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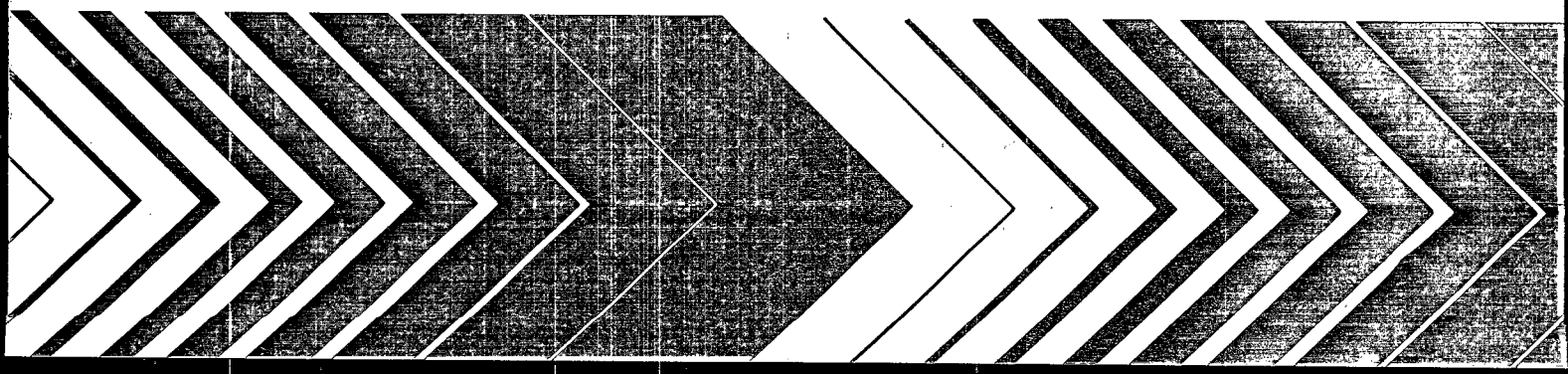
Bioremediation of Hazardous Wastes



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BIOREMEDIATION OF HAZARDOUS WASTES

by

Biosystems Technology Development Program
Office of Research and Development
U.S. Environmental Protection Agency

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

EPA's Office of Research and Development (ORD) initiated the Biosystems Technology Development Program to anticipate research needs that can be applied to our nation's waste management problems. In April 1991, ORD hosted the fourth annual Symposium on Bioremediation of Hazardous Wastes: U.S. EPA's Biosystems Technology Development Program in Falls Church, Virginia, to discuss recent achievements of the program and research projects aimed at bringing bioremediation into more widespread use.

At this year's conference, papers were presented in six key media-based or process-oriented research areas and on three current programs.

1. **Ground-Water Treatment.** Effective bioremediation of ground water is constrained by the geology, hydrology, and geochemistry of the subsurface environment. Design of any effective remedial action for contaminated ground water requires a great deal of site-specific information. Current ground-water research is focused on enhancement of degradation using bioventing to treat gasoline-contaminated vadose zones.

2. **Treatment in a Reactor.** In reactors, hazardous pollutants are brought into contact with microorganism to accelerate the degradation process. Landfill leachates are a good example of a liquid waste that is amenable to reactor treatment. In addition, toxic waste from Superfund sites can be treated in reactors. EPA researchers are exploring use of reactors at publicly owned treatment works (POTWs) as a model for such reactors.

3. **Soil/Sediment Treatment.** Decontamination of soils and sediments is one of the most difficult problems found at hazardous waste sites. This is due to the cost involved, heterogeneity of the media, and adequate transfer of amendments (e.g., oxygen nitrates).

4. **Combined Treatment.** Most hazardous waste sites contain complex mixtures of biologically persistent organic and inorganic contaminants that can be remediated only by a combination of treatment techniques. EPA researchers are developing methods to combine various physical, chemical, and biological treatment technologies, and comparing the effectiveness of the various combinations.

5. **Sequential Treatment.** Sequential treatment is generally applied to two waste types: compounds that degrade into stable intermediates that can be further degraded under different conditions than those used for the parent compound; and complex mixtures of wastes, which are generally degraded in order of their thermodynamic behavior. EPA researchers are investigating the most effective coupling and sequence of treatments for such wastes.

6. **Metabolic Process Characterization.** EPA's metabolic process research generates a better understanding of the processes by which microorganisms degrade chemicals, expanding the range of organisms that can be used in biosystems technologies. Based on the insights gained from this research, scientists can then choose indigenous organisms or enhanced organisms to meet needs in pollution cleanup and control.

7. **Risk Assessment.** A number of the high-priority compounds that require disposal are known carcinogens or precarcinogens. Since biodegradation does not necessarily result in total degradation to carbon dioxide and water, researchers need to assess whether ultimate or procarcinogens are created by a given biological treatment. Public health evaluations must be conducted to determine the toxicity of substances at Superfund sites, assess the safety of nonindigenous organisms, and compare bioremediation with other potential technologies.

8. **Bioremediation Field Initiative.** As part of its overall strategy to increase the use of bioremediation to treat hazardous wastes, EPA implemented the Bioremediation Field Initiative. This program assists the regions and the states in conducting field tests and evaluations of this technology. At more than 140 sites in the United States, treatability studies are being conducted and bioremediation is being planned, is in full-scale operation, or has been completed.

9. **Oil Spill Bioremediation Project.** EPA's Oil Spill Bioremediation Project in Prince William Sound, Alaska, examined whether the addition of nutrients to oil-contaminated beaches would sufficiently enhance oil degradation rates to enhance biodegradation. The success of this project demonstrated that bioremediation should be considered as a key component in any cleanup strategy for future oil spills impacting the shoreline.

In pursuing research in these areas, the Biosystems Technology Development Program has identified a number of avenues through which to enhance the use of this technology. These are:

- **Process characterization.** Isolate and identify microorganisms that carry out biodegradation processes. Search out and characterize biodegradation processes in surface waters, sediments, soils, and subsurface materials in order to identify those that may be used in biological treatment systems and develop process-based mathematical models to evaluate potential treatment scenarios.

- **Process development.** Develop new biosystems for treatment of environmental pollutants. Biosystems would include naturally selected microorganisms, consortia, bioproducts, and genetically engineered microorganisms.

- **Process engineering.** Determine, evaluate, optimize, and demonstrate the engineering factors necessary for applying biological agents to detoxify or destroy pollutants in situ or at a centralized treatment facility.

- **Environmental risk.** Determine environmental fate and effects of, as well as risks involved in the use or release of, degrading microorganisms or their products to detoxify or destroy pollutants.

- **Mitigation of adverse consequences.** Develop means to mitigate adverse consequences resulting from the accidental or deliberate release of microorganisms for pollution control.

- **Technology transfer.** Transfer information on advances in the technology to the user community. Provide evaluation of full-scale projects and a central repository of field projects.

The Biosystems Technology Development Programs 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
Environmental Research Laboratory—Gulf Breeze, Florida
Health Effects Research Laboratory—Research Triangle Park, North Carolina
Risk Reduction Engineering Laboratory—Cincinnati, Ohio
Center for Environmental Research Information—Cincinnati, Ohio

As hazardous wastes become increasingly more diverse with respect to contaminants and contaminant mixtures, our nation will need to rely more and more on innovative technologies for improved treatment

efficiency at lower costs. In February 1990, EPA Administrator William Reilly held a meeting with over 70 representatives of biotreatment companies, contractors, environmental organizations, academia, and other federal agencies. The purpose of the meeting was to develop an agenda outlining strategies for increasing the use of bioremediation for cleaning up hazardous waste sites and petroleum products. As a result, ORD together with the Office of Solid Waste and Emergency Response (OSWER) launched a new Bioremediation Field Initiative with three primary goals to be carried out over the following two years.

The first goal is to more fully document the performance of full-scale bioremediation field applications. OSWER and ORD will evaluate treatment effectiveness, operational reliability, and costs at both in situ and ex situ bioremediation projects, focusing on in situ biological treatment for surface and subsurface contamination. The second area covered by the initiative is to provide technical assistance to EPA and states overseeing bioremediation projects or considering the use of bioremediation. ORD Technical Support Centers in Ada, Oklahoma, and Cincinnati, Ohio, will provide assistance with site characterization, treatability study design, or the interpretation of results. The third part involves the creation of a data base, which will be a central repository of current data on progress in the field in determining the treatability of various contaminants.

To date, the Bioremediation Field Initiative has identified over 140 sites across the country where bioremediation projects are being considered, planned, or are currently underway. These sites include Comprehensive Emergency Response, Compensation, and Liability Act (CERCLA), Resource Conservation and Recovery Act (RCRA), and Underground Storage Tank (UST) sites. Approximately one-third of these projects are in the planning stages, one-third are undergoing or have completed treatability studies, and one-third are being designed or implemented. Data gathered from the initiative indicates that bioremediation is being undertaken for three major waste categories: petroleum, creosote, and solvents. These three types of waste comprise about two-thirds of wastes being biologically remediated.

According to data gathered by the Bioremediation Field Initiative, soil alone and soil and ground water together are the media most often treated with bioremediation. Bioremediation is less often used to treat ground water only, sediments, and surface water. The most frequently applied bioremediation technique is in situ treatment (over 80 sites), followed by land treatment and liquid treatment in a reactor (over 60 sites each).

The Field Initiative is also considering four sites for performance evaluations: 1) a creosote site, 2) a site contaminated with trichloroethylene, dichloroethylene, and vinyl chloride, 3) and ethylene glycol site, and 4) an underground storage tank.

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. Each law—including 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)—requires 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 asssessments, 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 and enhancement to groups that apply technologies at specific sites.

Some of the most promising new technologies for solving hazardous waste problems involve the use of biological treatment systems. Biological treatment uses microorganisms, such as bacteria or fungi, to transform harmful chemicals into less toxic or nontoxic compounds. These microorganisms can break down pollutants to obtain energy to live and reproduce. They have a wide range of abilities to metaboloize different chemicals; scientists can tailor technology to the pollutants at specific sites and in specific media (e.g., contaminated aquifers, waste lagoons, contaminated soils) by using an organism in the treatment system that breaks down a particular pollutant. Where possible, technologies are developed to utilize native microorganisms that have been demonstrated to metabolize the pollutants on the site. In other cases, organisms known to metabolize the pollutants can be introduced and supplemented if necessary to accelerate biodegradation.

Biodegradation is an attractive option because it is "natural," 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 are also carefully monitored to reduce the possibility of a product of 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. Other methods of applying biological treatments, such as spreading contaminated soils on controlled land plots or mixing sludges with water for treatment in contained vessels, are currently used with varying degrees of success, depending on the chemical contaminants and environmental conditions. Additional research in such technologies will broaden their applicability and effectiveness. 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.

Perhaps one of the most well-known recent application of bioremediation technology was in cleaning up portions of the shoreline of Prince William Sound, Alaska, in the wake of the March 1989 *Exxon Valdez* tanker accident. This project demonstrated EPA's ability to work with private industry and academia to develop and transfer expertise in handling hazardous wastes. The Federal Technology Transfer Act of 1986 (FTTA) provided a mechanism whereby industry and EPA could share research costs and results.

The FITTA encourages the development of commercial pollution control technologies by making possible cooperative research and development among federal laboratories, industry, and academic institutions. Under the FITTA, EPA and industry can cooperate in developing and marketing biological treatment technologies in any of the six key areas of biosystems research identified by ORD's Biosystems Technology Development Program: ground-water treatment, liquid reactors, soil/sediment treatment, combined treatment, sequential treatment, and metabolic processes research.

This document is divided into six sections based on research performed in these media-based or process-oriented areas. Each section contains an introduction to the specific technology and summary of recent research, followed by abstracts of EPA projects presented at the April 1991 Symposium on Bioremediation of Hazardous Wastes.

SECTION ONE
GROUND-WATER TREATMENT

Effective bioremediation of ground water is limited by the geology, hydrology, and geochemistry of the subsurface environment. Engineers must control the hydrology of the system to direct the biological treatment to the area of contamination. They also must understand the geochemistry of the site to avoid mineral precipitation and the subsequent plugging of aquifers. Thus, the design of any effective remedial action for contaminated ground water requires a great deal of site-specific information.

Because oxygen has limited solubility in water, contaminated ground water is frequently anoxic. This problem can be offset by artificially supplying oxygen to the pollutant plume so that aerobic degradation is not limited by a poor oxygen supply. Nutrients can also be added to make environmental conditions suitable for microbial activity. Oxygen can be delivered to ground water by withdrawing the ground water, adding oxygen, and reinjecting the water using injection wells and trenches.

In a series of research projects managed by the U.S. EPA R.S. Kerr Environmental Research Laboratory, the U.S. Coast Guard in Traverse City, Michigan, has been evaluating the biodegradation of hydrocarbon-contaminated vapors within the unsaturated zone using bioventing. Bioventing is the engineered advection of air blown through contaminated soil to promote aerobic degradation. The Bioventing Reclamation Pilot Study is being performed on subsurface contamination resulting from an aviation gasoline spill of about 35,000 gallons that occurred in 1969. One study area is using injected aeration only; the other employs injection as well as extraction/reinjection. Fuel hydrocarbons were reduced by about 40 percent in both treatment areas during the first 3 months of operation. Laboratory and field studies of kinetics under natural and engineered advective conditions are helping EPA researchers determine the optimal conditions for bioventing.

