USING A MOLECULAR APPROACH TO MONITOR A BIOAUGMENTATION PILOT

Edwin R. Hendrickson, Mark G. Starr, Margaret A. Elberson, Jo Ann. Payne, E. Erin Mack, Hui-Bin Huang (DuPont Co. Newark, DE), Michaye L. McMaster (GeoSyntec Consultants, Guelph, Ontario, Canada) and David E. Ellis (DuPont Co., Wilmington, DE.)

ABSTRACT: The Bioremediation Consortium of the Remediation Technologies Development Forum (RTDF) carried out a successful anaerobic bioaugmentation pilot to bioremediate a chloroethene Tetrachloroethene (PCE), Trichloroethene (TCE) and 1,2 *cis-dichloroethene* (cDCE) contaminated aquifer at Kelly Air Force Base near San Antonio, Texas (TX). An anaerobic dechlorinating enrichment culture, KB-1, was injected into the ground to duplicate the successful bioaugmentation pilot at Dover AFB, Delaware (DE) (1997-1999). The KB-1 community structure has been analyzed and shown to have a *Dehalococcoides ethenogenes*-like species present in its community structure. *Dehalococcoides ethenogenes* (DHE) is an organism described by Maymo-Gatell et al. (Science 276, 1568-1571, 1997). DHE was shown to dechlorinate PCE and TCE by removing all the chlorine atoms to form ethene, through a process known as dehalorespiration. *Dehalococcoides ethenogenes*-like (DHE-like) organisms detected in samples from approximately 30 different sites in North America and Europe have shown 16S rRNA gene sequences (rDNA) with signature sequences that are unique to the sampling site. This was found true for the KB-1 DHE-like organism. We have developed a specific 16S rRNA polymerase chain reaction (PCR) assay to detect DHE-like organisms. Using the PCR assay and 16S rDNA sequence information, groundwater samples were monitored during the course of the Kelly pilot study. The DHE-like organism was not detected in the control groundwater that had been given electron donors (methanol and acetate). After bioaugmentation, PCR analysis of groundwater from monitoring wells detected the DHE-like organism. Detection first appeared in the injection well and then in down gradient monitoring wells (first in the nearest well and then in the well wells further down gradient). The DHE-like organism was detected in the extraction wells, two months after bioaugmentation. Together, with field data from monitoring wells that have demonstrated dechlorination of PCE to ethene, the PCR and sequence data suggest that the bioaugmentation culture, KB-1, had colonized the test plot in the Kelly AFB chloroethene contaminated aquifer.

INTRODUCTION

Chloroethene solvents, perchloroethene (PCE) and trichloroethene (TCE), are widely used as solvents, degreasing, and cleaning agents. Because of past disposal practices and spills, these agents are contaminants in groundwater, sediments and soil. Standard remedial approaches have proven to be ineffective and costly for removing these substances from the environment. Within the past 15 years, dechlorination mechanisms by natural microbial populations have

suggested that the destruction of chlorinated compounds can be practically achieved by stimulating bacterial reductive dechlorination in the field (Beeman et al., 1994; McCarty, 1997). The Remediation Technologies Development Forum (RTDF), a government and industry research consortium, conducted a successful pilot field dechlorination study at Dover Air Force Base (AFB), Dover, Delaware (DE) (1997 to 1999) (Ellis et al., 2000). The study demonstrated that biostimulation (the addition of nutrients and electron donors into the subsurface) and bioaugmentation (the injection of a dechlorinating culture into the subsurface) could be used to stimulate the dechlorination of TCE beyond 1,2-*cis*-dichloroethene (cDCE) to ethene. The biostimulation stimulated the formation of anaerobic environment needed for dechlorination and the bioaugmentation culture provided the organisms to complete the process (Ellis et al., 2000).

