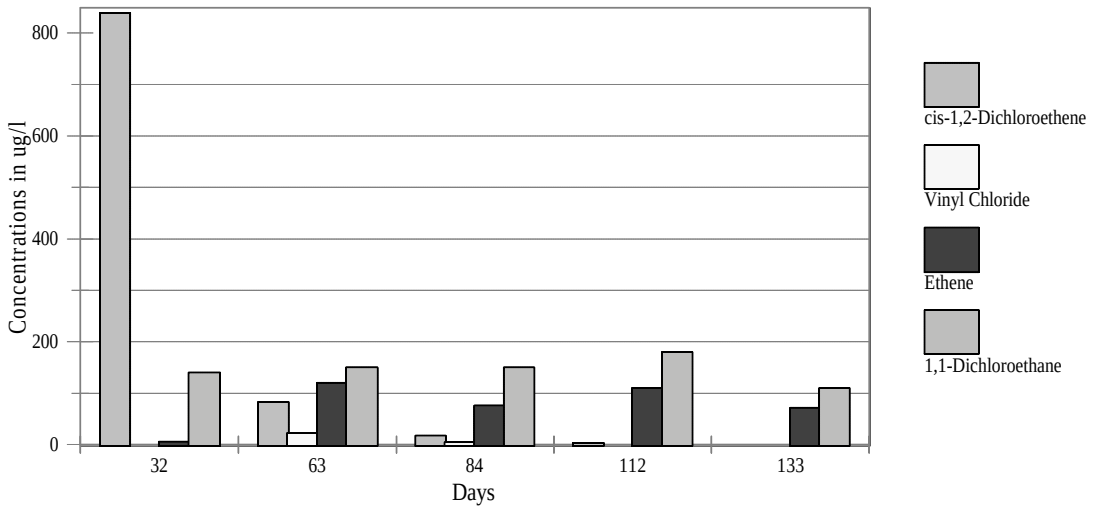

FIGURE 3: OW15 VOC concentrations.

increase is clear evidence that a large amount of biodegradation was taking place in the vicinity of OW7 and that the decrease observed in cis-1,2-DCE and VC concentrations was not simply a result of non-destructive processes such as dilution and dispersion.

At OW15, cis-1,2-DCE concentrations dropped from 840µg/l in the first post-injection sampling event (day 32) to below detection by day 133 (Figure 3). VC was not detected (<40µg/l) in the first post-injection sampling event at OW15, but was detected (22µg/l) in the next monitoring event (day 63). This is understandable as a rapid decrease in cis-1,2-DCE occurred during this same time period, resulting in additional VC production. By day 112, the concentration of VC at OW15 was not detected at a reporting limit of 5µg/l. During the first post-injection sampling event (day 32), ethene was detected at 5.9µg/l at OW15. By day 63, the amount of ethene produced in the vicinity of OW15 had increased to 120µg/l and remained rather stable throughout the remainder of the pilot test. The significant decrease in VOC concentrations and increase in ethene production at OW15 is an indication that biodegradation is occurring downgradient of the treatment area as well as directly within the treatment area.

In both wells, the concentrations of 1,1-DCA did not decrease appreciably during the pilot test and appear to have increased at OW7 (Figure 3). This can be attributed to the fact that BAV1, a key dechlorinating species in BDI, uses all DCE isomers and VC as growth-supporting electron acceptors but is not capable of metabolically degrading 1,1-DCA. As discussed above, concentrations of cis-1,2-DCE and VC, which BAV1 is capable of metabolizing, decreased significantly during the pilot test. This strongly suggests that the rapid biodegradation that took place at the site during the pilot test may be directly attributed to the BDI bioaugmentation and not just biostimulation alone.

Bio-Dechlor CENSUS Results. Bio-Dechlor CENSUS results for both OW7 and OW15 are presented in Tables 1 and 2, respectively. At OW7, the level of DHC increased by almost three orders of magnitude immediately following the BDI application. The DHC count was down slightly on day 63, but nearly doubled by the next sampling event (day 84). The greatest increase of DHC organisms at OW7 occurred between 84 and 112 days after sampling, when the DHC count reached 153,000 genomes per bead. On day 133, the

DHC count had reached 192,000 genomes per bead. This phenomenon follows a classical microbial growth curve in which concentrations rapidly increase and then reach an asymptotic level that is governed by the specific characteristics of the aquifer. A DHC concentration of 192,000 genomes per bead is one of the higher concentrations that has been observed with the Bio-Dechlor CENSUS test.

As expected, there was a much smaller post-injection DHC population in the vicinity of OW15 (Table 2) than OW7, but VOC results show that the population was able to degrade the small amount of contaminant mass that existed near OW15. It is possible that the presence of DHC observed in OW15 could be attributed to the biostimulation of the naturally occurring population since the DHC applied during the BDI injection may not have reached the vicinity of this well in such a short period of time. However, because the concentration of total organic acids increased in this well shortly after the HRC injection, it is possible that groundwater is moving faster than previously predicted and the DHC presence observed in OW15 is a direct result of the injection of BDI that occurred upgradient of the well. Although DHC are sessile organisms by nature and tend to live on the surface of the soils, there are several mechanisms which can lead to microbial transport within the subsurface including hydronamic shear, change in groundwater chemistry, and *in situ* growth. Because it was not possible to do a baseline round of sampling at OW15, we cannot determine whether DHC was present in the vicinity of the well prior to injection or only after the injection occurred.

