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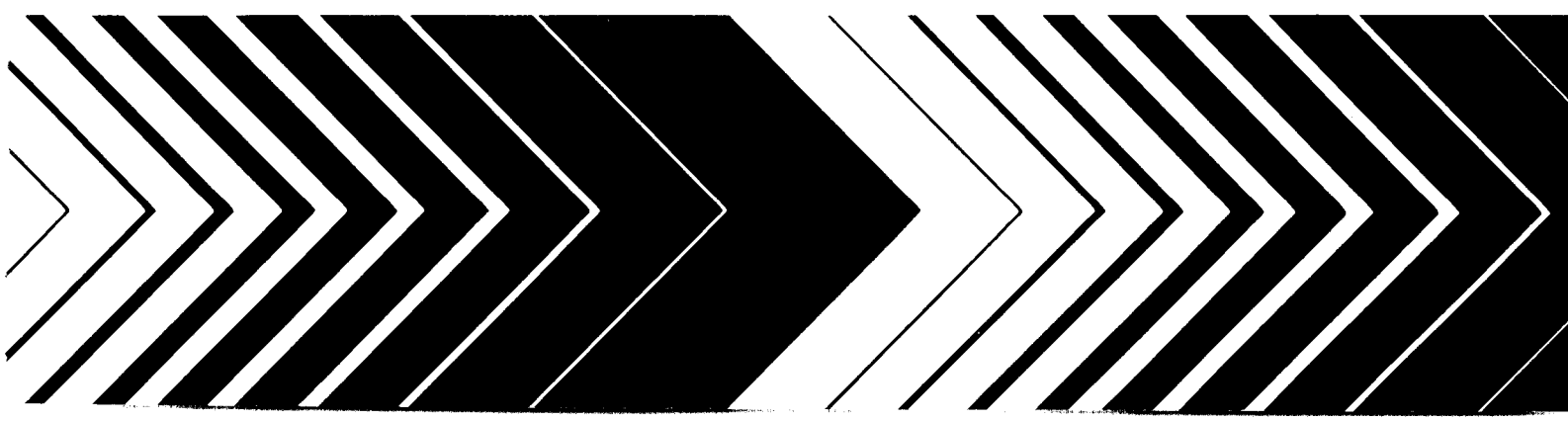
Bioremediation of Hazardous Wastes



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Bioremediation of Hazardous Wastes

by

Biosystems Technology Development Program

Office of Research and Development
U.S. Environmental Protection Agency
Ada, OK; Athens, GA; Cincinnati, OH;
Gulf Breeze, FL; and Research Triangle Park, NC

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Executive Summary

In 1987, the U.S. Environmental Protection Agency's (EPA) Office of Research and Development (ORD) initiated the Biosystems Technology Development Program to anticipate and address research needs in managing our nation's hazardous waste. The Agency believes that bioremediation offers one attractive alternative to conventional methods of cleaning up hazardous waste and has developed a strategic plan for its acceptance and use by the technical and regulatory communities. The Agency's 5-year strategic plan is centered around site-directed bioremediation research to expedite the development and use of relevant technology.

In May 1992, ORD hosted the fifth annual Symposium on Bioremediation of Hazardous Wastes: U.S. EPA's Biosystems Technology Development Program, in Chicago, Illinois. This was the first time this symposium had been held in a regional office, and it drew the largest audience in 5 years. At the conference, 28 papers and 9 poster exhibits were presented on recent program achievements and research projects aimed at bringing bioremediation into more widespread use.

These Proceedings comprise an Executive Summary, an Introduction, and papers and poster presentations in five key research and program areas, which, taken as a whole, represent a comprehensive approach to bioremediation of hazardous waste sites. The five research and program areas are:

1. **Site Characterization.** EPA's Biosystems Program recognizes the basic need for more complete site characterization techniques as a cornerstone to the application of *in situ* technology. Site characterization includes detailed descriptions of the surface and subsurface, including hydrology, geology, and the physics and chemistry of a site. The four topic papers presented covered research on petroleum-spill cleanup, the efficacy of *Pseudomonas* to remediate chemical contamination, and on constraints to the proposed use of methane-oxidizing bacteria for a TCE plume.

2. **Bioremediation Field Initiative.** This initiative was instituted in 1990 in response to research needs to expand the nation's field experience in bioremediation techniques and to collect and disseminate performance data from field application experiences. The Agency assists the regions and states in conducting field tests and in carrying out independent evaluations of site cleanups using bioremediation. Through this initiative, tests are under way at Superfund sites, RCRA corrective action facilities, and Underground Storage Tank sites. Eight papers and two poster presentations were devoted to this key program area and covered field evaluations currently under way at sites utilizing bioventing, biochemical techniques, and bioremediation under a variety of aerobic and anaerobic conditions.

3. **Performance Evaluation.** Performance evaluation of bioremediation technologies involves determining the extent and rate of cleanup by a particular bioremediation method as well as the environmental fate and effects of the parent compounds and their by-products. Because remediation efforts at a contaminated site can produce additional compounds during the remediation process, a key component of performance evaluation is evaluating potential health effects. The particular purview of this area is to develop bioremediation approaches that protect public health. Four presentations discussed risks related to bioremediation and potential genotoxicity.

4. **Process Research (including laboratory, pilot-scale, and field research).** Process research focuses primarily on identifying microorganisms that could degrade contaminants and developing methods for their effective delivery. The work involves isolating and identifying microorganisms that carry out biodegradation processes and developing new biosystems for treatment of environmental pollutants in surface waters, sediments, soils, and subsurface materials. Eleven papers and five poster presentations

addressed this critical area. Although the majority of presentations dealt with laboratory-scale work, both pilot-scale and field research also were discussed. Pilot-scale research will become increasingly important in the near future to provide critical information on process operation and control and residuals/emissions management. Field research, in turn, is essential for evaluating the performance of bioremediation processes and for accelerated site-directed research of process concepts that lend themselves to accelerated testing.

5. **Modeling.** Mathematical modeling assists researchers in extrapolating field results to predict and guide the application of technologies at other sites. Models provide assessments of the opportunities for successful site bioremediation, provide cost-effective designs for field bioremediation, design appropriate sampling strategies to support performance evaluations, and provide effective guidance with which to apply research results to other sites. Two presentations concerned the mechanisms by which granular-activated carbon degrades hazardous waste. A third presentation dealt with biodegradation kinetics.

The Biosystems Technology Development Program draws on ORD scientists who possess unique skills and expertise in biodegradation, toxicology, engineering, modeling, biological and analytical chemistry, and molecular biology. Participating laboratories and organizations are:

- Environmental Research Laboratory-Ada, Oklahoma
- Environmental Research Laboratory-Athens, Georgia
- Center for Environmental Research Information-Cincinnati, Ohio
- Risk Reduction Engineering Laboratory-Cincinnati, Ohio
- Environmental Research Laboratory-Gulf Breeze, Florida
- Health Effects Research Laboratory-Research Triangle Park, North Carolina

Introduction

The U.S. Environmental Protection Agency (EPA) is responsible for protecting public health and the environment from the adverse effects of pollutants. EPA's authority to develop regulations and to conduct environmental health research is derived from major federal laws passed over the last 20 years that mandate broad programs to protect public health and the environment. The Clean Air Act; the Safe Drinking Water Act; the Clean Water Act; the Toxic Substances Control Act; the Federal Insecticide, Fungicide, and Rodenticide Act; the Resource Conservation and Recovery Act; and the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA, known as Superfund) all require that EPA develop regulatory programs to protect public health and the environment.

For the control and cleanup of hazardous wastes, the Superfund law gives EPA broad authority to respond directly to releases of hazardous materials that endanger public health or the environment. Also, the Superfund Amendments and Reauthorization Act of 1980 (SARA) expands EPA's authority in research and development, training, health assessments, community right-to-know, and public participation. EPA's Office of Research and Development (ORD) conducts basic and applied research in health and ecological effects, hazardous wastes, and remediation development and demonstration of control technologies. Technologies are designed to provide efficient, cost-effective alternatives for cleaning up the complex mixtures of pollutants found at Superfund sites or at other locations, such as oil spills. As the technologies advance, ORD transfers information on their use to groups that apply technologies at specific sites.

Some of the most promising new technologies for solving hazardous waste problems involve the use of bioremediation, an engineered process that relies on microorganisms, such as bacteria or fungi, to transform hazardous chemicals into less toxic or nontoxic compounds. These microorganisms have a wide range of abilities to metabolize different chemicals; scientists can tailor the technology to the pollutants at specific sites and in specific media (e.g., contaminated aquifers, waste lagoons, contaminated soils) by using organisms in the treatment system that break down a particular pollutant under specific conditions.

Bioremediation is an attractive option because it is a natural process, and the residues from the biological processes (such as carbon dioxide and water) are usually geochemically cycled in the environment as harmless products. These processes also are carefully monitored to reduce the possibility of a product or a process being more toxic than the original pollutant. Another advantage of biological treatments-particularly *in situ* treatment of soils, sludges, and ground water-is that they can be less expensive and less disruptive than options frequently used to remediate hazardous wastes, such as excavation followed by incineration or landfilling. Finally, bioremediation holds another clear advantage over many technologies relying on physical or chemical processes: instead of merely transferring contaminants from one medium to another, biological treatment can degrade the target chemical.

Until recently, the use of bioremediation was limited by lack of thorough understanding of biodegradation processes, their appropriate applications, their control and enhancement in environmental matrices, and the engineering techniques required for broad application of the technology. The Agency recognized that, along with basic understanding of biological processes, comprehensive mechanistic process control, engineering design, and cost data are necessary for the acceptance and use of bioremediation by the technical and regulatory communities. In response to these needs, ORD developed an integrated Bioremediation Research Program, whose mission is to advance the understanding, development, and application of bioremediation solutions to hazardous waste problems threatening human health and the environment. The program was designed to strike a balance between basic research activities leading to a fundamental understanding of biological degradation processes and engineering activities leading to practical scientific applications of the technology. The Bioremediation Research Program is made up of three major research components: the Biosystems Technology Development Program, the *In Situ* Application Program, and the Bioremediation Field Initiative.

EPA's bioremediation research efforts to date have already produced significant results in the laboratory, at the pilot scale, and in the field. Accomplishments range from aquifer restoration to soil cleanup to process characterization to technology transfer.

Section One
Site Characterization

The bioremediation community has focused major research on site characterization. EPA's Biosystems Program recognizes the basic need for improved site characterization, which includes detailed descriptions of the surface and subsurface, involving hydrology, geology, and the physics and chemistry of a site. These descriptions will clarify the opportunities for and constraints to bioremediation.

Cleanup of petroleum spills is a subject of much current research. A recent project focused on the constraints to bioremediation due to the hydraulic and geochemical nature of a site. A case study of a site contaminated with trichloroethylene also found constraints to bacterial remediation under the design condition originally planned. Two other studies described promising results from innovative strategies using *Pseudomonas* to facilitate bioremediation of ground water contaminated with hydrocarbons.

Site Characterization at Pipeline Spill at Park City, Kansas: Estimating Hydraulic and Geochemical Constraints on Bioremediation

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Engineering design for bioremediation of spills of petroleum hydrocarbons is strongly influenced by (1) the vertical distribution of the oily-phase hydrocarbon, (2) the position of the water table, (3) the hydraulic and pneumatic conductivity of the contaminated geological material, (4) the volumetric demand for electron acceptor to remediate hazardous components of the spill, (5) geochemical constraints on compatibility of nutrient amendments in ground water, and (6) respiratory constraints on the concentrations of amendments to ground water.

Geological Context

In the late 1970s, refined petroleum hydrocarbons were spilled from a buried pipeline in a rural area in south central Kansas. The pipeline carried a variety of refined products, including various grades of gasoline and diesel. The resulting combination of fuels resembles JP-4 jet fuel. The leak was detected when low concentrations of petroleum hydrocarbons were detected in a nearby municipal water well in 1980.

The site is located within the floodplain of the Little Arkansas River. Figure 1 is a schematic representation of the geology of the site. The land surface was originally backswamp muds. Below the clay is a layer of sand and gravel containing the water table aquifer. Within the sands there is a general increase in sediment grain size with depth. Below the aquifer is consolidated bedrock.

Petroleum hydrocarbons usually behave as LNAPLs, meaning they drain through the unsaturated zone under the influence of gravity, then accumulate near the water table. At the water table, they spread laterally in the capillary fringe. The hydrocarbons moved laterally over 400 ft to the water well, but the maximum vertical extent of contamination near the spill was only 10 to 15 ft.

Emergency Response

The original amount of hydrocarbon released is unknown; however, several thousand gallons of free-phase product were pumped from two interceptor trenches installed after the problem was identified. The remaining hydrocarbon that collected in the trenches was ignited and allowed to burn for about a year. By 1984 both trenches had been backfilled.

In an attempt to remove dissolved-phase hydrocarbon from the aquifer, the municipal water well was pumped for 6 years beginning in 1980. Pumping was discontinued when hydrocarbon could no longer be detected in the extracted water. Unfortunately, when pumping ceased, concentrations of alkylbenzenes (BTEX) rebounded in the water. Considerable amounts of dissolved- and oily-phase hydrocarbon remained in the aquifer; these problems are common with the pump-and-treat approach to aquifer remediation (3).

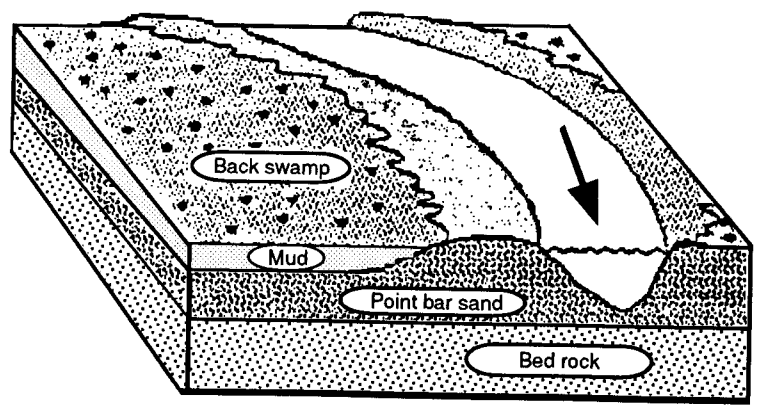

Figure 1. Geological setting of the site.

Coastal Remediation Company (CRC) began a feasibility study for *in situ* bioremediation. CRC joined with the U.S. Environmental Protection Agency's Robert S. Kerr Environmental Research Laboratory (Kerr ERL) in a Cooperative Research Demonstration Agreement to evaluate nitrate as an electron acceptor for bioremediation of the spill.

Distribution of the Hydrocarbon

CRC began a site investigation in 1989. Eighteen monitoring wells and two sets of five clustered piezometers were installed to define the extent of contaminated ground water and the direction of ground-water flow. In the spring of 1991, CRC and staff of the Kerr ERL installed 12 additional monitoring wells and two additional sets of clustered piezometers to define further the distribution of oily-phase material. All the monitoring wells were approximately 25 ft deep with well screens set 5 ft above and 5 ft below the water table. All wells were drilled using a hollow stem auger drilling rig with continuous coring capabilities. Each well was fully cored from surface to its greatest depth. The lithologically distinct intervals were identified and described, and the core extracts were analyzed for total petroleum hydrocarbons and alkylbenzenes (BTEX).

Figure 2 shows the relationship among the layers of geological materials, the ambient water table, and the vertical distribution of spilled hydrocarbon in an area adjacent to the spill. The hydrocarbon is roughly confined to an interval between the base of the clay layer and the lowest water table since the spill. The hydrocarbon penetrates the present water table only a few feet; most of the spill lies above the present water table.

Delivery of Nutrients and Electron Acceptors

Wells were installed over the spill to distribute nutrients and electron acceptors. Each well is screened in sandy, unsaturated material below the clay layer, but above the water table. To prevent failure (blowout) of the recharge well, the rule of thumb requires that pressure in a recharge well should not exceed 20 percent of the pressure head between the land surface and the water table (2). For the demonstration site, this constitutes a value no greater than 2.0 per square in. (psi) as measured in the discharge manifolds to the injection wells. Many injection wells on a fairly tight spacing are needed to deliver the necessary volume of water required to meet the nutrient and electron acceptor demand of the spill within a reasonable time period.

More than 400 recharge wells have been installed on a 20-ft grid spacing. Two large-capacity production wells have been installed to recirculate ground water through distribution pipelines back to the recharge wells. The production wells, pipelines, and recharge wells are designed to recharge 125 gallons per minute (gpm) per acre of surface area.

Aquifer tests indicate that the hydraulic conductivity ranges from 0.17 to 0.36 cm/sec. Ground-water flow models were used to estimate the variations in elevation of the water table during pumping. The predicted rise in the water table, assuming uniform conditions, is less than 1 ft, which is inadequate to flood the

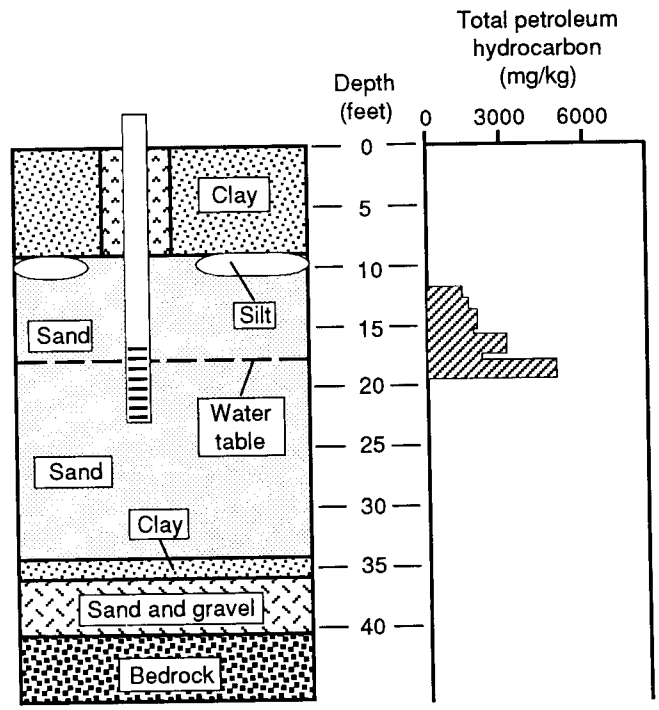

Figure 2. *Relationship among spilled hydrocarbons, layers of geological materials, the water table, and monitoring wells.*

contaminated interval totally (compare Figure 2). The local conductivity at the depth of the recharge wells may be less than average, resulting in a higher rise in the water table. Limitations on water supply and the position of the water table, however, probably make it impossible to reach all the spill with nitrate-amended ground water. Remedial technology using air, such as bioventing, may be required to reach the oily-phase hydrocarbon in the unsaturated zone.

Supply of Electron Acceptor and Nutrients

Ground water in contact with the spill has a high concentration of ferrous iron. To minimize problems with oxidation and precipitation of iron, nitrate was selected as the primary electron acceptor. The rate of supply of nitrate (N) is limited by the flow of water and the concentration of nitrate allowed by the state of Kansas in its permit (10 mg/L as N).

The volumetric electron acceptor demand has been estimated from the alkylbenzene content of the oily-phase material in the spill. The relationship between the supply of nitrate and the average demand, the demand of the most contaminated depth interval, the rate of expression of nitrate demand, and the rate of depletion of alkylbenzenes is discussed by Hutchins (1991). In general, the rate of cleanup is controlled by the rate of supply of nitrate, which is controlled, in turn, by hydrologic, geochemical, and regulatory considerations.

A batch desorption isotherm demonstrated that the aquifer solids could maintain solution concentrations of orthophosphate

above 0.1 mg/L against very strong phosphate demands. Phosphate should not be limiting for biodegradation of hydrocarbons. Because phosphate precipitates impose a risk of plugging the recharge wells, no phosphate salts will be added to the recharged ground water.

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Field Demonstration of Innovative Bioremediation Strategies for PCP and Creosote

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In earlier papers, we had described the isolation and characterization of microorganisms capable of utilizing (mineralizing) high-molecular-weight polycyclic aromatic hydrocarbons (HMW PAHs) and other recalcitrant creosote constituents as sole sources of carbon and energy for growth (3, 5, 6). In addition, an axenic culture of *Pseudomonas* sp. strain SR3 was shown to mineralize pentachlorophenol (PCP) when supplied as a sole carbon source in liquid medium (12).

The object of the current study was to evaluate, at bench- and pilot-scale levels, the ability of these specially selected microorganisms to facilitate the bioremediation of ground water contaminated with creosote and PCP. The performance of the bioremediation process was evaluated according to chemical analyses of system influent, effluent, and bioreactor residues by performing a mass balance evaluation and comparative biologi-

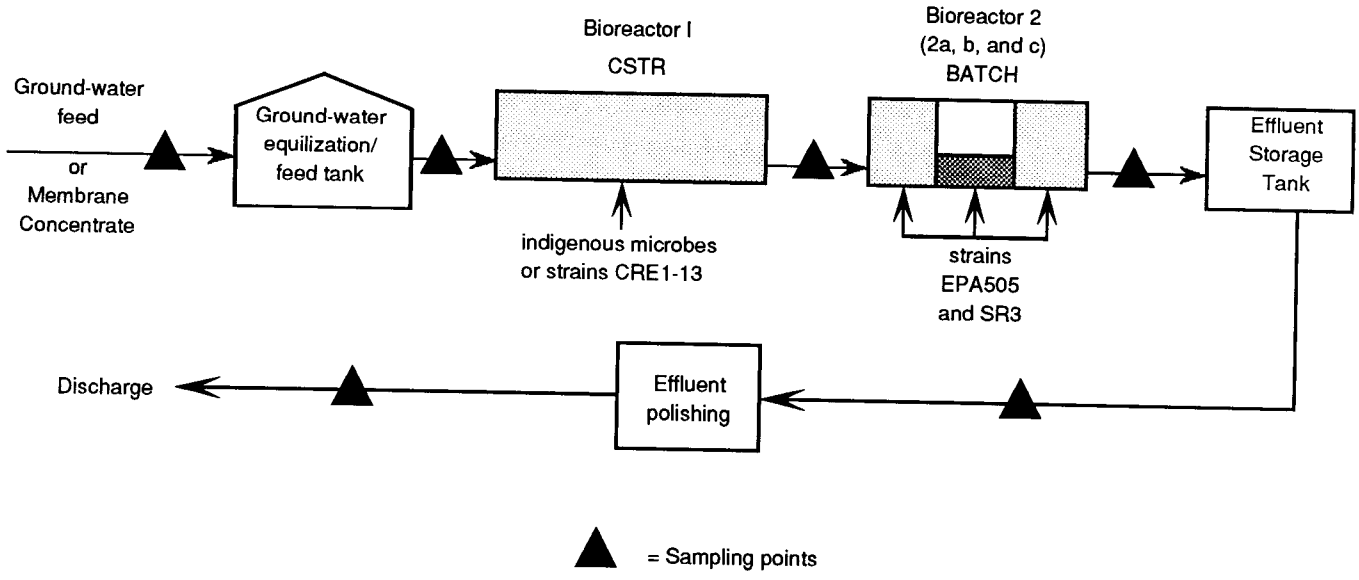

Figure 1. *Ground-water bioremediation system.*

Table 1. *Summary of Toxicological Analyses*

Bioreactor Sample	Microtox EC_{50} 5 min	Mysids LC_{50} 96h	Ceriodaphnia LC_{50} 48h	Embryonic Menidia (1% solutions)
Specially selected microorganisms				
Feed	0.0185	0.01	0.007	embryotoxic
Biorx 1	2.3645	0.34	0.710	teratogenic
Effluent	1.2956	0.75	0.530	teratogenic
Non-specific microorganisms				
Feed	0.1456	0.02	0.079	embryotoxic
Biorx 1	25.057	0.32	0.292	teratogenic
Effluent	54.107	0.16	0.230	teratogenic

cal toxicity measurements. Results obtained upon the addition of specialty microorganisms were compared with those obtained when nonspecific microorganisms (i.e., obtained from soil at the site and activated sludge) were employed for bioreactor operations.

The bioremediation process was field-tested with ground water contaminated by creosote and PCP recovered from the American Creosote Works (ACW) Superfund Site, Pensacola, Florida. A two-stage, continuous-flow, sequential inoculation system was developed for the efficient biodegradation of creosote and PCP (Figure 1).

The first stage of the pilot-scale system used a 120-gallon EIMCO reactor (bioreactor 1). Ground water was delivered to this bioreactor for 8 days at an average rate of 30 gallons per day (4-day hydraulic retention time). Effluent from bioreactor 1 was delivered to one of three, 60-gallon, completely mixed batch reactors that served as the second bioreactor (bioreactors 2a, 2b, and 2c). Once each chamber of bioreactor 2 was filled with effluent from bioreactor 1, material was held in the batch mode for 4 days. At the end of the 4-day batch reaction, the effluent was clarified by settling biomass, and was placed into a holding tank for final analysis and testing prior to discharge.

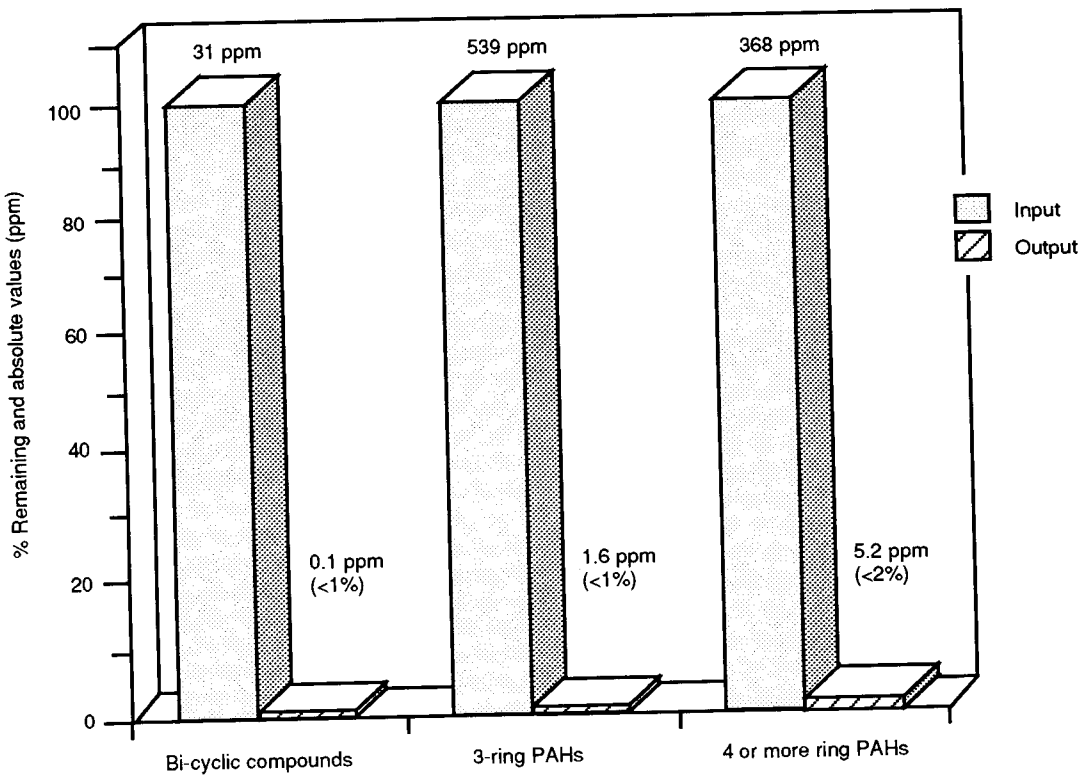

Figure 2. *Reduction of PAH contaminants in creosote-contaminated ground water.*

When specially selected bacteria were added to the system, a one-time inoculum of a 13-member bacterial community with known degradative abilities was reconstituted from frozen stock cultures (strains CRE1 through CRE13) and added to bioreactor 1 at a concentration of approximately 1 x 10^6 cells of each strain/mL. Daily additions of *Pseudomonas paucimobilis* strain EPA 505 and *Pseudomonas* sp. strain SR3 (approximately 1 x 10^6 cells/mL) were added to each filled chamber of bioreactor 2. Daily measurements were taken of TCOD, SCOD, TSS, VSS, DO, pH, oxygen uptake, temperature, NH3-N, reactive-P, total creosote, and PCP (GC-FID, GC-ECD analyses), and select biological toxicity assays to monitor bioreactor activity and performance.

With the two-stage, continuous-flow, sequential inoculation design, the concentration of creosote constituents was reduced from about 1,000 ppm in the ground-water feed to <7 ppm in the system effluent (removal efficiency of >99 percent). Also, the toxicity and teratogenicity of the bioreactor effluent was significantly reduced as determined by Microtox™, embryonic *Menidia beryllina, Mysidopsis bahia,* and *Ceriodaphnia dubia* assays (Table 1). Notably, the cumulative concentration of 9 HMW PAHs (each containing 4 or more fused rings) was reduced from 368 ppm in the ground-water feed to 5.2 ppm in the system effluent (Figure 2). However, mass balance evaluation found approximately 20 percent of the HMW PAH associated with residues in the flow lines, biomass, and reactor sludges.

When nonspecific (indigenous) microorganisms were used, bioreactor performance was much reduced. It was consistently observed that indigenous microbes were unable to degrade PCP. Upon the addition of the PCP-degrading bacterium (*Pseudomonas* sp. strain SR3) to the bioreactor system, however, the concentration of PCP in ground-water feed was reduced from 256 ppm to 31 ppm in the bioreactor effluent. Hence, in the absence of SR3 inoculation, recovery of PCP from the bioreactor system could be used as a surrogate standard demonstrating an 85 to 90 percent recovery of the chemical tracer.

Acknowledgements

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Case Study on Site Characterization at a TCE Plume: St. Joseph, Michigan, NPL Site

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A plume of TCE in a sandy water table aquifer originates from an industrial facility in St. Joseph, Michigan. The origin of the plume is on a ground-water divide. The plume bifurcates; part drains to the east and part to the west. Based on concentrations of compounds in conventional monitoring wells, bioremediation through co-oxidation by methane-oxidizing bacteria was proposed as the remedy for the west plume. To calibrate a design model for bioremediation, it was necessary to estimate the average concentration of TCE and its dechlorination products in the ground water, and the flux of TCE and dechlorination products along the plume.

Ground-Water Sampling

Estimates of contaminant concentrations were obtained from two transects extending across the plume roughly perpendicular to the flow of ground water. To sample ground water, an auger slotted over the first 5 ft of its length was drilled into the earth. Starting at the water table about 40 ft below land surface, the auger was advanced 5 ft, water was pumped until conductivity and redox stabilized, and samples were taken for chemical analysis; then the auger was advanced another 5 ft to take the next sample. This depth-discrete sampling was continued to the bottom of the aquifer 80 ft below the land surface. Borings were spaced along the transects at intervals of 20 to 40 ft. Both transects extended all the way across the plume.

Estimates of Contaminant Flux

Concentrations in the plume were estimated by averaging the individual depth-discrete samples. More than 50 such samples were included in each transect. The hydraulic gradient was multiplied by the hydraulic conductivity to determine the flow of water in the plume. The flow was multiplied by the concentrations of contaminants to estimate the flux. The flux of TCE off the industrial facility was 60 kg/year. The flux of cis DCE and vinyl chloride was 71 and 11 kg/year, respectively.

Implications for Bioremediation

The design model strongly indicated that bioremediation could not reach acceptable concentrations for TCE or cis DCE when methane was used as the primary feedstock for aerobic biotransformation. As a result, plans for a pilot-scale evaluation of bioremediation were abandoned. This case study illustrates the importance of site characterization for proper implementation of bioremediation. If data from the conventional monitoring wells had been used to design the remedy, it would not have performed as expected.

Approaches to the Characterization of Trichloroethylene Degradation by *Pseudomonas Cepacia* G4

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Gulf Breeze, FL

Introduction

In the presence of aromatic compounds, *Pseudomonas cepacia* G4 expresses a novel pathway for the metabolism of toluene (4). The first two steps of the pathway are mediated by toluene *ortho*-monooxygenase (Tom, Figure 1), an enzyme that also attacks trichloroethylene (TCE). To determine the genetic location of the Tom gene for further study, a series of Tn5 mutants was made in *P. cepacia* G4 (Table 1). One of the mutants, *P. cepacia* G4 5223, lost the ability to degrade TCE, but gave rise to spontaneous "revertants" (e.g., strain Phe1), which express Tom activity regardless of the presence of aromatics (2,3). While the use of *P. cepacia* G4 in the bioremediation of TCE-contaminated sites is limited by its requirement for aromatic compounds to induce Tom activity, *P. cepacia* G4 5223 Phe1 has no such require-

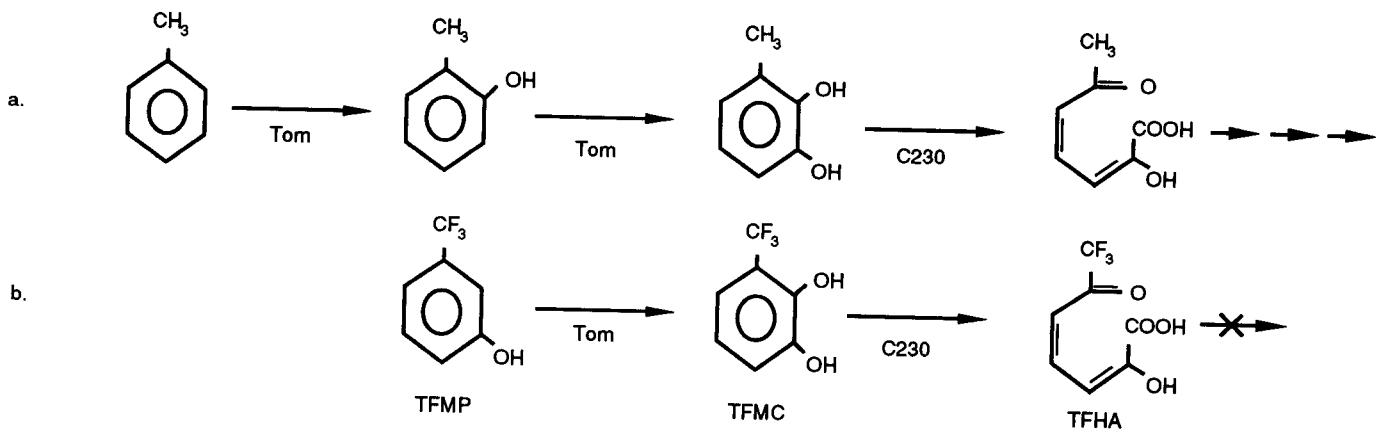

Figure 1. *The pathway of toluene utilization in* ***P. cepacia****. a. Toluene is hydroxylated first to ortho-cresol then to 3-methylcatechol via toluene ortho-monooxygenase (Tom). The ring is then cleaved by catechol-2,3-dioxygenase (C230). b. Trifluoromethylphenol (TFMP) is hydroxylated to trifluorom ethylcatechol (TFMC) via Tom activity, after which the ring is cleaved by C230 to yield 2-hydroxy-6-oxo-7,7,7-trifluoroheptadienoic acid (TFHA), which is not further metabolized by* ***P. cepacia*** *G4. Tom activity is responsible for TCE co-metabolism in* ***P. cepacia****. The appearance of TFHA (bright yellow color) from TFMP indicates the presence of both Tom and C230 activities.*

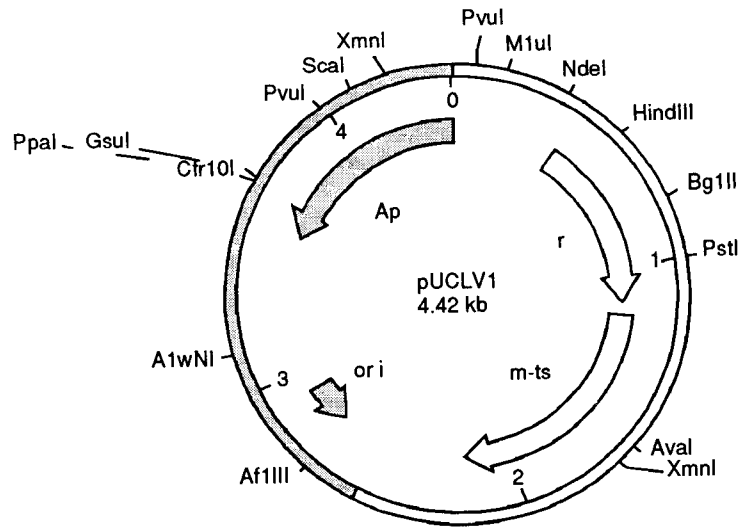

Figure 2. *Restriction map of pUCLV1 showing positions of the EcoRI gene (r), the temperature sensitive EcoRI methylase (m-ts), the ß-lactamase gene (Ap), and the origin of replication (ori). Portions of the vector derived from pUC19 are shaded. Portions of the vector derived from pLV59 are unshaded.*

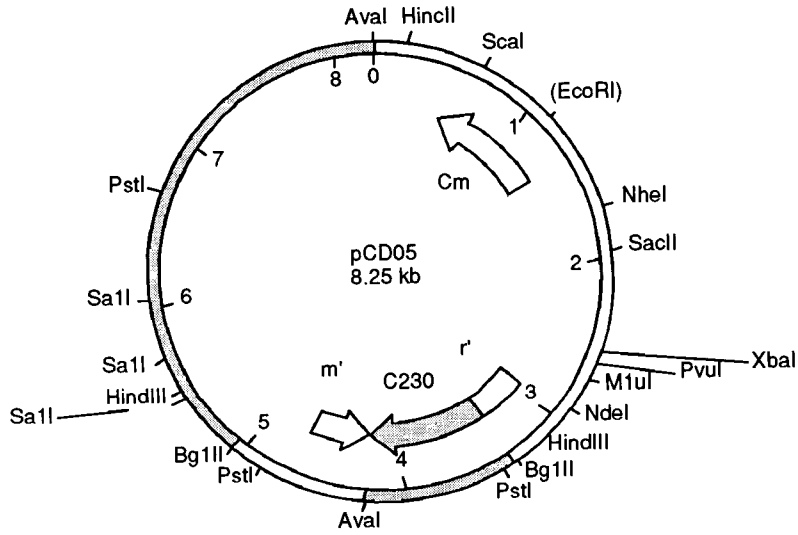

Figure 3. *Restriction map of pCDO5 showing positions of the chloramphenicol acetyltransferase gene (Cm), remnants of the EcoRI gene (r ') and the EcoRI methylase (m '), and the catechol-2,3-dioxygenase gene of **P. cepacia** (C230). Portions of the plasmid derived from **P. cepacia** DNA are shaded. Portions of the plasmid derived from pLV59 are unshaded.*

ment. Phe1, therefore, provides significant advantages over other bacteria for the bioremediation of TCE-contaminated aquifers (2). Further study of the genetic basis for TCE degradation in *P. cepacia* G4 and the mechanism of Tom expression in Phe1 is necessary to optimize their potential for application to bioremediation.

