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Task 7 - Development of Degradation Processes

Topical Report

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1000 Independence Avenue
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U.S. Department of Energy
Office of Fossil Energy
Morgantown Energy Technology Center
P.O. Box 880
Morgantown, West Virginia 26507-0880

By
Monsanto Company
800 N. Lindbergh Boulevard
St. Louis, Missouri 63167

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Development of an Integrated *in-situ* Remediation Technology

DOE Contract Number: DE-AR21-94MC31185

Topical Report for *Task* #7 entitled: "Development of Degradation Processes" (September 26,1994-May 25,1996)

Submitted by:

M. Joan Brackin, Michael A. Heitkamp and Sa V. Ho
Monsanto Company
800 N. Lindbergh Boulevard
St. Louis, Missouri 63167

Zachary C. Houston, Veena Warikoo and Perry L McCarty
Western Region Hazardous Substance Research Center
Stanford University
Stanford, California 94305-4020

Abstract: Contamination in low permeability soils poses a significant technical challenge to in-situ remediation efforts. Poor accessibility to the contaminants and difficulty in delivery of treatment reagents have rendered existing in-situ treatments such as bioremediation, vapor extraction, pump and treat rather ineffective when applied to low permeability soils present at many contaminated sites. The Lasagna™ technology is an integrated in-situ treatment in which established geotechnical methods are used to install degradation zones directly in the contaminated soil and electro-osmosis is utilized to move the contaminants back and forth through those zones until the treatment is completed. The general concept of the technology is to use electrokinetics to move contaminants from the soils into "treatment zones" where the contaminants can be removed from the water by either adsorption or degradation. The focus of technical task #7 was to optimize the conditions required for electro-osmotic movement of contaminants and microbial degradation in the treatment zones. This topical report summarizes the results of aerobic microbial research performed to evaluate the feasibility of incorporating the chemical-degrading organisms into biotreatment zones in laboratory-scale

electro-osmosis units and to demonstrate the combination of electrokinetics and aerobic microbial degradation for the removal of contaminants from clay. Also included in this report are the results of investigating microbial movement during electro-osmosis and studies involving the optimization of the microbial support matrix in the biozone.

The Stanford study was conducted in order to obtain a better understanding of rates of anaerobic reductive dehalogenation of TCE to ethylene and of factors affecting these rates in order to determine the potential for application of TCE biodegradation as part of the Lasagna™ technology. In the anaerobic process, TCE is used as an electron acceptor by microorganisms and converted sequentially to cis-1,2-dichloroethylene (cis-DCE), vinyl chloride (VC), and finally, ethene. The main objective of this study was to determine the effect of temperature and pH on the rates of TCE conversion to cis-DCE, and on the rates of cis-DCE conversion to ethene. Here, a mixed culture of TCE degradaing bacteria was developed from a seed taken from DuPont's Victoria, Texas, site where active dehalogenation of tetrachlorothylene (PCE) to ethene has been occurring. This culture was repeatedly transferred and grown on yeast extract and TCE. In addition, parallel rate experiments were conducted using a facultative microorganism (strain MS-1) that was isolated from the Victoria aquifer material and converts either PCE or TCE to cis-DCE. Strain MS-1 was also grown on yeast extract. Batch rate studies were conducted in which about 100 mg/L of biomass under anaerobic conditions was supplemented with 0.008 M to 0.013 M TCE at different temperatures (range of 20^0 to 45^0 C) and pH (range of 5 to 8), and the rates of degradation were monitored with time.

B. Table of Contents

C. List of Figures and Tables

C	Celsius
cfu	colony forming unit
cm	centameter
g	grams
GAC	granular activated carbon
GC	gas chromatography
hr	hours
HPLC	high pressure liquid chromatography
K_f	Freundlich coefficient
L	liters
lacZY	lactose operon genes
mM	millimolar
mg	milligram
ml	milliliter
N	normal
nm	nanometer
PNP	p-nitrophenol
ppb	parts per billion
rpm	revolutions per minute
TCE	trichloroethylene

Management Plan

A Management plan for this project was prepared by Monsanto and submitted on November 30, 1994. That plan summarized the work plan which was developed in conjunction wiht DuPont, GE, EPA's Risk Reduction Laboratory (RREL), Martin Marietta Energy Systems, (MMES), and the Department of Energy. The DOE Gaseous Diffusion Plant in Paducah, Kentucky, has been chosen as the site for the initial field tests. CDM Federal Programs Corporation was chosen as the site for the initial field tests. CDM Federal Programs Corporation was chosed to provide the on-site support of the field tests which were installed at the DOE site in November 1994. This experiment tested the combination of electro-osmosis and *in-situ* sorbtion in the treatment zones. In 1994 and 1995, technology development was carried out under the present contract by Monsanto, DuPont and General Electric. These studies evaluated various degradation processes and their integration into the overall treatment scheme at the bench and pilot scales.

Technical Deliverables

Tables 1 and 2 summarize the 13 technical tasks and the 8 topical reports which will be written describing the results obtained in the technical tasks. These two tables show which organization is primarily responsible for the tasks and for preparing the topical reports. This report summarizes Task #7 (8.1.6) "Lab-scale Electrokinetic and Microbial Degradation".

Statement of the Problem

Contamination in low permeability soils poses a significant technical challenge to *in-situ* remediation efforts. Poor accessibility to the contaminants and difficulty in delivery of treatment reagents have rendered existing *in-situ* treatments such as bioremediation, vapor extraction, and pump and treat, rather ineffective when applied to low permeability soils present at many contaminated sites.

The Solution

The proposed technology combines electro-osmosis with treatment zones that are installed directly in the contaminated soils to form an integrated *in-situ* remedial process. Electro-osmosis is an old civil engineering technique and is well known for its effectiveness in moving water uniformly through low-permeability soils with very low power consumption. Conceptually, the integrated technology could treat organic and inorganic contamination, as well as mixed wastes. Once developed, the technology will have tremendous benefits over existing ones in many aspects including environmental impacts, cost effectiveness, waste generation, treatment flexibility, and breadth of applications.

Consortium Description

A Consortium has been formed consisting of Monsanto, E. I. du Pont de Nemours & Co., Inc. (DuPont) and General Electric (GE), with participation from the Environmental Protection Agency (EPA) Office of Research and Development and the Department of Energy's (DOE) Environmental Management Office of Science and Technology. The five members of this group are leaders in their represented technologies and hold significant patents and intellectual property which, in concert, may form an integrated solution for soil treatment. The Consortium's activities are being facilitated by Clean Sites, Inc., under a Cooperative Agreement with EPA's Technology Innovation Office.

Table 1. List of Tasks and Responsible Company

Task	Company
Task 1 - Evaluation of Treatment Zone Formation Options (5.1.2)	DuPont
Task 2 - Electrokinetic Model Validation and Improvement (6.5)	GE
Task 3 - Design Guidance for Field Experiments (6.6)	GE/DuPont
Task 4 - Analysis of Electrode Geometry and Soil Heterogeneity (6.7)	GE/DuPont
Task 5 - Cost Analysis (7)	Monsanto/DuPont
Task 6 - Lab-Scale Development of Microbial Degradation Process (8.1.2)	DuPont
Task 7 - Lab-Scale Electrokinetic and Microbial Degradation (8.1.6)	Monsanto
Task 8 - Lab-Scale Tests of Lasagna Process Using DOE Paducah Soil (8.1.7)	Monsanto
Task 9 - TCE Degradation Using Non-Biological Methods (8.2.1, 8.2.2.2, 8.2.3.2)	GE/Monsanto
Task 10 - Bench- and Pilot-Scale Tests (9.3)	Monsanto
Task 11 - Establish Contamination Conditions Before and After Tests (10.1.2)	DuPont/MMES
Task 12 - Design and Fabrication of Large-Scale Lasagna Process (12.1, 12.2)	Monsanto/DuPont/Nilex
Task 13 - Large-Scale Field Test of Lasagna Process (12.3, 12.4)	Monsanto/CDM

Table 2. List of Topical Reports and Responsible Company

Topical Report	Company
Task 1 - Evaluation of Treatment Zone Formation Options	DuPont
Tasks 2 - 4 Electrokinetic Modeling	GE
Task 5 - Cost Analysis	Monsanto
Task 6 - Laboratory-Scale Microbial Degradation	DuPont
Tasks 7, 8, 10 - Bench- and Pilot-Scale Tests of Lasagna Process	Monsanto
Tasks 9 - TCE Degradation Using Non-Biological Methods	GE
Task 11 - Contamination Analysis, Before and After Treatment	Monsanto
Tasks 12 and 13 - Large-Scale Field Test of Lasagna Process	Monsanto

F. INTRODUCTION

During the last decade, a great deal of research has been conducted to develop *in-situ* technologies for treating contaminated soils and groundwater. Attractive characteristics of *in-situ* technologies include potential lower costs and less disruption to the environment. However, promising *in-situ* treatments, which include bioremediation, vapor extraction and pump and treat, are rather ineffective when applied to low permeable soils present at many contaminated sites.

A novel, *in-situ* technology aimed at cleaning up contamination in heterogeneous or low-permeability soils is being developed. This new approach involves the synergistic combination of electro-osmosis (EO) with other technologies. Electro-osmosis, or more generally electrokinetics (EK), has recently received increasing attention as an *in-situ* method for soil remediation (5-10). Electrokinetics includes the transport of water (electro-osmosis) as well as ions (electromigration) as a result of an applied electric field. For remediation applications, water injected into the soil at the anode flows by electro-osmosis through the contaminated soil, bringing the contaminants to the surface at the cathode region for further treatment or disposal.

Monsanto's new approach is an integrated *in-situ* treatment in which established geotechnical methods are used to install treatment zones directly into the contaminated soil and electroosmosis is utilized to move the contaminants back and forth through those zones (11). As contaminants are transported into the treatment zones, they can be removed from the water by (bio)degradation, immobilization or adsorption. The process is called "Lasagna"™ due to the many layers created by the electrodes, soil and treatment zones.

One possible configuration of the Lasagna™ process is to create highly permeable zones in close proximity sectioned through the contaminated soil region and turn them into biotreatment zones by introducing specific chemically-degrading microbes. The microbes added to the biozone can be either aerobic or anaerobic species. Electrokinetics can then be utilized for transporting chemical contaminants from the soil into the biozones. Liquid flow can be periodically reversed, if needed, simply by switching the electrical polarity. This mode would enable multiple passes of the contaminants through the biozones for complete microbial degradation. Essential nutrients could be added to the injected water at the anode to enhance long-term stability of the microbes in the biotreatment zones.

G. OBJECTIVES

The purpose of Task #7 was to study the feasibility of coupling electroosmotic removal of chemicals from contaminated soil with *in-situ* biodegradation by specific aerobic microbes contained in biozones. The important task in development of the degradation processes was to optimize the conditions required for effective degradation rates in the biozone while maintaining efficient electroosmotic movement of chemicals. The main objectives of Task #7 were as follows:

1. Develop and characterize the anaerobic process for reductive dehalogenation of TCE to ethylene. Determine rates of TCE transformation for anaerobic mixed cultures developed in the Stanford Environmental Engineering and Science Research Laboratory.

