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

This second annual report covers the period between June 2000 and May 2001. We reported last year that biological degradation of pure PCE DNAPL was possible by an anaerobic mixed culture enriched

from a PCE-contaminated groundwater site (*Environ. Sci. Technol.*, 2000, **34**, 2979). Expanded studies conducted this past year were 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. Four parallel continuous-flow columns were constructed to simulate aquifers contaminated with PCE DNAPL (2% residual saturation). One column was used as a negative control to monitor PCE dissolution alone. The other three were pentanol-fed (soluble substrate, fed continuously), oleate-amended (slow hydrogen release following calcium precipitation, placed in column initially), and vegetable oil-amended (mixed with PCE and placed in column initially). During eight months of operation, near saturation concentration of PCE (0.8 mM) was observed in the effluent of all columns. PCE dehalogenation began after 1-2 months in the three substrate-supplied columns, with *cis*-1,2-dichloroethene (cDCE) exceeding the effluent PCE concentration. Vinyl chloride (VC) and ethene production were also found in significant amounts (0.2 to 0.4 mM) although batch studies suggested that significant toxicity from PCE should have occurred. Compared with the control column, the DNAPL dissolution rate was enhanced by about three times when directly coupled with biological degradation. High concentrations of PCE, cDCE, and ethene inhibited methanogens in batch studies, but active methanogenesis occurred in both the pentanol-fed and oleate-amended columns, but not in the vegetable oil-amended column. This suggests that methanogens managed to colonize the column niches where PCE DNAPL was not present. This was confirmed in the pentanol-fed column as methane production decreased significantly when the feeding solution was changed so that it was pre-saturated with PCE. This change was accompanied by a greatly enhanced dechlorination to ethene. These results suggest possible DNAPL remediation strategies to enhance dehalogenation while controlling competitive methanogenic utilization of donor substrates.

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. Initial efforts used the technique of error-prone PCR, which randomly mutagenizes a target gene, to generate DhlA variants. Current efforts have shifted to a novel form of family shuffling, which restricts mutations only to those found in other related dehalogenating enzymes. This technique produced more functional mutants at even higher error rates. In one case, an error rate of 3.4% generated a library of mutants of which about 18% retained activity against 1,2 DCA. This demonstrates that the family shuffling technique generates more productive mutants than error-prone PCR. Furthermore, regio-specific mutagenesis experiments were done to map out regions of DhlA that are more tolerant to mutations. This “plasticity map” of DhlA, in complement with the family shuffling technique, promises to raise the odds of finding useful DhlA variants.

Research under objective three is aimed at determining potential cell components in *Pseudomonas stutzeri* strain KC that bring about carbon tetrachloride. Pyridine-2,6-dithiocarboxylic acid (PDTC), a compound excreted by *Ps. eudomonas stutzeri* strain KC under iron-limiting conditions, is now known to be responsible for transforming carbon tetrachloride without the formation of chloroform. PDTC must be reduced by cell components for this purpose, but no specific cell types are required to reduce

PDTC. Thus, some common cell component(s) are likely to be involved in the reduction and regeneration of PDTC. Towards finding such cell component(s), we are assessing a known carbon tetrachloride transforming bacterium, *Shewanella oneidensis* MR-1 for its ability to reduce PDTC. Several MR-1 mutants lacking menaquinone formation capability were created and tested to determine if menaquinone was involved in the reduction. In experiments with the wild type MR-1, the addition of PDTC greatly enhanced the rate of carbon tetrachloride transformation, producing only trace amounts of chloroform. These results indicate that *Shewanella oneidensis* MR-1 is capable of reducing PDTC. In an experiment with various menaquinone-deficient mutants of MR-1, all showed the capability of reducing PDTC with increased rate of carbon tetrachloride transformation and decreased chloroform formation. These results show that menaquinone is not involved in reducing PDTC for carbon tetrachloride transformation.

Planned Activities

A numerical model of biologically enhanced DNAPL dissolution is being developed in order to determine the important processes involved in addition to continued column and toxicity studies related to this process. The enzyme research is focused on the iterative family shuffling of functional mutants generated from previous family shuffling experiments, and investigation of state of the art molecular modeling software for refining the design of DhlA mutants. Efforts with carbon tetrachloride are now focused on method development for accurate detection and measurement of the excreted PDTC molecule, which will enable us to assess the PDTC-cell component interaction more effectively.

Project Publications

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