Results and Discussion

P. cepacia G4 and its derivatives carry two cryptic plasmids of approximately 50 and 150 kb. In an effort to enhance the fitness of Phe1 for use in bioremediation, a third plasmid, pRO101, was introduced via conjugation. Plasmid pRO101, which carries the genes of the 2,4-D degradative pathway, was intended to enable Phe1 to eliminate potentially toxic intermediates from the metabolism of halogenated aromatics that it would be likely to encounter in polluted aquifers. Subsequent plasmid assays, however, indicated that some recipients of pRO101 had lost the large resident plasmid (pPhe1L). Those organisms, designated Phe1 cure, lost the ability to degrade TCE and to transform trifluoromethylphenol (TFMP) to trifluoroheptadienoic acid (TFHA), a colorimetric reaction used to detect Tom activity (Figure 1). Phe1 cure remained resistant to kanamycin, however, suggesting that

Table 1. *Relevant Characteristics of P. cepacia G4 and Tn5-derivative Strains.*

Designation	TCE [a]	TFMP [b]	Stability [c]	Phenotype	Putative Tn5 Location
P. cepacia G4	*+i* [d]	*+i*	*N/A*	*N/A*	*N/A*
P. cepacia G4 5220	*+i*	*-*	*stable*	*C230⁻*	*cdo*
P. cepacia G4 5223	*-*	*-*	*yields TCE +c*	*Tom⁻*	*tom regulatory*
P. cepacia G4 5227	*-*	*-*	*stable*	*Tom⁻*	*tom*
P. cepacia G4 5223 Phe1	*+C* [e]	*+C*	*stable*	*Tom+c*	*unknown*

[a] Ability to degrade trichloroethylene

[b] Ability to produce hydroxytrifluoroheptadienoic acid from trifluoromethylphenol.

[c] Relative stability of phenotype after several passages on a nonselective medium.

[d] Inducible activity.

[e] Constitute activity.

Tn5 does not reside on the large plasmid in Phe1. Matings between wild-type G4 and Phe1 cure yielded putative Phe1 recipients of the large plasmid from G4 (pG4L), which had regained wild-type (inducible) activity against TCE. Clearly, the large plasmid plays a role in toluene metabolism and TCE co-metabolism in *P. cepacia* G4 and its derivatives. However, there may also be involvement of the chromosome and/or the smaller plasmid, as indicated by loss of TCE activity in strain 5223 (parent of Phe1), which does not seem to carry the Tn5 insertion on the large plasmid.

While Phe1 is currently being tested for its ability to remove TCE from contaminated water, efforts are also being directed toward cloning genes involved in Tom activity from plasmid and chromosomal DNAs. The advantages of cloned Tom activity include the ability to alter expression of the gene, to place the gene in a well-defined genetic background and the potential to produce purified enzyme(s) for specialized field applications. To facilitate screening for Tom activity in potential clones, a novel host-vector system has been constructed. The vector, pUCLV1 (Figure 2), carries the *Eco*RI endonuclease and methylase genes from pLV59 (1), and the ß-lactamase gene and replication origin of pUC19 (5). The *Eco*RI methylase gene is temperature sensitive and functions only at temperatures below 28°C. At high temperatures, therefore, the methylase is inactive, and the cell is rapidly killed due to the expression of *Eco*RI. *Eco*RI expression can be inactivated by cloning into one of three unique restriction sites within the *Eco*RI gene. The result is a very tightly controlled selection against nonrecombinant transformants on a high copy-number plasmid. High copy number was considered desirable in the cloning vector to increase gene dosage and, therefore, to increase the chances of detecting a weakly expressed gene product.

The host cell for cloning is an *E. coli* strain that carries the gene for catechol-2,3-dioxygenase (C230, see Figure 1) on a low copy-number plasmid (pCDO5). Plasmid pCDO5 (Figure 3) is an over-producer of C230, which carries the Cm^r gene and origin of replication from pLV59. The high level of expression of the C230 gene is fortuitous in that it will facilitate the detection of Tom activity carried on the primary cloning vector.

In this system, Tom activity carried on the cloning vector can be detected by the conversion of TFMP to trifluoromethylcatechol (TFMC). TFMC is, in turn, a substrate for the C230 expressed by pCDO5, producing the bright yellow-ring cleavage product TFHA. Thus, the combination of plasmids pUCLV1 and pCDO5 allow for a rapid and sensitive detection of recombinant Tom activity. While *P. cepacia* G4 5223 Phe1 has strong potential for immediate use in bioremediation, the use of this new host-vector system to isolate and characterize the gene involved in TCE removal may provide even more powerful ways to address the problem in the future.

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Section Two
Bioremediation Field Initiative

The Bioremediation Field Initiative is one of the major research components of EPA's Bioremediation Research Program. The initiative was undertaken in 1990 to expand the nation's field experience in bioremediation techniques. Objectives of the initiative are to more fully document the performance of full-scale applications of bioremediation; to provide technical assistance to site managers; and to provide regular information on treatability studies, design, and operation of bioremediation projects, including cost and performance data. The initiative is currently tracking bioremediation activities at more than 130 Superfund sites, RCRA corrective action facilities, and Underground Storage Tank sites nationwide.

Full-scale performance evaluation is the subject of several recent projects. At a Montana Superfund site, researchers studied the bioremediation of contaminated ground water and soil both *in situ* and in aboveground facilities.

EPA researchers reported results of the first 6 months of a 2-year field study of bioremediation using bioventing begun in 1991 at two sites in differing climatic zones: near Fairbanks, Alaska, and near Salt Lake City, Utah. Bioventing is the process of supplying oxygen *in situ* to soil microbes to stimulate biodegradative activity with the goal of destroying toxic compounds in the ground.

A treatability study and design assessment examined the use of nitrate for *in situ* bioremediation of an aquifer contaminated with petroleum hydrocarbons. Results showed that remediation is controlled by the rate of application of nitrates and microbial reaction rates. Researchers recently studied a method for delivering methane and oxygen nutrients directly to a microbial population for *in situ* bioremediation of an aquifer contaminated with trichloroethylene. Another recent project involved a full-scale site characterization and treatability study of trichloroethylene in a ground-water plume and the potential for enhanced anaerobic rather than aerobic biological transformation.

A demonstration project at a confined treatment facility (CTF) is simulating field conditions on the rate and extent of polychlorinated biphenyl (PCB) biotransformation under varied biotreatment scenarios. In another project, researchers are characterizing the nutritional, physiological, and environmental factors that enhance the growth of desired microbial populations for dechlorinating hazardous wastes. EPA is also investigating the effects of toxic heavy metals on the anaerobic biotransformation of organic compounds.

Progress in the Field Applications of Bioremediation

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and

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The U.S. Environmental Protection Agency (EPA) has developed an initiative to expand the nation's field experience in the use of bioremediation. The EPA's Office of Solid Waste and Emergency Response and the Office of Research and Development have instituted the Bioremediation Field Initiative to provide solid performance data to sufficiently define the capabilities of this technology. The initiative provides assistance to the Regions and states in conducting field tests and carrying out independent evaluations of site cleanups using bioremediation. Sites presently considered in this initiative include Superfund, RCRA corrective action facilities, and Underground Storage Tank (UST) sites. This initiative has three objectives. The first is to more fully document performance on full-scale applications of bioremediation. Seven field evaluations are being conducted on petroleum, wood preserving, and solvent wastes contaminating soil and ground water. The second is to provide technical assistance to site managers for sites in a feasibility or design stage to facilitate conducting treatability studies, field pilot studies, and other studies through the Agency's Technical Support Centers. The third objective is to provide information regularly on treatability studies, design, and full-scale operations of bioremediation projects. Current cost and performance data will be available on the operation of biological treatment systems for a variety of wastes and contaminated matrices.

Evaluation of Full-Scale *In Situ* and *Ex Situ* Bioremediation of Creosote Wastes

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The bioremediation field initiative has three main objectives: (1) to document more fully the performance of full-scale bioremediation field applications in terms of treatment effectiveness, operational reliability, and cost; (2) to provide technical assistance to EPA and state agency site managers that are overseeing or considering the use of bioremediation; and (3) to develop a treatability data base that will be available through EPA's Alternative Treatment Technology Information Center (ATTIC). The performance evaluation project in Libby, Montana, focuses primarily on the first objective. Champion International Superfund Site in Libby, Montana, was nominated by the Robert S. Kerr Environmental Research Laboratory as a candidate site for performance evaluation.

The first phase of the bioremediation performance evaluation (which has been completed) is to summarize previous and current remediation activities. This summary is to be followed by identification of critical site characterization and treatment parameters that are important to evaluate bioremediation for each of the treatment units identified. The final objective is to evaluate bioremediation performance based on the information obtained.

Three biological treatment processes will be addressed: (1) surface soil bioremediation in a prepared-bed, lined land treatment unit (LTU); (2) oil/water separation of extracted ground water and treatment of the aqueous phase in an above-ground fixed-film bioreactor; and (3) *in situ* bioremediation of the upper aquifer at the site. Each biological treatment process will be addressed with regard to design, performance, and monitoring activities.

Two forms of wood preservative were used at the site: creosote and pentachlorophenol. Polycyclic aromatic hydrocarbons are the primary components of concern at the site and are associated with the soil phase primarily by adsorption. Contaminated soil from three primary source areas (tank farm, butt dip, and waste pit) has been excavated and moved to one central location (waste pit). The soil is pretreated in the waste pit area and is further treated in a prepared-bed, lined LTU. Planned activities associated with the field initiative are (1) statistical sampling of the soil treatment unit, (2) field-scale treatment kinetics, (3) toxicity reduction, (4) clean-up levels achieved, (5) influence of moisture and soil structure, and (6) mass balance of contaminants by soil and leachate analyses.

The upper aquifer ground-level treatment unit is provided for the separation of LNAPL and DNAPL from ground water extracted for subsequent biological treatment prior to reinjection via the infiltration trench. The subsequent biological treatment is two, fixed-film reactors that will be operated in series. The first reactor is for roughing purposes, while the second is for polishing and reoxygenation of the effluent prior to reinjection. Planned activities include (1) flow composited sampling, (2) evaluation and prediction of reactor performance, (3) analysis of biofilm dynamics, (4) mass balance of contaminants, and (5) treatment optimization.

The upper aquifer *in situ* bioremediation system involves the addition of hydrogen peroxide and inorganic nutrients to stimulate the growth of contaminant-specific microbes (Figure 1). Planned activities include (1) dissolved oxygen profiles, (2) aquifer material sampling to distinguish abiotic and biotic effects, (3) dissolved oxygen uptake evaluation and correlation to the rate of biodegradation, and (4) toxicity reduction.

The current status of the three biological treatment processes is as follows.

Above-Ground Bioreactor

The preliminary performance analysis of the existing bioreactors indicates the following: (1) removal of PAHs and PCP is strongly influenced by flow rate through the reactor (10 gallons per minute versus 15 gallons per minute); (2) areas within the reactor may be anaerobic as indicated by dissolved oxygen measurements taken; (3) removal of PAHs (80 to 90 percent) is more efficient than removal of PCP (40 to 80 percent); (4) nutrients in the form of nitrogen and phosphorus, although added to the reactor, are not proportioned either to flow rate or to mass loading of contaminants, and are generally insufficient; (5) it was possible to increase the removal of PCP within the bioreactor by lowering the flow rate through the reactor from 15 gpm to 10 gpm.

A pilot-scale bioreactor has been designed and constructed for physical placement beside the full-scale bioreactor at the site. The pilot-scale reactor will simulate the behavior of the full-scale reactor and allow modification of nutrients, flow rate, and dissolved oxygen to evaluate the effect of process parameters, without interfering with the operation of the full-scale reactor. The pilot-scale bioreactor has been hydraulically tested in the

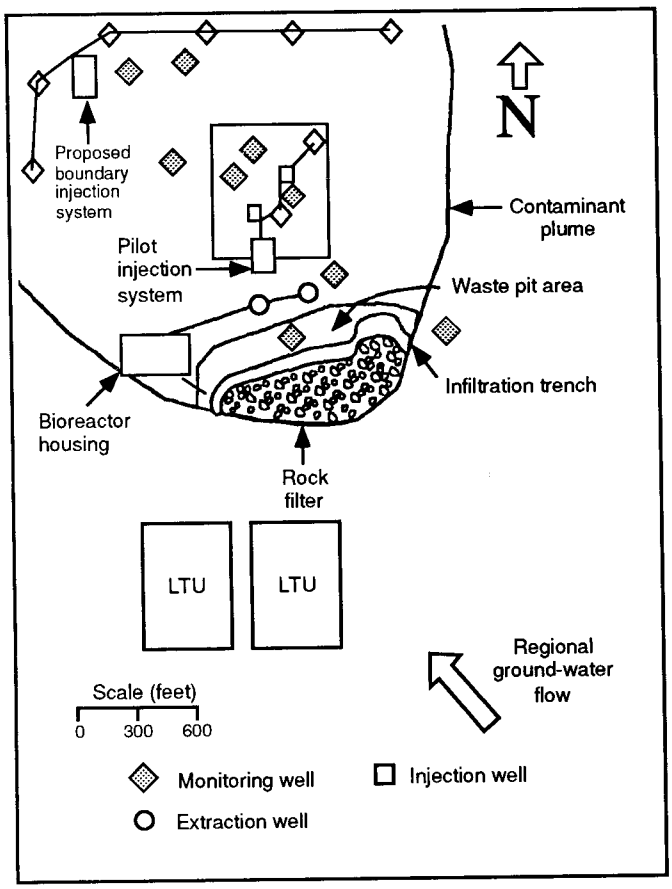

Figure 1. In situ bioremediation of upper aquifer (from Woodward Clyde).

laboratory and will be transported to the site in the spring of 1992.

In Situ Ground Water

Results of both sampling trips indicate that the aquifer materials below the site are in a chemically reduced condition (presence of reduced iron and manganese); therefore, the site has an abiotic oxygen demand in addition to any biological oxygen demand. This situation provides challenges to designing *in situ* evaluations for determining how much uptake of oxygen is due to microorganisms. Therefore, the absence of hydrogen peroxide in one well and the presence of oxygen in a downstream well that has received hydrogen peroxide upgradient may indicate that the abiotic oxygen demand, rather than the biological oxygen demand, has been met. It is likely that both abiotic and biotic demands for oxygen are occurring simultaneously.

No additional ground-water samples have been taken since October 1991 because of the onset of winter. Present plans are to take aquifer material in the spring (if possible) for PAH analysis and for reducing potential. A single-well, "push-pull" test, as well as a multiple-well test, will be performed to determine oxygen consumption at contaminated and background wells at the site.

Land Treatment Units

Total estimated contaminated soil volume for treatment is 45,000 yd^3 (uncompacted). Contaminated soil clean-up goals (dry-weight basis) are (1) 88 mg/kg total (sum of 10) carcinogenic PAHs, (2) 8 mg/kg naphthalene, (3) 8 mg/kg phenanthrene, (4) 7.3 mg/kg pyrene, (5) 37 mg/kg PCP, and (6) $\leq$ 0.001 mg/kg dioxin equivalency.

The LTU comprises two adjacent 1-acre cells. Each cell is lined with low-permeability materials to minimize leachate infiltration from the unit. When reduction of contaminant concentrations in all lifts placed in the LTU has reached an acceptable level, a protective cover will be installed over the total 2-acre unit and maintained in such a way as to minimize surface infiltration, erosion, and direct contact.

Contaminated soil is applied to and treated in lifts (approximately 9 in. thick) in the designated LTU cell until target soil contaminant levels are achieved for a given lift. Degradation rates, volume of soil to be treated, initial contaminant concentration, yearly degradation period duration, and LTU cell size determine time required for remediation of a given lift. Based on an estimated 45-day timeframe for remediation of each applied lift to acceptable contaminant levels, an estimated 45,000 yd^3 of contaminated soil, and a 2-acre total LTU surface area, projected time to complete soil remediation is 8 to 10 years.

The primary purpose of the LTU soil sampling programs being carried out in this project is to determine statistical significance, confidence of rate, and extent of contaminated soil biodegradation at this site. A quantitative expression for data variability is necessary to determine an accurate estimate of biodegradation of these contaminants **at field scale**. Such an expression will allow data generated to be used by others to help estimate biodegradation potential of similar types of wastes under similar conditions at other sites. A critical element in design of this project involves obtaining a good representation of mass balance for those compounds being monitored. Correspondingly, assessment of degradation intermediates, humification, leachate, soils, and air quality is planned. If Phase II sampling and analytical program design will allow tracking of target contaminants through the system, investigators can determine an accurate fate and transport profile that denotes biodegradation performance. Completion of other project activities, such as degradation kinetics and toxicity reduction studies, will generate data that can be used to help assess overall bioremediation effectiveness and, subsequently, help predict performance of bioremediation processes at other, similar sites.

Three field sampling events directed toward Phase II performance evaluation studies were completed during 1991. The first event, in early May, consisted of collecting soil core samples for analyses immediately following placement of treatment lift #3 (first 1991 lift; lifts #1 and #2 were applied in 1990) in LTU cell #1. A second sampling event in late June involved collection of 32 soil core samples during treatment process for lift #3. In late July, treatment lift #4 (second 1991 lift) was placed in LTU cell #1, and lift #1 was placed in cell #2. A third sampling event in late

September involved collection of 32 core samples from both LTU cell #1 and LTU cell #2. All 32 core samples collected for each sampling event penetrated to the bottom of treatment lift #1.

Performance Evaluation Data–Year 1

Data on the concentrations of pentachlorophenol are presented in Table 1, on phenanthrene in Table 2, on pyrene in Table 3, and on total carcinogenic PAHs in Table 4. Data currently available from the three 1991 LTU sampling events are presented. Full results from these sampling events will be available after data quality checks are completed for the remaining data.

These data indicate that the variability inherent in lift application and sampling is high enough to account for any differences between discrete sampling regimes, as in the performance evaluation, and composite sampling regimes, as in the operations monitoring.

It may be noted that the pentachlorophenol concentrations have a lower variability in lifts #1 and #2 than in lift #3, applied just before sampling. Lifts #1 and #2 had been tilled many times since application, and perhaps this tilling had mixed the soil sufficiently to reduce the spatial heterogeneity of the PCP concentrations. No doubt some of the lower variability is due to the lower concentrations of PCP in lifts #1 and #2 at the sampling date being considered. If it is assumed that the initial concentrations of PCP at application were similar for all lifts, then there is some indication that the PCP concentrations are being reduced.

Phenanthrene concentrations are lower than treatment levels, except for a small number of the discrete samples. One high value in lift #1 (151 mg/kg) markedly increases the variability of the data in this lift. Otherwise, phenanthrene levels were low even immediately after application of the lift (lift #3).

Pyrene concentrations in the LTU are somewhat more variable than pentachlorophenol and phenanthrene concentrations. This may be partly due to the tendency of the larger molecule to be more strongly adsorbed on the soil, causing the measured concentrations to be more sensitive to variations in the efficiency of the extraction step in the analytical process. The concentrations in lifts #1 and #2 are clustered near the treatment level, with many just above or just below the treatment level. The reduction in concentration is apparently good, especially in lifts #1 and #3. Since lift #2 concentrations were low at application of the lift, the reduction is not as apparent as in lifts #1 and #3.

The variability of the total carcinogenic PAHs concentration, being the sum of the variability of 10 individual components, is high. The data variability precludes any definitive conclusion concerning concentration reductions for this parameter. Most of the concentrations are around the 88 mg/kg treatment level.

Table 1. Pentachlorophenol Concentrations In The Libby Soil Treatment Facility (mg/kg)

Sampling Date 5/6/91									
Sampling Location		Feet South							
Feet East	Sample Depth	0	28.3	56.6	84.9	113.2	141.5	169.8	198.1
84.9	Lift 3	229	313	500	49	0	43	435	169
	Lift 2	30	155	33	12	12	144	79	9
	Lift 1	76	9	29	33	30	147	19	7
56.6	Lift 3	257	217	42	167	303	325	761	275
	Lift 2	4	15	21	0	0	58	40	15
	Lift 1	31	28	38	17	29	380	94	9
28.3	Lift 3	189	321	367	204	440	336	1243	1121
	Lift 2	0	9	21	17	11	11	35	2
	Lift 1	42	17	35	28	64	27	34	52
0	Lift 3	275	891	798	710	212	55	667	663
	Lift 2	226	26	177	18	24	12.5	67	29
	Lift 1	156	13	165	176	136	24	18	56

Table 2. Phenanthrene In The Libby Soil Treatment Facility (mg/kg)

Sampling Date 9/18/91

Sampling Location		Feet South							
Feet East	Sample Depth	0	28.3	56.6	84.9	113.2	141.5	169.8	198.1
		32	31	30	29	28	27	26	25
84.9	Lift 4	5.6	6.6	6.4	4.4	7.4		7.5	10.9
	Lift 3	1.7	2.6	0.9	1.4	2.0	ND	4.0	3.1
	Lift 2	0.2	4.1	1.0	3.6	0.6	ND	2.7	7.0
	Lift 1	0.3	1.9	ND	0.8	0.6	ND	3.8	ND
		17	18	19	20	21	22	23	24
56.6	Lift 4	3.1	5.6	6.3	7.2	10.2	8.7	10.5	4.5
	Lift 3	2.8	4.3	0.9	3.9	2.8	6.8	2.8	7.0
	Lift 2	1.6	1.6	2.7	8.1	4.7	2.1	1.2	4.8
	Lift 1	1.2	2.6	1.0	3.0	0.2	1.6	4.2	1.6
		16	15	14	13	12	11	10	9
28.3	Lift 4	12.0	4.8	3.8	2.4	4.9	3.3	ND	4.3
	Lift 3	3.3	0.8	3.7	ND	ND	3.0	ND	2.5
	Lift 2	13.0	1.6	ND	ND		1.7	ND	1.1
	Lift 1	2.7	1.4	3.5	3.1	2.8	3.7	1.4	1.9
		1	2	3	4	5	6	7	8
0	Lift 4	1.4	3.0	2.8	ND	ND	2.7	4.7	4.0
	Lift 3	0.2	0.9	2.9	ND	ND	0.8	2.3	ND
	Lift 2	6.1	2.9	4.6	0.2	2.0	ND	2.2	ND
	Lift 1	ND	2.5	3.3	5.5	3.6	3.5	3.5	1.6

Table 3. Pyrene In The Libby Soil Treatment Facility (mg/kg)

Sampling Date 5/6/91

Sampling Location		Feet South							
Feet East	Sample Depth	0	28.3	56.6	84.9	113.2	141.5	169.8	198.1
		32	31	30	29	28	27	26	25
84.9	Lift 4								
	Lift 3								
	Lift 2								
	Lift 1								
		17	18	19	20	21	22	23	24
56.6	Lift 4								
	Lift 3								
	Lift 2								
	Lift 1								
		16	15	14	13	12	11	10	9
28.3	Lift 4								
	Lift 3	79.4	159.6	75.9	92.7	118.0	82.0	105.3	110.0
	Lift 2	ND	23.7	17.1	13.4	10.1	7.9	ND	10.8
	Lift 1	266.6	26.4	3.8	14.4	ND	18.8	ND	9.1
		1	2	3	4	5	6	7	8
0	Lift 4								
	Lift 3	8.9	62.7	95.9	66.1	39.6	80.1	28.4	44.9
	Lift 2	11.3	13.3	15.4	ND	19.8	ND	17.2	6.1
	Lift 1	23.3	3.3	8.1	28.2	14.3	133.6	30.7	ND

Table 4. Total Carcinogenic PAHs In The Libby Soil Treatment Facility (mg/kg)

Sampling Date 9/18/91

Sampling Location		Feet South							
Feet East	Sample Depth	0	28.3	56.6	84.9	113.2	141.5	169.8	198.1
		32	31	30	29	28	27	26	25
	Lift 4	352	194	187	131	241		282	247
84.9	Lift 3	53	134	101	83	51	78	79	228
	Lift 2	47	81	84	93	59	66	60	206
	Lift 1	38	74	86	53	35	19	105	23
		17	18	19	20	21	22	23	24
	Lift 4	165	352	250	317	201	192	241	110
56.6	Lift 3	165	217	42	160	115	187	88	172
	Lift 2	94	94	117	90	114	129	75	174
	Lift 1	35	232	43	102	22	116	104	117
		16	15	14	13	12	11	10	9
	Lift 4	223	254	150	130	223	172	185	244
28.3	Lift 3	108	370	106	159	193	149	229	151
	Lift 2	357	86	51	124		72	220	71
	Lift 1	116	92	71	131	125	113	68	114
		1	2	3	4	5	6	7	8
	Lift 4	44	187	128	151	17	111	211	134
0	Lift 3	81	120	136	63	19	131	150	113
	Lift 2	143	154	274	59	88	172	113	71
	Lift 1	23	116	188	119	151	192	212	68

Optimizing Bioventing in Shallow Vadose Zones and Cold Climates: Eielson AFB Bioremediation of a JP-4 Spill

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Introduction

Bioventing is the process of supplying oxygen *in situ* to oxygen-deprived soil microbes by forcing air through unsaturated contaminated soil at low flowrates (1). Unlike soil venting or soil vacuum extraction technologies, bioventing attempts to stimulate biodegradative activity while minimizing stripping of volatile organics, thereby destroying the toxic compounds in the ground. Bioventing technology is especially valuable for treating contaminated soils in areas where structures and utilities cannot be disturbed because the bioventing equipment (air injection/withdrawal wells, air blower, and soil gas monitoring wells) is relatively noninvasive.

The U.S. EPA Risk Reduction Engineering Laboratory, with resources from the U.S. EPA Bioremediation Field Initiative, began a 2-year field study of *in situ* bioventing in the summer of 1991 in collaboration with the U.S. Air Force at Eielson Air Force Base (AFB) near Fairbanks, Alaska. The site has JP-4 jet fuel-contaminated unsaturated soil where a spill occurred in association with a fuel distribution network. The contractor operating the project is Battelle Laboratories, Columbus, Ohio. With the pilot-scale experience gained in these studies and others, bioventing should be available in the very near future as a reliable, inexpensive, and unobtrusive means of treating large quantities of organically contaminated soils. The following is a report on progress through January 1992.

Methodology

At Eielson AFB, we are studying bioventing in shallow soils in a cold climate in conjunction with soil-warming methods to enhance the average biodegradation rate during the year. Roughly 1 acre of soil is contaminated with JP-4 from a depth of roughly 2 ft to the water table at 6 to 7 ft. Initial (prebioventing) soil gas measurements taken in July 1991 ranged from 600 to 40,000 ppm total hydrocarbons, 0 to 13 percent O_2, and 10 to 18 percent CO_2, indicating oxygen-limited biological activity and a high degree of contamination. Thus, addition of oxygen as air to the site would be expected to increase the rate of biodegradation. In comparison, dry atmospheric air composition contains 20.9 percent O_2 and 0.03 percent CO_2.

The test area was established by laying down a relatively uniform distribution of air injection/withdrawal wells and constructing three 50-ft square test plots within this test area (see Figure 1). Air is injected from 2 to 6 ft below grade at a rate of 2.5 ft^3/min/well, spaced 30 ft apart. Thus, the test plots should receive relatively uniform aeration. One plot is a control, i.e., bioventing only (no heating). Two plots are being used to evaluate the following two strategies of combining bioventing with warming of the soil above ambient temperature to increase the rate of biodegradation year-round:

- *Passive warming*: enhanced solar warming in late spring, summer, and early fall using plastic covering (mulch) over the plot and passive heat retention the remainder of the year by applying insulation on the surface of the plot.

- *Active warming*: warming by applying heated water from soaker hoses 2 ft below the surface. Water is applied at roughly 35°C and at an overall rate to the plot of roughly 1 gal/min. Five parallel hoses 10 ft apart deliver the warm water. The surface is covered with insulation year-round.

In addition to the network of air injection/withdrawal wells, three-level soil gas monitoring wells at 2.5, 4.5, and 6 ft below grade, and three-level temperature probes at 2.5, 4, and 5 ft below grade were installed to provide independent measurement at three depths throughout the site. The venting of air and the trickling of unheated water to the actively warmed plot began in September 1991. Warming of the water began in October 1991. A plan view of the installation is presented in Figure 1. A cross-section of a portion of the actively warmed plot is shown in Figure 2. An air injection well, a soil gas monitoring well, and a temperature probe were installed in a nearby uncontaminated area of similar geologic structure east of the site shown in Figure 1 for a background control.

Periodically, *in situ* respirometry tests (2) are conducted to measure the *in situ* oxygen uptake rates by the microorganisms. These tests involve temporarily (4 to 8 days) shutting off the air and monitoring the soil gas oxygen concentration with time. Oxygen uptake due to oxygen demands other than biological activity is calculated by conducting a parallel shutdown test in the background (uncontaminated) area. The rate of decrease in oxygen concentration with time, relative to the rate observed in the background area, indicates a relative biodegradation rate. Thus, these tests allow estimation of the biodegradation rate as a function of time, and therefore, as a function of ambient temperature and soil-warming technique. Quarterly comprehensive and monthly abbreviated *in situ* respiration tests are planned. Final soil hydrocarbon analyses will be conducted in summer 1993 and

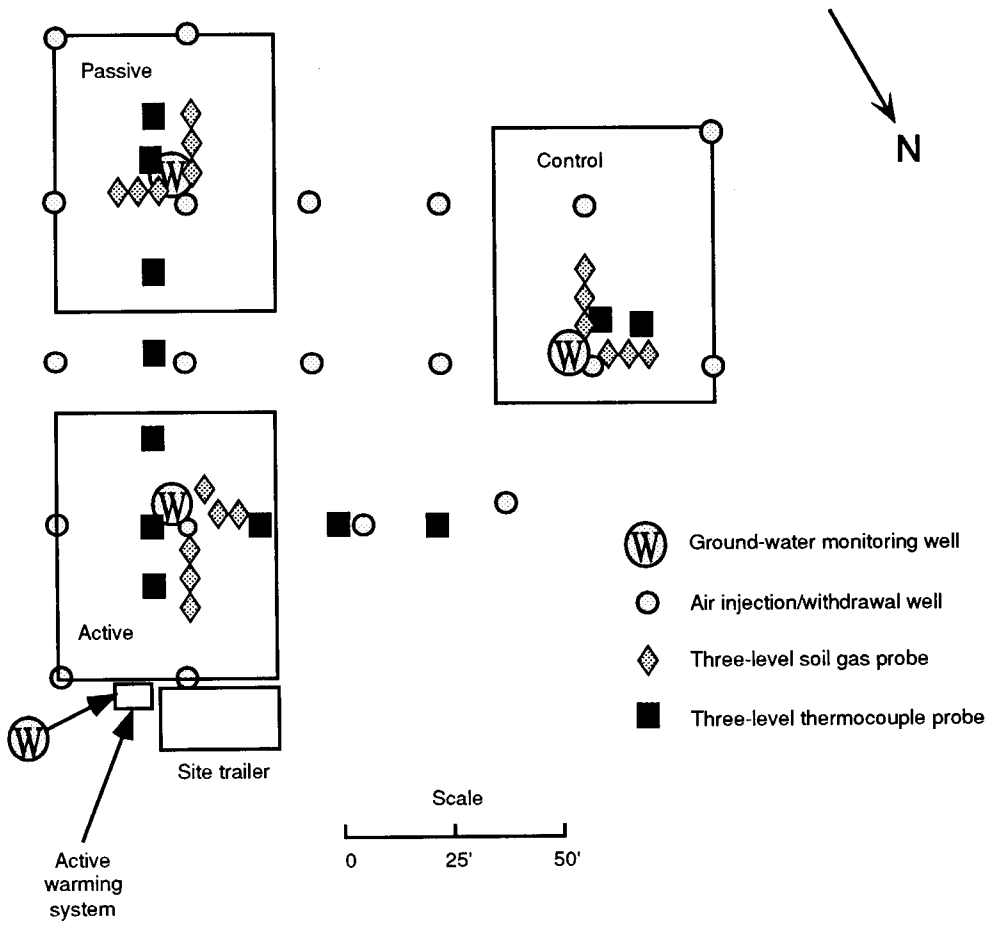

Figure 1. *Plan view of the joint U.S. EPA and U.S. Air Force bioventing activities at Eielson AFB, near Fairbanks, Ak.*

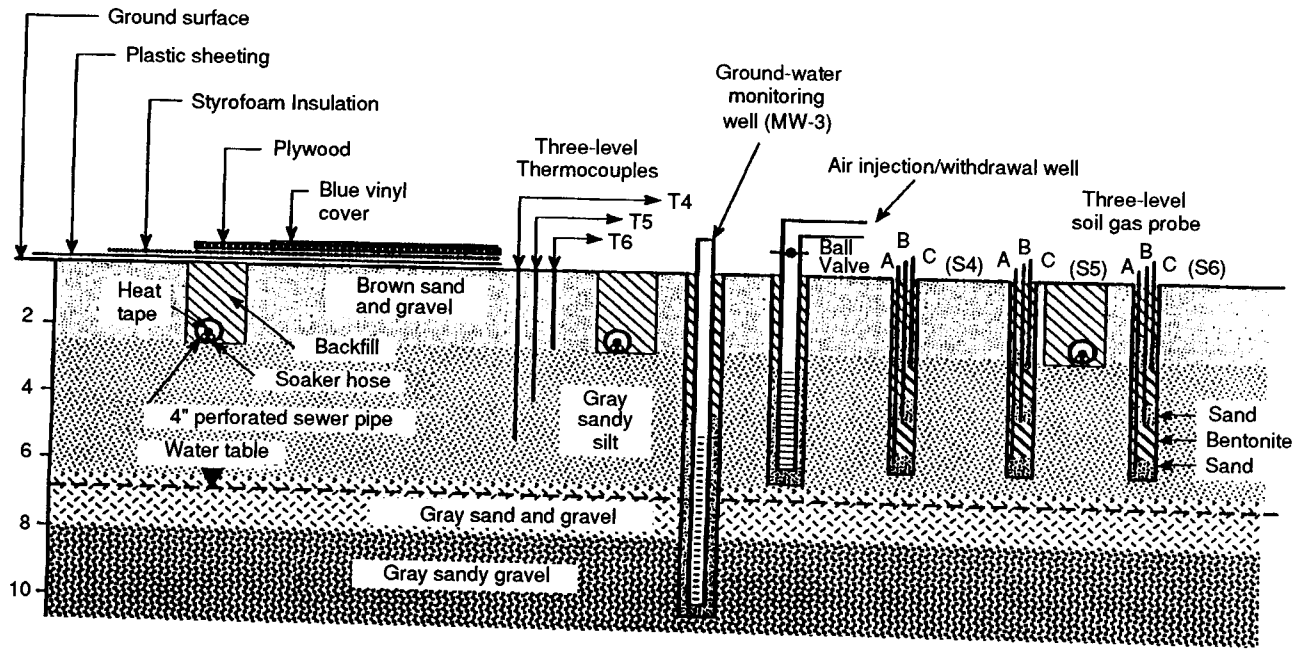

Figure 2. *Cross-sectional view of a portion of the actively warmed test plot at Eielson AFB showing the various wells, the temperature monitoring devices, the heated water delivery system and the ground coverings. Vertical axis is depth from the surface in feet.*

compared with the initial soil analysis to document actual hydrocarbon loss due to bioventing.