BIOVENTING OF AN AVIATION GASOLINE SPILL: DESIGN AND OPERATION OF A FIELD DEMONSTRATION

John M. Armstrong, Ph.D. and Christopher J. Griffin, P.E., The Traverse Group, Inc., Ann Arbor, MI.

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, which is the site of a spill of about 35,000 gallons of aviation gasoline which occurred in 1969. After 20 years, a major portion of the spill still persists in the subsurface as a plume that is about 1,100 feet long and 250 feet wide. This study is being conducted as a cooperative effort between the U.S. Coast Guard and the U.S. Environmental Protection Agency's Robert S. Kerr, Environmental Research Laboratory.

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

The 90- by 75-foot study area has been divided into two equal areas of 45 by 75 feet to evaluate the effects of different flows and extraction patterns. The northern area has an injection system, while the southern area has an injection and extraction/reinjection system. The pneumatic properties of the

unsaturated zone were evaluated by the performance of a pneumatic pump test, resulting in a design radius of influence of 10 feet. The work plan calls for ambient air to be injected into both areas at an initial rate that would replace the calculated volume of air-filled pore space over 24 hours. The flow rate would be increased to a vapor recharge rate of 8 hours or less as the system becomes acclimated.

The blower package, therefore, has to be capable of extracting vapors in the south study area, at depths of 15 to 18 feet (depth of the water table) and flow rates ranging from 5 to 63 cubic feet per minute (cfm), then reinjecting the vapors at the same rate, at a depth of 10 feet. Additionally, 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 ambient air injected will be placed in twice the area (two test plots), the blower has to be able to inject air at flow rates ranging from 10 to 128 cfm.

The construction of the Bioventing Project consisted of installing, in the north area, 15 aeration injection points placed on 10-foot centers in a 3- by 5-foot grid and screened just above the water table. In the south area, eight sets of injection points coupled with seven extraction points, 10 feet on center, were installed, with screens placed just above the water table. Eight reinjection wells were installed, with the screens placed at a depth of 10 feet.

The blower package used is a 45 URAI Roots vacuum pump with a maximum flow rate of 130 cfm. This system extracts vapors at a vacuum of 4 to 6 inches of mercury and reinjects the vapors at a pressure of 6 pounds per square inch (psi). The vacuum pump is driven by a 10 HP 3-phase electric motor. Similarly, an additional 45 URAI Roots pump, also with a maximum flow rate of 130 cfm, is used for the ambient air injection. This blower is driven by a 7.5 HP 3-phase electric motor at an operating pressure of 6 psi. All the equipment is explosion proof.

The monitoring requirements of the EPA work plan called for the installation of several different types and depths of monitoring equipment and/or sample points. To monitor vapor hydrocarbon and oxygen concentrations, six 5-point cluster wells were installed with three cluster wells per plot. The cluster wells consisted of 1/4-inch diameter tubing with a wire mesh screen covering the tip. The five points of each cluster well were installed at 3.28-foot (1-meter) depth increments throughout the unsaturated zone. Additionally, we installed three 14-point cluster monitoring wells (well screens at 1.5-foot intervals from ground surface to 21 feet—one per plot and one at an upgradient location) and one set of moisture/temperature probes per plot. 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 feet below grade.

The development of a sufficient microbial population to degrade the hydrocarbon vapors requires adequate quantities of nitrogen, phosphorous, and potassium. The EPA Bioventing Work Plan called for an initial application of 64 pounds of nitrogen, 13 pounds of phosphorus, and 5 pounds of potassium to be applied to each area prior to startup. Additionally, during the growing season, 10 pounds of nitrogen, 2 pounds of phosphorous, and 1 pound of potassium were to be applied to each area monthly. These nutrients were applied in an aqueous solution by sprinklers until they were detected in the ground water, indicating that they had moved completely through the treatment zone.

The Bioventing Project is sampled and/or monitored daily, biweekly, 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 flow line is determined daily with a Bacharach Threshold Limit Value (TLV) combustible gas meter.

Biweekly 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 gas meter, and the oxygen concentration is determined using a Bacharach Oxygen indicator. Additionally, the soil moisture content and soil temperature are measured biweekly in the moisture/temperature probes.

The surface emissions are sampled at two locations within each of the study areas biweekly and at two upgradient locations weekly. The samples, taken over a 4-hour period, are pulled using an Ismatec peristalic pump set for a flow of approximately one liter per hour. A 19-inch diameter stainless steel bowl having a volume of 4.3 gallons (16 liters) is inverted and placed flush on the ground. The sample is pulled from the bowl through flexible vinyl tubing that is attached to the bowl by a 1/4-inch diameter steel ball valve tapped into the bottom of the bowl. Any water that collects within the emission chamber is removed by a water trap, located upstream from the sample trap, consisting of a flask containing a drying agent (Drierite).

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

BIOVENTING OF AN AVIATION GASOLINE SPILL: PERFORMANCE EVALUATION OF A FIELD DEMONSTRATION

Don Kampbell, U.S. Environmental Protection Agency, Ada, OK.

A spill of about 35,000 gallons of aviation gasoline occurred in 1969 at a U.S. Coast Guard air station. Much of the spill persists after 22 years as an oily phase residue at the water table near a depth of 5 meters. The subsurface matrix is a fairly uniform beach sand to 15 meters.

Aerobic soil microcosms were used in the laboratory to simulate the ability of the spill-site soil to biodegrade aviation gasoline vapors. Reaction rates with acclimated microcosms were rapid, with disappearance curves showing typical first-order kinetics. Degradation rates within a temperature range of 12 to 23° C were high. A nutrient addition of ammonia, nitrate, phosphorus, and potassium increased by several fold the bacteria count, degradation rate, and active biomass. A suppressive effect was shown on degradation of dimethyl and trimethyl pentanes mixtures when compared to singular components. A Lineweaver-Burk reciprocal plot was used to calculate a biochemical reaction kinetic maximum velocity value of 5.7 mg fuel/kg soil-hour and half saturation constant of 7.0 mg fuel/kg soil for aviation gasoline vapors. Extrapolation of the rates to field conditions suggests consumption of the gasoline vapors during bioventing within 8 hours in the unsaturated zone.

The pilot demonstration systems have been operational slightly more than 3 months. One area has injected aeration only; the second has injection, extraction, and reinjection. Turf was established to cover both treatment areas. A nutrient solution of nitrogen and phosphorus was dispersed throughout the unsaturated zone. Subsurface flow characteristics will be defined with a sulphur hexafluoride tracer test. Core material, soil gas, and underground water are being analyzed to determine the extent of remediation. Core material fuel hydrocarbons have been reduced about 40 percent in both treatment areas during the first 3 months of operation. Surface emissions have been less than 1 percent of total volatile hydrocarbons detected in the soil gas aeration stream at a 1-meter depth.

Objectives of the project are to demonstrate that:

- Remediation will be completed in a reasonable time.
- Surface emissions of gasoline do not occur.
- Remediated core material will be <10 mg fuel carbon/kg.
- Final benzene levels in the ground water will not exceed 5 µ/g liter.
- Performance and economical advantages will be applicable to full-scale remediation.

The project described in this presentation is jointly funded by the U.S. Environmental Protection Agency and the U.S. Coast Guard. The work described is in the initial phase of data compilation. Therefore, the contents have not been subjected to the Agency's review policy and no official endorsement should be inferred.

LABORATORY AND FIELD STUDIES OF THE KINETICS OF BIOVENTING

David W. Ostendorf, Associate Professor, Civil Engineering Department, University of Massachusetts, Amherst, MA; Don H. Kampbell, Research Chemist, R.S. Kerr Environmental Research Laboratory, U.S. EPA, Ada, OK; and Ellen E. Moyer, Research Assistant, Civil Engineering Department, University of Massachusetts, Amherst, MA.

The interaction of laboratory and field investigations of biodegraded hydrocarbon vapors are discussed under naturally diffusive and engineered advective conditions.

The coupled transport of aviation gasoline and oxygen vapors has been measured and modeled at the U.S. Coast Guard Air Station in Traverse City, Michigan, as part of a series of research projects managed by the R.S. Kerr Environmental Research Laboratory of the U.S. EPA. The data consist of a group of stainless steel tubing clusters set at 1-meter depth increments over the 5-meter thick unsaturated zone in the uniform sand at the site. The clusters have been sampled over a 13-month period with hydrocarbon and oxygen meters, calibrated against known headspace standard gases. The model is a time averaged steady balance of diffusion and Michaelis-Menton kinetics, coupled stoichiometrically under the assumption of abundant oxygen. An implicit solution is put forth by Ostendorf and Kampbell:

$$z = \left(\frac{DK}{2V}\right)^{1/2} I\left(\frac{H}{K}\right) \qquad \text{(diffusion)} \qquad (1)$$

with elevation z above the contaminated capillary fringe, soil moisture diffusivity D, half saturation constant K, maximum reaction rate V, and hydrocarbon concentration H (1). The integral function I(H/K) is evaluated by Ostendorf and Kampbell (1), and the corresponding oxygen concentration follows from the stoichiometry of the reaction. This natural diffusion model is calibrated with the field soil gas data at four clusters, yielding the following kinetics:

Unacclimated sandy soil

$V = 8.6 \times 10^{-9}$ kg/m^3-s

K = 0.10 mg/L

Bioventing is the engineered advection of air blown through contaminated soil, subject to aerobic biodegradation. Ostendorf and Kampbell model this process as a balance of advection and Michaelis-Menton kinetics, yielding a simple prediction for the effluent hydrocarbon vapor concentration H_E:

$$\frac{t_p V}{K} = \ln\left(\frac{H_I}{H_E}\right) + \frac{H_I - H_E}{K} \qquad \text{(advection)} \qquad (2)$$

with pneumatic residence time t and influent concentration H_I (2). Thus the reaction kinetics play a major role in determining the removal efficiency of the bioventing reactor. The foregoing theory describes field data from an acclimated clay soil bioreactor treating a propane/butane waste gas mixture at Racine, Wisconsin (3).

Ostendorf and Kampbell apply laboratory microcosm data to the diffusion and advection transport models cited above (2). Aseptic soil samples from the Wisconsin and Michigan sites were dosed with appropriate gases in headspace vials, and the subsequent decay of concentration was measured in a gas chromatograph (subject to abiotic control). The temporal decay of the initial concentration H_O is described by a balance of storage, headspace sorption, and Michaelis-Menton kinetics:

$$t = \frac{R_D K}{V}\left[\ln\left(\frac{H_O}{K}\right) + \frac{H_O - H}{K}\right] \qquad \text{(microcosm)} \qquad (3)$$

with retardation factor R_D. The microcosm-based kinetics for Traverse City agree fairly well with the field calibrated values listed above. The Wisconsin soil kinetics, obtained from the microcosms, are:

Acclimated clay soil

$V = 4.1 \times 10^{-5}$ kg/m^3-s

K = 0.088 mg/L

These values, when substituted into the bioventing advection model (Equation 2) yield a 92 percent predicted removal rate that compares favorably with the observed range of 90 to 99 percent (3). We conclude that laboratory microcosms yield kinetics that are consistent with field values, implying no transport limitations on the field or microcosm scale in unsaturated soil.

We note the close correspondence of half saturation constants for two dramatically different soil types. The wide range of maximum reaction rates may be due to biomass variation in clays and sands, and also suggests that biostimulation may be possible in natural soils. Ostendorf and Kampbell explored the latter possibility by running a series of microcosm studies for stimulated soil samples at Traverse City with the results (2):

Acclimated sand with nutrients

$V = 2.8 \times 10^{-6}$ kg/m^3-s

K = 0.25 mg/L

These kinetics are input to a series of simulations of bioventing effectiveness at the site. The optimal conditions, consisting of a high concentration influent and a biostimulated soil, result in very effective bioventing, with complete removal of product in about 1 month of system operation. Natural kinetics are too slow for effective bioventing at Traverse City, and an auxiliary exhaust air treatment process would have to be considered at the site.

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SECTION TWO
TREATMENT IN A REACTOR

Work is ongoing for treatment of liquid and gaseous waste streams in a reactor. In reactors, pollutants are brought into contact with microorganisms to accelerate the degradation process. Landfill leachate is a good example of a type of liquid waste that is amenable to reactor treatment. Over 130 million tons of solid waste are disposed of each year in landfills across the nation, and various organic chemicals and ions often leach from the waste.

A current laboratory-scale project is exploring the use of white rot fungus (Phanerochaete chrysosporium) in a reactor to degrade organic pollutants in wastewater. This fungus has been found to decolorize the bleach plant effluent that results from the manufacture of white paper. It can also dechlorinate the organic chlorine compounds in the effluent, and can transform high molecular weight compounds into lower molecular weight compounds. It may be possible to use immobilized P. chrysosporium as a pretreatment for a variety of wastewaters prior to conventional secondary treatment.

In recent years, granular activated carbon (GAC) has received increased attention for removing synthetic organic compounds (SOCs) from water. One study tested two anaerobic GAC expanded-bed bioreactors as pretreatment units for decontaminating hazardous leachates. One leachate was representative of those from old, stabilized waste landfill sites; the second was typical of younger, more active sites. In both reactors, the majority of the SOCs were removed by biological activity. Another study investigated the effects of molecular oxygen on the adsorptive capacity of GAC for different organic compounds found in wastewater.