2. Examine the ability of the microbe *Pseudomonas cepacia* G4 to efficiently degrade TCE under cometabolic conditions. Study the growth and culture conditions necessary to produce maximum degradation by *Ps.* G4 during electroosmosis.

3. Utilize Monsanto's proprietary lacZY genetic marking technology to insert a phenotypic gene marker into *Pseudomonas cepacia* G4 and *Pseudomonas putida* PNP1, an aerobic degrader of p-nitrophenol.

4. Use genetically marked *Ps.* G4 and *Ps.* PNP1 in lab-scale electroosmosis experiments for the detection and tracking of the microbes to determine survival rates, movement and chemical degradation efficiency.

5. Investigate the use of various microbial support materials, such as sawdust, sand and granular activated carbon in the treatment zone of bench-scale electroosmosis unit.

6. Examine parameters such as electroosmosis flow rate, chemical loading in soil, biozone aeration and duration of electroosmosis treatment to optimize biodegradation of soil contaminants.

Bacterial Strains and Culture Conditions

Pseudomonas cepacia G4 was obtained from the USEPA (Environmental Research Lab, Gulf Breeze, Florida) and was previously characterized (2). A 2,000 ml Bellco™ microcarrier spinner flask was used in the construction of a chemostat for the growth and maintenance of the *Ps.* G4. The chemostat was fitted with influent and effluent lines, filtered oxygen line, pH and oxygen probes and sampling port. The chemostat contained 1,000 ml of L-salts and was started by inoculating the flask with 200 ml of a 18-hr culture of Ps. G4 grown in a L-salts/5mM lactate/2mM phenol media. After incubation for 24 hrs at 28^0 C, continuous growth was initiated by starting the influent flow of a L-salts media containing 5 mM phenol and 5 mg/L yeast extract. The working volume of the chemostat was maintained at 1,200 ml and the dilution rate was held at 0.07 h^{-1}. The chemostat was aerated by replacing the head space with pure oxygen twice each day.

The p-nitrophenol (PNP) degrading microbe, *Pseudomonas putida* PNP1, was previously isolated in our laboratory and stored as 1 ml glycerol cultures in a -70^0 C freezer. For each electroosmosis study, a 1 ml culture was added to 50 ml of L-salts containing 100 mg/L PNP. Cells were grown for 18 hrs at 28^0 C and 200 rpm agitation. After initial growth, the 50 ml culture was centrifuged, the supernatant discarded and the pellet resuspended in another 50 ml of L-salts/100 mg/L PNP. The cells were incubated with agitation for another 18 hrs, then pelleted and resuspended in 30 ml of L-salts only. This culture was then used to inoculated the biozone of the bench-scale electroosmosis unit.

Triparental Mating Procedure for Production of Genetically Marked Microbes

The Monsanto proprietary *lac*ZY marking system relies on the introduction of the *E. coli lac* operon genes *lac*Z and *lac*Y into the host pseudomonad strain. These genes code for the expression of b-galactosidase and allow bacteria to transport and utilize lactose as a carbon source. A broad host-range delivery plasmid, containing the *lacZY* genes linked to a TN7 transposition element and also containing the gene for gentamicin resistance, was used for the

marking of rifampicin-resistant *Ps.cepacia* G4 and *Ps. putida* PNP1. The plasmid containing the *lacZY* genes was mobilized from *E. coli* to the two parental *Pseudomonas* strains by a triparental mating procedure using a pRK2013 helper plasmid consisting of the RK2 transfer genes and a ColE1 replicon (4).

Exponential cultures (1ml) of *E. coli HB* 101 containing pRK2013 and the *E. coli* host carrying the *lacZY* plasmid to be transferred were combined with 1 ml of the recipient Rif^R-*Pseudomonas* strain. The combined culture was pelleted, the supernatant removed, and the pellet was spotted onto a nutrient plate. After a 24 hr incubation at 30^0 C, the cell mass was diluted into L-salts and plated onto a Pseudomonas F agar (Difco) containing 50 mg/L rifampicin and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-B-d-galactoside (X-gal). Parental strains containing a stable chromosomal insertion of the lacZY genes produced b-galactosidase, which cleaved the X-gal and gave colonies which appeared blue on the test media. Colonies which exhibited the *lacZY* phenotype were screened by Southern Analysis to confirm the chromosomal insertion of the *lacZY* genes. The genetically marked microbes *Ps. cepacia* G4 *lacZY* and *Ps.putida* PNP1 *lacZY* were used for all degradation and electro-osmosis experiments and were quantitated for each experiment by plating onto a Pseudomonas F agar with 50 mg/L rifampicin and the chromogenic substrate X-gal.

TCE Degradation Assays

All degradation experiments were conducted in 12 ml glass vials containing 6 ml of L-salts media. Typical TCE concentration was 2.5 mg/L and 200 mg/L of phenol was added to each assay vial as an enzyme inducer. Degradation of TCE was achieved by adding chemostat-grown *Ps. cepacia* G4 *lacZY* cells at a final concentration of 1×10^9 cfu/ml of media.The vials were sealed with teflon lined rubber septa and screw-threaded caps. Each assay was incubated up to 32 hr at 28^0 C with low agitation (approximately 100 rpm). Duplicate degradation assays were performed for each experiment and a non-inoculated set of test vials was added to monitor the abiotic loss of TCE. Hexane extraction (1:1) of the test media allowed for determination of residual TCE concentrations and analysis was performed by gas chromotography (GC) with an electron capture detector. The level of detection for the GC analysis was 10 ppb.

Set-up for Electroosmosis Experiments.

Electroosmosis (EO) studies designed to understand the factors that influence microbial movement and chemical degradation were carried out in a standard bench-scale EO cell, as shown in Figures 1and 2 ..and described previously (11). The cylindrical tube used granular activated carbon as electrodes and kaolinite clay as the test soil. The left portion on the cell (cathode side) was packed with approximately 1000 g of clean clay with a 37.5% wt. % soil moisture content. The right portion (anode) was packed with the same amount of clay contaminated with p-nitrophenol (PNP). The contamination loading was either 400 µg PNP/g wet weight of clay or 75 µg PNP/g of wet clay. The clean clay zone and the contaminated clay zone were seperated by a 1.25 cm biozone. The biozone had a volume of 100 ml and was packed with either granular activated carbon (GAC), sawdust, sand or a combination of GAC and sawdust. All EO experiments utilized *Ps. putida* PNP1*lac*ZY as the test organisms. The biozone was inoculated directly by injecting 30 ml of a 1 x 10^9 cfu/ml suspension of bacteria through a port in the bottom of the biozone. Oxygen was supplied for biodegradation by sparging air through tubes connected to the bottom of the treatment zones. Each EO run was performed by passing an amount of water equivalent to 1 pore volume for the contaminated clay zone (about 375 ml) through the EO unit. EO was carried out in the direction of anode to cathode and the duration of each EO run was 7-9 days.

At the end of each run, the unit was disassembled and the anode and cathode clay zone were divided into 10 sections each. For microbial analysis, eight clay sample of 1g each were taken from both the clean and contaminated clay sections. Also, three samples of 1 g each were taken from the biozone. Each clay and biozone sample was diluted from 10^{-3} -10^{-6} in L-salts and plated onto Pseudomonas media containing 50 mg/L rifampicin and 0.006% X-gal. The plates were incubated two days and microbes were quantitated based on the appearance of blue colonies on the selective media.

The clean clay, contaminated clay and biozone material were also analysed for residual PNP. The analysis involved extracting the sectioned clay zones and biozone material with 0.1N NaOH and measuring the level of PNP in solution by spectrophotometric absorption at 400 nm or by high-performance liquid chromatography (HPLC). Results of PNP analysis were used for mass balance calculations to determine PNP degradation efficiency during electroosmosis.

Carbon Adsorption Studies with TCE

Carbon adsorption experiments were performed to determine the ability of two different granular activated carbons to adsorb TCE. Standard GAC was an un-seived 30-mesh obtained from Calgon Corp. and mesopore GAC was a 14 x35 mesh carbon obtained from Westvaco Co. Variable amounts of each carbon type, ranging from 0.75 mg to 2.85 mg, were placed in 250 ml glass bottles fitted with teflon lined rubber septa and screw-threaded caps. Each bottle was filled with 220 ml of a 1,000 mg/L aqueous solution of TCE. The bottles were agitated at 150 rpm for two weeks, then a 50 µl sample of the TCE solution was diluted into hexane for determination of residual TCE.

TCE Degradation by *Ps. cepacia* G4

The primary chemical currently under investigation for bioremediation using the Lasagna technology is the chlorinated solvent TCE. The microbial strain *Ps. cepacia* G4 fortuitously oxidizes TCE using catabolic enzymes associated with an aromatic degradative pathway to degrade TCE to CO_2 and
chloride (1). This strain was used in closed-vial biodegradation studies to examine its TCE degradative capacities and the phenol-induction phenomenon.

For efficient chemical degradation, it was important to use a cell culture that was optimally induced for TCE degradation. Cells grown on 5 mM phenol in a continuous culture system demonstrated the ability to degrade TCE at both low (2 mg/L) and high (20 mg/L) concentrations. Fully induced *Ps. cepacia* G4 was capable of degrading 85-95% of a 2.5 mg/L solution of TCE within 12 hrs and also demonstrated the ability to degrade 95% of a 20 mg/L solution of TCE within 24 hrs. The genetically 'tagged' *Ps. cepacia* G4 *lacZY* produced the same TCE degradation efficiencies. This confirmed that the chromosomal insertion of the *lacZY* genes into the microbe did not disrupt the enzymatic pathway for cometabolism of TCE. The ability of *Ps. cepacia* G4 to efficiently degrade TCE was dependent on the presence of phenol for enzyme induction and on high concentrations of active cells. Degradation experiments which utilized less than a 5×10^8 cfu/ml concentration of whole cells showed minimal TCE degradation and optimum TCE degradation was achieved with a cell concentration of 1×10^9 cfu/ml.

A series of degradation studies were performed to determine the length of time a phenol-induced culture of *Ps. cepacia* G4 could efficiently degrade TCE when phenol is not present and to determine the minimal concentration of phenol needed to produce good TCE degradation efficiency. These tests were performed by adding a known concentration of induced *Ps. cepacia* G4 to septa-capped vials containing 10 mls of L-salts and incubating the mixture for 0-36 hrs before adding TCE to a final concentration of 10 mg/L. After a final incubation period of 20 hrs, two vials for each 2 hr time point were extracted with hexane and anaylzed for residual TCE. An analysis of TCE concentration over time showed that a degradation efficiency of 90-95% was maintained for 24 hrs. There was a gradual decrease in degradation efficiency from 24-36 hrs, with only 40% activity remaining after 36 hrs. A similar experiment was performed in which an uninduced culture of *Ps. cepacia* G4 was added to a series of septa vials containing a 10 mg/L

solution of TCE and a variable level of phenol, ranging from 0-200 mg/L. After incubating the vials for 20 hrs, the solution was analyzed for residual TCE. A plot of remaining TCE vs. phenol content in the growth media showed that the culture required a 150 mg/L level of phenol for complete TCE degradation.