Results

Progress to date includes installation of the pilot-scale equipment and initial soil sampling for total hydrocarbons during July and August 1991, continuous soil temperature monitoring since August 25, 1991, weekly soil gas monitoring, and *in situ* respiration tests conducted periodically since early October 1991. As of early January 1992, all test plots were being adequately aerated. The active, passive, and control plots, and the background location showed average soil gas oxygen levels of 12, 20, 15, and 20 percent, respectively. Soil gas oxygen levels should be maintained above at least 5 percent oxygen to avoid oxygen limitation of microbial activity.

Figure 3 shows average soil temperatures for each plot and the background location as a function of time through early January 1992. Clearly, the active-warming strategy is functioning successfully: as ambient temperatures fell during the fall to an average winter value of about -20°C, the actively warmed plot remained above 10°C (except for a short period near 8°C), while the temperature in the other plots and the background dropped steadily toward 0°C.

The passively warmed plot is not maintaining a significantly higher temperature than the control plot. However, this plot did not benefit from a summer of enhanced solar heating using the plastic mulch because installation and startup occurred late in the summer. Insulation, not mulch, was laid down during the installation in anticipation of the upcoming winter. The success of the passive heating strategy will be evaluated during the winter of 1993 after a full season of solar heating.

The temperatures in Figure 3 are reflected in the measured oxygen uptake rates and biodegradation rates from an *in situ* respirometry test conducted in early December 1991. The results shown in Table 1 reveal the biodegradation rate in the actively warmed plot is at least twice the rate in either of the other two plots. In addition, the biodegradation rate in the actively warmed plot of 2.9 mg/kg/day is consistent with biodegradation rates observed in other bioventing studies at various locations (1). The conversion from oxygen uptake rates to biodegradation rates is accomplished by assuming that the biodegradation reaction follows the stoichiometry in Equation 1:

$$C_6H_{14} + 9.5O_2 = 6\,CO_2 + 7H_2O \qquad (1)$$

The biodegradation rate associated with the control plot was greater than the rate associated with the passively warmed plot. This difference is unexplainable at this time. Another year of data will be needed to determine if this difference is a consistent trend or random variation.

Reduced water flow rate from the buried soaker hoses due to the accumulation of silt around the hoses caused inefficient performance of the actively warmed plot in its early operation. The low water flow rate resulted in the steady decline of the temperature between Day 30 and 56 (Figure 3). Our goal is to maintain the soil in the actively warmed plot between 10° and 20°C in the winter. To remedy this situation, weekly high-pressure pulses are employed to clear the hoses. The pulsing began on Day 56 (noted in Figure 3) and resulted in increased soil temperature. Discovering the water flow rate that maximizes heating and maintains adequate soil oxygen levels will be an important outcome of this field study.

Table 1. *Results of an In Situ Respiration Test in Early December 1991 at Eielson AFB*

Plot	Oxygen uptake rate (% O_2/hr)	Biodegredation rate (mg/kg-day)
Actively warmed	*0.15*	*2.9*
Passively warmed	*0.039*	*0.74*
Control	*0.071*	*1.4*
Background	*0*	*0*

Biodegradation rate is calculated from the oxygen uptake rate assuming the reaction stoichiometry in Equation 1.

Conclusions

This paper summarizes the first 6 months of a 2-year joint U.S. EPA and U.S. Air Force study of *in situ* bioventing. Already, the work at Eielson AFB has shown that the active soil-warming techniques are successful at maintaining soil at summer-like temperatures during cold (winter) ambient temperatures. The elevated temperatures have allowed at least a doubling of the biodegradation rate in the heated plot relative to the other test plots. The most efficient means of delivering the warm water to avoid blockage of the buried hoses and the optimal water and air flowrates that provide adequate warming and aeration continue to be investigated. Additional data over the next 1-1/2 years are required to fully evaluate the soil warming strategies and bioventing at Eielson AFB. Operational costs of the soil warming techniques will be evaluated at the completion of the study.

These bioventing studies are generating valuable pilot-scale performance data and operational experience for a technology that in the near future could provide a very economical means of *in situ* cleanup of organically contaminated unsaturated soils. In addition, the soil warming techniques investigated here will be applicable to enhancing biological treatment rates of unsaturated soils for any bioremediation technology at any location where a significant portion of the year is too cold to allow satisfactory biological activity.

We thank Dr. Andrea Leeson for preparing the graphical portions of the paper.

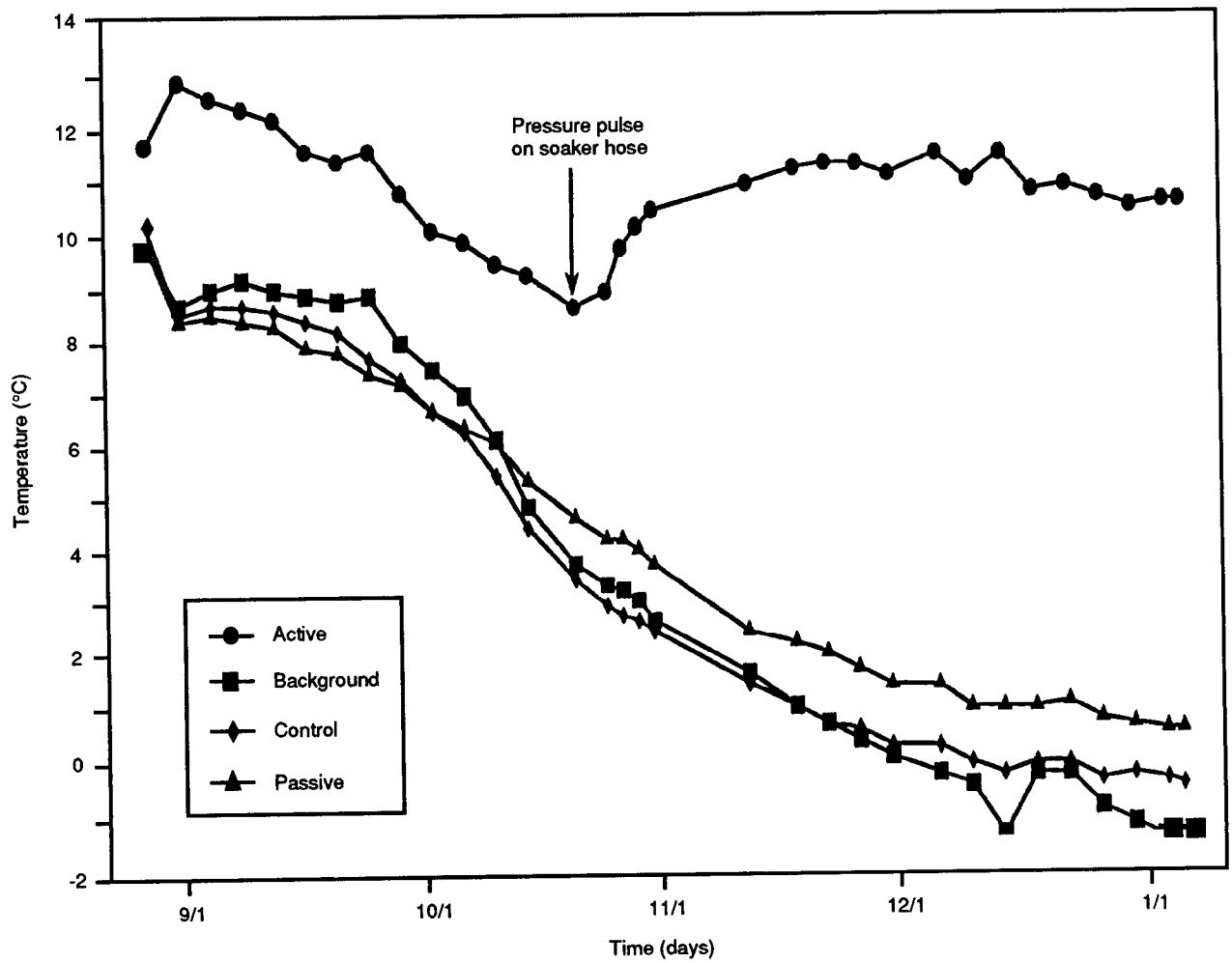

Figure 3. *Average temperature of each test plot and background as a function of time at Eielson AFB. Time scale is date beginning in late August 1991 through early January 1992.*

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1. R.E. Hoeppel, R.E. Hinchee, and M.F. Arthur. 1991. Bioventing soils contaminated with petroleum hydrocarbons. J. Indust. Microbiol. 8:141.

2. S.K. Ong, R.E. Hinchee, R. Hoeppel, and R. Schultz. 1991. *In situ* respirometry for determining aerobic degradation rates. In *In Situ* Bioreclamation, R.E. Hinchee and R. F. Olfenbuttel, eds. Butterworth-Heinemann, Boston, pp. 541-545.

Optimizing Bioventing in Deep Vadose Zones and Moderate Climates: Hill AFB Bioremediation of a JP-4 Spill

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Introduction

Bioventing is the process of supplying oxygen *in situ* to oxygen deprived soil microbes by forcing air through unsaturated contaminated soil at low flow rates (1). Unlike soil venting or soil vacuum extraction technologies, bioventing attempts to stimulate biodegradative activity while minimizing stripping of volatile organics, thus destroying the toxic compounds in the ground. Bioventing technology is especially valuable for treating contaminated soils in areas where structures and utilities cannot be disturbed because bioventing equipment (air injection/withdrawal wells, air blower, and soil gas monitoring wells) is relatively noninvasive.

The U.S. EPA Risk Reduction Engineering Laboratory, with resources from the U.S. EPA Bioremediation Field Initiative, began a 2-year field study of *in situ* bioventing in the summer of 1991 in collaboration with the U.S. Air Force at Hill Air Force Base (AFB) near Salt Lake City, Utah. The site has JP-4 jet fuel-contaminated unsaturated soil where a spill has occurred in association with a fuel distribution network. The contractor operating the project is Battelle Laboratories, Columbus, Ohio. With the pilot-scale experience gained in these studies and others, bioventing should be available in the very near future as a reliable, inexpensive, and unobtrusive means of treating large quantities of organically contaminated soils.

The objectives of this project are to increase our understanding of bioventing large volumes of soil and to determine the influence of air flow rate on biodegradation and volatilization rates. The following is a summary of progress through January 1992.

Methodology

The site is contaminated with JP-4 from depths of approximately 35 ft to perched water at roughly 95 ft. Here, bioventing, if successful, will stimulate biodegradation of the fuel plume under roads, underground utilities, and buildings without disturbing these structures. A plan view of the installation is shown in Figure 1. The air injection well is indicated; "CW" wells are soil gas cluster wells where independent soil-gas samples can be taken at 10-ft intervals from 10 to 90 ft deep, and "WW" wells are ground-water wells. A cross-sectional view along the path AA' in Figure 1 is shown in Figure 2. Air is currently being injected from one well into the plume at a rate of 40 ft^3/min depths from 30 to 95 ft.

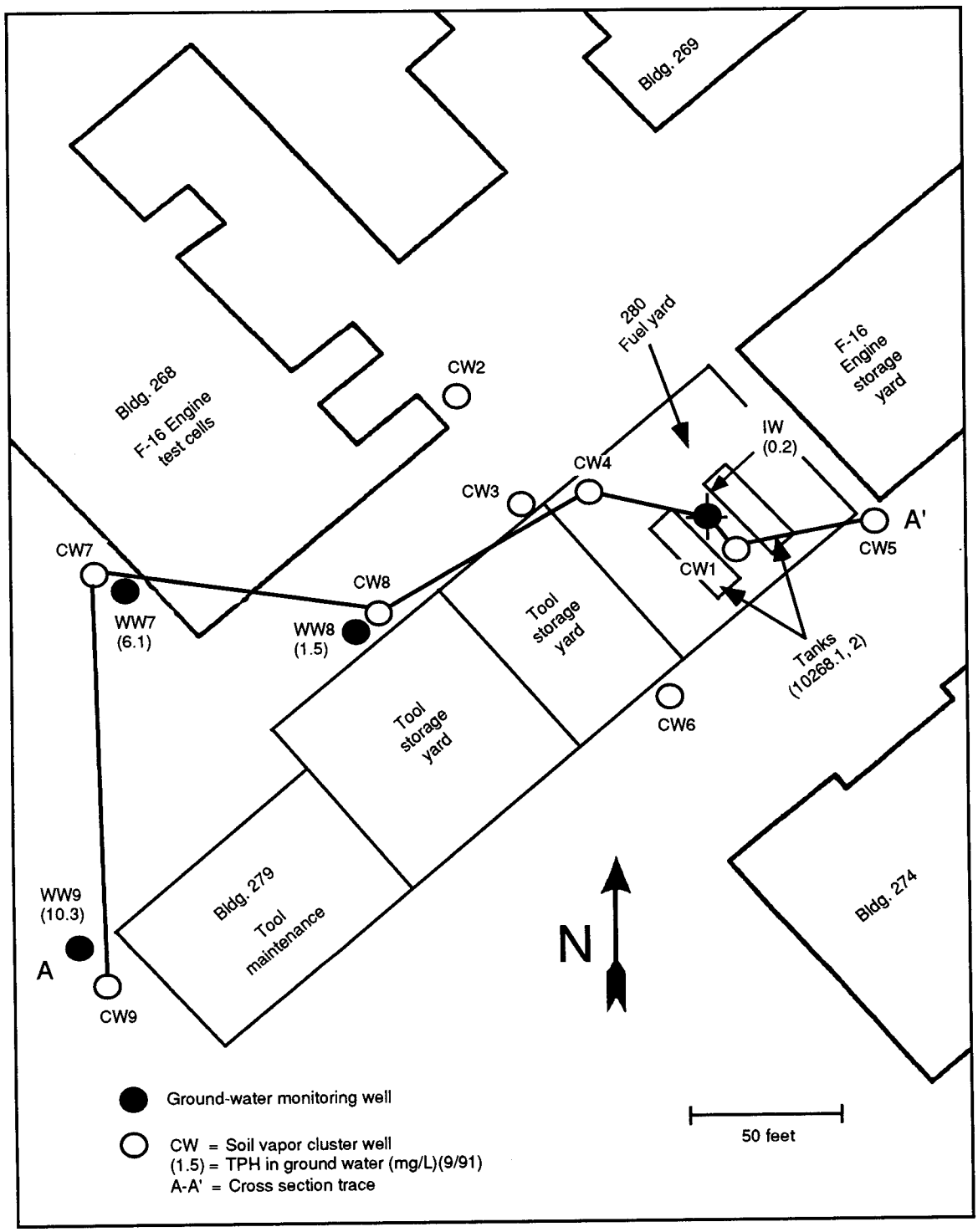

Figure 1. *Plan view of the joint U.S. EPA and U.S. Air Force bioventing activities at Hill AFB, near Salt Lake City, Utah. CW are cluster soil-gas monitoring wells, WW are ground-water monitoring wells, and the air injection well is indicated. The path AA' indicates the location of the cross-sectional view shown in Figure 2.*

An inert gas tracer study, regular soil gas measurements at several locations and depths, and semiannual *in situ* respiration tests (2) are planned to demonstrate the effectiveness of delivering oxygen and stimulating biodegradation in a large volume of soil of substantial depth. The *in situ* respirometry tests involve temporarily (4 to 8 days) shutting the air off to the injection well and monitoring the soil-gas oxygen concentration with time. Oxygen uptake due to oxygen demands other than biological activity is calculated by conducting a parallel shut-down test in the background (uncontaminated) area. The rate of decrease in

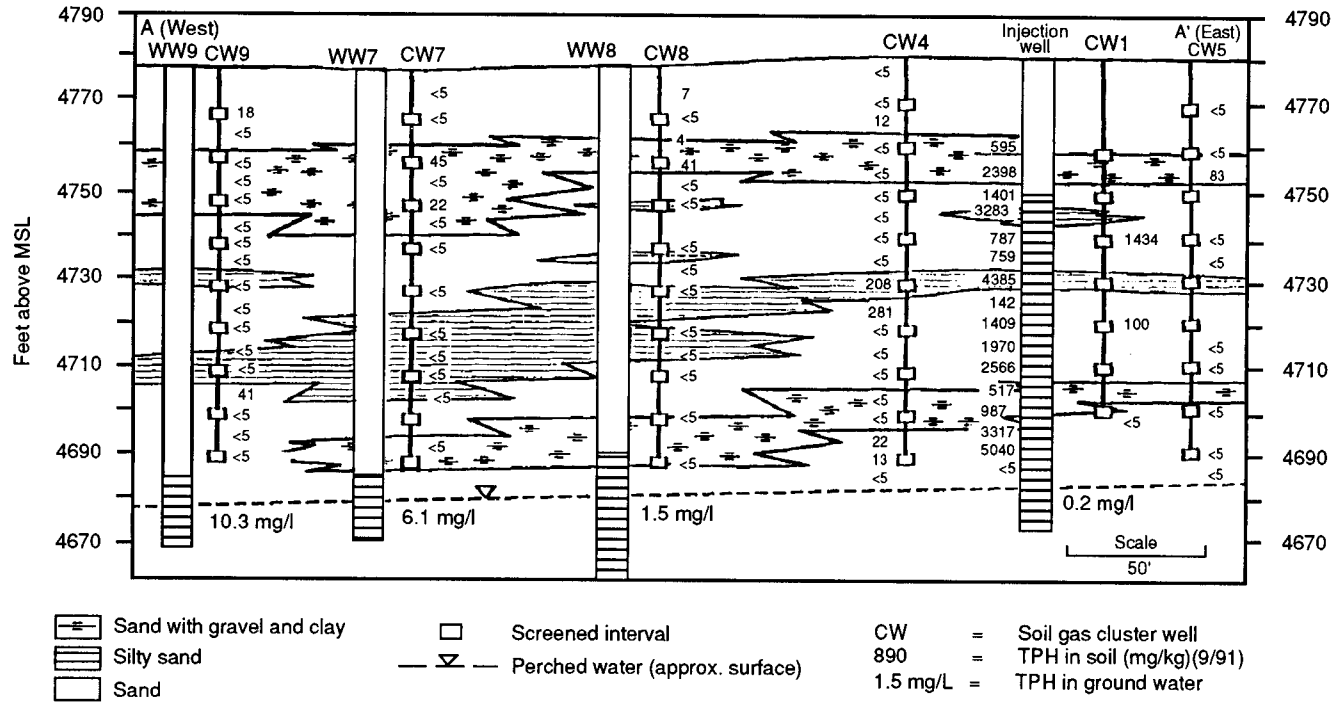

Figure 2. *Cross-sectional view at Hill AFB along the path AA' shown in Figure 1, showing the relative locations of the air injection well, soil-gas cluster wells, ground-water wells, and some geological features of the site. Vertical axis indicates elevation above sea level in feet. Numbers along wells indicate soil total petroleum hydrocarbon concentrations (TPH) (mg/kg) from soil samples taken at the depth shown during drilling in September 1991.*

oxygen concentration with time, relative to the rate observed in the background area, indicates a relative biodegradation rate at that time during the study. Thus, these tests allow estimation of the biodegradation rate as a function of time.

The inert gas tracer study involves temporarily replacing the injection of air with the injection of helium and observing the transport of gas in the soils by monitoring for the inert gas at the various soil-gas wells with time. The spatial distribution of tracer with time will indicate the uniformity (or lack thereof) of aeration of the site. The air injection rate will be decreased semiannually to evaluate the tradeoff between the loss in area of influence of the injected air for bioremediation and the decrease in volatilization of volatile organics to the atmosphere at the soil surface.

Final soil hydrocarbon analyses will be conducted in summer 1993 and compared with the initial soil analysis to document actual hydrocarbon loss due to bioventing.

Results

Progress to date at Hill AFB includes completion of the installation of the wells shown in Figures 1 and 2, initial soil sampling for total hydrocarbon levels as a function of depth, and commencement of air injection on September 5, 1991. Total petroleum hydrocarbon (TPH) soil analysis results also are shown in Figure 2. The jet fuel contamination is most highly concentrated in a 50-ft radius around the injection well.

Thus, the cleanup strategy being investigated here is (1) to aerate the highly contaminated zone near the injection well to stimulate biodegradation of the jet fuel in this zone, and (2) to allow the organics that are volatilized, due to aeration of the plume, to travel through a significant volume of soil outside the plume to provide biodegradation before the air stream reaches the surface.

Budgetary constraints have not allowed significant measurements to date. However, the inert gas tracer study and the first *in situ* respiration test and air flow rate change are scheduled for spring 1992.

We thank Chris Perry for preparing the graphic portions of the paper.

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1. R.E. Hoeppel, R.E. Hinchee and M.F. Arthur, 1990. Bioventing soils contaminated with petroleum hydrocarbons. J. Indust. Microbiol. 8: 141.

2. S.K. Ong, R.E. Hinchee, R. Hoeppel, and R. Schultz, 1991. *In situ* respirometry for determining aerobic degradation rates. In *In Situ* Bioreclamation; R.E. Hinchee and R.F. Olfenbuttel, eds. Butterworth-Heinemann, Boston, pp 541-545.

Use of Nitrate to Bioremediate a Pipeline Spill at Park City, Kansas: Projecting From a Treatability Study to Full-Scale Remediation

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Microcosm tests were conducted to determine whether an aquifer contaminated with petroleum hydrocarbons at Park City, Kansas, could be remediated *in situ* under denitrifying conditions, and to determine the rate and extent of nitrate consumption and alkylbenzene biodegradation. These rates, in conjunction with detailed core analyses from the site, were then used to estimate nitrate demand and assess the current design of the remediation system.

Discrete 15-cm cores were obtained over continuous lengths from 3 to 10 m below land surface to define the vertical extent of contamination, using methods described previously (1). These cores were extracted and analyzed for benzene, toluene, ethylbenzene, and xylenes (BTEX) by GC/MS and for total petroleum by GC using JP-4 jet fuel as a reference standard. Aquifer material was then obtained for microcosm preparation from both contaminated and uncontaminated intervals at the site using aseptic sampling techniques under anaerobic conditions. Microcosms were prepared in an anaerobic glovebox using 13-mL headspace vials and sterile diluted spring water, and were amended with ammonium and phosphorus nutrients. Sets prepared with both contaminated and uncontaminated aquifer material received either nitrate alone, BTEX spike alone, nitrate plus BTEX spike, or nitrate plus BTEX spike with biocides. Microcosms were sealed without headspace using Teflon-lined septa and incubated in an anaerobic glovebox at 20°C. Periodically, three replicates were sacrificed for each set and analyzed for aqueous BTEX, nitrate, nitrite, and nutrients.

Toluene, ethylbenzene, *m*-xylene, *p*-xylene, 1,3,5-trimethylbenzene, and 1,2,4-trimethylbenzene were degraded to less than 5 µg/L within 20 days in the clean-aquifer microcosms amended with nitrate and BTEX. About half of the *o*-xylene was removed, whereas benzene and 1,2,3-trimethylbenzene were recalcitrant. There was no degradation of the compounds in the microcosms without nitrate addition during the same time period, relative to controls. However, nitrate consumption occurred without BTEX addition, indicating a background nitrate demand in the uncontaminated aquifer material. Alkylbenzene biodegradation also occurred in the contaminated-aquifer microcosms, although sorption and leaching effects mediated by the residual petroleum hydrocarbons precluded a direct assessment of the rates of removal. Although the measured nitrate-consumption rate was equivalent to that observed in the clean-aquifer microcosms, the actual nitrate demand was at least four times as high in the contaminated-aquifer microcosms. Zero-order rate constants were obtained for removal of the alkylbenzenes (BTEX) and nitrate and were expressed on a dry-weight basis (Table 1). It is important to note that this method of rate expression is preferable, because rates based on solution analyses only, where unrealistic water/solid ratios are employed, as is the case with most microcosm studies, can underestimate the absolute rates by as much as an order of magnitude. Rates expressed on a dry-weight basis can be extrapolated easily to the natural water content of an aquifer.

Although benzene was recalcitrant in the microcosms, previous work had shown benzene to be degraded at field scale, presumably due to the presence of residual oxygen in the system (1). It is expected to be degraded at Park City as well, and hence these rates were used to calculate the penetration zone for nitrate application and to estimate the time required for remediation. Calculations were made for Cell #2, a 3,400-m^2 area that is to receive ground-water recharge containing 10 mg/L NO_3-N at an application rate of 680 m^3/d through a network of injection wells. Continuous cores were obtained from two locations within this cell; the summarized data are shown in Table 2. Based on these analyses, Cell #2 contains 1,300 kg BTEX and 58,000 kg total petroleum hydrocarbons (TPH). The nitrate demand of the contaminated zone can then be calculated using stoichiometric relationships discussed elsewhere (2), and the time required for remediation can be estimated based on the projected application rate. These estimates were compared to those obtained using the intrinsic microbial rates of BTEX degradation and nitrate removal, considering either the average values for the entire depth interval or the most contaminated interval within the aquifer profile.

Table 1. *Zero-Order Rate Constants for BTEX and Nitrate-Nitrogen Removal in Microcosms Prepared with Uncontaminated and Contaminated Park City Aquifer Material (all units in mg/kg dry weight/day)*

Parameter	Treatment	Uncontaminated	Contaminated
BTEX	Nitrate only	–	0.248
	Nitrate plus BTEX	1.65	0.275
Nitrate-N	Nitrate only	2.80	2.92
	Nitrate plus BTEX	2.80	3.06

Table 2. *Summary Core Data for Locations 60B and 60H, Cell #2*

Parameter	*Units*	*60B*	*60H*
Depth interval	*m BLS**	*2.4-8.5*	*2.7-8.9*
Contaminated zone(s)	*m BLS*	*2.4-6.9*	*6.0-7.2*
Uncontaminated zone(s)	*m BLS*	*6.9-8.5*	*2.7-6.0*
			7.2-8.9
*Most contaminated interval***	*m BLS*	*6.4-6.6*	*6.7-6.8*
Mean BTEX concentration	*mg/kg dry wt*	*42.6*	*24.3*
Mean TPH concentration	*mg/kg dry wt*	*2,360*	*686*
Peak BTEX concentration	*mg/kg dry wt*	*353*	*412*
Peak TPH concentration	*mg/kg dry wt*	*10,500*	*13,400*

* Meters below land surface

** For BTEX, not necessarily Total Petroleum Hydrocarbon (TPH).

Based on projected nitrate demand alone, 210 days would be required to supply enough nitrate to remediate the aquifer. This assumes that BTEX constitutes the bulk of the nitrate demand, and that desorption and reaction rates are instantaneous relative to the application rate. The reaction rates, at least, should be fast. Only 13 days would be required to satisfy the average theoretical nitrate demand if nitrate were continuously present, based on the microbial reaction rate for nitrate removal observed in the microcosm tests. Similarly, only 21 days would be required on the average to degrade all of the BTEX, based on the observed microbial reaction rate for BTEX biodegradation. This observation illustrates that the main factor affecting remediation time will be the rate of application of nitrate.

This result can also be shown by calculating the penetration zone of nitrate once the infiltrate reaches the water table. Based on the application rate, the design nitrate concentration, and the microbial rate of nitrate removal, the penetration zones would be 5.8 to 6.1 m for the Core 60B location and 6.3 to 6.6 m for the Core 60H location. As shown in Table 2, the actual contaminated interval extends to below these zones in both locations, and hence nitrate breakthrough is not expected until substantial remediation has occurred.

Although remediation will be controlled by the nitrate application rate, microbial reaction rates could become the important process parameters, if the rate of remediation is controlled by contributions from the most contaminated interval. For example, it would take 270 days to remediate the most contaminated interval in Cell #2, even given the fast rates of TEX removal by the microorganisms. Of course, if this interval is small relative to the entire volume being treated, it may not contribute significantly to the final average BTEX concentration in the ground water after remediation.

In summary, these microcosm tests have shown that many of the aklylbenzenes can be degraded under denitrifying conditions in this aquifer, although benzene may be recalcitrant unless some oxygen is available. These data can then be used to predict the limiting factors for remediation, and indicate that regulatory constraints on the permissible nitrate loading will govern the length of time required for remediation, since the *in situ* microbial processes are sufficiently rapid not to be a limitation.

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Design and Treatability Study of *In Situ* Bioremediation of Chlorinated Aliphatics by Methanotrophs at St. Joseph, Michigan

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Introduction

The St. Joseph, Michigan, National Priority List industrial site is a relatively homogeneous fine-sand aquifer contaminated with mg/L concentrations of trichloroethylene (TCE), cis- and trans-1,2-dichloroethylene (c-DCE, t-DCE), and vinyl chloride (VC). Laboratory, field, and modeling studies indicate that conditions were ideal for evaluating *in situ* bioremediation at this site using methanotrophic bacteria (1). To develop an appropriate treatment system design, additional site characterization is required, alternative technologies need to be researched, and modeling studies for alternatives need to be evaluated. These tasks are being carried out by a team of researchers at Stanford University in cooperation with Allied Signal Corporation, Engineering Science, and Region 5 and the Kerr Laboratory of the U.S. Environmental Protection Agency. This abstract presents a summary of the status of this study.

Background

Methanotrophic bacteria have the potential for cometabolically oxidizing chlorinated aliphatic compounds to nontoxic end products. In the Moffett Field pilot-scale study, methanotrophs biostimulated in a 2-m zone through oxygen and methane addition achieved the following percentages of biodegradation: TCE, 20 percent; c-DCE, 50 percent; t-DCE, 85 percent; and VC, 95 percent (2). The Saint Joseph site appeared ideal for studying the potential of this process at full scale since the aquifer is relatively homogeneous and the major contaminants present from the source of contamination are c-DCE, t-DCE, and VC (1).

Results of a preliminary feasibility study of the potential for methanotrophic treatment at the site were presented by McCarty et al. (1). Soil microcosm studies in columns packed with St. Joseph aquifer solids showed methanotrophs were present. Upon stimulation through the batch addition of 3.5 mg/L of methane and 25 mg/L of oxygen, the following extents of degradation were achieved: VC, 95 percent; t-DCE, 80 percent; and TCE, 20 percent, at contaminant concentrations of 100 µg/L, 160 µg/L, and 70 µg/L, respectively. Sorption studies indicated the contaminants were weakly sorbed onto the aquifer solids with retardation factors ranging from 1.2 to 2.9 for VC, to 2.7 to 5.2 for TCE.

Simulations performed of a potential *in situ* treatment scheme using laboratory-derived parameters indicated that *in situ* treatment of VC and t-DCE compared favorably to pump-and-treat, while the more slowly degraded c-DCE and TCE were more rapidly removed by pump-and-treat. The feasibility study indicated the methanotrophic process has potential for treating the downgradient portion of the plume that contained VC and the DCE isomers. Thus, the design of an *in situ* treatment system and more detailed characterization of the site were initiated.

Mixing System for Methane and Oxygen Addition

One of the challenges in *in situ* bioremediation is the delivery and mixing of growth substrate and nutrients, such as oxygen and methane needed for the development of the methanotrophic bacterial population. The mixing method being investigated uses a recirculation well with two screens and a pump, which induces flow through the bore and flow recirculation through the porous formation (Figure 1). Methane and oxygen would be introduced directly into the recirculating ground water. The method eliminates pumping the contaminated ground water to the surface,

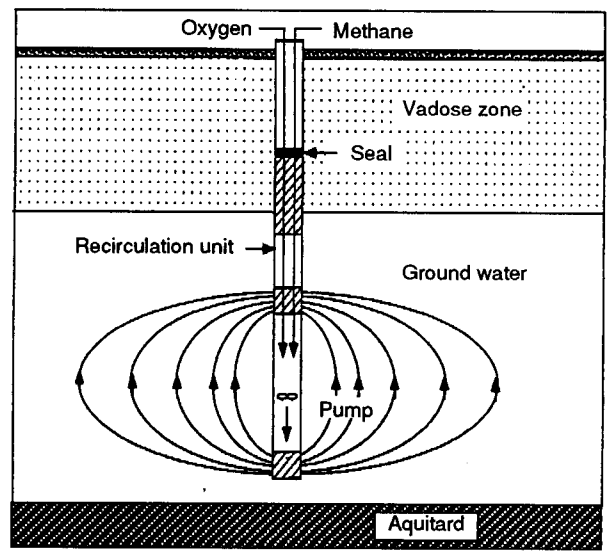

Figure 1. *Recirculation system for in situ remediation at the St. Joseph site.*

surface treatment, and the subsequent reinjection. Our design work for the recirculation system has focused on the mathematical modeling of the flow, modeling of the biostimulation and biodegradation process, and developing a mass transfer device for dissolving methane and oxygen in the recirculating ground water.

The flow modeling has been performed using an axisymmetric boundary element method, which for simple cases (no regional flow, homogeneous aquifer properties) reduces a three-dimensional problem to a one-dimensional problem. This procedure enables accurate representation of the dimensions of the well, the free surface, and the effects of uncertainty in the hydraulic conductivity. The results of a simulation with streamtubes from the recirculation unit are shown in Figure 2. Much of the recirculating flow occurs near the well, and the sphere of influence is approximately equal to the thickness of the aquifer. A three-dimensional boundary element code is being formulated for a more detailed analysis, including the effects of regional flow, multiple wells, and irregular-shaped boundaries.

Biostimulation and biotransformation modeling of the recirculation system has been performed by adapting the code developed in the Moffett Field study (3,4) for the flow geometry shown in Figure 2. Initial simulations indicate that an alternate pulse cycle of 2 days oxygen and 1 day methane provides the most favorable biological growth pattern and degradation rate. However, there is a potential for bioclogging of the aquifer near the well screens due to the recirculation of nutrients.

A Venturi mass-transfer device has been developed and studied in the laboratory as a means of dissolving methane and oxygen in the recirculating ground water. The tests indicate the device could achieve the desired methane and oxygen addition rates at the St. Joseph site. The study showed that the presence of other gases in the ground water (e.g., nitrogen) decrease the efficiency of oxygen and methane addition. Thus, measurements of trace gas composition of the ground water are required.