In addition to measuring the total DHC concentration at the site by targeting the 16S rRNA gene sequence, levels of the *bvcA* gene were also measured. Levels of the *bvcA* gene for each well are provided in Tables 1 and 2. As mentioned earlier, the *bvcA* gene has been shown to be directly involved in the VC to ethene degradation step and is absent in all strains of DHC that do not metabolize VC. In the baseline sampling event in OW7, testing with Bio-Dechlor CENSUS indicated that the aquifer contained low levels of DHC but that there were no strains of DHC present containing the *bvcA* gene. Following the injection of BDI, the *bvcA* gene was detected at levels of approximately 200genomes/bead. The appearance of the *bvcA* gene appears to be directly attributed to the addition of BDI, as the culture contains the BAV1 strain of DHC and therefore the *bvcA* gene. From this data, we can infer that the post-injection DHC increases observed in OW7 may be a direct result of bioaugmentation and not biostimulation alone.

Organic Acids. At OW7, the concentration of total organic acids rose slowly during the first two months of the pilot test (Table 1). This is likely a result of the rapid carbon utilization rate of the organisms in the subsurface. Upon the addition of a carbon source such as HRC, microorganisms enter their exponential stage of growth and it is possible that the utilization rate of carbon is often close to or even equal to the release rate at this stage. As microbial growth reaches an asymptotic level, however, the organic acid concentrations typically increase. This phenomenon was observed in the vicinity of OW7, as concentrations of total organic acids had increased significantly by the day 84 monitoring event and remained high during the remainder of the test. Based on the 12 to 18 month longevity of HRC, it is expected that the organic acid concentrations will remain high for a similar period of time. At OW15 (Table 2), organic acid concentrations increased markedly by the second round of post-injection monitoring (day 63), indicating that the HRC reached the well rather quickly and that groundwater, in the vicinity of the test area, appears to be moving faster than even the high end of the calculated flow

velocity range. The total organic acid concentration at OW15 dropped slightly from day 63 to day 84, but increased to levels even higher than those seen in OW7 in the final monitoring event (day 133).

CONCLUSIONS

The combined use of biostimulation and bioaugmentation to treat recalcitrant VOCs in groundwater during pilot testing was successful in accelerating the rate of cis-1,2-DCE and VC degradation in the vicinity of the injection area, as well as downgradient of the injection area, within the 133-day testing period. The elevated DHC levels in OW7 indicate that the organisms introduced as part of the BDI consortium were able to successfully adapt to their new environment. At OW7, located immediately downgradient of the area of injection, cis-1,2-DCE concentrations were reduced from 2,500µg/l to non-detect within 133 days of injection. Rather than seeing an accumulation of VC, produced by the degradation of cis-1,2-DCE, VC concentrations decreased from 2,500µg/l to 400µg/l within 70 days. Due to the presence of the *bvcA* gene at OW7 and the continuing release of lactic acid by HRC for 8 to 12 months beyond the testing period, it is anticipated that the concentration of VC in the vicinity of OW7 will be reduced to non-detect before the HRC is exhausted. Concentrations of ethene, an indicator of complete degradation, increased from 110µg/l to 990µg/l at OW7 during the 133-day test period.

Concentrations of 1,1-DCA at OW7 increased from 140µg/l to 200µg/l during the pilot test. At sites where both biostimulation and bioaugmentation have taken place, it is difficult to determine whether rapid chlorinated solvent degradation is a direct result of the bioaugmentation application or can be attributed to biostimulation only (and therefore would have occurred without the addition of organisms). However, the fact that 1,1-DCA is the only baseline compound in OW7 not affected by the pilot test application and is also the only compound not metabolized by the dechlorinating species present in BDI, indicates that the application of BDI may have been vital in accelerating the rate of dechlorination. Degradation of cis-1,2-DCE and VC might have occurred at a much slower rate without biaugmentation. The concentration of 1,1-DCA, however, is well below the remedial goal and does not require additional treatment.

Based on the results of the HRC/BDI pilot test, the technology was selected as the remedial alternative for groundwater in the Upper Outwash. Full scale implementation is planned for March 2005. HRC/BDI will be injected as a treatment curtain in the Upper Outwash. The natural channel feature, which contains the Upper Outwash, will be utilized to direct groundwater flow through the curtain before reaching the edge of the perched unit and infiltrating into the underlying regional aquifer. To address the source of VOCs in the glacial till, a HVDPE system will be installed and operated.