The culture used to bioaugment the pilot study plot was the "Pinellas Culture"; an enrichment culture developed from groundwater and soil taken from Department of Energy's Pinellas site in Largo, Florida. Its microbial population was enriched to dechlorinate PCE or TCE to ethene (DeWeerd et al., 1998; Harkness et al., 1999). We have developed a detection strategy that uses the Polymerase Chain Reaction (PCR) assay that uses species-specific primers to test for dechlorinating bacteria in the environment. The species-specific primers were designed using the variable region sequences in 16S rRNA gene sequences (rDNA) sequences from known dechlorinating bacteria found in GenBank. This assay was used to investigate the presence of dechlorinating bacteria in the Pinellas culture and their ability to survive in the environment (Elberson et al., 1999; Hendrickson et al., 2000).

Examination of 16S rDNA sequences from DNA extracted from dechlorinating organisms showed that the 16S rRNA variable sequences could be used to identify the organisms. By analyzing culture samples, the Pinellas culture's population has been partially characterized and shown to have three dehalorespirating-strains, *Dehalobacter restrictus*, *Dehalospirillum multivorans*, and *Dehalococcoides ethenogenes* (Elberson et al., 1999; Hendrickson et al., 2000). The latter strain *Dehalococcoides ethenogenes is* the only laboratory strain shown to completely dechlorinate PCE to ethene (Maymó-Gatell et al., 1997). These methods were used analyze pilot field samples and demonstrated that some of dechlorinating population in the Pinellas culture survived in the pilot's anaerobic subsurface and could be found throughout the pilot study area. Dehalorespirating organisms were not detected in samples taken outside the pilot area. It was concluded that some of the culture had indeed survived and colonized the pilot subsurface. The detection and sequencing assays were performed one, two and three years after the Pinellas culture had been injected into the ground. Results demonstrated that the organisms can survive for a long period after injection into the subsurface and continue to dechlorinate as long as the required anaerobic environment is maintained.

This paper discusses analysis of the microbiology component of the RTDF's second pilot to demonstrate microbiological dechlorination of chloroethene solvents in a contaminated aquifer at Kelly AFB, San Antonio Texas (TX). The site chosen was next to Building P360, which was historically used for

the maintenance and repair of jet engines. These activities for more than 25 years resulted in solvent spills and subsequent contamination of the groundwater. Like the Dover AFB bioaugmentation pilot, the subsurface was enriched with electron donors and nutrients to develop anaerobic conditions in the subsurface to

(iv) If it does colonize the subsurface, can we track its progress with by tracking the DHE-like strain in the subsurface with the PCR detection assay? (v) Does the apparent colonization of the aquifer subsurface correlate with complete dechlorination of PCE to ethene (if this could be stimulated to occur)?

MATERIALS AND METHODS

Groundwater Samples. One thousand-milliliter groundwater samples were taken from the injection, extraction, and monitoring wells of the chloroethene contaminated pilot using plastic sampling bottles, filled-up to the top, sealed, doubled bagged and shipped to the lab on ice. Upon arrival, the samples were either stored overnight at 4° C or immediately centrifuged using a GSA rotor in a RC5B Sorvall Superspeed centrifuge. The resulting cell/soil pellets were resuspended in 2 mL of 1x PBS (10 mM Na phosphate, 150 mM Na chloride, pH 7. 6) and either stored at -20°C or extracted for genomic DNA

Design of *Dehalococcoides ethenogenes*-specific Primers for the PCR Assay. The PCR primers were designed using unique sequence from variable and hypervariable regions of the *Dehalococcoides ethenogenes* 16S rDNA sequence using the procedure described in Hendrickson and Ebersole (2000).

DNA Extraction Procedure. DNA was extracted from the microcosm cultures or groundwater samples by a bead mill homogenization procedure, FastDNA® SPIN Kit Spin Kit for Soil (Bio 101, Vista, CA), that was designed to isolate genomic DNA from all cell types. For groundwater, 1 mL of the resuspended pellet in 1x PBS was used. For soil microcosm cultures, a 10 mL sample was centrifuged to a pellet and resuspended in 500 µL of the culture media. The genomic DNA from both types of samples were isolated using the silica matrix system of FastDNA SPIN Kit by following the manufacturer's protocol and recommendations. The isolated DNA sample was stored at -20° C until it is needed for the PCR assay.