Contaminant Characterization Results

A detailed characterization of the ground-water contamination was conducted at the location proposed for the *in situ* evaluation. Details of this characterization are provided by Wilson et al. (5). The distribution of the contamination is significantly different than we had anticipated from the data previously collected from the nearby monitoring wells. The concentrations of the chlorinated aliphatic compounds varied significantly with depth. Relatively high concentrations (several mg/L of TCE, c-DCE, and VC) exist at all locations within 20 m of the center of the plume. The maximum concentrations of the contaminants were much higher than expected (TCE, 133 mg/L; c-DCE, 128 mg/L; vinyl chloride, 56 mg/L). Ethylene, as well as methane, is present in relatively high concentrations. Higher ethylene concentrations tend to be associated with higher VC concentrations, suggesting the ethylene is a product of anaerobic VC transformation. The aquifer is anoxic at depth with a low redox potential, which also suggests that conditions are suitable for anaerobic methane-forming processes. These data, discussed in detail by McCarty and Wilson (6), strongly suggest that anaerobic microbial trans-

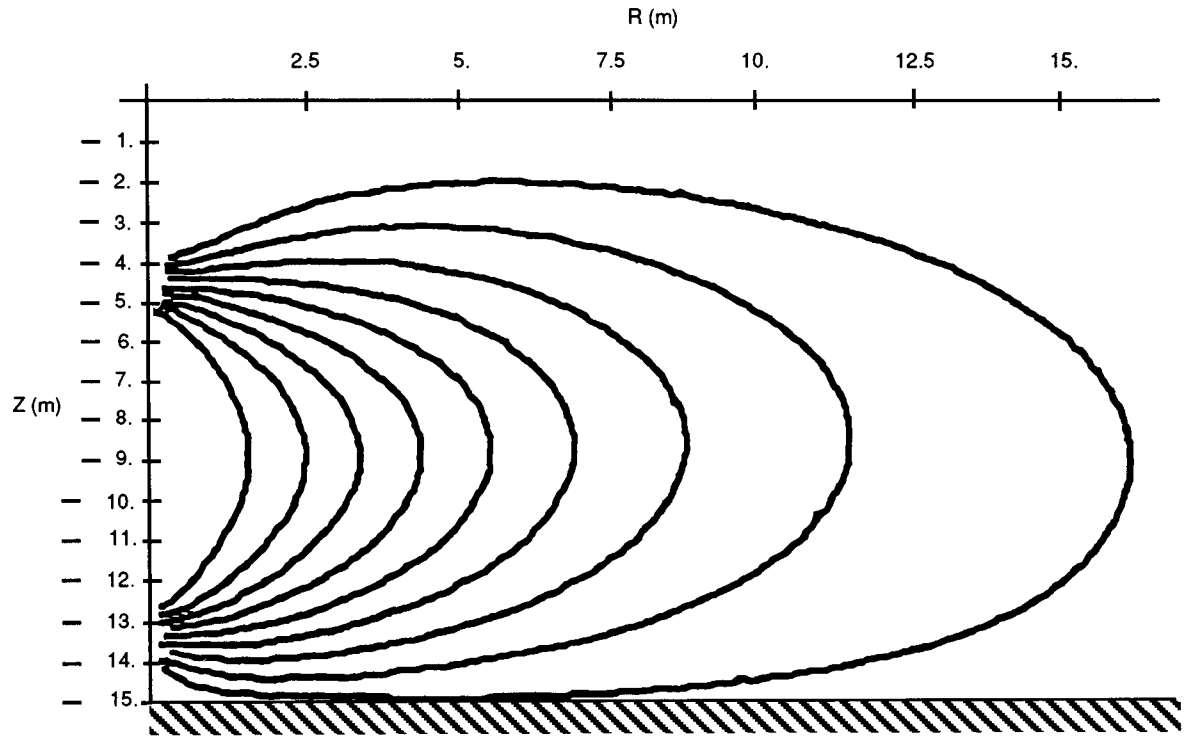

Figure 2 *Streamlines calculated for the recirculation unit. Each streamtube represents 10 percent of the total flow. Half of a symmetric profile is shown.*

formation processes have occurred in the aquifer, and not only are responsible for the presence of c-DCE and VC, but also for the complete dechlorination of a significant amount of TCE to form ethylene, a relatively nonharmful chemical.

Microcosm Experiments

The high concentrations of the chlorinated organics observed in the characterization studies might inhibit the biostimulation of a methanotrophic population because of product toxicity shown by Alvarez-Cohen and McCarty (7), and others. Microcosm studies with St. Joseph aquifer solids were initiated to investigate this possibility. VC and TCE degradation was studied at concentrations ranging from 0.8 mg/L to 8 mg/L. The results showed complete methane utilization in all of the enriched columns regardless of VC or TCE concentration. In the methane-stimulated columns, approximately 25 percent and 80 percent VC removal was observed in the columns fed 8 mg/L and 0.8 mg/L of VC, respectively. The TCE concentrations in all of the enriched columns remained approximately the same as in the control columns. Studies performed at 12°C, the aquifer temperature, indicated that enrichment of a mixed methanotrophic culture is possible and occurs within about 40 days and that the extent of VC transformation approaches than found at room temperature.

The results of the microcosm studies indicate that high TCE concentrations and lower temperatures do not inhibit methanotrophic growth, and that the extent of VC transformation appears to be the same with or without TCE present. TCE in mg/L concentrations, however, is not effectively transformed by the methanotrophic process; thus, efforts should be made to locate the proposed recirculation unit in an area where TCE concentrations are low and VC is the downgradient contaminant.

Summary and Conclusions

Studies leading to the design of a system for the methanotrophic *in situ* treatment of VC and DCE isomers have been initiated. A recirculation system has been chosen for study that would eliminate the need for pumping contaminated ground water to the surface. Initial hydrodynamic and biological modeling studies indicate that effective remediation of VC can be achieved with the system. A method for adding methane and oxygen directly to the recirculating ground water has been developed that could be implemented at the site.

A detailed characterization of ground-water contamination showed zones of high concentrations of CACs. The presence of ethylene indicated that anaerobic transformation to a nontoxic end product has occurred. Thus, enhanced anaerobic transformation should be explored. Microcosm studies indicated that methanotrophs would grow in the presence of mg/L concentrations of VC and TCE and would effectively degrade VC. The results indicate that a promising strategy would be to promote anaerobic transformation of TCE upgradient and use aerobic methanotrophic treatment downgradient as a final polishing step. This scenario is currently being investigated.

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Natural Anaerobic Treatment of a TCE Plume St. Joseph, Michigan, NPL Site

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Introduction

A ground-water plume containing trichloroethylene (TCE) and other chlorinated aliphatic compounds (CACs) is present at a National Priority List industrial site in St. Joseph, Michigan, approximately 750 m east of Lake Michigan. Lagoons covering about 2 hectares were used at the site between 1968 and 1976 for disposal of industrial wastes. Between 1976 and 1978, the lagoons had liquids removed from them, and were then backfilled and capped. Whether the TCE in ground water resulted from discharge into the lagoon or from spills elsewhere on the site is not known. Surface drainage from the site is controlled by both Lake Michigan to the west and Hickory Creek, which lies about 350 meters to the east, with a ground-water divide running north and south through the plant site. The water table lies about 10 m below the ground surface in a fine sand aquifer that is about 20 m deep.

TCE and other chlorinated aliphatic compounds were found in ground-water plumes emanating from the site in 1982. Subsequent studies indicated that the ground-water plume is divided, with a segment moving toward Hickory Creek and another toward Lake Michigan. Both segments contained cis- and trans-1,2-dichloroethylene (cDCE, tDCE), vinyl chloride (VC), and a few other CACs in addition to TCE. The possibility of *in situ* treatment of the VC portion of the plume by methanotrophic bacteria was proposed through injection of methane and oxygen (1). To provide additional information for the pilot study, a detailed site characterization was undertaken at one location through a cooperative effort with Allied-Signal Corporation, Engineering Science, Region 5 and the Kerr Laboratory of EPA, and Stanford University. This study indicated that the ground water contained significant levels of ethene, a likely end product of anaerobic TCE transformation, plus high concentrations of methane. The extent of anaerobic transformation of TCE and the potential for enhanced anaerobic rather than aerobic biological transformation are being explored. A summary of information supporting *in situ* reduction of TCE follows.

Background

The anaerobic reduction of TCE to DCE and VC was reported in the early 1980s (2,3,4). Further reduction of tetrachloroethylene (PCE) to VC and ethene under methanogenic conditions was reported recently by Freedman and Gossett (5). In subsequent studies, DiStefano et al. (6) found that the reduction of PCE (as high as 55 mg/L) to ethene could take place under anaerobic conditions with methanol as the growth substrate, even in the absence of methanogenesis. In their study, about 70 percent of the methanol was converted to acetate, while about 30 percent was associated with the dechlorination reactions. They suggested this efficient reductive dechlorination might be exploited for bioremediation of PCE-contaminated sites. Major et al. (7) provided field evidence for the reductive transformation of low mg/L concentrations of PCE to VC and ethene at a chemical-transfer site in North Toronto where methanol and acetate were found as ground-water contaminants. Transformation of PCE to ethene was successful in methane-producing laboratory microcosms using site aquifer material supplemented with methanol and acetate. Since TCE is the first reduction product from PCE, these studies support the potential for TCE transformation to the environmentally benign ethene. Remaining VC might then be treated by the originally proposed methanotrophic treatment.

Charecterization Results

As discussed above, reductive dechlorination of CACs requires an electron donor, which may be native organic matter, or an added organic compound such as methanol or acetate. The capacity of the donor to supply electrons can be analytically determined as its chemical oxygen demand (COD). Figure 1 provides contours of COD in ground water at the St. Joseph site based upon monitoring data reported in 1986 (8). Figure 2 is a

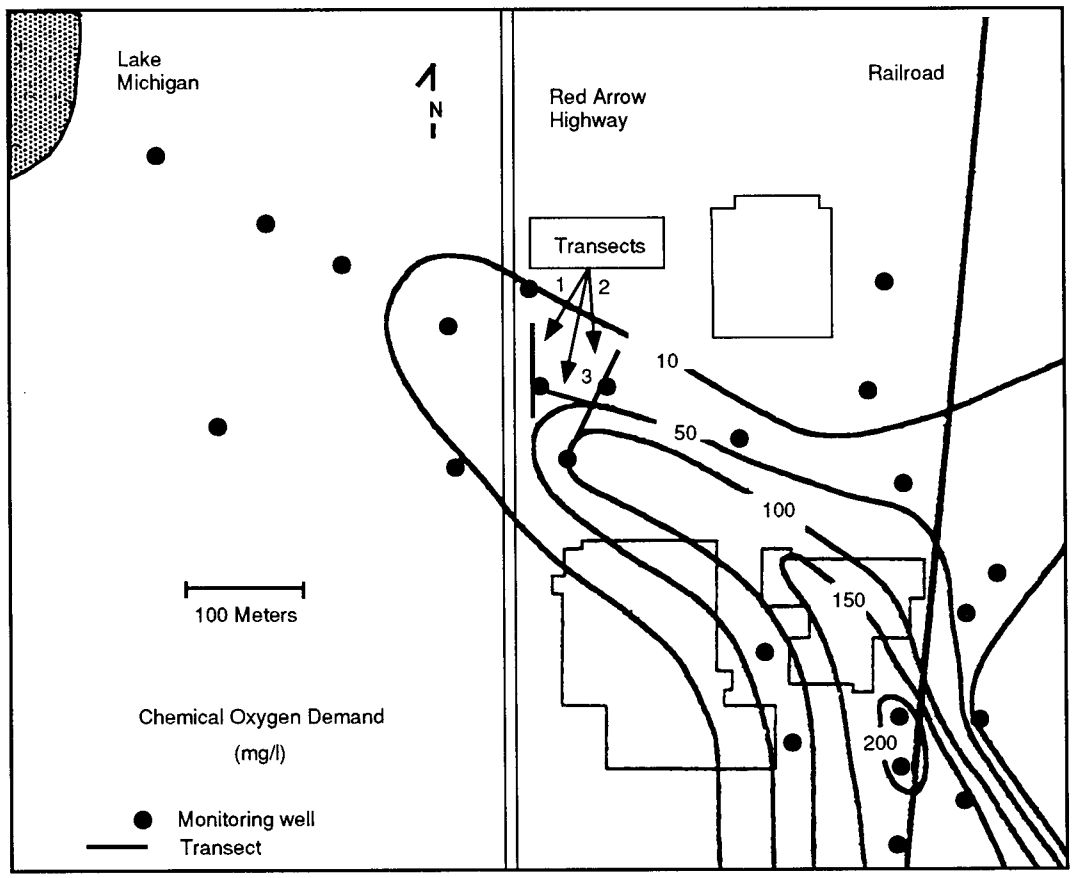

Figure 1. *COD contours for the St. Joseph site (after Keck (8)).*

composite of 10 mg/L contours for chlorinated aliphatic compounds, also reported previously. A correlation between COD decrease in Figure 1 and transformations of TCE to VC in Figure 2 is apparent. Also shown in Figure 1 are the locations of the three transects taken for the detailed characterization, which consisted of 17 boreholes with ground-water sampling from each at 5-ft vertical intervals down to the aquitard at about 30 m.

Table 1 summarizes the electrons released by TCE reduction to different products, along with the equivalent amount of COD decrease required for the various transformations. COD decrease associated with methane production is indicated as well. An electron equivalent of reduction requires one-fourth mole molecular oxygen, or 8 g COD decrease. Since the reduction of one mole or 131 g TCE to ethene releases six electrons, an equivalent decrease of 48 g of COD is necessary. Table 2 summarizes the CACs, ethene, and methane found at selected sampling locations, and the equivalent COD decrease that would be associated with these products. The average concentration of methane found at all sampling depths of 25 m or more was 6 mg/L, which corresponds with an average COD conversion of 24 mg/L. Figure 1 indicates a COD decrease as high as 200 mg/L may have occurred at the site, which is four to six times the equivalent COD change to form products at the selected Table 2 locations. These observations may be consistent as the amount of COD dilution between the lagoon and the detailed characterization location is unknown, other possible electron acceptors such as nitrate and sulfate were not considered, the effect of sorption of chemicals on their distribution is unknown, and some methane may have escaped by volatilization or other processes. At the sampling locations where the greatest degrees of dehalogenation were found (Table 1), reductive dehalogenation itself was associated with 8 to 36 mg/L of the total equivalent COD decrease. This too represents a fairly high percentage of the COD reduction suggested by Figure 1 contours.

Summary and Conclusions

Relatively high concentrations of TCE, DCE, VC, ethene, and methane were found in ground water at the St. Joseph site. Organic chemicals represented by ground-water COD appear to have undergone biological removal, a significant portion being associated with methane production (as high as 25 percent) and dechlorination of TCE (as high as 18 percent). An active anaerobic dehalogenating population appears to be present in the aquifer. It appears that enhanced reduction of remaining CACs to ethene may be possible if additional organic compounds, such as methanol or acetate, were added to the aquifer. Microcosm studies to determine chemical requirements, reaction rates, and transformation end products are proposed.

Table 1. *Half Reactions Indicating Electron Equivalents of Change and Associated Equivalent COD Decrease Associated with Change*

Transform product	Mol. wt.	Half reaction	Equiv. COD decrease g COD/g product*
Methane	16	$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$	4
DCE	97	$CHCl{=}CCl_2 + H^+ + 2e^- \rightarrow CHCl{=}CHCl + Cl^-$	0.16
VC	62.5	$CHCl{=}CCl_2 + 2H^+ + 4e^- \rightarrow CH_2{=}CHCl + 2Cl^-$	0.51
Ethene	28	$CHCl{=}CCl_2 + 3H^+ + 6e^- \rightarrow CH_2{=}CH_2 + 3Cl^-$	1.71

* From [$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$], the half-reaction for oxygen, one electron equivalent of COD equals one-fourth mole of molecular oxygen or 8 grams, thus Equiv. COD Decrease = (8n/Mol. Wt), where n is the number of electrons in the half-reaction.

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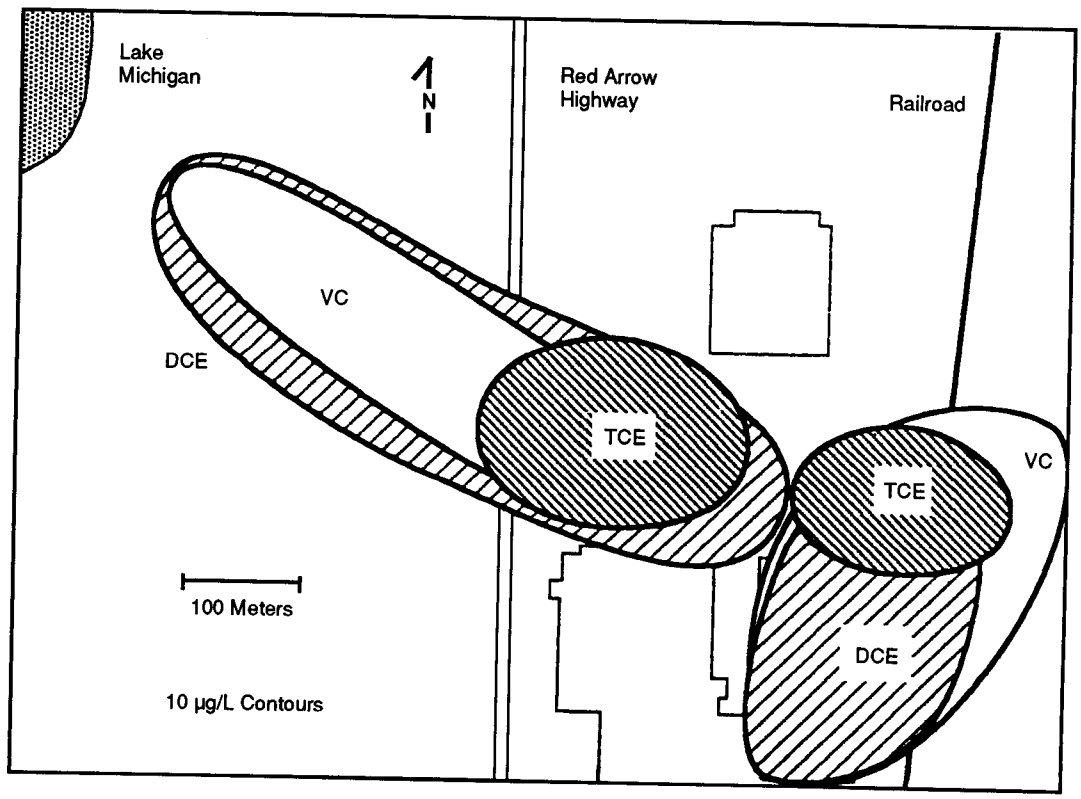

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Table 2. *Concentrations of CACs, Ethene, and Methane Found at Selected Sampling Locations Along Detailed Characterization Transects, and the Equivalent COD Decrease Associated with the Products*

Sample [@]		TCE	1,1DCE	cDCE	tDCE	VC	Ethene	CH_4	Total
1-2-70'	mg/l	4.03	0.09	4.14	0.81	3.19	6.62	4.61	
	Equiv. COD Decrease, mg/l	0.00	0.01	0.66	0.13	0.41	11.32	18.44	30.97
	% of Equiv. COD	0.0	0.0	2.1	0.4	1.3	36.6	59.5	
1-3-75'	mg/l	12.80	0.27	16.90	0.67	56.40	2.25	6.62	
	Equiv. COD Decrease, mg/l	0.00	0.04	2.70	0.11	28.80	3.84	26.00	61.5
	% of Equiv. COD	0.0	0.1	4.4	0.2	46.8	6.2	42.2	
2-1-75'	mg/l	0.44	0.09	13.40	0.21	1.46	3.15	7.43	
	Equiv. COD Decrease, mg/l	0.00	0.01	2.14	0.03	0.74	5.39	29.72	38.03
	% of Equiv. COD	0.0	0.0	5.6	0.1	1.9	14.2	78.1	
2-6-65'	mg/l	0.51		4.70	0.03	2.66	4.27	11.72	
	Equiv. COD Decrease, mg/l	0.00	0.00	0.75	0.00	1.36	5.98	46.88	54.97
	% of Equiv. COD	0.0	0.0	1.4	0.0	2.5	10.9	85.3	
3-2-80'	mg/l	2.53	0.01	0.90	0.03	0.27	4.87	11.59	
	Equiv. COD Decrease, mg/l	0.00	0.00	0.14	0.00	0.14	8.32	46.36	54.96
	% of Equiv. COD	0.0	0.0	0.3	0.0	0.3	15.1	84.4	

[@] First value is transect number, second value is borehole number, and third value is depth of sample below ground surface in feet.

Proposed PCB Biodegradation Study at The Sheboygan River Confined Treatment Facility

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The primary objective of the proposed field demonstration study at the Sheboygan River Confined Treatment Facility (CTF) is to examine the effects of alternating aerobic and anaerobic conditions, with and without the addition of inorganic nutrients, on the rate and extent of PCB biotransformation under simulated field conditions. Collecting data under conditions that closely resemble a large-scale treatment of PCBs will provide valuable information regarding the correlation of laboratory and field data and the development of scenarios for enhanced bioremediation.

The selection of treatment designs for the Sheboygan River CTF has been based, in part, on the outcome of laboratory bench-scale studies in the context of engineering feasibility. Two potential treatments, inoculation with acclimated organisms and augmentation with organic amendments, have not been demonstrated to enhance significantly the rate of dechlorination of PCBs in bench-scale laboratory studies. Addition of inorganic nutrients, however, is considered beneficial for enhancing microbial activity. Other strategies still under consideration are those designed to increase the physical-chemical availability of PCBs bound to sediments, including the addition of surfactants, and the application of aerobic/anaerobic cycling. Surfactants may increase the availability of "bound" PCBs to microorganisms, but identifying a surfactant that is nontoxic and not a preferred substrate for microbial growth is a challenging problem. Thus, surfactant treatment is not considered to be a viable option at this time.

Aerobic/anaerobic cycling is currently the most promising treatment option and merits trial at the field scale. During the aerobic phase, the bioavailability of PCBs may be enhanced through the degradation of naturally occurring organic compounds as well as contaminants such as oil and grease, all of which are known to bind PCBs. Further, biphenyl and lower chlorinated PCB congeners, primarily mono- and di-chlorinated PCBs, are degraded preferentially under aerobic conditions (1,2). Thus, an aerobic treatment phase contributes to more complete biodegradation. Anaerobic conditions favor the biotransformation of the more highly chlorinated PCB congeners, e.g., 6, 7, and 8 chlorines, through reductive dechlorination reactions (3,4), producing lower chlorinated products amenable to aerobic attack. Taken together, the alternating aerobic/anaerobic scheme seems promising for more extensive bioremediation of PCBs (5). This scenario is supported by preliminary laboratory studies indicating that aerobic/anaerobic cycling reduces the concentration of both higher and lower chlorinated PCBs. Furthermore, we have recent evidence of *ortho*-dechlorination of lower chlorinated congeners in lab-scale, aerobic/anaerobic cycling experiments, an observation not often cited.

The aerobic/anaerobic field experiments will be conducted for a minimum of 1 year and perhaps for 2 years to provide sufficient time for potentially significant *in situ* bioremediation. Anaerobic conditions will be established by natural means; that is, no externally added source of oxygen will be provided to the CTF during this phase of the treatment. Sediments will be aerated by pumping soluble oxidants, such as peroxides or oxygen-saturated water, into the CTF. Oxidant addition can be made either directly through the existing distribution piping system or to the CTF outflow liquid in a recycle fashion.

The effectiveness of either strategy will depend on the nature of dispersion of the added oxidant and its consumption within the sediments. Tracer studies have been proposed to test dispersion from the upflow nutrient distribution piping system. Results from that study are necessary to determine the adequacy of the existing distribution system for oxygen addition in the aerobic cycle. Oxidants under consideration include hydrogen peroxide and oxygen-saturated cold water. In the event the currently existing plumbing should be inadequate for the distribution of oxidant, flow and distribution of the oxidant could be augmented by sinking wells at points of low flow and pumping or withdrawing interstitial fluids.

Determination of the optimum cycle times for the system to be operated under aerobic and anaerobic conditions will have two components: the time required for the transition from anaerobic to aerobic conditions and vice versa, and the time required within each regime for the desired extent of PCB transformation. Indicators of aerobic status may include direct measurement of oxygen levels in liquid outflows and interstitial sediment fluids or such indirect measures as chemical oxygen demand (COD) or total soluble carbon of sediment samples. Adequate aeration would be expected to lower the soluble organic content of sediments. Sampling procedures will be designed to detect flow problems such as channeling/short-circuiting.

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Characterization of Microorganisms, Microbial Consortia, and Microbial Processes for the Reductive Dechlorination of Hazardous Wastes

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The purpose of this study is to determine the nutritional, physiological, and environmental factors that enhance the activity, growth, and enrichment of microbial populations capable of the transformation of chlorophenols. The ultimate goal is to gain sufficient knowledge for the development and characterization of enriched microbial consortia and pure microbial cultures responsible for the reductive dechlorination of chlorinated aromatic compounds.

To date, only one pure microbial culture has been described that is capable of the reductive dechlorination of a chlorinated aromatic compound. This isolate (*Desulfomonile tiedje*) is restricted to the dechlorination of meta-halogenated benzoates and some chlorophenols (1,2,3). Few studies have been reported that describe the physiological or nutritional conditions affecting reductive dechlorination of chloroaromatics. Gibson and Suflita (4) and Kuhn et al. (5) have demonstrated enhanced dehalogenation rates of 2,4,5-T and chloroanilines, respectively, by addition of organic carbon supplements to methanogenic aquifer slurries. Further, Nies and Vogel (6) reported that addition of organic substrates, such as methanol, acetone, glucose, and acetate, accelerated the dechlorination of PCBs in river sediments. The enhanced dechlorination rate is theorized to be related to the supply of potential electron donors to the dechlorinating population.

In this study, we report the effects of pH, nutrient supplements, and heat (pasteurization) on enriched microbial cultures affecting the dechlorination of mono- and di-chlorophenols (CP) primarily under methanogenic conditions. Results will be used in future studies to characterize microbes and microbial consortia responsible for reductive dechlorination.

In initial studies, both unadapted and 2,4-DCP-adapted freshwater sediment slurries were used as model systems to assess the effects of added nutrients on the onset and rate of reductive dechlorination. Additions of individual substrates, including formate, butyrate, propionate, acetate, and a vitamin mix, did not substantially enhance dechlorination in chlorophenol-adapted cultures or in adapted cultures with recently depleted activity. However, addition of yeast extract enhanced dechlorination activity as well as the onset of dechlorination in most experiments. Addition of acetate also stimulated dechlorination activity in fresh, unadapted sediment slurries. However, in some experiments, addition of organics, such as formate and propionate, extended the time before the onset of dechlorination. Studies are in progress to determine the source of additional (potential) electron donors.

The effects of heat and pH on the dechlorination of selected mono- and di-chlorophenols (DCP) were studied using CP-adapted freshwater sediment slurries and CP-adapted consortia. CP-adapted sediment cultures were enriched by the repeated addition of the mono- or di-CP over a period of 6 months and subsequent dilution into sterile, diluted (10 percent solids) sediment. Some cultures subsequently were transferred to filter-sterilized site water supplemented with 0.1 percent yeast extract and amended with the appropriate chlorophenol. For heat-treatment studies, most cultures were heated to 85°C for 15 minutes and diluted 10-fold into sterile sediment slurry (10 percent dry weight). Dechlorination was followed over time. In many experiments, the heat treatment was repeated without further dilution.

The initial heat treatment of DCP-adapted slurries did not significantly decrease the rate or extent of dechlorination activity. A second heat treatment had a more pronounced inhibitory effect. Dechlorination of the first addition of 2,3-DCP, for example, was complete by day 6 in control slurries (non-heated) and by day 9 in slurries heated for 15 and 30 minutes to 85°C. Additional DCP added to these cultures subsequently was dechlorinated within 2 days in control slurries, whereas the rate of dechlorination was decreased by 50 percent following the second

(identical) heat treatment. Cultures enriched with 2,4-DCP and 3,4-DCP were not affected as severely by the second heat treatment compared to 2,3-DCP heat-treated cultures.

Sediment slurries adapted to dechlorinate 2-, 3-, and 4-chlorophenol were completely inhibited by the initial heat treatment. Further, nonheated cultures (controls), which were also diluted into sterile 10 percent sediment, exhibited a long lag period before the onset of chlorophenol dechlorination. It is evident that physiological and environmental factors have differing effects on the various dechlorinating populations.

The effect of pH on the onset and rate of dechlorination of dichlorophenols was examined in sterile sediment slurries (10 percent w/v) adjusted to pH 5 to 9 and inoculated with either heat-treated, DCP-adapted sediment slurries or DCP-enriched cultures. In general, the rate of dechlorination of each of the DCPs was greatest at pH 7 over the range of pH tested (5 to 9). Dechlorination of all DCPs tested was complete within 6 days of incubation in experiments adjusted to pH 7. However, longer lag times and decreased dechlorination rates were observed for experiments incubated at the extremes of pH tested (pH 5 and 9).

These results indicate that physiological and environmental factors are important considerations in the development of strategies for remediation of hazardous wastes.

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Effects of Metals on the Reductive Dechlorination of Chlorophenols

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Recent interest in the use of bioremediation technology for the cleanup of contaminated soils and sediments has led to a greater understanding of the fate of a variety of toxic organic compounds in natural environments. Most of the research has focused on the biological transformation of a single organic contaminant in laboratory microcosms using naturally occurring microbial inocula. However, most polluted ecosystems and hazardous waste sites are most often contaminated with a mixture of organic compounds and toxic inorganic wastes such as metals. The fate and transport of organic compounds in natural environments such as soils and sediments has received considerable interest in recent years, but little is known about the potential toxicity of metals to naturally occurring microorganisms and microbial processes of importance to the biotransformation of organic compounds.

Soils and sediments are quite varied in composition but generally consist of an array of mineral particles, organic matter, microbial cells and debris, and inorganic solutes, all of which are important in the formation of metal complexes. Further, recent studies have indicated the importance of microbial cell surfaces in the sorption and immobilization of metals in natural ecosystems.

The purpose of this study is to investigate the effects of toxic heavy metals on anaerobic microbial processes affecting the biotransformation of organic compounds. As a model system, we are studying metal toxicity on the reductive dechlorination of chloroaromatic compounds in unadapted and substrate-adapted microbial communities from freshwater sediments.

The onset, rate, and extent of biotransformation of several mono-, di-, and trichlorophenols were examined using unadapted and chlorophenol(CP)-adapted freshwater sediment slurries (pH 7.0) in the presence and absence of added metal salts ($CuCl_2$, $CdCl_2$, $K_2Cr_2O_7$). The time required for unadapted control sediment slurries (no added metals) to dechlorinate the respective chlorophenol (10 mg/L) was as follows: 2,4-DCP and 2,4,6-TCP (16 to 17 days); 2,3-DCP (21 days); 2-CP (31 days); 2,4,5-TCP (37 days); 3-CP (80 days). Addition of a 20 ppm concentration of the chloride salts of Cu(II) and Cd(II) had little or no effect on the onset, rate, or extent of dechlorination for most chlorophenols tested. Addition of Cr(VI) at 20 to 40 ppm, however, increased the lag time before initiation of dechlorination for most chlorophenols tested. Addition of 100 ppm (or greater) Cr(VI), as $K_2Cr_2O_7$, caused total inhibition of dechlorination of 2- and 3-CP, 2,3-DCP, and 2,4,5- and 2,4,6-TCP. Addition of 100 ppm Cr(VI) increased the lag time of 2,4-DCP dechlorination from 13 days (control) to 40 days. Higher concentrations of Cd(II) (100 to 200 ppm) also caused complete inhibition of dechlorination for all chlorophenols with the exception of 2,4-DCP. The rate of dechlorination of 2,4-DCP was reduced by 70 percent and 100 percent at 100 and 200 ppm Cd(II) concentrations, respectively. Addition of $CuCl_2$ at 100 ppm increased the lag time before the onset of dechlorination for most chlorophenols tested, but dechlorination of 2,4-DCP was still evident at 200 ppm Cu(II). The distribution of the test metals between the aqueous and complexed (sediment) phase of the experimental samples was determined by inductively coupled plasma (ICP) spectrometry. In most experiments, the aqueous phase concentrations of the added metals were low and varied from 0 to 2 ppm for Cu(II), 0 to 30 ppm for Cd(II), and 0 to 28 ppm for Cr(VI) over the range of metal salts added (20 to 200 ppm).

The effects of metal salts (20 to 200 ppm of Cu(II), Cd(II)) on the reductive dechlorination of 3,4-DCP were compared in two distinct freshwater sediments of similar pH but of differing total organic carbon content. Control experiments (no added metals) for both sediment types differed slightly with regard to the onset of dechlorination but rates of dechlorination were similar. However, sediment cultures containing the higher organic carbon content (34 mg C/g) were, in general, more resistant to the

inhibitory effects of the added metal salts. The onset and rate of dechlorination of 3,4-DCP were only slightly affected in the higher organic carbon sediment cultures amended with Cu and Cd salts at total metal concentrations up to 150 ppm. Dechlorination in sediment cultures with lower organic carbon content (17 mg C/g) was completely inhibited (100 days incubation) in cultures amended with a total metal (Cu or Cd) concentration of 50 ppm. Further, longer lag times (40 days compared to 14 days) were observed in those cultures with the lower organic carbon content at a lower (20 ppm) added metal concentration. Results of recent experiments using 3,4-DCP-adapted sediment cultures of low organic carbon content indicated that dechlorination was more resistant to the inhibitory effects of the added metal salts than those reported above for the unadapted sediment cultures. Additional studies are in progress to determine the inhibitory nature of metals on reductive dechlorination of higher chlorinated chloroaromatics. These preliminary data suggest that the metal type, aqueous metal concentration, and organic carbon content may affect the transformation of chlorophenols in anoxic sediments.

Section Three
Performance Evaluation

Performance evaluation of bioremediation technologies involves determining the extent and rate of cleanup by a particular bioremediation method as well as the environmental fate and effects of the parent compounds and their by-products. Remediation efforts at a contaminated site can produce intermediate compounds that can themselves be hazardous. Performance evaluation projects determine the risk of potential health effects and the bioremediation approaches that best protect public health.

Researchers have determined that nonconventional pollutants (NCPs) are a potentially important source of hazardous exposure. In an EPA project, researchers documented the fact that NCPs limit the ability of scientists to perform risk characterizations through analytical chemistry and are developing bioassays from which to determine potential genotoxicity at remediation sites. Two other projects evaluated the performance of bioventing systems to determine their effectiveness and to monitor possible health risks from surface emissions of hazardous compounds.

A fourth study concerned potential health effects associated with microorganisms genetically engineered for bioremediation. Investigators exposed laboratory mice to a variety of strains of biotechnology agents and observed the results on their respiratory systems.

Development of Comparative Genotoxicity Risk Methods for Evaluating Alternative Hazardous Waste Control Technologies

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Most environmental remediation situations present mixtures of toxicants and other pollutants (1). Remediation efforts, especially biological ones, produce additional compounds that add to the complexity of evaluating the potential health effects of a contaminated site during and after remediation efforts. Exposures to toxicants from most hazardous waste sites through soil, water, and air are typically low-level chronic exposures. Therefore, one of the toxicological endpoints of greatest interest is genotoxicity, which includes both carcinogenicity and mutagenicity endpoints. Due to the presence of multiple pollutants and the potential production of other pollutants by remediation processes, most of the actual toxicants within a typical remediation site are not identified. The Committee on Environmental Epidemiology of the National Research Council states, "There is evidence that NCPs [nonconventional pollutants] are a potentially important source of hazardous exposure. Some preliminary toxicologic studies suggest that NCPs have important biologic properties, environmental persistence, and mobility... In the broadest sense, these unidentified substances represent risk of unknown magnitude" (1).

NCPs, therefore, limit the ability to do risk characterizations of remediation sites when only analytical chemistry is used for exposure assessment studies. By incorporating biological tests into assessment studies, it is possible to improve the estimations of potential human toxicity before, during, and after remediation efforts. When appropriately coupled with analytical chemistry, bioassays can also be used to identify the major toxic pollutants. In addition, during the development of remediation methods, bioassays can be used for comparative assessments between differing technological approaches. This presentation will discuss the use of bioassays for comparative assessments of complex mixtures, for identifying individual toxins within complex mixtures, and for enhancing exposure assessment.

In bioremediation research, the ideal situation is to avoid the production of additional toxicants and to demonstrate the safety of the expected products—even products for which analytical chemistry monitoring is not done. For determining whether or not bioremediation is an appropriate alternative for a remediation effort prior to its application, two approaches are available. The first approach is to test individual metabolites. Although this approach appears straightforward, it may take extended periods of time to elucidate the actual metabolic pathways and to test each metabolite produced. An alternative approach for examining health risks is to examine products produced in the culture medium when the biodegrading organism(s) is grown on the carbon source of interest. Complete knowledge of the degradation pathway is unnecessary for this evaluation. This method also lessens the likelihood that post-treatment analysis of a remediated site will identify new toxicants that were not a part of the original monitoring scheme.