Isotherm Analysis for TCE Adsorption to GACs

The adsorption properties for two different granular activated carbons (GAC) were examined to determine their capacity for TCE adsorption and to investigate the feasibility of using GAC as part of the EO bizone microbial support material. The two GACs investigated were a standard, 30-mesh GAC and a carbon termed mesopore GAC, which has a larger surface area and more total pore volume than standard GAC. Adsorption experiments were interpreted using the Freundlich adsorption equation: $\log q_e = \log K_f + 1/n \log C$ and a plot of this data is shown in Fig 3. The Freundlich coefficients, K_f and $1/n_f$, for standard GAC were 30.54 (mg/g)(mg/L) and 0.50, respectively, and 3.54 (mg/g)(mg/L) and 0.74, respectively, for mesopore GAC. A linearized Langmuir adsorption plot showed that standard GAC could adsorbed a maximum of 530 mgTCE/g carbon, while mesopore carbon had an extrapolated maximum capacity of 620 mg TCE/g carbon. The linear Langmuir plot also showed that the standard GAC was at adsorption equilibrium after a two-week incubation with TCE, while the mesopore carbon was not. Thus, the low K_f exhibited by the mesopore carbon was probably due to the non-equilibrium condition.

Microbial Movement and Biodegradation During Eletroosmosis

The coupling of microbial degradation with EO for *in-situ* removal of contaminants in clay was initially approached by performing a series of experiments designed to develop a fundamental understanding of the factors that influence bacterial migration during electroosmosis. Fourteen bench-scale EO studies were run to examine factors that might influence microbial movement and degradation such as: soil contaminant concentration, EO flow velocity, soil porosity and composition, microbial chemotactic processes, electromigration by microbes and composition of biozone microbial support material. These studies were performed using PNP as the representative soil contaminant . The studies were greatly facilitated by using the genetically tagged microbe *Ps. putida* PNP1 *laZY* .

Initial EO test runs using highly contaminated clay (400 µg PNP/g clay) caused the PNP-degrading microbes to consistently migrate away from the chemical contaminant. Microbial analysis of the clay in both the anode and cathode sides of the EO unit showed that the microbes demonstrated a chemoaversion effect to the toxic level of PNP and migrated away from the chemical into the cathode side of the EO unit. Degradation efficiencies were low, ranging from 40-50%. When the contaminant level was reduced to 85 µg PNP/g clay, the microbes exhibited a strong chemotactic response towards the chemical and consistently migrated only into the anode side of the EO unit, which contained the contaminated clay. Also, excellent degradation efficiencies were achieved at these more moderate chemical contamination levels, with degradation rates ranging from 94-98%. A summary of EO test runs is provided in Table 1.

Control EO studies with no PNP contamination in the clay showed that EO flow velocity played an important role in moving the microbes from their initial position in the EO biozone into the downstream side of the EO unit. Furthermore, lack of microbial movement towards the anode when PNP was not present in the clay indicated that electromigration of the bacteria (which have an inherent negative surface charge) towards the anode was not a factor in microbial movement.

Examination of different construction material for the biozone showed that pine sawdust worked well as a biosupport for the microbes, and also exhibited the ability to adsorb moderate levels of PNP. The addition of 5% GAC to the sawdust helped mitigate toxic effects when PNP was present at high levels (400 µg/g clay). However, degradation rates were not enhanced when GAC was added to the biozone due to irreversible binding of the PNP to the carbon, and thus, limiting the ability of the microbes to degrade the chemical. Sand was also used as a microbial support in the biozone, but was determined unsuitable as a microbial support due to its tendency to dessicate during aeration of the biozone.

Anaerobic Biodegradation of TCE

Kinetic experiment to study the dehalogenation rate of TCE were performed by using a mixed culture of TCE-degrading anaerobic bacteria. The bacterial consortium was grown in a reactor receiving hydrogen feed. Batch studies were conducted at 25^0 C by adding 220 ml of culture to 254 ml bottles to which various concentrations of TCE was added together with hydrogen gas as the electron donor. Studies with increased hydrogen gas concentrations were conducted with similar results, indicating that the hydrogen concentration used in the studies was not rate limiting

to the reaction. The initial rates of dehalogenation were measured, generally over a short period of one hour and before dehalogenation products rose to concentrations that my have affected the results. The effect of TCE concentration on reaction rate was considered to follow the Monod rate model for microbial reactions.

The rate of dehalogenation of TCE with different TCE starting concentrations is shown in Figure 1. of the Stanford Topical Report (see appendix). The maximum dehalogenation rate was 0.18 mg TCE/mgVSS/day, which occurred at a substrate concentration of 1000 ppb TCE. Analysis of Monod kinetics showed a K_s of 1.4 uM and 180 ug/l, and the observed k values corresponding to these K_s values were 1.5 mmol g^{-1} d^{-1} and 200 mg g^{-1} d^{-1}, respectively. It was noted that the TCE dehalogenation rate with hydrogen-grown culture was about the same as that as with pure culture MS-l (see Table 3 of Stanford report). This data suggests that the mixed microbial culture supported, on hydrogen alone as an electron donor had a higher percentage of dehalogenators that when yeast extract was used.

The mixed culture of TCE-degrading bacteria was also used to study the transformation rate of TCE as a function of temperature and pH. The temperature optimum was shown to be between 35^0 and 40^0 C, although good dehalogenation occurred with temperature as high as 45^0 C. The rates found at 21^0 C were about one-half of the maximum rates, which is a typical rate reduction for a temperature difference of 10^0 C. On a molar basis, the rate of vinyl cloride dehalogenation was up to three times that for cis-DCE dehalogenation, and the rate of vinyl cloride dehalogenation was about two-thirds of that for TCE dehalogenation. Thus, cis-DCE dehalogenation is shown for this mixed culture to be the rate limiting step in the overall conversion to ethene.

The results for pH effects are summarized in Table 4 of the Stanford Topical Report. These data indicate that dehalogenation rates were optimum at pH 6.5, but vary little between pH 5.5 and 7.5. Effective dehalogenation occurred over the entire pH range

Coupling of Electroosmosis with Biodegradation

Demonstration of the Lasagna™ technology on both a bench-scale and field-scale levels has shown that EO is a very promising process for remediating contaminated ground water (11,14). Coupling of EO with biodegradation allows for selected chemically-degrading microbes to be used in treatment zones for the purpose of *in-situ* biodegradation of soil contaminants. The microbe *Pseudomonas cepacia* G4 was shown to have the capacity to degrade low to moderate levels of trichloroethylene through a co-metabolic process. Microbial degradation of TCE by *Ps.* G4 is dependent on phenol induction and the required inducer ratio of phenol:TCE for effective TCE degradation is rather high. However, cells which have been induced for TCE degradation retain full TCE degradation capacity for 24 hrs. Thus, phenol can be added once a day instead of continuously to keep the organisms fully induced. Although *Ps.* G4 was shown to be an effective aerobic degrader of TCE in controlled shake-flask experiments, several difficulties occurred when attempting to couple TCE degradation with EO. Of primary importance was a lack of good methodology for keeping adequate levels of inducer (phenol) in the EO biozone. Secondly, proper aeration of the zone could not be achieved without volatilizing the TCE mobilized into the biotreatment zone during EO. Therefore, bench-scale EO studies for microbial destruction of TCE were not performed.

The coupling of EO removal of an organic contaminant from clay soil with *in-situ* biodegradation in treatment zones was successfully demonstrated when PNP was used as the model contaminant. PNP is a non-volatile soil contaminant that is aerobically degraded by a variety of microbes via primary enzymatic pathways (i.e.-does not require co-inducers Furthermore, the predominant soil microbes that degrade PNP (pseudomonad species) are closely related to the TCE-degrading microbe *Ps.* G4 . The microbe *Ps. putida* PNP1

lacZY , a highly efficient PNP degrader, was used in a series of bench-scale EO runs designed to study both *in-situ* microbial degradation and the factors which influence microbial migration during EO. Although the efficiency of PNP degradation was low (40-50%) when soil contamination was high (400 ug/g soil), excellent chemical removal rates of 96-98% degradation of PNP were achieved at moderate (85 ug/g soil) levels of soil contamination. Furthermore, these rates were achieved with a single pore-volume pass through the biotreatment zone.

Studying the use of selected microorganisms as bioremediating agents requires some understanding of the migration behavior of microbes during electroosmosis. Research approaches for modeling microbial transport to predict the rate and extent of microbial movement in consolidated media usually involves the use of advection-dispersion equations adapted to include factors such as bacterial random motility, chemotactic response and cell growth and death (13). Although these models can be useful for the fundamental understanding of microbial migration behavior, specific data was needed to examine microbial movement during EO. The tracking, presence and distribution of *Ps. putida* PNP1 in the EO cell was greatly facilitated by the use on the Monsanto proprietary 'reporter gene' technology for tagging microbial strains. Microbial movement studies in bench-scale EO units showed that microbes do not exhibit a random movement pattern during electroosmosis but that chemotaxis was the primary factor responsible for the migration of microbes. When a chemical is present in the soil which is toxic to the microbes, they respond by moving away from the contaminated soil. However, when chemicals are present in soil at tolerable concentrations, chemically-degrading microbes will sense these as potential food source and respond by moving out of the biotreatment zone and into the chemically contaminated soil. Electro-osmotic flow may facilitate the movement of the microbes from the biotreatment zone into the soil. However, analysis of

the concentration of microbes maintained in the biotreatment zone at the end of each 9-day EO run showed that >98% of the total microbial inoculum remained in the biotreatment zone.

Field-trial demonstration of the Lasagna™ technology has shown that high levels of soil chemical contamination can be moved into treatment zones (14). The use of microbial degradation for removal of soil contaminants during EO requires that chemicals be present in concentrations tolerable to the microbes. Therefore, packing materials for the biotreatment zone need to have some adsorptive capacity to protect the microorganisms from toxic levels of contaminant. The use of granular activated carbon as a microbial support in the biotreatment zone allows for excess chemical contamination to be adsorbed onto the carbon, and thus, mitigate the toxicity to the microbes. Studies with different granular-activated carbons showed that a mesopore type GAC, which has a larger surface area and greater chemical adsorption capacity than standard GAC, could be a useful addition to the biotreatment zone. A mixture of GAC and other microbial supports, such as sand or sawdust, could provide a good environment for microbial attachment and growth as well as mitigate levels of contaminant in excess of what the microbes can degrade. Proper design of the biotreatment zone is important for the overall process of achieving high rates of microbial degradation during EO, and thus, demonstrating the effective coupling of EO with biodegradation.