PCR Procedure. The 16S rDNA sequences were amplified from the samples using *Dehalococcoides*-specific 16S rDNA primers as previously described (Hendrickson and Ebersole, 2000). All PCR amplifications were performed using the GeneAmp PCR kit with Taq DNA polymerase (PE Applied Biosystems, Branchburg, NJ) in a Perkin Elmer 9600 thermal cycler as previously described by Hendrickson et al. (2001). A "direct detection" protocol used 1 µL of the microcosm culture was directly added to the PCR reaction, which was conducted as previously described above (Hendrickson and Ebersole, 2000).

Analysis of *Dehalococcoides ethenogenes* Related Sequences. After the *Dehalococcoides ethenogenes*-like sequences are detected in the groundwater or microcosms developed from contaminated soil, they were amplified, cloned, sequenced and analyzed as previously described by Hendrickson and Ebersole. (2000). .

Groundwater Modeling and Design. Groundwater modeling and design was previously discussed by Major et al. (2001). A layout of the monitoring wells is shown in Figure 2. Monitor wells (B1, B2 and B3) were spaced along the central plot's downgradient flow path from the injection well (I1) to the ejection well (E1)

at distances of 8, 12 and 23 feet. Groundwater was extracted from three downgradient wells (E2, E1 and E3) spaced 5 feet apart where E1 is on the plot centerline. Two transgradient (T1 and T2) wells were place in a perpendicular line to the centerline of the plot, 10 feet from either side of monitoring well B2. These were used to measure pilot test area pore volume and estimating the area of influence. Water extracted was combined, amended, and re-injected into I1. The pumping rate, subsurface residence time, electron donors/nutrients delivery, sampling schedule and analysis of parameters were previously discussed (Major et al., 2001). Additional samples outside the pilot area were sampled to assess the recalculation system's impact outside of the pilot area.

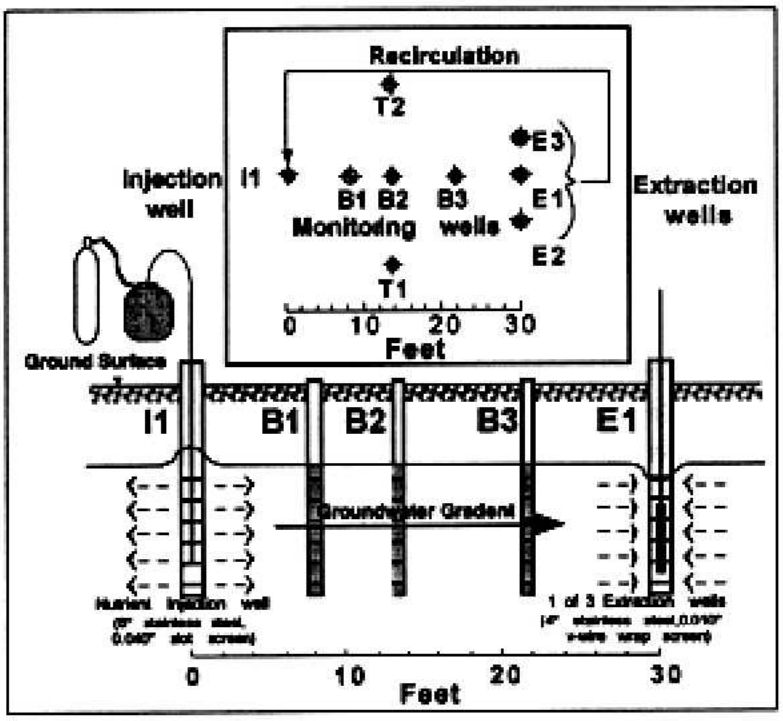

FIGURE 2. Schematic of Pilot test Area's Well Systems and Locations.

