# FINAL REPORT

## Reductions in DNAPL Longevity through Biological Flux Enhancement

ESTCP Project ER-0438

JANUARY 2009

C. Herb Ward
Pedro J. J. Alvarez
Diego E. Gomez
**Rice University**

Joseph B. Hughes
**Georgia Institute of Technology**

Marcio L. B. da Silva
**Universidade Federal de Santa Catarina**

Approved for public release; distribution unlimited.

Environmental Security Technology Certification Program

# TABLE OF CONTENTS

**LIST OF ACRONYMS** ..... **iv**

**ACKNOWLEDGEMENT** ..... **1**

**EXECUTIVE SUMMARY** ..... **2**

**1. INTRODUCTION** ..... **3**

BACKGROUND ..... 3
OBJECTIVES AND DEMONSTRATION ..... 3
REGULATORY DRIVERS ..... 4
STAKEHOLDER/END-USER ISSUES ..... 4

**2. TECHNOLOGY DESCRIPTION** ..... **5**

TECHNOLOGY DEVELOPMENT AND APPLICATION ..... 5
PREVIOUS TESTING OF THE TECHNOLOGY ..... 8
FACTORS AFFECTING COST AND PERFORMANCE ..... 9
ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY ..... 9

**3. DEMONSTRATION DESIGN** ..... **9**

PERFORMANCE OBJECTIVES ..... 9
SELECTING TEST SITE ..... 10
TEST SITE DESCRIPTION ..... 10
PRE-DEMONSTRATION TESTING AND ANALYSIS ..... 11
TESTING AND EVALUATION PLAN ..... 14
**3.5.1 Demonstration Installation and Start-Up** ..... **14**
**3.5.2 Period of Operation** ..... **14**
***3.5.3 Amount/Treatment Rate of Material to be Treated*** ..... **15**
**3.5.4 Residuals Handling** ..... **15**
**3.5.5 Operating Parameters for the Technology** ..... **16**
**3.5.6 Experimental Design** ..... **16**
**3.5.7 Sampling Plan** ..... **19**
**3.5.8 Demobilization** ..... **22**
SELECTION OF ANALYTICAL/TESTING METHODS ..... 22
SELECTION OF ANALYTICAL/TESTING LABORATORY ..... 22
HEALTH AND SAFETY PLAN (HASP) ..... 22

**4. PERFORMANCE ASSESSMENT** ..... **22**

PERFORMANCE CRITERIA ..... 22
PERFORMANCE CONFIRMATION METHODS ..... 23
DATA ANALYSIS, INTERPRETATION, AND EVALUATION ..... 24

**5. COST ASSESSMENT** ..... **47**

COST ANALYSIS ..... 48

**6. IMPLEMENTATION ISSUES** ..... **48**

ENVIRONMENTAL CHECKLIST ..... 48
OTHER REGULATORY ISSUES ..... 48
END-USER ISSUES ..... 49

**7. REFERENCES** ..... **50**

**8. POINTS OF CONTACT** ..... **54**

**APPENDIX A** ..... **55**
**APPENDIX B** ..... **64**
**APPENDIX C** ..... **72**
**APPENDIX D** ..... **75**

## LIST OF FIGURES

**Figure 2-1. Schematic of the Experimental Design ..... 7**
**Figure 3-1. Picture of the ECRS ..... 12**
**Figure 3-2. Example of Cumulative CAH Removal Sata to Assess Impact of Biologically Enhanced Dissolution ..... 18**
**Figure 4-1. Cumulative Effluent COD for the Bioaugmented Tank (A) and Biostimulated Tank ..... 26**
**Figure 4-2. Effluent Concentrations of PCE, TCE, cis-DCE, VC, and Ethene ..... 28**
**Figure 4-3. Cumulative Effluent Concentrations of PCE, TCE and cis-DCE in the Bioaugmented Tank and Biostimulated Tank ..... 29**
**Figure 4-4. Effluent PCE Breakthrough Curves for Column Studies ..... 31**
**Figure 4-5. Comparison of Electron Donor Equivalents used for Methanogenesis Versus Reductive Dechlorination in Bioaugmented Tank and Biostimulated Tank .... 32**
**Figure 4-6. Effluent PCE, TCE, DCE, VC and Ethene Concentrations in the bioaugmented and Non-bioaugmented Tanks ..... 35**
**Figure 4-7. Comparison of Electron Donor Equivalents used for Methanogenic and Dechlorination Activities in the Bioaugmented Tank ..... 36**
**Figure 4-8. DGGE with Universal Bacterial Primers on Samples from Biostimulated Tank ..... 37**
**Figure 4-9. DGGE with Universal Bacterial Primers for the Inoculum and Samples from Bioaugmented Tank ..... 38**
**Figure 4-10. DGGE with *Dehalococcoides*-specific Primers on Samples from the Biostimulated Tank and from the Inoculum and Samples from Bioaugmented Tank ..... 40**
**Figure A-1. Schematic Representation of the ECRS Showing Sampling Wells, DNAPL Source and Injection Points ..... 56**
**Figure A-2. Bromide Breakthrough Data for the Bioaugmented Tank and Biostimulated Tank ..... 58**
**Figure A-3 Schematic Representation of the Tanks. Showing the Locations of the Sampling Wells,DNAPL Source and Inoculation Injection ..... 61**
**Figure A-4. Chloride Breakthrough Data from the Bioaugmented and Control Tank. Pore Hydraulic, Retention Time were 14 and 12 days for Bioaugmented and Control Tank, Respectively ..... 61**
**Figure A-5. Bioaugmentation of the Tank ..... 62**
**Figure B-1. Schematic of ECRS with Groundwater Sampling Wells and Core Sampling Points ..... 65**

# LIST OF TABLES

**Table 3-1. Performance Objectives ..... 10**
**Table 3-2. Laboratory Analytical Methods for Technology Demonstration ..... 20**
**Table 3-3. Laboratory Analyses Required for Technology Demonstration ..... 21**
**Table 4-1. Performance Criteria ..... 22**
**Table 4-2. Expected Performance and Performance Confirmation Methods ..... 24**
**Table 4-3. Comparison of Total Dechlorinating Organisms (*Dehalococcoides*, *Dehalobacter* spp. and *Desulfuromonas* spp.) to All Other Organisms in Both the Bioaugmented Tank and Biostimulated Tank ..... 42**
**Table 4-4. Comparison of *Dehalococcoides* to Archaea Cell Numbers in Both the Bioaugmented Tank and Biostimulated Tank ..... 44**
**Table 5-1. Cost Tracking ..... 47**
**Table A-1. Properties of Sandy Material used as Matrix in the Experimental Control Release Systems (ECRS) ..... 57**
**Table A-2 Phase 2 Soil Analysis ..... 60**
**Table B-1. Quantitative Real-time PCR Primers and Probes used to Target 16S rRNA Genes ..... 68**
**Table B-2. Characterization of the Culture used for Bioaugmentation ..... 70**
**Table C-1. Examples of Incompatible Chemicals ..... 77**

## LIST OF ACRONYMS

CAH chlorinated aliphatic hydrocarbon
cDCE cis-1,2-dichloroethene

DGGE denaturing gradient gel electrophoresis
DNAPL dense non-aqueous phase liquid
DoD Department of Defense

ECRS Experimental Controlled Release System
EEL Environmental Engineering Laboratories
EHSP Environmental Health and Safety Plan
ESTCP Environmental Security Technology Certification Program

FID flame ionization detector

HASP Health and Safety Plan
HRC® Hydrogen Releasing Compound

MSDS Material Safety Data Sheet

NAS National Academy of Science

OSHA Occupational Safety and Health Administration

PCE tetrachloroethene
PCR polymerase chain reaction

QA/QC quality assurance/quality control
qPCR real-time quantitative polymerase chain reaction

RT-PCR reverse transcriptase polymerase chain reaction

SERDP Strategic Environmental Research and Development Program

TCE trichloroethene

USEPA United States Environmental Protection Agency

VC vinyl chloride
VFA volatile fatty acid

## Acknowledgement

This work was supported by the Texas Advanced Technology Program (TATP 003604-0087-2003), the U.S. Department of Defense (DoD) Environmental Security Technology Certification Program (ESTCP), and by Regenesis Bioremediation Products, Inc. (San Clemente, California). We thank Robert Dawson for valuable technical assistance in operating the ECRS used in this research and Neal Aronson, Abe Salinas and Adam Guerra, undergraduate students at Rice University, who helped perform the laboratory analyses.

The final report was executed at Rice University, Department of Civil and Environmental Engineering, 6100 Main Street, MS-317, Houston, Texas 77005.

## Executive Summary

In the first phase of this project (Phase I), two 11.7 $m^3$ Experimental Controlled Release Systems (ECRS), packed with sandy model aquifer material and amended with tetrachloroethene (PCE) DNAPL source zones, were operated in parallel with identical flow regimes and electron donor amendments. Hydrogen Releasing Compound® (HRC®), and later dissolved lactate, served as electron donors to promote dechlorination. One ECRS was bioaugmented with an anaerobic dechlorinating consortium directly into the source zone, and the other served as a control (biostimulated only) to determine the benefits of bioaugmentation. The presence of halorespiring bacteria in the aquifer matrix prior to bioaugmentation, shown by nested PCR with phylogenetic primers, suggests that dechlorinating catabolic potential may be somewhat widespread. PCR analyses demonstrated that the bacteria present in the culture used for bioaugmentation in the ECRS prevailed for almost a year. Unfortunately, even with *Dehalococcoides* present, complete dechlorination to ethene was achieved at minimum (<1μM). Results demonstrated that the low concentration of ethene produced in this first phase was not due to washout of the dechlorinating organisms. It was also demonstrated that as long as the electron acceptor was not limiting, there was greater energy flow to the dechlorinating populations than to the methanogens. Overall, the results obtained in the Phase I corroborate that source zone reductive Dechlorinating of PCE is possible at near field scale, and that a system bioaugmented with a competent halorespiring consortium can enhance DNAPL dissolution and dechlorination processes at significantly greater rates than in a system that is biostimulated only.

The second experiment, Phase II, compared the fate of a mixed DNAPL source zone under a natural attenuation scenario (no treatment, natural rates of dissolution) with a most probable engineering approach that included biostimulation and bioaugmentation. The same experimental ECRS tanks used on Phase I described above were emptied and repacked with uncontaminated sandy soil. HRC® was continuously added in the influent as a pre-hydrolized (diluted) mixture consisting of 50:50 v/v HRC® : deionized water. The effluent concentration of ethene measured in the biostimulated and bioaugmented tank (~ 4μM) was 4 fold higher than Phase I. This suggests a more complete dechlorination activity that was most likely the result of the slower groundwater seepage velocity used in this experiment (0.4 m/d) compared to the Phase I experiment (1.6 m/d). Cumulative mass balance calculations showed that the total mass removed at the end of the experiment in the biostimulated and bioaugmented tank was near 47% of the total mass of PCE added to the tank. Of this removal, 26% was removed by dissolution (as measured by the mass of PCE in the effluent) and 21% by dechlorination to lesser chlorinated products, mainly TCE and *cis*-DCE. In the control tank, 34% of the PCE added to the tank was removed, with 31% being removed by dissolution and 3% by dechlorination. The benefit of biostimulation and bioaugmentation was observed with higher (7 fold) dechlorination activity compared to the control tank.

# 1. Introduction

## Background

Remediation of aquifers contaminated with chlorinated solvents (CAH) such as tetrachloroethene (PCE) and trichloroethene (TCE) is a multi-billion dollar problem for industry and state and federal government. Estimates of the number of DoD-administered sites containing CAH contamination has been placed near 3000, and a large number of these are believed to contain non-aqueous phase liquids (NAPL) (USEPA 1997). Numerous other sites under Superfund jurisdiction are either identified or believed to contain residual source contamination. The most frequently used treatment technologies (pump-and-treat, zero-valent iron barriers) focus on management of CAH plumes rather than DNAPL source zones that slowly dissolve over years to produce plumes (Stroo et al. 2003). Present technology treats the symptoms rather than the cause. Remediation times once estimated at a few years are now known to be in the hundreds of years for many sites. While DNAPL source zones can be contained hydraulically, cleanup to acceptable levels has not been achieved with presently available "innovative" technology (*in situ* oxidation, *in situ* thermal treatment, surfactant/cosolvent flooding).

Published laboratory studies conducted at Rice University have demonstrated that rapid dechlorination activity can occur in the immediate vicinity of pure CAH DNAPL, cause dramatic changes in the mass transfer and partitioning characteristics of the DNAPL, and result in rapid DNAPL dissolution (Cope et al. 2001; Adamson et al. 2004). Source zone bioremediation harnesses the natural metabolism of dehalorespiring organisms, capable of thriving at high concentrations of chlorinated solvent contamination, to modify the dissolution characteristics of DNAPLs. If source zones could be effectively treated using low cost bioremediation technology, significant reduction in remediation life cycle costs could be achieved at DoD-administered sites (Stroo et al. 2003). Carefully controlled near-field scale demonstrations are required to validate this benefit. A controlled test (Adamson et al. 2003) of source zone bioaugmentation at a near-field scale using PCE DNAPL has recently been successfully completed in the Experimental Controlled Release System (ECRS) (Reeves et al. 2000) at Rice University. Use of this system for assessing the potential for DNAPL source zone bioremediation provides a means for avoiding many of the difficulties inherent in field-scale work (adequate estimation of the mass and composition of DNAPL initially present, an inability to operate a parallel independent control study, and the high costs generally associated with experimental work at this scale).

## Objectives and Demonstration

Having demonstrated the ability to construct, bioaugment, and monitor DNAPL source zones in a controlled release system, we have a unique capability to conduct a quantitative demonstration of DNAPL source zone bioremediation in a cost-effective manner with a known initial DNAPL mass and composition and a parallel independent control. Conducting this demonstration was the objective of the study proposed herein. Furthermore, the objectives of this work were, 1) to provide a basis for critical analysis of the extant field data from ongoing tests to determine if source zone longevity is being biologically impacted at these sites, 2) to allow for the characterization of microbial ecology in the DNAPL source zone and downgradient using molecular techniques for tracking and enumerating critical populations, 3) to determine whether

PCR based analysis targeting phylogenetic or catabolic biomarkers could be a reliable and cost-effective tool to estimate dechlorination rates and, 4) develop a basis for cost and effectiveness considerations at field scale.

### Regulatory Drivers

Reports from the U.S. Environmental Protection Agency (USEPA) and the National Academy of Sciences (NAS) have acknowledged the technical difficulties in dealing with NAPL-contaminated sites and pressed for the development of innovative remediation approaches (USEPA 1996; NRC 1994; NRC 1999). A 2001 expert panel workshop sponsored jointly by the Strategic Environmental Research and Development Program (SERDP) and the Environmental Security Technology Certification Program (ESTCP) was held to identify key research and development needs for addressing source zone remediation. According to published reports from this workshop (SERDP/ESTCP 2001), research into remediation of source zone contamination has been labeled "a more pressing need at this point than research on plume restoration". Participants identified source zone bioremediation and bioaugmentation as among the highest priorities.

### Stakeholder/End-user Issues

Demonstration of source zone bioremediation is intended to provide unequivocal evidence that dissolution of DNAPL can be enhanced biologically. The use of two parallel systems with known inputs and operating conditions allows for a direct quantification of this effect. Use of this type of flux enhancement parameter provides an estimate of the impact on source longevity, and therefore cost comparisons with regards to duration of site monitoring/closure are possible. This report provides information that could be followed for initiating and implementing a source zone bioremediation system, as well as the type of monitoring and data analysis required. If implemented on the field scale, this remediation scheme requires considerable expertise in terms of gathering and interpreting subsurface hydrological and biogeochemical data, but no more than is typically required in enhanced bioremediation (biostimulation) strategies. All components used were available commercially and can be modified to meet particular site-specific needs.

## 2. Technology Description

### Technology Development and Application

Highly chlorinated solvents such as PCE and TCE contain relatively oxidized carbon and can be reductively dechlorinated biologically in the presence of a suitable electron donor. Typical remediation schemes have involved engineering methods to stimulate natural or introduced microbes to dechlorinate within a contaminant plume some distance downgradient of a perceived or delineated source zone. These strategies rely on the ability to dechlorinate aqueous-phase contaminant in the presence of a suitable electron donor, and success is measured by the production of ethene as the terminal product of the process. While there have been a number of cases where enhanced bioremediation has proven successful in plume management, there has been concern that biologically-based strategies are unable to address those sites that contain significant amounts of contaminant present as free product. Because nearly all highly chlorinated solvents are only sparingly soluble in water, these compounds have a limited ability to dissolve into the aqueous phase when introduced into the subsurface. That portion of the mass that does not dissolve remains as a DNAPL that can serve as a long-term source of contamination. The strategies used to date have focused on (often costly) means of removing mass. The ability for bioremediation to achieve substantial mass reduction has been given little consideration. For the most part, reductive dechlorination has not been considered an effective treatment strategy for these sites because of concerns about the ability to impact dissolution via transformation of aqueous phase contaminant. Additionally, the ability of organisms to remain active in regions of high concentrations of PCE or TCE has not been clear.

A number of recent studies have increased the interest in source zone bioremediation by demonstrating the ability to promote rapid dechlorination in the presence of NAPL, often in the presence of specialized microbial populations. Source zone bioremediation harnesses the natural metabolism of dehalorespiring organisms, capable of thriving at high concentrations of chlorinated solvent contamination, to modify the dissolution characteristics of DNAPLs. The effect of this metabolic activity on dissolution processes is two-fold. First, rapid dechlorination of the dominant DNAPL constituents (typically either TCE or PCE) near the oil-water interface creates a favorable concentration gradient for rapid mass transfer to the aqueous phase. Secondly, the NAPL composition rapidly changes from a single-component DNAPL to a mixture that includes more soluble and less chlorinated daughter products (i.e., *cis*-1,2-dichloroethene, cDCE, and vinyl chloride, VC) with a net dissolution rate (i.e., the sum of the dissolution rate of each DNAPL constituent) much greater than the DNAPL originally present. Laboratory-scale studies have quantified the collective effect of these processes on DNAPL dissolution. Under equilibrium dissolution conditions, PCE in a DNAPL source was reduced by 83% when compared to abiotic controls (Carr et al. 2000). In a companion study investigating NAPL removal in columns, PCE removal rates were enhanced by up to 16 times when compared to dissolution alone in abiotic controls (Cope et al. 2001). In both studies, the reduction in source longevity was highly correlated with the ability of the dechlorinating populations to produce more soluble metabolites. Furthermore, these studies established that high dechlorination rates observed in previous research with an anaerobic enrichment culture (Carr et al. 1998; Zheng et al. 2001) can be maintained in close proximity to a NAPL zone, and that

traditional perceptions about the associated toxicity and inhospitableness of these regions should not rule out the potential for bioremediation. Subsequently, similar studies have validated this observation at the laboratory scale (Yang et al. 2000; Yang et al. 2002; Adamson et al. 2004).

To test these findings on a larger scale, a recent near-field scale study was conducted with the objective of evaluating the ability to inoculate a non-dechlorinating porous medium containing a pure PCE NAPL under controlled conditions (Adamson et al. 2003). In this experiment, an active and stable laboratory-scale dechlorinating culture was used as inoculum to develop large volumes of culture needed to seed a previously verified field-scale simulated aquifer system, ECRS (Reeves et al. 2000). Prior to culture addition, a known mass of pure PCE had been added to the simulated aquifer. Results show that the introduced culture rapidly began to dechlorinate PCE even in regions containing NAPL. Moreover, activity (measured as both rate and extent of dechlorination) improved over the course of the monitoring period, suggesting that the culture was stable and that the need for re-augmentation in these conditions was minimal.

Assessing the potential of biological methods in source zone remediation in the subsurface suffers from the general lack of suitable metrics or diagnostic tools for accurately quantifying success. Mass reduction stands as the goal of all strategies, and numerous physical-chemical methods such as surfactant flooding have been effective in removing significant portions of contaminant from the subsurface. However, rarely is a site sufficiently characterized such that there is certainty about the exact amount and location of free product in a heterogeneous aquifer. Therefore, the actual extent of mass reduction is only an estimate. An alternative approach to assess success of source zone bioremediation is to calculate the macro-scale flux of contamination from the DNAPL to the aqueous phase. Flux of a contaminant in an aquifer can be quantified in terms of a mass transfer rate across a given cross-sectional area ($M/T/L^2$), and it is a function of the difference between the saturation concentration at an interface and the bulk concentration (Adamson et al. 2004). The ability to change contaminant flux is useful as a basis for comparison in situations where biological activity is stimulated. Specifically, the goal of bioremediation of source zones is to increase the local flux of contaminant into the aqueous phase via the production of more soluble and less hydrophobic metabolites. This process yields compounds that partition into the aqueous phase and are transported from the source zone. This increase in flux is characterized by a higher cumulative concentration of chlorinated ethenes in the aqueous phase than otherwise would be observed without biological activity (Cope et al. 2001).

The use of dissolution flux as a performance parameter can greatly aid in quantifying the impact of source zone bioremediation, particularly if this effect is quantified on a near-field scale. A test platform such as the ECRS has the potential to demonstrate this effect at a sufficient scale. Operation of two parallel tanks with one serving as an independent uninoculated control is a direct indication of the impact of an actively dechlorinating culture on flux. Furthermore, use of this system for assessing the potential for DNAPL source zone bioremediation provides a means for avoiding many of the difficulties inherent in field-scale work, in particular the adequate estimation of the mass and composition of DNAPL initially present and the high costs generally associated with experimental work at this scale.

An overall schematic of the technology demonstration is provided in Figure 2-1. Tests were conducted using two ECRS platforms where temperature and all inputs were controlled and migration of components out of (and intrusion of unwanted components into) the test zone was eliminated. These platforms were aquifer simulation systems developed by the DoD to evaluate remediation technologies after controlled releases of subsurface contaminants (Reeves et al. 2000). They are comprised of metal tanks (5.5 m long x 2.1 m wide x 1.8 m high) packed with a sandy matrix and fitted with an extensive network of sampling lines and influent and effluent controls. The data obtained from the effluent was representative of the entire cross-sectional area perpendicular to the groundwater flow as determined by bromide tracer studies.

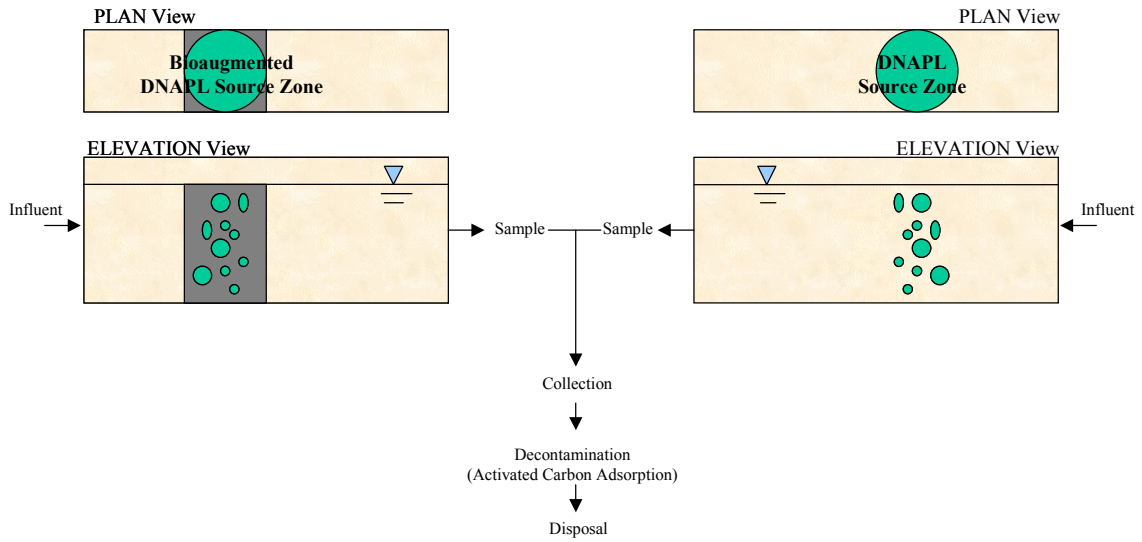


**Figure 2-1. Schematic of the Experimental Design. Two ECRS Tanks were Operated in Parallel with Identical Aquifer, Electron Donor, and DNAPL Constituents. In Phase I, one was Biostimulated and Bioaugmented and the Other Served as a Control (**biostimulated only**). In Phase II, one was Biostimulated and Bioaugmented and the other Served as Control (**simulating natural attenuation**). In Phase I, the DNAPL was added 30 cm from the Bottom of the Tanks, Creating a Pool. In Phase II, the DNAPL was Added from the Top to Form a Dispersed Plume Downgradient.**

In this project, two ECRS tanks were operated in parallel with identical aquifer, electron donor, and DNAPL constituents. In Phase I, one tank served as a non-bioaugmented control. The other was biostimulated and bioaugmented by the addition of 15L of an anaerobic dechlorinating consortium directly into the source zone. Carbon and electron donor requirements were satisfied initially by the addition of HRC® and later by the addition of lactate directly upstream of the DNAPL region. Following culture establishment and development, the dissolution flux from residual saturation in the inoculated tank was compared to the control. Subsequently, both systems were emptied and repacked. One tank was biostimulated and bioaugmented directly into the source zone and the other tank served as a control (simulating natural attenuation). Because the mass, composition, and location of the DNAPL were known in all cases, it was possible to quantify, through data collection and modeling, the effect of bioremediation on DNAPL source zones.

The culture used for bioaugmentation has consistently demonstrated the ability to dechlorinate high concentrations of PCE (including mixed NAPL) to ethene. Extensive molecular characterization studies have demonstrated that the culture contains multiple dechlorinating species, including *Dehaloccoides* spp., that appear to be key in dechlorinating beyond cDCE (Hendrickson et al. 2002; Major et al. 2002). Successful scale-up procedures have already been developed and were followed in this work. A previous bioaugmentation trial with this culture demonstrated survival and activity of a diverse microbial population *in situ* (Adamson et al 2003).