Recent efforts with 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) illustrate the application of these two approaches (2). 2,4,5-T is well known because it is one of the principal components of the defoliant Agent Orange (4). The herbicide 2,4,5-T is a pollutant in the United States; it was used for weed control from the 1940s until banned in 1969, after a study indicated that it is teratogenic (3). Because 2,4,5-T is persistent in the environment, it remains an environmental problem. Some soil microorganisms, however, have been shown to degrade chlorinated phenoxyherbicides (4,5). One study reported that, aerobically, 2,4,5-T is metabolized to 2,4,5-trichlorophenol (2,4,5-TCP), 2,5-dichlorohydroquinone (DCHQ), and 5-chloro-2-hydroxyhydroquinone (CHHQ) before the arene ring is cleaved (6). Another study indicated that the principal 2,4,5-T intermediates are 3,5-dichlorocatechol and 2-chlorosuccinate (CS) (7). A strain provided by Doctors A.M. Chakrabarty and U. Sangodkar, *Pseudomonas cepacia* AC1100, has the ability to degrade 2,4,5-T. Dr. Sangodkar indicates that AC1100 metabolizes 2,4,5-T through the intermediate CHHQ and that catechol is a by-product of the metabolism. This information is more than is typically available for the degradation products of a pollutant.

To test the utility of these two approaches, two genotoxicity bioassays (the *Salmonella* plate incorporation mutagenicity assay (8) and the prophage-induction bioassay (9)) were used to detect mutagens and potential carcinogens. Initially, 2,4,5-T and its commercially available metabolites were tested with both assays. When tested up to 10 mg per plate using *S. typhimurium* strains TA98, TA100, TA102, and TA104 in the presence and absence of an exogenous metabolic activation system, none of the compounds was mutagenic. Two of the compounds (2,4,5-T and 2,4,5-TCP) were positive in the prophage-induction assay when the metabolic activation system was present. DCHQ was considered a weak positive in this assay when the metabolic activation system was absent. These results indicate that one of

the proposed pathways produced genotoxic metabolites and that one of these metabolites (2,4,5-TCP) is more biologically active than the parent compound. These results, in turn, bring into question whether or not to apply this bacterial strain in remediation, and they might make all bioremediation efforts suspect. The question remains, do toxic metabolites persist?

In the second portion of the study, *P. cepacia* strain AC1100 was used to degrade 2,4,5-T as described by Kilbane et al. (4). At selected times over a 60-hour period, aliquots were withdrawn, cell growth was monitored, and the level of 2,4,5-T was measured. In addition, aliquots from the different time intervals were bioassayed using both methods. In no case did the *Salmonella* strains TA100 and TA98 indicate any mutagenicity. This would indicate that mutagenic hydrocarbon metabolites were not produced. When the prophage-induction bioassay was used in the presence of metabolic activation, the growth of AC1100 was inversely related to the genotoxicity of the 2,4,5-T concentration. The mutagenicity of the uninoculated 2,4,5-T culture did not decline over the same time period. This indicates that the 2,4,5-T was metabolized by AC1100 and that mutagenic metabolites, if produced, did not accumulate within the media.

The ideal situation for evaluating a biodegradation process prior to use would be to determine the toxicity associated with a particular process by (1) understanding the metabolic pathways of the process and the toxicity of each metabolite, and (2) examining the toxicity of a process during a bench-scale operation. This is not always possible. The estimation of toxicity through the use of bioassays, however, provides a powerful tool for the development of bioremediation approaches that protect public health.

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Performance of Bioventing at Traverse City, Michigan

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Two pilot-scale bioventing systems in adjacent plots were evaluated during a 9-month operating period at an aviation gasoline spill site. The unsaturated subsurface was bioremediated to less than 100 mg TPH/kg soil within 5 months. Benzene was less than 5 ppb in the underlying ground water when bioventing was concluded after 9 months of operation. Minimal surface emissions were indicative of a biological cleansing process near the soil surface. Considerable oily-phase residue remained below the water table. Biosparging by air injection below the depth of contamination in the saturated subsurface presently is being evaluated.

Objective

The objective of the project was to design, install, operate, and evaluate two pilot-scale bioventing systems. Performance of the two systems included demonstrating that surface emissions were minimal, total fuel hydrocarbons in the remediated core material would be less than 100 mg/kg, final benzene in the underlying ground water would not exceed 5 µg/L, remediation would be completed in a reasonable time, and the technique would be applicable to full-scale reclamation.

Treatability Studies

Prior to design of the pilot-scale bioventing systems, laboratory soil microcosm studies were conducted using surface soil from the spill site. Microcosms have been used to simulate biodegradation of volatile gasoline components as reported by Kampbell (2).

Aerobic microcosms of 160 mL glass serum bottles and 32 g dry-weight basis Rubicon sand were used to determine biodegradation of fuel vapors under variable parameters of moisture, nutrients, vapor concentrations, and temperature. Degradation under favorable conditions was rapid and complete, showing a curve typical of first-order kinetics. An NPK nutrient response was obtained. Differential responses occurred for temperature ranges from 4°C to 37°C and moisture levels of 3.5 percent to 24 percent, but none greatly hindered the biodegradation process. The rates indicated that at a vapor exposure concentration of 14.5 mg aviation gasoline/kg soil, consumption would be complete during 8 hours of retention in the subsurface.

Introduction

Bioventing is an *in situ* bioremediation process that provides an air flow to vaporize and transport volatile organic pollutants upward from the subsurface to more amenable media for mineralization. The air also provides oxygen for microbial degradation processes. Naturally occurring soil microbes, once acclimated, can biodegrade the volatiles, such as fuel hydrocarbon contaminants.

An aviation gasoline spill of about 35,000 gallons 20 years earlier had an 80 x 360 m surface area plume in 1989. Much of the spill has remained as oily globules in subsurface capillary pores near the ground-water table. Water table changes have resulted in a vertical contaminant smear of nearly 1 m. The water table depth has been near 5 m. The vertical profile was relatively uniform beach sand from the surface to thick clay at 15 m. Detailed descriptions of the surface and subsurface at the field site are presented in Ostendorf et al. (1) and Twenter et al. (3).

Experimental Design

Turf was established on a 75 ft x 90 ft rectangular area overlying the plume of contamination. A nutrient solution was applied for dispersion throughout the unsaturated subsurface. Two blowers in a building shown in Figure 1 were connected to aeration transfer piping and to screened air-injection wells with adjustable depth to emit air flow just above the water table. The north plot venting system was injection only, and the south plot was injection, extraction, and reinjection. The venting systems began operating in October 1990. A blower rate of 5 cpm to each plot was calculated to average an injected-air retention time of 24 hours.

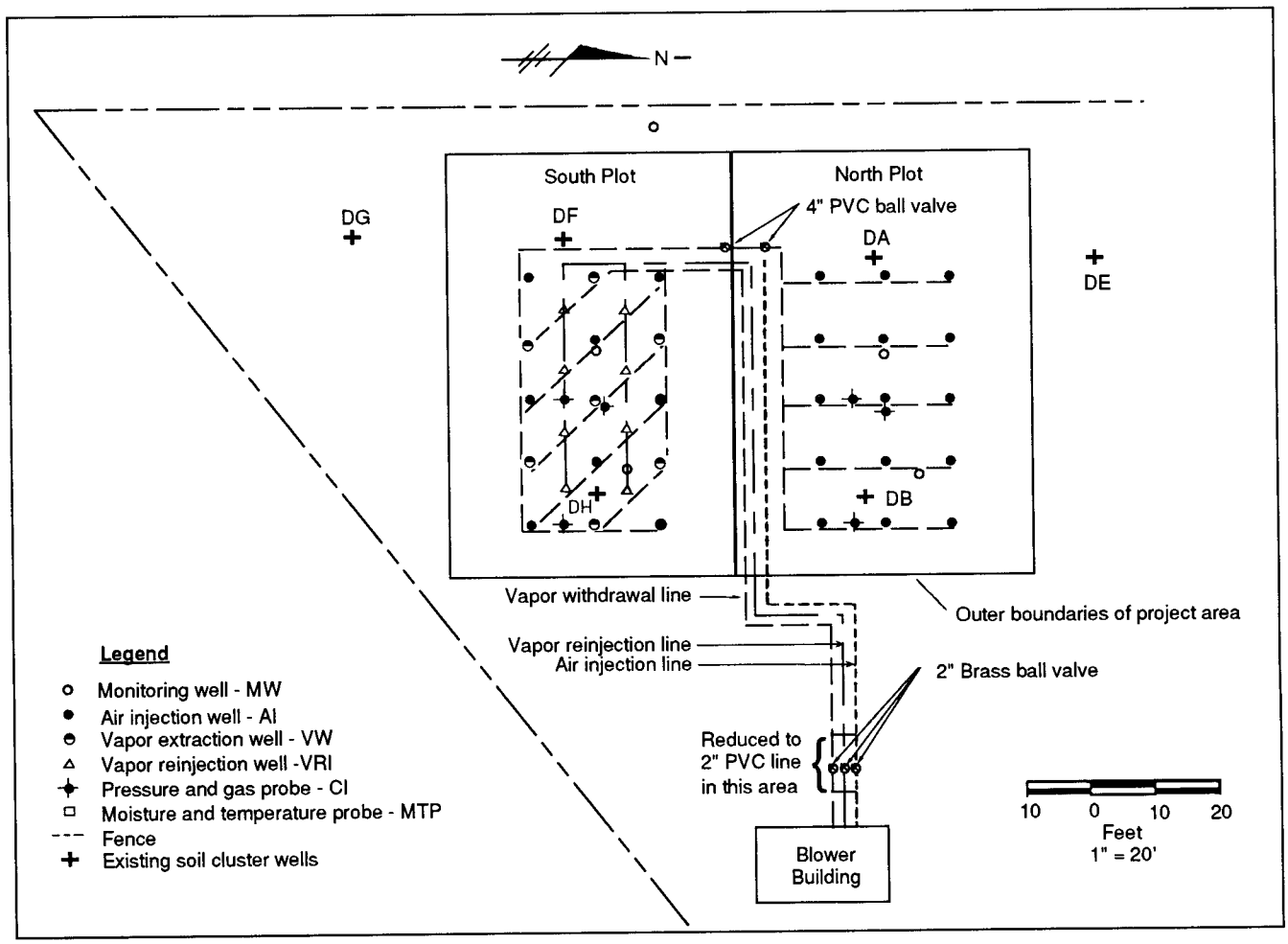

Figure 1. *Bioventing flow line plan.*

System Monitoring

Blower flow rates, water table levels, monitoring well nutrients + aromatic hydrocarbons (BTEX) + dissolved oxygen, vertical profile oily residue, subsoil moisture + temperature, soil gas carbon dioxide + oxygen + combustible gas, and hydrocarbon surface emissions were assayed on a regularly scheduled basis.

Time Requirement

Venting of the subsurface by both pilot-scale systems was done at a rate equivalent to a calculated residence time of 24 hours. Gasoline vapor throughout the unsaturated subsurface increased to a maximum of near 6,000 mg/L total petroleum hydrocarbons in 3 days. A gradual decline to near 600 mg/L occurred 3 weeks later, after which the decline was much less. The bioventing systems were shut down in January for the winter because the upper subsurface was frozen. When the soil had thawed, the venting was restarted in April (Table 1). After 5 months of venting, the soil gas hydrocarbon concentration of 50 mg/L was attained. This level was selected as the clean-up requirement for two reasons. Uncontaminated subsurface meter readings were usually in the 10 to 40 mg/L range. Very low concentrations of less than 50 mg/L may not be sufficient to stimulate enzyme production needed to degrade the gasoline hydrocarbons.

Extent of Treatment

Total petroleum hydrocarbon concentration in the subsurface above the water in the bioventing area ranged up to 2,060 mg/kg in September 1990, up to 38 mg/kg in October 1991. A comparison of vertical profile fuel carbon mass in the north plot for September 1990 and October 1991 is shown in Table 2. Unsaturated subsurface reduction in oily-phase residue was more than 99 percent between the two sampling periods. Oily-phase residue in the saturated subsurface below the water table was reduced 22 percent during the same time period. Some natural losses likely occurred from volatilization, dissolution, and biodegradation.

South plot data (Table 3) compared to the control showed a large reduction in unsaturated subsurface oily-phase residue, but only a slight reduction below the water table.

From January 1991 to October 1991, total BTEX concentrations in monitoring well water decreased tenfold (Table 4). All benzene levels in October 1991 were less than 5 ppb. Several

Table 1. *Soil Gas Hydrocarbons (mg/L)*

Date	North Plot		South Plot	
	One Meter	Three Meters	One Meter	Three meters
10/22/90	1,940	2,060	4,220	2,960
10/29/90	860	910	1,960	1,210
11/12/90	430	490	760	550
01/10/91	180	200	200	260
04/04/91*	400	990	600	1,580
04/18/91	310	820	480	1,160
05/02/91	78	52	66	38
08/08/91	32	40	20	20
10/07/91	16	16	14	14

* System started after 3-month shutdown for winter.

Table 2. *Fuel Carbon Mass in North Plot (mg/ft²)*

	09/25/90		
	Rep 1	Rep 2	$\bar{x}$
Avove water table	43,640	54,020	48,830
Below water table	221,630	232,800	227,220

	10/16/91			
	Rep 1	Rep 2	Rep 3	$\bar{x}$
Avove water table	253	172	480	302
Below water table	147,980	234,390	150,360	177,580

Table 3. *Fuel Carbon Mass in South Plot and South Control (mg/ft²)*

	South Plot		
	Rep 1	Rep 2	$\bar{x}$
Avove water table	37	27	32
Below water table	105,335	62,502	83,920

	South Control		
	Rep 1	Rep 2	$\bar{x}$
Avove water table	12,090	4,510	8,300
Below water table	54,616	103,910	79,260

causative factors for the reductions were possible. Adsorption of air with oxygen by the ground water from overlying venting may have stimulated vaporization and biodegradation of the water-phase BTEX. Gasoline globules trapped in capillary pores remain as a liable pool for potential release of BTEX.

Discussion

Both bioventing systems performed satisfactorily to remediate the unsaturated test area subsurface contamination of aviation gasoline. Surface emissions, once acclimation was attained 1 month after system startup, did not exceed 10 µg TPH/L and were less than 1 µg TPH/L in most instances. Ground-water levels were essentially the same in October 1990 and October 1991, although variations near 0.4 m occurred during the bioventing operating period. Considerable oily-phase residue remained below the October 1991 water-table level. Final benzene concentrations were less than 5 µg/L compared to initial levels up to 133 µg/L in the underlying ground water near the water table. Large

reductions in gasoline hydrocarbons between 1 m-depth soil gas measurements and surface emissions indicated an active rhizosphere cleansing action near the soil's surface.

Costs

Frequent statements in the literature state that bioremediation is emerging as a cost-effective alternative to treat subsurface contamination. It does reduce capital costs by eliminating transportation and disposal of recovered contaminants. *In situ* bioremediation may be less costly, but the biological process can be difficult to control and, in many instances, slow. The cost of soil venting by a field-scale system has been reported to be roughly $50 per ton (U.S. EPA Report 540/A5-89/003, 1989), while incineration costs are more than 10 times this amount. A cost estimate of about $15 per yd^3 sandy soil for bioventing treatment at a JP-4 jet fuel contaminated site has been reported by Vogel (4).

Total costs incurred for the pilot-scale bioventing systems over the 6-month period for construction, operation, field monitoring, and sample analyses were near $147,000. Expansion of the injection well grid from 3,000 ft^2 to 60,000 ft^2 for a field-scale system would increase construction costs about $69,400. Less extensive field monitoring and sample analyses could be instigated to offset the increase in construction cost. Maintaining total costs of $147,000 for the field-scale bioventing system with 2 ft of vertical oily-phase residue above the water table would result in a clean-up cost estimation of $33 per yd^3 of contaminated subsurface.

A grid of air injection biosparging wells was installed in the north plot during the latter part of October 1991 to a depth of 3 m below the water table. Evaluation of biosparging effectiveness will be completed in May 1992.

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Table 4. *Ground-Water BTEX*

Sample	Well depth	Benzene (mg/L)	Total BTEX (mg/L)
		January 1991	
MW2N	*17*	*<5*	*1,018*
MW2N	*21*	*<5*	*440*
MW2S	*18*	*<5*	*9*
MW2S	*20*	*43*	*1,028*
Total		*≤43*	*≤2,495*
		April 1991	
MW2N	*17*	*<5*	*149*
MW2N	*21*	*174*	*492*
MW2S	*18*	*<5*	*39*
MW2S	*20*	*7*	*444*
Total		*≤181*	*≤1,124*
		October 1991	
MW2N	*17*	*<5*	*51*
MW2N	*21*	*<5*	*159*
MW2S	*18*	*<5*	*19*
MW2S	*20*	*<5*	*31*
Total		*≤0*	*≤260*

Bioventing of a Gasoline Spill at Traverse City, Michigan: Practical Engineering Considerations

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The Bioventing Reclamation Pilot Study is designed to evaluate the biodegradation of hydrocarbon-contaminated vapors within the unsaturated zone during induced volatilization. This study is being conducted at the U.S. Coast Guard Air Station in Traverse City, Michigan, the site of a spill of about 35,000 gallons of aviation gasoline that occurred in 1969. After 20 years, a major portion of the spill still persists in the subsurface as a residual plume that is about 1,100 ft long and 250 ft wide. This study is a cooperative effort between the U.S. Coast Guard and the U.S. Environmental Protection Agency's Robert S. Kerr Ground Water Research Laboratory.

The subsurface conditions at the site consist of a uniform beach sand extending to depths of about 50 ft, underlain by a gray, glacial silty clay. The water table is located at a nominal depth of about 15 ft below the ground surface. Over the past 6 years, the water table elevation has fluctuated 6 to 8 ft.

The 90- x 75-ft study area has been divided into two equal areas of 45 x 75 ft to evaluate the effects of different flows and extraction patterns. A conceptual design is presented in Figure 1. The northern area has an injection system, while the southern area has an injection and extraction/reinjection system. The

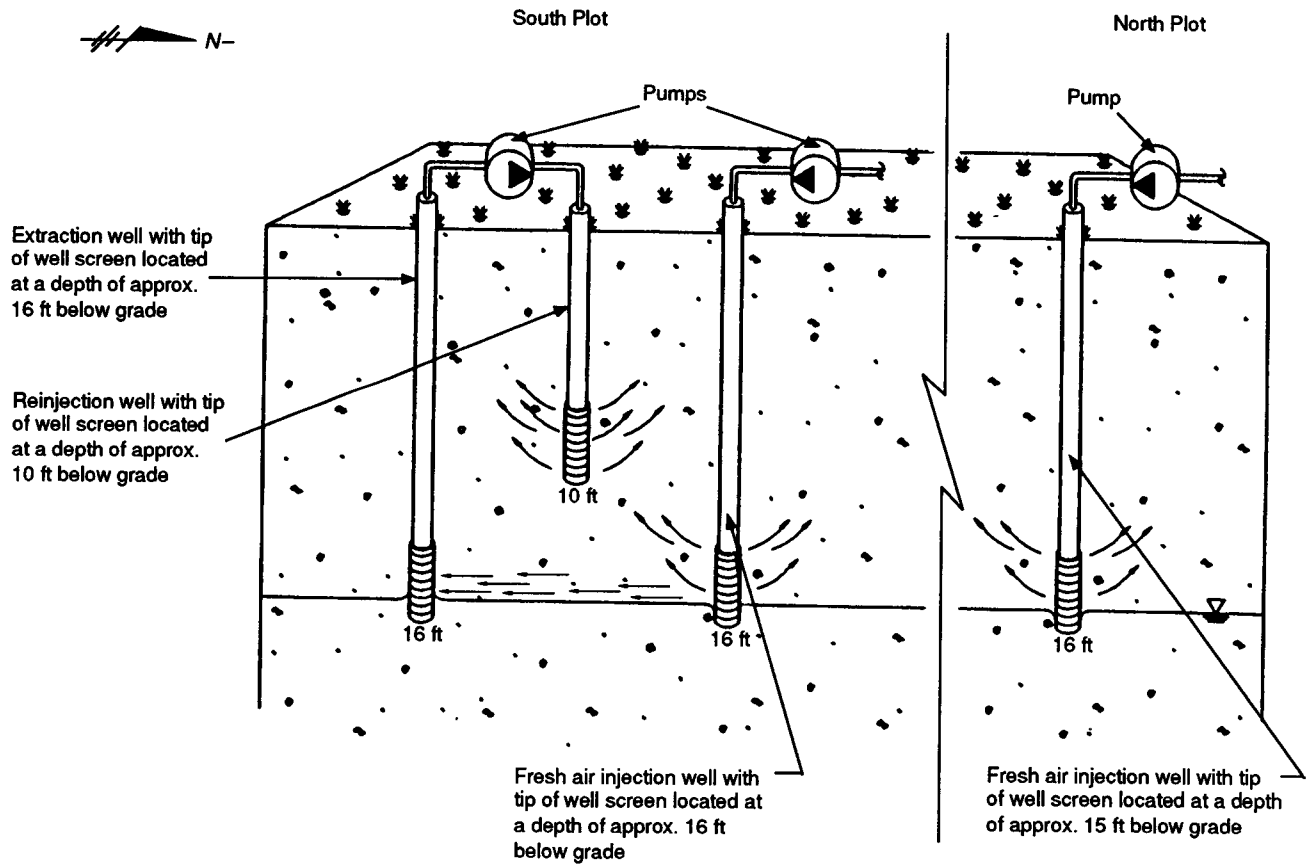

Figure 1. Conceptual design.

pneumatic properties of the unsaturated zone evaluated by the performance of a pneumatic pump test resulted in a design radius of influence of 10 ft. Ambient air is injected into both areas at an initial rate that replaces the volume of calculated air-filled pore space in 24 hours. The flow rate will be increased to a vapor recharge rate of 2 hours or higher as the system becomes acclimated. Based on the treatment area size, depth to water, and air-filled porosity, the recharge rates correspond to flows ranging from 5 cubic feet per minute (cfm) for the 24-hour recharge to 63 cfm for the 2-hour recharge.

The blower system, therefore, has to be capable of extracting vapors in the south study area from the water table (depths of 15 to 18 ft, at the corresponding flow rates; ranging from 5 to 63 cfm) and then reinjecting the vapors at the same rate at a depth of 10 ft. In addition, the system has to be able to inject ambient air at the same flow rate within both the extraction/reinjection plot (south area) and the air injection plot (north area). Accordingly, because the injected ambient air will be placed in twice the area (both test plots), the blower has to be able to inject air at flow rates ranging from 10 cfm to 128 cfm.

As previously mentioned, the project is divided into two different treatment areas. Construction consisted of installing 15 aeration injection points placed on 10-ft centers in the north area. These wells were placed in a 3- x 5-grid pattern and screened just above the water table. In the south area, eight sets of injection points coupled with seven extraction points, 10 ft on centers, were installed with screens placed just above the water table. Eight reinjection wells were installed with the screen placed at a depth of 10 ft.

Root 45 vacuum blowers with a maximum flow rate of 130 cfm were used to inject or extract and reinject the contaminated vapors. These blowers extract at vacuum pressures of 4 to 6 in. of mercury and inject (or reinject) at pressures of 6 pounds per square in. (psi). The extraction/reinjection blower is driven by a 10 HP 3-phase electric motor, while the injection blower is driven by a 7-1/2 HP 3-phase electric motor. All of the equipment is explosion-proof.

The monitoring requirements of the EPA Bioventing Work Plan called for the installation of several different types and depths of monitoring equipment and/or sampling points. To monitor hydrocarbon vapor and oxygen concentrations, three 5-point cluster wells were installed per plot. The cluster wells consisted of 1/4-in. diameter copper tubing with a wire mesh screen covering the tip. The five sampling points of each cluster well were installed at 3.28-ft (1 m) depth increments throughout the unsaturated zone. In addition, we installed three 14-point cluster monitoring wells (well screens at 1.5-ft intervals from ground surface to 21 ft—one per plot and one at an upgradient location), and one set of moisture temperature probes per plot. The monitoring wells are of the same construction as the cluster wells. These wells were installed for water-quality sampling below the water table and relative-humidity monitoring above the water table. The moisture temperature probes are Soil Test Series 300 moisture temperature cells consisting of thermistor soil cells buried at depths of 5, 10, and 15 ft below grade.

The development of a sufficient microbial population to degrade the hydrocarbon vapors requires adequate quantities of nitrogen, phosphorus, and potassium. In accordance with the EPA Work Plan, 64 pounds of nitrogen, 13 pounds of phosphorus, and 5 pounds of potassium were applied to each area prior to startup. Also, during the growing season, 10 pounds of nitrogen, 2 pounds of phosphorus, and 1 pound of potassium are applied to each area monthly. These nutrients were added as an aqueous solution by sprinklers.

The Bioventing Project is sampled and/or monitored daily, twice a week, and monthly. Daily monitoring consists of measuring the blower's operating parameters, such as flow rate, pressure, and vapor temperature. Combustible gas concentration within the vapor reinjection line is determined daily using a Bacharach Threshold Limit Value (TLV) combustible gas meter.

Twice-a-week monitoring includes determining the combustible gas and oxygen concentration within the three 5-point cluster wells located in each plot. The combustible gas concentration is determined using the TLV meter, while the oxygen concentration is determined using a Bacharach oxygen meter. The soil moisture content and temperature are measured twice a week using the soil test moisture temperature probes.

Surface emissions are sampled twice a week at two locations within each study area and at two upgradient locations weekly. To measure surface emissions, a 19-in. diameter stainless steel bowl, which has a volume of 4.3 gallons (16 liters), is inverted and placed flush on the ground as a vapor collection chamber. The sample is pulled from the chamber through teflon tubing that is attached to the bowl by a 1/4-in. diameter steel ball valve tapped into the top of the chamber. Any water that collects within the emission chamber is removed by a water trap, consisting of a flask containing a drying agent (Drierite), located upstream from the sample trap. Samples are drawn over a 4-hour period, using an Ismatec peristaltic pump set for a flow of approximately 1 L/hour.

Water quality samples analyzed for nutrients and BTEX are obtained monthly by sampling at two depths in each of the 14-point monitoring wells.

Clearance and Pulmonary Inflammatory Response After Intranasal Exposure of C3h/HeJ Mice to Biotechnology Agents

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Environmental application of engineered microorganisms has initiated research into health effects potentially associated with these organisms. Pulmonary exposure to biotechnology agents may occur during their production or application. In this study, survival of the dosed microorganisms and pulmonary inflammatory response were explored in 30-day-old male C3h/HeJ mice exposed intranasally (i.n.) to each agent. Mice were administered 10^6 colony-forming units of *Pseudomonas maltophila* BC6 or *P. aeruginosa* strains BC16, BC17, BC18, or AC869. Strains BC6, BC16, BC17, and BC18 were isolated from a commercial product designed for environmental application and strain AC869 was engineered for the ability to degrade 3,5-dichlorobenzate. Two clinical isolates, *P. aeruginosa*, strains PA01 and DG1, were included as positive controls. None of the isolates were detected in the lungs 14 days following treatment. However, strains BC6 and BC16 were recovered from nasal washings. The two clinical isolates and strain BC16 induced a strong inflammatory response that was evident throughout the duration of the experiment (14 days). With the exception of strain BC17, all of the isolates were recovered from the intestinal tract 14 days following treatment. Translocation to the liver, spleen, and/or mesenteric lymph nodes was observed, depending on the dosed strain.

Section Four
Process Research

Process research involves isolating and identifying microorganisms that carry out biodegradation processes and developing new biosystems for treatment of environmental pollutants. Although the majority of conference presentations dealt with laboratory-scale work, it is clear that both pilot-scale and field research are becoming increasingly important. Pilot-scale research provides critical information on process operation and control and residuals/emissions management. Field research is essential for evaluating the performance of full-scale bioremediation processes and for accelerated testing on technologies that lend themselves to field testing.

In one project, researchers studied the ability to stimulate an indigenous aerobic microbial population in a shallow aquifer to enhance its bioremediation characteristics. Another paper described three experimental and theoretical studies on biodegrading volatile organic compounds (VOCs) in aerobic biofilters. Two additional studies focused on design criteria for biofilter treatment of VOCs.

Researchers at a field project evaluated the effectiveness of a fungal treatment to deplete pentachlorophenol in the soil at a former wood treating plant. Two groups studied the effectiveness of anaerobic bacteria to remediate river and lake sediments contaminated with PCBs. River sediments were also used to investigate the effects of four reducing conditions on the biodegradability of halogenated aromatic compounds.

The subject of another research presentation was the unique metabolic capabilities of *Pseudomonas* to remove trichloroethylene from contaminated air and ground water using gas-phase bioreactors. Another study covered the biodegradation of paper-milling effluents by anaerobic microorganisms. A Florida wood preserving plant was the site for a study of bioremediation of contaminated ground water by indigenous microorganisms. One presentation discussed the chemical interactions in microbial biofilms used in water quality control.

Researchers observed bioremediation of soils and sediments contaminated with aromatic amines, important environmental contaminants that can have toxic effects on microbes as well as on animals. EPA presented a report on developing small-scale evaluation techniques for bioremediation of soils by fungi. A new project has as its objective the development of sequential anaerobic-aerobic technologies for biotreatment of soils or sediments contaminated with highly chlorinated aromatic compounds. Researchers also evaluated the effects of surfactant action on microbial degradation.

Evaluation of Enhanced *In Situ* Aerobic Biodegradation of CIS- and Trans-1-Trichloroethylene and CIS- and Trans-1,2-Dichloroethylene by Phenol-Utilizing Bacteria

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Introduction

Recent research has demonstrated that aerobic microorganisms grown on phenol or toluene can initiate the cometabolic oxidation of chlorinated aliphatic compounds (CACs) to stable nontoxic end products (1,2,3,4). Such microorganisms possessing toluene oxygenase (TO) (2,3,4) have good potential for bioremediating aquifers contaminated with CACs and their anaerobic and abiotic transformation products. In this study the ability to stimulate an indigenous phenol-utilizing population in a shallow aquifer that can degrade cis- and trans-1,2-dichloroethylene (c-DCE, t-DCE) and trichloroethylene (TCE) was evaluated. In addition, microcosm studies were performed to evaluate the ability of organisms with other oxygenase systems to degrade CACs. Growth substrates and the assumed enzyme systems studied include: phenol (TO); toluene (TO); methane (methane monooxygenase, MMO) (5); and ammonia (ammonia monooxygenase, AMO) (6).

Background

Previous evaluations at the Moffett test site of enhanced *in situ* biodegradation of CACs focused on stimulating indigenous methanotrophic bacteria through methane and oxygen addition (7). In these tests the following percentage transformations were achieved in a 2-m biostimulated zone: TCE, 20 percent; c-DCE, 50 percent; t-DCE, 90 percent; and vinyl chloride, 95 percent. TCE and c-DCE degradation were limited, indicating other oxygenase systems should be evaluated. The objective of this study was to evaluate the TO system for *in situ* biodegradation of

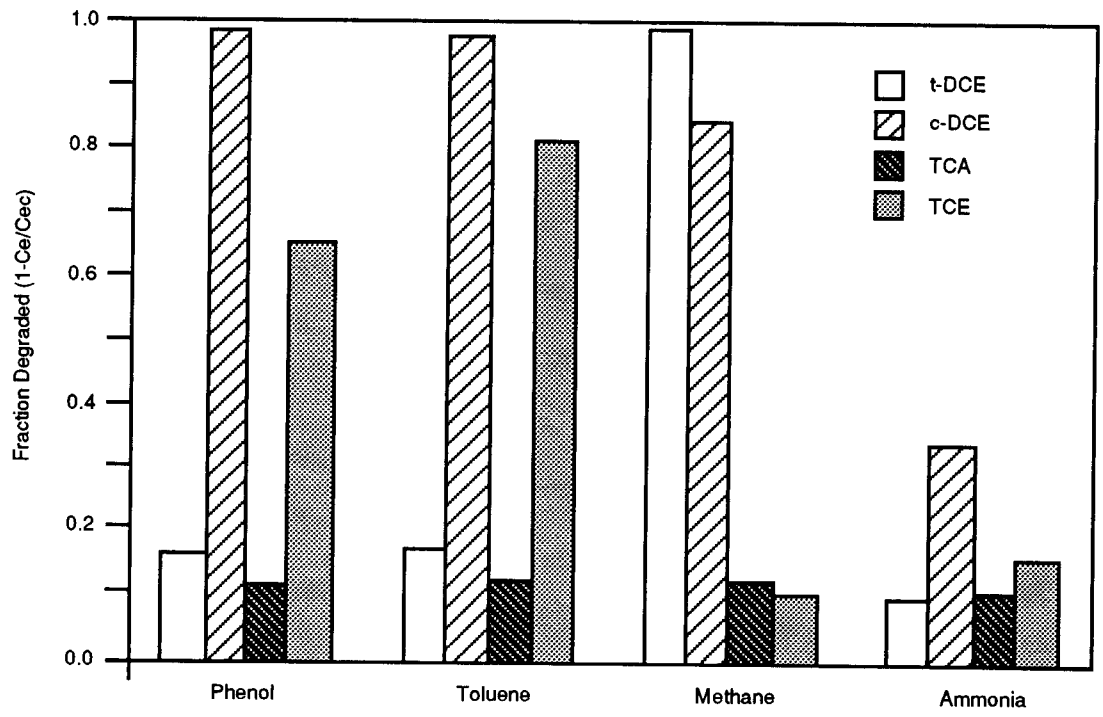

Figure 1. *Relative fractions of CACs degraded in the ground-water microcosms following stimulations with different primary growth substrates.*

TCE, c-DCE, and t-DCE at the Moffett test site by phenol and oxygen addition. Performing the evaluation at the same site permits a direct comparison of the TO system with the MMO system previously studied

Microcosm Studies

Microcosm studies were performed at the field site with columns fed oxygenated ground water from the test zone. The 1-m, 3-in. O.D. columns were filled with 5/8-in. Flexrings as a biological support medium. The columns were inoculated with microbes in ground water from the test zone. Five columns were batch fed twice weekly. Each was fed a different substrate to induce different enzyme systems: methane, MMO; phenol, TO; toluene, TO; ammonia, AMO, and a control-fed dissolved oxygen only. The ammonia addition was to stimulate nitrifiers that possess AMO. The substrates were added at the following concentrations: phenol, 11 mg/L; toluene, 8.5 mg/L; methane, 6.7 mg/L; and ammonia, 7.1 mg/L.

The DO consumption as a function of time indicated microbes growing on phenol and toluene were most rapidly stimulated, followed by methane oxidizers, and then ammonia oxidizers. DO consumption in each column was approximately 20 mg/L, consistent with the amount of substrate added. After 50 days of primary substrate addition, a mixture of TCE, c-DCE, and t-DCE at concentrations of 40 µg/L, 20 µg/L, and 20 µg/L, respectively, were added, while continuing to feed the primary substrates and DO. After several exchanges pseudo-steady-state decreases in concentrations of the CACs were observed in the stimulated columns compared to the control. Figure 1 shows the estimates of the percentage degradation observed, correcting for losses in the control column. The columns fed phenol or toluene were most effective in degrading TCE and c-DCE, while the methane-fed column was more effective in degrading t-DCE. The ammonia-fed column was the least effective.

The relative extent of transformation in the methane column — (t-DCE > c-DCE > TCE) — is consistent with our earlier *in situ* studies with methane-utilizing bacteria. These results indicate that the ground-water-fed columns do mimic the *in situ* conditions. Thus we anticipated that the *in situ* tests with phenol would result in more effective transformation of TCE and c-DCE than was observed previously with methane.

Results of *In Situ* Biostimulation with Phenol

The *in situ* tests were performed along SSE, a new experimental leg that was offset approximately 1 m from the SI leg used in previous experiments with methanotrophic bacteria. The same stimulus-response experimental methodology was used as in our previous studies. The stimulus was the injection of ground water amended with the chemicals of interest and the response was the chemical concentration history at the monitoring locations. Experiments were performed under the induced gradient conditions created by the injection and extraction of ground water.

Bromide tracer tests indicated the new test zone had good hydraulic characteristics, as the injected fluid completely permeated the regions around the observation wells. The fluid transport times were rapid, ranging from 6 hours to the SSE1 observation well (1 m from the injection well), to 24 hours to the SSE3 observation well (4 m from the injection well).