Anaerobic Application to Lasagna™ Technology

The purpose of the First Phase of the Stanford Study was to determine the kinetics of TCE dehalogenation by microorganisms. Some calculations were made to indicate feasibility of applying anaerobic treatment to the Lasagna™ Technology. Since the rate of

cis-DCE dehalogenation was governing, the typical rate for this transformation of 0.1 mmol per day per gram of bacteria might be used. This would correspond to a TCE transformation of 0.1 mmol per day per gram of bacteria. It was then assumed that a 2.5 cm wide biotreament zone containing support media and a void volume of 0.3 would be used. It was also assumed there would be a groundwater induced velocity by Lasagna of 2.5 cm per day in clay, also with a void volume of 0.3. The velocity through the bioreactor would thus be the same as through the clay. This means that the hydraulic detention time in the reactor would be 1.0 day. The last assumption was an organism concentration of 1000 mg/L (volatile suspended solids dry weight).

With the above listed maximum TCE degradation rate assumed, the reactor would be able to degrade 13 mg/L of TCE in the water passing though the reactor. The reactor would need to be maintained through the introduction of a primary substrate such as yeast extract or hydrogen. Based upon the measured effect of TCE concentration of reaction rates, the maximum TCE degradation rates are obtained when TCE concentrations are 1mg/l and higher, or well above the K_s values determined.

Conclusions of the Stanford Project

Toxicity of cis-DCE to mixed dehalogenating cultures was found to be a potential limitation of the biodehalogenation process when treating water with high TCE concentrations. Exposure of microorganisms to more than 8 mg/l cis-DCE caused inhibition problems, which corresponds to about 11 mg/L TCE. However, much higher TCE concentrations can be treated provided that cis-DCE is degraded as rapidly as it is formed, thus preventing a buildup to the inhibitory cis-DCE concentration. Temperature optimum for dehalogenation by the mixed culture was in the normal mesophilic range of

35^0C, with rates reduced to about one-half of this at a temperature of about 20^0C. The pH optimum was found to be 6.5, with rates at pH 5.5 or 7.5 being about one-half the maximum rates. For complete dehalogenation of TCE to ethene, it is the cis-DCE rate that will govern reactor and in-situ dehalogenation design.

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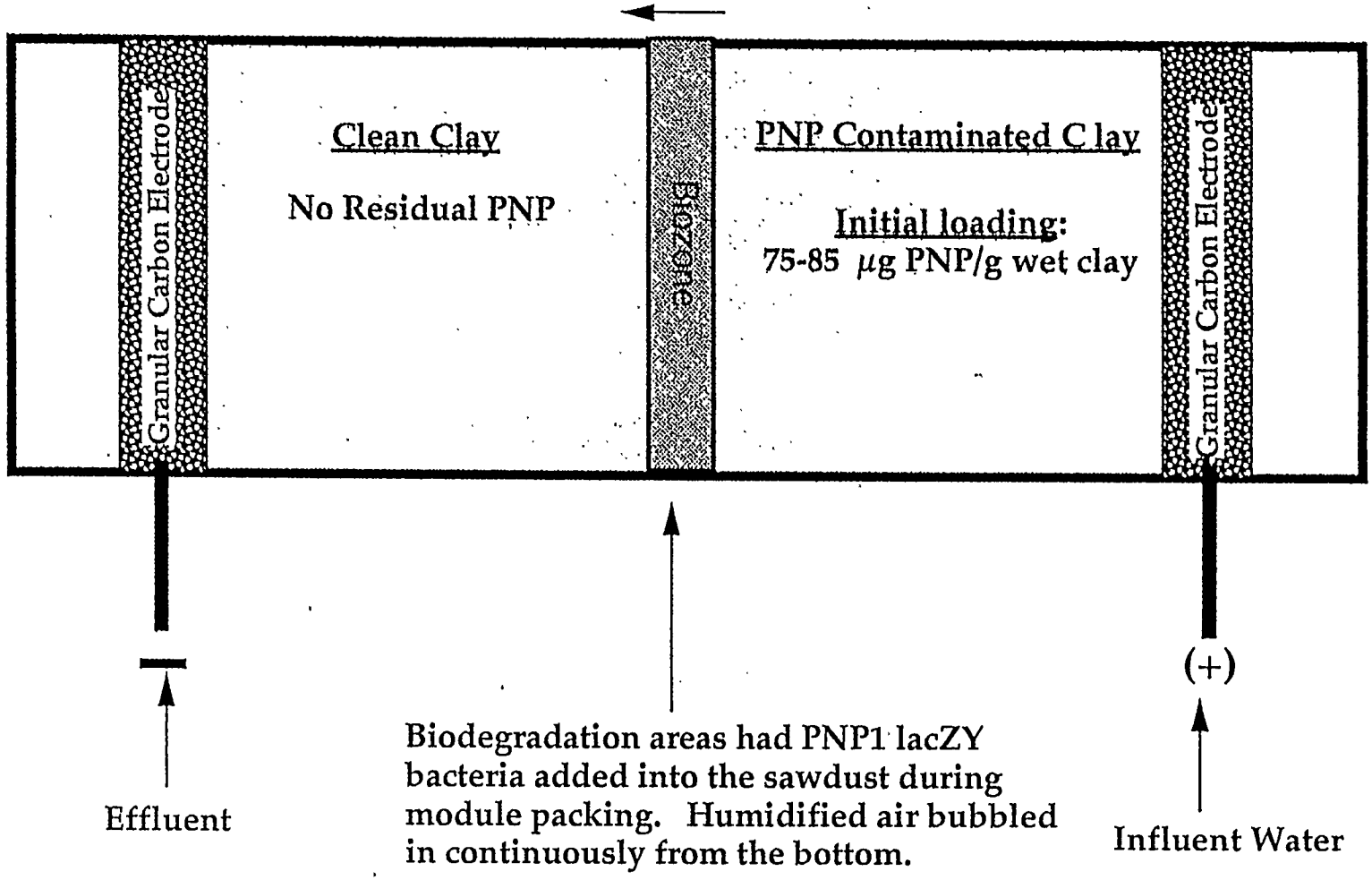
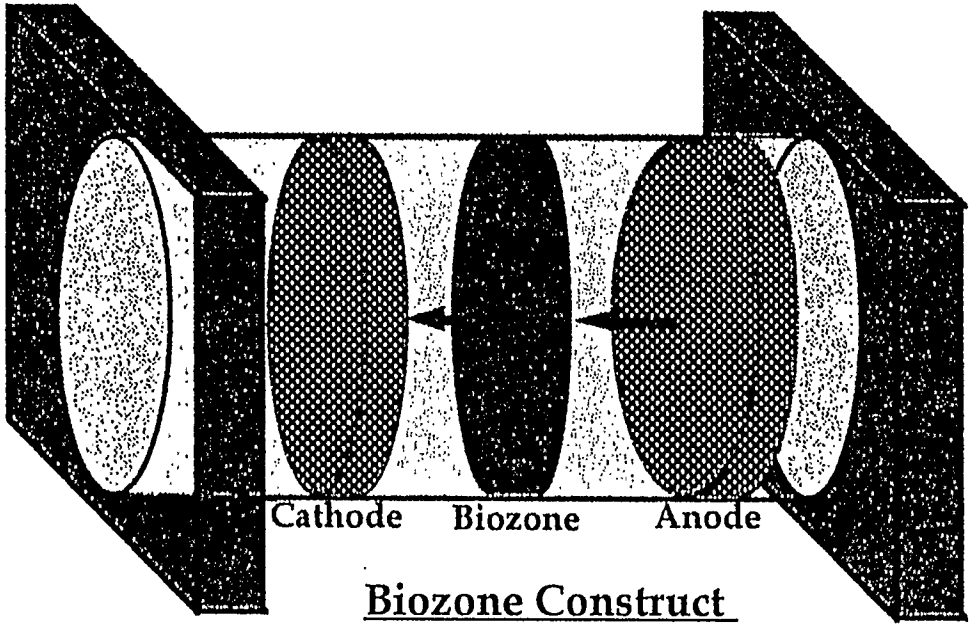

COMBINED EO & BIODEGRADATION
EO FLOW:
Granular Carbon Electrode
Clean Clay
No Residual PNP
Biozone
PNP Contaminated C lay
Initial loading:
75-85 μg PNP/g wet clay
Granular Carbon Electrode
(+)
Effluent
Biodegradation areas had PNP1 lacZY bacteria added into the sawdust during module packing. Humidified air bubbled in continuously from the bottom.
Influent Water

Figure 1.

Test Parameters for Studying Microbial Movement During EO



Soil Characteristics

Porosity: .4-.62

Tortuosity: 2.0

Pore Size: 1-10 μm

EO Conditions

Volt Gradient: 1V/cm

EO Velocity: 1 cm/d

Pore Volume: 1-1.5

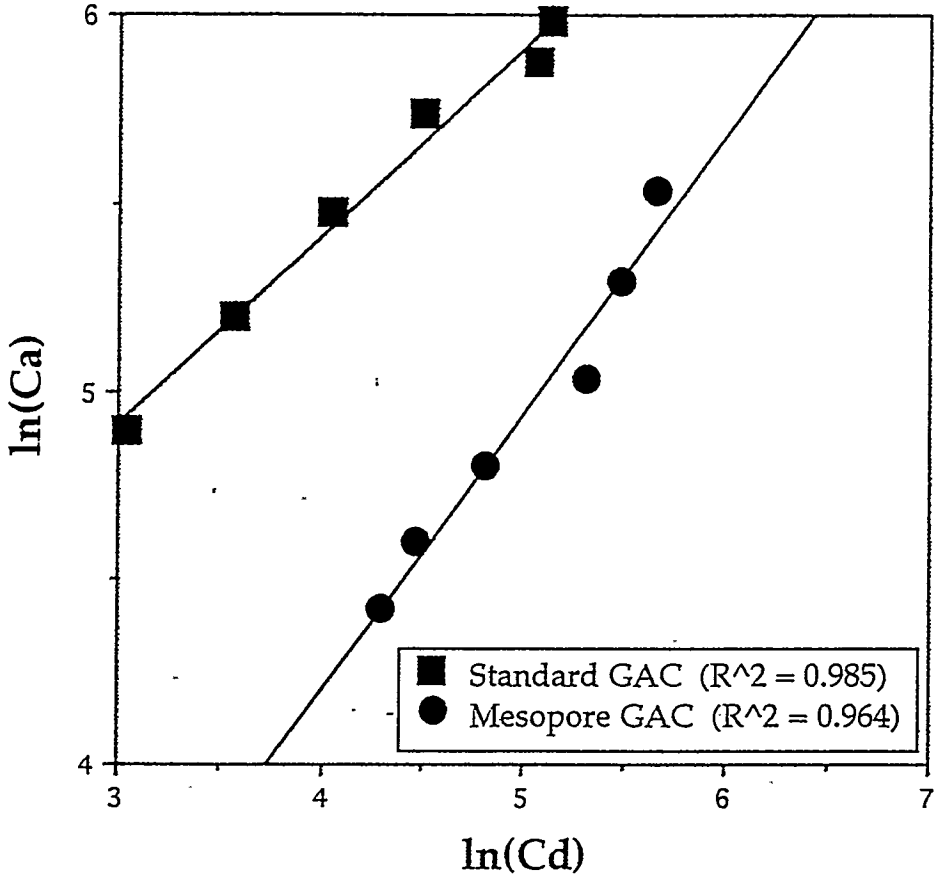
Run Duration: 7-9 d

Biozone Construct

Sand
Sand + 2% GAC
Pine Sawdust
Sawdust + 2% GAC
Monsanto Biocarrier

Figure 2.