Design parameters included water table depth, flow rate (and resulting water velocity), mass of DNAPL added, culture density, mass of electron donor added, and locations for additions and sampling.

Applications of the technology include any DNAPL-contaminated site that has suitable hydrogeologic characteristics to be a candidate for biostimulation and/or bioaugmentation. The project was designed to provide quantitve evidence that enhanced dissolution is possible in the presence of an actively dechlorinating microbial population, and thus should increase the acceptability of source zone bioremediation as an practical remediation alternative.

### Previous Testing of the Technology

The ECRS platforms have been used to test chlorinated solvent and surfactant fate and transport on several occasions. The most pertinent is the recent demonstration of the inoculation and dechlorination of an introduced PCE DNAPL, conducted at Rice University in 2001-2002 (Adamson et al. 2003). This research was partially funded under a SERDP project titled "Foam Delivery of Hydrogen for Enhanced Aquifer Contacting and Anaerobic Biodegradation of Chlorinated Solvents" (project number ER-1203), part of as an on-going project that also included use of the ECRS as a partial means of validating surfactant-promoted foam formation and transport. A second SERDP project titled "Low-Volume Pulsed Biosparging of Hydrogen for Bioremediation of Chlorinated Solvent Plumes" (ER-1206) relied on a second ECRS tank during the investigation. This project (concluded in 2003) further demonstrated the effectiveness of this system in studying bioaugmentation in treating subsurface contamination, and a

component of the study involved establishment of a DNAPL source zone. Hydrogen sparging was an effective means of stimulating dechlorination and did not result in excessive tunneling or stripping of contaminants.

### Factors Affecting Cost and Performance

Costs for this demonstration were fairly well delineated based on previous experiments. The use of two parallel tanks and flow distribution systems provided additional unit costs with some opportunities for favorable economies of scale (particularly with regard to personnel time allotted to design and monitoring). Because most analytical work was conducted with existing equipment, the need for greater than anticipated sample analysis resulted in only marginally higher costs.

Due to the controlled nature of the near-field scale system (inputs and outputs are known and quantifiable), many of the factors that could affect performance were negligible, especially those associated with hydraulic control. The main factor that negatively impacted performance was the clogging of the effluent lines due to microbial growth and the accumulation of byproducts such as hydrogen sulfide. This issue was solved by monitoring the water table and cleaning the effluent lines on a daily basis using either bleach and/or pressure ($N_2$ gas).

### Advantages and Limitations of the Technology

The primary strength of source zone bioremediation as a technology is the ability to impact source zone longevity through relatively inexpensive means. The process relies on enhancement of dissolution of contaminant into aqueous phase via the formation of more soluble metabolites. Other source zone remediation technologies exist, including *in situ* oxidation, *in situ* thermal treatment, and surfactant/cosolvent flooding. However, there is concern that none are capable of removing sufficient mass to reach desired clean-up levels. Furthermore, each of these technologies rely on costly inputs, either in terms of chemical additions or electrical requirements. Source zone bioremediation does not require removal or manipulation of sediments, and chemical additions are restricted to compounds that generally have unit costs below $1 U.S. /kg. The technology demonstration described in this plan was intended to quantify the effect of biological activity on dissolution, thus providing a true indication of the promise and applicability of source zone bioremediation.

A potential limitation of the technology was the formation of chlorinated metabolites during the remediation process. While the production of these compounds can be considered desirable in terms of transferring mass from NAPL to aqueous phase, the potential formation of significant amounts of cDCE and VC means that downgradient remediation of these contaminants must be taken into account as part of a comprehensive plan.

## 3. Demonstration Design

### Performance Objectives

The objectives for project performance are summarized in Table 3-1.

**Table 3-1. Performance Objectives.**

| Type of Performance Objective | Primary Performance Criteria | Expected Performance (Metric) | Actual Performance Objective Met? |
|---|---|---|---|
| **Qualitative** | 1. Enhance contaminant mobility through production of metabolites | Increase in cumulative mass in effluent, specially by the formation of cDCE | Yes |
| | 2. Faster remediation | Increase in cumulative mass in effluent | Yes |
| **Quantitative** | 1. Reduce contaminant mass | > 20% relative to control | Yes |
| | 2. Enhance dissolution | > 20% relative to control | Yes |
| | 3. Microbial colonization and enumeration | +/- of dechlorinating organisms in/near source zone coupled with enumeration of critical species | Yes |
| | 4. Cost comparison | > 25% savings relative to control | Yes |

### Selecting Test Site

The test site selected for this project was the Rice University campus. Because of the unique nature of the testing system, there was no need to screen candidate demonstration sites. The demonstration required sufficient space to place two ECRS tanks, the associated influent and effluent lines, and flow control equipment. The tanks were housed inside the Ryon Laboratory to ensure constant temperature conditions, and the existing water supply and sewer capabilities were utilized. Most of the analytical equipment was located on campus; therefore, all analyses were performed on campus, with the exception of some microbial testing that was conducted at the Georgia Institute of Technology.

### Test Site Description

Because the test site was not a subsurface location, there was no true contaminant history for the site. However, the controlled release system had been used in a previous demonstration and many of the associated hydrogeologic characteristics were similar.

The experimental system consisted of two metal tanks (5.49 m long, 2.13 m wide, 1.83 m high) open to the atmosphere (Figure 3-1). These are the same ECRS systems that were described by Adamson, *et al.* (2003). Fine masonry sand (New Caney, Texas) was emplaced to provide model aquifer material. The physical-chemical properties of the sand used are shown in Appendix A (Table A-1). Packing was performed by saturated, continuous fill to a depth of

approximately 1 m. This sand-water saturation strategy was designed to enhance distribution of the sand and to minimize mounding, channeling, and other heterogeneities that can occur during packing. The tanks were then drained at a rate of 500 mL/min to induce compaction and then saturated to a depth of 1 meter.

Multiple internal sampling or injection points (0.6 and 1.3 cm ID, respectively) were installed using stainless steel tubing during tank packing. The source water for the ECRS was from the Rice University (Houston, Texas) tap water supply. The tap water was not dechlorinated before use because no inhibitory effects were observed previously (Adamson *et al.*, 2003). Each tank was fitted with two influent and two effluent lines. Effluent lines were placed on both sides of each end of the tanks to minimize preferential flow and channeling. Flow was controlled using electronic flow meters (McMillan Co., Georgetown, Texas) in the influent and effluent lines to maintain a near constant rate (22-30 L/h). Activated carbon canisters (liquid phase activated carbon; total surface area of 1,050 $m^2/g$, TIGG Corp., Heber Springs, Arkansas) were installed in the effluent lines to remove chlorinated solvents before discharge to the sewer.

**Pre-Demonstration Testing and Analysis**

This project did leverage heavily against two recently projects funded by SERDP (ER-1203 and ER-1206) that validated the appropriateness of the ECRS in demonstrating enhanced bioremediation. The opportunity to simultaneously operate two tanks allows for the integration of data and techniques from these studies.

The ability to construct, bioaugment, and monitor DNAPL source zones in this controlled release system was demonstrated in the preliminary trial (Adamson et al. 2003), and many of the same techniques were utilized in the current study. Establishment of anaerobic conditions occurred after approximately 1 month of electron donor addition. The mass of PCE added (1 L) as well as the injection location proved suitable in establishing a source zone and residual concentration in the effluent (≈10 mg/L). The inoculum mass (≈1500 mg/L) resulted in colonization by the dechlorinating consortium without the need for re-inoculation. Dechlorination to TCE (immediate formation), cDCE (dominant product after 225 days), and VC (dominant product after 302 days) occurred over the course of the monitoring period. These milestones were used as guidelines for assessing the expected onset of microbial activity within the system.

**Figure 3-1. Picture of the ECRS.**

The dechlorinating culture used was developed from an anaerobic methanogenic consortium that had shown dechlorination activity for over nine years in the laboratory (Zheng et al., 2001). This culture is capable of rapid and complete dechlorination of PCE to ethene (240 µmol/L/d). The culture was maintained in a 20-L high-density polyethylene carboy equipped with ports for injection of nutrients, sodium hydroxide, and PCE. The carboy also had fittings for culture mixing and headspace analysis. The culture was fed 0.25 mM PCE and 3 mM MeOH daily and maintained with an 80-day retention time using a draw-and-fill method. This method allowed for higher cell densities than used in previous bioaugmented ECRS experiments (Adamson et al., 2003), because the culture was fed daily. The total bacterial and archaea concentrations in the consortium, determined by real time quantitative PCR (qPCR), were $3.1 \times 10^9$ cell/mL and $2.0 \times 10^8$ cell/mL, respectively. Assuming a mass of $1.33 \times 10^{-9}$ g/cell (Bratbak, 1985), 6390 mg of biomass was added for tank bioaugmentation.

Extensive molecular characterization studies have concluded that the culture contained multiple dechlorinating species, including *Dehaloccoides* spp., that appear to be key in dechlorination

beyond cDCE. The previous bioaugmentation trials with this culture demonstrated survival and activity of a diverse microbial population *in situ*. The number of organisms were quantified using established primers for qPCR (Lendvay et al. 2003), an extension of the standard PCR analysis. Population dynamics were assessed by doing a preliminary analysis of the entire microbial community using DGGE to identify distinct populations. Once bioaugmentation of the ECRS tank was completed, the colonization and distribution of the identified organisms, as well as microbial community shifts, were determined using the same techniques.

Flow-through aquifer columns were used to determine whether the anaerobic culture used for bioaugmentation could enhance dissolution of the DNAPL by biosurfactant production, as a possible mechanism for the high concentration of PCE observed in the bioaugmented tank early in the experiment. Three glass columns (15 cm long, 1.5-cm internal diameter) (Da Silva and Alvarez, 2002) were packed with the same sandy material used in the ECRS. All tubing and fittings were Teflon-lined to minimize adsorption losses. Feed solutions were dispensed from gas-tight syringes (100 mL) (SGE, Austin, Texas) at constant flow (1 mL/h) using a syringe pump (Harvard Apparatus). The effluent tubing was adapted for sampling with a 0.64-cm (0.25-in. #28) male Luer Lock adapter and a thin (30-gauge) disposable syringe needle. A bicarbonate-buffered (1000 mg/L) synthetic groundwater (Vongunten and Zobrist, 1993) was fed continuously (1 mL/h). Synthetic groundwater was used to reproduce similar ionic strength encountered in groundwater. One pore volume was displaced in 7 hours, with a seepage velocity of 5.1 cm/d. The DNAPL source in the columns consisted of neat PCE (0.8 mg) injected with a glass gas-tight syringe (10µL) below the effluent cap of the column (4 cm). One column was fed continuously with the synthetic groundwater plus 50% v/v ethanol to enhance the dissolution of PCE (positive control). The second column was fed continuously with synthetic groundwater alone (negative control), to define a PCE dissolution baseline. A third column was fed with the same bacterial consortium used to bioaugment the ECRS. Samples (1 mL) were taken over time from each column by attaching the needle from the effluent lines to gas chromatography vials (5 mL), previously sealed with Teflon-lined rubber septa and aluminum crimps. Headspace samples (100 µL) were analyzed for PCE immediately after collection using gas chromatography, as described previously.

**Testing and Evaluation Plan**

**3.5.1 Demonstration Installation and Start-Up**

Refer to section 3.3 Test Site Description.

**3.5.2 Period of Operation**

| | | Planned Completion | Status |
|---|---|---|---|
| **Task 1:** | **Baseline data collection** | | |
| | 1.0 ECRS preparation, design effluent and influent lines, flow control devices, installment of monitoring wells, and pack the tanks. | 09/2004 | Completed |
| | 2.0 Delineation of hydraulic and geochemical Characteristics | 09/2004 | Completed |
| | 3.0 PCE and HRC® injection. | 09/2004 | Completed |
| | 4.0 Monitoring chlorinated solvents, VFA's, pH, dissolved oxygen, and characterization of microbial ecology prior to bioaugmentation. | 10/2004 | Completed |
| **Task 2:** | **Inoculation and Assessment of Enhanced Dissolution (*Phase 1*)** | | |
| | 1.0 Scale-up and characterization of the dechlorinating culture | 08/2005 | Completed |
| | 5.0 Microbial characterization of the inoculum | 12/2004 | Completed |
| | 6.0 Comprehensive microbial community analysis prior to inoculation. | 12/2004 | Completed |
| | 4.0 Culture inoculation and bioaugmentation. | 10/2004 | Completed |
| | 5.0 Monitoring chlorinated solvents, VFA's, pH, methane, ethane, $H_2$, dissolved oxygen, and characterization of microbial ecology. | 9/2005 | Completed |
| | 6.0 Comprehensive microbial community analysis at the end of *Phase 1*. | 9/2005 | Completed |
| | 7.0 Determine cumulative removal of CAHs and dissolution rates. | 9/2005 | Completed |

**Task 3: Assessment of Enhanced Dissolution: Impact of Source Zone Architecture and evaluation of DNAPL flux enhancement through biostimulation and bioaugmentation of the source zone compared to natural CAH flux from DNAPL (*Phase 2*).**

| | | |
|---|---|---|
| 1.0 Unload ECRS tanks, design effluent and influent lines, flow control devices, and installment of monitoring wells. | 10/2005 | Completed |
| 2.0 Repack ECRS the tanks. | 12/2005 | Completed |
| 3.0 PCE and HRC® injection. | 12/2005 | Completed |
| 4.0 Monitoring PCE concentration to assess DNAPL distribution and allow establishment of source zones. | 01/2006 | Completed |
| 5.0 Bioaugmentation. | 01/2006 | Completed |
| 6.0 Monitoring chlorinated solvents, VFA's, pH, methane, ethane, $H_2$, dissolved oxygen, and characterization of microbial ecology. | 07/2006 | Completed |
| 7.0 Microbial analysis (Biomarkers studies) at the end of *Phase 2*. | 12/2006 | Completed |
| 8.0 Determine cumulative removal of CAHs and dissolution rates. | 01/2007 | Completed |
| 9.0 Final Report | 06/2007 | Completed |

### 3.5.3 Amount/Treatment Rate of Material to be Treated

Each tank was amended with ≈ 1L of PCE to establish a region of DNAPL contamination. Pumping rates resulted in the delivery and disposal of approximately 528 L (Phase I) and 216 L (Phase II) of water per day per tank.

### 3.5.4 Residuals Handling

Effluent water from the ECRS tanks was treated via activated carbon adsorption. Activated carbon canisters (liquid-phase activated carbon; total surface area 1050 $m^2/g$, TIGG Corp., Heber Springs, Arizona) were installed in the effluent lines to remove chlorinated solvents before discharge to the sewer. Disposal of these carbon canisters was handled by the manufacturer. Aqueous samples were collected and disposed by the Environmental Health and Safety Department at Rice University. Following completion of the monitoring phase, CAH-contaminated soil was collected and analyzed by USA Environmental (Houston, Texas). According to standard USEPA procedures, sediment were divided into subunits, and

each container was then sampled for CAHs. The data collected during this sampling dictated whether disposal in a sanitary landfill, a hazardous waste landfill, or incineration was the appropriate option for each subunit.

### 3.5.5 Operating Parameters for the Technology

The technology demonstration was operated on a continuous basis over the course of approximately 8 months per phase. Most of the personnel and labor requirements were focused on the set-up and start-up portions of the demonstration, the most time-consuming of which were involved with culture development and testing and design and implementation of flow control systems. Following bioaugmentation with the dechlorinating culture, the operating parameters shifted to monitoring and analytical requirements. This required daily measurements (flow rates, pH, $O_2$, CAH) as well as periodic comprehensive sampling (molecular characterizations). Problems with clogging of the effluent lines were observed and required monitoring on a daily basis. Thus, personnel were required to routinely unclog the effluent lines (using bleach or pressurized nitrogen) and maintain the flow.

### 3.5.6 Experimental Design

The technology demonstration consisted of three operational tasks. The first of these was the establishment of baseline conditions in both tanks which occurred immediately after pre-demonstration set-up and start-up. The second task included the Phase I comparison of dissolution in the biostimulated and bioaugmeted source zone versus the biostimulated only control. The third task involved the Phase II comparison of biostimulation and bioaugmentation versus a pump-and-treat control.

Baseline data were collected during the period after injection of PCE DNAPL and the addition of HRC®. This monitoring period was anticipated to last 4 to 8 weeks. Constant flow rates were maintained between both tanks, ensuring that seepage velocities and residence times were roughly equal. The primary conditions to be monitored during this process included 1) dissolved oxygen concentrations, 2) volatile fatty acid (VFA) concentrations, and 3) PCE concentrations. Based on the most relevant previous trial with the ECRS, it was demonstrated that the initial bulk dissolved oxygen concentrations of 2.0 mg/L could be decreased to less than 0.5 mg/L in approximately 16 days. This was accomplished by a one-time flush of 0.7 mM of acetate through the system followed by the addition of 3.0 mM of lactate through sampling lines. The lactate amendments were continued on a regular cycle (every 4 days) until HRC® was injected. The current technology demonstration did utilize an earlier injection schedule for HRC® (roughly the same date as PCE addition) and thus did not need to rely on the manual addition of alternate electron donors. This provided a continuous source of electrons and carbon and greatly reduced the amount of labor-associated time necessary to achieve anaerobic conditions. Because HRC® is hydrolyzed to lactate, it was anticipated that this compound could serve a similar role in depleting the residual oxygen, and that the duration would be on the order of 2 to 3 weeks. Bulk oxygen concentrations (in the effluent and at locations in the source zone) below 0.5 mg/L were an indicator that the redox environment had shifted to sufficiently anaerobic conditions and that inoculation could proceed. A second indicator was the

formation of VFA from the fermentation of lactate, specifically propionate and acetate formation. The onset of fermentation coincided with depletion of oxygen in the previous trial and provided evidence that native anaerobes were active. The third condition to be monitored as part of the first task was the PCE concentration. Establishment of a well-distributed source zone was assessed by sampling in and around the region of injection as well as via effluent measurements. The sampling lines in the source zone region were expected to contain globules of neat PCE during the period of distribution and mobilization in the days following DNAPL addition. These globules may be small enough in mass to eventually dissolve in sampling bottles, but the measured concentrations should remain above 10% of the aqueous solubility of PCE. Unsuccessful source zone establishment would be characterized by no recovery of DNAPL or PCE concentrations below 10% of solubility. Measured effluent PCE concentrations should remain relatively constant, and any notable increase or decrease following this plateau was indicative of excessive mobilization of the added DNAPL.

The initiation of Phase I of the demonstration was the second operational task. Scale-up and characterization of the dechlorinating culture was completed by this point. One of the ECRS tanks was inoculated with culture in lines located upstream and within the source zone (Figure 3-1). The cell mass added was dependent on the amount available by this date, but it was anticipated that the mass was in the range of 1,000 to 1,500 mg. This mass was diluted for ease of delivery and to ensure distribution throughout the tank. The relative numbers of BAV1 and other select *Dehaloccoides* spp. present in the mixed culture were established prior to inoculation. The inoculation was conducted by providing positive pressure ($N_2$) into the culture carboy.

Flow rate and the associated hydraulic characteristics remained constant during this operational phase. Concentrations of PCE and metabolites in the effluent were monitored on a daily basis, and cumulative removal of CAH in each tank was estimated based on these measurements and the cumulative flow rate. This was the primary means of validation for the technology, and a quantitative indication of the biological dissolution enhancement was calculated using these data. An example of model data is displayed in Figure 3-2.

The cumulative removal over time was plotted for an inoculated and a control system. The dissolution rate at any particular time can be estimated based on the slope of the line, and the impact on source longevity can be estimated by comparing the removal rate (or total mass removed) versus the initial mass of PCE added. This metric should be significantly higher in the bioaugmented reactor when compared to the pump-and-treat control. An alternate means of measuring flux was employed by taking periodic samples from a set of down-gradient lines lying perpendicular to the direction of flow. The data from these transect lines can be combined and averaged to determined the flux at a near down-gradient location. Because advection-dispersion and dilution are less prominent factors in the vicinity of the source zone (relative to the effluent end), this method provides a more localized measurement of the dissolution effect and may result in a more clear demonstration of the biological impact. Measurements within the source zone also aided in delineating DNAPL distribution and provided evidence about the type and quantity of metabolites formed in this region.

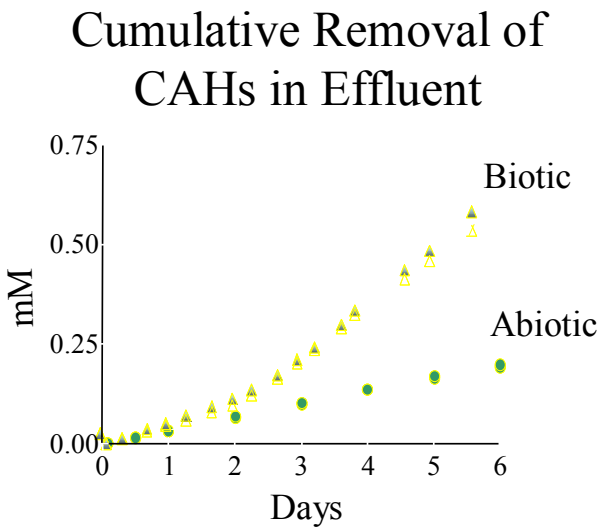


**Figure 3-2. Example of Cumulative CAH Removal Sata to Assess Impact of Biologically Enhanced Dissolution** (from Carr et al. 2000).

Samples were collected and stored for molecular analysis on a regular basis. A preliminary assessment determined the presence/absence of targeted species after a single hydraulic residence has elapsed. This provided baseline data for the original distribution of the added culture. Subsequent samples were then analyzed to determine both movement and proliferation from this original ecological footprint. Comprehensive community analysis (using DGGE) was conducted 1) pre-inoculation, 2) immediately post-inoculation, and 3) at the conclusion of Phase I. Comparisons were aided by quantitative measurements (real time polymerase chain reaction, qPCR) of targeted species. These were used to determine if organisms had colonized the source zone region, particularly isolates such as BAV1 that are capable of growing via the reductive dechlorination of cDCE and VC. Presence of these organisms in and around the source zone is a strong indication that dechlorination beyond cDCE can occur in the vicinity of PCE DNAPL.

Following the completion of Phase I, the systems were repacked with fresh sand and prepared for the Phase II demonstration. This experiment utilized identical operational parameters to those described for Phase I, with the exception that changes in the source zone architecture were artificially imposed.

In this experiment, the source zone was placed 1 foot below the aquifer matrix surface so the PCE DNAPL injected would not pool on the bottom of the ECRS in a manner similar to the formation of DNAPL pools on impermeable layers of an aquifer. The more homogenously distributed source zone in the second ECRS tank was established in a manner identical to the Phase I experiment. Over the course of the first 4 to 8 weeks, effluent PCE concentrations were monitored to assess distribution of the DNAPL and steady-state dissolution.

After establishment of source zones, one tank was bioaugmented, using identical cell masses and injection locations. Pre-hydrolyzed HRC® was added as a source of electrons to each system. Monitoring events for each system followed similar schedules. Molecular characterizations of the respective microbial communities were conducted to assess the survival of the introduced microorganisms, their distribution and correlation with dechlorination activities.

Detailed information about analytical methods supporting the experimental design is included in Appendix A.

### 3.5.7 Sampling Plan

The sampling plan followed the model used in the previous ECRS source zone study (Adamson et al. 2003). In addition to the summary provided below, modifications made according to the requirements of this technology demonstration are detailed.

The majority of samples were aqueous, either from the effluent or from the interior of the tank. All aqueous samples from the interior of the tank were collected from the metal lines driven into the sand vertically from above. Mesh screens were placed at the opening of each line to prevent intrusion of sediment. Lines were anchored in place by the surrounding compacted aquifer matrix. The opposite end of the metal line was linked to non-sorptive Tygon tubing that can was connected to a syringe. All interior aqueous samples were collected by hand via glass syringe. Lines were flushed according to the total volume of space between the two openings (typically 120 mL). This volume was discarded and the subsequent sample collected and transferred to the appropriate sampling container. Effluent samples were collected by switching a bypass valve, connected Tygon tubing and a glass syringe to the effluent spigot.

Aqueous analyses required sample volumes of 25 mL. Because of the volatility of many of the analytes, collection bottles (70 mL glass serum bottles) were sealed (Teflon septa and crimp caps) prior to transfer. A vacuum of 25 mL was imposed on the bottle before transfer to ensure that pressure following the liquid addition was near atmospheric. Liquid samples were transferred from glass syringes to the sealed bottles using 25 gauge needles to minimize the size of the resulting septum hole.

Aqueous samples were analyzed rapidly after collection, aided by the on-site location of the analytical laboratory. For volatile organic compounds, complete partitioning between liquid and gas phases requires approximately 30 minutes, but analysis was compromised after several days because of volatile losses. Headspace samples for VOC should not be stored for long periods of time, and therefore no sample preservation methods were used. In general, no samples that required analysis via gas chromatography were stored. The same is true for dissolved oxygen and pH measurements; these analyses were conducted immediately after sample collection. In cases when less volatile analytes could not be measured on the same day, aliquots of the original 25-mL sample were stored for a maximum of 4 days. In these

cases, samples were preserved by lowering pH to < 2 (through addition of HCl), filtered through 0.2 μm syringe filter, and stored at 4°C or lower to minimize growth of organisms.

With the exception of a portion of the molecular work, all analyses were conducted on-site. This minimized the opportunity for damage or deterioration of the samples, and ensured that consistent methods of sample collection, identification, and analysis were employed. All analyses were done without replicates; however, duplicate injections were performed on a daily basis to ensure that samples were equilibrated and that analytical equipment was functioning and utilized properly. Duplicate injections served as quality control, along with a daily blank for each type of instrumentation/analysis. Instruments were calibrated on a weekly basis, with check standards run daily to make certain that results were representative.