The test zone was not contaminated with the CACs of interest. TCE, c-DCE, and t-DCE were added to the reinjected ground water at concentrations of 30 µg/L, 40 µg/L, and 40 µg/L, respectively, prior to biostimulation. The transport of the CACs was retarded compared to the bromide tracer, due to sorption onto the aquifer solids. c-DCE was the least retarded, while TCE was the most, which is consistent with our previous observations. The steady-state concentrations indicated essentially no transformation in the presence of dissolved oxygen alone.

Active biostimulation was initiated through phenol addition after steady-state contaminant concentrations were achieved. Phenol was pulse injected for 1 hour in an 8-hour pulse cycle at a concentration of 50 mg/L (6 mg/L time averaged). The phenol injection concentration was doubled after 520 hours, and then was reduced to the original concentration after 840 hours.

The responses of DO, TCE, and c-DCE at the SSE2 well, 2 m from the injection well, are shown in Figure 2. The biostimulation with phenol is indicated by the DO decreases, which were small during the periods of low phenol addition, but increased after higher phenol concentrations were added. Decreases in c-DCE and TCE concentrations were associated with decreases in DO, indicating cometabolic transformations resulted from biostimulation. Significant degradation of c-DCE and TCE were observed, with c-DCE being more rapidly degraded than TCE. The c-DCE concentration decreased by approximately 60 to 70 percent and TCE by 20 to 30 percent during the period of low phenol addition. Doubling the phenol injection concentration resulted in a greater transformation of both TCE and c-DCE, with 85 to 90 percent, and over 90 percent, transformed, respectively. Least transformed of the three compounds studied was t-DCE (not shown), consistent with the microcosm studies. Decreasing the amount of phenol increased the TCE concentration, indicating that the extent of transformation was related to the amount of phenol added.

The field results also showed evidence for competitive inhibition of cometabolic transformation by phenol. When phenol concentrations were high due to its pulse addition, the concentrations of c-DCE and t-DCE, which were not pulsed, also increased in concentration. These results are consistent with those observed for the methanotrophic study and indicate that competitive inhibition affects the cometabolic transformation rates.

Summary

An indigenous phenol-utilizing population that effectively degraded TCE and c-DCE was easily stimulated *in situ*. The phenol-utilizing population degraded up to 90 percent of the TCE in a 2-m biostimulated zone, compared to 20 to 30 percent observed previously with methane-utilizing bacteria. The phenol-utilizing population more effectively degraded c-DCE, but was less effective in degrading t-DCE.

Microcosm studies performed under conditions similar to the field tests agreed qualitatively with the *in situ* tests. The results are promising, indicating that microcosm studies are of use in evaluating the potential for cometabolic *in situ* treatment at contaminated sites. Future studies at the site will explore TCE concentration effects on degradation efficiency, and will determine whether a compound more environmentally acceptable

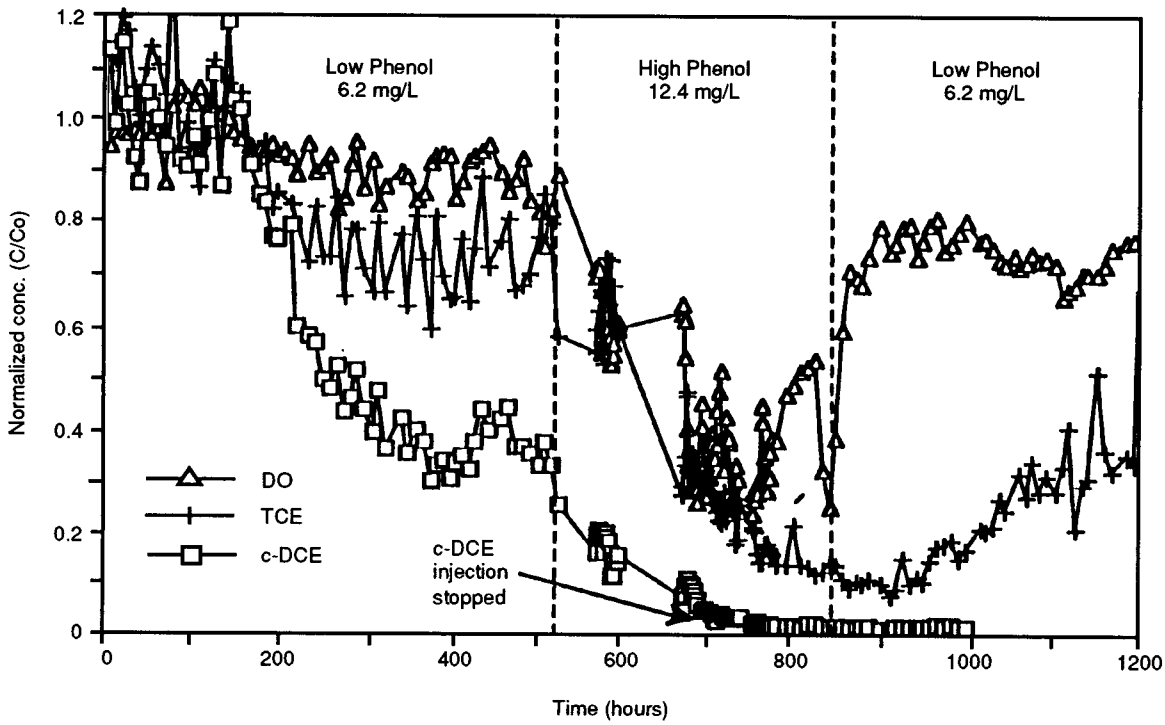

Figure 2. *Response of DO, TCE, and c-DCE at the SSE2 well during in situ biostimulation with phenol at Moffett Field.*

than phenol or toluene can be used to induce an indigenous TO population that effectively degrades TCE.

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Fundamental Studies on the Treatment of VOCs in a Biofilter

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The Superfund Amendments and Reauthorization Act (SARA) emission summary for petroleum and chemical manufacturing companies reveals that the largest environmental releases of chemicals are volatile organic compounds (VOCs) into air. Current technologies for treating VOCs include activated carbon adsorption, wet scrubbing, and incineration. An emerging technology for treatment of VOCs involves the use of biofilters. This paper describes three experimental and theoretical studies on the biodegradation of toluene, methylene chloride, trichloroethylene (TCE), ethylbenzene, and chlorobenzene conducted in aerobic biofilters.

The first study on three compounds describes the performance of a pelletized, activated carbon biofilter (1). Complete degradation of toluene, methylene chloride, and TCE was demonstrated at the bench-scale at 2 minutes gas retention time. After about 180 days of operation the biofilter flooded due to plugging by biomass accumulation. Thereafter, the carbon pellets had to be cleaned mechanically.

The second study on the above five compounds describes the performance of a bench-scale biofilter packed with ceramic pellets (celite, Manville Corporation, California). Complete degradation of the five compounds was obtained in the experiments. The TCE concentration was reduced by about 35 percent.

The third study on the five compounds describes the performance of a biofilter constructed from corrugated plates of celite. The straight corrugations form straight passages, which, unlike the tortuous packed-bed system, offer an unobstructed means for biomass release from the biofilter. The performance of the straight passages system exhibited self-regulation of biomass and provided essentially complete degradation of four compounds. The TCE concentration was reduced by about 40 percent.

Two models for a biofilter system were developed; the first model quantitates biofilter performance at low biomass loadings (biofilm regime), and the second at high biomass loadings (plug flow regime) of biofilter operation. These models were applied to the experimental data that had been obtained from the above three studies. Insights into biofilter design and operation gained from this analysis are presented. Preliminary design procedures for biofilters and cost comparison with other technologies are included in the presentation.

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Fungal Treatment of Pentachlorophenol

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The investigation of fungal treatment systems has been mainly confined to laboratory or bench-scale studies. A systematic series of investigations (1, 2, 3) has permitted us to consider and engage in a treatment effectiveness study under field conditions at an abandoned wood-treating site in Mississippi. The study site is located in Brookhaven, Mississippi, 60 miles south of Jackson, and was identified as a removal action site for EPA Region 4. Ownership of the operation changed hands several times between 1980 and 1991, when the owners filed for bankruptcy. The wood-treating operation was based on both creosote and pentachlorophenol technology. As a result of a treatment chemical spill in the facility's operations, surrounding soil was contaminated to some depth. The soil was excavated and mounded above the ground surface in a RCRA hazardous waste treatment unit.

The field study was undertaken as a SITE Program demonstration involving two phases. The first phase was designed to evaluate the ability of three different fungal species to deplete pentachlorophenol in soil. Three inoculum loadings were used with the required controls. The experimental design consisted of a randomized complete block (RCB) without replication and a balanced incomplete block (BIB) with treatment replicated four times (Figure 1). Treatments (Table 1) were applied to screened 25 cm/deep soil in 3.05-m x 3.05-m plots. Six of the plots were allocated to the RCB design, and the four plots for the BIB design were subdivided into four 1.53- m x 1.53-m subplots.

After inoculation with fungi, as outlined by the design, each plot was irrigated and tilled with a garden rototiller. The tiller was cleaned as it was moved throughout the plots to prevent cross contamination between treatments and controls. Woodchips were added to the soil plots, in accordance with the study design, to provide a substrate to sustain growth of the fungi. Soil moisture was monitored daily throughout the study and maintained at a

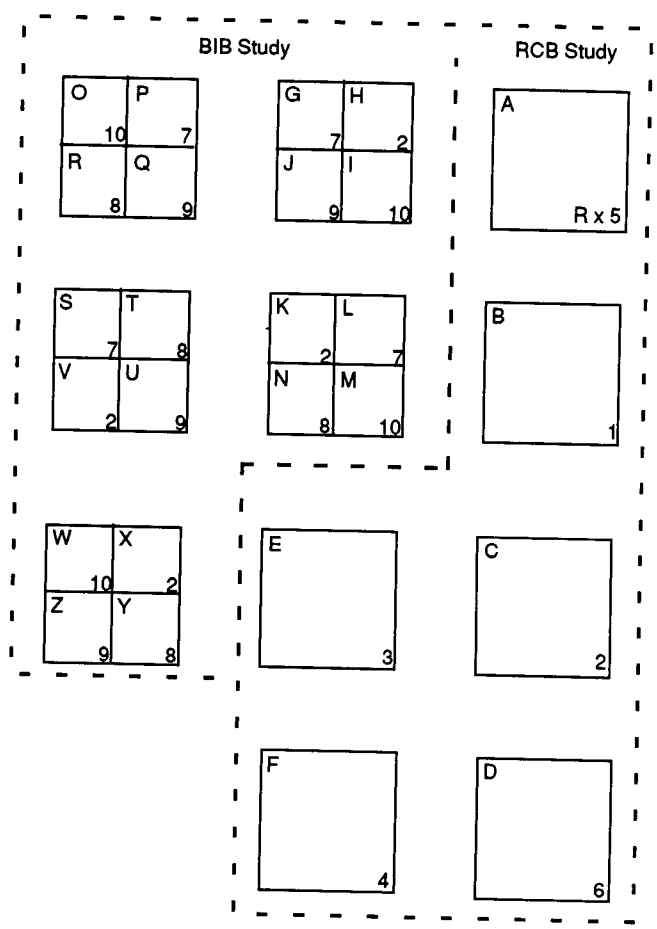

Figure 1. *Treatment plot arrangement.*

prescribed level. Both ambient and plot temperatures were recorded daily throughout the study. Weekly plot tilling was scheduled for the duration of the study. A time series analysis of treatment performance was accomplished by sampling the plots before application of the treatments, immediately after treatment application, and then after 1, 2, 4, and 8 weeks of operation.

The study was conducted over a 2-month span from September 18 through November 13, 1991. The analysis of treatment data has identified the *P. sordida* treatment as the most effective to biotransform pentachlorophenol (Figure 2). This fungal treatment has been selected as the treatment for the SITE demonstration in 1992 when the period of operation is designed to be 5 months.

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Table 1. *Experimental Design for Fungal Treatment Effectiveness*

Treatment	Fungus/Control	Inoculum Loading	Plots
1	*P. chrysosporium*	5%	B
2	"	10%	C,H,K,V,X
3	*P. sordida*	10%	E
4	*P. chrysosporium*/ *T. hirsuta*	5% 5%	F
5	Inoculum carrier control	10%	A
6	No treatment control	–	D
7	*T. hirsuta*	10%	G,L,P,S
8	*P. chrysosporium*	13%	N,R,T,Y
9	"	10% (Day O) 3% (Day 14)	J,Q,U,Z
10	Wood chip control	–	I,M,O,W

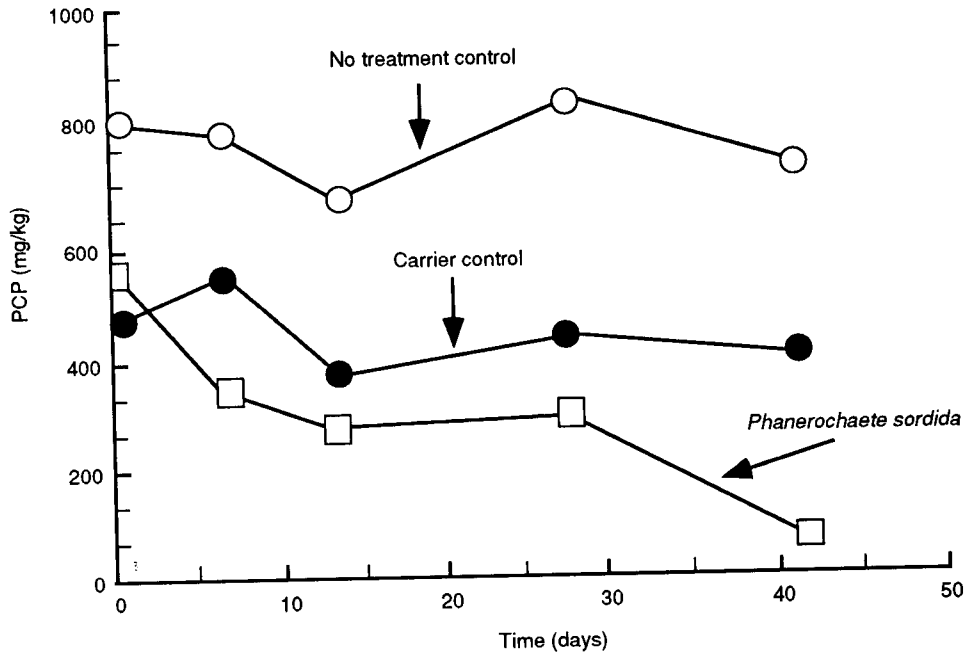

Figure 2. *Pentachlorophenol depletion by Phanerochaete sordida.*

Anaerobic Degradation of Highly Chlorinated Dibenzo-*p*-Dioxins and Dibenzofurans

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Methanogenic Hudson River sediments contaminated with 100 mg/kg "weathered" Aroclor© 1242 were incubated anaerobically and spiked with 144 ± 14 µg/kg of the following polychlorinated dioxin (PCDD) and dibenzofuran (PCDF) congeners: 1,2,3,4,7,8-H_xCDD; 1,2,4,6,8,9/1,2,4,6,7,9-H_xCDD; 1,2,3,4,6,7,9-H_pCDD; 1,2,4,6,8-PentaCDF; and 1,2,3,4,6,7,8-H_pCDF. Previously, a hexachlorinated congener was identified as a metabolite in H_pCDD incubations. No further PCDD/F metabolites were detected in final extracts of Hudson River microcosms, yet the methanogenic population exhibited dechlorinating activity on Aroclor© 1242, suggesting a concentration or bioavailability threshold below which no reductive dehalogenation occurs or at rates not measurable within the time of this project.

Background

Reductive dehalogenation of polychlorinated biphenyls (PCBs) with a high degree of chlorine substitution has been demonstrated to occur in previously contaminated Hudson River sediments (1) and in methanogenic microcosms containing pristine or adapted sediments, spiked with mg/kg concentrations of either Aroclor© mixtures (2,3) or individual PCB congeners (3,4). The PCBs presumably serve as an alternative "electron sink" to CO_2 for methanogenic bacteria. Because of the structural similarity between dioxins or furans and PCBs, the potential for susceptibility of the PCDD/F to reductive dehalogenation processes was investigated.

Substrate disappearance plots expressed as C/C_o of HexaCDD-, HexaCDDi-, and HeptaCDD-spiked microcosms indicated that biotransformation at least contributed to substrate disappearance in the live replicates, relative to the killed or chemical controls (Figure 1, A-C). Previously, it was reported that after 2 months, approximately 9 µg/kg of hexa-chlorinated dioxin was formed in Hudson River sediment-inoculated microcosms spiked with HeptaCDD (5). The concentration of the intermediate decreased to 6 µg/kg after 9 months (Figure 1, D), but no lesser chlorinated congeners resulting from further dehalogenation were detected (5). In a parallel series of experiments, partitioning the PCDD/F in microcosms inoculated with aquifer material was shown to influence the interpretation of long-term incubation results, as 60 to 90 percent of the substrate was found to be associated with the settled sediment, which was only a minor fraction of the solids sampled at any given time.

In this paper we describe dechlorinating activity on Aroclor©1242 and partitioning PCDD/F in Hudson River sediments during long-term incubations.

Results

The analyses of Hudson River extracts spiked with PCDD/F for lower chlorinated congeners (penta- and tetrachlorinated) were impeded by interferences in the PCDD/F window by the higher chlorinated PCB congeners of Aroclor© 1242. Most of these interferences can be eliminated by GC/MS analyses of M^+, (M^++2), and (M^+-2) isotopic ratios (6). No products were conclusively identified, however, in part because of the low intensities obtained relative to background values. The recovery of PCDD/F in Hudson River sediment-inoculated microcosms, as exemplified for the dioxins, is given in Figure 2. All values are normalized with respect to octachloronaphthalene recovery (52 to 65 percent) after 24-hour Soxhlet extraction with hexane:acetone (1:1). The low PCDD/F recovery in both live and killed incubations follows the same pattern observed in the C/Co plots, indicating sorption to the high organic carbon river sediments.

The lack of further dehalogenation of PCDD/F was not due to inactivity of the methanogenic populations present. The distribution of PCB homologs in an Aroclor© 1242 standard, in the "weathered" Aroclor© 1242 present in Hudson River sediments (time 0), and in the active microcosms after 16 months, is shown in Figure 3. Since a mixture of fatty acids was added to the microcosms to augment the indigenous methanogenic populations, the already dechlorinated Aroclor© 1242 (B) was further dehalogenated, resulting in the accumulation of di- and trichlorobiphenyls at the expense of tetra- and pentachlorinated congeners (C).

Conclusions

None of the PCDD/F congeners other than H_pCDD showed accumulation of lesser chlorinated isomers, although all decreased over time relative to the killed controls. This may be explained by the presence of "weathered" Aroclor© 1242 as an alternative electron acceptor. Aroclor© 1242 was present at much higher concentrations (100 mg/kg) and was further dehalogenated by the methanogenic population. Assuming the same populations are responsible for dechlorinating activity on PCDD/F and PCBs, a threshold value might be invoked below which no

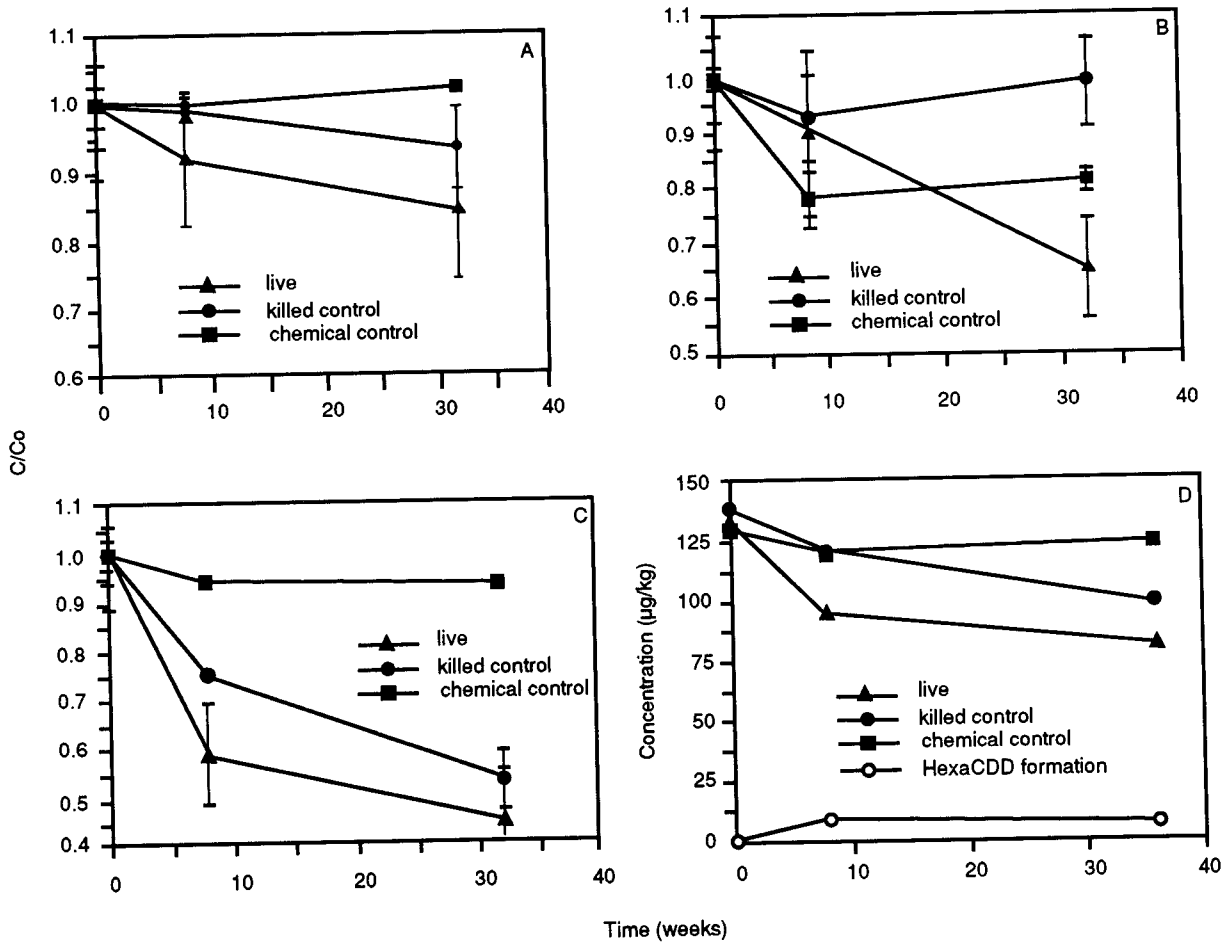

Figure 1. *Fate of H_xCDD (A), H_xCDDi (B), and H_pCDD (C) in Hudson River sediments, and product formation in H_pCDD-spiked microcosms (D). The values for the C/Co plots represent averages of three and two replicates for active microcosms and killed or chemical controls, respectively.*

dehalogenation occurs. Moreover, the low extraction efficiencies obtained for all PCDD/F after extended incubation in a relatively high organic carbon inoculum (1.5 percent) may indicate that the (higher) chlorinated congeners are strongly sorbed and rendered biologically unavailable. To test this hypothesis, some microcosms were spiked with 2 mg/kg PCDD/F, but data are not available at the time of this report.

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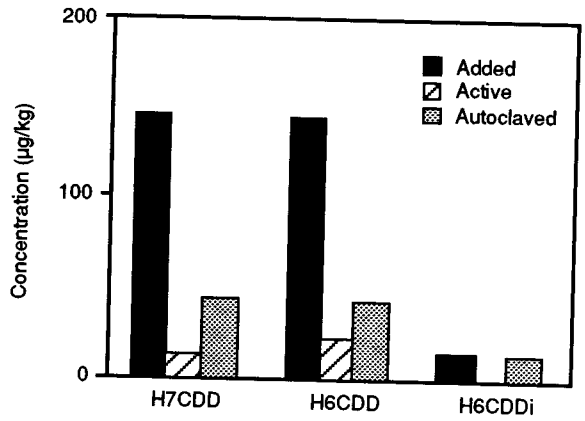

Figure 2. *PCDD/F added to and recovered from final Hudson River extracts after a 16-month incubation period.*

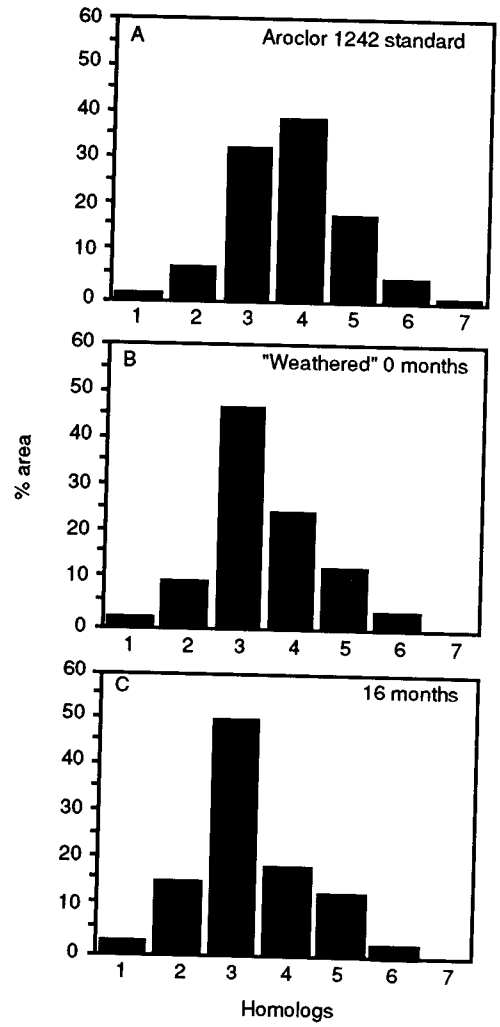

Figure 3. *PCB homolog profile of an Aroclor© 1242 standard (A), and Hudson River sediment-inoculated microcosms at time 0 (B) and after 16 months (C).*

Bacterial Degradation of Trichloroethylene In a Gas-Phase Bioreactor

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Introduction

The application of the constitutive trichloroethylene (TCE)-degrading bacterium, *Pseudomonas cepacia* G4 5223 Phe1, in bioreactors designed for the removal of TCE from contaminated air or ground water was investigated. To exploit the unique metabolic capabilities of this bacterium, two bioreactor designs were used; both employed a fixed biofilm associated with the surface of an inert support material. Various materials were assessed for their abilities to support a TCE-degrading population of Phe1. One reactor received air-entrained TCE along with a continuous inflow of nutrient medium. Strain G4 (the non-constitutive parent of G4 5223 Phe1) was unable to affect TCE concentration in such a vapor phase bioreactor (100 percent re-feed), whereas Phe1 (the constitutive TCE degrader) completely removed the 80 µm TCE present. Tests of strain Phe1 in a continuous flow vapor phase reactor resulted in an average of 92.1 percent removal of TCE at an average input concentration of 12.6 µm (0 percent re-feed) over a 72-hour test.

Results and Discussion

Ground water contamination by TCE and related chlorinated compounds (dichloroethylenes and vinyl chloride) is a subject of overwhelming concern. Currently, treatment of TCE-contaminated soil and water relies primarily on pump-and-treat systems in which TCE is distilled away from the water under vacuum or is air stripped and transferred onto an adsorbent such as charcoal. In either event, the result is simply the transfer of the pollutant to another location. The capacity to destroy the contaminant at the site via bioremediation could provide significant environmental and economic benefits.

All TCE-degrading, non-recombinant bacterial systems characterized to date, require chemical induction. Because of this co-metabolic requirement, the bioremediation potential of these bacteria is limited to situations in which a constant input of inducer can be maintained. The use of such organisms in a bioreactor requires a sensitive balancing of the inducer chemical, growth substrate (if different from the inducer), TCE, and oxygen. The simplifications inherent to a bioreactor colonized by a constitutive TCE degrader are obvious. *P. cepacia* strain G4 5223 Phe1 is an excellent candidate for use in bioremediation systems in view of its ability to mineralize TCE constitutively (2,3,4).

Because *P. cepacia* G4 5223 Phe1 does not use TCE as a growth substrate, it was necessary to consider bioreactor designs that would allow continual nutritional supplementation of the biofilm. Consequently, six different physical support materials were tested for the ability to maintain an active population of Phe1. Several colonizable surfaces were found to serve in this role. One bioreactor achieved colonization of the support material to $\geq 1.4 \times 10^8$ bacteria gram^{-1}.

The toxicity of TCE and its breakdown products in the bioreactor were also analyzed. Tests with Phe1 indicated a toxicity due to the metabolism of TCE at concentrations above 500 µm (similar to the metabolite toxicities reported for isoprene oxidizing bacteria (1)).

Due to the inactivity of the wild-type strain toward TCE in the absence of an aromatic inducer, *P. cepacia* G4 was an ideal negative control for the bioreactor studies. Air-entrained TCE was introduced to columns containing an axenic biofilm of either *Pseudomonas cepacia* G4 5223 Phe1 or *Pseudomonas cepacia* G4 with 100 percent re-feed, and no supplementation of oxygen or nutrients (Figure 1). Gas chromatographic analysis of the column atmospheres indicated TCE breakthrough occurred at approximately 0.3 hours in both columns. While the column containing G4 attained a concentration of approximately 50 µm (approximately 6,000 ppb) and maintained this concentration throughout the 20.3-hour test period, the G4 5223 Phe1-containing column never achieved a return TCE concentration above 13 µm (approximately 1,700 ppb) at the initial breakthrough, and declined to undetectable levels by 20.3 hours. In addition, an

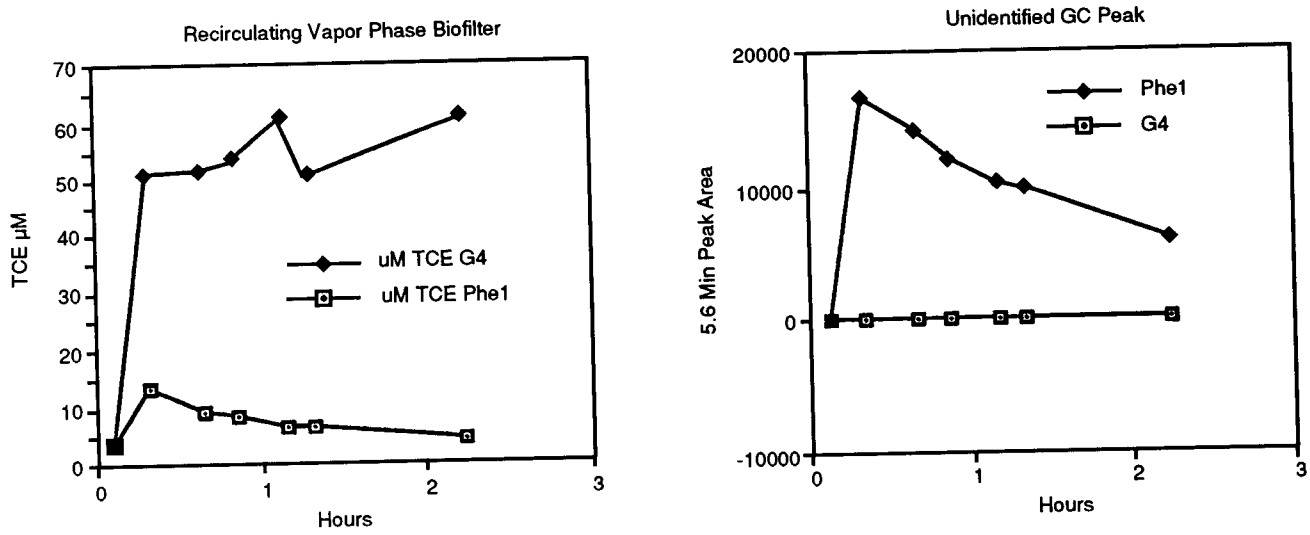

Figure 1. TCE concentration in the atmospheres of two independent recirculating gas-phase biofilters inoculated with Pseudomonas cepacia G4 or G4 5223 Phe1.

unidentified transient compound, which we interpreted to be a metabolite, was produced only in the G4 5223 Phe1 column. It is represented in Figure 1 as the area under a cryptic peak occurring at a retention time of 5.6 minutes.

The second bioreactor tested was a continuous flow, gas-phase biofilter that allows constant addition of nutrients and air-entrained TCE to the biofilm. Nutrients were pumped to the top of the column where they flowed down through the inert support. Spent medium was pumped from the bottom of the column to a waste bottle, where it was sampled for residual TCE. Air and TCE vapor were pumped into the column at the bottom and allowed to exit at the top. Input and output ports were sampled to assess TCE concentrations before and after exposure to the biofilm. Input and output concentrations over a 72-hour trial are shown in Figure 2. Air-entrained TCE was fed at 3.25 mL/min, through a column packed with 231 cm^3 of crushed oyster shell colonized by *P. cepacia* G4 5223 Phe1. Continuous input of medium into the column was maintained at 0.37 mL/min. The average TCE input and output concentrations over this time were 12.6 and 1.0 µm, respectively, yielding an overall TCE removal of 92.1 percent with 0 percent re-feed. The overall efficiency during the test period was found to correspond to 0.51 mg TCE/hour/kg support material (at 2.5 g/cm^3).

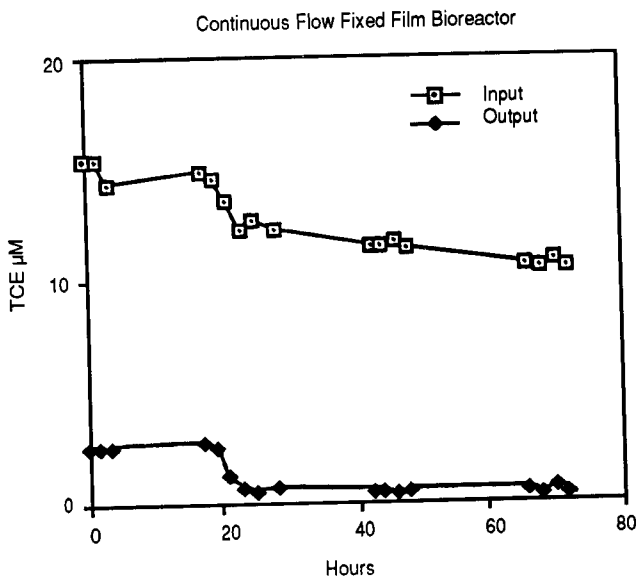

Figure 2. TCE degradation within a continuous feed vapor-phase bioreactor.

Clearly, *P. cepacia* G4 5223 Phe1 growing in a thin aqueous biofilm is capable of degrading air-entrained TCE. These results indicate that the potential exists for coupling bioremediation with methods that generate TCE-air mixtures at contaminated sites. Such a coupling of physical and biological processes may greatly enhance current efforts to reduce the amount of TCE in contaminated aquifers.

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Anaerobic Bidegradation of 5-Chlorovanillate as a Model Substrate for the Bioremediation of Paper-Milling Waste

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5-chlorovanillate (5CV) was chosen as a model compound for studying the biodegradation of paper-milling effluents because it is readily available and it contains the methoxyl-, chloro-, and carboxyl side groups that are present on the aromatic chlorinated compounds in paper-milling effluent. Using this approach, an anaerobic enrichment was developed that degraded 5CV. 3-chlorocatechol (3CC) and catechol were detected as intermediates of degradation (Figure 1). If 5mM bromoethanesulfonic acid (BESA) was included in the enrichment, 5-chloroprotocatechuate (5CP), protocatechuate, and vanillate were also detected (Figure 2). From the temporal sequence and amounts of intermediates formed, we concluded that the major pathway of 5CV degradation was stepwise demethoxylation to 5CP, decarboxylation to 3CC, and dechlorination to catechol, which was subsequently completely degraded (Figure 3). The addition of BESA appeared to inhibit decarboxylation and demethoxylation, allowing us to detect 5CP, as a major intermediate, and vanillate and protocatechuate as minor products. Enrichments adapted to these intermediates will be studied using 16S rRNA analysis to identify bacterial species responsible for a particular degradative activity without having to isolate pure cultures. Phylogenetic placement of the species may also aid in developing enrichment strategies for obtaining the various bacterial species in pure culture. Once obtained in pure culture, the role of individual species in the degradation of 5CV will be assessed. A defined mixed culture of known bacterial species responsible for the complete degradation of 5CV can be devised for further study.