Freundlich Isotherm for Standard and Mesopore GAC



Parameters: 1/n=0.50 for Standard GAC
1/n=0.74 for Mesopore GAC

K=30.64 mg·l/g·mg for Standard GAC
K=3.45 mg·l/g·mg for Mesopore GAC

Figure 3.

Microbial Movement During Electroosmosis
Moderate Chemical Loading: 75μg PNP/g clay

Biozone Construct	Direction of Movement		Degradation of PNP (% of Total Load)
	Anode	Cathode	
Pine Sawdust	1 cm top[c] 2 cm bottom[c]	1 cm top	94
Pine Sawdust	1 cm top & bottom	1 cm top & bottom	72
Pine Sawdust[a]	1 cm top	ND	98
Pine Sawdust[b]	ND	1 cm top & bottom	NA

a. Dormancy Study: No electroosmosis for 4.5 days post-inoculation.

b. Electromigration Study: No chemical contamination in EO unit.

c. Refers to position along the top or bottom of the EO cell

Monsanto Environmental Sciences Center

Table 1.

L. Appendix

ANAEROBIC BIOPROCESS DEVELOPMENT FOR LASAGNA TECHNOLOGY

Topical Report

January 1, 1995 to December 31, 1995

Zachary C. Haston, Veena Warikoo, and Perry L. McCarty

January 1996

Award Number DE-AR21-94MC31185

For: U.S. Department of Energy
Office of Fossil Energy
Morgantown Energy Technology Center
Morgantown, West Virginia

By: Western Region Hazardous Substance Research Center
Department of Civil Engineering
Stanford University
Stanford, California 94305-4020

and

Monsanto Company
800 N. Lindbergh Boulevard
St. Louis, MO 63167

and

E.I. du Pont de Nemours & Company
140 Cypress Station Drive
Suite 135
Houston, TX 77090

and

General Electric Company
GE Corporate Research and Development
River Road at Van Antwerp Road
Building K-1, Room 3C21
Schenectady, NY 12301

and

CDM General Programs Corporation
800 Oak Ridge Turnpike
Suite 500
Oak Ridge, TN 37830

DISCLAIMER

ABSTRACT

This study was conducted in order to obtain a better understanding of rates of anaerobic reductive dehalogenation of trichloroethylene (TCE) to ethylene and of factors affecting these rates in order to determine the potential for application of TCE biodegradation as part of the Lasagna technology. In the anaerobic process, TCE is used as an electron acceptor by microorganisms and converted sequentially to cis-1,2-dichloroethylene (cDCE), vinyl chloride (VC), and finally, ethene. The main objective of this study was to determine the effect of temperature and pH on the rates of TCE conversion to cDCE, and on the rates of cDCE conversion to ethene. Here, a mixed culture of TCE degrading bacteria was developed from a seed taken from DuPont's Victoria, Texas, site, where active dehalogenation of tetrachloroethylene (PCE) to ethene has been occurring. This culture was repeatedly transferred and grown on yeast extract and TCE. In addition, parallel rate experiments were conducted using a facultative microorganism (strain MS-1) that was isolated from the Victoria aquifer material and converts either PCE or TCE to cDCE. Strain MS-1 was also grown on yeast extract. Batch rate studies were conducted in which about 100 mg/L of biomass under anaerobic conditions was supplemented with 0.008 M to 0.013 M TCE at different temperatures (range of 20° to 45° C) and pH (range of 5 to 8), and the rates of degradation were monitored with time.

For TCE, the maximum transformation rates in mmol per gram bacteria per day were found to be about 2.1 for strain MS-1 and 0.68 for the mixed culture. For cDCE the maximum rate in the same units for the mixed culture was about 0.16, and that for VC was 0.45. For complete conversion of TCE to ethene, the cDCE rate is thus slowest and thus would govern for the overall transformation from TCE to ethene. The temperature range where maximum rates of TCE and cDCE decomposition occurred with the mixed culture was between 30° C and 40° C. The rate at 45° C was comparable to the maximum rates. Rates at a temperature of 20° C were about one-half of the maximum. The temperature optimum for TCE decomposition with strain MS-1 was similar. The temperature relationship found is typical for growth of mesophilic microorganisms.

The effect of pH on the rates of TCE and cDCE transformation was examined with the mixed culture only. Optimum dehalogenation for both compounds occurred under somewhat acidic conditions and in the range between 6.0 and 7.0. Dehalogenation occurred even with the lowest pH studied of 5.0, but the rate with TCE was about two-thirds of the maximum while with cDCE it was about one-half of the maximum. At the high end of pH 8.0, the TCE dehalogenation rate was about one-third of the maximum and cDCE dehalogenation was about two-thirds of the maximum rate. These data indicate that somewhat acid conditions and somewhat elevated temperatures that may occur with use of the Lasagna process may not be excessively detrimental to the process. Monod kinetic coefficents were determined for cultures grown primarily on hydrogen and PCE. It was found that the half-velocity coefficient, K_s, was in the range of 1 to 4 μM for all CAHs except PCE, which was about 0.06 μM.

ACKNOWLEDGMENTS

The research reported herein built upon a three-year research effort supported by DuPont Chemical Company to characterize anaerobic microorganisms in aquifer material from a site in Victoria, Texas, and to evaluate factors affecting their rates of chlorinated solvent reductive dehalogenation. The studies were conducted through the U.S. Environmental Protection Agency supported Western Region Hazardous Substance Research Center at Stanford University. Since these groups have not reviewed this report, no endorsement by them should be inferred.

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ANAEROBIC BIOPROCESS DEVELOPMENT FOR LASAGNA TECHNOLOGY

INTRODUCTION

This research was conducted in response to a request from Monsanto Company to help evaluate the potential of anaerobic reductive dehalogenation of trichloroethylene (TCE) to ethene for destroying TCE removed from soil with electrokinetics, which is the principal upon which the Lasagna technology is based (Ho et al., 1995). Here, TCE can be removed from clay by a movement of current between two electrodes introduced into the subsurface. The electrical current and chemical changes occurring at the electrodes can change pH and increase soil temperature. The effect of such changes on bacterial activity is thus of interest. TCE is removed by Lasagna technology along with water contained in the aquifer material at a velocity on the order of 1 to 3 cm per day. The interest was to evaluate the potential for treating the TCE so removed within a 2 to 5 cm wide treatment zone constructed perpendicular to the direction of fluid flow.

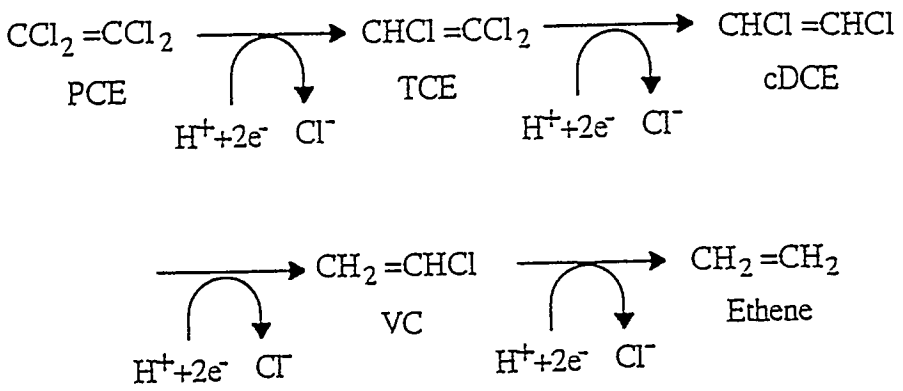
The proposal here was to conduct an evaluation of anaerobic TCE biodegradation in two phases. The first phase was to obtain a better understanding of rates of reductive dehalogenation of TCE to ethylene and of factors affecting these rates. The second phase was to use the information from phase one to develop a model for the reductive dehalogenation process for predictive evaluation of process performance and feasibility and for bench-scale pilot studies of the process. Funding only for phase one has been authorized and received. This report summarizes the results of the phase one studies.

PURPOSE

The goal of this phase one study was to develop and characterize the anaerobic process for reductive dehalogenation of TCE to ethylene. Rates of TCE transformation were to be determined for anaerobic mixed cultures developed in the Stanford Environmental Engineering and Science Research Laboratory from aquifer samples obtained by the DuPont Chemical Company at their Victoria, Texas, site. These cultures had been undergoing organism isolation and characterization for a three year period. The particular interest of this project was to determine how rates of dehalogenation were affected by temperature and pH, since these would be variables of importance in the Lasagna process. Aquifer temperatures normally vary from 8° to 20° C, depending upon latitude. However, the energy input from the Lasagna process can increase aquifer temperature as high as 45° C and can cause pH to drop as low as 4.5 or as high as 10. It was thus of interest to determine the tolerance of the dehalogenating microorganisms over the range of pH and temperature conditions of interest for this process.

BACKGROUND

The biological anaerobic reduction of chlorinated aliphatic hydrocarbons (CAHs) such as tetrachloroethylene (PCE) and trichloroethylene (TCE) to cis-1,2-dichloroethene (cDCE), trans-1,2-dichloroethylene (tDCE), and vinyl chloride (VC) was reported in the early 1980s (Barrio-Lage et al., 1986; Parsons and Lage, 1985; Vogel and McCarty, 1985) Further reduction of PCE and its intermediates to ethene was reported by (Freedman and Gossett, 1989). In such transformations, CAHs act as electron acceptors and are reduced as indicated in the following:



(1)

Such reduction requires that an electron donor be present. Generally, this is organic matter that is oxidized by the microorganisms as a primary substrate for energy, but it can be inorganic compounds as well, such as hydrogen.

In more recent studies, the conversion of PCE at concentrations as high as 55 mg/l to ethene has been reported (DiStefano, et al., 1991) with methanol used as a primary substrate, even in the absence of methane production or methanogenesis. Here, about 70% of the methanol was converted to acetate, and about 30% was associated with the dechlorination reactions.