Because this was not a field site, there were no background concentrations to measure, but the non-bioaugmented tank served as an independent control. All data from this control system was reported at the same frequency as the bioaugmented system.

Tables 3-2 and 3-3 summarize the methods of analysis and the sampling frequency.

**Table 3-2. Laboratory Analytical Methods for Technology Demonstration.**

| Parameter | Method | Sample Volume (mL) | Detection Limits |
|---|---|---|---|
| VOCs[1] | GC/FID | 25 | < 0.01 mM |
| Methane | GC/FID | 25 | < 0.005 mM |
| Ethene | GC/FID | 25 | < 0.01 mM |
| VFAs | GC/FID | 25 | < 0.1 mM |
| pH | | 25 | |
| Dissolved Oxygen | | 25 | 0.1 mg/L |
| Species Identification and/or quantification | PCR/ qPCR | 1 gram of aquifer material or $50\text{-}10^3$ mL | > 1000 copies/ mL |
| Microbial Community Analysis | DGGE | 1 gram of aquifer material or $50\text{-}10^3$ mL | |

[1] VOC include PCE, TCE, cDCE, VC

**Table 3-3. Laboratory Analyses Required for Technology Demonstration.**

| Experimental Phase | Analysis[2] | # of Sampling Events | # of Samples per event: Effluent | # of Samples per event: Interior | Total # of Samples |
|---|---|---|---|---|---|
| **Preliminary** | **Establishment of PCE residual** | | | | |
| | VOCs, methane | 40 | 2 | | 80 |
| | VOCs, methane | 2 | | 24 | 48 |
| | **Depletion of $O_2$** | | | | |
| | Dissolved $O_2$ VFAs | 40 | 2 | | 80 |
| | Dissolved $O_2$ VFAs | 2 | | 24 | 48 |
| | **Microbial community analysis** | | | | |
| | PCR/qPCR/DGGE | 2 | 2 | 2 | 6 |
| **Phase I** | **Monitoring of biological enhancement** | | | | |
| | VOCs, Methane, VFAs, $O_2$, pH | 180 | 2 | | 360 |
| | VOCs, Methane, VFAs, $O_2$, pH | 6 | | 24 | 144 |
| | **Microbial community analysis** | | | | |
| | PCR/RTm-PCR | 4 | 2 | 12 | 56 |
| | PCR/T-RFLP/DGGE | 1 | | 4 | 4 |
| **Phase II** | **Monitoring of biological enhancement** | | | | |
| | VOCs, Methane, VFAs, $O_2$, pH | 180 | 2 | | 360 |
| | VOCs, Methane, VFAs, $O_2$, pH | 6 | | 24 | 144 |
| | **Microbial community analysis** | | | | |
| | PCR/qPCR | 4 | 2 | 12 | 56 |
| | PCR/ /DGGE | 1 | | 4 | 4 |

[1] Analytes listed in the same entry can either be measured using the same method or a subsequent analysis can be conducted on the same sample volume

[2] Determination of inorganic anion concentrations is not expected to be a routine measurement but will be conducted on an as-needed basis to support primary analyses

Additional detail concerning sample collection, analysis, experimental controls, data quality and calibrations is contained in Appendix A. All procedures complied with the quality assurance plan detailed in Appendix B.

### 3.5.8 Demobilization

Disposal of aquifer material was carried out by a licensed independent group (USA Environmental, Houston, Texas).

### Selection of Analytical/Testing Methods

Analytical methods are detailed in Appendix A. All methods were developed from standard USEPA procedures and modified based on available sample volumes.

### Selection of Analytical/Testing Laboratory

The analytical capabilities at Rice University have been tested and successfully implemented for a number of similar projects. Analyses conducted at Georgia Tech utilized their ability to identify and quantify specific (and not publicly available) microbial isolates of interest. Because similar instrumentation was also available at Rice University (specifically qPCR), a portion of these analyses were also conducted at Rice University. DGGE analyzes were conducted at Microbial Insights, Inc. (Rockford, Tennessee).

### Health and Safety Plan (HASP)

The HASP for this technology demonstration is located in Appendix C.

# 4. Performance Assessment

### Performance Criteria

The general performance criteria are summarized in Table 4-1.

**Table 4-1. Performance Criteria.**

| Performance Criteria | Description | Primary or Secondary |
|---|---|---|
| Enhance contaminant mobility | PCE and metabolites (TCE, cDCE, VC, ethene) transferred to aqueous phase. | Primary |
| Faster remediation | Decrease the longevity of PCE added as DNAPL. | Primary |
| Reduce contaminant mass | Remaining PCE in the bioaugmented system must be less than PCE in pump-and-treat control at conclusion of demonstration. | Primary |
| Enhance dissolution | Dissolution rates in effluent and in transect across source zone region should be higher in bioaugmented system relative to the control. | Primary |
| Microbial colonization and enumeration | Establishment of targeted species throughout tank and near source zone. | Primary |
| Cost comparison | Lowered cost as a result of decreased source longevity. | Primary |

| | | |
|---|---|---|
| Process waste | CAHs in aqueous effluent (200-580 L/d) are removed prior to disposal via activated carbon adsorption . | Secondary |
| Hazardous materials | DNAPL-contaminated sediment (50 $m^3$) will be analyzed and disposed of by an independent contractor at conclusion. | Secondary |
| Reliability | Daily monitoring to ensure no flow disruption. Valves could be replaced when this occurs with minimal skill level required to perform this task. Sensitivity to environmental conditions was not an issue because the systems were maintained in climate-controlled building. | Secondary |
| Factors affecting technology performance | All inputs and outputs were controlled compared to a typical field site. | Secondary |
| Scale-up constraints | Flow control did not utilize pumps as would be typical in a full-scale implementation, but groundwater velocity was similar. | Secondary |

### Performance Confirmation Methods

Sampling locations and frequency were previously summarized in Figure 3-1 and Table 3-1. These were used to assess performance based on the methods detailed in Table 4-2.

**Table 4-2. Expected Performance and Performance Confirmation Methods.**

| Performance Criteria | Expected Performance Metric (pre-demo) | Performance Confirmation Method[1] | Actual (post demo) N/A [2] |
|---|---|---|---|
| **Primary Criteria (Performance Objectives): Qualitative** | | | |
| Enhance contaminant mobility | Increase in cumulative mass in effluent, ethene formation in bioaugmented system | Effluent concentration (PCE/TCE/cDCE/VC/ethene) | **N/A** |
| Faster remediation | Increase in cumulative mass in effluent | Effluent concentration (PCE/TCE/cDCE/VC/ethene) | **N/A** |
| **Primary Criteria (Performance Objectives): Quantitative** | | | |
| Reduce contaminant mass | > 20% relative to control | $\frac{(\text{Initial Mass - Cumulative Mass in Effluent})_{bioaugmented}}{(\text{Initial Mass - Cumulative Mass in Effluent})_{control}} > 1.25$ | **N/A** |
| Enhance dissolution | > 20% relative to control | $\frac{(\text{Cumulative Mass in Effluent/Day})_{bioaugmented}}{(\text{Cumulative Mass in Effluent/Day})_{control}} > 1.25$ | **N/A** |
| Microbial colonization and enumeration | Dechlorinating organisms in/near source zone coupled with enumeration of critical species | +/- in effluent and source zone sampling lines using targeted PCR<br><br>RTm-PCR | **N/A** |
| Cost comparison | > 25% relative to control | $\frac{(\text{Cost/Day})_{bioaugmented}}{(\text{Cost/Day})_{control}} < 0.8$<br>(extrapolated over lifetime of source zone) | **N/A** |
| **Secondary Performance Criteria: Qualitative** | | | |
| Reliability | No major breakdowns or interruptions in flow | Monitoring and record keeping | **N/A** |
| Process waste | none | Periodic monitoring of effluent from activated carbon canisters | **N/A** |
| Hazardous materials | Contaminated sediment at completion of each phase | Independent analysis by disposal company | **N/A** |
| Factors affecting technology performance | None because of climate control | Experience from demonstration operation | **N/A** |
| Scale-up constraints | Flow rate consistency | Monitor during demonstration operation | **N/A** |

[1]See Appendix A and Appendix B for more detail; [2] Not Applicable

### Data Analysis, Interpretation, and Evaluation

Analysis were based on a comparison of performance between the two systems. One tank served as the inoculated (bioaugmented) system, while the second tank was not bioaugmented (control). Testing of two tanks in parallel with identical operating conditions allowed for the most direct comparison possible. Design and operation of Phase II were conducted based on lessons learned

during Phase I of the technology demonstration. While the majority of operating variables remained very similar during both phases (with the exception that the control tank was not biostimulated and bioaugmented), there was an opportunity to improve certain aspects of the design such as decreasing the seepage velocity in Phase II and increasing the diameter of the effluent lines to avoid clogging.

## Phase I

ECRS tanks were monitored for 276 days. HRC® was largely depleted in the systems after 40 days as indicated by a decrease in the effluent COD concentration to negligible levels and the resulting stabilization of the cumulative mass of COD exiting the tanks (Figure 4-1). Possible explanations for the rapid HRC® depletion are the enhancement of dissolution rates due to the relatively fast groundwater velocity used (seepage velocity = 1.6 m/d), the higher water temperatures (23ºC) in the ECRS than would be encountered in the field (typically 10-15ºC), or possibly the relatively soluble HRC® formulation used (glycerol tripolylactate). Most of the added HRC® was recovered (73.2 and 80.5% in the bioaugmented and biostimulated tank, respectively) in the effluent as fermentation byproducts such as acetate and propionate (Figure 4-1).

On day 118, lactate feeding to both systems was initiated and sustained as an alternative electron donor. Most of the added lactate was recovered (86.5 and 89.6% in the bioaugmented and biostimulated tank, respectively) in the effluent during the following 20 days (up to day 138) (Figure 3). Acetate and propionate (byproducts of lactate fermentation) were detected in the effluent of both tanks and concentrations increased over time (from day 118 to day 148) during the 30 days after lactate addition. Figure 4-1 shows a difference in total COD and the effluent acetate plus propionate concentrations, which could be due to the production of other byproducts of lactate fermentation that were not monitored. The missing COD was likely associated with $CO_2$ production and biomass formation during metabolism of the electron donor(s). It is unlikely that other biochemical processes were involved in consuming the missing COD. Based on the bioavailable iron concentration in the sand, Fe (III) reduction would have consumed 1 g COD (i.e., < 0.01 % of the added COD). Cumulative methane production accounted for 300 g as COD (i.e., 1.3 %). The influent tap water contained some sulfate (44 mg/L), but its utilization as electron acceptor would also account for negligible COD consumption (i.e., < 0.01 %).

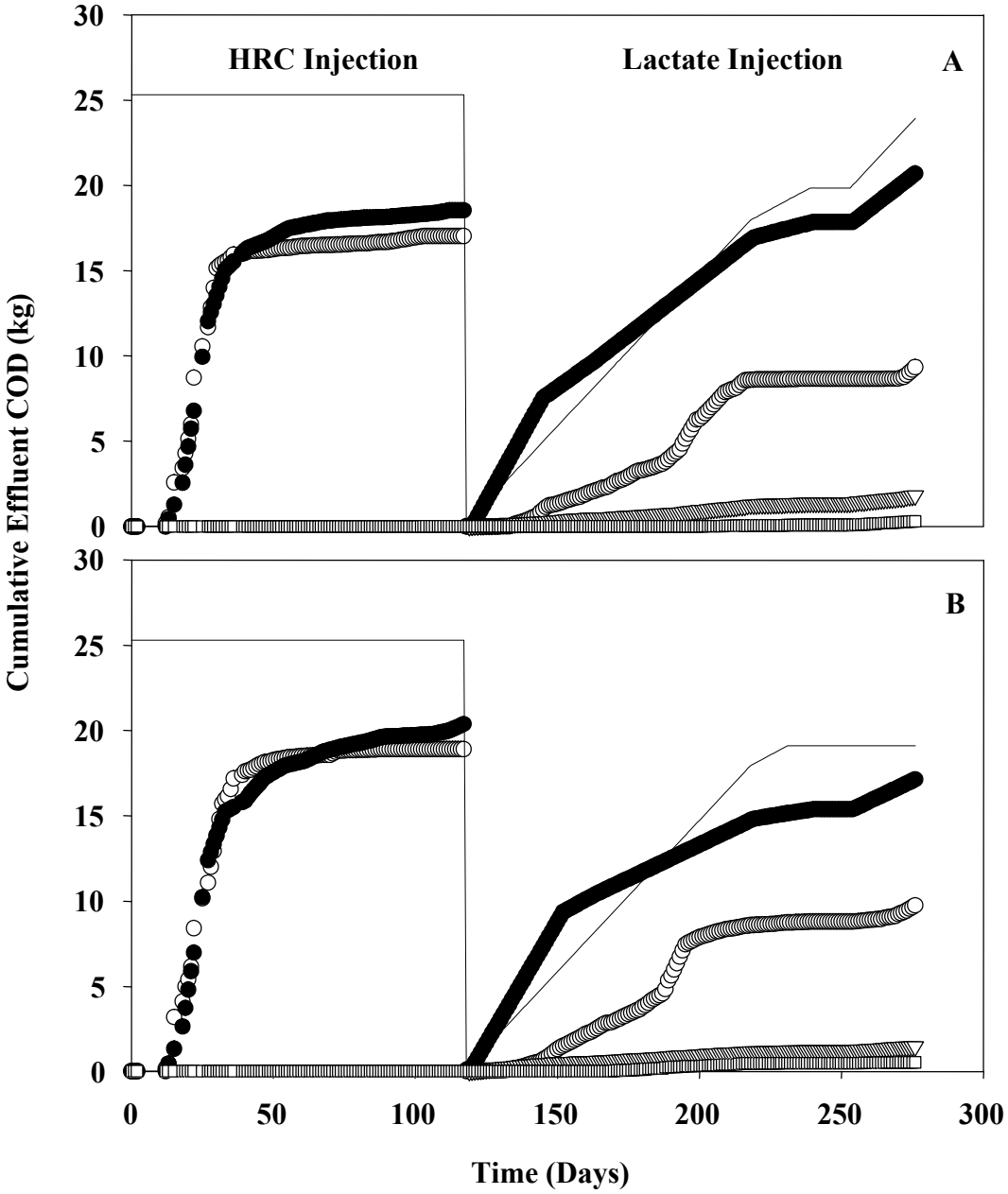


**Figure 4-1. Cumulative Effluent COD for the Bioaugmented Tank (A) and Biostimulated Tank (B). Symbols: – Influent COD, ● Effluent COD, ○ Effluent Propionate and Acetate, ∇ Effluent Lactate, and □ Effluent Methane.**

Based on effluent concentrations, both the bioaugmented tank and biostimulated tank demonstrated the step-wise dechlorination of PCE to TCE to *cis*-DCE to VC and then to small amounts of ethene (Figure 4-2). In the biostimulated tank, PCE was first detected in the effluent at day 13 of the experiment, when an initial spike in the PCE concentration was observed (Figure 4-2A). After this spike, PCE concentrations remained between 50-100 μM for the duration of the experiment. TCE was detected on day 35 and its concentration increased over time until day 125 (Figure 4-2B). *cis*-DCE was first detected in the effluent after day 75 (Figure 4-2C), but its concentration increased significantly after day 125 when the TCE concentration started to diminish. VC appeared in the effluent after day 124 at a very low concentration, but increased after day 150 (Figure 4-2D). Ethene was first detected in the effluent at day 152 (Figure 4-2E). After suspending lactate injection in this tank (on day 232), the effluent concentrations of VC and ethene decreased below detection limit. This implies that the dechlorination activity decreased due to the discontinued addition of an electron donor.

In the bioaugmented tank, PCE concentrations in the effluent followed a similar trend to the biostimulated tank; an initial PCE spike almost three times greater than in the biostimulated tank was seen around day 13 and then the concentration of PCE fell sharply (Figure 4-2A). TCE was first observed in the effluent near day 35, reached a maximum concentration of 38 μM on day 80, and then started to decrease (Figure 4-2B). *cis*-DCE was first detected in the effluent on day 100 (Figure 4-2C), but the concentrations rapidly increased after day 118 when the TCE concentrations began to decline. VC and ethene were detected at day 159 and 167, respectively (Figure 4-2D and E). Both VC and ethene reached their maximum concentrations in the effluent around day 225 and then stabilized.

Cumulative mass balance calculations showed that a significant quantity of the PCE source zone was removed within the first 50 days in the bioaugmented tank (Figure 4-3). The total mass removed at the end of the experiment was near 90% of the total mass of PCE added to the tank. Of this removal, 59% was removed by dissolution (as measured by the mass of PCE in the effluent) and 31% by dechlorination to lesser chlorinated products such as TCE and *cis*-DCE. In the biostimulated tank, only 68% of the PCE added to the tank was removed, with 48% being removed by dissolution and 20% by dechlorination (Figure 4-3). The lower residual mass of PCE in the bioaugmented tank was partly due to the high concentration of PCE exiting the tank in the beginning of the experiment just after bioaugmentation. This high concentration of PCE measured in the effluent of the bioaugmented tank was initially thought to be caused by biosurfactant properties of the culture that could have enhanced DNAPL dissolution. However, column tests conducted under conditions similar to that in the tanks failed to confirm this hypothesis (see below), contrary to similar phenomena observed in the biodegradation of petroleum hydrocarbons (Francy *et al.*, 1991).

The culture used to bioaugment the tank was capable of complete dechlorination of PCE to ethene, but the concentration of ethene observed in the bioaugmented tank was relatively low (< 4 μmol/L). A main cause of the slow ethene production could have been the short contact time resulting from the relatively high groundwater velocity in the tanks (1.6 m/d). Such a fast velocity could have also caused washout of some of the added dechlorinating organisms, which

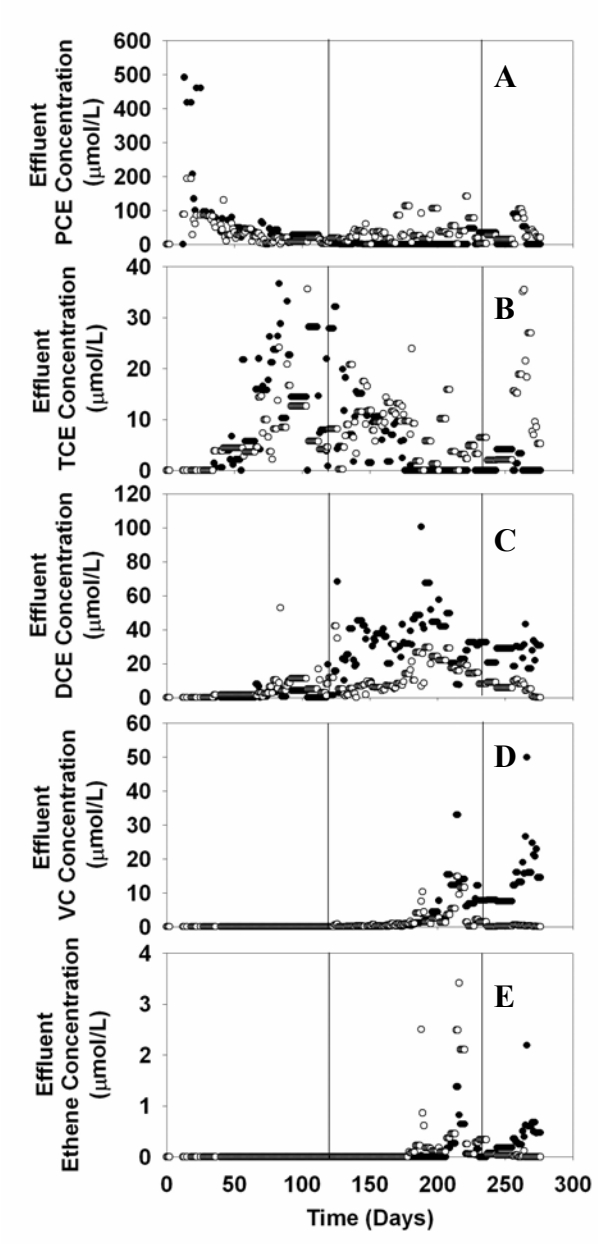


**Figure 4-2. Effluent Concentrations of PCE (**A**), TCE (**B**), *cis*-DCE (**C**), VC (**D**), and Ethene (**E**). Symbols: ● Bioaugmented Tank and ○ Biostimulated Tank. PCE Injection was on Day 0, HRC® was added Six Days before the PCE Injection, and Bioaugmentation was on Day Eight. Lactate Injection Started in both Tanks at Day 118 (**left line**) and Ceased in the Biostimulated Tank at the Day 232 (**right line**).**

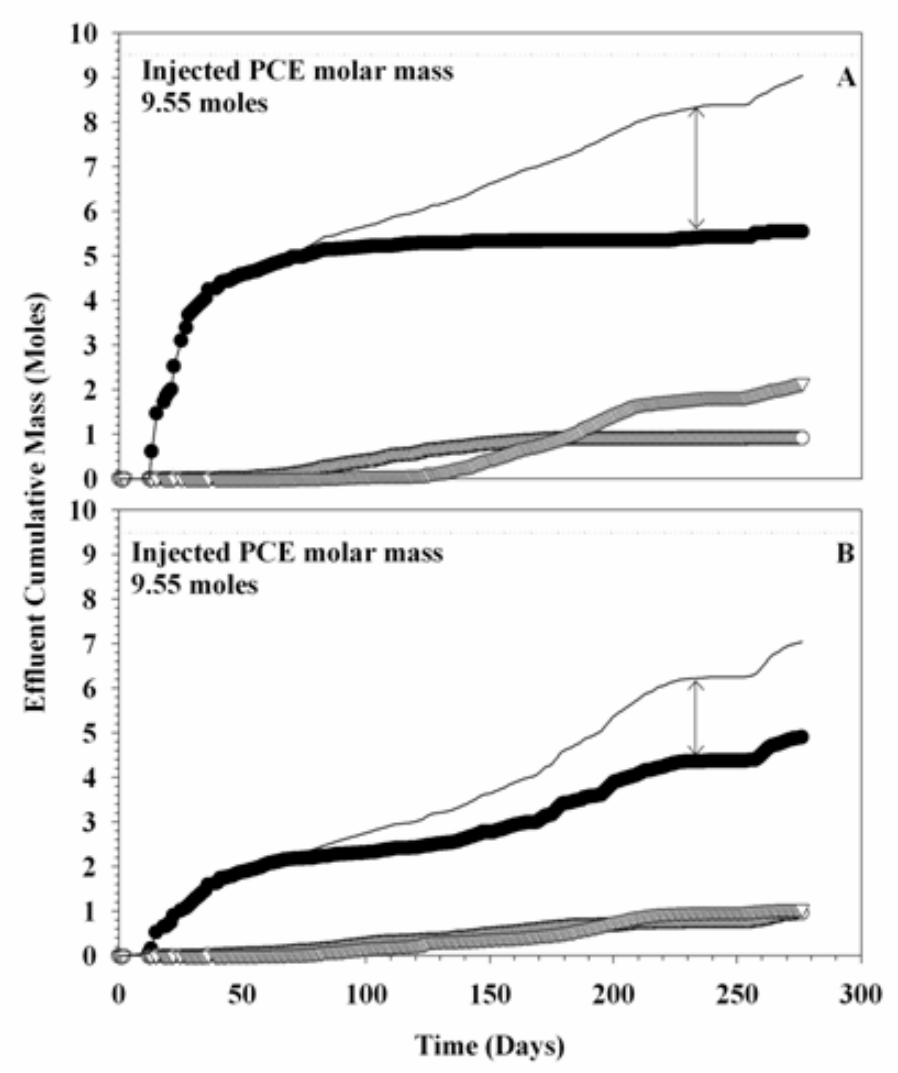


**Figure 4-3. Cumulative Effluent Concentrations of PCE, TCE and cis-DCE in the Bioaugmented Tank (A) and Biostimulated Tank (B). Symbols: ● PCE, ○ TCE, ∇ *cis*-DCE, and – Total Chlorinated Mass. Arrows Show Difference in Extent of Dechlorination between the Two Tanks.**

would be conducive to lower ethene production rates. Insufficient supply of electron donor was also a likely factor that hindered the extent of dechlorination, especially during the time after HRC® was depleted (day 40) and before lactate was added (day 118). Although the pH (6.33 ± 0.20 and 6.28 ± 0.14 for the biostimulated tank and bioaugmented tank, respectively) was below the optimum value (6.8-7.8) reported for dechlorinating organisms (Middeldorp *et al.*, 1999), it is unlikely that such a small difference hindered ethene production. Interestingly, ethene concentrations observed in this work were much higher than the concentration of ethene observed in a similar ECRS bioaugmentation study conducted by Adamson *et al.* (2003).

Column studies were performed to test the hypothesis that biosurfactants produced by the culture were capable of mobilizing and/or increasing the solubilization of DNAPL. The column tests conducted under conditions similar to that in the tanks failed to confirm this hypothesis, contrary to similar phenomena observed in the biodegradation of petroleum hydrocarbons (Francy *et al.*, 1991). Almost all of the PCE added to the positive control column fed groundwater plus ethanol was recovered (>98%). But there was not a significant difference between the columns fed groundwater only (negative control) or with the culture used for bioaugmentation. Nearly 50% of PCE was recovered in both effluents (Figure 4-4). Injection of 15 L of culture directly into the DNAPL source zone (conducted under positive pressure) in the bioaugmented tank may have displaced some DNAPL and increased surface to volume ratio of the DNAPL. This could have increased dissolution of PCE and the amount of soluble PCE in the effluent.