RESULTS AND DISCUSSION

The injection methanol and nutirents began on Day 1. The effect of the addition of methanol in monitoring well (MW) B1 are shown in Figure 3 (Major et al., 2001). By Day 89, analytical data revealed that that the dissolved oxygen had not declined and redox potential had not been reduced. Further, the PCE was not being converted to cDCE. Acetate was added with methanol as a second electron donor to induce anaerobic conditions. Consequently, on Day 125, the dissolved oxygen concentrations (1.1 mg/L) and redox potential (-233 mV) became indicative of anaerobic conditions. Concentration of cDCE had begun to increase. Similar results were found on Days 164 and 173. By this time, near stoichiometric conversion of PCR to cDCE had occurred; however, no evidence for dechlorination of PCE beyond cDCE was observed (Major et al., 2001).

Dehalococcoides-like organisms had not been detected in the original sample at Day 1 and were not stimulated by the addition of nutrients and electron donors. As

shown in Table 1 and in Figure 4, DHE-like strains could not be detected by the PCR assay prior to bioaugmentation with culture KB-1. In Figure 4A, the PCR assay did not yield a detectable product in the electrophoresis gel for MWs B1 and B2 and the injection well I1 at Day 173 (3 days before bioaugmentation). Bioaugmentation was conducted on Day 176 with injection of 14 Liters of the KB-1 culture into the subsurface, which was characterized as dechlorinating culture (able to convert TCE to ethene) at the time of injection.

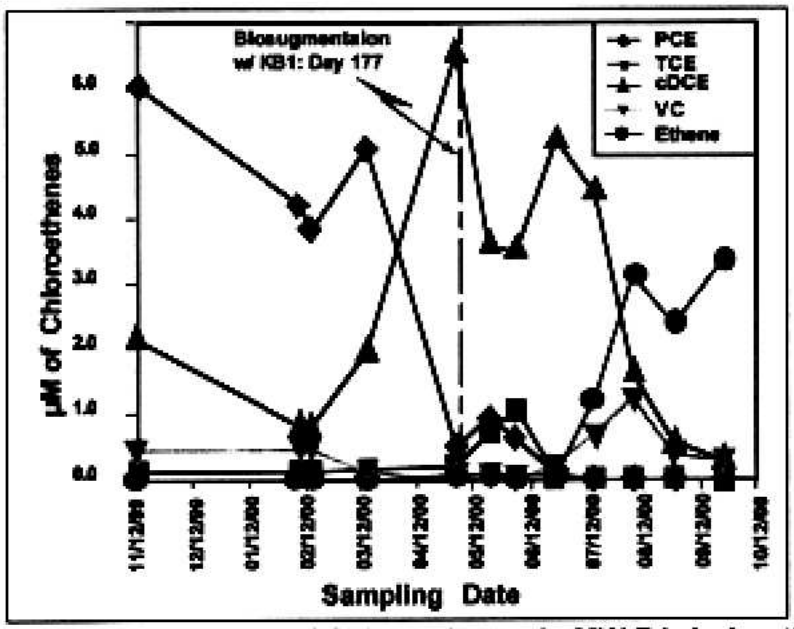

FIGURE 3. Concentration of Chloroethenes in MW B1 during the Bioaugmentation Pilot Test

TABLE 1. Detection of the KB1 Dehalococcoides-Like Organism in the Bioaugmentation Pilot Plot and Chloroethene and Ethene Concentrations in MW B2 on Sampling Dates