Several pure cultures of anaerobic bacteria have been found to reductively dehalogenate PCE to TCE (Fathepure and Boyd, 1988; Fathepure, et al., 1987; Egli et. al., 1987, 1988). In these cases, the rate of PCE dehalogenation was slow, and the biochemical mechanisms of PCE transformation was not clear.

Several pure cultures of microorganisms are now available that can also reduce PCE further to cDCE (Holliger, et al., 1993; Neumann, et al., 1994; Scholz-Muramatsu, et al., 1995; Holliger and Schumacher, 1995; Sharma and McCarty, 1995). Holliger et al. (1993, 1995) reported on a strictly anaerobic microorganism called *Dehalobacter restrictus* that grows only on hydrogen or formate as electron donors and PCE or TCE as electron acceptors.

The newly isolated microorganism reported by Neuman et al. (1994) and Scholz-Muramatsu (1995) is called *Dehalospirillum multivorans* and is also a strict anaerobe that can use PCE as an electron acceptor for growth, but it can use a variety of organic substrates as electron donors as well as hydrogen. Acetate is used as a carbon source. Organic electron donors on which it can grow includes pyruvate, lactate, ethanol, formate, and glycerol. When using PCE and H_2 for energy and acetate for cell carbon, growth yields are 1.4 g of cell protein per mole chloride released. When using pyruvate as an electron donor, the dechlorination rate of up to 0.22 mmol/mg protein/day and a doubling time of 2.5 h were obtained at an initial PCE concentration of 300 μM. Concentrations higher than 300 μM were inhibitory. When using pyruvate, acetate and lactate were produced as fermentation products. Dehalogenation did not occur with acetate. The temperature optimum for this organism ranged from 25 to 37°C, with dechlorination completely inhibited at 42°C. PCE dehalogenation had a narrow pH optimum between 7.0 and 7.5, and was inhibited at pH 6.0 or 8.5. This organism can alternatively use nitrate or fumurate as electron acceptors, but if present, they prevent dehalogenation, as does polysulfide. The dehalogenase is active in cell extracts, and was found to be soluble. Evidence suggests that the dehalogenase is constitutive.

Sharma and McCarty (1995) through the research on Victoria aquifer material and supported by DuPont Chemicals isolated the first reported facultative microorganism capable of converting PCE

to cDCE. Based upon cellular fatty acid composition and metabolic characteristics, this organism matches closely to *Enterobacter agglomerans*. This rapidly-growing facultative bacterium (0.83/h in triglycollate medium), termed strain MS-1, transforms PCE to cDCE at rates as high as 0.024 mmol/g bacteria/day in a defined growth medium. This transformation requires the absence of oxygen and nitrate, but does not require the highly reducing conditions associated with sulfate reduction or methanogenesis. Strain MS-1 can transform high concentrations of PCE (up to 1 mM) via TCE to cDCE, which is a much higher concentration tolerance than yet reported by others. Ongoing research with MS-1 is directed towards determining its physiological characteristics, the biochemistry of dehalogenation, whether it can obtain energy from dehalogenation (preliminary data suggests it can), and its chemistry and dehalogenation kinetics. PCE dehalogenation results from growth on a wide variety of organic donors, including acetate, formate, and lactate.

No pure culture of an organism has yet been reported that is capable of reducing cDCE to VC or VC to ethene, although there are numerous reports of such conversions in mixed cultures. As a comparison between rates for conversion of cDCE to ethylene with that of PCE to cDCE noted above, (Tandoi, 1994) reported on the kinetics of conversion of PCE to ethylene with a highly enriched mixed culture growing on methanol. They reported conversion of 0.55 mM PCE to VC within 20 hr, or 0.005 mmol PCE/mg cells/day in the presence of 1.6 mmol methanol and 10 mg/l yeast extract. This rate is much lower than that for conversion of PCE to cDCE noted above. VC conversion to ethene did not occur until PCE was gone. In contrast, added VC was converted without delay in the absence of PCE. A mathematical model that they developed was consistent with VC conversion to ethene being delayed when other intermediates such as TCE or cDCE were present. No inhibition was present with concentrations of up to 0.5 mM of any of the chlorinated aliphatic hydrocarbons studied (PCE, TCE, cDCE, tDCE, 1,1-DCE, VC).

The group that is currently closest to obtaining a pure culture that dehalogenates CAHs completely to ethylene is the Cornell group, which were the first to report on PCE conversion to ethene (DiStefano et al., 1991; DiStefano, et al., 1992; Freedman and Gossett, 1989; Tandoi, 1994). The highly enriched strictly anaerobic organism that they have obtained is capable of complete conversion of PCE to ethene while using hydrogen as an electron donor and acetate as a source of carbon for cell synthesis. Yeast extract is used in the media as a source of required trace nutrients. This organism is a strict anaerobe.

The evidence available from the pure culture studies reported so far indicates that reductive dehalogenation is coupled with energy production, an observation that suggests it may be an efficient mechanism for PCE and TCE dehalogenation. These studies also suggest that hydrogen may be a key intermediate in the anaerobic catabolism of organics that is used by the dehalogenating bacteria as an electron donor for dehalogenation. A general observation is that yeast extract is required to satisfy trace organic nutrient requirements, and an organic carbon source such as acetate is required for general cell synthesis. Such a hypothesis suggests that the dehalogenating microorganisms would occupy a niche in an anaerobic system somewhat similar to that occupied by the hydrogen-consuming methanogens. Here, complex organics are fermented to simpler compounds, which are then oxidized further by removal of hydrogen and associated electrons. The hydrogen is an end product of such oxidation, and in methanogenesis and/or sulfidogenesis (sulfate reduction) is used as an electron donor by methanogens or sulfate reducers. Evidence is accumulating that the dehalogenating bacteria also compete for such hydrogen. It appears that hydrogen oxidation coupled with dehalogenation is much more favorable thermodynamically than coupling with carbon dioxide reduction (methanogenesis) or sulfidogenesis (Vogel, et al., 1987). This is illustrated by the thermodynamic values for various reactions with hydrogen as electron donor as listed in Table 1. Here it can be seen that the free energy per mole of hydrogen for the reductive dehalogenation of CAHs is less than that for oxygen and nitrate as electron acceptors, but much higher than that for sulfate reduction and

methanogenesis. Theoretically, then, reductive dehalogenation is quite favorable energetically for the microorganisms.

Table 1. Thermodynamics of reactions with hydrogen as electron donor

Reaction	ΔG kJ/mol H_2*
$H_2 + \frac{1}{2}O_2 \rightarrow H_2O$	-211.5
$H_2 + \frac{2}{5}NO_3^- + \frac{2}{5}H^+ \rightarrow \frac{1}{5}N_2 + \frac{6}{5}H_2O$	-194.5
$H_2 + \frac{1}{4}SO_4^{2-} + \frac{3}{8}H^+ \rightarrow \frac{1}{8}H_2S + \frac{1}{8}HS^- + H_2O$	-16.7
$H_2 + \frac{1}{4}CO_2 \rightarrow \frac{1}{4}CH_4 + H_2O$	-8.5
$H_2 + CCl_2{=}CCl_2 \rightarrow CHCl{=}CCl_2 + H^+ + Cl^-$	-167.7
$H_2 + CHCl{=}CCl_2 \rightarrow CHCl{=}CHCl + H^+ + Cl^-$	-167.9
$H_2 + CHCl{=}CHCl \rightarrow CH_2{=}CHCl + H^+ + Cl^-$	-133.6
$H_2 + CH_2{=}CHCl \rightarrow CH_2{=}CH_2 + H^+ + Cl^-$	-143.7

*ΔG values based upon pH = 7, $H_2 = 10^{-4}$ atm, N_2 = 1 atm, O_2 = 0.2 atm, CO_2 = 1 atm, $Cl^- = 10^{-3}$M, $SO_4^{2-} = 10^{-3}$M, $H_2S = 10^{-4}$M, $HS^- = 10^{-4}$M, and all chlorinated hydrocarbons and ethylene = 10^{-4}M.

A question of importance is the quantity of electron donor that must be added in order to achieve conversion of PCE to ethylene. As indicated in Table 1, an equivalent of four moles of hydrogen (8 g) is required at a minimum to obtain conversion of one mole (131 g) of PCE to ethylene. If the source of hydrogen were acetate, and if all electrons available in acetate could be used for this purpose (not presently shown by existing data), then one mole of acetate (59 g) would be sufficient as a minimum for complete conversion of one mole of PCE to ethylene as four moles of hydrogen would could be produced:

$$CH_3COO^- + H^+ + 2H_2O \rightarrow 2CO_2 + 4H_2 \quad (2)$$

However, if as is more likely to be the case, the hydrogen potentially available in mixed cultures is that released by acetogenic bacteria, then more electron donor would be required. This is illustrated for lactate by the following equation for its conversion to acetate and hydrogen:

$$2CH_3CHOHCOO^- + 2H_2O \rightarrow 2CH_3COO^- + 2CO_2 + 4H_2 \quad (3)$$

This equation indicates that 2 moles of lactate (178 g lactate or 224 g sodium lactate) would be required as a minimum to provide the necessary electron donor. The acetate produced could be

used for cell synthesis, and probably would be, but there would be excess acetate which would need to be consumed by other organisms, such as methanogens, or else it will build up in the system. Interestingly, the findings of DiStefano, Gossett and Zinder (1991) is consistent with a model of acetogenesis from methanol, and then dehalogenation with the resulting hydrogen:

$$2CH_3OH \rightarrow CH_3COO^- + H^+ + 4H_2 \quad (4)$$

$$4H_2 + CCl_2{=}CCl_2 \rightarrow CH_2{=}CH_2 + 4H^+ + 4Cl^- \quad (5)$$

Net:
$$4CH_3OH + CCl_2{=}CCl_2 \rightarrow 2CH_3COO^- + CH_2{=}CH_2 + 6H^+ + 4Cl^- \quad (6)$$

Here, 33% of the methanol is associated with dehalogenation while 67% is with acetate formation, values that are close to those measured.

The available evidence suggests that the presence of oxygen and nitrate prevent dehalogenation from occurring, even with pure cultures of dehalogenating bacteria. Thus, sufficient donor must also be added to effect their removal.

Evidence contradicting the above hypothesis that the hydrogen released through acetogenesis is the only hydrogen available, and not that from acetate, is the fact that in some of our mixed culture soil-column studies, acetate was effective at dehalogenation, about as effective as methanol (Haston, et al., 1994). In this case, a large excess of acetate was added (about 65 times that indicated by the stoichiometry of Eq. 3) and methane fermentation was highly active. It may have been that there was sufficient hydrogen within side reactions to satisfy the needs. Studies by Pramod and McCarty (1995) in our laboratory with pure culture MS-1 indicate that acetate is also an effective electron donor for PCE reduction to cDCE, suggesting that acetate may be useful for this purpose. Again, excess amounts were used.