Methane concentrations were monitored throughout the experiment and the quantity of electron donor used for methanogenesis was compared to the amount used for dechlorination. Previous studies demonstrated that methanogens can out compete dechlorinating organisms in the presence of high hydrogen concentrations (He *et al.*, 2002; Yang and McCarty, 1998). In this study, more electron equivalents (COD) were used for methanogenesis than halorespiration in both tanks (Figure 4-5). However, in the bioaugmented tank, less methane was produced in relation to the amount of dechlorination as compared to the biostimulated tank. Even with the methanogens utilizing more electron equivalents than the dechlorinating organisms in the biostimulated tank, *cis*-DCE, VC and small amounts of ethene were all produced in both tanks. But the benefit of bioaugmentation was observed with higher (1.6 times) dechlorination activity compared to that in the biostimulated tank (Figure 4-5). The higher utilization of equivalents by the halorespiring organisms in the bioaugmented tank was most likely due to the higher biomass of dechlorinating organisms in this tank.

Overall, dechlorination activity was observed in both the bioaugmented tank and the biostimulated tank. Microbial analysis (nested PCR), conducted prior to bioaugmentation, showed the presence of specific bacteria capable of dechlorinating PCE to *cis*-DCE (*Dehalobacter* spp., *Sulfurospirillum* spp., *Desulfuromonas* spp.) and PCE to ethene (*Dehalococcoides* spp.) in both tanks. It is unlikely that the dechlorination activity observed in the biostimulated tank was caused by cross-inoculation from the bioaugmented tank since all the influent and effluent pipes were run separately. Cross-inoculation caused by spray during bioaugmentation was also unlikely since the culture was added from a closed container and all the lines were sealed. The most plausible explanation for the dechlorination activity in the

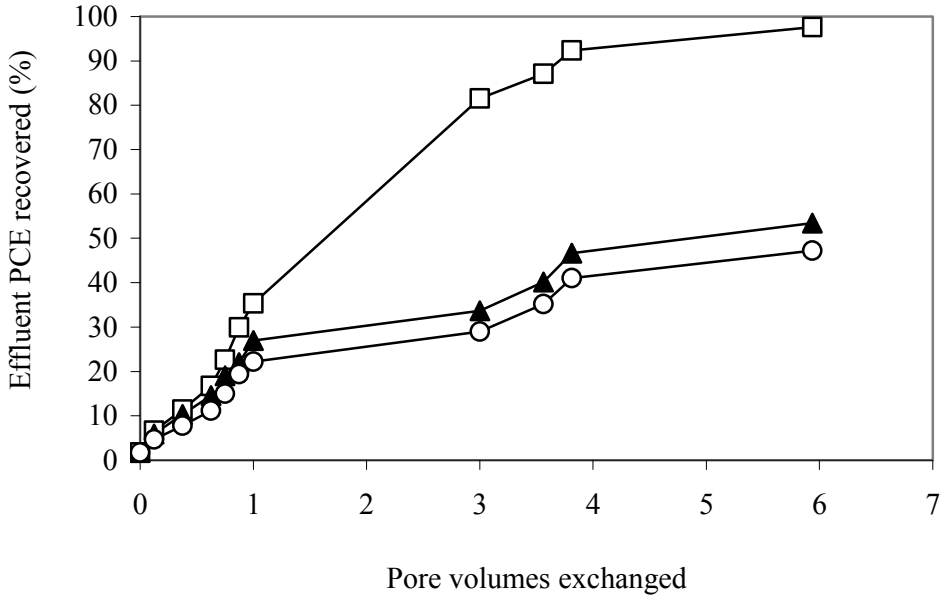


**Figure 4-4. Effluent PCE Breakthrough Curves for Column Studies. Symbols: □ 50%** (v/v) **Ethanol, ▲ Groundwater, and ○ Dechlorinating Culture.**

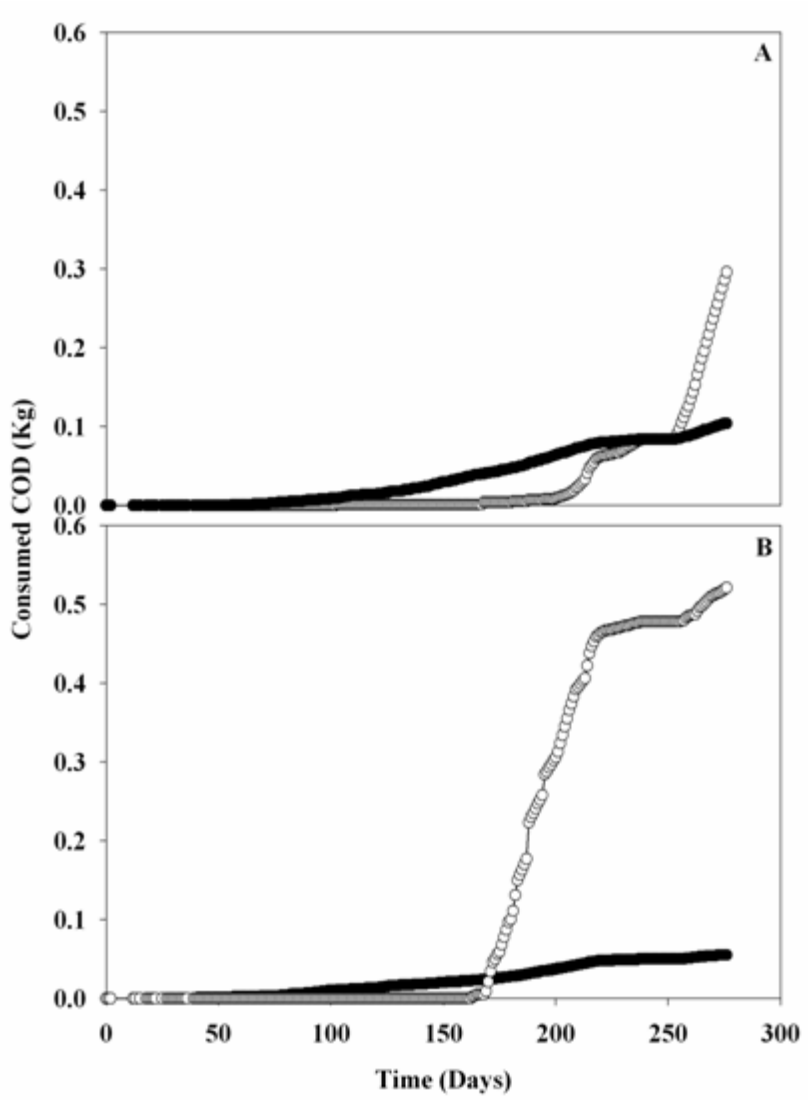


**Figure 4-5. Comparison of Electron Donor Equivalents (**as COD**) Used for Methanogenesis Versus Reductive dechlorination in Bioaugmented Tank (**A**) and Biostimulated Tank** (B**). Symbols: ○ Methane COD and ● dechlorination COD**.

biostimulated tank is that the sand initially contained low concentrations of dechlorinating organisms that eventually proliferated due to selective pressure by PCE and electron donor amendments.

The sandy material used in this work was obtained from the Brazos River in south Texas which drains several urban areas, and the possibility that this material had previous exposure to trace levels of chlorinated solvents or naturally occurring chloroorganic compounds cannot be ruled out (Keppler *et al.*, 2002). Our results support the notion of that halorespiring bacteria may be widely distributed in nature. Hendrickson *et al.* (2002) demonstrated that *Dehalococcoides* organisms are widely distributed in the environment and can survive in a wide range of geographical locations, geological matrices, and climatic zones, possibly consuming naturally

produced chloroorganic compounds. Microbial analysis of the groundwater conducted after 160 days of experiment showed that the concentration of *Dehalobacter* spp. was one order of magnitude higher in the bioaugmented tank ($10^4$ cells/ mL) compared to the biostimulated tank ($10^3$ cells/mL). Because *Dehalobacter* spp. cannot dechlorinate past *cis*-DCE, these results corroborate the higher concentrations of *cis*-DCE observed in the bioaugmented tank.

In this study, biostimulation was performed by adding a solid electron donor (HRC®) and later a liquid electron donor (dissolved lactate). Our results were insufficient to distinguish which electron donor delivery approach is more cost-effective for source bioremediation. Whereas liquid delivery systems (e.g., lactate) can generally achieve good hydraulic control, one potential concern is clogging in areas near the injection well due to excessive microbial growth. In addition, continuous delivery can result in relatively high operation (energy and labor) costs. On the other hand, solid-phase delivery systems (e.g., HRC®) provide for a long-term source of electron donor with negligible energy and labor requirements, resulting in lower operational costs. However, depletion of the stimulatory material and the potential for contaminated water to bypass the biostimulated zone, due to lack of hydraulic control, are potential concerns that need to be addressed on a case-by-case basis (Alvarez and Illman, 2005).

Overall, it was demonstrated that the dechlorination of a PCE DNAPL can be achieved utilizing both bioaugmentation and biostimulation. Although dechlorination in the biostimulated and bioaugmented tanks followed similar patterns, and some PCE DNAPL may have been displaced during injection of the microbial culture, it was clear from the overall mass balance of dechlorination products that bioaugmentation enhanced PCE mass removal (1.6 times) by increasing the local flux of contaminants into the aqueous phase via the production of more soluble and less hydrophobic metabolites (mainly *cis*-DCE). These results suggest that bioaugmentation could significantly aid in the removal of DNAPL source zones in aquifers compared to biostimulation alone.

## Phase II

The objective of Phase II was to evaluate DNAPL flux enhancement through bioaugmentation and biostimulation of the source zone. Two ECRS tanks were operated in parallel with identical aquifer material, and DNAPL constituents. One tank was biostimulated and bioaugmented by the addition of a suitable mass of an anaerobic dechlorinating consortium (same as used in Phase I) directly into the source zone. The other tank served as control (natural attenuation) to discern the benefits of bioaugmentation plus biostimulation.

Monitoring of the ECRS tanks was carried out for 225 days. Effluent PCE concentrations decreased over time in both tanks (Figure 4-6). TCE and DCE were first detected in the effluent of the biostimulated (prior to bioaugmentation) tank after 67 days of experiment (Figure 4-6).

Therefore, it seems that the indigenous microorganisms were capable of dechlorinating PCE to TCE, DCE, and VC, but not to ethene.

Dechlorination activity (as measured by the production of TCE, DCE, VC and ethene) increased significantly just after bioaugmentation. More importantly, VC and ethene, which are often the targeted dechlorination byproduct that measures the success of DNAPL bioremediation, were only detected (after 120 days) following bioaugmentation. VC and ethene concentrations that increased over time. Dechlorination activity was not observed in the control tank as indicated by the absence of measurable concentrations of TCE, DCE, VC and ethene (Figure 4-6), until day 150. After 150 days, however, TCE and DCE started to be detected as dechlorination products in the control tank.

Figure 4-7, shows the fate of COD as electron equivalents in the bioaugmented tank. A noticeable increase in electron acceptor equivalent (as COD) consumption was observed after bioaugmentation.

Effluent methane concentration in the biostimulated and bioaugmented tank were below detection limits until day 170. After 170 days effluent methane concentration increased and persisted at about 80 mg/L.

The low concentrations of acetate and propionate (typical byproducts of the HRC® fermentation) observed in the tanks suggested that the available $H_2$ source is being used completely for dechlorination processes rather than methanogenic process. For example, previous studies demonstrated that the population of methanogenic bacteria cannot outcompete dechlorinating organisms in the presence of low hydrogen concentrations (He*, et al.*, 2002, Yang and McCarty, 1998).

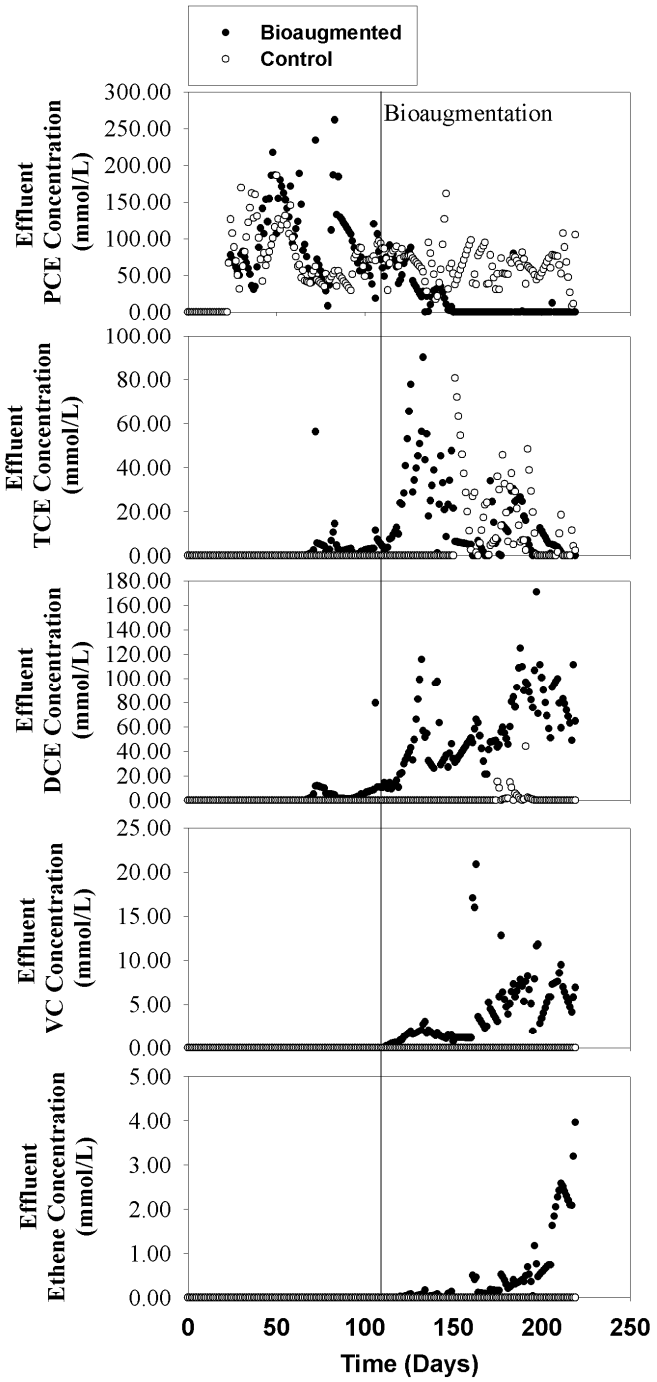


**Figure 4-6. Effluent PCE, TCE, DCE, VC and Ethene Concentrations in the bioaugumented and Non-bioaugmented (**control**) Tanks. Bioaugmentation was Performed on Day 106.**

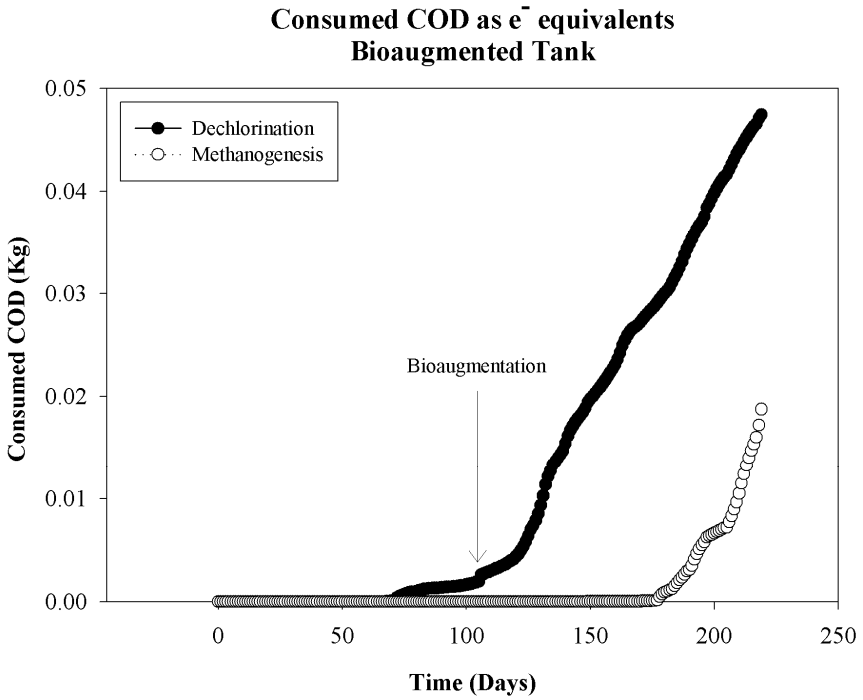


**Figure 4-7. Comparison of Electron Donor Equivalents (**as COD**) used for Methanogenic and Dechlorination Activities in the Bioaugmented Tank.**

## Microbial Analysis (Phase I)

Groundwater samples from all the regions of the bioaugmented tank and the biostimulated tank (Phase I) were analyzed to compare the initial microbial populations present in the sand. Using qPCR, it was determined that the cell numbers for total bacteria (~$10^4$ cells/mL), *Dehalobacter* spp. (~$10^1$ cells/mL), *Desulfuromonas* spp. (~$10^1$ cells/mL), archaea (~$10^2$ cells/mL) and *Dehalococcoides* (~$10^1$ cells/mL) were all approximately the same order of magnitude.

DGGE profiles with universal bacterial primers on samples taken from the biostimulated tank initially, at Day 161 and at the end of the experiment (Final) is shown in Figure 4-8. Initially, there were no major populations detected by DGGE in the biostimulated tank. On Day 161, in the plume region, bands appear in the DGGE profile. The final samples analyzed demonstrated that in the source zone region detectable levels of organisms were present in the bottom 0.3 m of the tank and that the plume region had a higher number of bands and therefore a wider diversity than the source region. Bands from the DGGE were sequenced and the results show that *Dehalococcoides* and *Dehalobacter* spp. were both present in the tank and are labeled on Figure 4-8.

The DGGE profile with universal bacterial primers on samples taken from the bioaugmented tank and on the culture used to inoculate the bioaugmented tank is shown in Figure 4-9.

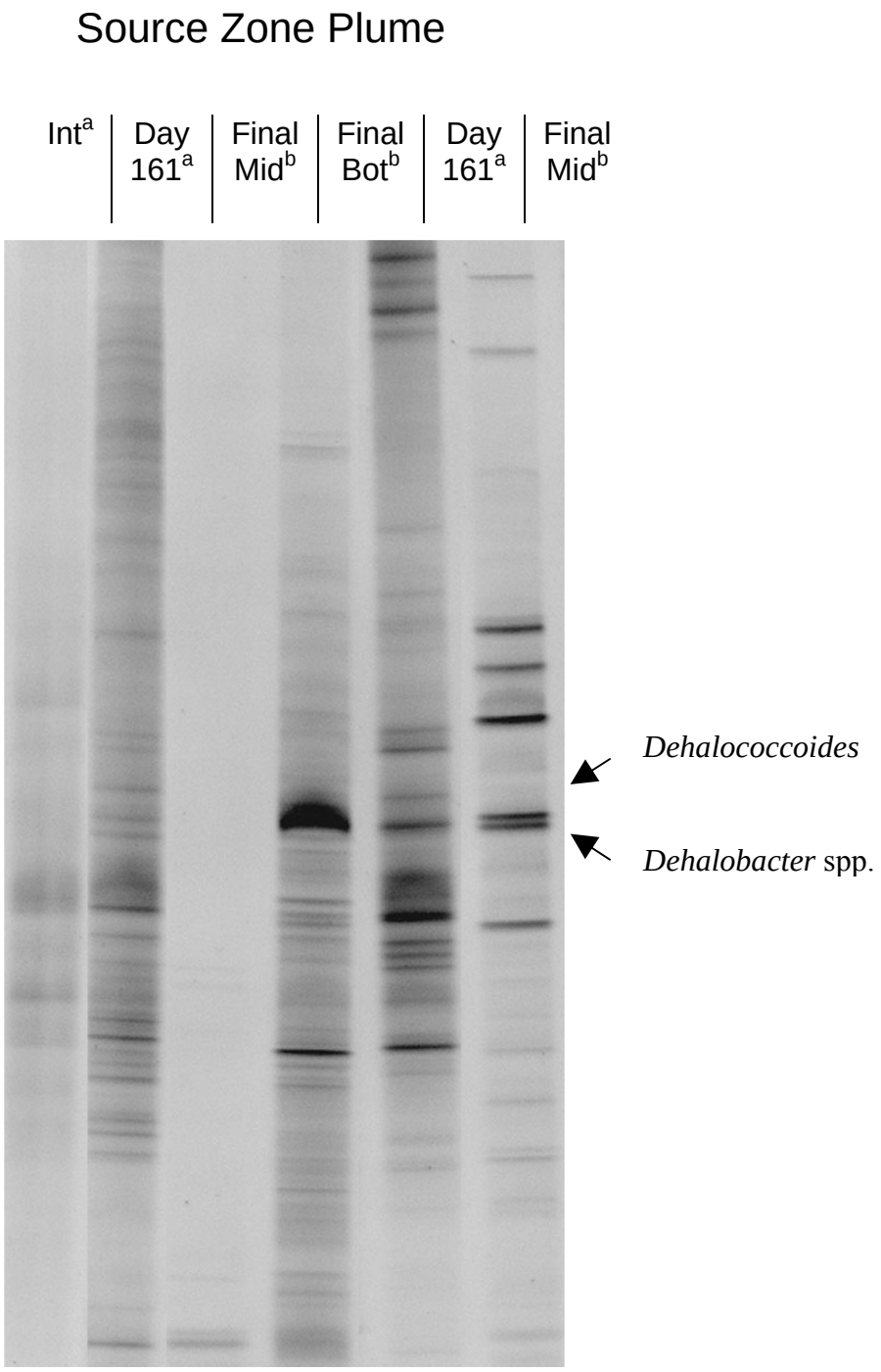


**Figure 4-8. DGGE with Universal Bacterial Primers on Samples from Biostimulated Tank. int = Initial, Mid = (**0.3-0.6 m**), Bot = (**0 – 0.3m**). a = Groundwater Sample, b = Soil Sample.**

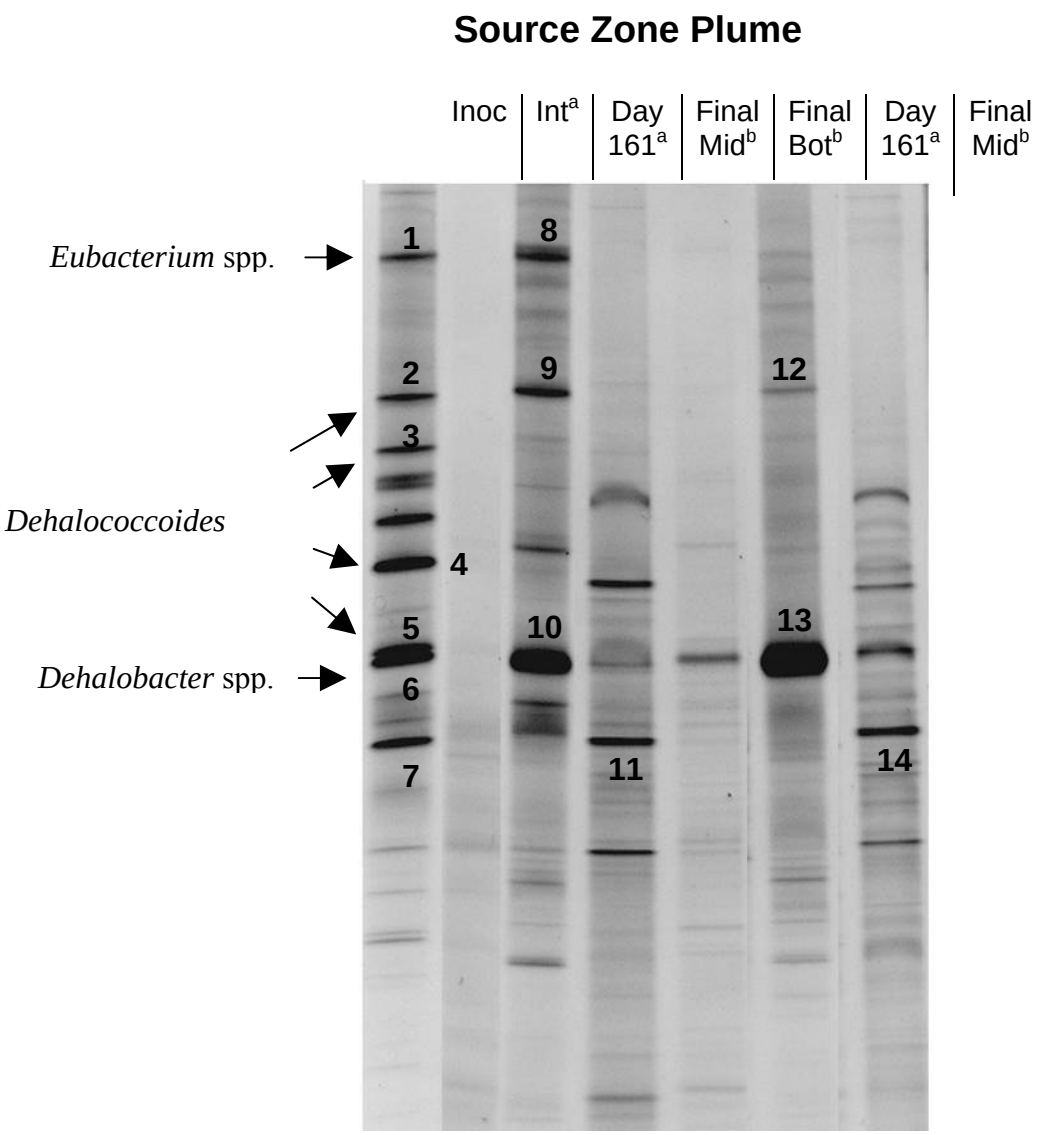


**Figure 4-9. DGGE with Universal Bacterial Primers for the Inoculum and Samples from Bioaugmented Tank. Inoc = Inoculum, Int = initial, Mid = (**0.3-0.6 m**), Bot = (**0 – 0.3m**). a = Groundwater sample, b = soil sample.**

The DGGE analyses demonstrate that the culture used to inoculate the tank contained approximately 10 dominant organisms. These bands were sequenced and the ones that yielded identifiable organisms are labeled: *Dehalococcoides* (bands 2, 3, 4, and 5) *Dehalobacter* spp. (band 6) and *Eubacterium* spp. (band 1).