Other research is exploring the treatment of ground water containing trichloroethylene (TCE) and degradation products in bioreactors using Pseudomonas cepacia Strain G4. Another recent EPA study analyzed the biodegradation of three volatile organic compounds (VOCs) in an aerobic biofilter containing acclimated biomass. Nearly 100 percent removal of the three compounds was achieved.

TREATMENT OF WASTEWATER WITH THE WHITE ROT FUNGUS *PHANEROCHAETE CHRYSOSPORIUM*

Thomas W. Joyce and Hou-min Chang, Department of Wood and Paper Science, North Carolina State University, Raleigh, NC., and John A. Glaser, U.S. EPA, Risk Reduction Engineering Laboratory, Cincinnati, OH.

The purpose of this summary is to provide an overview of the use of the white rot fungus *Phanerochaete chrysosporium* in the treatment of aqueous wastewaters, with emphasis on wastewaters from paper manufacture bleach plants.

White rot fungi, and in particular *P. chrysosporium*, are able to degrade a wide variety of organic pollutants (1, 2, 3). Those studies were extensions of research that found that the fungus was able to decolorize the bleach plant effluent resulting from the manufacture of white paper (4). As the chromophoric material in the effluent was destroyed, the organic chlorine-containing compounds were also degraded (5). The degradation is believed to occur as the result of secondary metabolic activity, i.e., the fungus cannot utilize the substrate for carbon or energy purposes (6). The degradation of pollutants is now known to be mediated by a family of enzymes excreted by the fungus (7).

Treatment in a Reactor

The initial treatability studies on bleach plant effluent were done using 125-mL shake flasks. After evaluation of numerous reactor designs, it was determined that a rotating biological contactor (RBC) was best suited to the physiological needs of the fungus. Our standard laboratory-scale reactor has a capacity of about 2.1 L. It contains eight partially wetted (40 percent) disks rotating at 1 rpm (8). It has also been found that a higher-than-atmospheric oxygen tension promotes organic pollutant destruction. Typically, the fungus is grown at 37°C; after substantial mycelial growth, the temperature is lowered to 30°C for enzyme production (9). The pH is maintained in the range of 3 to 5. After 2 to 4 days of fungal growth, the nitrogen is removed from the wastewater and the fungus soon enters secondary metabolism, when the enzymes actually responsible for organic pollutant destruction are excreted. Other researchers have found that the fungus can be immobilized on porous plastic (10) or in alginate (11), or that the enzymes themselves can be immobilized and remain viable (12).

With respect to bleach plant effluents, the use of *P. chrysosporium* may be particularly advantageous. Not only does the fungus reduce the color of the effluent, but it also can dechlorinate the organic chlorine compounds as measured by AOX. At present, typical biological treatment systems, aerated lagoons and activated sludge, do not remove substantial amounts of color or AOX from the effluent. The immobilized fungi can remove some 70 percent of the color and about 50 percent of the AOX. We have found that the high molecular weight organic compounds in bleach plant effluent can be transformed into lower molecular weight compounds that exhibit more toxicity as measured by the MICROTOX bioassay (13).

We believe the most logical use for the immobilized fungus may be in a two-stage system whereby the fungus is used as a pretreatment prior to conventional secondary treatment (14). The fungus would thus be used to dechlorinate the AOX compounds and reduce the average molecular weight of the remaining lignin compounds. The conventional secondary treatment could then remove a significant portion of the remaining organic compounds.

In some cases, it may be economically advantageous to concentrate by ultrafiltration the effluent to be treated by the fungus. We have found that the rate of color removal is generally proportional to color concentration. In one example case, the cost of fungal treatment for a bleach plant effluent was $12.37/ton pulp; when ultrafiltration was added to the treatment flowsheet, the cost decreased to $4.46/ton pulp despite the added cost of the UF system (15).

To date, the immobilized fungus has not been tested at even pilot plant scale. We believe, however, that it can be effectively integrated into an existing treatment scheme to pretreat a wide variety of wastewaters to make them more amenable to conventional treatment.

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TREATMENT OF CERCLA LEACHATES BY CARBON-ASSISTED ANAEROBIC FLUIDIZED BEDS

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Rainfall and surface runoff percolating through landfill are contaminated with a number of organic and inorganic compounds, and direct discharge of exiting leachate to municipal wastewater treatment plants can result in inadequate removal of many hazardous substances. Many volatile and semivolatile synthetic organic chemicals (SOCs) used as solvents, degreasers, and components in industrial products are present in leachates originating from hazardous waste sites. These SOCs are often inadequately treated in aerobic wastewater treatment processes; as volatiles are subject to air stripping, many semivolatiles simply pass through untreated, and highly chlorinated compounds are difficult to degrade aerobically.

In this study, two anaerobic granular activated carbon (GAC) expanded-bed bioreactors were tested as pretreatment units for the decontamination of hazardous leachates. Two municipal leachates, rendered hazardous with the addition of 10 volatile and four semivolatile organic compounds commonly found in leachates, were fed to two identical bench-scale (10.2 cm diameter x 96.5 cm high) expanded-bed reactors. One leachate, with a chemical oxygen demand (COD) of approximately 1,100 mg/L, was representative of those that emanate from old, stabilized waste landfill sites where only a small fraction of the leachate COD is made up of volatile fatty acids. Sulfate concentrations in this leachate averaged 89 mg SO_4/L. The second leachate, with a COD of approximately 3,800 mg/L, was typical of younger, more active landfill sites, where the majority of the COD is attributable to volatile fatty acids. This leachate had a very low sulfate content. The different characteristics of the two leachate feed streams resulted in one reactor operating in a sulfate-reducing mode and the second in a strictly methanogenic environment. Both reactors were operated with a 6-hour unexpanded empty-bed contact time and achieved SOC removals acceptable for pretreatment units. In both reactors, the majority of the SOCs were removed by biological activity, with GAC adsorption providing stability to each system during startup and buffering against load fluctuations during long-term operation.

The SOCs and their respective concentrations added to the two leachates are summarized in Table 1. During the first phase of the study, chloroform was deleted from the SOC supplement because of toxicity problems associated with this volatile organic compound. The effect of chloroform on the performance of the treatment systems was evaluated during the second phase of the study.

Phase One (No Chloroform Addition)

The first phase of the study extended over a period of 400 days, during which both reactor systems exhibited excellent removal efficiencies for all the SOCs listed in Table 1. A summary of the performance of the two systems averaged over the last 260 days of operation is given in Table 2. A comparison of the two systems indicates that the sulfate-reducing environment may produce equal or better performance than a methanogenic environment in removing a consortium of hazardous chemicals from waste streams. All three volatile aromatic compounds (toluene, chlorobenzene, and ethylbenzene) in the SOC consortium were removed at higher rates in the sulfate-reducing environment. Also, the persistence of the intermediate biodegradation product of nitrobenzene, aniline, was only significant in the methanogenic reactor.

Phase Two (Chloroform Addition)

During the second phase of the study, chloroform was added to the SOC supplement at an initial feed concentration of 2 mg/L. This feed concentration was maintained for a period of 105 days. During this period, chloroform was not observed to exert an adverse effect on the performance of either the sulfate-reducing or the methanogenic reactors. Encouraged by the ability of both reactors to handle this feed level of chloroform, the influent chloroform concentration was raised to 3.5 mg/L. The methanogenic reactor responded with a rapid decline in methane production and COD reduction, and corresponding increases in effluent SOC concentrations. The sulfate-reducing reactor, on the other hand, was able to withstand this increased feed concentration of chloroform, and continued to maintain excellent removal of all the SOCs for a period of 156 days. The current plan is to continue increasing the feed concentration of chloroform to the sulfate-reducing reactor. Recovery of the methanogenic reactor was rapidly achieved after chloroform addition was terminated.

Table 1. Composition of SOC supplement added to the leachates.

Compound	Concentration (*pg*/L)
VOLATILE ORGANIC COMPOUNDS	
Acetone	10,000
Methyl Ethyl Ketone	5,000
Methyl Isobutyl Ketone	1,000
Trichloroethylene	400
1,1-Dichloroethane	100
Methylene Chloride	1,200
Chloroform	0 to 3,500
Chlorobenzene	1,100
Ethylbenzene	600
Toluene	8,000
SEMIVOLATILE ORGANIC COMPOUNDS	
Phenol	2,600
Nitrobenzene	500
1,2,4-Trichlorobenzene	200
Dibutyl Phthalate	200

Table 2. Summary of SOC concentrations in reactor effluents.

Compound	Sulfate Reducing Reactor Effluent		Methanogenic Reactor Effluent	
Acetone	189*	(216)#	410*	(577)#
Methyl Ethyl Ketone	70	(68)	220	(150)
Methyl Isobutyl Ketone	35	(16)	58	(25)
Trichloroethylene	8	(10)	5	(3)
Methylene Chloride	65	(50)	46	(44)
1,1-Dichloroethane	20	(17)	14	(8)
Chlorobenzene	67	(42)	165	(86)
Ethylbenzene	34	(19)	85	(41)
Toluene	436	(303)	1,102	(744)
Phenol	22	(23)	93	(63)
Nitrobenzene	6	(16)	8	(17)
1,2,4-Trichlorobenzene	10	(15)	14	(16)
Dibutyl Phthalate	26	(29)	36	(30)

*All concentrations in µg/L.
#Standard deviation.

IMPROVED PREDICTION OF GAC CAPACITY IN A BIOLOGICALLY ACTIVE FLUIDIZED BED

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The first applications of activated carbon for water quality control involved the addition of powdered activated carbon (PAC) to drinking water treatment plants for taste and odor control. In recent years, granular activated carbon (GAC) has received increasing attention for removing synthetic organic compounds from water. Moreover, the possibility of GAC reuse have accentuated the applicability of granular activated carbon to drinking water treatment.

Interest in removing biologically resistant organic contaminants led to the application of activated carbon in wastewater treatment. PAC has been used in activated sludge systems (1), while GAC found application in anaerobic fixed film processes (2) and in aerobic wastewater treatment (3). In addition to its adsorptive properties, activated carbon has been reported to provide an excellent surface for microbial attachment (4, 5).

Nakhla (6) found the adsorptive capacity of GAC for o-cresol in an expanded-bed anaerobic GAC bioreactor treating a synthetic mixture of phenol, acetic acid, and o-cresol to be much lower than that determined from an adsorption isotherm conducted on virgin carbon using the standard bottle-point technique. Further experimentation revealed that the adsorptive capacity obtained from the biological anaerobic reactor agreed very well with capacities of virgin GAC determined from isotherm experiments conducted in the absence of molecular oxygen. The adsorptive capacity of vir-

gin GAC exhibited for o-cresol in the presence of molecular oxygen (oxic conditions) was almost 200 percent above the adsorptive capacity of virgin GAC attainable in the absence of oxygen (anoxic conditions). Since both aerobic and anaerobic biological GAC reactors are in use, this study was designed to further investigate the effects of molecular oxygen on the adsorptive capacity of activated carbon for different organic compounds.

To evaluate the effect of the same functional group substituted at different positions on the parent phenol molecule, both oxic and anoxic adsorption isotherm experiments were conducted using 16 x 20 U.S. Mesh virgin F-400 GAC (Calgon Carbon, Pittsburgh, PA) as an adsorbent and 2-, 3-, and 4-methylphenol as adsorbates. Experimentally determined data on adsorption capacity for these three compounds on GAC are presented in Figure 1. All the adsorption isotherms were found to be well described by the Freundlich isotherm equation $q_e = K * C_e^{1/n}$. The straight lines on Figure 1 were obtained by nonlinear least square fit of experimental data to the Freundlich isotherm equation. As is apparent from this figure, the absence of molecular oxygen from the test environment resulted in almost identical GAC adsorptive capacities for all three compounds. On the other hand, the presence of molecular oxygen had a diverse impact on the adsorptive capacity of GAC for different compounds. This is well illustrated in Figure 2, where the ratio of oxic and anoxic adsorptive capacities for all three compounds is plotted as function of the respective equilibrium adsorbate liquid phase concentrations. The most significant increase in oxic adsorptive capacity was observed in the case of 2-methylphenol followed by 4-methylphenol, while the adsorptive capacity of GAC for 3-methylphenol was the least affected by the presence of molecular oxygen. Another important conclusion from this figure is that the two adsorption isotherms (oxic and anoxic) have very different values of the coefficient 1/n in the Freundlich isotherm equation, since the ratio of the two adsorptive capacities depends on the liquid phase concentration of the adsorbate.

Further investigation of the observed phenomenon was continued by extracting GAC that was preloaded with each of the adsorbates during the adsorption isotherm experiments. Extraction was performed in a soxhlet extraction apparatus using methanol for 1 day followed by further extraction with methylene chloride for an additional period of 3 days. The results of these experiments are presented in Figure 3. Extraction efficiency was calculated as the ratio of the mass of adsorbate in the extract to the mass of adsorbate loaded on the carbon during the adsorption isotherm experiment.