Date	Exp. #Day	Bgmnt. #Day	Monitoring Well Number									[Chloroethenes] in MW B2			
			I1	B1	B2	B3	E1	T1	T2	E2	E3	µM PCE	µM cDCE	µM VC	µM Ethene
3-May-00	173	-3	–	–	–	NT	–	NT	NT	NT	NT	0.17	5.40	nd	nd
7-May-00	177	1	++	–	NT	NT	–	NT	NT	NT	NT	NT	NT	NT	NT
22-May-00	192	17	++	–	NT	NT	–	NT	NT	NT	NT	0.68	4.33	nd	nd
5-Jun-00	206	30	++	+/–	NT	NT	–	NT	NT	NT	NT	0.23	3.40	nd	nd
27-Jun-00	228	51	++	++	NT	NT	–	NT	NT	NT	NT	0.16	4.78	0.13	nd
18-Jul-00	249	72	++	++	++	+/–	–	NT	NT	NT	NT	0.03	4.84	0.93	1.25
7-Aug-00	268	93	++	++	++	+/–	+/–	NT	NT	NT	NT	0.06	1.88	0.74	3.14
29-Aug-00	291	116	++	++	++	++	++	++	++	–	NT	0.03	0.74	0.38	2.66
25-Sep-00	318	143	+/–	++	++	++	++	++	++	+/–	NT	0.1	0.24	0.19	3.07

Exp. = RTDF pilot experiment
Bgmnt. = bioaugmentation w/ KB1
#Day = number of days
nd = non-detect
NT= No test was done
– = DHE not detected
++ = DHE Detected
+/– = DHE detected in a trace band

The first indication of dechlorination beyond cDCE was the occurrence of trace amounts of VC on Day 228 or 51 days after bioaugmentation. Ethene was detected at 21 days later on Day 249. Vinyl chloride was now readily detected. By Day 318, all of the PCE and most of the cDCE and VC had been converted to Ethene.

After the injection of KB-1 for bioaugmentation, 1 liter samples of groundwater were taken for "*Dehalococcoides* analysis" as part of the sampling

regime of monitoring wells for measuring the changes in the concentration of chloroethenes and other geochemical parameters. As shown in Table 1 and Figure 4 A, DHE-like organism was detected in the in the injection well (I1) on the first day after bioaugmentation. It was not detected in MW B1 or the extraction well E1.

Examination of the data in Table 1 demonstrates that *Dehalococcoides*-like organism was gradually detected, with respect to time, down gradient from the injection well after bioaugmentation. It was first detected in MW B1, 30 days after the KB-1 injection. DHE was later detected in samples from wells, simultaneously in B2 and B3 after 72 days, in E1 after 93 days, simultaneously in T1 and T2 after 115 days and in E2, 143 days after bioaugmentation. Figure 4 shows an example of a gel, detecting PCR products obtained with one set of DHE-specific PCR primers. Sequences amplified with DHE-specific 16S rRNA primers were sequenced and found to have the same signature sequences in the