The initial sample from the source zone of the bioaugmented tank contained no detectable levels of bacteria. Comparing the banding pattern of the inoculum to the banding pattern of the samples taken from the bioaugmented tank shows that on Day 161 both the plume region and the source zone area contained the dominant bands (bands 10 and 12) that represented *Dehalobacter* spp. and *Dehalococcoides* in the inoculum. Sequencing of these bands was used to identify species

but as dechorination proceeded and the organisms increased in number, the bands became larger and it was impossible to separate them with DGGE. The other dominant *Dehalococcoides* bands in the inoculum (2, 3, and 4) do not appear in any of the samples taken from the bioaugmented tank, except for band 2, which is seen again as a dominant band (band 9) on Day 161 in the source zone and as a faint band (band 12) on Day 161 in the plume region. The *Eubacterium* spp. identified was only present on Day 161 in the source zone region. There was also a band in the inoculum (band 7) that appeared in final samples for both the source zone and the plume region. Sequencing of this band identified it as most closely related to "unculturable organisms".

DGGE was also performed with *Dehalococcoides*-specific primers on both the bioaugmented tank and the biostimulated tank and those results are shown in Figure 6.4. Initially, in the biostimulated tank there was a strain 195-type organism (PCE-to-VC and ethene cometabolically) (Figure 4-10A). This organism is present in the source zone on Day 161, but then was not seen in any other samples taken. In the final sample in the plume region of the biostimulated tank, a strain VS-type organism (*cis*-DCE-to-ethene) and a strain FL2-type (TCE-to-VC and ethene cometabolically) or a strain GT-type organism (*cis*-DCE-to-ethene) was identified. It is impossible to separate strain FL2 or strain GT by DGGE because they have identical 16S rRNA sequences (He et al., 2005; Sung et al., 2006a).

In the inoculum for the bioaugmented tank, there were three possible *Dehalococcoides* strains identified in the DGGE with *Dehalococcoides*-specific primers: strain 195-type, strain FL2-type and/or a strain GT-type (Figure 4-10B). All three strains were seen in all the samples from the bioaugmented tank, but in the final sample for the source zone region and the sample taken on Day 161 in the plume region, the strain FL2-type or strain GT-type band was no longer dominant.

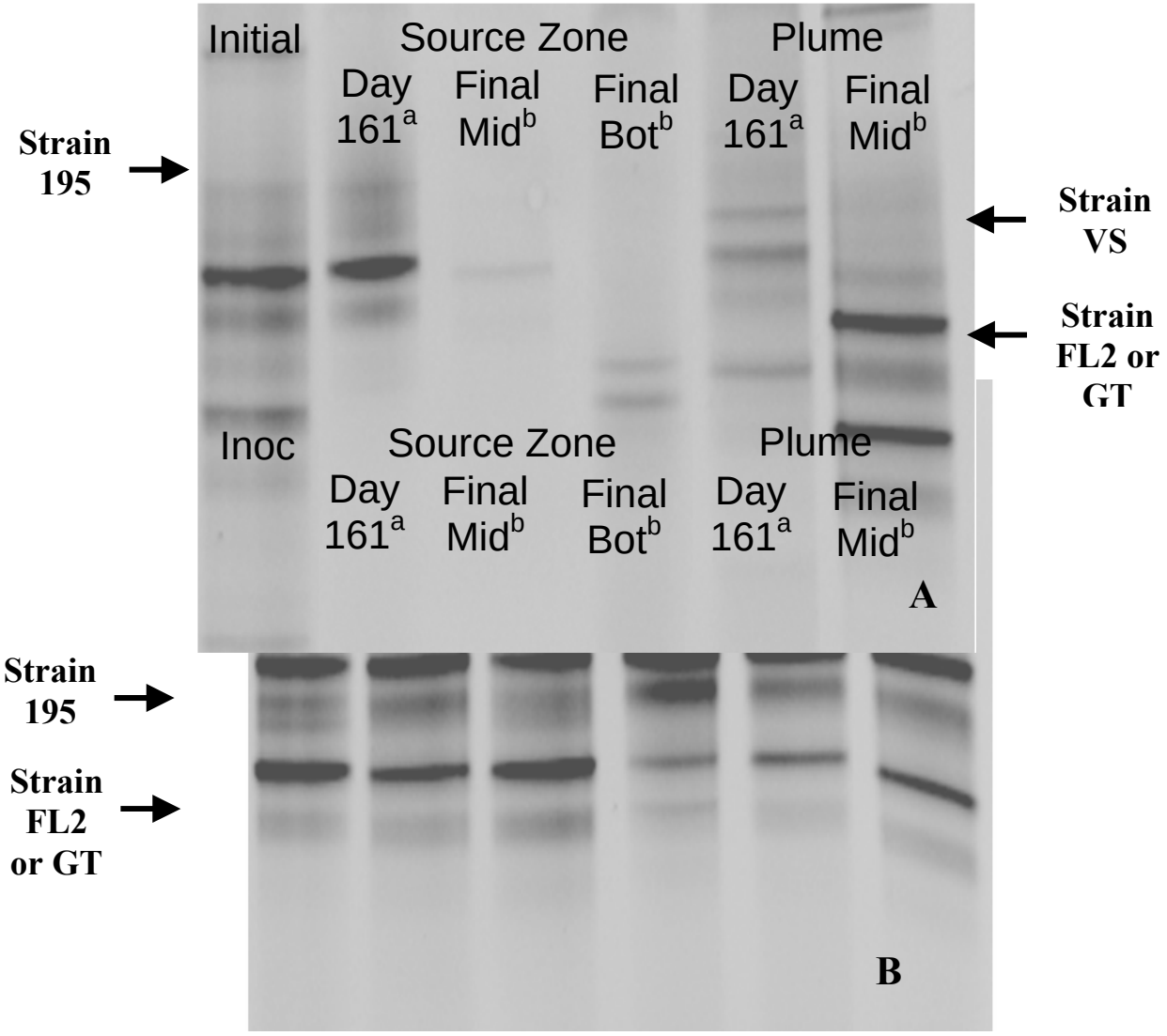


**Figure 4-10. DGGE with *Dehalococcoides*-specific Primers on Samples from the (**A**) Biostimulated Tank and from the (**B**) inoculum and samples from bioaugmented tank. Inoc = Inoculum, Int = initial, Mid = (**0.3-0.6 m**), Bot = (**0 – 0.3m**). a = Groundwater sample, b = soil sample.**

A comparison of the total numbers of dechlorinating organisms (*i.e.*, the sum of the cell numbers for *Dehalococcoides*, *Dehalobacter* spp., and *Desulfuromonas* spp.) to the total bacteria in both the bioaugmented tank and biostimulated tank is given in Table 4-3. In both the bioaugmented tank and the biostimulated tank, the dechlorinating organisms are less than 1% of the total organisms present for all samples taken, except the final sample in the upgradient region of the biostimulated tank, the final sample in the source zone for the bioaugmented tank, and the final sample in the plume region for both the bioaugmented tank and the biostimulated tank. In these samples, the dechlorinating organisms were still only 1-2% of the population. It is interesting that the majority of the dechlorinating organisms in the biostimulated tank were in the upgradient region and in the plume region, but in the bioaugmented tank the majority of the dechlorinating organisms were in the source zone and the plume region.

The total cell numbers of archaea and *Dehalococcoides* were compared and are listed in Table 4-4. Initially, the biostimulated tank contains approximately an order of magnitude more archaea than the bioaugmented tank, but both tanks contain the same order of magnitude of *Dehalococcoides*. On Day 161, there was only a slight increase in the numbers of *Dehalococcoides* for both tanks in all samples, but from Day 161 to the final sample, there was at least a two orders of magnitude increase in the *Dehalococcoides* cell numbers, except for in the source zone of the biostimulated tank where no *Dehalococcoides* were detected in the final sample.

It was demonstrated by both the qPCR and DGGE results that the dechlorinating populations were not washed out of either tank. It was also determined that at least one of the dominant *Dehalococcoides* identified in both tanks was capable of growth on VC (Sung et al., 2006a; Duhamel et al., 2004), so the lack of ethene production was not due to conditions restricted to slow cometabolic transformation of VC to ethene.

**Table 4-3. Comparison of Total Dechlorinating Organisms (*Dehalococcoides*, *Dehalobacter* spp. and *Desulfuromonas* spp.) to All Other Organisms in Both the Bioaugmented Tank and Biostimulated Tank.**

| | **Bioaugmented Tank** | | **Biostimulated Tank** | |
|---|---|---|---|---|
| | **Percent of Total Bacteria** | | **Percent of Total Bacteria** | |
| **Upgradient Region** | **Dechlorinators** | **Others** | **Dechlorinators** | **Others** |
| **Initial**[a] | 0.49 ± 0.24 | 99.51 ± 0.42 | 0.11 ± 0.01 | 99.89 ± 0.29 |
| **Day 161**[a] | 2.56e-3 ± 7.28e-4 | 100.00 ± 0.40 | 0.01 ± 2.14e-3 | 99.99 ± 0.20 |
| **Final**[b] | 0.07 ± 0.03 | 99.93 ± 0.69 | 2.83 ± 0.60 | 97.17 ± 0.09 |
| **Source Zone** | | | | |
| **Initial**[a] | 0.57 ± 0.15 | 99.42 ± 0.36 | 0.01 ± 0.002 | 99.99 ± 0.32 |
| **Day 161**[a] | 0.02 ± 0.01 | 99.97 ± 0.23 | 0.04 ± 0.01 | 99.96 ± 0.35 |
| **Final**[b] | 2.20 ± 0.69 | 97.79 ± 0.36 | 0.10 ± 0.04 | 99.90 ± 0.32 |
| **Plume Region** | | | | |
| **Initial**[a] | 0.57 ± 0.05 | 99.43 ± 0.50 | 0.22 ± 0.03 | 99.78 ± 0.09 |
| **Day 161**[a] | 0.08 ± 0.01 | 99.92 ± 0.13 | 0.17 ± 0.02 | 99.83 ± 0.19 |
| **Final**[b] | 1.72 ± 0.63 | 98.28 ± 0.10 | 1.01 ± 0.16 | 98.99 ± 0.19 |

[a] = groundwater sample
[b] = soil sample

Archaea cell numbers were at least two orders of magnitude higher than the *Dehalococcoides* cell numbers in all regions of the bioaugmented tank and in the upgradient region and plume region of the biostimulated tank on Day 161. For the final sample, the archaea cell numbers were still 1-2 orders of magnitude higher than the *Dehalococcoides* cell numbers, but during this time frame, methanogenesis was consuming 3-5 times more electron donor equivalents. These results suggest that a direct comparison of cell numbers between archaea and *Dehalococcoides* is not an indicator of which process is consuming the most electron donor equivalents. In Phase I it was demonstrated that dechlorination consumed a majority of the electron donor equivalents in the bioaugmented tank for approximately 250 days of the experiment, compared to only 170 days in the biostimulated tank. By Day 250 in Phase I, approximately 88% of the PCE was removed from the bioaugmented tank; combining this previous result with the qPCR results of this study implies that even when bioaugmenting with an archaeal rich culture (~$10^8$ cells/ml) there is enough electron donor equivalents to support both dechlorination and methanogenesis until the electron acceptor (chloroethenes) concentration becomes limiting.

Another interesting finding of this work is that qPCR demonstrated the dechlorinating populations in both the bioaugmented tank and the biostimulated tank were approximately 1-2% of the total bacterial populations present in both tanks, even after dechlorination occurred. It is only possible to compare this percentage with one other study because quantification of total bacteria present at chloroethene contaminated sites is not available (Ellis et al., 2000; Lendvay et al., 2003; Major et al., 2002). Sleep et al. (2006) found that *Dehalococcoides* were approximately 10% of the total bacterial population in the effluent of their bioaugmented system, but only about 1% of the effluent in their biostimulated system. It appears that when *Dehalococcoides* cell numbers are relatively high (> $10^6$ cells/ml) in remediation systems, they are still only a small portion of the overall microbial population.

Utilizing DGGE with *Dehalococcoides*-specific primers on samples from the biostimulated tank demonstrated initially that the biostimulated tank contained a dominant band whose sequence was most closely related to *Dehalococcoides ethenogenes* strain 195, and two faint bands below this one. By the end of the experiment the dominant *Dehalococcoides* in the biostimulated tank shifted to a strain VS-type and a strain GT-type and/or a strain FL2-type organism in the plume region. It is feasible that the strain 195-type organism was out competed by the strain VS-type and strain GT-type, since both of these organisms can gain energy from the dechlorination of *cis*-DCE-to-ethene (Sung et al., 2006a; Duhamel et al., 2004) (after Day 161 mostly *cis*-DCE was detected in the effluent (Da Silva et al., 2006). If the indigenous organisms were to become dominant in the bioaugmented tank, the same shift in the dominant species of *Dehalococcoides* should be identified.

**Table 4-4. Comparison of *Dehalococcoides* to Archaea Cell Numbers in Both the Bioaugmented Tank and Biostimulated Tank.**

| | **Bioaugmented Tank (cells/mL or cell/g)** | | **Biostimulated Tank (cells/mL or cells/g)** | |
|---|---|---|---|---|
| **Upgradient Region** | ***Dehalococcoides*** | **Archaea** | ***Dehalococcoides*** | **Archaea** |
| **Initial[a]** | (4.97e+1) ± (4.82e+0) | (1.11e+2) ± (2.66e+1) | (3.79e+1) ± (4.84e+0) | (8.53e+2) ± (1.13e+1) |
| **Day 161[a]** | (5.11e+1) ± (5.86e+0) | (8.60e+4) ± (2.35e+4) | (8.57e+1) ± (1.77e+1) | (1.57e+5) ± (2.41e+4) |
| **Final[b]** | (2.54e+3) ± (5.25e+2) | (1.20e+6) ± (2.44e+5) | (1.14e+4) ± (8.19e+2) | (1.98e+4) ± (4.30e+3) |
| **Source Zone** | | | | |
| **Initial[a]** | (1.94e+1) ± (9.41e-1) | (1.45e+2) ± (1.04e+2) | (4.85e+1) ± (1.07e+0) | (1.89e+3) ± (1.33e+2) |
| **Day 161[a]** | (3.20e+2) ± (7.96e+1) | (2.47e+4) ± (6.52e+2 ) | (8.19e+1) ± (9.02e+0) | (2.56e+0) ± (2.80e-1) |
| **Final[b]** | (1.03e+5) ± (3.55e+3) | (6.02e+5) ± (1.03e+5) | 0.00 ± 0.00 | (2.94e+5) ± (3.23e+4) |
| **Plume Region** | | | | |
| **Initial[a]** | (5.87e+1) ± (4.66e+0) | (3.57e+1) ± (4.70e+0) | (1.48e+1) ± (4.69e+0) | (1.71e+3) ± (2.54e+2) |
| **Day 161[a]** | (6.89e+2) ± (1.36e+2) | (3.44e+4) ± (5.28e+3) | (9.68e+0) ± (8.55e-2) | (5.83e+3) ± (1.54e+2) |
| **Final[b]** | (4.16e+4) ± (5.03e+3) | (2.44e+5) ± (1.80e+4) | (2.12e+3) ± (3.65e+2) | (5.37e+5) ± (8.05e+4) |

[a] Units are cells/mL
[b] Units are cells/g

It was determined initially that a strain 195-type *Dehalococcoides* was present in the sand used to pack the ECRS. Since the inoculum contained a strain 195-type organism, it is impossible to differentiate between the augmented and indigenous strain 195-type organism in the bioaugmented tank. But, the DGGE with *Dehalococcoides* specific primers implied that the *Dehalococcoides* in the inoculum remained the dominant *Dehalococcoides* in the bioaugmented tank, since there was no evidence of a strain VS-type organism in any of the samples. This suggests that the bioaugmented organisms became the dominant populations in the bioaugmented tank.

Another line of evidence to demonstrate that the organisms contained in the augmented culture became the dominant community in the bioaugmented tank was the results from the DGGE with universal bacterial primers. It is clear that some dominant bands from the inoculum are seen in the banding patterns from samples taken from the tank overtime. Comparing the DGGE with universal bacterial primers on samples from the biostimulated tank to samples from the bioaugmented tank demonstrated that they have very different banding patterns and therefore, different communities.

Interestingly, results from the DGGE analysis suggest that there is evidence of DNAPL migrations in the biostimulated tank. The DGGE with *Dehalococcoides*-specific primers on samples from the biostimulated tank demonstrated that there were no *Dehalococcoides* present in the source zone at the end of the experiment, and qPCR analysis confirmed these results showing no *Dehalococcoides* in the source zone region. qPCR did demonstrate there were *Dehalococcoides* in the upgradient region of the biostimulated tank and the plume region, which could be where the DNAPL migrated. These results demonstrate the difficulty in locating the source of DNAPL, even in a controlled system.

DGGE with *Dehalococcoides*-specific primers for the bioaugmented tank also demonstrated that the GT-type and/or strain FL2-type organisms in the bioaugmented tank were not dominant in the source zone (bottom) for the final sample or in the plume region for the sample on Day 161. It would be expected that the strain GT-type organism would flourish in the plume region, since only *cis*-DCE was present in the effluent (after Day 161). It is possible that PCE DNAPL was still present in the source zone and the strain 195-type organism was dechlorinating this remaining PCE DNAPL. Another possibility is that the strain 195-type organism grew on VC as demonstrated by the presence of the *vcrA* gene. The *vcrA* gene was detected in the inoculum for the bioaugmented tank. These findings could explain why the strain 195-type organism became dominant in the bioaugmented tank over the VC respiring strain GT-type *Dehalococcoides*.

DGGE with universal bacterial primers identified multiple *Dehalococcoides* bands in the inoculum used for the bioaugmented tank. DGGE separates bands based on the GC content of the sequence, and if there are multiple organisms with identical sequences, in theory, they should be represented by a single band in the gel. This multiple banding phenomenon is one of the limitations of DGGE and has been seen by other researchers (Nakatsu et al., 2006; Calvo-Bado et al., 2003). One explanation for multiple bands with identical sequences is the formation of heteroduplexes (Kanagawa, 2003). Heteroduplexes occur during multiple template (samples with DNA from multiple organisms) PCR when there is cross-hybridization between the target sequence and primers and/or other templates. When the PCR product is run on the gel, the

heteroduplexes show up as extra bands on the gel (Kanagawa, 2003). This could explain the multiple bands seen for *Dehalococcoides* in the DGGE with universal bacterial primers.

The results of this study demonstrate that the bioaugmentation of the ECRS system was successful and that the organisms present in the inoculum became the dominant organisms in the ECRS. Unfortunately, even with *Dehalococcoides* present, complete dechlorination to ethene was not achieved and analysis of the groundwater and sand from the ECRS systems with molecular biology techniques did not give any insight into why dechlorination was incomplete. Results demonstrate that the lack of ethene production was not due to washout of the dechlorinating organisms. It was also demonstrated that there was no correlation between cell numbers and activity of methanogens and dechlorinators, and that as long as the electron acceptor was not limiting, there was greater energy flow to the dechlorinating populations than to the methanogens.

## Cost Assessment

### Cost Reporting

As part of the comprehensive cost assessment that is presented in the ESTCP cost and performance report at the conclusion of the technology demonstration, there are a number of capital and operational costs that were tracked and reported throughout the project. A summary of these is included in Table 5-1.

**Table 5-1. Cost Tracking.**

| Cost Category | Sub Category | Details |
|---|---|---|
| **START-UP COSTS** | Culture Development | (growth reactors, growth medium constituents) |
| | Test system preparation | |
| | Mobilization | (planning and labor) |
| **CAPITAL COSTS** | Flow control | (feed reservoirs, valves, flow meters, piping, sampling lines) |
| | Porous medium | (sand) |
| | Process stream treatment | (activated carbon canisters) |
| | Sample collection | (syringes, sample bottles) |
| | Raw material installation | (installation of HRC® by Regenesis personnel) |
| **OPERATING COSTS (Direct)** | Process raw materials | (PCE, HRC®) |
| | Nutrients | Sodium-lactate |
| | Sample analysis | GC analytical column, consumables for molecular work including primer construction and sequencing |
| | Ongoing operation and supervision | (labor) |
| | Monitoring | $0 |
| | Maintenance and Utilities | (none)[1] |
| | Travel and Sample Delivery | (molecular analyses off-site) |
| **INDIRECT COSTS** | Environmental Health and Safety Training | (none)[1] |
| **DEMOBILZATION** | Sediment disposal | (includes testing and disposal) |

[1] Costs associated with these categories are paid through overhead or indirect costs to Rice University

### Cost Analysis

The cost comparison was aided greatly by the use of an independent control during this demonstration. This control serves as a direct indication of the costs associated with a pump-and-treat plume management strategy.

Initial DNAPL mass and source longevity provided the primary cost basis for determining scale-up costs for full-scale implementation. A quantitative measure of the impact of the addition of a dechlorinating culture could be used to re-evaluate the operating requirements for a site that has been estimated as 100-year pump-and-treat remediation. Cost drivers include pumping rates (and resulting process streams) as well as monitoring requirements, both of which would decrease if the source longevity is favorably impacted. Life cycle considerations for the implemented technology would differ significantly from the described demonstration, primarily in terms of the capital costs associated with a field site versus those associated with a simulated aquifer. Regulatory considerations would also need to be accounted for, although start-up and operating and maintenance costs would be relatively similar in such a life-cycle assessment. One major difference is that the technology could theoretically result in the formation of significant levels of chlorinated metabolites, all of which would be necessary to treat further in a full-scale implementation. It is likely that this liability would be dealt with directly by designing a down-gradient remediation scheme and that the costs of this type of strategy would be built into a life cycle analysis. Given the generally accepted persistence of contamination in DNAPL source zones, a minimum of a 100-year lifetime would be necessary in conducting a comprehensive life cycle analysis.

# 5. Implementation Issues

## 6.1. Environmental Checklist

No permit procurement was necessary to conduct this technology demonstration. All rules and regulations set forth by the Rice University Environmental Health and Safety Department (EHSD) were followed, and this department served in an oversight capacity for this project. Aqueous samples collected for analysis were combined and disposed of according to the regulations of the Rice University EHSD (hazardous waste landfill or incineration of chlorinated solvent-contaminated waste). All aqueous discharges to the sewers were free of organic contaminants, with regular and systematic checks to ensure complete compliance. Disposal of sediment was contracted out to a licensed independent group (USA Environmental, Houston, Texas).

## 6.2. Other Regulatory Issues

The data and information resulting from this technology demonstration were freely disseminated via publications (Da Silva, et. al, 2006) and presentations at national conferences (Battelle). No plans have been made yet to discuss this information specifically with regulatory agencies.

## 6.3. End-User Issues

This technology demonstration were intended to test and demonstrate the viability of source zone bioremediation. The experimental systems (ECRS) were not designed to be directly used in a full-scale remediation plan. Therefore, many of the procurement and hydraulic issues associated with the ECRS are not necessarily of concern to a site manager interested in implementing the technology. Of particular interest to the end user would be the degree of dissolution enhancement (and therefore the impact on source longevity) that could be achieved biologically when compared to the pump-and-treat alternative. In addition, the inoculation mass and survival of particular dechlorinating species is key to designing at a larger scale.

In terms of a full-scale implementation, little expertise is required to analyze results to determine if the technology is successful. Monitoring for the formation of chlorinated metabolites and ethene is an indication that dissolution has been enhanced. More comprehensive flux enhancement data can be obtained by placing monitoring wells closer to the delineated location of a source zone.

The primary issues (regulatory and procurement) that will need to be dealt with by those wishing to implement the technology are the 1) injection of a suitable organic donor, and 2) development or procurement of a suitable dechlorinating culture. Both of these steps must be done in compliance with the framework of federal, state, and local regulations that apply to a given site location. Because this technology is not markedly different than traditional biostimulation (in the absence of NAPL) in terms of basic design considerations and requirements, there is increasingly less resistance encountered in receiving approval for this type of remediation plan. Obtaining a suitable culture for bioaugmenting a source zone requires a certain degree of skill in terms of culture development, although there are numerous research institutions as well as commercial operations that can be used for consultation or as a potential source of organisms. One of the electron donors used in this project is a proprietary compound (HRC®), and the manufacturer (Regenesis) intends to take the technology to the marketplace if the demonstration proves successful.

## 7. References


Adamson, D. T. and J. B. Hughes (2004) "Flux and Product Distribution During Biological Treatment of PCE DNAPL", Environmental Science and Technology, (38), 2021-28.

Aiello, M. R. Quantitative Environmental Monitoring of PCE Dechlorinators in a Contaminated Aquifer and PCE-fed Reactor. M.S., Michigan State University, East Lansing, Michigan, 2003.

Bratbak, G. (1985) Bacterial Biovolume and Biomass Estimations. Apllied Environmental Microbiology, 49, 1488-1493.

Cope, N. and J. B. Hughes (2001) Biologically-enhanced Removal of PCE from NAPL Source Zones. Environmental Science and Technology, 35, 2014-2021.

Carr, C.S., S. Garg, and J.B. Hughes (2000) "Effect of Dechlorinating Bacteria on the Longevity and Composition of PCE-Containing Nonaqueous Phase Liquids Under Equilibrium Dissolution Conditions." Environmental Science and Technology, Vol. 34, No. 6, pp. 1088-1094.

Calvo-Bado, L. A.; J. A. W. Morgan, M. Sergeant, T. R. Pettitt, and J. M. Whipps. Molecular Characterization of Legionella Populations Present within Slow Sand Filters Used for Fungal Plant Pathogen Suppression in Horticultural Crops. Applied Environmental Microbiology, **2003,** 69, 533-541.