The extraction efficiencies obtained from the carbons used in anoxic isotherm experiments showed very little dependence on the carbon loading. On the average, 90 percent of the adsorbed compound was extracted from these carbons. On the other hand, the extraction efficiencies for the carbons used in the oxic isotherm experiments were significantly lower and exhibited strong dependency on the equilibrium carbon loading. Furthermore, the least amount of adsorbate in the oxic isotherm experiments was extracted from the GAC in the case of 2-methylphenol, followed by 4-methylphenol and 3-methylphenol. This arrangement is in agreement with the degree of influence of molecular oxygen on the adsorptive capacity of GAC for these three adsorbates.

Gas chromatographic/mass spectroscopic analyses of the extracts from several samples of carbon that were preloaded with o-cresol under oxic conditions revealed the presence of significant amounts of dimers, trimers, and even tetramers of o-cresol. The discovery of polymers of o-cresol suggested that some polymerization reactions are taking place on the surface of GAC in the presence of molecular oxygen. These polymerization reactions offer a possible explanation for the increased removal of adsorbate from the liquid phase under oxic conditions.

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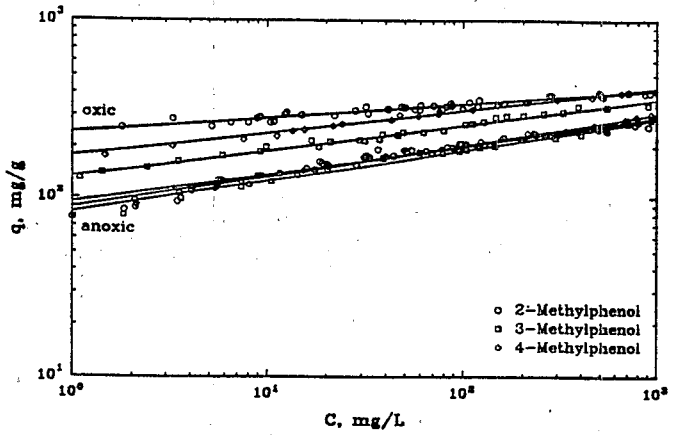

Figure 1. Oxic and anoxic adsorption isotherms of methylphenol on GAC.

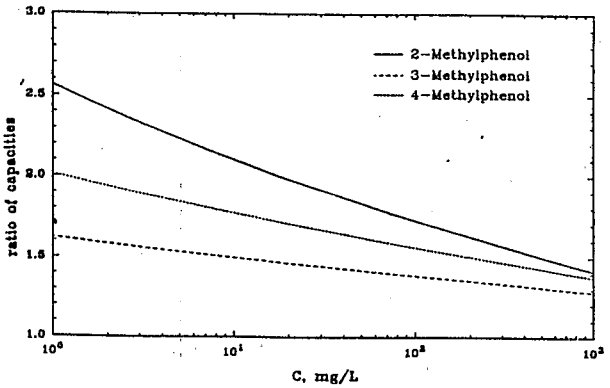

Figure 2. Ratio of oxic to anoxic adsorptive capacities of GAC for methylphenol.

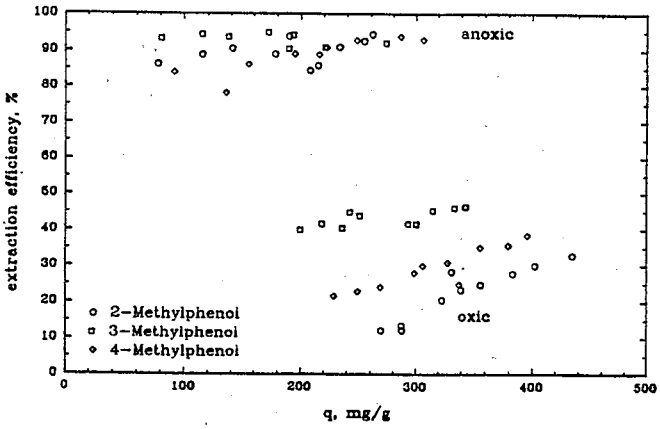

Figure 3. Extraction efficiency of methylphenol from GAC.

TREATMENT OF TCE AND DEGRADATION PRODUCTS USING *PSEUDOMONAS CEPACIA*

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Summary

The constitutive trichloroethylene (TCE)-degrading *Pseudomonas cepacia* strain G4 Phe1 was found to be very stable, with no detectable loss of activity (or marker) after 100 generations of nonselective growth. Toluene monooxygenase activity towards trifluoromethyl phenol was constant throughout the growth curve as measured by the rate of production of 2-hydroxy-7,7,7-trifluoroheptadienoic acid. TCE degradation rates measured for G4 Phe1 (0.5 - 1 nmole TCE min^{-1} mg^{-1} protein) are approximately 15 to 30 percent of the maximal wild type G4 phenol induced activity. G4 Phe1 was found to degrade TCE over a fairly wide range of physical conditions: 4 to 30°C, pH 4 to 9, 3 to 30 mg l^{-1} oxygen, and 0 to 20 0/00 salinity. A slight lowering of growth rate in G4 Phe1 was observed in the presence of TCE at an aqueous concentration of 530 µM. Chlorobenzene and 2-chlorophenol were found to be inhibitory to TCE degradation at 1 to 10 µM. Following introduction of pRO101 (a transposon derivative of pJP4) 2-chlorophenol and chlorobenzene levels inhibitory to TCE degradation were increased to 100 µM.

Results and Discussion

Ground-water contamination by organic pollutants is a subject of overwhelming concern throughout the industrialized world. Chief among these pollutants are those categorized as volatile organics. These include the chloroethylenes: TCE, tetrachloroethylene, *trans*-1,2-dichloroethylene (DCE), 1,1-DCE, and vinyl chloride (ranked first, second, third, fifth, and more than tenth, respectively, of all volatiles detected as ground-water contaminants in the United States) (3). The constitutive TCE degrader, *Pseudomonas cepacia* G4 Phe1, that employs a unique toluene monooxygenase (4) for the degradation of TCE (5), was investigated for its bioremediation potential.

Rates of TCE degradation were determined using a glass syringe with a Teflon plunger and no air headspace in the bioreaction chamber as previously described (1). This assay was adopted for rate analysis because it more closely resembles a contaminated aquifer, and multiple samples can be taken without the introduction of an air headspace. The range of certain physical variables (temperature, oxygen concentration, pH, and salinity) likely to affect the rate of TCE degradation by *P. cepacia* G4 Phe1 under environmental conditions was investigated using this technique.

The most profound effect on the rate of TCE degradation was found at the lowest temperatures used (Figure 1a) where despite cooling to 4°C, G4 Phe1 maintained approximately 30 percent of the rate of TCE degradation measured at 30°C. TCE degradation rates of approximately 20 and 45 percent (relative to the maximal pH 7 value) were evident at pH extremes of 4 and 9, respectively (Figure 1b). Little effect on TCE degradation rates was observed over the ranges of oxygen concentration (2.8 to 31.3 mg L^{-1}) (Figure 1c) or salinity (0 to 20 0/00) tested (Figure 1d).

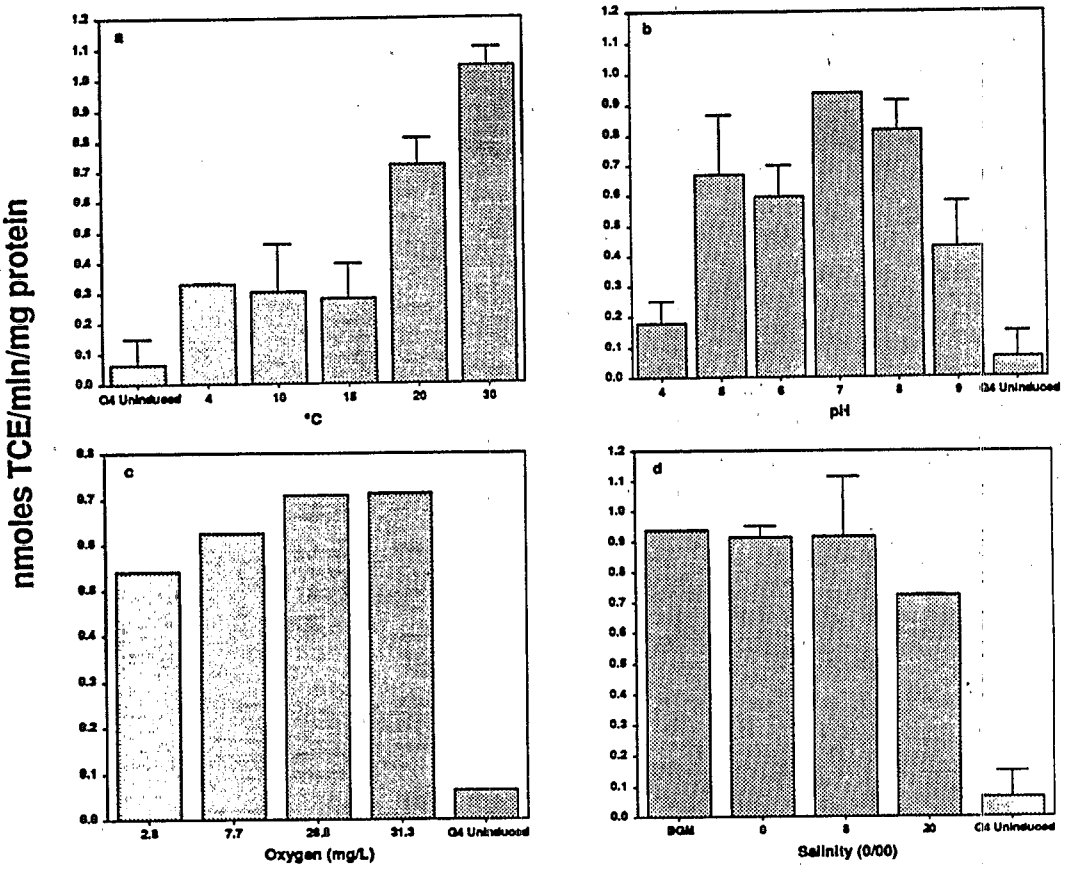

Figure 1. Effects of varying temperature, pH, dissolved oxygen, and salinity on the rate of TCE degradation by *Pseudomonas cepacia* G4 Phe1.

Possible toxicity of a metabolite of TCE towards G4 Phe1 was investigated. G4 does not affect TCE in the absence of an aromatic inducer (2). Therefore, any toxicity in the presence of TCE would be due to direct toxicity. G4 Phe1 differs only in one significant respect from G4 (i.e., the constitutive expression of the toluene and TCE degradative enzymes). Any toxic effects beyond those seen with G4 would therefore be attributable to the active metabolism of TCE. A slight toxic effect was measured as a depression of growth rate for G4 Phe1 on glucose and yeast extract as a result of the metabolism of TCE at levels c.a. 2 mM as if all were in aqueous solution (measured at 530 μM).

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AEROBIC BIODEGRADATION OF VOLATILE ORGANIC COMPOUNDS IN A BIOFILTER

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In recent years the emission of volatile organic compounds (VOCs) has received increased attention from EPA, OSHA, and other government agencies due to the serious human health hazards these compounds present as pollutants. The origins of these VOCs can be from manufacturing processes or wastewater treatment plants, where the waste stream is stripped of the VOCs during aeration. Another significant source of these pollutants is landfill leachate.

The conventional physical/chemical treatment methods for these gaseous pollutants are adsorption on a solid, absorption in a solvent, incineration, or catalytic conversion. An alternative to these conventional treatment methods is the biological destruction of the VOCs. This method has the advantages of pollution destruction (as compared to transfer to another medium) and lower operation and maintenance costs.

The biodegradation can be carried out in a biofilter. A biofilter consists of a packed column containing biologically active mass. The biologically active matter (biomass) can exist either as a biofilm on the support medium or as biomass particles trapped in the void spaces between the support material.

Biodegradation of three volatile organic compounds in an aerobic biofilter was studied. The three chemicals (substrates) were studied at the following concentrations: toluene: 520 ppm; methylene chloride: 180 ppm; trichloroethylene: 25 ppm. The substrates were fed upflow to the biofilter through the gas phase. The requisite composition of the substrates in the gas phase was achieved by making the synthetic gas mixtures in a cylinder and subsequently blending them with air. The biofilter was packed with pelletized activated carbon support material. Nutrient solution was circulated counter to the gas through the bed. The inlet and outlet gas streams were analyzed for the above three chemicals.

The biofilters contained active acclimated biomass. The results showed that nearly 100 percent removal of the three compounds was achieved in the biofilter.

SECTION THREE
SOIL/SEDIMENT TREATMENT

Soil and sediment contamination is one of the most difficult problems encountered at hazardous waste sites. Soils at industrial sites are often contaminated with a complex mixture of pollutants. Biologically treating these soils in situ is much more effective and inexpensive than excavating the soils — in itself a major task — and then performing the treatment. EPA's biosystems research on soils and sediments currently focuses on pentachlorophenol (PCP), hexachlorobenzene, and other chlorinated aromatic compounds.

PCP is a common soil contaminant at wood-preserving facilities. Recent EPA studies have shown that a lignin-degrading fungus, Phanerochaete chrysosporium, can effect a rapid and extensive depletion of PCP from soil and other contaminated materials. Current research is underway to further investigate the feasibility of using P. chrysosporium and other lignin-degrading fungi to remediate soils contaminated with wood-preserving chemicals.