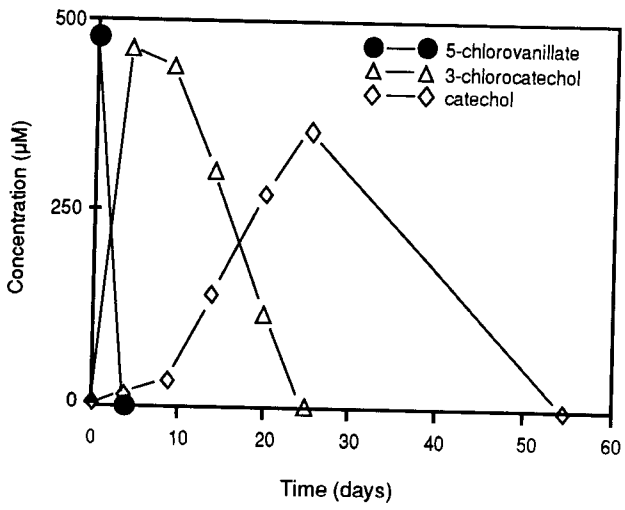

Figure 1. Degradation of 5-chlorovanillate in the first transfer of an Eleven Mile Creek primary anaerobic 5CV enrichment.

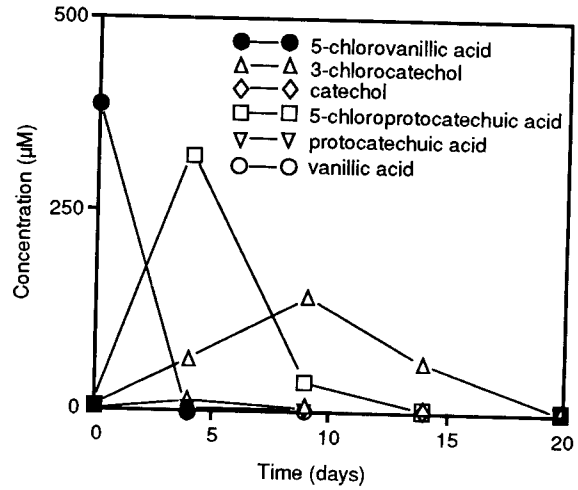

Figure 2. Degradation of 5-chlorovanillate in the first transfer of an Eleven Mile Creek primary anaerobic enrichment with 5mM BESA added.

In addition to the 5CV enrichment described above, an anaerobic bacterial co-culture that dechlorinates 3-chlorobenzoate (3CB) was shown stepwise to dechlorinate 5CV to vanillate and demethoxylate vanillate to protocatechuate. Protocatechuate was not further degraded. This co-culture contained a gram-negative small diplobacillus, and curved rod. The diplobacillus was iso-

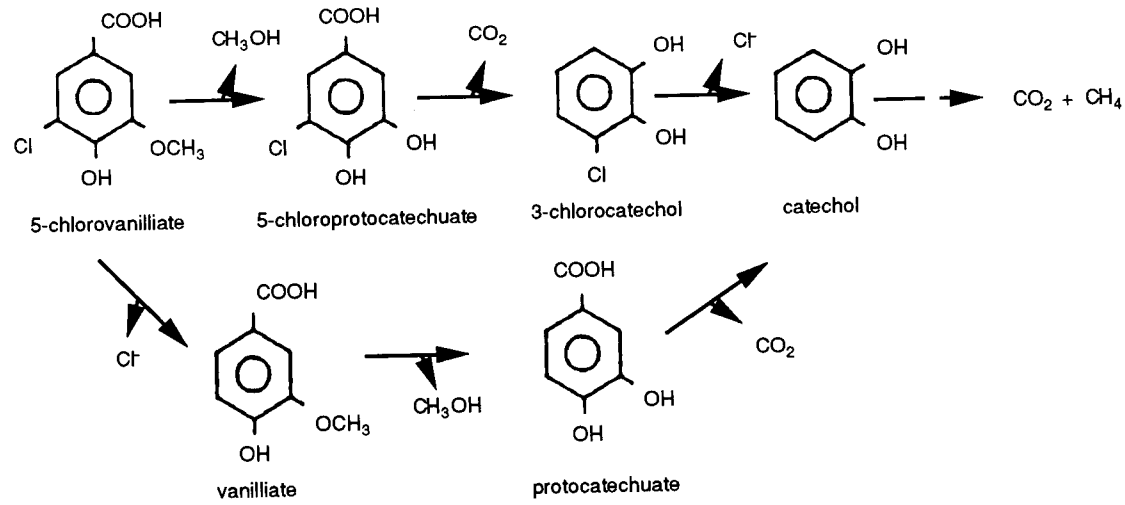

Figure 3. Proposed pathway for the complete degradation of 5-chlorovanillic acid.

lated in defined pyruvate medium and designated strain 3162pyr. Physiological studies placed strain 3162pyr in the desulfoviridin-negative, desulfovibrio-like group of rod-shaped sulfate reducers that include *Desulfovibrio* strain Norway 4, and the newly formed genus *Desulfomicrobium*. Placement of strain 3162pyr as a new species in this group was confirmed by 16S rRNA analysis. 16S rRNA analysis of the co-culture is currently underway to identify the curved rod.

Strain 3162pyr degraded benzoate, 3CB, and 3-bromobenzoate in the presence of pyruvate, but did not degrade 5CV. Benzoate has not been detected as an intermediate in the degradation of 3CB or 3-bromobenzoate. These data suggest that the curved rod is responsible for dechlorination of 5CV. Studies are underway to confirm the role of the two species in 5CV transformation.

The original 5CV enrichment, its derivatives, and the co-culture will be tested for their ability to detoxify paper-milling waste obtained from a local paper-milling company. Untreated and treated waste samples will be assayed in a test for toxicity/tetratogenicity with embryonic inland silversides, *Menidia beryllina*. Comparisons can be made to determine the efficacy of the various consortia in detoxification.

Methanogenic Degradation of Heterocyclic Aromatic Compounds by Aquifer-Derived Microcosms

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Introduction

The ultimate fate of heterocyclic aromatic compounds in subsurface environments is controlled by various transport and transformation processes. Potentially the most important but currently one of the least understood processes affecting groundwater quality is biotransformation of these pollutants by indigenous microorganisms under anoxic conditions. Heterocyclic compounds are frequently encountered in the environment because they are major constituents of both fossil and synthetic fuels and of many pesticide mixtures (e.g., creosote). Heterocyclic aromatic hydrocarbons that contain nitrogen or sulfur in their ring structure have been shown to degrade under methanogenic, denitrifying, and sulfate-reducing conditions.

The mechanism of the initial methanogenic oxidation of benzothiophene was shown to be hydroxylation of the heterocyclic ring, followed by reduction and cleavage (1). After cleavage, the substituent side chains and the remaining homocyclic ring were subjected to various reactions, including oxidation, decarboxylation, desulfurylation, and O-methylation. These reactions were followed by reduction and cleavage of the homocyclic ring, ß-oxidation, and mineralization to CH_4, CO_2, and H_2S. The major degradation pathway intersected segments of both the phenol and benzoic acid pathways. A minor pathway was observed starting with the oxidation of the homocyclic ring with subsequent ring reduction, ring cleavage, degradation of the remaining S-heterocyclic ring, and mineralization (1).

There have been several reports of the persistence of intermediate compounds during laboratory anaerobic degradation studies of heterocyclic aromatic compounds. Oxindole has been shown to be an intermediate of the anaerobic biodegradation of indole (2). 2(1H)-quinolinone and 1(2H)-isoquinolinone were observed as intermediates of quinoline and isoquinolinone, respectively (3). Godsy et al. (3) also observed that during downgradient travel in an aquifer and in laboratory microcosms, quinoline and isoquinoline were very rapidly oxidized to 2(1H)-quinolinone and 1(2H)-isoquinolinone, respectively. The latter two compounds persisted for some time before they were mineralized to CH_4 and CO_2.

In this study, we present evidence that the major factor affecting the fate of creosote-derived heterocyclic compounds in methanogenic environments is microbial conversion to CH_4 and CO_2. The pathways and thermodynamics of microbial conversion by a complex, aquifer-derived, methanogenic consortium is determined for a number of these compounds.

Materials and Methods

The sample site is located in Pensacola, Florida, adjacent to an abandoned wood-preserving plant (3). The wood-preserving process consisted of steam pressure treatment of pine poles with creosote and/or PCP (pentachlorophenol). For more than 80 years, large but unknown quantities of wastewater, consisting of extracted moisture from the poles, cellular debris, creosote, PCP, and diesel fuel from the treatment processes, were discharged to nearby surface impoundments. The impoundments were unlined and in direct hydraulic contact with the sand-and-gravel aquifer. Contamination of the ground water resulted from the accretion of wastes from these impoundments.

Microcosms used for the study were prepared in 4 L glass sample bottles and contained approximately 3 kg of aquifer material anaerobically collected from the approximate centroid of the active methanogenic zone (3). The compounds of interest were added to 2.5 L of mineral salts solution at concentrations similar to those found in the aquifer: 10 to 40 mg/L. Amorphous FeS was used as the reducing agent (4) to ensure methanogenic conditions. The microcosms were prepared, stored, and sampled in an anaerobic glove box containing an O_2-free Ar atmosphere at 22°C.

Degradation pathways for the various compounds were determined by computer-aided gas chromatography-mass spectrometry (GC/MS/DS) analysis of intermediate compounds that appeared in the growth liquor during biodegradation (3). Concen-

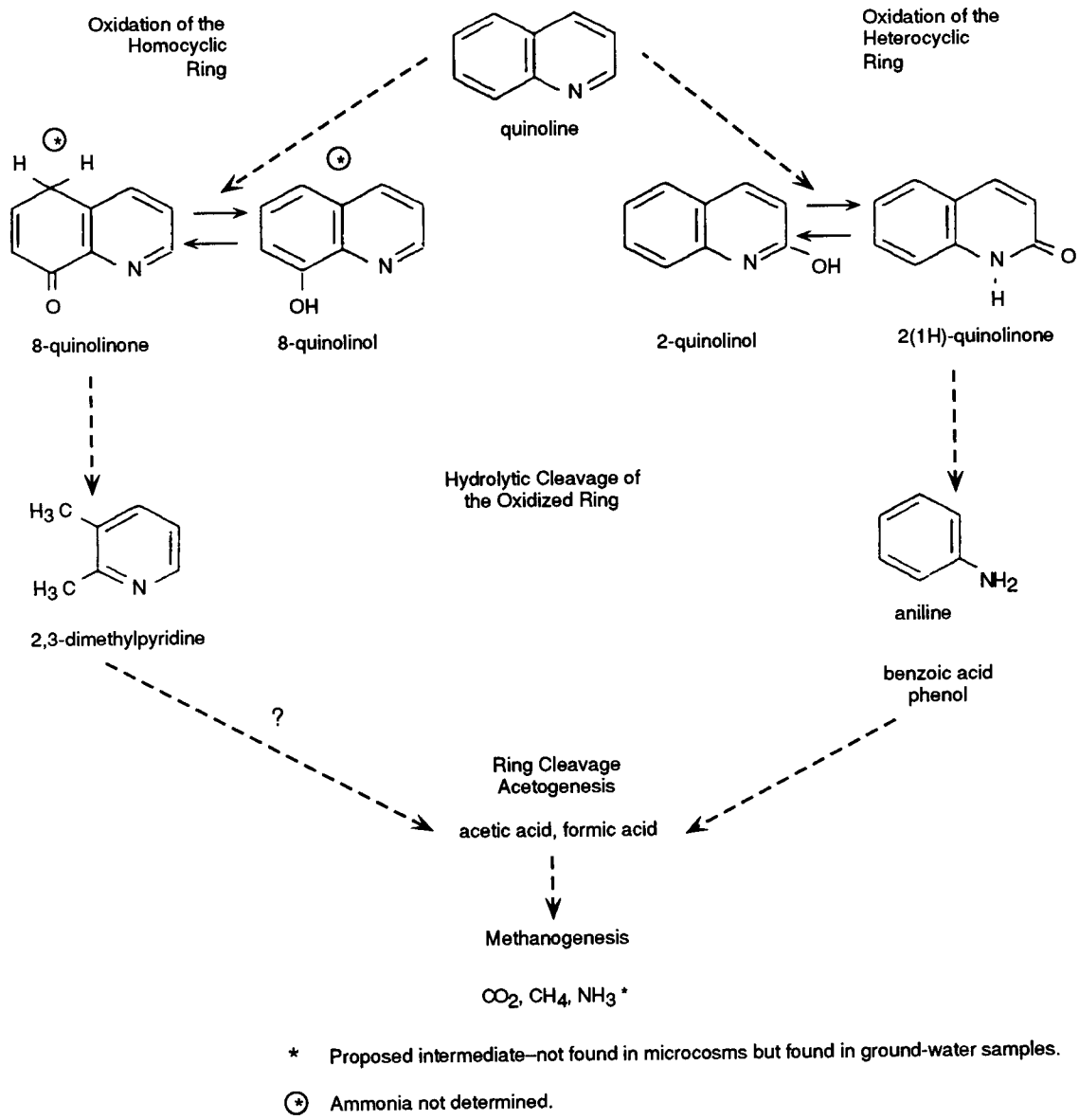

Figure 1. Methanogenic degradation pathway for quinoline in aquifer-derived microcosms.

trations of CH_4 and CO_2 in the head space and dissolved in the mineral salts were determined by gas chromatography (GC) (5).

Results

Compounds detected in the growth liquor just before the onset and during methanogenesis of quinoline are shown in Figure 1. Compounds that were found in the organic-free controls are not shown and presumably do not arise from the degradation of quinoline. Autoclaved controls did not produce any detectable amounts of CH_4 and CO_2.

During oxidation of the nitrogen-containing heterocyclic compounds indole, quinoline, and isoquinolinone to oxindole, 2(1H)-quinolinone, and 1(2H)-isoquinolinone, respectively, the parent compounds were initially stoichiometrically oxidized. The oxidation did not support growth. The oxidized intermediates persisted for some time after the initial oxidation of the parent compounds before they were degraded to CH_4 and CO_2.

The mass balances on the complete degradation of the above compounds yielded 87.6 percent of theoretical gas production for oxindole, 91.7 percent for 2(1H)-quinolinone, and 88.5 percent for 1(2H)-isoquinolinone. The conversion values obtained for the above compounds are well within the expected range.

Discussion and Conclusions

It is evident, based on the report by Pereira et al. (6) of the incorporation of water into the homocyclic ring, that the first step in biodegradation consisted of oxidation, followed by the cleavage of the N-heterocyclic ring. After cleavage of this ring, the substituent side chains and remaining homocyclic rings were subjected to various reactions, including oxidation, decarboxyla-

Table 1. *Free Energy Changes During Oxidation of the Tested Heterocyclic Compounds*

Compound	$\Delta G^{o'}_{(aq)}$ kJ.mol^{-1}(8,9)
Indole to Oxindole	
$C_8H_7N + H_2O \rightarrow C_8H7NO + 2\ H^+ + 2e^-$	*168*
Quinoline to 2(1H)-quinolinone	
$C_9H_7N + H_2O \rightarrow C_9H_7N) + 2\ H^+ + 2e^-$	*84*
Isoquinoline to 1(2H)-isoquinolinone	
$C_9H_7N + H_2O \rightarrow C_9H_7NO + 2\ H^+ + 2\ e^-$	*56*
Oxindole to CH_4 and CO_2	
$C_8H_7NO + 6.5\ H_2O + H^+ \rightarrow 3.75\ CO_2 + NH + 4.25\ CH_4$	-362
2(1H)-quinolinone to CH_4 and CO_2	
$C_9H_7NO + 7.5\ H_2O + H^+ \rightarrow 4.25\ CO_2 + NH + 4.75\ CH_4$	-298
1(2H)-isoquinolinone to CH_4 and CO_2	
$C_9H_7NO + 7.5\ H_2O + H^+ \rightarrow 4.25\ CO_2 + NH + 4.75\ CH_4$	-303

Note: $\Delta G^{o'}_{(aq)}$ from (8) or estimated using the method of Jobak (9).

tion, and deamination, with ultimate conversion to benzoic acid and phenol. The conversion to benzoic acid and phenol most likely follows the previously described pathways (2, 7), consisting of the reduction of the homocyclic ring, cleavage of this ring, ß-oxidation, and mineralization to CH_4, CO_2, and NH_3. A minor pathway first reported by Godsy and Grbić-Galić (1) for benzothiophene was also observed for quinoline, presumably for isoquinoline, but not for indole. The pathway consists of oxidation of the homocyclic ring with subsequent ring reduction, ring cleavage, degradation of the remaining N-heterocyclic ring, and mineralization. Nitrogen-containing aliphatic acids, however, were not detected in the microcosms to confirm that 2,3-dimethylpyridine was converted to CH_4 and CO_2. Nitrogen-containing aliphatic acids were detected in ground-water samples. The corresponding compounds during benzothiophene degradation were detected, confirming that thiophene-2-ol was degraded to CH_4 and CO_2 (1). 2,3-dimethylpyridine was removed from the contaminated ground water faster than dilution or dispersion could account for during downgradient movement in the aquifer at the study site. This was presumably due to biodegradation.

The oxidation of the parent compounds was found to be a process that requires energy (Table 1); therefore, these reactions must be coupled to the reduction of an unidentified compound(s). The first step in the anaerobic degradation of aromatic compounds is the reduction of the aromatic ring (7). The electrons from the oxidation of the nitrogen heterocycles are quite possibly used for this purpose. It is not apparent why the parent compounds are rapidly oxidized, then remain for long periods of time (days to months) before they are converted to CH_4 and CO_2 (3), if not for the purpose of obtaining electrons for the reduction of other aromatic compounds (e.g., phenol and benzoate). It is not clear at this time whether the organism(s) that are responsible for the oxidation of the parent compound are involved in the methanogenic degradation of the oxidized products.

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Chemical Interactions and pH Profiles in Microbial Biofilms

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Biofilm processes are important to water quality control and are applied in numerous areas such as in ground-water treatment, in conventional and hazardous-wastewater treatment, and in the prediction of fate and effect of pollutants in natural stream systems. Substrate utilization in biofilms has been modeled traditionally by coupling Fick's law with Monod reaction kinetics. An inherent assumption in these models is that the pH remains constant within the biofilm. However, biological reactions can effect changes in the local pH. Nitrification and the utilization of chlorinated organics produces acid equivalents and causes a decrease in pH, while denitrification consumes acid equivalents and causes an increase in pH. Production and utilization of carbon dioxide also alters chemical equilibrium. The rates of substrate utilization and growth of microorganisms are pH dependent and may vary significantly from the bulk solution to the attachment wall of biofilms. This is specially important for methanogens, which can tolerate only a very narrow pH range, and for nitrifying bacteria, which cannot tolerate low pH. Furthermore, diffusional resistance to mass transfer creates pH gradients within the biofilm and alters the speciation of compounds into forms unavailable for microbial consumption (e.g., an increase in pH transforms carbon dioxide into bicarbonate and inhibits autotrophic organisms).

A detailed understanding of the chemical interactions within the biofilm is essential for the optimization of reactor operation. An approach was developed to analyze the effect of pH on substrate transport. This approach incorporates ionic mass transport effects accurately and accounts for corrections for the activity, electrophoresis, relaxation, and hydration of ions. Models will be developed for general autotrophic and heterotrophic biofilms, including nitrifying, denitrifying, and dechlorinating systems. Preliminary results for an autotrophic biofilm are described.

Inorganic carbon in the form of dissolved CO_2 is a major nutrient required by autotrophs (such as algae) and may easily become limiting in systems used for wastewater treatment. Using Monod kinetics to describe the rate of substrate utilization of inorganic carbon and assuming the rate of CO_2 production through endogenous respiration to be first order with respect to the biomass density, a steady-state mass balance on inorganic carbon yields,

$$\frac{dJ_{CO_2}}{dx} + \frac{dJ_{HCO_3^-}}{dx} + \frac{dJ_{CO_3^{2-}}}{dx} - \frac{bX_f}{Y} - \frac{kX_f[CO_2]}{K_s + [CO_2]} \qquad (1)$$

where J is the molar flux of the species; x is the direction perpendicular to the biofilm surface; b is the respiration coefficient; X_f is the biomass density; Y is the yield of microorganisms per mass of substrate; k is the maximum utilization rate; K_s is the Monod half-saturation constant for CO_2; and $[CO_2]$ refers to the molar concentration of CO_2. The characteristics of the system dictate the appropriate expressions for the flux to be used. In this study, three scenarios utilizing various expressions for the flux are compared. Case 1 refers to a dilute system where Fick's law is used for neutral solutes and the Nernst-Planck equation is used for ionic solutes; case 2 incorporates activity corrections using Davies' equation; and case 3 includes activity corrections with additional corrections for electrophoretic effects, relaxation, and hydration using the Stefan-Maxwell equations. Equation (1) with relationships for chemical equilibrium and electroneutrality was solved assuming HCO_3^-, CO_3^{2-}, H^+, OH^-, and Na^+ as the ions present within the system.

The profiles of CO_2, HCO_3^-, CO_3^{2-}, and pH for a biofilm depth of 1,000 µm with a bulk pH of 7.0 and an alkalinity of 1 meq/L for case 1 are shown in Figure 1. As dissolved CO_2 is consumed, the pH increases dramatically within the first 100 µm of the biofilm. Consequently, the dissolved CO_2 concentration decreases rapidly in the first 50µm due to the coupled effects of pH on the speciation of inorganic carbon and the net substrate utilization of CO_2. HCO_3^- remains relatively constant during the

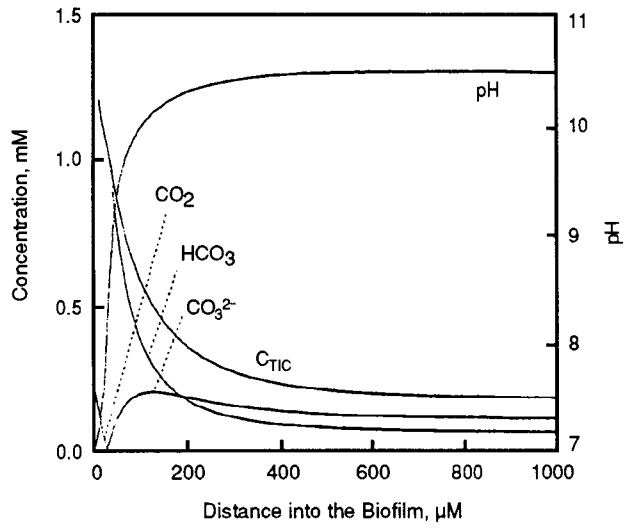

Figure 1. Concentration profiles of the inorganic carbon species and pH for L_f = 1,000 μm, bulk pH = 7.0, bulk alkalinity = 1 meq/L (case 1).

first 50 μm of the biofilm and subsequently decreases throughout the biofilm as the rise in pH transforms the dominant inorganic carbon species to CO_3^{2-}. Finally, the CO_3^{2-} concentration increases due to the shift in chemical equilibrium and eventually decreases because CO_2 is still utilized even in the deeper portions of the biofilm. Thus, the utilization of a substrate whose speciation is dependent on pH may be severely limited in deep biofilms.

Figure 2 shows the variation of the total flux as a function of the overall biofilm depth for a bulk alkalinity of 1 meq/L for various bulk pH values for case 1. The increase in flux is linear with the biofilm depth at small values of L_f while the flux approaches a constant value at large L_f. The flux is limited by the rate of reaction within the biofilm when L_f is small and is limited by the rate of mass transfer for large values of L_f. A decrease in pH, while retaining the same alkalinity, provides a higher flux when the biofilm is deep because a greater fraction of the inorganic carbon in the bulk is CO_2. Thus, simple strategies such as altering the bulk conditions by changing the pH or increasing the buffer intensity of the system will optimize utilization of the biofilm depth.

Other results of the study show that incorporating activity corrections in the analysis (case 2) results in a decrease in the calculated fluxes for deep biofilms. The activity of each species is reduced in systems with an ionic strength less than 1 m and results in an overall decrease in the driving force for the flux. This decrease is proportional to the bulk alkalinity for the system investigated and a larger difference develops between the fluxes at high and low alkalinity values. When the biofilm depth is small, a decrease in the driving force is insignificant because the flux is limited by the rate of reaction within the biofilm. The calculated fluxes for cases 2 and 3 are identical and corrections for electrophoretic effects, relaxation, and hydration are negligible.

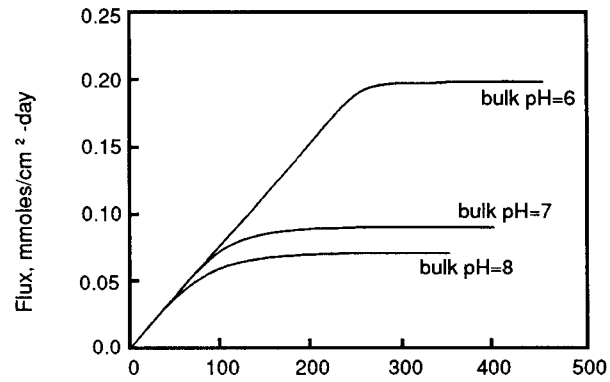

Figure 2. Total flux of inorganic carbon into the biofilm for various L_f and bulk pH, and for a bulk alkalinity = 1 meq/L (case 1).

Decontamination of PCB-Contaminated Sediments Through the Use of Bioremediation Technologies

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Polychlorinated biphenyl (PCB) congeners are relatively inert, stable to oxidation and heat, and have high viscosities, high refractive indices, low vapor pressures, high hydrophobicities, and excellent dielectric characteristics. As a result of these properties, PCBs were used in a variety of industrial applications, including organic diluents, plasticizers, hydraulic fluids, heat exchange and transfer fluids, solvent extenders, and microscope immersion oils (1). Some of these properties also contributed to their accumulation in soils, various elements of the food chain, and sediments of streams, lakes and estuaries (2). During the past two decades, concern has increased with respect to the ecotoxicological and human implications of the accumulation of PCBs (3, 4). In 1971, PCBs were restricted to closed systems in the United States. Eight years later, a total ban on their manufacture and use was ordered by the Congress. Residual contamination of PCBs continues to be a problem, however, particularly along the banks of the Great Lakes where PCBs were used.

Pollution control agencies have launched a concerted effort to reduce the levels of accumulated PCBs in the environment because of their persistence, toxicity, and tendency to bioconcentrate. Polychlorinated biphenyls can be transformed into less harmful compounds by both chemical and physical methods, e.g., ultraviolet radiation, microwave treatment, supercritical water processes, and incineration (5). However, microbial degradation appears to offer the most cost-efficient method of reducing levels of PCBs in contaminated sediments. Consequently, the coupling of anaerobic dechlorination to aerobic metabolism has been suggested as a possible method to reduce the levels of accumulated PCBs in the environment.

Results from bench-scale studies suggest that PCBs can be biodegraded by both aerobic and anaerobic microbial processes (4, 6). Aerobic bacteria usually degrade congeners with one to five chlorine atoms, but not the higher chlorine-substituted congeners. However, the rate and extent of degradation are influenced by the position of the chlorine atoms on the biphenyl ring (7, 8). For example, congeners with open 2,3- or 3,4-positions are degraded faster than congeners with chlorine atoms at these positions. These sites are necessary for the attachment of a dioxygenase enzyme to effect the fission of the biphenyl ring. Several bacterial strains that can biodegrade PCBs aerobically have been isolated from enrichments of soils and sediments from contaminated sites. These strains vary in their abilities to degrade PCBs; some degrade only mono-, di-, and trichlorobiphenyls, and others degrade tetra- and pentachlorobiphenyls (7). Anaerobic reductive dechlorination of PCBs was first proposed by Brown and coworkers (9). Later reports from laboratory studies provided unequivocal evidence that reductive dechlorination could occur in anaerobic sediments (10). Microorganisms that are capable of anaerobic dechlorination often degrade the highly chlorinated PCB congeners, but not the less chlorinated congeners and the biphenyl nucleus (4). There is also a preferential removal of chlorine from the *para* and *meta* positions. The products of anaerobic dechlorination, therefore, are mainly *ortho*-substituted and have fewer chlorine atoms on the biphenyl ring. Such congeners are less toxic and may serve as substrates for aerobic dechlorinating bacteria (10).

The objective of this study is to determine the dechlorinating capacity of PCB-contaminated sediments from selected Great Lakes sites. The dechlorination of indigenous PCB congeners in sediment from the Saginaw River was determined under aerobic conditions. The initial chromatographic profile of Saginaw sediment indicated a preponderance of congeners with one to four chlorine atoms, which suggests that the original Aroclor mixture had been transformed. An additional aerobic incubation for 6 months resulted in a further net loss in the concentration of total congeners reported to be amenable to aerobic degradation. These congeners include 4-chlorobiphenyl, 2,2'4-trichlorobiphenyl (tri-CB), 2,4',5-tri-CB, 2,4,4'-tri-CB, 3,4,4'-tri-CB, 2,3',5',6-tetrachlorobiphenyl (tet-CB), and 2,2',3,4'-tet-CB.

Sediments collected from the Ashtabula River and the Sheboygan Harbor and bay area were incubated under anaerobic conditions. The Ashtabula sediment was spiked with 2,3,3',4,4'-pentachlorobiphenyl (penta-CB), 2,3,3',4,4',5-hexachlorobiphenyl (hexa-CB), and 2,2',3,4,5,6,6'-heptachlorobiphenyl (hepta-CB). Each congener was added individually and in combination with the other two congeners. Reductive dechlorination was observed after lag periods of 5, 3, and 4 months for the penta-CB, hexa-CB, and hepta-CB, respectively. The Sheboygan sediment was spiked with three concentrations of 2,2',3,3',4,5,6,6'-octachlorobiphenyl (octa-CB). After a lag phase of 5 months, a decrease in the higher chlorinated congeners, including the added octa-CB, and a concurrent increase in the lower chlorinated congeners were observed in all the samples amended with the octa-CB, but not in the unspiked and autoclaved sterile controls. These preliminary data suggest the presence of actively dechlorinating microorganisms in the sediments from these three Great Lakes sites. Further research will include studies to determine the factors that will enhance the dechlorinating activity of these microorganisms.

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Bioremediation of Soils and Sediments Contaminated with Aromatic Amines

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Aromatic amines comprise an important class of environmental contaminants. Concern over their environmental fate arises from the toxic effects that certain aromatic amines exhibit toward microbial populations and reports that they can be toxic and/or carcinogenic to animals. Aromatic amines can enter the environment from the degradation of textile dyes, munitions, and numerous herbicides, including the phenylureas, phenylcarbamates, and acylanilides. Because these chemicals are synthesized from aromatic amines, loss of aromatic amines to the environment may also result from production processes or improper treatment of industrial waste streams. The high probability that contamination of soils, sediments, and ground-water aquifers with aromatic amines will occur necessitates the development of *in situ* bioremediation techniques for their treatment.

A number of studies suggest that aromatic amines become covalently bound to the organic fraction of soils and sediments through oxidative and/or nucleophilic coupling reactions (1,2). It generally is accepted that, once bound, the bound residue is less bioavailable and less mobile than the parent compound. Thus, procedures that enhance the irreversible binding of aromatic amines to soil constituents could potentially serve as a remediation technique.

Model studies suggest that oxidative enzymes derived from soil microorganisms play a significant role in catalyzing the formation of bound residues (3). Stimulation of these naturally occurring enzymes could provide an effective *in situ* method for the treatment of soils, sediments, and ground-water aquifers contaminated with aromatic amines. For example, Berry and Boyd (4) were able to enhance covalent binding of the potent carcinogen, 3,3'-dichlorobenzidine (DCB), in soil by the addition of highly reactive substrates (i.e., ferulic acid and hydrogen peroxide). They concluded that by providing the indigenous peroxidase enzymes with highly reactive substrates, the number of reactive sites for covalent binding was increased, which led to the enhanced incorporation of DCB.

The goals of our research are to gain a better understanding of the role that extracellular enzymes play in the covalent binding of aromatic amines to natural organic matter (NOM) and to develop effective methods for remediation of contaminated soils and sediments by the stimulation of indigenous oxidative coupling enzymes.

Initially, our research efforts have focused on elucidating the mechanisms by which aromatic amines (i.e., aniline and substituted anilines) bind irreversibly to sediments, soils, and dissolved organic matter. Accordingly, the sorption kinetics of a series of 2- and 4-substituted anilines were measured in a sediment-water system. In general, the rate and extent of sorption were found to increase with the pKa of the aniline. Decreasing pH enhanced the sorption of the 4-substituted anilines in a silt-clay system. Sequential extraction studies with ^{14}C-aniline suggested that reversible cation exchange processes do not contribute significantly to aniline sorption in the natural sediment-water system, and that

irreversible covalent binding to the organic matter of the sediment matrix dominates the sorption process. In other studies, treatment of a sediment-water system with 2,4-DNP or hydroxylamine, chemicals known to form stable adducts with carbonyl groups, 24 hours prior to the addition of the 4-methoxyaniline effectively blocked the sorption of the aniline, suggesting the importance of covalent binding through nucleophilic addition to carbonyl groups in the sediment matrix.

To provide direct spectroscopic evidence for the type of covalent linkages formed between aromatic amines and natural organic matter, ^{15}N NMR was used to analyze fulvic acid that had been treated with ^{15}N-aniline. INEPT and ACOUSTIC ^{15}N-NMR spectra exhibited resonances for imine, anilide, anilino-quinone, and anilino-hydroquinone nitrogens, providing further evidence that covalent binding of aniline occurs through nucleophilic addition to carbonyl moieties found in organic matter.

Experiments are currently being designed to increase the binding capacity of sediment-water systems by enhancing the activity of the indigenous enzymes through the addition of reactive substrates such as naturally occurring phenols and hydrogen peroxide. Future studies will focus on assaying soil, sediment, and aquifer materials for peroxidase activity and measuring the capacity of these systems to covalently bind representative aromatic amines. In addition, we will use techniques for isolating extracellular soil enzymes to transfer activity from soils with high activity to soils with low activity.

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Development of Small-Scale Evaluation Techniques for Fungal Treatment of Soils

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Standardized bioremediation protocols that mimic full-scale treatments will allow for consistent and economical evaluations and comparisons of remediation options under controlled conditions. These protocols are essential for developing technologies, such as lignin-degrading fungi (LDF) processes, where the data base of experience is limited. They not only help decrease the chance of failure but can also be a research tool for further development. In this research, a draft protocol to assess fungal treatment of contaminated soils has been developed and is currently being tested and modified. A tiered approach is being utilized (Figure 1), starting with simple visual laboratory analyses and progressing to bench-scale soil pans. The component experiments and preliminary results from the first test soil are described in the following paragraphs.

The protocol is initiated by examining data concerning site-specific conditions including the characteristics of the soil and concentrations of the pollutants. In addition, the feasibility of a full-scale land treatment (i.e., adequate space and temperature) at the site must be assessed, as land farming is currently the only method available to apply LDF technologies. If the LDF technologies appear to be feasible, soil samples will be characterized for the target concentrations, nutrient contents, and other important characteristics, and a simple sensitivity test will be initiated. In the sensitivity test, fungal strains that have been identified in the pre-screening stage are plated on a medium that has been amended with the target pollutants at varying concentrations. Since the conditions for growth are optimal for the fungus during this experiment, the lack of growth can be attributed to an inhibiting effect of the pollutants at the test concentration.

