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

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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 third annual report of a three year grant covers the period between June 2001 and June 2002. We have requested a no-cost one year extension for the grant to complete ongoing efforts. 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 last year 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. A manuscript describing the results of these studies was prepared this past year, was accepted for publication in *Environmental Science & Technology*, and is available as an ASAP Article on the ES&T website (see publications). 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. We have made progress in this effort and the first results have been submitted as a manuscript by Chu, Kitanidis, and McCarty (2002). 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. This effort is 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. 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 DNA shuffling protocol StEP in our laboratory and demonstrated the generating of recombinants from a number of parent dehalogenase genes. DNA sequencing of randomly chosen clones from the StEP library showed the protocol to be efficient at generating chimeras. In a StEP library made from 3 different parental dehalogenase genes (*dhlA*, *linB*, *dhaA*), 4 out of 7 (57%) clones sequenced were different from both the parental sequences and from each other. From this particular library, we estimate the recombination rate to be 6-12 crossovers per kilobase and the random error rate to be 8e-4 or 0.8 bp per kilobase. This is evidence that we can generate a library of dehalogenases with both a high crossover rate and with fidelity. We have developed and implemented a standardized procedure to clone a shuffled library of *dhlA* alleles. Briefly, the alleles that were generated by DNA shuffling are cloned into pETBlue-1, which is a cloning vector where expression of the cloned gene is under control of a T7 promoter. Plasmids are then introduced into *E. coli* (NovaBlue), which lacks T7 RNA polymerase, and, thus, does not express the cloned gene. We adopted and modified a pH indicator-based plate screening system to detect dehalogenation activity against the various target substrates, including 1,2-DCA, DCEs and VC. The active colonies identified are purified

and re-streaked according to the above protocol. Only clones that have been confirmed to be active in this second screening step are considered for further screening, sequencing and shuffling. 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 three is aimed at determining potential cell components in Pseudomonas stuzeri strain KC that facilitate the transformation of carbon tetrachloride without the formation of chloroform. Pyridine-2,6-dithiocarboxylic acid (PDTC), a compound excreted by *Pseudomonas stuzeri* strain KC under iron-limiting conditions, is known to be responsible for this transformation. The reactive species is a PDTC-Cu complex. This PDTC-Cu complex must be reduced by cell components, but no specific cell types are required to reduce PDTC-Cu complex. Thus, some common cell component(s) are likely involved in the reduction and regeneration of PDTC and in the activity of PDTC. Previous data suggested that proteins interact with the PDTC-Cu complex. (Tatara et al., 1993; Gurian, unpublished results). To identify such proteins, we used Surface Enhanced Laser Desorption Time-of-Flight Mass Spectrometry (SELDI TOF MS). This analytical tool can capture or dock one or more proteins on an addressable array chip, and a laser is applied to ionize the protein for sizing by mass spectrometry. Initial results showed no detectable proteins using cell lysates from *P.stuzeri* strain KC, presumably due to weak binding of the proteins to the chip surface. Additionally, commercially available techniques involving either matrix assisted laser desorption/ionization (MALDI) or SELDI showed only limited selectivity and detection for specific classes of proteins in complex mixture. To enhance specific protein binding to the chip surface, we developed a novel technique for the display of PDTC for the capture and detection of the protein(s) that binds with PDTC (collaboration with M. Bednarski). This technique involves the construction of polymerized thin film surfaces with PDTC-Cu complex embedded in the film from diacetylene-containing lipid monomers. The surface is coated on the SELDI MS chips to capture and can detect proteins that bind with PDTC from a complex cellular lysate. The thin film is an organic monomolecular film made from self-assembled monolayers. The film possesses good chemical stability and provides homogeneous coverage of the SELDI MS chips. Monomolecular thin film formation by polymerization of diacetylene monomers is stable to laser desorption during ionization in the SELDI MS and can embed or display binding substrates such as PDTC for potential enhanced protein capture and docking with low nonspecific binding. In construction of the film on SELDI chips, a 1:1 mixture of the monomeric solution of diacetylene and PDTC-Cu was applied to the chip surface, and polymerization was achieved by ultraviolet light irradiation at 254 nm. Freshly prepared cell lysate from Strain KC was added on top of the film surface and incubated for binding. After incubation, the energy active molecule, sinnapinic acid solution was placed on to the surface and allowed to air dry. The SELDI chips were subsequently analyzed in ProteinChip reader. Initial assessment of this technique for capture and detection of specific binding proteins to PDTC-Cu was encouraging. Although some nonspecific binding was observed in controls, their intensities were relatively low. Some binding signals were only associated with the surfaces treated with PDTC-Cu. The data demonstrated that display of PDTC using thin film formation is a promising technique for capture and detection of the protein(s) associated with interaction and reduction of PDTC in Strain KC and in other cell types. Further effort is needed to enhance the specificity of protein binding to PDTC-Cu and to decrease the nonspecific interactions. We are currently evaluating different modifications to improve the effectiveness of this technique. Once the protein(s) that binds specifically to PDTC-

Cu is captured, the protein will be isolated and sequenced.

Planned Activities

The numerical model of biologically enhanced DNAPL dissolution is further being developed to better determine the important processes involved. We will continue the research on screening of clones 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

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