Another recent project focused on the ability of unacclimated microorganisms and sediment microorganisms acclimated to dechlorinate 2,4- and 3,4-dichlorophenol to degrade 2,4-D, 2,4,5-T, DDT, hexachlorobenzene, and chloroanisoles. EPA researchers also recently examined the effects of various nonionic surfactants on the anaerobic dechlorination of hexachlorobenzene. The use of surfactants has been suggested as a way to enhance soil treatment, since surfactants can mobilize pollutants into micellar solution in the presence of soil or sediment solids, desorbing them from these media.

Another recent EPA study examined the anaerobic degradation of chlorophenol under methanogenic and sulfidogenic conditions. The same study also examined the degradation of chlorinated phenols and benzoic acids under methanogenic, sulfidogenic, and denitrifying conditions. The results indicate that degradation of these chlorinated aromatic compounds can take place under various reducing conditions.

Researchers have also examined the anaerobic transformation of phenol to benzoate using a bacterial consortium. Phenol was transformed to benzoate without complete mineralization of benzoate; the results showed that transformation was via para-carboxylation.

USE OF LIGNIN-DEGRADING FUNGI IN THE REMEDIATION OF PENTACHLOROPHENOL-CONTAMINATED SOILS

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Recently, we reported that a lignin-degrading fungus, *Phanerochaete chrysosporium*, was capable of depleting pentachlorophenol (PCP) from three soils in the laboratory. This depletion was found to result primarily from the transformation of PCP to nonvolatile products. The nature of these products—whether soil-bound or extractable—was greatly influenced by soil type. We believe that the soil-bound products are humic acid-PCP hybrid polymers that are formed as a result of the activity of extracellular enzymes (lignin peroxidases, Mn peroxidases, laccases) with phenol-oxidizing activity, which are produced by the lignin-degrading fungi. Our objective is to determine the feasibility of using lignin-degrading fungi to remediate soils that are contaminated with wood-preserving chemicals. The results of some of our studies are summarized below.

In investigations of white-rot fungal degradation of hazardous compounds, *P. chrysosporium* strain BKM-F-1767 has been used almost exclusively as the experimental organism. However, there are an estimated 1,400 species of lignin-degrading fungi, and there is great diversity among these organisms in their ability to degrade lignin. Thus, there is reason to believe that this same diversity will be seen in xenobiotic degradation.

Several studies were conducted to determine the degree of interspecific and intraspecific variation among selected *Phanerochaete* spp. in growth rate and in their ability to tolerate and degrade the wood preservative PCP. Mycelial extension rates of selected strains of *Phanerochaete chrysorhiza, Phanerochaete laevis, Phanerochaete sanguinea, Phanerochaete filamentosa, Phanerochaete sordida, Inonotus circinatus,* and *Phanerochaete chrysosporium,* and the ability of these organisms to tolerate and degrade PCP in an aqueous medium and in soil, were measured. Most of the tested species had maximum mycelial extension rates in the range of $\leq$0.5 to 1.5 cm d^{-1}, but there were large interspecific differences. A notable exception, *P. sordida,* grew very rapidly, with an average mycelial extension rate of 2.68 cm d^{-1} at 28°C. There were also significant intraspecific differences in mycelial extension rates. For example, mycelial extension rates among strains of *P. sordida* ranged from 1.78 to 4.81 cm d^{-1}.

Phanerochaete spp. were very sensitive to PCP in 2 percent malt agar. Growth of several species was prevented by the presence of 5 ppm PCP. However, *P. chrysosporium* and *P. sordida* grew at 25 ppm PCP, albeit at greatly decreased mycelial extension rates. We have observed lignin-degrading fungi in PCP-contaminated soils with PCP concentrations exceeding 500 ppm. Therefore, growth of lignin-degrading fungi on malt agar in the presence of PCP should be used as a screening tool for determining relative sensitivities and not as an indication of growth performance of these organisms in PCP-contaminated soil.

In an aqueous medium, mineralization of PCP by *P. sordida* strain 13 was significantly greater than that by all other tested *P. sordida* strains and *P. chrysosporium* (Table 1). This strain was tested in a field investigation with *P. chrysosporium* to determine the ability of these fungi to deplete PCP in contaminated soils.

Inoculation of a field soil contaminated with a commercial wood preservative product and containing 250 to 400 $\mu g\ g^{-1}$ PCP with either *Phanerochaete chrysosporium* or *P. sordida* resulted in an overall decrease of 88 to 91 percent of PCP in the soil in 6.5 weeks. This decrease was achieved under suboptimal temperatures for the growth and activity of these fungi, and without the addition of inorganic nutrie-

Table 1. Percentage of total [^{14}C] PCP mineralized and volatilized in liquid cultures of *P. chrysosporium* and several strains of *P. sordida* after 30 days[a].

Strain	Mineralization	Volatilization	Total ^{14}C Evolved
	(%)		
P. chrysosporium	1.97 (0.22)b	13.82 (1.20)b	15.79
P. sordida 7	2.67 (1.09)b	12.91 (2.79)b	15.58
P. sordida 8	1.92 (0.44)b	8.88 (2.21)b	10.80b
P. sordida 9	1.22 (0.54)b	11.92 (2.35)b	13.14
P. sordida 13	11.64 (2.54)c	8.48 (0.52)b	20.12
Control	0.17 (0.03)d	0.06 (0.03)c	0.23

[a] Figures in parentheses represent the standard deviation of three observations. Means followed by the same letter are not significantly different.

Table 2. Percentage of mineralization and volatilization of pentachlorophenol and pentachloroanisole in liquid cultures of *P. chrysosporium* or *P. sordida* 13 and from control (noninoculated) cultures.

Culture	Compound	Mineralization (%)	Volatilization (%)
P. chrysosporium	PCP	8.91	7.95
	PCA	7.95	9.54
P. sordida	PCP	16.13	8.25
	PCA	13.07	10.02
Control	PCP	1.05	0.08
	PCA	0.40	15.92

ents. A small percentage (8 to 13 percent) of the decrease in the amount of PCP was a result of fungal methylation to pentachloroanisole (PCA). However, both of these organisms can also transform PCA (Table 2), and thus it would also be expected to be depleted from soil over time.

For soil studies, inocula have consisted of aspen chips (0.65 to 1.3 cm) preinfested with pure cultures of a single fungus. We have found in laboratory studies and in a field study that aspen chips absorb PCP. Therefore, we have also investigated the metabolism of PCP by lignin-degrading fungi in wood chips.

Inoculation of PCP-contaminated softwood or hardwood chips (whether sterilized or not) with either *P. chrysosporium* or *P. sordida* resulted in a decrease in the PCP concentration of the chips. No decrease in the PCP concentration was observed in noninoculated chips, indicating that the observed PCP decreases were due to the activities of *P. chrysosporium or P. sordida*. Depletion in hardwood and softwood chips inoculated with *P. chrysosporium* was rapid and extensive (63 to 72 percent decrease after 6 weeks), except in nonsterile softwood chips. In nonsterile softwood chips, depletion of PCP was very slow and resulted in only a 30 percent decrease after 6 weeks. This lower rate of PCP depletion may have been the result of a lower rate of colonization of these chips by *P. chrysosporium* due to competition from indigenous microbes.

Depletion of PCP by *P. sordida* was also affected by sterilization. Inoculation of nonsterile softwood and hardwood chips resulted in only a 50 percent and a 45 percent decrease in the PCP concentration, respectively, after 42 days. However, the PCP concentration in both hardwood and softwood chips that had been sterilized was decreased by *ca.* 66 percent by *P. sordida* after 42 days. Again, this lower rate of decrease was probably due to competition from indigenous microbes.

Depletion of PCP was always accompanied by an increase in the concentration of PCA. Accumulation of PCA in sterile cultures was much greater than in nonsterile cultures of both fungi. This was particularly true for cultures inoculated with *P. sordida*. Only 7 percent and 19 percent of the PCP decrease in nonsterile softwood and hardwood cultures, respectively, was due to conversion of PCP to PCA. However, this low rate of conversion was associated with relatively low amounts of PCP depletion.

In nonsterile hardwood and softwood chips inoculated with *P. chrysosporium*, 65 percent and 72 percent, respectively, of the PCP decrease was due to conversion of PCP to PCA. In sterile chips inoculated with either fungus, virtually all of the PCP decrease was due to conversion to PCA. The difference between sterile and nonsterile chips in the amount of PCP loss due to methylation to PCA suggests that the indigenous microbes inhabiting the nonsterile chips, which were not able to metabolize PCP, were able to metabolize PCA and thus prevent its accumulation.

Inoculation of PCP-contaminated softwood chips with the lignin-degrading fungus *Trametes hirsuta* also resulted in a rapid and extensive removal of PCP (Figure 1). Approximately 62 percent of the PCP was removed after 28 days of incubation. This is similar to and greater than the amount of PCP removed by *P. chrysosporium* and *P. sordida*, respectively, after the same incubation time. However, the removal was not due to conversion of PCP to PCA. Since no PCA accumulated in cultures of this fungus, we are in the process of determining the fate of PCP in soils supporting growth of *Trametes hirsuta* to determine the ability of this organism to transform PCP in soils to innocuous products.

The results of these and other studies have shown that lignin-degrading fungi can effect rapid and extensive depletion of PCP from soils and other contaminated media (i.e., wood chips), and that there is a great diversity among fungal species in their ability to effect decreases of PCP and in their metabolism of PCP. Further studies are needed to confirm the incorporation of PCP into humic materials in soils, to assess the stability of these hybrid polymers, and to continue the screening process to identify fungi with superior PCP-degrading capabilities.

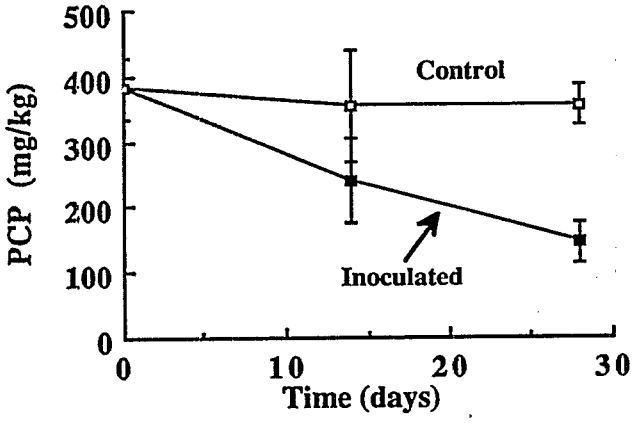

Figure 1. Effect of inoculation with lignin-degrading fungus *Trametes hirsuta* on the PCP concentration of PCP-contaminated softwood chips.

ANAEROBIC DEGRADATION OF CHLORINATED AROMATIC COMPOUNDS

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Over the last few years, our laboratory has been investigating the anaerobic degradation of a variety of nitrogen heterocyclic and chlorinated aromatic compounds. These compounds have included pentachlorophenol, 2,4-D, 2,4,5-T, DDT and chlorinated anisoles, anilines, benzenes, benzoic acids, biphenyls, and phenols. In 1991, we reported on the reductive dechlorination of pentachlorophenol by sediment microbial communities acclimated to dechlorinate 2,4- and 3,4-dichlorophenol (DCP). We report this year on the degradation of 2,4-D, 2,4,5-T, DDT, hexachlorobenzene, and chloroanisoles.

The ability of unacclimated sediment microorganisms and sediment microorganisms acclimated to dechlorinate 2,4- and 3,4- (DCP) to degrade 2,4-D and 2,4,5-T was investigated. Acclimated sediment microorganisms were prepared from sediment collected in November 1987 and March 1990. When the experiments were conducted using sediments from November 1987, the 2,4-DCP acclimated microorganisms dechlorinated 2,4-D without a lag to 4-chlorophenoxyacetic acid, the 3,4-DCP acclimated microorganisms did not dechlorinate 2,4-D over several months of exposure, a mixture of the two acclimated microbial populations paralleled the 2,4-DCP acclimated microorganisms, and the unacclimated sediment microorganisms paralleled the 3,4-DCP acclimated microorganisms. The 4-chlorophenoxyacetic acid was produced in stoichiometric quantities and was stable for the duration of the experiment. Similar results were observed with sediments collected in March 1990; however, 4-chlorophenoxyacetic acid was readily degraded in the 2,4-DCP acclimated sediment. 2,4,5-T was degraded at the same rate with acclimated sediment microorganisms and unacclimated sediment microorganisms; no degradation intermediates were detected and dechlorination followed a lengthy lag period (>14 days).

Previous studies in our laboratory (1) have shown that sediment microorganisms acclimated to dechlorinate 2,4- or 3,4-DCP could dechlorinate the respective chlorinated anilines but not the respective chlorinated benzoic acids. A partial explanation for the dechlorinating specificity was that the phenol and aniline are *ortho/para* directing, whereas benzoic acid is *meta* directing. To further test this hypothesis we have examined the dechlorination of chlorinated anisoles. Because of the similarity of the sigma values for the methoxy substituent of anisole and the hydroxy and amine substituents of phenol and aniline, the methoxy substituent should also be *ortho/para* directing for dechlorination. Therefore, we have examined the degradation of 2,4-dichloroanisole (DCAn) by the 2,4- and 3,4-DCP acclimated sediment microorganisms (March 1990). When added to unacclimated sediment microorganisms, 2,4-DCAn was slowly demethylated (50 percent in 24 days) to 2,4-DCP following a 5-day lag period. The 2,4-DCP was subsequently degraded over the next 2 weeks. Following a second addition of 2,4-DCAn, a 50 percent loss of 2,4-DCAn was observed in 2 days; however, the product observed was 4-chloroanisole. When 2,4-DCAn was added to an equal mixture of 2,4- and 3,4-DCP acclimated sediment microorganisms or to separate 2,4-and 3,4-DCP acclimated sediment microbial communities, greater than 50 percent of the 2,4-DCAn was lost in 2 days in all cases. The only products identified were 4-chlorophenol and phenol. Unfortunately, we were unable to determine whether dechlorination and demethylation occurred concomitantly or sequentially. The finding that 4-chloroanisole was the primary product from the second addition of 2,4-DCAn, however, is indicative of rapid reductive dechlorination of 2,4-DCAn by a microbial community acclimated to dechlorinate 2,4-DCP.