Carr, C.S., S. Garg, and J.B. Hughes (2000) Effect of Dechlorinating Bacteria on the Longevity and Composition of PCE-Containing Nonaqueous Phase Liquids Under Equilibrium Conditions. Environmental Science and Technology. 34: 1088-1094.

Cupples, A. M., A. M. Spormann, and P. L. McCarty (2003) Growth of a *Dehalococcoides*-like Microorganism on Vinyl Chloride and Cis-dichloroethene as Electron Acceptors as Determined by Competitive PCR. Applied Environmental Microbiology, **69,** 953-959.

Da Silva, M. L. B. and P. J. J. Alvarez (2004) Enhanced Anaerobic Biodegradation of Benzene-toluene-ethylbenzene-xylene-ethanol Mixtures in Bioaugmented Aquifer Columns. Applied Environmental Microbiology, **70,** 4720-4726.

Da Silva, M. L. B. and P. J. J. Alvarez (2002) Effects of Ethanol Versus MTBE on Benzene, Toluene, Ethylbenzene, and Xylene Natural Attenuation in Aquifer Columns. Journal of Environmental Engineering ASCE 128, 862–867.

Da Silva, M.L.B.; Rebecca Da Prato, Diego E. Gomez, Joseph Hughes, Pedro, J.J. Alvarez, and Herb Ward. (2006). "Reductions in DNAPL Longevity Through Biological Flux Enhancement." Water Environmental Research, 78(13): 2456-2465.

Da Silva, M.L.B., D. E. Gomez, R. Da Prato, J. Hughes, P. J. J. Alvarez, and C. H. Ward (2005) *Bioaugmentation to Enhance Source Zone Remediation Through Reductive Dechlorination*. 8th In Situ and On-Site Bioremediation Symposium, Battelle. Baltimore, Maryland, June 6-9.

Duhamel, M., K. Mo, and E. A. Edwards (2004) Characterization of a Highly Enriched *Dehalococcoides*-containing Culture that Grows on Vinyl Chloride and Trichloroethene. Applied Environmental Microbiology, **70,** 5538-5545.

Ellis, D. E., E. J. Lutz, J. M. Odom, R. J. Buchanan, C. L. Bartlett, M. D. Lee, M. R. Harkness, and K. A. Deweerd. Bioaugmentation for Accelerated In Situ Anaerobic Bioremediation. Environmental Science and Technology, **2000,** 34, 2254-2260.

Ebersole, R. C. and E. R. Hendrickson (2005) Nucleic Acid Fragments for Identification of Dechlorinating Bacteria, 6,894,156,USA.

El Fantroussi, S., J. Mahillon, H. Naveau, and S. N. Agathos (1997) Introduction and PCR detection of *Desulfomonile tiedjei* in Soil Slurry Microcosms. *Biodegradation*, **8,** 125-133.

Ellis, D. E., E. J. Lutz, J. M. Odom, R. J. Buchanan, C. J. Bartlett, M. D. Lee, M. R. Harkness, and K. A. Deweerd (2000) Bioaugmentation for Accelerated In Situ Anaerobic Bioremediation. Environmental Science and Technology, **34,** 2254-2260.

Francy, D. S., J. M. Thomas, R. L. Raymond, and C. H. Ward (1991) Emulsification of Hydrocarbons by Subsurface Bacteria. Journal of Industrial Microbiology, **8,** 237-245.

Gerritse, J., V. Renard, T. M. P. Gomes, P. A. Lawson, M. D. Collins, and J. C. Gottschal (1996) *Desulfitobacterium* sp. strain PCE1, an Anaerobic Bacterium that can Grow by Reductive Dechlorination of Tetrachloroethene or Ortho-chlorinated Phenols. *Archives of Micorbiology*, **165,** 132-140.

Greenberg, E. E., L. S. Clesceri, and A. D. Eaton (1992) *Standard Methods for the Examination of Water and Wastewater*, American Public Health Association/American Water Works Association/Water Environment Federation, 18th ed; Washington, D.C.

He, J. Z., K. M. Ritalahti, M. R. Aiello, and F. E. Löffler. Complete Detoxification of Vinyl Chloride by an Anaerobic Enrichment Culture and Identification of the Reductively Dechlorinating Population as a *Dehalococcoides* Species. Applied Environmental Microbiology, **2003b,** 69, 996-1003.

He, J. Z., Y. Sung, M. E. Dollhopf, B. Z. Fathepure, J. M. Tiedje, F. E. Löffler (2002) Acetate Versus Hydrogen as Direct Electron Donors to Stimulate the Microbial Reductive Dechlorination Process at Chloroethene-contaminated Sites. Environmental Science and Technology, **36,** 3945-3952.

He, J., Y. Sung, R. Krajmalnik-Brown, K. M. Ritalahti, and F. E. Löffler (2005) Isolation and Characterization of *Dehalococcoides* sp. Strain FL2, a Trichloroethene (TCE)- and 1,2-Dichloroethene-respiring Anaerobe. *Environmental Microbiology*, **7,** 1442-1450.

He, J. Z., K. M. Ritalahti, M. R. Aiello, and F. E. Löffler (2003b) Complete Detoxification of Vinyl Chloride by an Anaerobic Enrichment Culture and Identification of the Reductively Dechlorinating Population as a *Dehalococcoides* Species. Applied Environmental Microbiology, **69,** 996-1003.

He, J. Z., K. M. Ritalahti, K. L. Yang, S. S. Koenigsberg, and F. E. Löffler (2003a) Detoxification of Vinyl Chloride to Ethene Coupled to Growth of an Anaerobic Bacterium. *Nature*, **424,** 62-65.

Hendrickson, E. R., J. A. Payne, R. M. Young, M. G. Starr, M. P. Perry, S. Fahnestock, D. E. Ellis, and R. C. Ebersole (2002) Molecular Analysis of *Dehalococcoides* 16S Ribosomal DNA from Chloroethene-contaminated Sites Throughout North America and Europe. Applied and Environmental Microbiology, **68,** 485-495.

Holliger, C., D. Hahn, H. Harmsen, W. Ludwig, W. Schumacher, B. Tindall, F. Vazquez, N. Weiss, and A. J. B. Zehnder (1998) *Dehalobacter restrictus* gen. nov. and sp. nov., a Strictly Anaerobic Bacterium that Reductively Dechlorinates Tetra- and Trichloroethene in an Anaerobic Respiration. Archives of Microbiology, **169,** 313-321.

Kean, J. A., M. N. Lodato, and D. Graves (2000) Enhanced Biological Reductive Dechlorination at a Dry Cleaning Facility. Ground Water Currents, 37.

Keppler, F., R. Borchers, J. Pracht, S. Rheinberger, and H. F. Scholer (2002) Natural Formation of Vinyl Chloride in the Terrestrial Environment. Environmental Science and Technology, **36,** 2479-2483.

Krajmalnik-Brown, R., T. Holscher, I. N. Thomson, F. M. Saunders, K. M. Ritalahti, and F. E. Löffler (2004) Genetic Identification of a Putative Vinyl Chloride Reductase in *Dehalococcoides* sp. strain BAV1. Applied Environmental Microbiology, **70,** 6347-6351.

Kube, M., A. Beck, S. H. Zinder, H. Kuhl, R. Reinhardt, and L. Adrian (2005) Genome Sequence of the Chlorinated Compound Respiring Bacterium *Dehalococcoides* species strain CBDB1. *Natural Biotechnology*, **23,** 1269-1273.

Lendvay, J. M., F. E. Löffler, M. Dollhopf, M. R. Aiello, G. Daniels, B. Z. Fathepure, M. Gebhard, R. Heine, R. Helton, J. Shi, R. Krajmalnik-Brown, C. L. Major, M. J. Barcelona, E. Petrovskis, R. Hickey, J. M. Tiedje, and P. Adriaens (2003) Bioreactive Barriers: A Comparison of Bioaugmentation and Biostimulation for Chlorinated Solvent Remediation. Environmental Science and Technology, **37,** 1422-1431.

Löffler, F. E., Q. Sun, J. R. Li, and J. M. Tiedje (2000) 16S rRNA Gene-based Detection of Tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. Applied Environmental Microbiology, **66,** 1369-1374.

Luijten, M. L. G. C., J. de Weert, H. Smidt, H. T. S. Boschker, W. M. de Vos, G. Schraa, and A. J. M. Stams (2003) Description of *Sulfurospirillum halorespirans* sp nov., an Anaerobic, Tetrachloroethene-respiring Bacterium, and Transfer of *dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov. International Journal of Systematic and Evolutionary Microbiology, **53,** 787-793.

Major, D. W., M. L. McMaster, E. E. Cox, E. A. Edwards, S. M. Dworatzek, E. R. Hendrickson, M. G. Starr, J. A. Payne, and L. W. Buonamici. Field Demonstration of Successful Bioaugmentation to Achieve Dechlorination of Tetrachloroethene to Ethene. Environmental Science and Technology, **2002,** 36, 5106-5116.

Muyzer, G., E. C. Dewaal, and A. G. Uitterlinden. Profiling of Complex Microbial-Populations by Denaturing Gradient Gel-Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes-Coding for 16s Ribosomal-Rna. Applied Environmental Microbiology, **1993,** 59, 695-700.

Magnuson, J. K., M. F. Romine, D. R. Burris, and M. T. Kingsley (2000) Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes*: Sequence of tceA and Substrate Range Characterization. Applied Environmental Microbiology, **66,** 5141-5147.

Maymó-Gatell, X., Y. T. Chien, J. M. Gossett, and S. H. Zinder (1997) Isolation of a Bacterium that Reductively dechlorinates tetrachloroethene to ethene. *Science*, **276,** 1568-1571.

Middeldorp, P. J. M., M. L. G.C. Luijten, B. A. van de Pas, M. H. A. van Eekert, S. W. Kengen, G. Schraa, and A. J. M. Stams, (1999) Anaerobic Microbial Reductive Dehalogenation of Chlorinated Ethenes. *Biorem. J.*, **3,** 151-169.Kanagawa, T. Bias and Artifacts in Multitemplate Polymerase Chain Reactions (PCR). Journal of Bioscience and Bioengineering, **2003,** 96, 317-323.

Müller, J. A., B. M. Rosner, G. von Abendroth, G. Meshulam-Simon, P. L. McCarty, and A. M. Spormann (2004) Molecular Identification of the Catabolic Vinyl Chloride Reductase from *Dehalococcoides* sp. strain VS and Its Environmental Distribution. Applied Environmental Microbiology, **70,** 4880-4888.

Nakatsu, C. H., V. Torsvik, L. Ovreas. Soil Community Analysis Using DGGE of 16S rDNA Polymerase Chain Reaction Products. Soil Science Society of America Journal, **2000,** 64, 1382-1388.

Reeves, T., J. Miller, and P. C. Johnson (2000) *Modular Remediation Testing System*. Lewis Publishers: Boca Raton, FL.

Reinhard, M., S. Shang, P. K. Kitanidis, E. Orwin, G. D. Hopkins, and C. A. Lebron (1997) In Situ BTEX Biotransformation Under Enhanced Nitrate- and Sulfate-reducing Conditions. Environmental Science and Technology, **31,** 28-36.

Ritalahti, K. M. and F. E. Löffler (2004) Populations Implicated in Anaerobic Reductive Dechlorination of 1,2-dichloropropane in Highly Enriched Bacterial Communities. Applied Environmental Microbiology, 70, 4088-4095.

Ritalahti, K. M., B. K. Amos, Y. Sung, Q. Z. Wu, S. S. Koenigsberg, and F. E. Loffler. Quantitative PCR Targeting 16S rRNA and Reductive Dehalogenase Genes Simultaneously Monitors Multiple Dehalococcoides Strains. Applied Environmental Microbiology, **2006,** 72, 2765-2774.

Sleep, B. E., D. J. Seepersad, K. Mo, C. M. Heidorn, L. Hrapovic, P. L. Morrill, M. L. McMaster, E. D. Hood, C. LeBron, B. S. Lollar, D. W. Major, and E. A. Edwards. Biological Enhancement of Tetrachloroethene Dissolution and Associated Microbial Community Changes. Environmental Science and Technology, **2006,** 40, 3623-3633.

Smits, T. H. M., C. Devenoges, K. Szynalski, J. Maillard, and C. Holliger (2004) Development of a Real-time PCR Method for Quantification of the Three Genera *Dehalobacter*, *Dehalococcoides*, and *Desulfitobacterium* in Microbial Communities. Journal of Microbiological Methods, **57,** 369-378.

Stroo, H.F., C.H. Ward, M.C. Kavanaugh, C. Vogel, A. Leeson, M. Unger, J.A. Marqusee, and B.P. Smith (2003) *Remediation of Chlorinated Solvent Source Zones: Challenges and Research Needs*. Environmental Science and Technology. 37: 224A-230A.

Sung, Y., K. E. Fletcher, K. M. Ritalahti, R. P. Apkarian, N. Ramos-Hernandez, R. A. Sanford, N. M. Mesbah, and F. E. Loffler, (2006) Geobacter lovleyi sp. nov. Strain SZ, a Novel Metal-Reducing and Tetrachloroethene-Dechlorinating Bacterium. *Applied Environmental Microbiology*, **72,** 2775-82.

Sung, Y.; K. M. Ritalahti, R. P. Apkarian, and F. E. Löffler (2006) Quantitative PCR Confirms Purity of Strain GT, a Novel Trichloroethene-to-ethene Respiring *Dehalococcoides* Isolate. Applied Environmental Microbiology, **72,** 1980-1987.

Sung, Y., K. M. Ritalahti, R. A. Sanford, J. W. Urbance, S. J. Flynn, J. M. Tiedje, and F. E. Löffler (2003) Characterization of Two Tetrachloroethene-reducing, Acetate-oxidizing Anaerobic Bacteria and their Description as Desulfuromonas michiganensis sp nov. Applied Environmental Microbiology, **69,** 2964-2974.

Suzuki, M. T., L. T. Taylor, and E. F. DeLong (2000). Quantitative Analysis of Small-subunit rRNA Genes in Mixed Microbial Populations via 5'-nuclease Assays. Applied Environmental Microbiology 66:4605–4614.

Thomas, J. M. and C. H. Ward (1989) Insitu Biorestoration of Organic Contaminants in the Subsurface. Environmental Science and Technology, **23,** 760-766.

Vongunten, U. and J. Zobrist (1993) Biogeochemical Changes in Groundwater-infiltration Systems - Column Studies. Geochim Cosmochim Ac, 57, 3895-3906.

Yang, Y. R. and P. L. McCarty (2000) Biologically Enhanced Dissolution of Tetrachloroethene DNAPL. Environmental Science and Technology, 34, 2979-2984.

Yang, Y. R. and P. L. McCarty (1998) Competition for Hydrogen within a Chlorinated Solvent Dehalogenating Anaerobic Mixed Culture. Environmental Science and Technology, **32,** 3591-3597.

Yang, Y. and P.L. McCarty (2002) "Comparison Between Donor Substrates for Biologically Enhanced Tetrachloroethene DNAPL Dissolution." Environmental Science and Technology, Vol. 36, No. 15, pp. 3400-3404.

Zheng, D., C. S. Carr, and J. B. Hughes (2001) Influence of Hydraulic Retention Time on Extent of PCE Dechlorination and Preliminary Characterization of the Enrichment Culture. Bioremediation Journal, 5, 159-168.

## 8. Points of Contact

| Point of Contact | Organization | Phone/Fax/E-mail | Role in Project |
|---|---|---|---|
| C. Herb Ward | Rice University, 6100 Main St, MS-316, Houston, TX 77005 | (713) 348-4086<br>(713) 348-5948<br>wardch@rice.edu | Principal Investigator |
| Joseph Hughes | Georgia Institute of Technology, 790 Atlantic Drive, Atlanta, GA 30332 | (404) 894-2201<br>(404) 894-2278<br>jhughes@ce.gatech.edu | Co-Principal Investigator |
| Pedro Alvarez | Rice University, 6100 Main St, MS-317, Houston, TX 77005 | (713) 348-5903<br>(713) 348-5203<br>alvarez@rice.edu | Co-Investigator |
| Marcio L.B. da Silva | Universidade Federal de Santa Catarina. Dept. Eng. Sanitaria, Fpolis, SC, Brazil. 88040-970 | + 55 (48) 3331-7569<br>+ 55 (48) 3234-6459<br>silva@ens.ufsc.br | Project Manager/ Research Engineer |
| Steve Koenigsberg | Regenesis Bioremediation Products, 1011 Calle Sombra, San Clemente, CA 92673 | (949) 366-8001<br>(949) 366-8090<br>skoenigsberg@regenesis.com | Industry Sponsor |

# Appendix A: Analytical Methods Supporting the Experimental Design

## *Phase I*

*Experimental Controlled Release System (ECRS).* Two 11.7 $m^3$ near field scale ECRS were employed to evaluate the relative effects of bioaugmentation and biostimulation on the removal of PCE DNAPL source zones (Adamson et al., 2003; Reeves et al., 2000). One system was bioaugmented with 15 L ($3.1\times10^9$ bacteria/mL) of the anaerobic dechlorinating consortium directly into the source zone and biostimulated by the addition of electron donors upstream of the DNAPL region. This system is referred to as the bioaugmented tank. The other system was not bioaugmented, but the indigenous microbial community was biostimulated with the same electron donors. This system is referred to as the biostimulated tank.

The experimental system consisted of two metal tanks (5.49 m long, 2.13 m wide, 1.83 m high) open to the atmosphere (Figure A-1). These are the same ECRS systems that were described by Adamson, et al. (2003). Fine masonry sand (New Caney, Texas) was emplaced to provide model aquifer material. The physical-chemical properties of the sand used are shown in Table A-1. Packing was performed by saturated, continuous fill to a depth of approximately 1 m. This sand-water saturation strategy was designed to enhance distribution of the sand and to minimize mounding, channeling, and other heterogeneities that can occur during packing. The tanks were then drained at a rate of 500 mL/min to induce compaction and then saturated to a depth of 1 meter.

Multiple internal sampling or injection points (0.6 and 1.3 cm ID, respectively) were installed using stainless steel tubing during tank packing. The source water for the ECRS was from the Rice University (Houston, Texas) tap water supply. The tap water was not dechlorinated before use because no inhibitory effects were observed previously (Adamson et al., 2003). Each tank was fitted with two influent and two effluent lines. Effluent lines were placed on both sides of each end of the tanks to minimize preferential flow and channeling. Flow was controlled using electronic flow meters (McMillan Co., Georgetown, Texas) in the influent and effluent lines to maintain a near constant rate (22-30 L/h). Activated carbon canisters (liquid phase activated carbon; total surface area of 1,050 $m^2$/g, TIGG Corp., Heber Springs, Arkansas) were installed in the effluent lines to remove chlorinated solvents before discharge to the sewer.

The hydraulic characteristics of the tanks were determined using bromide breakthrough curves. Breakthrough data were obtained by continuous injection of a potassium bromide solution (1 kg/L) directly into the influent lines of the tanks using a syringe pump (Harvard Apparatus, Hollistion, MA), which gave an influent concentration of 1g/L. Bromide recovery was 100 ± 3%. One pore volume was displaced in 3 - 4 days (Figure A-2). The average hydraulic conductivity was 0.17 m/d, corresponding to a seepage velocity of 1.6 m/d. Similar bromide breakthrough curves for both effluent lines in each tank confirmed the absence of preferential flow paths.

To establish DNAPL source zones, neat PCE (1 L total per tank) was added 30 cm from the bottom of the tanks. PCE was introduced through two sample lines (500 mL each) perpendicular to flow and downgradient (2 m) from the inlet of the tanks (Figure A-1). PCE delivery was accomplished using glass syringes (100 mL) and manual injection under minimal positive

pressure. HRC® was added as electron donor directly upstream from the source zones. HRC® was injected into both tanks 7 days after PCE injection using a direct push geoprobe method developed by the supplier (Regenesis Bioremediation Products, Inc., San Clemente, California) (Figure A-1). Six locations were chosen for HRC® addition. The injection points (Figure A-1) were 0.9 m and 0.3 m upstream of the PCE addition, and were perpendicular to flow. The quantity of HRC® injected (22.5 L per tank; 25.3 kg as COD) into the subsurface was based on calculations made by the supplier (Regenesis) and it was identical (on a source area basis, 1.9 $L/m^2$) to the quantity of HRC® utilized for the treatment of a PCE contaminated site (Kean et al., 2000). This amount served to induce anaerobic conditions by depleting essentially all of the residual oxygen in the soil-water matrix.

HRC® was depleted in the systems after approximately 40 days of operation. Sodium lactate (600 mg/L), a surrogate electron donor, was continuously injected in the influent of both tanks beginning on day 118. Electron donor injection was performed using two syringe pumps (Harvard Apparatus, Holliston, Massachusetts) connected in-line with the influent of both tanks. Injection of lactate continued for 158 days (to day 277) in the bioaugmented tank. The injection of lactate in the biostimulated tank was discontinued at day 232, and the tank was monitored for 45 days to investigate the relationship between electron donor addition and dechlorination potential.

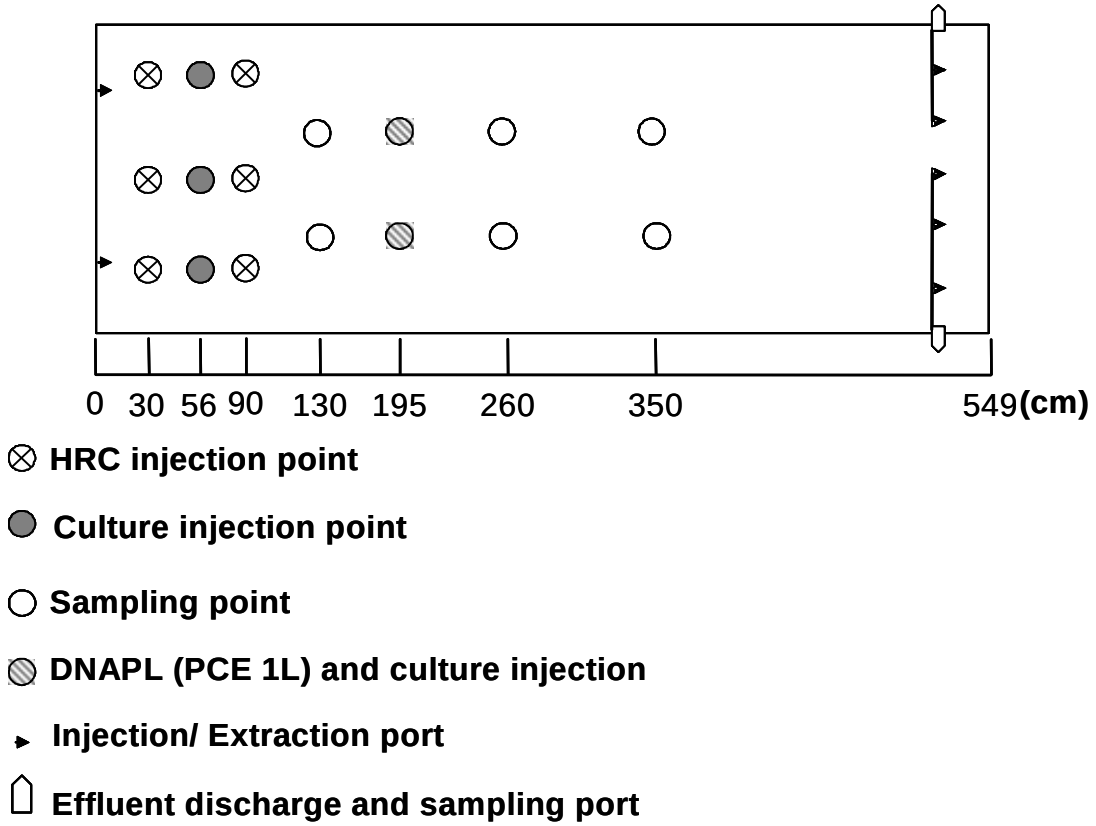


**Figure A-1. Schematic Representation of the ECRS Showing Sampling Wells, DNAPL Source and Injection Points.**

**Table A-1. Properties of Sandy Material used as Matrix in the Experimental Control Release Systems (**ECRS**).**

| **Parameter** [a] | **Units (ppm)** |
|---|---|
| Nitrate-N | 3 |
| Phosphorus | 2 |
| Calcium | 111 |
| Magnesium | 11 |
| Sulfur | 47 |
| Bioavailable iron | 31 |
| Total iron | 198 |
| Porosity | 0.32 |
| Conductivity | 0.97 mmohs $cm^{-1}$ |
| Organic matter | 0.09% |

[a] Soil analysis conducted by Soil, Water and Forage Testing Laboratory, Department of Soil and Crop Sciences, Texas A&M University, College Station, Texas.