However, compounds that are involved in acetogenesis have tended to be much more effective at dehalogenation (Haston et al., 1994). Included here are benzoate, lactate, sucrose and ethanol. Of these, benzoate was by far the best for complete conversion of PCE to ethene. Acetogenic conversion with benzoate is indicated as follows:

$$C_6H_5COO^- + 6H_2O \rightarrow 3CH_3COO^- + CO_2 + 2H^+ + 3H_2 \quad (7)$$

On this basis, 4/3 moles of benzoate (109 g benzoate or 132 g sodium benzoate) would be required as a minimum per mole of PCE converted to ethylene.

In most mixed culture studies reported with reactors or in soil systems, the amount of electron donor added has been much higher than the minimum stoichiometric amounts indicated above. A major factor has no doubt been that large additional amounts of electron donor are desirable in order to maintain a healthy mixed population that includes sulfate reducers and methanogens in order to maintain a suitable reducing environment for dehalogenating microorganisms. To some degree the excess amount required can be estimated from a knowledge of the oxygen and nitrate present in the waters and in need of reduction. Some sulfate reduction may be desirable to produce sulfide, which reduces the oxidation-reduction potential to a point suitable for strict anaerobes. These requirements generally are much in excess of the minimum stoichiometric amounts for dehalogenation, and must be factored into any practical scheme.

While progress is being made on understanding factors affecting dehalogenation of CAHs, the kinetics of the reactions are not yet well understood. This is partly a result of the complexity of the reactions involved and the lack of good knowledge about the biochemistry of the process. While

some organisms clearly can obtain energy from reductive dehalogenation, it is not clear that the reductive dehalogenation noted to occur at contaminated sites or in laboratory mixed cultures are also involved in energy metabolism. They may simply be fortuitous dehalogenations or transformations occurring by enzymes or cofactors produced for other purposes by microorganisms, a process termed cometabolism. In addition, various organisms, such as nitrate and sulfate reducers and also methanogens compete with dehalogenating bacteria for available electron donors in mixed cultures under anaerobic conditions. An additional factor is that an evaluation of microbial reaction kinetics requires that one know what constituents are limiting reaction rates. This may be the electron donor, the electron acceptor, or an other required nutrient such as nitrogen, phosphorus, or perhaps, some trace vitamin that is required for bacterial growth. Slowly, a better understanding of these processes is emerging, but there adequate resolution will depend upon our ability to isolate the relevant organisms involved and their study in pure culture without the presence of so many confounding factors. In the mean time, empirical studies such as conducted in the following can shed adequate light on the factors of importance so that rational decisions about process design can be made.

Anaerobic aquifer material for this study was obtained from a contaminated site in Victoria, TX, which is being bioremediated for PCE dechlorination to ethene under sulfate reducing conditions by the addition of benzoate (Haston et al., 1994). All experiments with this aquifer material were conducted under anaerobic conditions.

METHODOLOGY

Microcosms (125 ml) were initially used to simulate *in situ* conditions and to determine conditions under which complete dehalogenation of PCE to ethene could be obtained. Benzoate was found to be one of the best substrates for complete dehalogenation. These initial experiments supported by DuPont Chemicals also indicated that methane production was not required for the dehalogenation, and neither was sulfate. Indeed, the data suggest that organisms carrying out reductive dehalogenation compete with methane producers and sulfate reducers. Providing conditions that allow the dehalogenators to compete successfully with the other microorganisms for electron donor may be one of the keys to successful reductive dehalogenation.

Enrichment cultures were then developed in an attempt to create good conditions for chlorinated aliphatic hydrocarbon (CAH) dehalogenation and to obtain pure cultures of dehalogenating bacteria. From these studies, a new facultative bacterium, strain MS-1, was isolated that rapidly dehalogenates PCE to cDCE. Rates studies were conducted with this organism as part of this study to obtain a comparison with the mixed cultures evaluated for complete dehalogenation to ethene.

This past year with DuPont supported research a 3.6 liter semi-continuous anaerobic reactor was seeded with Victoria culture and operated at 25° C on a 60-day detention time and fed yeast extract. Every three days 180 ml of reactor mixed contents was removed and replaced with a feed solution. Initially, the feed solution contained 3.3 mg PCE and 430 mg of yeast extract along with 180 ml of defined basic salt media. Although added separately, if combined, the concentration in the feed solution would be 18 mg/L PCE and 2,400 mg/L yeast extract. The concentration of PCE was then increased from time to time until reactor failure resulted. The results of these studies are included here as they indicate concentration limitations of importance to the Lasagna process.

As part of the Monsanto-supported studies, a new 1.6 liter mixed reactor was then operated semicontinuously at 25° C with a 60-day detention time by exchanging 80 ml every three days. The 80 ml of basic mineral media feed contained 400 μM PCE and in addition, 1 mmol hydrogen gas was added to serve as the electron donor. The use of just hydrogen worked for a several month period, but after that, dehalogenation of cDCE and VC, but not PCE and TCE, began to

decline. It was determined from batch studies that some amount of yeast extract addition was essential. Another alternative semi-continuous two-liter reactor operating at 35° C with a 30-day detention time was then fed 250 mg/l yeast extract and 30 µM TCE and in basic salt medium. It maintained good dehalogenation of PCE to ethene throughout the period of this study. Results from these three reactor operating modes are described in the following.

RESULTS AND DISCUSSION

Reactor Operation at High PCE Concentration

Studies funded by DuPont Chemicals were conducted in order to determine the effect of the concentration of primary substrate and CAH on the rate of dehalogenation. First, the concentration of electron donor required for dehalogenation was evaluated. A very small enrichment culture was mixed in defined media and various concentrations of yeast extract were added along with about 15 µM PCE (2.5 mg/L). The results (Table 2) indicate that organism growth on only 25 mg/L yeast extract was sufficient to obtain reduction of PCE to cDCE. However, growth on more than 100 mg/L yeast extract was required for the complete reduction of PCE to ethene. Thus, much more donor is required for complete PCE conversion than for the initial conversion to cDCE.

Table 2. Results of varying concentrations of yeast extract on PCE dehalogenation.

mg/L Yeast Extract	Day	µM			
		PCE	cDCE	VC	Ethene
0	1	13	0	0	0
	4	12	0	0	0
	55	10	0	0	0
25	1	14	0	0	0
	4	0	15	0	0
	55	0	15	0	0
50	1	16	0	0	0
	4	0	15	0	0
	55	0	14	0.2	0.2
100	1	15	0	0	0
	4	0	16	0	0
	55	0	14	0.2	0.2
200	1	14	0	0	0
	4	0	15	0	0
	55	0	0.2	0.2	14
400	1	16	0	0	0
	4	0	16	0	0
	55	0	0.4	0.2	14

The effect of CAH concentration on dehalogenation was studied by slowly increasing PCE concentration in the reactor feed. Before this increase was started, the reactor contained about 200 mg/l of suspended bacterial solids (dry weight). With addition of 245 µmol cDCE in 3.65 L, or about 6.5 mg/L cDCE, complete dehalogenation to ethene required about 2 days. This represents a rate of about 16 µg cDCE/mg suspended solids/day. However, following an increase in the influent PCE concentration, reactor operation became poor. Methane production first stopped while dehalogenation continued to occur. Then, the conversion of PCE to cDCE stopped, but conversion of cDCE to ethene continued. Finally, conversion of cDCE itself slowed considerably. Subsequent studies indicated that cDCE was relatively toxic to the process compared with PCE, and buildup of cDCE during high PCE loadings was the cause of failure.

Through a series of studies, microorganism exposure to a saturation concentration of PCE of 180 mg/l was found to be toxic, but exposure to 40 mg/l was not and permitted conversion of the PCE to cDCE. However, an equivalent amount on a molar basis of cDCE (30 mg/l) was inhibitory to the process and affected the two separate processes of cDCE reduction to VC and methane production. A cDCE concentration of 14 mg/l was somewhat inhibitory, but a concentration of 8 mg/l was not. It is important to note that inhibition is related to what the microorganism are exposed to in the reactor, and not the concentration in the feed to the reactor. Feed concentrations can be much higher provided that the compounds and their intermediates are consumed fast enough so that inhibitory concentrations do not result in the reactor itself. This can be a special problem during reactor startup, and so caution here is needed until the reaction is proceeding well.

Dehalogenation Rates with Yeast Extract Feed

The enrichment culture maintained in the two-liter reactor fed yeast extract was used to study transformation rates of individual chlorinated aliphatic compounds as functions of temperature and pH. For temperature studies, 200 ml of the enrichment culture was dispensed inside an anaerobic glove box into five 254-ml amber bottles. Each bottle was amended with 10 mg/L yeast extract and incubated in water baths adjusted for different temperatures for one hour. Then, in order to start an experiment, a chlorinated compound was added at an initial concentration of 10 to 25 µM. Bottles were shaken thoroughly before a sample was taken. Samples were analyzed for CAH reduction and product formation with time. In addition, strain MS-1 was grown on yeast extract and its rate of dehalogenation of TCE to cDCE was measured in order to compare the pure culture and mixed culture rates for organisms grown on the same substrate. TCE and not PCE was studied as TCE was the subject of Monsanto's particular interest, and they supported this portion of the study.

In order to determine the effect of pH, a similar procedure was followed. The pH ranging from 5.0 to 8.0 was adjusted in these bottles by the addition of 1N NaOH or 1N HCl. A sterile control bottle containing TCE or cDCE in water was included in the experiments to determine possible loss of substrates due to sampling.

The results are summarized in Table 3 for temperature effects. The temperature optimum was generally between 35° and 40° C, although good dehalogenation occurred with temperature as high as 45° C. The rates found at 21° C were about one-half of the maximum rates, which is a typical rate reduction for a temperature difference of 10° C or so. Interestingly, on a molar basis the rate for VC dehalogenation was up to three times that for cDCE dehalogenation, and the rate of VC dehalogenation was about two-thirds of that for TCE dehalogenation. Thus, cDCE dehalogenation is shown for this mixed culture to be the rate limiting step in the overall conversion to ethene. Another interesting observation is that the rate of TCE dehalogenation by the pure culture strain MS-1 was about three times that by the mixed culture developed on the same substrate. This is also a reasonable difference between the two.

The results for pH effects are summarized in Table 4. These data indicate that dehalogenation rates are optimum at pH 6.5, but vary little between pH 5.5 and 7.5. Effective dehalogenation occurred over the entire pH range studied of 5.0 to 8.0.

Table 3. Effect of temperature on dehalogenation rates at pH 7.0.

Chemical	Culture	Temp. (°C)	Dehalogenation Rate	
			mmol g^{-1} d^{-1}	mg g^{-1} d^{-1}
TCE	MS-1	21	0.99	130
		30	1.58	210
		35	2.11	280
		40	1.73	230
		45	2.87	380
TCE	Enrichment	21	0.33	44
		30	0.48	63
		35	0.64	84
		40	0.68	89
		45	0.54	71
cDCE	Enrichment	21	0.08	8
		30	0.11	11
		35	0.09	9
		40	0.15	15
		45	0.24	24
VC	Enrichment	21	0.25	16
		30	0.45	28
		35	0.45	28
		40	0.39	24
		45	0.23	14

Table 4. Effect of pH on dehalogenation rates at 35° C.