To date, we have investigated the reductive dechlorination of several compounds having some structural resemblance to the acclimating substrates. Recently we have investigated the ability of acclimated sediment microorganisms to degrade compounds with little structural resemblance. DDT added to unacclimated sediment microorganisms, both autoclaved and nonautoclaved, was rapidly converted to DDE within 4 days. Similar results were observed for DDT added to 2,4- and 3,4-DCP acclimated sediment microorganisms, both autoclaved and nonautoclaved. However, 4-chlorophenol was observed as a significant product with acclimated microorganisms (autoclaved and nonautoclaved) but not with unacclimated microorganisms. The mechanism of 4-chlorophenol formation is currently under investigation. Hexachloro- benzene (HCB) was investigated in sediments collected from two different ponds. In both cases, a loss of HCB (70 ppm) was observed after a substantial lag phase (90 to 100 days). Less than 20 percent of the HB remained after 170 days of incubation. Mixtures of lesser chlorinated congeners were identified as intermediate degradation products. A different mixture was observed in each of the two sediments, and no one product dominated in either case. Hexachlorobenzene was stable in autoclaved sediment.

The reductive dechlorination of chlorinated aromatic compounds requires a source of reducing equivalents. Dolfing and Tiedje (2) have identified molecular hydrogen as a source of reducing equivalents for the dechlorination of 3-chlorobenzoate by an anaerobic consortium. Gibson and Suflita (3) and Nies and Vogel (4) have shown that organic substrates can also be the source of reducing equivalents for the reductive dechlorination of 2,4,5-T and PCBs. We are currently testing molecular hydrogen and a number of organic substrates as sources of reducing equivalents for the reductive dechlorination of 2,4- and 3,4-DCP by microorganisms in sediments slurries. In some cases, our results are different from previous reports. We found, for example, that changes in headspace gas composition resulted in the following order of increasing reductive dechlorination: $CO_2/N^2 > N^2 < H^2/N^2$. We also observed that propionate, formate, and butyrate, which have been shown to stimulate the degradation of 2,4,5-T, inhibited or did not enhance the reductive dechlorination of 2,4-DCP. In a parallel study, we have tested a number of complex organic substrates as possible sources of reducing equivalents. Addition of sterile sediment or a sediment extract supported reductive dechlorination of 2,4-DCP. Lake water collected from just above the sediment source only marginally supported 2,4-DCP degradation. Organic mixtures such as landfill leachate and rumen

fluid did not stimulate dechlorination of 2,4-DCP when added to sediments at low concentrations (1 to 2 percent) and inhibited activity at higher (15 to 20 percent) concentrations.

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INFLUENCE OF NONIONIC SURFACTANTS ON THE ANAEROBIC DECHLORINATION OF HEXACHLOROBENZENE

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Background

Surfactants can solubilize pollutants into micellar solution in the presence of soil or sediment solids, effectively desorbing them from these natural media (1,2). This phenomenon has been suggested as a possible tool to enhance the treatment of contaminated sediments or soils (3,4,5). The rationale is that surfactant micelles (or monomers or emulsions) will solubilize precipitated or sorbed compounds, making them more readily available for biological remediation, pump-and-treat operations, or soil washing operations.

The effectiveness of surfactants in removing contaminants from soils or sediments is largely a function of 1) the sorption reactions of pollutants to the sedimentary materials, 2) the solubilization of pollutants by the surfactant micelles (and/or monomers), and 3) the interactions of surfactant monomers and micelles with sediment or soil components. These processes have recently been examined in several freshwater sediments spiked with various polycyclic aromatic hydrocarbons (PAHs) and the anionic surfactant sodium dodecylsulfate (SDS) (1, 2). Also, we have recently examined the solubilization of PAHs and other compounds in solutions of various nonionic surfactants, including Brij 35, Tween 80, and Tween 20, as well as observing some of the interactions of these surfactants with sediment solids.

The structures of Tween 80 and Brij 35 are shown in Figure 1. Unlike SDS, which can be purchased in pure form (>99 percent), these surfactants are homolog mixtures of differing ethoxy chain lengths. Also, unlike SDS, whose primary interaction with sediment components under conditions of interest is the precipitation of its calcium salt, nonionic surfactants sorb to sediment solids—possibly through both hydrophobic and hydrophilic mechanisms. At surfactant concentrations around their critical micelle concentration (cmc), and at sediment concentrations of 5 to 10 percent, a large portion (>95 percent) of the surfactant is sorbed. However, because of their lower toxicity to microorganisms (relative to anionic surfactants), we chose to examine the effects of enhanced solubilization by these nonionic surfactants on microbially mediated transformation reactions of hydrophobic organic pollutants. Hexachlorobenzene (HCB) was chosen as a test compound, primarily because of its low water solubility, 5.0 μg/L (6) (i.e., high sediment-water partition coefficient), and because it is known to degrade relatively slowly in anaerobic sediments (7, 8).

Anaerobic Dechlorination of HCB

The dechlorination of low levels of hexachlorobenzene (2.8×10^{-3} mM) incubated in anaerobic pond sediments (7 percent solids) occurred after an initial lag period of 10 days at a rate of 5.2×10^{-5} mM/day. Fresh sediments inoculated with acclimated sediments dechlorinated HCB (2.2×10^{-3} mM) at a slightly faster rate of 6.5×10^{-5} mM/day with no initial lag phase. Initially, only one dechlorination pathway was observed with pentachlorobenzene (PCB) and 1,2,3,5-tetra chlorobenzene (1,2,3,5-TTCB) as intermediates, and 1,3,5-trichlorobenzene (1,3,5-TCB) as a possible end product. After 55 days, a second dechlorination pathway was observed with 1,2,4,5-TTCB and 1,2,4-TCB as intermediates, and all three dichlorobenzenes (DCBs) as possible end products. Both pathways have been observed during anaerobic incubation of fresh digester sludge (7). After 75 days, degradation of HCB is complete, leaving 1,3,5-TCB, 1,2,4,-TCB, and 1,3-DCB as the major products. In acclimated sediments, a shorter delay of 10 days occurs before onset of the second pathway.

With additions of Tween 80, the polyoxyethylene sorbitan monooleate surfactant, the aqueous phase concentration of HCB was increased by one to two orders of magnitude over that of controls, which were generally at half aqueous saturation (2 to 3 µg/L) in the sediment slurry. After 1 week, however, the surfactant was degraded, and the concentration of aqueous HCB decreased to control levels. Initially, the rates of HCB dechlorination and product formation were similar to that of controls for low levels of Tween 80 (1,500 mg/L). After 40 days, however, dechlorination ceased. As Tween 80 concentrations were increased to 5,000 mg/L, initial rates of dechlorination decreased to almost imperceptible levels. In addition, there was no evidence of the second pathway.

In acclimated sediments exposed to low levels of Tween 80 (900 and 1,200 mg/L), rates of HCB dechlorination are similar to those of controls. At even lower levels of Tween 80 (300 and 600 mg/L), dechlorination rates are slightly faster. Both pathways are evident in these acclimated sediments.

Contrasting the influence of Tween 80 on HCB dechlorination is the effect of Brij 35, a polyoxyethylene alcohol surfactant. HCB dechlorination is completely suppressed in acclimated sediment slurries in the presence of low levels of Brij 35 (1,000 mg/L). In fresh sediment slurries, the surfactant addition results in longer lag periods. Again, the surfactant appears to be readily degraded, thus preventing aqueous solubility enhancement effects from being observed. Currently, another alcohol surfactant, Brij 30 [$C_{12}H_{25}O(CH_2CH_2O)_4H$], is being tested and shows no signs of being degraded, itself, to date (after 4 weeks).

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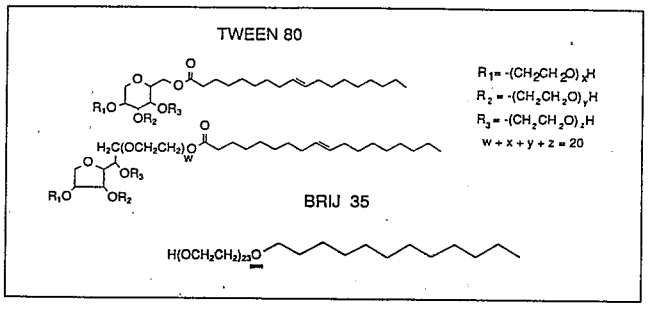

Figure 1. Tween 80 and Brij 35 structures.

ANAEROBIC DEGRADATION OF CHLOROAROMATIC COMPOUNDS UNDER DIFFERENT REDUCING CONDITIONS

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Chlorophenol Degradation under Methanogenic Conditions

If only dechlorination of a chloroaromatic substrate is taking place and the aromatic ring remains intact, no carbon is provided for microbial growth. In the environment, carbon sources in addition to a chloroaromatic are likely to be available, which may affect the metabolism of the chloroaromatic. In order to examine this, sediment enrichment cultures were set up with 2,4-dichlorophenol and 4-chlorophenol under methanogenic condition with and without the addition of a supplementary carbon source. Propionate was chosen as a readily utilizable carbon source, and *para*-cresol was used as a structurally similar nonchlorinated substrate.

2,4-Dichlorophenol was dechlorinated to 4-chlorophenol without a lag in 25 to 60 days in both the presence and absence of an auxiliary carbon source. It took approximately 50 days before the onset of 4-chlorophenol degradation. Degradation of 4-chlorophenol was stimulated by either *p*-cresol or propionate. In cultures without an auxiliary carbon source, 4-chlorophenol persisted. By repeated feedings of the chlorophenols and auxiliary substrates, the degradation rates were significantly enhanced. After 6 feedings of *p*-cresol and chlorophenol over a period of 450 days, the rate of 2,4-dichlorophenol and 4-chlorophenol degradation was enhanced over 10-fold to 40 and 10 μmol liter^{-1}day^{-1}, respectively.

Dechlorination at the *ortho*-position could be sustained, and by repeated dilution into fresh medium and refeeding, a stable microbial enrichment culture free of sediment, which degraded 2,6-dichlorophenol, was established. 2,6-Dichlorophenol was sequentially dechlorinated to 2-chlorophenol and phenol and ultimately mineralized to CH_4 and CO_2. On the other hand, the ability to degrade 4-chlorophenol was lost by transferring the culture into fresh medium, but, by refeeding, the 4-chlorophenol degrading culture could be maintained.

Cultures adapted to 2,4- and 2,6-dichlorophenol readily dechlorinated other dichlorophenols containing an *ortho*-chlorine. Dechlorination of 2,3- and 2,5-dichlorophenol yielded 3-chlorophenol. Dichlorophenols with no *ortho*-chlorines persisted. 2,3,6-Trichlorophenol was dechlorinated at the *ortho*-position yielding first 2,3- and 2,5-dichlorophenol, and then 3-chlorophenol. Similarly, 2,4,6-trichlorophenol was sequentially dechlorinated to 2,4-dichlorophenol and 4-chlorophenol. This preferential removal of *ortho*-chlorines, with *meta*- or *para*-chlorines removed at slower rates, appears to be characteristic for methanogenic cultures.

Chlorophenol Degradation under Sulfidogenic Conditions

Degradation of chlorophenols under sulfate-reducing conditions was studied with an estuarine sediment inoculum (East River). After an initial lag period of approximately 50 to 100 days, 2-, 3- and 4-chlorophenol and 2,4-dichlorophenol (0.1 mM) were completely removed in 120 to 220 days. 4-Chlorophenol was detected as a transient metabolite of 2,4-dichlorophenol, but no metabolites of the monochlorophenols were detected. The rate of chlorophenol degradation was greatly enhanced after repeated refeeding of the substrate to the sediment cultures. In acclimated cultures the monochlorophenols (0.16 mM) were degraded in 6 to 20 days, corresponding to rates of 8 to 40 μmol-liter^{-1}day^{-1}, which are similar to the degradation rates in methanogenic cultures. The relative rates of degradation were 4-chlorophenol > 3-chlorophenol > 2-chlorophenol, 2,4-dichlorophenol. No degradation of chlorophenols was observed in sterile controls.

During degradation of all three monochlorophenol isomers in the sediment cultures, there was a concomitant loss of sulfate, corresponding to the stoichiometric values expected for complete oxidation of the chlorophenol to CO_2, according to the following equation:

$$C_6H_5ClO + 3.25\ SO_4^{2-} + 4\ H_2O \rightarrow 6\ HCO_3^- + 3.25\ H_2S + Cl^- + 0.5\ H^+$$

Formation of sulfide was confirmed with 4-chlorophenols using a radiotracer technique. No methane was produced in the cultures, verifying that sulfate reduction was the main electron sink. Addition of molybdate, a specific inhibitor of sulfate reduction, inhibited chlorophenol degradation completely. These results indicate that chlorophenols can be mineralized under sulfidogenic conditions and that oxidation of the chlorophenol is coupled to sulfate reduction.