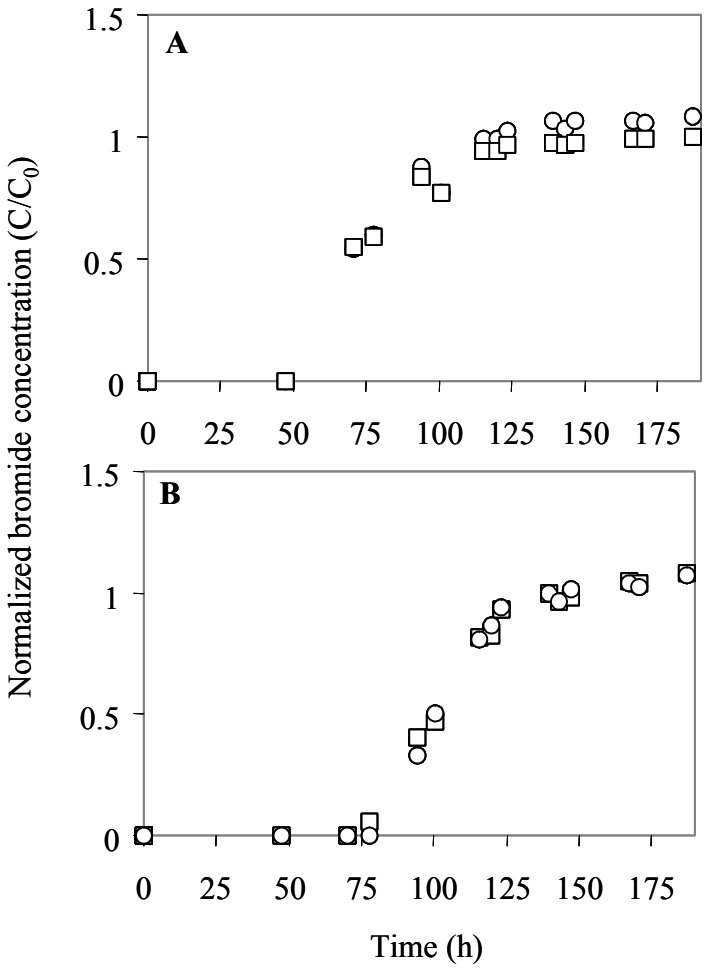


**Figure A-2. Bromide Breakthrough Data for the Bioaugmented Tank (**A**) and Biostimulated Tank (**B**). Each Tank had two Effluent Ports: □ Effluent 1 and ○ Effluent 2**.

## *Phase II*

The objective of Phase II was to evaluate DNAPL flux enhancement through bioaugmentation and biostimulation of the source zone. Two ECRS tanks were operated in parallel with identical aquifer material, and DNAPL constituents. One tank was biostimulated and bioaugmented by the addition of a suitable mass of an anaerobic dechlorinating consortium (same as used in Phase I) directly into the source zone. The other tank served as control (natural attenuation) to discern the benefits of bioaugmentation plus biostimulation. A known mass of electron donor (COD) was supplied by a continuous injection of a pre-hydrolyzed (50:50 v/v) HRC® into the influent of the bioaugmented tank.

The addition of a known mass of electron donor allowed a better intrinsic stoichiometric mass balance between electron equivalents and dechlorination processes. Differently from Phase I, the injection of the DNAPL source was moved closer to the influent of the tanks to allow a more complete dechlorination activity of the plume along the flow path in the tank. Moreover, DNAPL was applied 3 feet below the soil surface (in Phase I it was added 1 foot from the bottom of the tank as a DNAPL pool) to allow the formation of a residual NAPL downgradient.

The hydraulic characteristics of the tanks were also modified to have an overall seepage velocity of 0.46 m/d (in Phase I it was approx. 1.6 m/d). Decreasing the flow rates in the tanks likely increased the overall project's time frame as compared to Phase I, which took approximately 1 year of monitoring. However, decreasing flow rates allowed for a more realistic representation of groundwater flow and much higher dechlorination activity was expected.

*Experimental Controlled Release System (ECRS).* To evaluate the efficacy of bioaugmentation and biostimulation versus natural attenuation (mimicking pump-and-treat), two large-scale aquifer simulators (ECRS tanks) were built. They were comprised of metal tanks (5.49 m long, 2.13 m wide, 1.83 m high) open to the atmosphere. Fine masonry sand (from a quarry in New Caney, Texas) was used to pack the tanks. Soil analysis was conducted and is shown in Table A-2. Packing was performed by saturated, continuous fill to a depth of approximately 1 m. This sand-water saturation strategy was designed to allow for proper distribution of the sand and minimize mounding, channeling, and other heterogeneities that can occur during packing.

Multiple internal sampling or injection points were installed using ½ or ¼ inch stainless steel tubing, placed during the packing of the system (Figure A-3). The source water for the ECRS was from the Rice University potable water supply, consisting of a mix of surface water and groundwater. Each tank was fitted with two influent and effluent lines. Effluent lines were placed at both sides of each end of the tank to minimize preferential flow and channeling. Outflow lines were replaced by a larger diameter tubing (1 in) to prevent clogging as it occurred in phase I. Water flow (150 mL/min) was controlled using mechanical flow controllers (Gilmont GF-8521-1606) in the influent lines to maintain the flow at a rate of 4.2 – 4.5 L/h. Water levels were maintained at 2 feet from the soil surface in both tanks. Activated carbon canisters (3) (CANSORBXP polyethylene drums containing TIGG 5D virgin liquid phase activated carbon with total surface area of 1,050 $m^2/g$) were installed in series with the tanks effluent lines to remove any chlorinated solvents present before discharge into the sewer.

**Table A-2 – Phase 2 Soil Analysis[a].**

| | |
|---|---|
| Wet Digestion Moisture, Total | 1.90 % |
| Ash | 98.44 % |
| pH | 6.3 |
| Organic Matter | < 0.1 % |
| Sulfur | < 0.01 % |
| Iron (Total) | 198.1 mg/Kg |
| Nitrogen, Total-L | 1.93 % |
| Loss on Ignition | -0.34 % |

[a] Soil analysis conducted by Minnesota Valley Testing Laboratories, Inc, Minnesota.

The addition of PCE to establish a DNAPL source zone in each tank followed the hydraulic stabilization tests. Chloride breakthrough studies (conducted approximately 60 days prior to PCE injection) indicated that 12-14 days were required to displace one pore volume (Figure A-4). This is 4 times longer than the HRT used in phase I. Chloride breakthrough studies were conducted through continuous injection of a 188 mg/L NaCl solution in the influent of the tanks by using a 6-600 rpm peristaltic pump (Cole-Parmer Masterflex Console Drive Model 7521-40).

Neat PCE (1 L total) was added (day 0) in equal volumes (500 mL) through two sample lines approximately 2 m downgradient from the inlet of each ECRS tank. PCE delivery was accomplished via glass tight syringes under minimal pressure.

Hydrogen Releasing Compound (HRC®) was used as source of electron donor. HRC® injection started 46 days after PCE addition. A pre-solubilized mixture of 50:50 v/v deionized water: HRC® (COD = 540 mg/L) was injected using a syringe pump (Model Harvard Apparatus 22) connected directly in the influent of the bioaugmented tank.

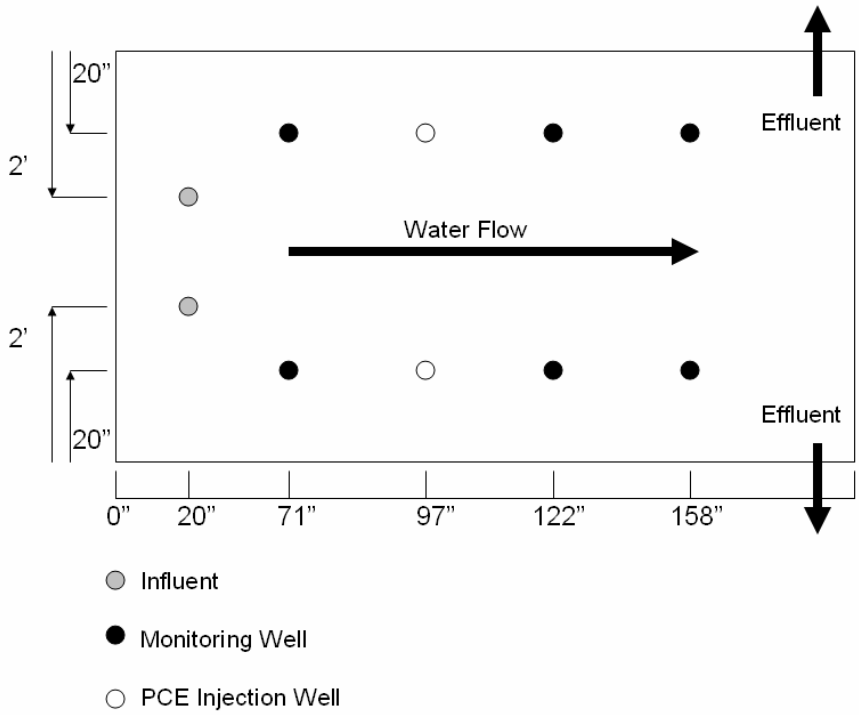


**Figure A-3 – Schematic Representation of the Tanks. Showing the Locations of the Sampling Wells, DNAPL Source and Inoculation Injection.**

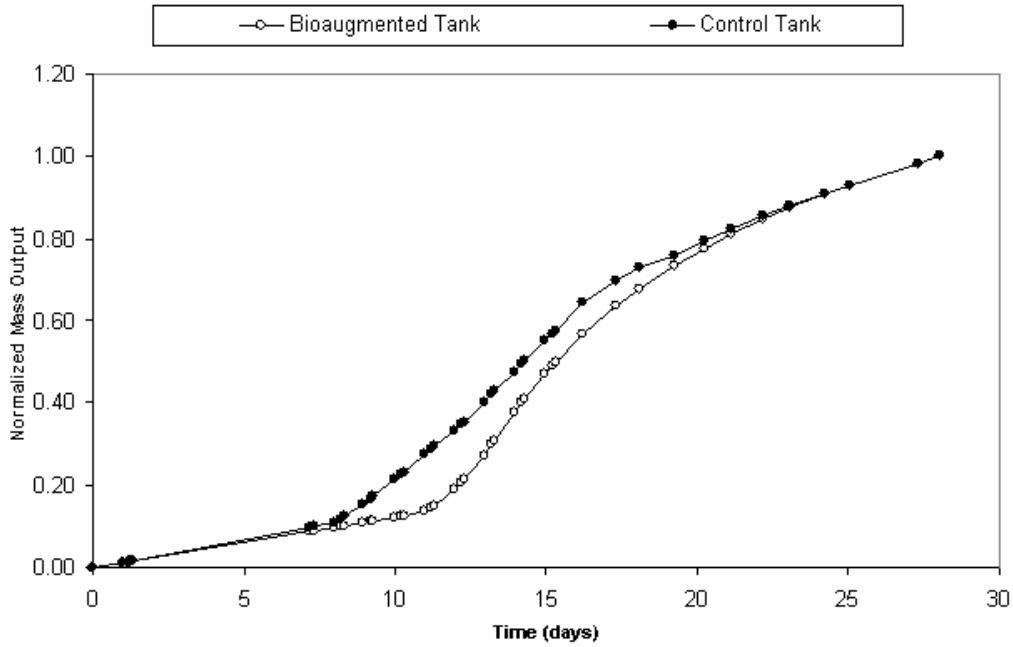


**Figure A-4. Chloride Breakthrough Data from the Bioaugmented and Control Tank. Pore Hydraulic Retention Time were 14 and 12 days for Bioaugmented and Control Tank, Respectively.**

The culture used to bioaugment the ECRS in Phase II was the same used for Phase I.

Bioaugmentation of one of the tanks was performed after 106 days of experiment after the successful establishment of a residual PCE concentration and depletion of oxygen due to BOD exerted by the HRC®. A bioreactor (20 L) was constructed to maintain and scale-up the volume of the culture. The bioreactor was constructed with a high-density polyethylene carboy tank equipped with ports for injection of nutrients, pH control, PCE addition, liquid sampling, recycling the contents of the bioreactor, and headspace analysis. Bioaugmentation was conducted by adding 15L of the culture (total of 6.4 g-cells) (7.5L per well) at 55.6 mL/ min in the same two lines used for PCE injection (Figure A-3, A-5).

**Figure A-5. Bioaugmentation of the Tank.**

Samples collected from the effluent lines of both tanks were monitored for chlorinated solvents (PCE, TCE, DCE, VC and ethene), methane, propionate, acetate, COD, DO, and pH. Chlorinated compounds were analyzed by the injection of 100 μL headspace samples directly into a gas chromatograph (gc) (Hewlett-Packard 5890) equipped with a flame ionization detector (FID) and a packed column (6 ft /1/8 in. OD) containing 60/80 Carbopack B/1% SP-1000 (Supelco). The operating parameters of the gc were 40°C for 2 minutes; 20°C/minutes to 150°C; 10°C/minutes to 200°C; held 10 minutes at 200°C; injector and detector temperatures were 200°C and 275°C, respectively. The flow rate for He (carrier gas) was 12 mL $min^{-1}$; air (460 mL/min) and $H_2$ (40 mL/min) were used as detector makeup gases. Standards were prepared by adding PCE, TCE, and *cis*-DCE dissolved in methanol, and VC, ethene, and methane gases, all at known volumes, to serum bottle (70 mL) containing deionized water (50 mL).

Volatile fatty acids (acetate, propionate, and lactate were analyzed by filtering aqueous samples (2.7 mL) through a syringe filter (0.22 μm) containing 0.3 M oxalic acid (0.3 mL). One μL of

this solution was then injected into a gc (Hewlett-Packard 5890) equipped with a FID that contained a glass packed column (2 m/ 2 mm i.d.) containing 80/120 Carbopack B-DA*/4% Carbowax 20 M (Supelco). The operating parameters for the gc were as follows: oven temperature was isothermal at 175°C, detector temperature was 200°C, and the injector temperature was 200°C. The flow rate for He (carrier gas) was 24 mL/min; air and $H_2$ were used as detector makeup gases.

COD was measured using the closed reflux colorimetric method using HACH COD vials. The input from HRC® addition was defined in terms of total COD, which included free aqueous lactate and polymerized lactate that had not undergone hydrolysis. The conversions for milligrams of acetate and propionate to milligrams of COD were: 1.09 mg-COD/ mg-acetate and 1.53mg-COD/mg-propionate.

DO and pH (Fischer Scientific Mod. Accumet 13636AP84A) were measured in aqueous samples.

## Appendix B: Analytical Methods Supporting the Sampling Plan

*Chemicals.* The following chemicals were obtained in liquid form: PCE (99+%, Sigma-Aldrich, St. Louis, MO), TCE (99+%, Sigma-Aldrich), cis-DCE (99+%, Sigma-Aldrich), methanol (MeOH) (HPLC grade, Fisher Scientific, Fairlawn, NJ), sodium hydroxide (NaOH) (1N, Fisher Scientific), sodium-DL-lactate (60% v/v, Sigma-Aldrich), and HRC® (glycerol tripolylactate, Regenesis Bioremediation Products, Inc., San Clemente, California). Gaseous chemicals obtained from Supelco included VC (8% VC, balance N2), nitrogen (Ultra High Purity), methane (99%), and ethene (99%).

*Analytical Methods.* Chlorinated compound concentrations in aqueous samples were determined using headspace analysis as described previously by Zheng et al. (2001). Standards were prepared by adding PCE, TCE, and cis-DCE dissolved in methanol, and VC, ethene, and methane gases, all at known volumes, to serum bottles (70 mL) containing deionized water (50 mL). Volatile fatty acids (acetate and propionate) were analyzed as described in Adamson et al. (2003). COD was measured with the closed reflux colorimetric method in Standard Methods for the Examination of Water and Wastewater (Greenberg et al., 1992) using COD vials (HACH Cat. 21259-15, Loveland, Colorado). The input from HRC® addition was defined in terms of total COD, which included free aqueous glycerol tripolylactate that had not undergone hydrolysis. Dissolved oxygen and pH (Fisher Scientific) were measured directly in aqueous samples.

*Culture Development.* A dechlorinating culture was developed from an anaerobic methanogenic consortium that had shown dechlorination activity for over nine years in the laboratory (Zheng et al., 2001). This culture is capable of rapid and complete dechlorination of PCE to ethene (240 μmol/L/d). The culture was maintained in a 20 L high-density polyethylene carboy equipped with ports for injection of nutrients, sodium hydroxide, and PCE. The carboy also had fittings for culture mixing and headspace analysis. The culture was fed 0.25 mM PCE and 3 mM MeOH daily, and maintained with an 80 day retention time using a draw-and-fill method. This method allowed for higher cell densities than used in previous bioaugmented ECRS experiments (Adamson et al., 2003) because the culture was fed daily. The total bacterial and archaea concentrations in the consortium, determined by real time quantitative PCR (qPCR) as described below, were $3.1 \times 10^9$ cell/mL and $2.0 \times 10^8$ cell/mL, respectively. Assuming a mass of $1.33 \times 10^{-9}$ g/cell (Bratbak, 1985), 6,390 mg of biomass was added to the tank.

*ECRS Sampling.* Samples from the ECRS were taken to determine how indigenous organisms in the ECRS sand matrix and the bioaugmented organisms distributed and performed in an active bioremediation system. Groundwater samples were taken initially (before bioaugmentation) and at Day 161 (when mostly *cis*-DCE was in the effluent). Groundwater samples were taken with sterile disposable syringes (60 mL) (Becton, Dickson and Company, Franklin Lakes, New Jersey) from each sampling well (Figure B-1) and placed into sterile Nalgene bottles (480 mL) (Fisher Scientific, Houston, Texas). These samples were

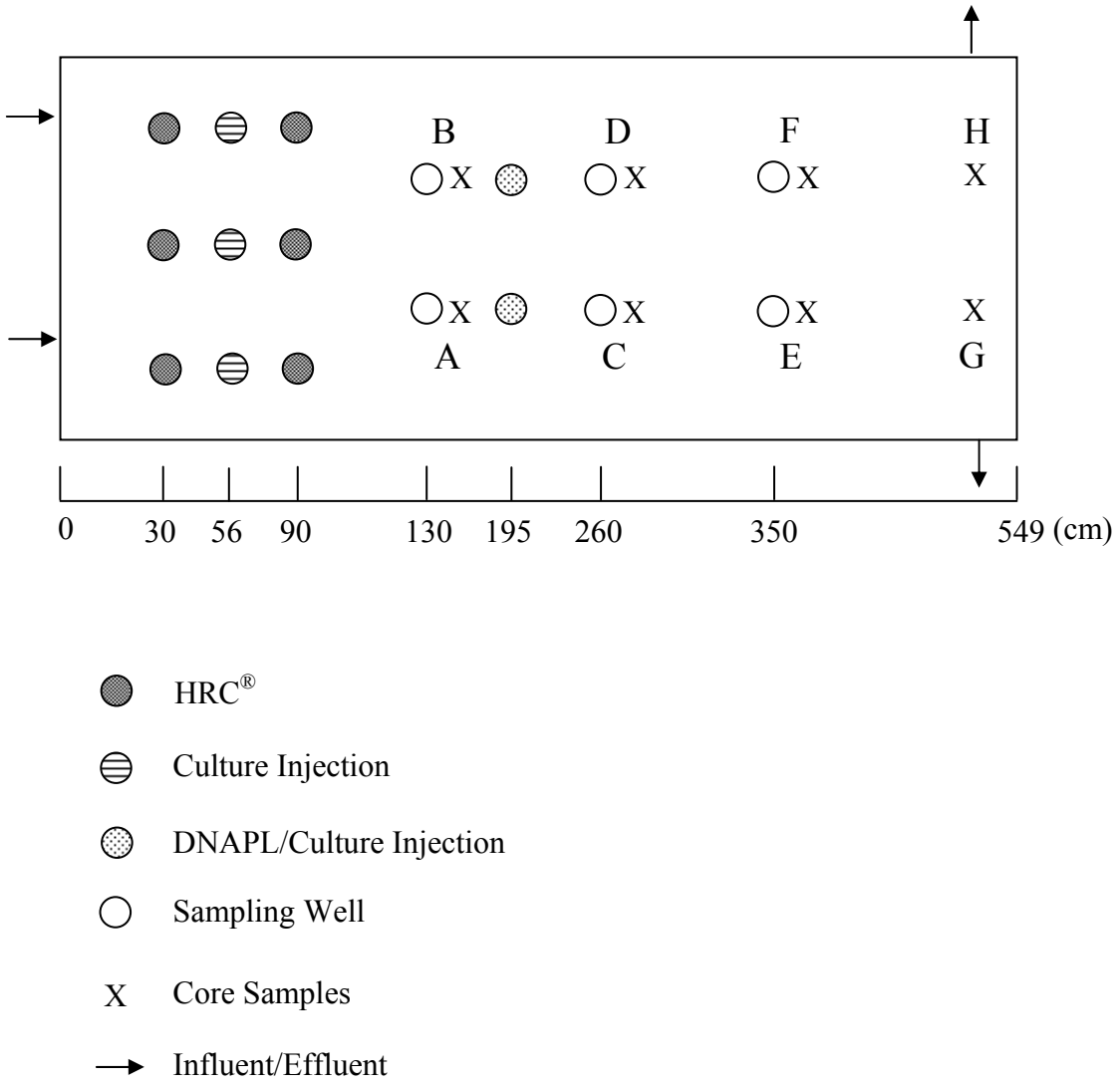


**Figure B-1. Schematic of ECRS with Groundwater Sampling Wells and Core Sampling Points. Modified from da Silva et al.** (2006).

vacuum filtered using MAGNA nylon (0.22 µm, 47 cm) filters and DNA was extracted directly from the filters.

Core samples were taken at the end of the experiment (~ Day 278) (VC and ethene were in effluent) (da Silva et al., 2006), and were taken from sampling points labeled on Figure 6.1 (as close to the sampling wells as possible). These samples were collected using PVC tubing (2.5 cm x 1.83 m). Once the core was taken, the tubing was cut into 0.3 m sections (the water table was 0.9 m from the bottom of the tank). The sand from inside the sections from the bottom (0 m) to 0.3 m and from 0.3 m to 0.6 m was emptied out onto weigh boats, homogenized, and allowed to dry. The sand was then placed into sterile centrifuge tubes (50 mL) and stored at -80 °C until DNA was extracted.

*DNA Extraction.* DNA was extracted using the MO BIO PowerSoil DNA Kit (MO BIO Laboratories, Inc., Carlsbad, California). For groundwater samples, the filter was placed directly into the tubes provided. For soil samples, sand (~0.25 g) was added to the tubes provided and triplicate sand samples were extracted for each core taken. The manufacturer's protocol was followed and a bead-beating device (BioSpec Products, Inc., Bartlesville, Oklahoma) was utilized for cell lysis. DNA was collected (60 µL) in microfuge tubes (1.5 mL) and stored at -80°C. DNA from the groundwater samples was combined to allow for analysis of regions of the tank. The combination of samples was as follows: samples A and B (upgradient region); samples C and D (source zone); and samples E and F (plume region). For the core samples E, F, G and H were combined to form the plume region.

*Quantitative Real-time PCR (qPCR).* qPCR was used to quantify the total number of bacteria, archaea, *Dehalococcoides*, *Desulfuromonas* spp., and *Dehalobacter* spp. The primers and probes used for qPCR reactions are listed in Table B-1. qPCR (30 µL) reactions contained 1 X TaqMan® PCR Master Mix (Applied Biosystems, Foster City, California), 300 nM of the forward primer, reverse primer and the probe and 3 µL template for the bacteria, *Dehalococcoides* and *Desulfuromonas* spp. The archaea qPCR reactions were set up as described above, except the primer and probe concentrations were modified to 450 nM for the forward primers, 900 nM for the reverse primer, and 250 nM for the probe as described by da Silva and Alvarez (2004). The conditions for the TaqMan® reactions were as follows: 2 minutes at 50°C, 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and one minute at 60°C (archaea and *Desulfuromonas* spp.) or 58°C (*Dehalococcoides*) or 52°C (bacteria).

The *Dehalobacter* spp. were quantified with a SYBR® Green approach and the qPCR reactions (30 µL) contained 1X SYBR® Green buffer (Qiagen, Valencia, California) and 300 nM of the forward and reverse primer. The conditions for the SYBR® Green reactions were as follows: 2 minutes at 50°C, 15 minutes at95°C and 40 cycles of 15 seconds at 94°C, 30 seconds of 58°C, and 30 seconds of 72°C, followed by a dissociation curve from 60°C – 95°C. All reactions (both TaqMan® and SYBR®) were run on an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, California) in standard 7500 mode (except for the bacteria reactions, which were run in 9600 emulation mode).
Standard curves for qPCR were prepared using a dilution series of quantified plasmids carrying one 16S rRNA gene from *Dehalococcoides* sp. strain BAV1, *Dehalobacter restrictus*, *Desulfuromonas* sp. strain BB1 or *Methanococcus maripaludis*. The linear range of

quantification was $10^1$-$10^7$ ($r^2$ = 0.99; amplification efficiency = 1.86), $10^1$-$10^9$ ($r^2$ = 0.99; amplification efficiency = 1.96), $10^2$-$10^9$ ($r^2$ = 0.99; amplification efficiency = 1.88), $10^2$-$10^9$ ($r^2$ = 0.99; amplification efficiency = 1.83), $10^2$-$10^8$ ($r^2$ = 0.99; amplification efficiency = 1.86) for *Dehalococcoides*, *Desulfuromonas* spp., *Dehalobacter* spp., bacteria, and archaea, respectively.

