Annual Report
Biodegradation of Chlorinated Solvents: Reactions near DNAPL and Enzyme Function

Recipient: Stanford University

DOE Grant Number DE-FG07-99ER62883
EMSP Project Number 70063
June 3, 2003

Lead PI: Perry L. McCarty (PI), Department of Civil and Environmental Engineering, Stanford University, Stanford, CA 94305-4020, phone: (650).723-4131, e-mail: mccarty@ce.stanford.edu

Co-PI: Alfred M. Spormann (Co-PI), Department of Civil and Environmental Engineering, Stanford University, Stanford, CA 94305-4020, phone: (650).723-3668, e-mail: spormann@ce.stanford.edu

Co-PI: Craig S. Criddle (Co-PI), Department of Civil and Environmental Engineering, Stanford University, Stanford, CA 94305-4020, phone: (650).723-9032, e-mail: criddle@ce.stanford.edu

Graduate Students: Ph.D. candidates: , Min-Ying Chu, Alison Cupples, Michael M. C. Liu; Post-Doctoral Scholars: Edward M. Driggers, Q. Shiang Fu, Laurence H. Smith, Yanru Yang

Research Objectives

The anaerobic biodegradation of chlorinated solvents is of great interest both for natural attenuation and for engineered remediation of these hazardous contaminants in groundwater. Compounds to be studied are carbon tetrachloride (CT) and the chlorinated ethenes, tetrachloroethene (PCE), trichloroethene (TCE) cis-1,2-dichloroethene (cDCE), and vinyl chloride (VC). The chlorinated solvents often are present as dense non-aqueous-phase liquids (DNAPLs), which are difficult to remove. Biodegradation of DNAPLs was previously thought not possible because of toxicity, but recent evidence indicates that under the right conditions, biodegradation is possible. Anaerobic biodegradation of DNAPLs is the major subject of this research.

The specific objectives of this multi-investigator effort are:

1. Evaluate the potential for chlorinated solvent biodegradation near DNAPLs,
2. Provide a molecular understanding of the biological mechanisms involved,
3. Determine cellular components involved in carbon tetrachloride transformation by *Pseudomonas stutzeri* strain KC without chloroform formation.

Research Progress and Implications

This fourth annual report of a three year grant that was extended for one year covers the period between June 2002 and June 2003. Concerning objective one, in our first year we reported on findings that enhanced reductive dehalogenation of tetrachloroethene dense non-aqueous phase liquids was possible (*Environ. Sci. Technol*., 2000, **34**, 2979). We reported in year two on expanded

studies to evaluate donor substrates that offer different remediation strategies for DNAPLs as well as studies on toxicity to different steps in the dehalogenation process by PCE and intermediate degradation products (*Environ. Sci. Technol.*, 2002, **36**, 3400. We have found that the presence of NAPL and the competition among many different types of bacteria makes it difficult to predict the performance of enhanced dissolution. For example, the transport of compounds may be retarded to different degrees at NAPL zones. The composition of PCE NAPL might be significantly changed due to the partitioning back of dehalogenation products. It is important to gain a general understanding of the behavior of this complex microbial NAPL system using numerical modeling. Progress on this aspect has been accepted for publication recently, and is available on the web (*Jour. Contaminant Hydrology*, In Press). With a good numerical model, the responses of such a system using different substrate delivery methods can be quickly evaluated; thus, it is possible to optimize the performance of a particular method before conducting actual experiments. One difficulty in modeling the biological reactions under dual transverse mixing is that high reaction rates occur in small regions (<0.2cm); therefore, extremely fine grids are required to correctly model the behaviors of the system. Because our goal is to stimulate a high PCE transformation rate exclusively near DNAPLs, detailed modeling is required to understand the extent of bio-enhanced DNAPL destruction and the dynamics of microbial communities around DNAPLs. A new manuscript directed towards this aspect is underway. One critical limiting factor for enhanced DNAPL dissolution is the toxicity of reaction end products, particularly cDCE. Experimental studies to determine the nature and extent of cDCE toxicity to reductive dehalogenation are also underway.