The sulfidogenic cultures were propagated by repeated refeeding of chlorophenols and dilution into fresh media, and are able to utilize the chlorophenol as a source of carbon and energy. This is the opposite of what was observed under methanogenic conditions, where 4-chlorophenol degrading cultures could not be subcultured without loss of activity. The sulfidogenic cultures were very specific and only degraded the monochlorophenol isomer to which they were acclimated. However, all the cultures rapidly degraded phenol, suggesting that it may be an intermediate in chlorophenol degradation.

Degradation of Chlorinated Phenols and Benzoic Acids under Three Reducing Conditions

Anaerobic enrichment cultures, under methanogenic, sulfidogenic, and denitrifying conditions were established on each of the three monochlorophenol and monochlorobenzoate isomers with Hudson River sediment from two different sites (HR1, HR2). In addition, denitrifying cultures were also established on the same compounds with East River sediment. Initial results monitoring substrate loss indicated that all three monochlorophenols and 3-chlorobenzoate were degraded under methanogenic conditions in HR1 cultures. Sulfidogenic HR1 cultures were active against all three monochlorophenols and 3- and 4-chlorobenzoate. Transient accumulation of phenol was detected in some of the chlorophenol-amended cultures under both methanogenic and sulfidogenic conditions, indicating that reductive dechlorination is taking place. HR2 cultures, in general, showed the same activity but at a much slower rate. The rate of degradation was enhanced with refeeding of the substrates. Under denitrifying conditions, degradation of 3- and 4-chlorobenzoate and 2-chlorophenol was observed in HR1 sediment cultures. These results indicate that degradation of these chlorinated aromatic compounds can take place under more than one reducing condition.

PARA-HYDROXYBENZOATE AS AN INTERMEDIATE IN THE ANAEROBIC TRANSFORMATION OF PHENOL TO BENZOATE

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Summary

Anaerobic transformation of phenol was studied using a bacterial consortium which transformed phenol to benzoate without complete mineralization of benzoate. Products of monofluorophenol transformation indicated *para*-carboxylation. Phenol and benzoate were detected during *para*-hydroxybenzoate (*p*-OHB) degradation. *p*-OHB was detected in phenol-transforming cultures containing 6-hydroxynicotinic acid (6-OHNA), a structural analogue of *p*-OHB, or at elevated initial concentrations of phenol (≥5 mM), or benzoate (≥10 mM).

Results and Discussion

The original phenol-degrading consortium (2) first transformed phenol to benzoate followed by complete mineralization of benzoate. Subculture B-1 transformed all of the phenol to benzoate, but failed to completely mineralize the resulting benzoate.

Subculture B-1 stoichiometrically transformed 2-fluorophenol (2FP) to 3-fluorobenzoate (3FB) in the presence or absence of phenol. The rate of 3FB formation (12.9 moles·l^{-1}·d^{-1}) was similar to the rate of 2FP decline (13.5 moles·l^{-1}·d^{-1}). In the presence of phenol, a small amount (3 percent) of 3-fluorophenol was transformed to 2-fluorobenzoate. 4-Fluorophenol was not transformed in the presence or absence of phenol. Neither 2-, 3-, nor 4-fluorobenzoate was used as an energy source. These results are the same as those obtained with the original phenol consortium (3). Thus, transformation was via *para*-carboxylation as previously concluded for the original phenol consortium.

para-Carboxylation of phenol implies the formation of *p*-OHB as an intermediate of transformation. Therefore, we examined the degradation of *p*-OHB by both the original phenol consortium and subculture B-1. After 3 days, *p*-OHB was completely degraded in both cultures. Phenol was the only compound detected during *p*-OHB degradation by the original phenol consortium (Figure 1a). By contrast, both phenol and benzoate were detected in subculture B-1 (Figure 1b). After 2 weeks, phenol was no longer detected in either consortium, but 900 _M benzoate was present in subculture B-1. Thus, the original phenol consortium had completely degraded the phenol formed via decarboxylation of *p*-OHB, whereas subculture B-1 had subsequently transformed phenol benzoate. Failure to detect benzoate during *p*-OHB degradation by the original phenol consortium may be a result of rapid benzoate turnover. The detection of benzoate during *p*-OHB degradation by subculture B-1 may be the result of diluting out a bacterial species essential for complete benzoate degradation.

p-OHB was not detected in the original phenol consortium, but small amounts (2 uM) of a compound with the retention time of *p*-OHB were detected in subculture B-1 during transformation of phenol. This suggested that benzoate accumulation inhibited *p*-OHB breakdown. Consequently, studies were devised to enhance *p*-OHB formation using analogue-, product-, and substrate-inhibition. The highest concentrations of *p*-OHB (14 to 43≥M) were detected in the original phenol consortium (data not shown) and in subculture B-1 at initial phenol concentrations ≥5 mM (Figure 2a). The intermediate was also detected (6 to 8 ≥M) in the presence of ≥100 ≥M 6-OHNA or ≥10 mM benzoate (Figures 2b and 2c, respectively).

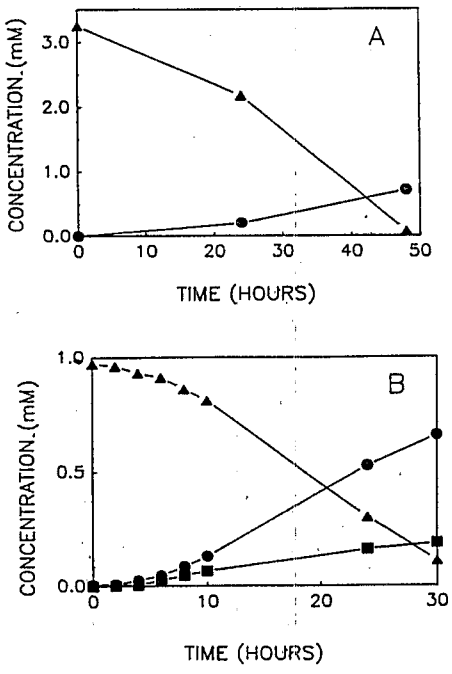

Figure 1. Degradation of *para*-hydroxybenzoate by the original phenol consortium (a) and subculture B-1 (b). Symbols: (●) phenol; (▲) *para*-hydroxybenzoate; (■) benzoate.

The intermediate in subculture B-1 grown with 10.5 mM phenol cochromatographed with authentic *p*-OHB under the two sets of separation parameters. Its UV spectrum matched that of authentic *p*-OHB. Its identity was confirmed by GC/MS analysis. The mass spectrum matched that of the TMS-derivative of authentic *p*-OHB with peaks at m/e 282 and m/e 267 (-CH_3). Neither 2- nor 3-hydroxybenzoate was detected during this analysis. Identifying *p*-OHB in cultures that were transforming phenol provided a direct indication of *para*-carboxylation. Since *p*-OHB was not detected in the original phenol consortium with initial phenol concentrations <5 mM, it is possible that *p*-OHB was not detected in other studies (1, 4, 5) because of the use of lower phenol concentrations (≥2 mM).

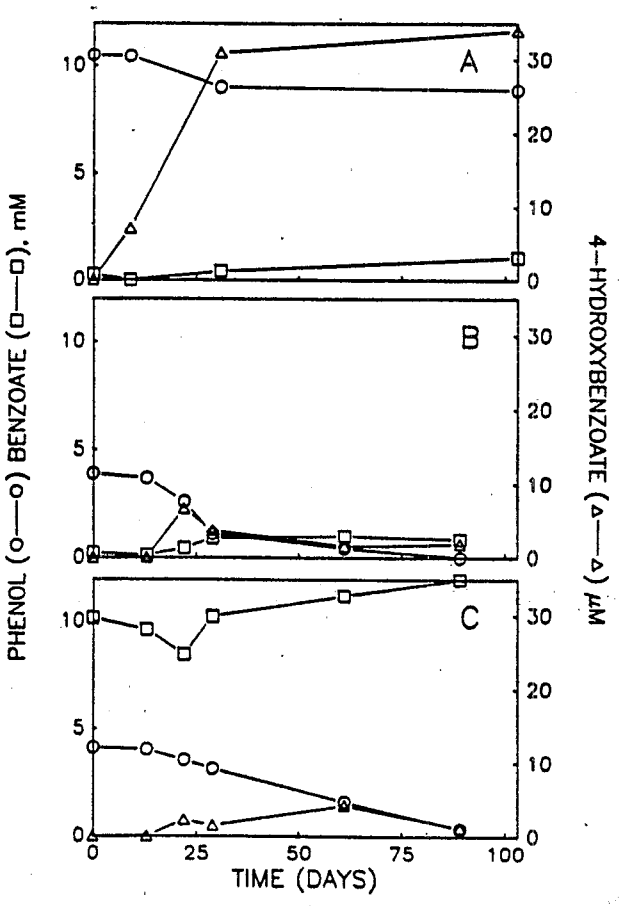

Figure 2. Detection of *para*-hydroxybenzoate (▲) as an intermediate in the transformation of phenol(●) to benzoate (■) in the presence of (a) 10 m M phenol, (b) 6-hydroxynicotinic acid (1 mM), or (c) 10 mM benzoate.

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AEROBIC DEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS

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Polycyclic aromatic hydrocarbons (PAHs) are chemicals possessing three or more aromatic rings found in complex mixtures in fossil fuels and their combustion products. Because a number of these chemicals are of concern to human health, their removal from contaminated sites is an important priority for remediation.

Many studies (reviewed in references 1, 2 and 3) have documented the abilities and mechanisms by which different axenic bacterial cultures degrade individual chemicals of this class, particularly those of lower molecular weight. However, the widespread occurrence of these chemicals as constituents of complex PAH mixtures, often together with chemicals of other types, requires an assessment of the abilities and conditions under which microorganisms can best effect degradation of mixtures of these chemicals, many of which have very limited aqueous solubility. Laboratory studies were undertaken to study the biodegradation of PAHs, as they occur in crude fossil fuels, and their products and wastes. In developing appropriate approaches and methods for studying biodegradation of mixed chemicals, it was anticipated that research would also lead to the isolation of PAH-degrading bacteria of value in other areas of biodegradation research. Initially, the neutral fraction of coal tar creosote, which contains all of the PAHs (Table 1) found in this lumber preservative (approximately 85 percent by weight of original creosote) together with a number of neutral oxygen- and sulfur-containing heterocycles, was chosen for study of its microbial degradation.

The plan of this study was to determine how readily various PAH constituents were degraded by aerobic bacterial cultures, which had been enriched by growth in a mineral salts medium containing the neutral creosote fraction as a carbon and energy source. It was anticipated that this

Table 1. Predominant polycyclic aromatic hydrocarbons in coal tar creosote*.

Compound	Coal Tar Creosote (% of Total PAHs (range)
Naphthalene	3.0 - 15.8
2-MeNaphthalene	2.1 - 14.2
1-MeNaphthalene	2.1 - 14.2
Biphenyl	2.3 - 2.8
2,6-DiMeNaphthalene	2.0 - 2.3
2,3-DiMeNaphthalene	2.0 - 2.4
Acenaphthene	4.1 - 9.0
Fluorene	9.6 - 10.0
Phenanthrene	4.6 - 21.0
Anthracene	1.5 - 2.0
2-Me Anthracene	0.5 - 2.6
Anthraquinone	0.1 - 1.0
Fluoranthene	6.8 - 10.4
Pyrene	2.2 - 8.5
2,3-Benzo[b]Fluorene	2.0 - 4.6
Chrysene	2.8 - 3.0
Benzo[a]Pyrene	0.1 - 1.0

**For sources see reference 6.*

approach would facilitate establishment of different cultures that could be isolated for their ability to utilize individual aromatic hydrocarbons and that could be used in defined mixed cultures to simulate the degradative performance of undefined enrichment cultures. In this way, contributions to the overall process of PAH degradation by individual axenic cultures, singly and in different combinations, could be assessed.