Fungal strains that are not relatively resistant to the target pollutants will be tested in the growth tier of the protocol. This stage allows for the selection of fungal species, inoculum loadings, nutrient loadings and compositions, and soil blending (to reduce pollutant concentration) that appear to have the most potential to remediate the soil. Fungal-inoculated and non-inoculated site soils are incubated for 4 to 6 weeks. The endpoint analyses depend on the experience with the target compounds and the soil's characteristics. If extensive LDF data are already

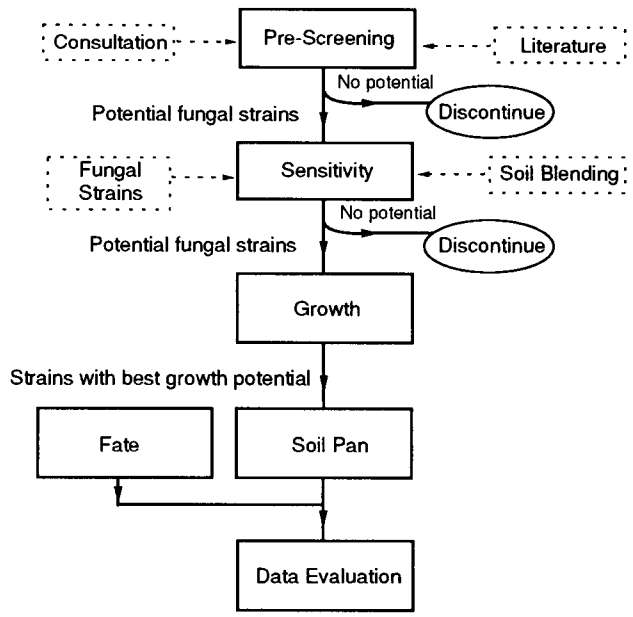

Figure 1. *Tiered treatability approach.*

available for the target pollutant(s), a visual growth evaluation might suffice. For a pollutant that has been studied to a lesser extent, disappearance of the parent compound, analysis of suspected intermediates, and simple toxicity assays are necessary.

The results from the growth studies will determine the number of fungal strains and other bioremediation variables that will need to be examined during the fate and soil pan stages of the protocol. The fate study seeks to determine how the target compounds are removed, i.e., by bioremediation or abiotic mechanisms. Radio-labeled compounds are spiked into clean reference soils and incubated in reactors similar to those used in the growth study. All possible routes of target compound disappearance are monitored during a 4- to 6-week incubation period, thus allowing for mass balances.

The soil pan study attempts to mimic field conditions. Twelve-in. square, 18-in. deep stainless steel pans with gravel under drains and leachate collection systems are currently being utilized. The pans are housed in glove boxes to protect researchers and also to control air flow through the box. Moisture and temperature are controlled to reflect field conditions. Common hand-held garden tools are used periodically to mix and aerate the soil. The performance of fungal treatments is reported as target compound disappearance and soil detoxification relative to the various control pans. The controls are treated identically to fungal treatments, except no inoculum is added. No attempt to eliminate indigenous microorganisms is needed as the advantages gained by the fungal treatment are sought. Controls that contain wood chips and/or sawdust/bran fungal growth matrix (used as part of the fungal treatment) are utilized. Each treatment is duplicated in two independent pans. The soil in the pans can be subsampled without a significant change in soil quantity so that the kinetics of degradation can be determined. Leachate samples are also easily obtained for analyses.

The use of toxicity assays to determine if the treatments are reducing the toxicity of the soil and to predict appropriate times to conduct expensive chemical analyses is also being considered for inclusion in this protocol. Assays that are currently being studied include plant germination, root elongation, earthworm lethality and reproduction, earthworm strand break, plant genetic damage using *Tradescantia paludosa*, and the Microtox system. When interpreting toxicity data, care must be exercised as the toxicity data from an individual assay only apply to the organism tested and inference to another is not valid. In addition, the effect of long-term toxicity cannot be assessed from short-term assays.

The evaluation of the technology is, therefore, based on the fate studies, soil pan experiments, and toxicity assays. Caution in the interpretation at these early stages of the development of LDF technologies is paramount as the protocol is not intended to verify the success of the LDF treatment in the field to detoxify the soil or to provide full-scale design parameters. Rather, it is intended to provide an economical means to determine if more detailed fate and field-scale treatability studies are warranted.

The original draft protocol was tested on creosote-contaminated soil from a Superfund site. The protocol described above reflects modifications that were deemed necessary from experience with this soil. Preliminary results found clear distinctions between the control and fungal treated pans, pollutant removal rates for different inoculum loadings, and different removal rates for lower and higher molecular weight compounds.

Additional soils will be tested using the revised protocol before a finalized guidance document is prepared. The protocol, however, should not be limited to fungal treatments, but could be modified to apply to virtually any land treatment application. The possibility of simplifying the protocol should also not be discounted. A comparison of results from this protocol with field data would be valuable in assessing its true predictive powers and is planned as part of this program.

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Development of Aerobic Biofilter Design Criteria for Treating VOCs

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The objective of this project is to reduce aerobic biotreatment by biofiltration of air contaminated with hazardous volatile organic compounds (VOCs). The VOCs to be studied are toluene, ethylbenzene, methylenechloride, trichloroethylene, and chlorobenzene.

Previous investigations have demonstrated that such VOCs can be rapidly and efficiently biodegraded in biofilters. Reactor plugging due to excess biomass was identified as a complicating problem. This research will evaluate different biological attachment media, as well as operational strategy alternatives for the purpose of optimizing reactor performance and design.

The experimental apparatus will be started in April 1992 at the EPA Test and Evaluation Facility. This apparatus consists of four separate biofilters, complete with all necessary support equipment. Each biofilter system is provided with independent temperature and humidity control, and is insulated to prevent temperature fluctuations. The simplified process description is as follows. Standard 100 psi utility air is purified by separating out particulates, CO_2, H_2O, and VOCs, producing essentially bone dry, pure air at ambient conditions (4 to 40°C). After let-down to 10 psi, the air is chilled to 4°C and then reheated to 15°C to ensure the constant temperature in the air, regardless of ambient conditions. From this point, air to each biofilter is independently metered on mass flow control. The air flows to a co-current, recirculated, temperature-controlled, packed column for humidification. A bypass permits independent control of relative humidity. The humidified air flows to the VOC feed, where liquid VOCs are injected and evaporated. A bypass permits adjustment of the flow conditions at the feed injection point. The completed feed air flows to the biofilter, which is configured for operation in either a co- or counter-current mode. A temperature sensor in the feed air line feeds back to control the humidifier circulation heater. Buffer and nutrient solutions are sprayed as needed onto the top of the biofilters.

Initially, each biofilter will be operated in different modes. Biofilters "A" and "B" have square cross sections of a 5.74 in. inner wall, and contain 48 in. of Corning Celcor® channelized media. One will be operated in a co-current mode and the other in a counter-current mode. Biofilters "C" and "D" have circular cross sections of a 5.74 in. ID. Both will be operated in a co-current mode. Biofilter "C" contains 48 in. of Manville Celite BioStars® pelletized attachment media. Biofilter "D" contains Clairtech Bioton® peat mixture.

Sequential Anaerobic-Aerobic Treatment of Contaminated Soils and Sediments

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Introduction

Many highly chlorinated aromatics and aliphatics can be destroyed microbiologically most rapidly by sequential anaerobic-aerobic treatment. In general, the biochemical pathway providing the highest rate for the initial steps of microbial destruction of the highly chlorinated organics is anaerobic reductive dechlorination. Once partially dechlorinated, the resulting compounds typically degrade faster under aerobic, oxidizing conditions.

For example, polychlorinated biphenyls (PCBs) are candidate contaminants for this sequential treatment (1,2,3). It is known that the *meta* and *para* chlorines are removed by anaerobic reductive dechlorination; however, the *ortho* chlorines are only very slowly removed by the same bioprocess. Aerobic organisms remove the *ortho* chlorine and complete the mineralization of the compound relatively quickly. Thus, sequential anaerobic-aerobic treatment should provide relatively rapid destruction of PCBs. This process should also be applicable to other highly chlorinated aromatics, such as pesticides, and the wood preservative PCP.

The objective of this project (begun in spring 1992) is to conduct fundamental and applied research in order to develop sequential anaerobic-aerobic landfarming and composting technologies capable of biologically treating soils or sediments contaminated with highly chlorinated aromatic compounds and other low-solubility compounds susceptible to sequential treatment. Because no experimental results are available yet, only the proposed methodology is discussed below.

Methodology

The project will commence with bench-scale studies conducted with aqueous slurries of soils or sediments to minimize mass transfer limitations. Samples of soils from contaminated sites will be screened for adapted microorganisms and from other sources such as other research groups. Each test will be initiated by spiking the soil or sediment with (1) contaminant of interest, (2) co-substrate, (3) adapted microorganisms, and (4) minimal salts media. The bench-scale studies will be conducted with the following run-in parallel: (1) serum-bottle reactors dedicated to soil and aqueous sampling, and (2) custom-designed flask reactors dedicated to on-line pH, oxidation-reduction potential (ORP), and gas-production measurements. Table 1 lists the system parameters that will be varied to provide a fundamental understanding and optimization of the sequential process.

Success of the anaerobic and aerobic phases of the treatment will be determined by observing the disappearance of the parent contaminant and following the appearance and disappearance of daughter compounds. Rates of degradation of parent and daughter compounds as a function of time during the degradation process will indicate optimal time for switching from anaerobic to aerobic conditions. Degradation rates will also be correlated with ORP.

Later, soil columns or pans simulating *in situ* or landfarming techniques and lab-scale composting reactors will be employed to demonstrate the sequential treatment technology under field-like conditions.

Table 1. *Potential Variations in Experimental Parameters*

Parameter	Potential Variations
Compound	*DDT, other pesticides, PCBs, PCP*
Co-substrate	*Pure compounds: acetate, methanol, ethanol Complex source: primary and anaerobic sludge*
ORP (anaerobic)	*Methanogenic, sulfate-reducing, nitrate-reducing*
Organism Source	*Enriched from contaminated materials, waste treatment facilities, other research groups*
Temperature	*Ambient (20°C), mesophilic (35°C), thermophilic (70°C)*
Soil	*High/low organic content, sand, silt, clay*

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Influence of Low Levels of Nonionic Surfactants On the Anaerobic Dechlorination of Hexachlorobenzene

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Surfactants solubilize pollutants into micellar solution in the presence of soil or sediment, effectively desorbing them from these solids (1). This phenomenon has been suggested as a potential tool to enhance the treatment of contaminated sediments and soils (2,3,4). The rationale is that surfactant micelles or emulsions will solubilize precipitated or sorbed compounds, making them more readily available for a variety of treatment processes: biological remediation, pump-and-treat, and soil-washing operations.

The potential of surfactants to enhance the bioavailability of highly sorbed compounds to microbial organisms has recently been investigated (5,6,7,8). Generally, surfactant concentrations greater than that required for micelle formation, i.e., the critical micelle concentration (cmc), are examined. With few exceptions, microbial degradation rates are slowed or completely inhibited at these high surfactant levels. Similarly, investigations of the effectiveness of antibacterial agents on microorganisms demonstrate that, at high concentrations of nonionic surfactants (0.01 to 0.1 percent w/v), the activity of the agent is suppressed. Two possible explanations for these results are that the surfactant is solubilizing components of the microbial membrane, or the solute or agent is less available to the organisms. Interestingly, at low surfactant concentrations (0.001 to .01 percent w/v), i.e., those less than the cmc, the activity of the agents is enhanced. To further explore the effects of various levels of nonionic surfactants on the capacity of microorganisms to degrade hydrophobic organic compounds, the dechlorination of hexachlorobenzene (HCB) in anaerobic sediment suspensions was examined.

Anaerobic pond sediments (5 to 7 percent w/v) were amended with one of three nonionic surfactants: Tween 80 and 85, polyethoxylated sorbitan monooleate, and trioleate, respectively, and Brij 30, a polyethoxylated lauryl alcohol. Surfactant concentrations ranged from 3 x 10^{-4} percent (w/v), well below the cmc, to 5 percent (w/v), well above the cmc. The addition of Tween 80 at concentrations less than the cmc, and just above it (<0.1 percent), enhanced the dechlorination rate of HCB compared to unamended sediments. The enhanced activity is characterized by a dramatic increase in 1,3,5 trichlorobenzene (TCB) after 55 to 60 days. Complete dechlorination of HCB occurs between 75 to 100 days. Generally, a similar response was observed for all surfactant concentrations less than 0.1 percent. By comparison, unamended suspensions were fairly inactive, producing only small amounts of TCB. Sediment suspensions previously acclimated to HCB also demonstrated enhanced activity with low levels of Tween 80. The more hydrophobic Tween 85 sorbed to a greater extent, and lowered the aqueous concentration of HCB by an order of magnitude. This reduced level of aqueous HCB may explain why enhanced dechlorination activity in these suspensions occurred more sporadically relative to those receiving Tween 80 amendments.

At concentrations well above the cmc for Tween 80, the aqueous concentration of HCB was enhanced one to two orders of magnitude over that of controls, which were generally at half-aqueous saturation (2 to 3 μg/L). Within a week, however, Tween 80 was degraded enough that the aqueous HCB concentration had decreased to control levels. The HCB dechlorination activity was initially similar to that of the controls, but after 40 days it ceased.

Additions of Brij 30 at concentrations less than the cmc (<0.03 percent) did not alter the dechlorination activity of HCB in fresh sediments. At concentrations greater than the cmc, aqueous concentrations of HCB remained elevated, indicating that the surfactant was not being degraded. However, additions of Brij 30 at these high concentrations totally inhibited HCB dechlorination activity in both fresh and acclimated sediments.

Our results demonstrate that, although micellar concentrations of nonionic surfactants have the capacity to transfer hydrophobic compounds from solid surfaces to a colloidal pseudo-phase, the addition of the surfactants suppressed, rather than enhanced, the

microbial degradation of the compound. At low surfactant concentrations, without any apparent enhanced solubility of HCB, dechlorination activity is increased—albeit surfactant-dependent. This may be explained by fluidization of the cell membrane by surfactant monomers resulting in enhanced mass transfer of the solute (observed by others). Further investigation of surfactant interactions with microbial membranes is required to assess whether higher concentrations are responsible for membrane solubilization or reduction of the more available, truly dissolved solute. In addition, although surfactant structure was shown to be an important factor, little is known of structure-activity relationships for these systems.

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Anaerobic Transformation and Degradation of Chlorobenzoates and Chlorophenols Under Four Reducing Conditions

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The anaerobic degradation of halogenated aromatic compounds under methanogenic conditions is well documented. However, there is little information on the biodegradability of these compounds under other reducing conditions. We therefore investigated the anaerobic biodegradation of chlorinated phenols and benzoic acids coupled to methanogenesis, sulfidogenesis, denitrification, and iron reduction. Anaerobic enrichment cultures were established on each of the three monochlorophenol and monochlorobenzoate isomers using sediment samples from the East River (New York), two locations of the Hudson River (New York), and two locations of the Nile (Egypt) as inoculum.

The anaerobic biodegradability of the chlorinated phenols and benzoic acids depended both on the position of the chlorine substituent and the electron acceptor available. In general, similar activities were found in all sediments, but with varying rates of metabolism. Under methanogenic and sulfidogenic conditions, 2-, 3-, and 4-chlorophenol (100 µm) were completely removed in 30 to 200 days. Methanogenic conditions generally allowed for chlorophenol degradation more rapid than under sulfidogenic conditions. Under denitrifying conditions, significant loss was only observed with 2-chlorophenol. Iron-reducing conditions promoted the degradation of all three monochlorophenols in 90 days. To our knowledge this is the first report of chlorophenol metabolism under denitrifying or iron-reducing conditions.

Rapid degradation of 3- and 4-chlorobenzoate under denitrifying conditions was observed in all the enrichment cultures established with the different sediment samples, with complete substrate loss observed in 21 days or less. Degradation of 2-chlorobenzoate only occurred under methanogenic conditions, with no substrate loss observed under sulfidogenic, denitrifying, or iron- reducing conditions for over 100 days. Degradation of 4-chlorobenzoate, on the other hand, only occurred under nitrate-reducing conditions. 3-chlorobenzoate was the only compound for which degradation was observed under all four reducing conditions examined.

Degradation of 2-, 3-, and 4-chlorophenol under methanogenic and sulfidogenic conditions could be sustained with repeated refeeding of the respective compound. Degradation of 3- and 4-chlorobenzoate under denitrifying conditions was also sustained with repeated refeeding. Although metabolites of chlorophenol or chlorobenzoate were not detected under sulfate- or nitrate-reducing conditions, under methanogenic conditions phenol appeared as a transient metabolite of 2-chlorophenol, and benzoate was detected as a metabolite of 3-chlorobenzoate. Hence, reductive dechlorination takes place as an initial step under methanogenic conditions.

The complete mineralization of the chlorophenols and chlorobenzoates coupled to methanogenesis, sulfidogenesis, denitrification, or iron reduction can be described by the stoichiometric equations given below.

Chlorophenols:

1. $C_6H_5OCl + 4.5\ H_2O \longrightarrow 3.25\ CH_4 + 2.75\ CO_2 + H^+ + Cl^-$
2. $C_6H_5OCl + 3.25\ SO_4^{2-} + 4\ H_2O \longrightarrow 6\ HCO_3^- + 3.25\ H_2S + 0.5\ H^+ + Cl^-$
3. $C_6H_5OCl + 5.2\ NO_3^- + 4.2\ H^+ \longrightarrow 6\ CO_2 + 2.6\ N_2 + 4.6\ H_2O + Cl^-$
4. $C_6H_5OCl + 27\ Fe(III) + 17\ H_2O \longrightarrow 6\ HCO_3^- + 27\ Fe(II) + 33\ H^+$

Chlorobenzoates:

5. $C_7H_5O_2Cl + 5\ H_2O \longrightarrow 3.5\ CH_4 + 3.5\ CO_2 + H^+ + Cl^-$
6. $C_7H_5O_2Cl + 3.5\ SO_4^{2-} + 5\ H_2O \longrightarrow 7\ HCO_3^- + 3.5\ H_2S + H^+ + Cl^-$
7. $C_7H_5O_2Cl + 5.6\ NO_3^- + 4.6\ H^+ \longrightarrow 7\ CO_2 + 2.8\ N_2 + 4.8\ H_2O + Cl^-$
8. $C_7H_5O_2Cl + 29\ Fe(III) + 19\ H_2O \longrightarrow 7\ HCO_3^- + 29\ Fe(II) + 36\ H^+$

Degradation of 2-, 3-, and 4-chlorophenols in the methanogenic enrichments was coupled to stoichiometric production of methane (Equation 1). In the sulfidogenic enrichments, monochlorophenol degradation was coupled to loss of sulfate corresponding to that expected for complete oxidation of the chlorophenol to CO_2 (Equation 2). Under denitrifying conditions, 3- and 4-chlorobenzoate were degraded coupled to sto-

ichiometric reduction of nitrate (Equation 7). Analysis on chlorophenol degradation coupled to iron reduction is in progress.

Our work indicates that chlorinated aromatic compounds can be degraded under a variety of anaerobic conditions. The anaerobic biodegradability of the different monochlorophenol and -benzoate isomers is dependent on the availability of an electron acceptor, either carbonate, sulfate, nitrate, or Fe(III). These are all environmentally significant electron acceptors. For example, sulfate and iron reduction are important anaerobic processes in marine environments, while nitrate is found as input into agricultural soils. The availability and the role of these anaerobic electron acceptors should be considered in understanding the fate of halogenated aromatic compounds in the environment.

Characterization of Biofilter Microbial Populations

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This project is a component of research by the University of Cincinnati/U.S. Environmental Protection Agency to develop aerobic biofilters capable of the biodegradation of volatile organic compounds (VOCs). Standard microbial methods and DNA probe technologies will be used to characterize the microorganisms that grow and operate as biodegradation agents in the biofilters. This information will contribute to the successful optimization of reactor design and its operational criteria in the biodegradation of VOCs in aerobic and anaerobic configurations. The specific objectives of this project are to (1) provide a rapid index of the microbial diversity of biofilter samples based upon DNA amplification fingerprinting (DAF) techniques, (2) identify the predominant organisms in biofilter samples, and (3) isolate, identify, culture, and characterize specific microorganisms or microbial subpopulations recognized as having an important active function in the desired biodegradative processes.

The profile patterns generated by the DAF method will be useful in providing an index of biodiversity that would be representative of the particular sample taken from the biofilter. Approaches to the second and third objectives will include the use of PCP-amplified DNA for pathway-specific gene probe sequences available from the literature, and for 16S rDNA sequence-specific probes for particular microorganisms identified from the biofilters.

Identification of the microbial strains and mixtures that are characteristic of efficient bioreactor operation is a long-term research aspect of this project.

Section Five
Modeling

A key to successful application of bioremediation technology is mathematical modeling. Modeling allows scientists to extrapolate the quantified results of laboratory and pilot research to select the technologies predicted to be most successful at particular sites.

Two papers presented modeling studies of granular-activated carbon (GAC). The first study further investigated a previously observed phenomenon, the major effect of molecular oxygen on the adsorptive capacity of GAC for aromatic organics. The second study is part of continuing research to evaluate quantitative techniques to characterize biofilms attached to GAC. These biofilms have proven highly effective for treating hazardous waste, but the treatment process has not been completely measured. The objective of another modeling project is to quantify biodegradation kinetics of certain organic compounds under anaerobic conditions. The investigators then intend to mix the compounds with sand, soil, and more complex matrices to measure the resulting products of biodegradation.

Prediction of GAC Adsorptive Capacity With and Without Molecular Oxygen

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Previously reported results by the authors (1,2) revealed that the presence of molecular oxygen has a major effect on the adsorptive capacity of GAC for several aromatic compounds (phenol, o-cresol, and 3-ethylphenol), as well as for natural organic matter. The adsorptive capacity exhibited in the presence of molecular oxygen can, in some instances, be up to threefold the capacity that was attainable under anoxic conditions. Experimental results have demonstrated that this increase in GAC adsorptive capacity was not due to biodegradation of these organic compounds in the presence of molecular oxygen (oxic conditions). It was also discovered that the presence of molecular oxygen promotes polymerization of these compounds on the surface of the carbon, which provides an explanation for the observed phenomenon. Polymerization of phenolic compounds is achieved through oxidative coupling reactions (3).

The effect of molecular oxygen on the adsorptive capacity of GAC is now further investigated for several phenolic and other aromatic organic compounds listed in Table 1. These compounds were chosen to study the effect of different functional groups substituted on the parent phenol molecule, as well as to represent a variety of recalcitrant compounds of special interest in wastewater treatment.

The effect of the same functional group, substituted on different positions of the parent phenol molecule on GAC adsorptive capacity exhibited under oxic and anoxic conditions, was investigated for 2-, 3-, and 4-methylphenol. The absence of molecular oxygen from the test environment yielded very similar GAC adsorptive capacities for all three compounds. On the other hand, the substitutional position of the methyl functional group had a significant impact on the adsorptive capacity of activated carbon that was exhibited in the presence of molecular oxygen. Oxic GAC adsorptive capacity for these compounds increased in the order: 3-methylphenol < 4-methylphenol < 2-methylphenol, indicating that substituting the methyl group on the *meta* position of the parent phenol molecule induced the least susceptibility to this polymerization reaction.

Table 1. *Organic Compounds Chosen for This Study*

2-Methylphenol	*Catechol*
3-Methylphenol	*Resorcinol*
4-Methylphenol	*Hydroquinone*
2-Ethylphenol	*2,4-Dichlorophenol*
3-Ethylphenol	*2,4,6-Trichlorophenol*
4-Ethylphenol	*Pentachlorophenol*
2-Propylphenol	*Benzene*
3-Propylphenol	*Toluene*
4-Propylphenol	*Nitrobenzene*
2-Nitrophenol	*Chlorobenzene*
3-Nitrophenol	*2,4-Dinitrotoluene*
4-Nitrophenol	
	Indole
2-Chlorophenol	*Quinoline*
3-Chlorophenol	*Methylquinoline*
4-Chlorophenol	
	Aniline
2-Hydroxybenzoic Acid	*Pyridine*
3-Hydroxybenzoic Acid	*2-Naphthol*
4-Hydroxybenzoic Acid	

Extraction experiments conducted with carbons used in oxic and anoxic isotherm experiments with these compounds yielded 90 percent recovery of the original compound when the adsorption occurred under anoxic conditions. On the other hand, the highest amount of adsorbate was extracted from the GAC used in the test with 3-methylphenol, which was the compound that showed the least increase in the GAC adsorptive capacity in the presence of molecular oxygen.

Anoxic adsorption isotherms for three *ortho*-substituted phenols (methyl, ethyl, and propyl groups) are compared in Figure 1. The increase in molecular weight of a substituted functional group induced an increase in the anoxic GAC adsorptive capacity since

the higher molecular weight compounds are usually more hydrophobic. However, the relative increase in the adsorptive capacity in the presence of molecular oxygen decreased as a result of an increase in the molecular weight of the substituted functional group (see Figure 2). This behavior is attributed to the stearic hindrance of the polymerization reaction. Further as the molecular weight of adsorbate increased, the extraction efficiency obtained for the carbons used in the oxic isotherm tests increased. This confirms the hypothesis of the diminished polymerization reaction for the higher molecular weight adsorbates.

Ongoing adsorption isotherm experiments are designed to evaluate the effects of the solution pH, the GAC type, and the size of the GAC particle on the extent of the adsorptive capacity increase that results in the presence of molecular oxygen. Furthermore, attempts will be made to relate the observed phenomena to chemical properties of adsorbates listed in Table 1.

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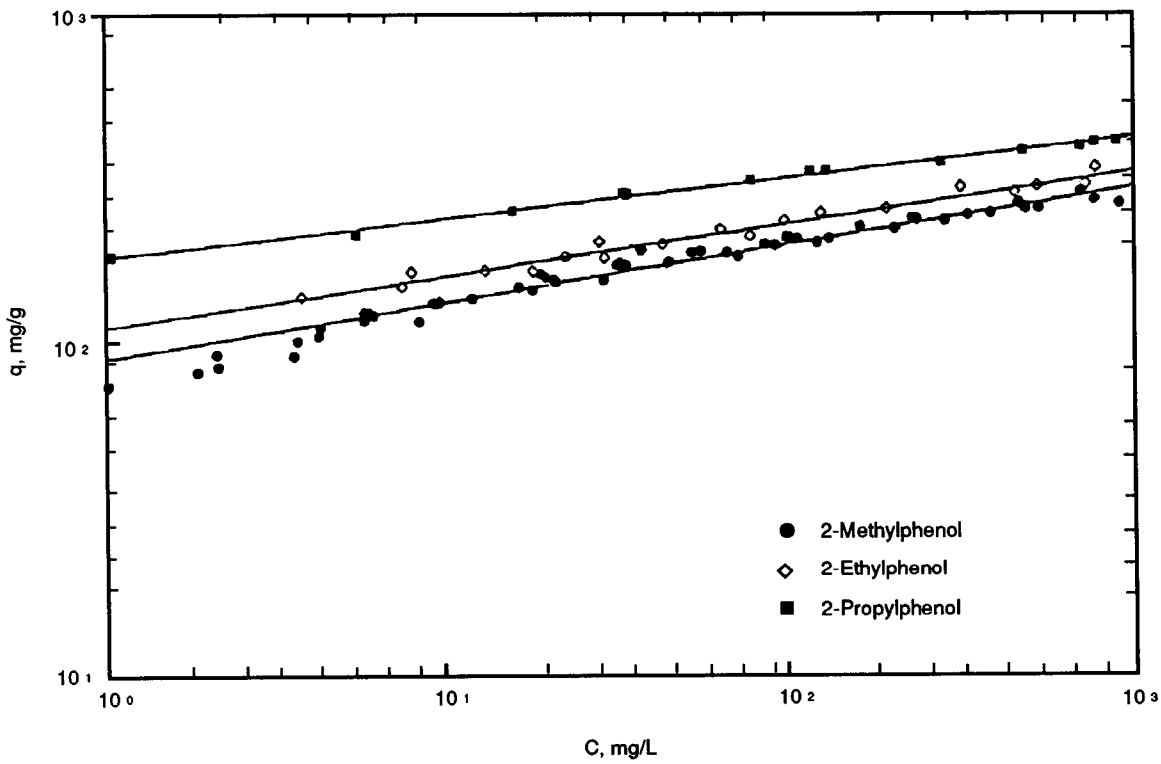

Figure 1. Anoxic adsorption isotherms for three ortho-substituted phenols.

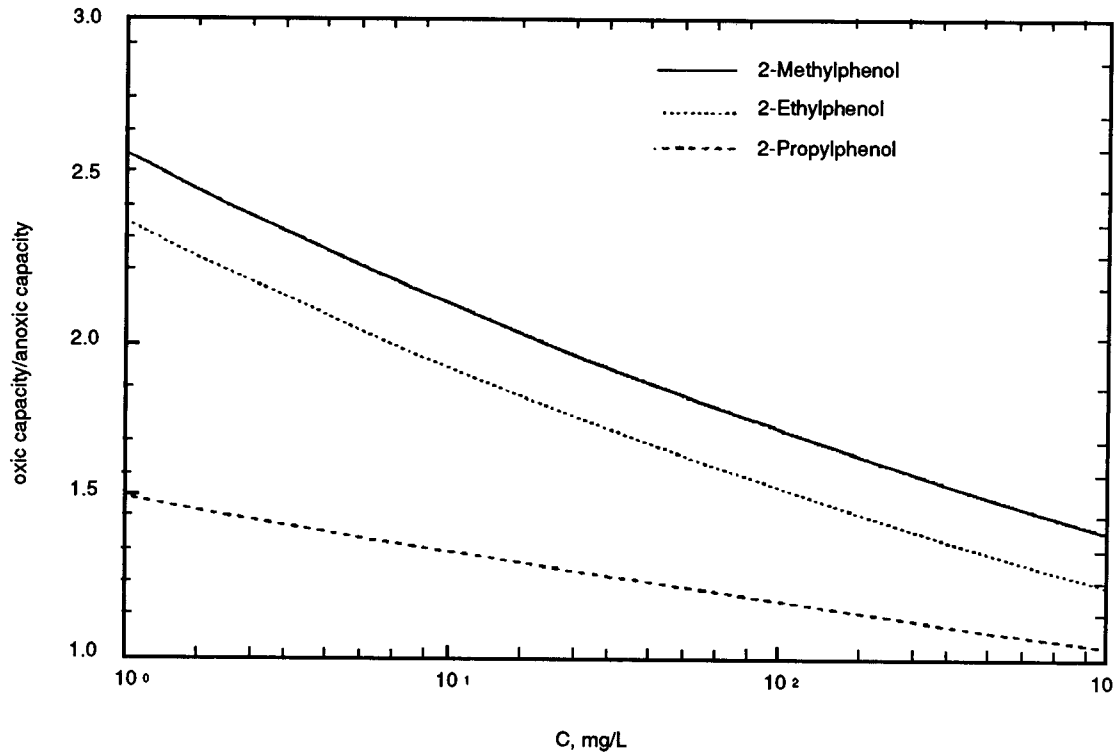

Figure 2. *Ratio of oxic and anoxic GAC adsorptive capacity for three ortho-substituted phenols.*

Development of Tools for Monitoring Biofilm Processes

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The engineering design, process control, and evaluation of microbial fixed-film processes can be challenging because of the lack of methods to measure biomass attachment characteristics. A particularly troublesome biofilm process to monitor, yet one that is very effective for treating hazardous waste, employs biological granular-activated carbon (GAC), i.e., biofilms attached to GAC. The adsorptive characteristics of the GAC and its organic nature preclude using many traditional techniques. The objective of this continuing research is, therefore, to identify and evaluate a variety of unique quantitative and semi-quantitative techniques to characterize biofilm attachment, with emphasis on biological GAC.

Based on a literature survey and consultation with researchers in various disciplines, several techniques with potential to achieve this project's objectives have been identified. These include a direct measure of biomass and biological activity, semi-quantitative visual assessments of attachment characteristics, and simple physical characteristics of the media affected by biological activity. Methods that have been eliminated include chemical oxygen demand, total organic carbon, washing of the GAC particle followed by enumeration of the microbial population in the wash water, volatile solids analyses, and direct microscopic counting.

All cell walls contain phospholipids and, consequently, the analysis for phosphorus from an extract can be directly correlated to the quantity of viable biomass. The application of existing techniques to GAC required some modifications but are effective. The variability of the test results and the effect of sample size are currently being evaluated.

A simple method to measure activity utilizes fluorescein diacetate (FDA). Fluorescein is released from FDA as the acetate is utilized as a substrate by the microbial populations. As fluorescein is released, the solution changes color as a function of the microbial activity. The GAC will, however, adsorb fluorescein and corrections are required to utilize this technique for biological GAC. Two important test variables are the size of the sample and the incubation time, both of which will affect the variability and sensitivity of the results.

The results from the FDA and lipid measurements are best interpreted in conjunction with one another. The ratio of FDA to lipid provides a measure of the activity per unit biomass. Consequently, changes observed in activity per unit biomass can signal recent changes in the microbial ecology within a reactor.

The three-dimensional image of a specimen's surface revealed by a scanning electron microscope (SEM) allows direct visual examination of the attachment characteristics of biomass attached to GAC. SEM examination of several biological GAC processes has revealed causes of reactor operating difficulties and provided insight to attachment characteristics. The technique has been qualitative to date, however, and comparisons of specimens or changes in attachment characteristics with time can be subjective. A semi-quantitative technique has been developed as part of this research. A rating system was developed that provides a framework to evaluate important fluidized-GAC bed reactor characteristics, including the type and quantity of microorganisms attached to the GAC and the physical characteristics of the GAC. Verification of the protocol is ongoing and involves the independent evaluation of GAC samples by several researchers to determine the extent to which evaluation techniques are objective.

The media's physical characteristics, i.e., porosity, settling velocity, and GAC diameter, are all easily measured by simple laboratory techniques. These parameters are not direct measurements of biological conditions but changes in the physical properties of the media caused by biological activity. The methods do not appear to be sensitive to slight changes in biological

conditions; however, they may be useful for designing certain biological GAC processes.

To develop, evaluate, and compare these biomass monitoring tools, bench-scale GAC-fluidized beds are being utilized. The SEM analyses have been found to be effective in monitoring developing biofilms but the lipid and FDA measurement do not appear to be sensitive when using reasonable sample sizes during this developmental stage. However, once a dense biofilm coats the GAC particles, the lipid and FDA analyses become sensitive and can discriminate between varied operating conditions. The SEM is also useful in identifying the general category of microorganism present and in trouble-shooting reactor-operational difficulties.

Future research should focus on assessing the variability and sensitivity of the techniques described above and determining their ability to predict the microbial environment within biological GAC as well as within other types of media. The use of the FDA and lipid measurements would also appear to be well suited for use in mathematical models as a quantitative biomass activity measure. Replacement of more typical biomass measures with these measures should be evaluated to determine if improved predictions are obtained.

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Evaluation of Anaerobic Respirometry to Quantify Intrinsic Anaerobic Biodegradation Kinetics of Recalcitrant Organic Compounds

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The objective of the study is to determine the kinetics of biodegradation of compounds difficult to degrade under strict anaerobic conditions using a standard respirometer specially modified for anaerobic operation.

A 12-position N-Con respirometer has been purchased to conduct the studies. The respirometer, which was originally designed for use under aerobic conditions, has been modified by researchers at New Mexico State University to be functional under anaerobic conditions. Instead of connecting the reaction vessel to an oxygen cylinder under positive pressure, this vessel is connected to a vacuum pump under a negative pressure of approximately 5 psig. As microbes degrade the substrate and produce gas, the increase in pressure is sensed by the pressure sensor, which signals the computer to open the exit valve venting the gas from the vessel headspace. Before release to the sensor, the product gas is passed through a dryer and a CO_2 scrubber. The total volume of gas exiting the vessel is computed automatically and recorded on the computer disk for determination of biodegradation kinetic constants. This operating principle can be used to quantify the biokinetic constants of recalcitrant compounds such as pentachlorophenol (wood preserving wastes) and 2,4-dinitrotoluene (ammunition production wastes).

A simple compound such as phenol dissolved in distilled water will be selected first to test the concept and gain experience with the operation. Liquor from a conventional anaerobic digester will serve as the inoculum. Once the operation has been mastered, more recalcitrant compounds such as pentachlorophenol or 2,4-dinitrotoluene will be investigated in a clean water medium. Sediments and soils contaminated with PCP will be used as the source of inoculum.

When the anaerobic biokinetic constants have been determined for the test compounds, a degree of complexity will be introduced by adding these compounds to a sand matrix and determining their anaerobic biodegradability while sorbed onto sand particles. More complexity will be introduced later by using a loam or clay soil matrix. Finally, mixtures of compounds will be tested to determine biodegradability in a complex mixture. Variables to be measured in addition to total gas production include CO_2, CH_4, disappearance of parent compound by chromatographic assay, COD, and pH. An important control variable will be temperature. Radio-labeled compounds also will be studied to determine the fate of the carbon from the parent compound.

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