Research under objective two has focused on the natural enzyme haloalkane dehalogenase A (DhlA), which is capable of hydrolytically dehalogenating short-chain haloalkanes such as 1,2 dichloroethane (1,2 DCA). Directed evolution experiments with DhlA are currently underway with the goal that functional mutants generated will provide a greater understanding of the molecular mechanism of hydrolytic dehalogenation as well as the evolution of environmentally important enzymes. Specifically, isolation of DhlA variants that are capable of dehalogenating structurally similar EPA priority pollutants such as 1,1,1 Trichloroethane (1,1,1 TCA) and 1,1,2 Trichloroethane (1,1,2 TCA) will result in immediate applicability and also guide the future design of synthetic catalysts for dehalogenation. We have successfully established the several DNA shuffling protocols in our laboratory and demonstrated the generating of recombinants from a number of parent dehalogenase genes. In our expression experiments using the generated diversity of *dhlA* alleles, we noticed that a reduced specific activity towards 1,2 DCA, even with the wild type allele. We examined whether protein folding might be a limiting step in our expression and subsequent screening conditions. Protein misfolding can lead to a high fraction of inactive protein and, thereby, decrease the specific activity of DhlA towards the test substrate. We explored the co-expression of our library in strains with the chaperonins GroEL from *E. coli*. The *groEL* genes were carried on a plasmid different from the *dhlA* containing petBlue-1, induced simultaneously with the *dhlA* alleles, and screened for activity. We discovered a significant improvement in specific activity in clones co-expressing *groEL*. In conjunction with our pH indicator-based screen, we can now detect small changes in specific activity in clones from our library. Currently, we are employing this modified experimental system towards detecting small changes in substrate specificity of DhlA. We feel we are making excellent progress towards our goal, and will continue with this approach during the coming year of study.

Research under objective 3 is aimed at determining potential cell components in *Pseudomonas stuzer*i KC that facilitate the transformation of carbon tetrachloride into carbon dioxide and nonvolatile

products, without chloroform formation. This is a unique detoxification reaction mediated by the secreted molecule pyridine-2,6-(bis)thiocarboxylate (PDTC) when it is chelated to copper. Previously we reported on the use of surface enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-MS) to identify and size cellular components involved in the transformation. We continued those efforts over this past year. Work by Dybas et al. (1995) demonstrated that addition of micromolar levels of ferric iron to an actively transforming culture inhibits CT transformation activity, but the level of iron required to inhibit CT transformation was more than 100-fold higher for washed cells reconstituted with a 10,000-Da supernatant filtrate. Thus, the inhibitory effects of iron are due largely to a supernatant factor or factors with a molecular weight greater than 10,000 Da. We hypothesize that this factor(s) is a protein that binds with ferric iron and possibly to the PDTC-Cu complex. Data from proteinase-treated culture dialysate indicates that this factor consists of one or more proteins. Specific binding assays based on binding interactions of proteins to surfaces containing iron, copper, and nickel were used along with SELDI-MS to identify supernatant proteins that may interact with PDTC-bound metals, and the molecular weights of these proteins were determined. The results indicate that several supernatant proteins bind specifically to surface-bound ferric iron, copper, and nickel. SDS-PAGE gel results showed proteins with sizes that align with those observed in the SELDI-MS spectra. We are currently isolating and sequencing these proteins. Once the sequences are known, we expect to be able to determine the protein functions and to develop an overall model of extracellular processes involved in CT transformation.

Planned Activities

The numerical model of biologically enhanced DNAPL dissolution is further being developed to better determine the important processes involved, and experimental studies are being conducted to determine the toxicity of cDCE to dehalogenation. We will continue the research to obtain functional mutants that will help better understand hydrolytic dehalogenation. Efforts will continue towards defining the protein(s) that binds to PDTC-Cu.

Information Access

Fu, Q. S., Hobbs S. K., Shi, G., Bednarski, M.D., and Criddle. C. S. 2003. SELDI-MS Characterization of Extracellular Proteins Involved in Carbon Tetrachloride Transformation by *Pseudomonas stutzeri KC*, Abstract, ASM 103rd General Meeting, Washington DC

Chu, M., Kitanidis P. K. and McCarty P. L. 2003. Effects of Biomass Accumulation on Microbially Enhanced Dissolution of a PCE Pool: a Numerical Simulation, *Journal of Contaminant Hydrology*. In Press. Corrected Proof now available on the web at http://www.sciencedirect.com/science?_ob=GatewayURL&_method=citationSearch&_uoikey=B6V94-48FCG54-1&_origin=EMFR&_version=1&md5=c6bba559b0e5aa24098994de8c69e133

Yang, Y. and McCarty, P. L. 2000. Biologically Enhanced Dissolution of Tetrachloroethene DNAPL, *Environmental Science & Technology*, **34**(14), 2979-2984.

Yang, Y., and McCarty, P. L. 2002. Comparison of Donor Substrates for Biologically Enhanced Tetrachloroethene (PCE) DNAPL Dissolution, *Environmental Science & Technology*, **36**(15), 3400-3404.