Chemical	Culture	pH	Dehalogenation Rate	
			mmol g^{-1} d^{-1}	mg g^{-1} d^{-1}
TCE	Enrichment	5.0	0.56	73
		5.5	0.70	92
		6.0	0.68	89
		6.5	0.78	102
		7.0	0.60	79
		7.5	0.62	82
		8.0	0.27	35
cDCE	Enrichment	5.0	0.07	7
		5.5	0.10	10
		6.0	0.13	12
		6.5	0.18	18
		7.0	0.14	14
		7.5	0.11	10
		8.0	0.10	10

Dehalogenation Rates with Hydrogen Feed

During the three month period that the reactor receiving hydrogen feed was operating well, kinetic studies were conducted. Here, batch studies were conducted at 25° C by adding 220 ml of culture to 254 ml bottles to which various concentrations of CAHs were added together with hydrogen gas as the electron donor. Studies with increased hydrogen gas concentrations were conducted with similar results, indicating that the hydrogen concentration used in the studies was not rate limiting to the reaction. The initial rates of dehalogenation were measured, generally over a short period of one hour and before dehalogenation products rose to concentrations that may have affected the results. The effect of CAH concentration on reaction rate was considered to follow the Monod rate model for microbial reactions, which assumes a single rate-limiting substrate, in this case the CAH of interest:

$$r_{dehalogenation} = k\frac{C}{K_s + C}X \qquad (8)$$

where $r_{dehalogenation}$ is the CAH dehalogenation rate in μM/day, k is the maximum dehalogenation rate in mmol per gram microorganisms per day, K_s (μM) is the affinity constant of the microorganisms for the CAH or the CAH concentration at which the rate is one-half the maximum rate, C is the CAH concentration in μM, and X is the microorganism concentration in the reactor, here taken to equal the volatile suspended solids concentration (dry weight) in mg/l.

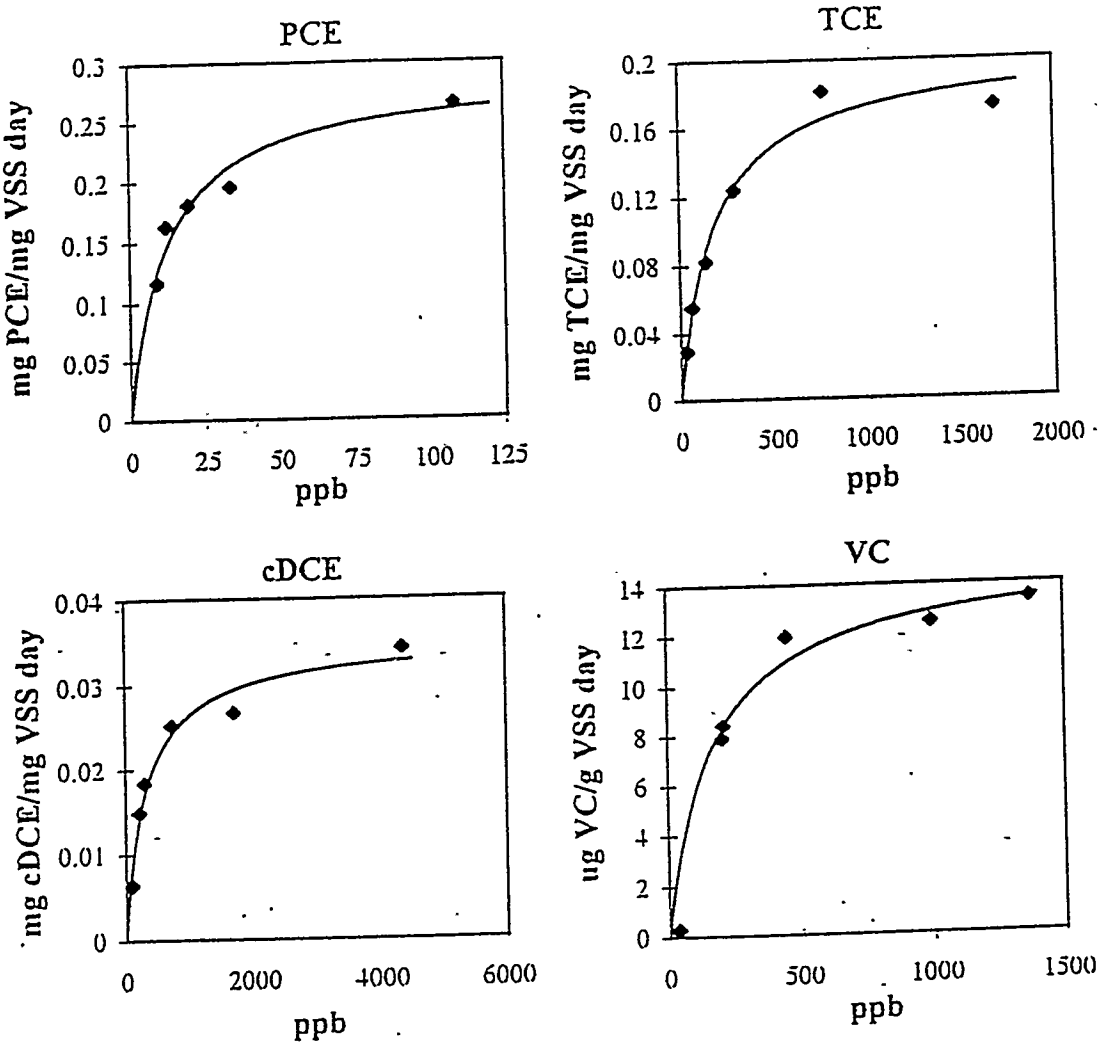
The rates obtained with different starting concentrations are illustrated in Figure 1, and Monod reaction rate coefficients that were derived from these data are summarized in Table 5. The values shown for PCE, TCE, and cDCE were obtained before the reactor experienced problems from lack of trace nutrients (yeast extract). However, the very low rates with VC were obtained after this and are thus not considered representative of the potential for this culture. The curves drawn in Figure 1 were made using the coefficients from Table 5 to illustrate the fit obtained.

The K_s values listed in Table 5 are all well below 1 mg/L. Thus, rates measured at concentrations above 1 mg/l, such as those listed in Tables 2 and 3, are close to maximum rates and representative of k values. By comparing the Table 3 rates in the 21° to 30° C range with the k values in Table 5 obtained at 25° C, it is seen (with the exception of VC) that the rates with hydrogen-grown cultures were three to four times the rates with growth on yeast extract. It is of interest to note that the TCE dehalogenation rate with hydrogen-grown culture was about the same as that as with pure culture MS-1 (Table 3). These data suggest that the mixed microbial culture supported on hydrogen alone as an electron donor had a higher percentage of dehalogenators than when yeast extract was used.

Table 5. Monod kinetics for dehalogenation by enrichment culture grown on hydrogen at 25° C.

	K_s		k	
Chemical	μM	μg/l	mmol g^{-1} d^{-1}	mg g^{-1} d^{-1}
PCE	0.06	10	1.7	280
TCE	1.4	180	1.5	200
cDCE	3.4	330	0.35	35
VC	2.7	170	0.00024	0.015

Figure 1. Dehalogenation rates for hydrogen-grown culture at 25° C as function of CAH concentration.



Another point of interest is that the concentration of PCE in the feed to the hydrogen reactor was 400 μM, or about 66 mg/l. This is higher than the toxic concentration found for PCE, but presented no problem as long as it was consumed rapidly when added to the reactor and as long as cDCE concentration within the reactor did not increase above about 8 to 10 mg/l.

APPLICATION TO THE LASAGNA TECHNOLOGY

The purpose of the First Phase of study was to determine the kinetics of TCE dehalogenation by microorganisms. The proposed Second Phase of study was to develop a model for evaluating the feasibility of applying anaerobic treatment to the Lasagna Technology and for conducting a bench-scale study of the process. While time to make such an evaluation was not available in the Phase One study, it appeared appropriate to make some rough calculations to indicate what might be anticipated if a more detailed study were undertaken. Since the rate of cDCE dehalogenation was governing, the typical rate for this transformation of 0.1 mmol per day per gram of bacteria might be used. This would correspond to a TCE transformation rate of 13 g per day per gram of bacteria. We then assume a 2.5 cm wide biotreatment zone containing support media and a void volume of 0.3. We also assume a groundwater induced velocity by Lasagna of 2.5 cm per day in clay, also with a void volume of 0.3. The velocity through the bioreactor would thus be the same as through the clay. This means that the hydraulic detention time in the reactor is 1.0 day. Next, we assume an organism concentration (volatile suspended solids dry weight) of 1000 mg/l. This serves as a starting point for our calculations.

With the maximum TCE degradation rate assumed above, this reactor would be able to degrade 13 mg/l of TCE in the water passing though the reactor. The amount degraded would change in direct proportion to a change in organism concentration, and inversely proportional to an increase in fluid velocity. In an actual reactor then, the organism concentration could be increased in order to obtain an increase in the rate and extent of TCE dehalogenation. The organisms would need to be maintained through the introduction of a primary substrate such as yeast extract, or perhaps hydrogen. Hydrogen addition would reduce the required population of microorganisms to obtain a given degree of treatment. Based upon the measured effect of TCE concentration on reaction rates, the maximum TCE degradation rates are obtained when TCE concentrations are 1 mg/l and higher, or well above the K_s values determined. These rough approximations provide information that might be used to consider the potential of biodegradation with other possible methods for treatment of TCE removed from a contaminated zone by the Lasagna process.

CONCLUSIONS

Toxicity of cDCE to mixed dehalogenating cultures was found to be a potential limitation of the biodehalogenation process when treating water with high TCE concentrations. Exposure of microorganisms to more than about 8 mg/l cDCE caused inhibition problems, which corresponds to about 11 mg/l TCE. However, much higher TCE concentrations can be treated provided that cDCE is degraded as rapidly as it is formed, thus preventing a buildup to the inhibitory cDCE concentration. Temperature optimum for dehalogenation by the mixed culture was in the normal mesophilic range of about 35° C, with rates reduced to about one-half of this at temperature of about 20° C. The pH optimum was found to be 6.5, with rates at pH 5.5 or 7.5 being about one-half the maximum rates. Dehalogenation of cDCE was found to be rate limiting in the complete conversion of PCE to ethene, with a dehalogenation rate of only about one-fifth of that for PCE or TCE. For complete dehalogenation of PCE or TCE to ethene, it is the cDCE rate that will govern reactor and in-situ dehalogenation design. Mixed cultures grown on hydrogen have a greater activity towards PCE than those grown on yeast extract, but require at least some yeast extract or perhaps some other trace nutrient source in order to maintain viability over long time periods.

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