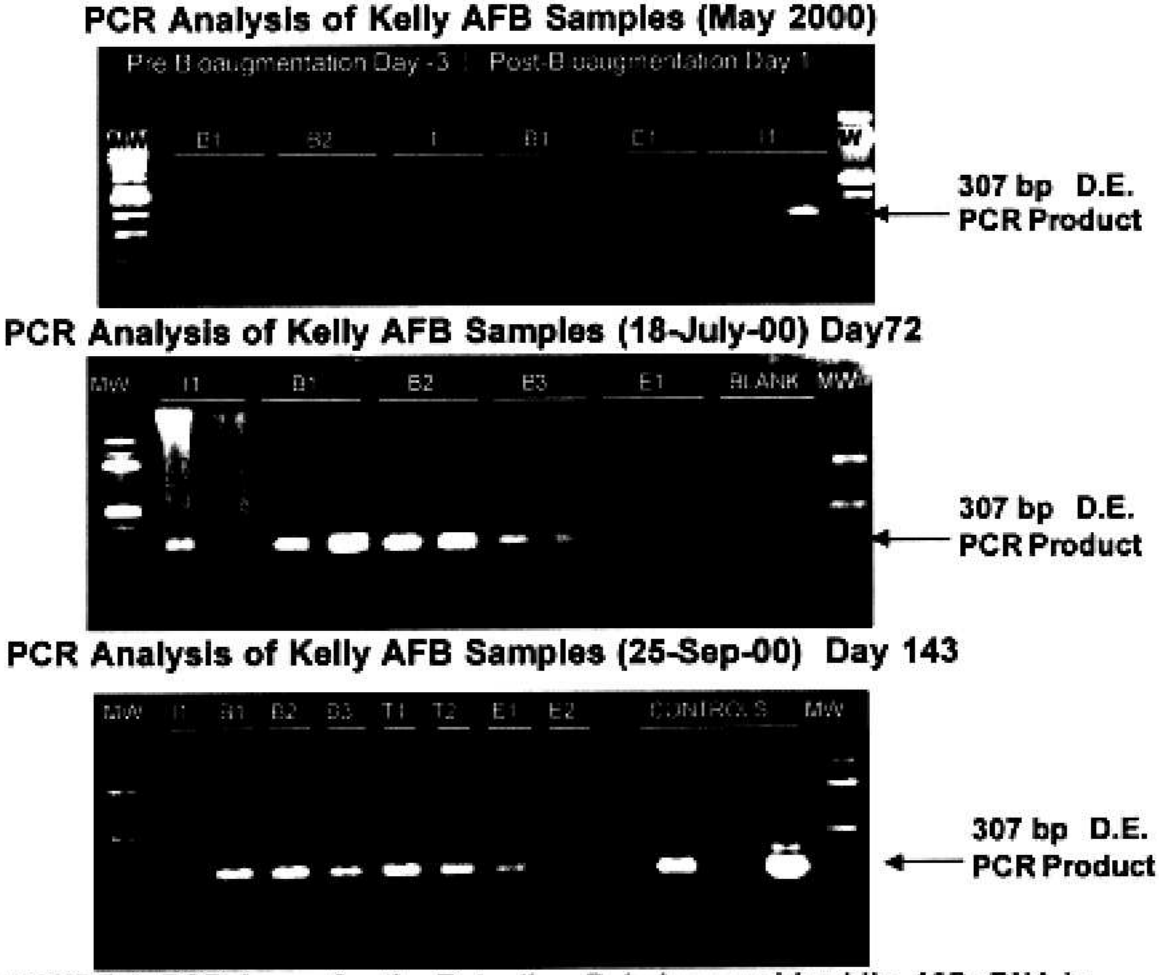

FIGURE 4. PCR Assay for the Detection *Dehalococcoides*-Like 16S rDNA in Groundwater Samples Taken from the Wells in the Pilot Test Area during Bioaugmentation Study.

variable 16S rDNA regions as the KB-1 DHE-like organisms. This indicted that the Kelly DHE-like sequences originated from the KB-1 culture. This is similar to data from the Dover AFB bioaugmentation study, where the signature sequences 16S rDNA of the Dover DHE-like test pilot organisms were found to

be the same as those found in the Pinellas bioaugmentation culture. One hundred-twenty nine days after bioaugmentation, background control groundwater samples, SB235 and SB236, were taken from control two wells approximately 40 yards, north and south, perpendicular to the bioaugmentation *Dehalococcoides*-like organism. These controls indicated that the plot. In addition, a third groundwater sample, SB237, was taken 6 feet down gradient from E1. As shown in Figure 5, all samples were negative for the *Dehalococcoides*-like organism was not naturally occurring in the aquifer as a separate coincidence from the pilot experiment.

FIGURE 5. PCR Assay Results for DHE In Pilot Back ground Control Samples taken from Area outside the Plot Area. The PCR control Is a Cloned *Dehalococcoides*-Like 16S rDNA Isolated from an Industrial Chloroethene Dechlorinating Site

CONCLUSIONS.