**Table B-1. Quantitative Real-time PCR Primers and Probes used to Target 16S rRNA Genes.**

| Target Organism | Primer Designation | Sequence | Reference |
|---|---|---|---|
| **Bacteria** | Forward | 5'-ATGGYTGTCGTCAGCT | ( Ritalahti et al., 2006) |
| | Reverse | 5'-ACGGGCGGTGTGTAC | |
| | Probe | 5'-FAM-CAACGAGCGCAACCC-TAMRA | |
| | | | |
| **Archaea** | Forward | 5'-CGGTGAATACGTCCCTGC-3' | (Suzuki et al., 2000) |
| | Forward | 5'-CGGTGAATATGCCCCTGC-3' | |
| | Reverse | 5'-AAGGAGGTGATCCTGCCGCA-3' | |
| | Probe | 5'-FAM-CTTGTACACACCGCCCGTC-TAMRA-3' | |
| | | | |
| ***Dehalococcoides*** | Forward | 5'-CTGGAGCTAATCCCCAAAGCT-3' | (He et al., 2003b) |
| | Reverse | 5'-CAACTTCATGCAGGCGGG-3' | |
| | Probe | 5'-FAM-TCCTCAGTTCGGATTGCAGGCTGAA-TAMRA-3' | |
| | | | |
| ***Desulfuromonas* spp.** | Forward | 5'-GACATCCCGATCGCACCTTA-3' | (Aiello, 2003) |
| | Reverse | 5'-CCATGCAGCACCTGTCACC-3' | |
| | Probe | 5'-FAM-AACATAGGGGTCAGTTCGGCTGGIT-TAMRA-3' | |
| | | | |
| ***Dehalobacter* spp.** | Forward | 5'-GTTAGGGAAGAACGGCATCTGT-3' | (Smits et al., 2004) |
| | Reverse | 5'- CCTCTCCTGTCCTCAAGCCATA-3' | |

The gene copy numbers were calculated as described by Ritalahti et al. ( Ritalahti et al., 2006). Genome and genomic analyses demonstrated that the 16S rRNA and the RDase genes exist as single copy genes on *Dehalococcoides* genomes (Kube et al., 2005; Seshadri et al., 2005). The numbers of 16S rRNA genes for *D. restrictus*, *Desulfuromonas* sp. strain BB1 and for *M. maripaludis* have not been determined. For both *D. restrictus* and *Desulfuromonas* sp. strain BB1 it was assumed there is one copy of the 16S rRNA gene per genome. The closest relatives to *M. maripaludis* contain 1 - 4 copies of the 16S rRNA gene according to the Ribosomal RNA Operon Copy Number Database (http://rrndb.cme.msu.edu/rrndb/servlet/controller), and our cell number estimates assumed two 16S rRNA gene copies per archaeal genome.

*Denaturing Gradient Gel Electrophoresis (DGGE).* DGGE with universal bacterial primers described by Muyzer et al. (1993) was performed on samples from the tank by Microbial Insights Inc. (Rockford, Tennessee; http://www.microbe.com). DGGE with *Dehalococcoides*-specific primers developed by Duhamel et al. (2004) was also performed on the samples by Microbial Insights Inc. It was assumed that the *Dehalococcoides* numbers would be low in the samples, so to increase the detection limit the samples were first amplified with universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541 R (5'-AAGGAGGTGATCCAGCCGCA-3') as described by Ritalahti et al. (2004), and then further amplification was performed with the *Dehalococcoides*-specific primers. The fragments from the gel were excised and placed into nanopure water (50 µL). PCR was performed using the excised gel band (2µL) with the same *Dehalococcoides*-specific DGGE primers or the universal bacterial primers. The resulting PCR product was purified using the Ultraclean™ PCR clean-up kit (Mo Bio Laboratories, Inc.) and sequenced. These sequences were compared to other 16S rRNA gene sequences using BLAST (http://www.gl.iit.edu/frame/genbank.htm).

*Bioaugmentation.* The dechlorinating culture was added to the bioaugmented tank after establishment of a residual PCE source zone and depletion of oxygen (DO < 0.1 mg/L). The characterization of the culture used is described in Table B-2.

**Table B-2. Characterization of the Culture used for Bioaugmentation.**

| Target Population or Gene | Gene copies $mL^{-1}$ |
|---|---|
| Bacteria | $3.1 \times 10^9$ |
| *Archaea* | $2.0 \times 10^8$ |
| *Dehalobacter* spp. | $3.1 \times 10^9$ |
| *Dehalococcoides* spp. | $1.0 \times 10^9$ |
| *Sulfurospirillum* spp. | Present[a] |
| *Desulfuromonas* spp. | ND[bc] |
| *Desulfitobacterium* spp. | ND[c] |
| *Desulfomonile tiedjei* | ND[c] |
| *Geobacter* sp. strain SZ | ND[c] |
| *tce*A<br>TCE → ETH<br>(*Dehalococcoides* spp.) | Present |
| *bvc*A<br>DCEs →ETH<br>(*Dehalococcoides* sp. strain BAV1) | ND[c] |
| *vcr*A<br>DCEs → ETH<br>(*Dehalococcoides* sp. strain VS or GT) | Present |

[a] Detected with nested PCR
[b] ND = Not detected
[c] Not detected with PCR or nested PCR

Bioaugmentation was performed by purging the carboy reactor containing the microbial consortium with nitrogen gas to provide positive pressure in the vessel and to maintain anaerobic conditions. A total of 15 L of the culture was added to the bioaugmented tank, which was divided between five injection wells (3 L per well). Two of the lines used for culture injection were the same lines used for the PCE addition (bioaugmentation occurred as close to the DNAPL source as possible); the other three lines used were located upgradient (1.5 m) of the PCE injection wells. The amount of culture injected represented 0.4% of the aquifer's pore volume.

*Column Studies.* Flow-through aquifer columns were used to determine whether the anaerobic culture used for bioaugmentation could enhance dissolution of the DNAPL by biosurfactant production, as a possible mechanism for the high concentration of PCE observed in the bioaugmented tank early in the experiment. Three glass columns (15 cm long, 1.5 cm ID) (da Silva and Alvarez, 2002) were packed with the same sandy material used in the ECRS. All tubing and fittings were Teflon lined to minimize adsorption losses. Feed solutions were dispensed from gas tight syringes (100 mL) (SGE, Austin, Texas) at constant flow (1 mL/h) using a syringe pump (Harvard Apparatus, Holliston, Massachusetts). The effluent tubing was adapted for sampling with a 0.64 cm (1/4 inch #28) male Luer Lock adapter and a thin (30-gage) disposable syringe needle. A bicarbonate buffered (1,000 mg/L) synthetic groundwater (Vongunten and Zobrist, 1993) was fed continuously (1 mL/h). Synthetic groundwater was used to reproduce similar ionic strength encountered in groundwater. One pore volume was displaced in 7 hours with a seepage velocity of 5.1 cm/d. The DNAPL source in the columns consisted of neat PCE (0.8 mg) injected with a glass gas-tight syringe (10μL) below the effluent cap of the column (4 cm). One column was fed continuously with the synthetic groundwater plus 50% V/V ethanol to enhance the dissolution of PCE (positive control). The second column was fed continuously with synthetic groundwater alone (negative control) to define a PCE dissolution baseline. A third column was fed with the same bacterial consortium used to bioaugment the ECRS. Samples (1 mL) were taken over time from each column by attaching the needle from the effluent lines to gas chromatography vials (5 mL), previously sealed with Teflon-lined rubber septa and aluminum crimps. Headspace samples (100 μL) were analyzed for PCE immediately after collection using gas chromatography, as described previously.

# Appendix C: Quality Assurance Project Plan

The research project was conducted at Rice University in the Department of Civil and Environmental Engineering under the supervision of Professors C. Herb Ward and Pedro Alvarez. Dr. Marcio da Silva was responsible for laboratory quality assurance/quality control (QA/QC) concerns. Some of the microbial analyzes were conducted at Georgia Tech, under the supervision of Professor Joseph Hughes. Dr. Rebecca Daprato was responsible for the QA/QC at Georgia Tech.

### *Data Quality Assurance*

The quality of all measurement data generated and processed was assessed for precision, accuracy, representativeness, comparability, and completeness. Most analysis involved in this project was performed using USEPA-recommended procedure. The procedures listed in *Standard Methods for the Examination of Water and Wastewater* (1992) were also consulted. Multiple samples from replicate tests were regularly analyzed to establish the precision of the sampling methods. The accuracy of the data was established using USEPA-approved procedures. The data was also compared to literature data to determine the accuracy and representativeness of the data.

### *Precision*

The reproducibility of quantification techniques (gc) was determined periodically by comparison of triplicate independent standard samples. For analytical methods, acceptable levels of precision were < 10% relative standard deviation (RSTD). Microbial counts are inherently more variable and the target RSTD for these measures was < 20%.

### *Accuracy*

The accuracy of all chemical analysis was determined by periodic triplicate samples of matrix spikes. This process was repeated on a regular basis to ensure data quality in different experimental phases of the research.

### *Method Detection Limits*

Detection limits were determined for individual measurements using USEPA-approved procedures, as well as those listed in *Standard Methods for the Examination of Water and Wastewater* (1992).

### *Comparability/ Representativeness*

When necessary, split samples were analyzed in-house.

### *Site Selection and Sampling Procedures for Critical Measures*

USEPA-approved collection methodologies were followed for collection and handling of sediment and water samples. Sediment and water samples were stored in a refrigerator at 4°C until use. The majority of the samples were analyzed within 2 h of collection.

### *Calibration Procedures and Frequency*

Instrument calibration was provided for the gas and ion chromatographs and pH meters. Sample calibrations were done with USEPA-approved calibration standards. Calibration was made with external standards within appropriate concentration ranges. Check standards were run daily for the gas chromatograph, and full calibration (using 6 to 8 standards across a wide concentration range) occurred on a weekly basis.

### *Analytical Procedures*

Glassware used in any aspect of an experiment were cleaned thoroughly (minimum 2 hours soaking in an anti-microbial detergent) and rinsed in organic free deionized water. For all solvents and chemicals, reagent grade or higher quality was a basic selection criterion. All consumables and supplies were obtained from reputable scientific suppliers. The highest grade of chemicals necessary for the task was selected.

Detailed analytical procedures are located in Appendix A.

Some proposed analyses, such as molecular-based microbial characterizations, were conducted at Georgia Institute of Technology in cooperation with the laboratory of Dr. Joe Hughes. Because Rice University personnel were involved in this off-site analysis, identical QA/QC procedures were followed.

### *Data Reduction, Reporting and Analysis*

All statistical analyses were performed using standard methodologies. Unless specifically reported otherwise, all hypothesis testing (comparison of means and variances, comparison of least-squares estimates for model coefficients, determining zero and non-zero coefficients, etc.) were done using a significance level of 5%. Sample tracking, beginning with a transfer of data from log books to a summary form indicating sample status, were done to ensure the timely analysis of each sample and the completeness of the data set. Data reduction was conducted by computer spreadsheets and recorded electronically and as hard copies. This information was available to the project officer upon request and is presented in the final report by use of summary figures and tables in the text and inclusion of all of the data in appendices.

### *Preventative Maintenance*

Routine instrument service was provided by service trained personnel (HP and Dionex).

### *Training*

All students and staff took courses in chemistry and analytical techniques pertinent to this project. Lectures were given through the Department of Civil and Environmental Engineering, the Chemistry Department, and the Environmental Health and Safety Department on campus. The manufacturers provide training specific to individual instruments.

### *Record Keeping*

All analytical data, instrument maintenance log sheets, sample records, and laboratory notebooks are available in the principal investigator's office for future reference.

### *References*


1) American Chemical Society, *Safety in Academic Chemistry Laboratories*, 4th edition, 1985.
2) Greenberg, E.E., L.S. Clesceri, A.D. Eaton. 1992. *Standard Methods for the Examination of Water and Wastewater*. American Public Heath Association/American Water Works Association/Water Environment Federation, Washington, D.C.

# Appendix D: Health and Safety Plan (HASP)

As part of the safety program, Rice University requires regular, thorough inspections by the Building's Safety Officer and Rice's EHSD. Protective equipment and waste disposal procedures follow Rice's EHSD's policy, which is based on both Federal and Texas Department of Health guidelines.

Worker safety in the laboratory is a primary concern of the Department of Civil and Environmental Engineering at Rice University. Laboratory practices affect the health and safety of everyone working in the laboratory. The faculty and staff attempt to provide a safe working environment, but workers are ultimately responsible for their safety. It is assumed that investigators and students are capable of acting responsibly to protect themselves and others when provided with adequate information. The focus of the safety program is on providing workers with adequate information. The rules and guidelines listed below, in conjunction with the document titled *Hazard Communication and Chemical Hygiene Program,* comprise the Environmental Engineering Laboratories' (EEL) Chemical Hygiene Program as required by 29 CFR Part 1910 (www.osha.gov).

***General Guidelines***

(Adapted from *Safety in Academic Chemistry Laboratories*, American Chemical Society, 1985)

1. Eye protection is required when performing hazardous techniques or when in close proximity to someone performing a hazardous technique in the laboratory and where chemicals are stored and handled. This includes any visitors to the laboratories.
2. Horseplay in the laboratories is especially dangerous and is prohibited.
3. Work only with materials of known flammability, reactivity, corrosiveness, and toxicity.
4. No eating, drinking, or smoking in the laboratories. No food or drink should be stored in refrigerators or freezers designated for chemical storage.
5. Confine long hair and loose clothing in the laboratories. Open-toed shoes or sandals are not permitted.
6. Mouth suction should never be used to fill pipettes, to start siphons, or for any other purpose.
7. Never perform experimental work in the laboratory alone, or at least without another person within easy call. Always inform someone, e.g. security personnel or telephone switchboard operator that you are in the laboratory if you will be alone. There should be a time limit of no more than one hour for contact between working laboratory personnel and people outside the lab.

In addition, all personnel must be able to address the following four questions for every experiment. If an individual cannot answer them, he or she should ask for assistance from the laboratory director.

1. What are the hazards associated with this experiment?
2. What are the worst possible outcomes?
3. What must I do to be prepared for such outcomes?
4. What are the prudent practices, protective equipment, and facilities needed to reduce the risk?

### *Safety Equipment*

All workers should know the location and proper usage all safety equipment including eye wash stations, spill control equipment, first aid kit, safety showers, fire extinguishers, and exits for every laboratory in which they work. Access to safety equipment should never be blocked. If an experimental set-up involves the chance of an explosion or implosion, a tip resistant blast shield should be used.

### *Dilutions*

To avoid violent reaction and splattering while diluting solutions, laboratory personnel should always pour concentrated solutions slowly into water or less concentrated solutions while stirring. This procedure is particularly applicable in preparing diluted acids. Goggles should always be worn and a fume hood used when diluting concentrated acids.

### *Compressed Gases*

A gas cylinder should always be secured by a strap, chain, or stand. A gas cylinder should never be left standing unsupported for any period of time, no matter how brief. It should be moved on a gas cart and immediately chained into its proper place with its cap securely in place to protect the valve stem. Always use the correct regulator. Promptly remove the regulator from an empty cylinder, replace the cap, and chain the cylinder in the proper location for pick-up.

### *Hoods*

Fume hoods are important safety devices in the laboratory, and will be checked on an annual basis by EHSD. Chemicals with PEL or TLV of less than 50 ppm or 100 $mg/m^3$ should be used in the hood (check the Material Safety Data Sheets, [MSDS]). Before use, verify that the hood is turned. In general, sash openings should be kept to a minimum, and sashes should be closed when not in use. Sources of emission should be kept at least 6 in inside the hood. Users should keep their faces outside the plane of the hood sash. Exhaust ports from hood and supply air vents should not be blocked, and should be checked regularly. Large pieces of equipment should be elevated at least 2 in to allow free airflow underneath them. Use traps and scrubbers to minimize release of toxic or noxious materials into the hood. A fume hood is not designed for intentional chemical releases such as evaporation of large amounts of waste chemicals.

### *Transporting Chemicals*

Chemicals should be carried from lab to lab in a cart or secondary spill container, if practical. Carry one large glass bottle at a time; it is easy to hit two of them together and break one.

### *Incompatible Chemicals*

Certain chemicals should not be stored with each other because of the possibility of violent reaction if they were accidentally allowed to mix. Check the MSDS of specific chemicals for

more information. A few common chemicals and some of their incompatibilities are given in the Table C-1.

**Table C-1. Examples of Incompatible Chemicals.**

| Chemical | Keep out of contact with |
|---|---|
| Acetic acid | Nitric acid, glycol, peroxides, permanganates |
| Acetone | Conc. nitric and sulfuric acids |
| Ammonia, anhydrous | Halogens, calcium hypochlorite (bleach), HF |
| Ammonium nitrate | Acids, metal powders, flammable liquids, chlorates, nitrites, sulfur |
| Activated carbon | Oxidizing agents |
| Flammable liquids | Ammonium nitrate, peroxides, nitric acid, halogens |
| Hydrogen Peroxide | Most metals and their salts, combustible materials, aniline, nitromethane |
| Nitric acid (conc.) | Acetic acid, aniline, chromic acid, hydrogen sulfide, flammable liquids and gases |
| Oxalic acid | Silver, mercury |
| Perchloric acid | Organic materials, metals |
| Potassium permanganate | Glycerine, ethylene glycol, benzaldehyde, any free acid |
| Sulfuric acid | Potassium chlorate, potassium perchlorate, potassium permanganate (also sodium and lithium salts of the above) |

In general, acids and bases should be stored separately. Organic acids should be stored with flammable materials, separate from oxidizers (including oxidizing acids - particularly nitric acid).

***Special Precautions***

Placing chemical orders through the lab director allows particular hazards to be pointed out specific to the chemicals being used. The MSDS should also be consulted for specific precautions to take when working with any given chemical. However, for the following classes of compounds, appropriate special precautions should always be taken.

Flammables - Check the area for sources of ignition before beginning to work.

Carcinogens - Substances that have been regulated as carcinogens by OSHA, designated as Group 1 or Group 2 (A or B) by IARC, or classified as "known to be carcinogens" or "reasonably anticipated to be carcinogens" by the NTP must be handled with gloves and in a hood as much as possible. Only instrumental analysis should take place outside the hood.

Reproductive Hazards - Substances that have been identified as reproductive hazards (embryotoxic, teratogenic) should be used in the hood and with gloves. Pregnant women and women of child-bearing age should be particularly careful when working with, or near, chemicals believed to be teratogenic, carcinogenic, or mutagenic. Talk to the lab director for more information. (Shane, B.S., *Environmental Science and Technology*, 1989, *23*, 1187-95 for an excellent overview of reproductive hazards.)

Severe Acute Hazards - Substances ranked as 4 in health by the NFPA should **never** be used in the laboratory without informing the lab director, and without another person nearby in the laboratory who is aware of what you are doing. Substances with a 4 in any of the other categories should also only be used with extreme caution.

Radioactive Materials - As much as possible, radioactive materials are to be handled in hoods or in a glove box designated for that purpose. Radioactive materials are only to be used in areas that are designated and posted for such usage.

All of the above classes of chemicals should be used in as small a quantity as is practical, and devices such as filters or scrubbers should be used to minimize releases.

***Emergencies***

In case of an emergency such as a chemical spill, notify EHSD . If EHSD is unavailable, call the campus police department . Evacuate the area if necessary. Assemble and check to make sure that everyone is out of the affected areas.

In case of an emergency requiring medical treatment or ambulance, contact the campus police department. They will contact the necessary emergency personnel.

***Fires***

If a fire is small and appears controllable, put it out by smothering it with an inverted beaker or watch glass, or, if necessary, by using a fire extinguisher. Direct the fire extinguisher at the base of the flame. If the fire is too large to immediately control, leave the laboratory area immediately, pull the fire alarm, and call the campus police department.

***Spills***

When possible, prevent spills by using secondary containment. For example, store chemicals in plastic bins so that if the primary container breaks, the spill will be contained. Clean spills up immediately. "Spill pillows" or "pads" are provided to absorb spills that are too large to clean up with paper towels. These spill pillows contain an absorbent which makes them effective on spills of acids (excluding HF), caustics, or solvents. Be sure to use gloves, and to properly dispose of the resulting waste. Be extremely careful if the spill is large and the material is volatile - the worker much make sure that he or she is not overcome by the vapors! If the spill is too large to be cleaned up by an individual, evacuate the area and contact the lab director. Spills of solid materials can be cleaned up with broom and dustpan, and disposed of as solid chemical waste.

***First Aid***

Workers are strongly encouraged to take first aid training. Individuals should be familiar with first aid measures recommended on the MSDS for the particular chemicals they are working with. A few generalizations can be made about first aid in the laboratory:

- Any chemical splashed on the skin should be flooded with large quantities of water immediately. Use the safety shower if necessary.
- Similarly, any chemical splashed into the eye should be washed out with copious amounts of water.
- If there is a release of gas or vapors in the laboratory that overcomes someone, get that person to fresh air as soon as possible.
- In any of these situations, when the person is out of immediate danger, get professional assistance as soon as possible.

It is far better to prevent an accident than respond to one.

***Medical Consultation***

If exhibiting signs or symptoms associated with exposure to a hazardous chemical, or after being in the vicinity of a spill, leak, or other likely significant exposure to a hazardous chemical, a worker may be examined, at no cost, by physicians at the Texas Medical Center.

***Reporting***

Report all laboratory accidents and near-accidents - even minor ones - to the lab director. Similar accidents may be avoided in the future by modifying a procedure or changing a piece of equipment. Injuries that are not reported immediately are not eligible for worker's compensation.

*Hazard Communication and Chemical Hygiene Program*

The purpose of the Hazard Communications Program in the Environmental Engineering Laboratories is to let workers know what hazardous substances are in the work environment and what they need to know about handling and storing these substances. The Environmental Engineering Laboratories are complying with the OSHA Hazard Communication Standard by compiling a hazardous chemicals list, by using MSDSs, by ensuring that containers are labeled, and by providing workers with training. Deviations from the guidelines set forth in this document require prior approval from a PI.

Training of employees will include:

1. Requirements of the Hazard Communication Standard and the Occupational Health Standard
2. Identification and explanation of operations within work areas where hazardous chemicals are present or being used
3. Location and availability of:
   a. Written Hazard Communication Program
   b. Material Data Safety Sheets
4. Methods, detection and monitoring practices used to warn of the presence or release of a hazardous chemical
5. Potential physical and health hazards of chemicals in the work area
6. Emergency procedures and use of protective equipment
7. Explanation of the labeling system in use in the EEL

8. How to read a Material Safety Data Sheet
9. Criteria which would invoke the use of specific exposure control measures
10. Procedures, activities or operations which are of a sufficiently hazardous nature to warrant prior approval from the lab director or faculty advisor prior to implementation
11. Procedures for insuring proper functioning of fume hoods

This training will take place in three ways - training of new graduate students and faculty, training of individuals, and updates at monthly laboratory meetings.

***General Training***

Initial training of graduate students, faculty members, and employees who work in the Environmental Engineering Laboratories will be in the form of a training class offered by Rice's EHSD with mandatory attendance. Further training will be conducted within EEL in the form of a seminar with mandatory attendance. A record of attendance at this general training session will be kept in the laboratory director's files. Topics that will be covered include:

1. The requirements of the Hazard Communication Standard, and how EEL's written Hazard Communication Program meets those requirements. The full text of 29 CFR 1910.1200, the OSHA Hazard Communication Standard, is on file in the EHSD and available on the web (www.osha.gov).
2. The requirements of the Occupational Exposures to Hazardous Chemicals in Laboratories regulation, and how EEL's written Hazard Communication Program meets those requirements. The full text of 29 CFR 1910 is on file in the laboratory and on the web (www.osha.gov).
3. Location and use of MSDS and other hazard-related resources. Terms used on Material Safety Data Sheets such as "PEL-OSHA", "TLV-ACGIH", and "flashpoint" will be explained. Material Safety Data Sheets will be a fully completed OSHA Form 174 or equivalent.
4. Potential physical and health hazards of chemicals if not handled properly. The presentation will focus on evaluating the likelihood and possible consequences of an accidental release of chemicals used in the EEL. This section will include discussion of terms including (but not limited to) "carcinogen", "teratogen", "mutagen", "$LD_{50}$" , "acute", "chronic", "corrosive", and "sensitizer". Target organ effects will be discussed.
5. Emergency procedures and use of protective equipment. Location and use of evacuation routes, fire alarms, fire extinguishers, safety showers, eyewash stations, first aid kits, and spill control kits will be explained.
6. The EEL labeling system will be defined. Included will be an explanation of hazard warnings and rankings on chemical containers, and a statement of EEL rules regarding labeling of secondary containers.
7. Circumstances which would require the use of special measures to control exposure to a hazardous chemical, and procedures, activities or operations which would require prior approval from the lab director or faculty advisor prior to implementation
8. The type of information that will be provided in individual training will be discussed.

### *Individual Training*

When an individual working in the EEL purchases or obtains a chemical for the first time, that individual will be required to receive instruction from the lab director regarding the proper use, potential hazards, and appropriate precautions specific to that chemical. If another employee is working in the same area, that person will also receive this training. This training will include a review of the MSDS for that compound, how to recognize an accidental release, spill cleanup procedures, and evaluation of possible necessary emergency procedures if an accident should occur. This training will also include specification of protective equipment to be worn while using the chemical, the location of valves or switches in the laboratory necessary to minimize the effects of an accident, and the proper disposal method for the chemical. An MSDS will be on file in the lab director's office. A record of all individual training sessions will be kept in the laboratory director's files. Individual training will also be provided for employees performing non-routine hazardous tasks.

### *Continuing Training*

Monthly lab meetings will provide an opportunity to reinforce the above-mentioned training and serve as a reminder of the importance of safe working conditions. Time will be devoted to updates on hazards involved with laboratory operations, review of accidents that have taken place in the EEL or in similar laboratories, and other related topics of general interest to laboratory workers. A record of attendance at these meetings will be kept in the laboratory director's files.

The laboratory director will be available to answer questions from workers and provide daily monitoring of safe work practices. As part of the assessment of the training program, faculty, staff, and students will provide input to the program coordinator regarding the training they have received and their suggestions for improving it.

### *Special Hazards*

Students and faculty working with radioactive materials receive a special training course. A monitoring program within the labs, including regular wipe tests of the area, will also be maintained.

### *Non-departmental Personnel*

When it is necessary for non-departmental personnel to enter the EEL, MSDSs and individual training will be made available to them for chemicals to which they could be exposed. Records of these individual training sessions will be kept in the laboratory director's files. Contractors will also be asked to provide MSDSs for any hazardous chemicals that they may use in the EEL.

### *Evaluation and Record-keeping*

Evaluation of the effectiveness of training will take place on an on-going basis. The laboratory director will continually review safety and health procedures and practices in the laboratory. Records of all training sessions, both individual and general, will be on file in the laboratory director's office.

### *Additional Information*

All employees, or their designated representatives, can obtain further information on this written program, the hazard communication standard, applicable MSDSs, and chemical information lists at the EHSD office.