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Accelerate the process of complete dechlorination

Bio-Dechlor INOCULUM (Dechlorinating Microbes)

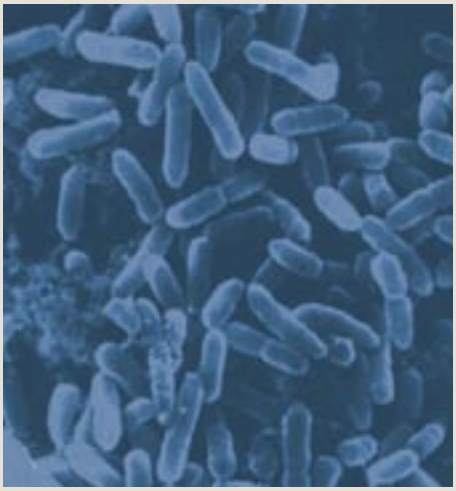
BIO-DECHLOR INOCULUM

BIOAUGMENTATION TO ACCELERATE THE PROCESS OF COMPLETE DECHLORINATION

Bio-Dechlor INOCULUM® is an enriched natural microbial consortium containing species of *Dehalococcoides* sp. (DHC). This microbial consortium has since been enriched to increase its ability to rapidly dechlorinate contaminants during in situ bioremediation processes. Bio-Dechlor INOCULUM has been shown to stimulate the rapid and complete dechlorination of compounds such as tetrachloroethene (PCE), trichloroethene (TCE), dichloroethene (DCE), and vinyl chloride (VC). The most current culture of Bio Dechlor INOCULUM PLUS(+) now contains microbes capable of dehalogenating halomethanes (e.g. carbon tetrachloride and chloroform) and haloethanes (e.g. 1,1,1 TCA and 1,1, DCA) as well as mixtures of these halogenated contaminants.

Bio-Dechlor INOCULUM PLUS(+) is provided in a liquid form and is designed to be injected directly into the contaminated subsurface. Once in place, this microbial consortium works to accelerate the extant rate of chlorinated ethene degradation. When faced with an insufficient quantity of critical dechlorinating microbes, Bio-Dechlor INOCULUM PLUS(+) supplies many beneficial chlorinated solvent degraders including the all important DHC required to achieve complete and rapid dechlorination.

This microbial consortium is compatible with most electron donors however it is often optimized with the addition of any of Regenesis' Hydrogen Release Compound (HRC®) products.



SPECIES OF *DEHALOCOCCOIDES* SP. (DHC)

BIO-DECHLOR CENSUS

DETECTION AND QUANTIFICATION OF *DEHALOCOCCOIDES* (DHC) IN THE SUBSURFACE

The advent of modern biotechnology has allowed the development of unique and rapid genetic assays for the detection of microorganisms. Bio-Dechlor CENSUS℠, an example of this advance, offers a state-of-the-art technique for the quantitative detection of *Dehalococcoides*, the microbe shown to be required for complete biodegradation of higher chlorinated compounds through to ethene.[1, 2]

Existing analytical technologies offer only a crude qualitative assessment (+/-) of the presence of the required *Dehalococcoides* species. These tests utilize a common technique known as the Polymerase Chain Reaction (PCR), whereby traces of DNA specific only to microbes of interest (their "fingerprint") are amplified from environmental samples such that they can be detected. This approach, unfortunately, does not allow for specific quantification of the existing and present microbial population, leaving the environmental professional with insufficient information for complete site assessment and management.

Regenesis now offers a solution to the quantification dilemma, Bio-Dechlor CENSUS. This census of critical microorganisms is a proprietary analysis and is provided by specialized laboratories in the environmental industry. Bio-Dechlor CENSUS utilizes a process termed "Real-Time PCR" in which the DNA amplification step is actually quantified with a fluorescent signal, indicating the number of target microbes in the sample (Figure 1). This valuable quantitative information allows environmental professionals to properly assess project sites for the potential for natural biodegradation of chlorinated contaminants and the degree of bioaugmentation that may be required.

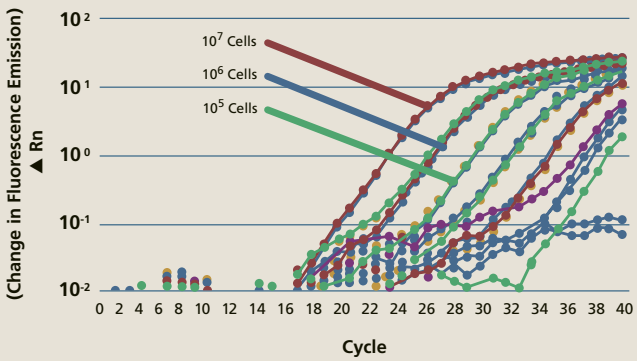


FIGURE 1: REAL-TIME PCR AMPLIFICATION OF 10-FOLD DILUTIONS OF GENOMIC DNA DERIVED FROM *DEHALOCOCCOIDES*

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2. Löffler, F.E.; Q. Sun; J. Li; J.M. Tiedje; Applied Environmental Microbiology 2000, 66(4), 1369-1374.