Dehalococcoides-like organisms were not detected in soil and groundwater taken from the Kelly AFB Pilot, suggesting that DHE-like organisms do not naturally inhabit the Kelly aquifer. The DHE-like 16S rRNA isolated from groundwater samples from the Kelly AFB bioaugmented pilot had the same signature sequences as the *Dehalococcoides*-Like species found in the KB-1 bioaugmentation culture indicating that its origin was from KB-1. This data suggest that 16S rDNA specific PCR primer assay was successful in "tracking" the KB-1 *Dehalococcoides* species in the bioaugmented aquifer because DHE was detected with respect to time, down gradient from the bioaugmentation injection well. This observation occurred simultaneously with demonstration of complete dechlorination of PCE to ethene in the pilot test plot. Together, the dechlorinating data, the PCR data and the sequence data suggests that the KB-1 culture had colonized the Kelly AFB PCE-contaminated aquifer. The RTDF's Kelly AFB and Dover AFB bioaugmentation pilot's data provides evidence to substantiate the use of dechlorinating consortia for the bioaugmentation of chloroethene contaminated aquifers that are not naturally attenuating.

REFERENCES

Beeman, R. E., J. E. Howell, S. H. Shoemaker, E. A. Salazar, and J. R. Buttram. 1994. "A field evaluation on *in situ* microbial reductive dehalogenation by biotransformation of chlorinated ethylenes." p. 14-27. *In* R. E. Hinchee, A.

Leeson, L. Semprini, and S. K. Ong (ed.), *Bioremediation of chlorinated and polycyclic aromatic hydrocarbon compounds*. Lewis Publishers, Boca Raton, FL.

DeWeerd, K. A., W. P. Flanagan, M. J. Brennan, J. M. Principe, and J. L. Spivack. 1998. "Biodegradation of Trichloroethylene and Dichloroethylene in Contaminated Soil and Groundwater." *Biorem. J.* 2:29-42.

Elberson, M. A., E. R. Hendrickson, J. A. Tabinowski, and D. E. Ellis. 1999. "Detection of Dechlorinating Bacteria in Groundwater and Soils from Waste Sites Contaminated with PCE and TCE." *Abstract of the Proceedings of the 99th General Meeting Of the American Society for Microbiology*, Chicago, Illinois, USA, May 3-June 3, 1999.

Ellis, D. E., E. J. Lutz, J. M. Odom, R. J. Buchanan, Jr., M. D. Lee, C. L. Bartlett, M. R. Harkness, and K. A. DeWeerd. 2000. "Bioaugmentation for Accelerated In Situ Anaerobic Bioremediation." *Environ. Sci. Technol.* 34(11): 2254-2260.

Harkness, M. R., A. A. Bracco, J. Brennan, M. J., K. A. DeWeerd, and J. L. Spivack. 1999. "Use of Bioaugmentation to Stimulate Complete Reductive Dechlorination of Trichloroethene in Dover Soil Columns." *Environ. Sci. Technol. 33*:1100 -1109.

Hendrickson, E. R., M. A. Elberson, J. A. Tabinowski, and D. E. Ellis. 2000. "Molecular Confirmation of Aquifer Colonization by an In Situ Bioaugmentation Culture." *Abstract. Bioaugmentation and Biomonitoring* The Second International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA, USA, May 22-25, 2000:E10.

Hendrickson, E. R., and R. C. Ebersole. October 2000. Nucleic acid fragments for the identification of dechlorinating bacteria. PCT: WO 0063443 A 32. pp 1-55

Major, D.W., M.L. McMaster, E.E. Cox, B.J. Lee and E.E. Gentry, E.R. Hendrickson, E.A. Edwards and S. Dworatzek. "Successful Field Demonstration of Bioaugmentation To Degrade PCE And TCE to Ethene" *Abstract. The Sixth International Symposium on In Situ and On-Site Bioremediation.* , San Diego, CA, USA, June 4-7, 2000.

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.

McCarty, P. L. 1997. "Breathing with Chlorinated Solvents." *Science 276*:1521-1522.