Phenols and basic N-heterocycles were removed from crude creosote by successive extraction of its methylene chloride solution with sodium hydroxide and sulfuric acid. After washing, drying, and solvent removal, the resulting creosote neutral fraction (CNF) was added (0.1%) to a mineral salts medium (4) also containing dimethyl sulfoxide (DMSO) at 0.02% to enhance the bioavailability of chemicals with low aqueous solubility. Soils from two contaminated lumber treatment sites (Live Oaks, Florida, and American Creosote Works, Florida) were used as sources of microorganisms. Mixed soil samples were used to inoculate shake-flask cultures (25°C, 200 rpm) for enrichment of CNF-degrading bacteria. Methylene chloride extraction of the entire flask contents after 14 days of incubation followed by capillary GC-FID analysis (5) of the concentrated extracts showed that, once established, sequential enrichment cultures effected significant and reproducible breakdown of a large proportion (70 percent) of CNF constituents, principally those having three rings or less. Compounds with four or more rings, e.g., pyrene or fluoranthene, were not significantly affected (Figure 1). By comparison, 14-day incubations of uninoculated flasks showed losses only of the more volatile constituents such as naphthalene. A number of axenic cultures were isolated from these enrichment cultures, and also directly from the site soils, by using individual aromatic hydrocarbons as selective substrates. Microorganisms able to utilize biphenyl, naphthalene, and phenanthrene were obtained from enrichment cultures while acenaphthene-, fluorene-, pyrene-, and fluoranthene-utilizing isolates were isolated directly from site soils. Organisms isolated from the enrichment cultures were shown to degrade their respective growth substrates when cultured in CNF-mineral salts media. By contrast, organisms obtained directly from soil were generally devoid of action towards their growth substrates when added to CNF-medium either singly or in combination with others. By systematically assembling defined mixtures of isolates, it was shown that the performance of the undefined enrichment cultures could be simulated by a five-member mixed culture (Figure 2). Attempts to construct defined cultures with fewer microbial members resulted in more limited degradation of aromatic hydrocarbons, such as acenaphthene, whether the organisms omitted were biphenyl-, naphthalene-, or phenanthrene-utilizers. These and other observations suggest that in mixed cultures, cooxidation may be an important mechanism of degradation of certain constituents of CNF and that consequently the rates of degradation of such chemicals will depend upon the numbers and activities of cells whose growth is supported by other chemicals.

Evaluating the extent to which enrichment and defined cultures employ cooxidation processes and accumulate cooxidation products is continuing, as is work to establish and define new biological systems that degrade the more recalcitrant four- and five-ring PAHs and higher molecular weight members of this series.

Acknowledgements

Technical assistance was provided by Walter Randall and Sol Resnick, Technical Resources, Inc. (TRI). Maureen Downey and Beat Blattman (TRI) assisted with chromatographic analyses, as did Suzanne Lantz who together with Jim Mueller (both of SBP Technologies, Inc.), provided valuable discussion and insights.

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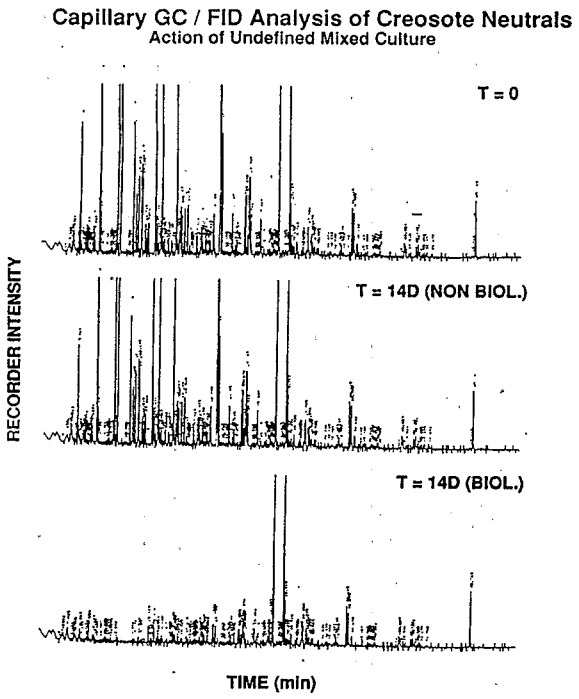

Figure 1. Action of undefined enrichment culture on creosote neutrals after 14 days of incubation (lower trace) compared to starting material (upper trace) and 14 days incubation without incubation (middle trace).

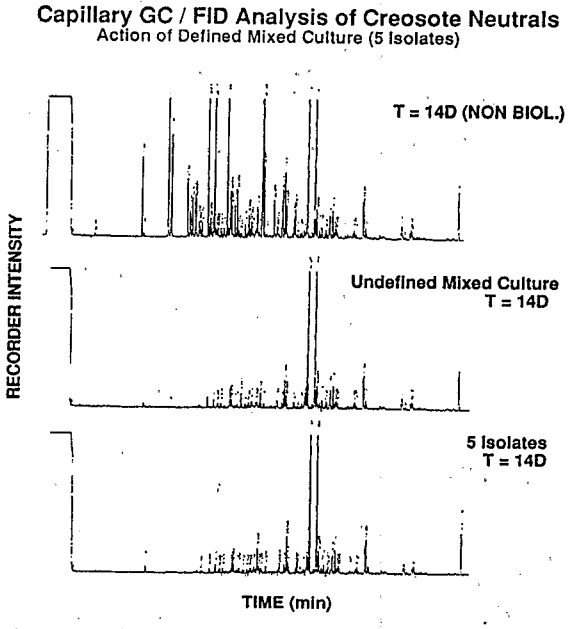

Figure 2. Action of defined mixed culture (5 organisms) on creosote neutrals after 14 days (lower trace) compared with 14 day incubation of undefined enrichment culture (middle trace) and starting material (upper trace).

SECTION FOUR
COMBINED TREATMENT

Most hazardous waste sites contain complex mixtures of persistent organic and inorganic contaminants that can be cleaned up only by a combination of treatment techniques. Researchers are developing methods to combine various physical, chemical, and biological treatment technologies, and comparing the effectiveness of the various combinations. For example, a chemical treatment—adding potassium polyethylene glycol (KPEG) to the soil at a site —may be used to dechlorinate PCBs. Then, biological treatment, which is more effective after dechlorination, can be used to complete the soil restoration. In one project, researchers are studying the degradation of KPEG-treated PCBs in both aerobic and anaerobic primary enrichment cultures. Current data support the possibility of biodegradation of PCB-KPEG products as part of a combined chemical/biological treatment of PCBs, at least under aerobic conditions.

Combined treatment is also being examined as an alternative to conventional treatment methods for the destruction of volatile organic compounds (VOCs). Researchers are studying the effectiveness of fixed-film anaerobic biological processes for treating and decontaminating leachates containing synthetic organic chemicals (SOCs). This process can result in less air stripping of VOCs than with aerobic processes. Two types of anaerobic pretreatment processes are being evaluated: an upflow anaerobic filter reactor and a GAC anaerobic fluidized bed reactor.

Researchers also conducted bench-scale tests of solid-phase (land treatment) and slurry-phase bioremediation of pentachlorophenol- and creosote-contaminated sediment and soil. The data indicate that slurry-phase bioremediation can be used effectively to treat materials contaminated with creosote and possibly PCP. Slurry-phase biotreatment technologies have been integrated into a multi-phasic treatment process, currently under evaluation at the pilot-scale level.

BACTERIAL DEGRADATION OF KPEG-MODIFIED PCBS IN ANAEROBIC AND AEROBIC ENRICHMENT CULTURES

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In the 1960s and 70s it became apparent that polychlorinated biphenyls (PCBs) were recalcitrant to biological degradation, and were accumulating in organisms throughout the food chain. Subsequently, PCBs were phased out of use; but now it is estimated that at least a half million tons of PCB currently exists in landfills and closed systems awaiting detoxification (1). Destruction is accomplished by transport of contaminated materials to incinerators. An alternative method is treatment of contaminated materials with a formulation of KOH and polyethylene glycol 400 (KPEG). KPEG attacks chlorine-bearing carbons, resulting in the formation of chloroarylpolyglycols (2,3,7). These compounds resemble PEG-based nonionic surfactants, and it appeared probable that PCB reacting with KPEG would be rendered soluble in water. Considerations such as these led to the suggestion that KPEG treatment coupled with biological degradation could be an alternative method of PCB disposal. We have been testing the feasibility of this idea by studying metabolism of a KPEG-treated PCB congener in both aerobic and anaerobic primary enrichment cultures.

Reaction of KPEG with a polychlorinated biphenyl may yield numerous products, since nucleophilic attack of KPEG can occur at several different chlorine-bearing carbons and result in a mixture of chloroarylpolyglycols with different degrees of PEG substitution, as well as small amounts

of chlorobiphenylols (2, 3). Therefore, we elected to carry out our studies using uniformly ^{14}C-labeled 2,2',4,4',5,5' hexachlorobiphenyl (^{14}C-HCBP) in order to facilitate identification of the products of the initial chemical reaction, as well as biodegradation products. This congener is 8.2 percent of Arochlor 1260 and 3.3 percent of Arochlor 1254 (4), and has been identified as a major congener found in both human adipose tissue (5) and milk (6).

KPEG was formulated as described by Kornell and Rogers (7) except that 1.3 mol of KOH was dissolved with heating into 1.0 mol of PEG 400. The mixture added to ^{14}C-HCBP absorbed to glass at a ratio of 100 mg congener to 1 mL KPEG. The reaction was incubated at 84°C and quenched by addition of water. Figure 1 details a timecourse of the reaction which demonstrates that KPEG effects a rapid phase transfer of ^{14}C-HCBP.

After the reaction had proceeded for 60 minutes, 95 percent of recovered radioactivity had been converted to a form that remained in the aqueous phase following repeated extractions with hexane. The products formed from ^{14}C-HCBP after a 48-hr reaction with KPEG remained soluble in water. In control experiments where ^{14}C-HCBP was incubated with PEG, rather than KPEG, phase transfer was not observed and the congener remained hexane soluble. This experiment indicated that KPEG reaction did convert HCBP to a water-soluble form, presumably the chloroarylpolyglycols first described by Brunelle and Singleton (3). Reverse-phase HPLC analysis (Figure 2) using an instrument equipped with an on-line radioactivity detector demonstrated increasing product complexity with time. Two major water -soluble products were formed within minutes; at later times, these were converted to forms which eluted relatively quickly from the reverse- phase column. These products accumulated and, with a 12-hr reaction time, six products from the aqueous phase could be resolved. These may represent chloroarylpolyglycols, with higher degrees of PEG substitution. On the basis of these experiments, a reaction time of 1 hour was chosen in order to minimize product homogeneity in the subsequent biodegradation studies.

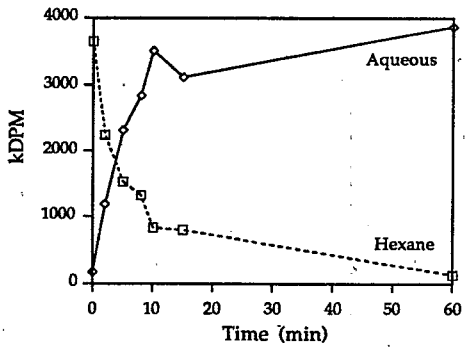

Figure 1. Phase transfer of HCBP during reaction with KPEG. Vials containing 100 mg of ^{14}C-HCBP (20 mCi/mg) and 1 mL of KPEG reagentwere incubated at 84°C before the reaction was quenched with water at the indicated times.

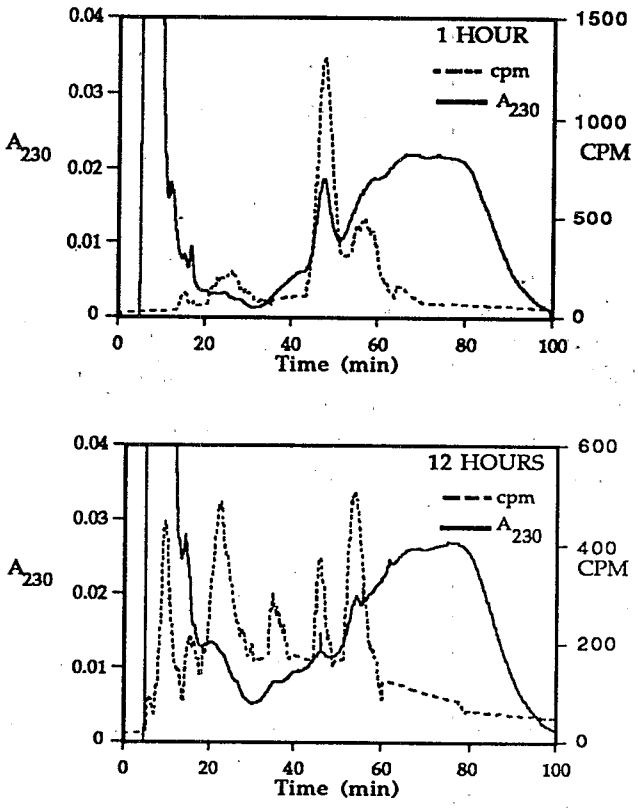

Figure 2. ^{14}C-HCBP/KPEG products after 1 and 12 hours of reaction time analyzed on a silica-based C-18 HPLC column eluted at 0.35 mL/min with a 30 to 50 percent isopropanol gradient.

Biodegradation experiments were initiated with anaerobic sewage sludge taken from the Jackson Pike municipal waste treatment plant (Columbus, OH). ^{14}C-arylpolyglycols were incubated with either undiluted sludge or primary enrichment cultures. Enrichment cultures were made using a mineral medium and incubated under a nitrogen headspace. The KPEG/^{14}C-HCBP reaction mixture was neutralized with HCL and added to cultures to final concentrations of 0.2 mM total arylpolyglycols and 25 mM PEG. Enrichments were set up in duplicate under methanogenic (no additions), sulfidogenic (15 mM Na_2SO_4), iron-reducing (0.1 percent amorphous iron oxide), or denitrifying (20 mM KNO_3) conditions. Controls were established for each condition investigated by inoculating cultures with autoclaved sludge. The fate of the labeled arylpolyglycols was followed by periodic ethyl acetate extraction of aliquots from each culture. Both arylpolyglycols and unreacted ^{14}C-HCBP could be extracted using this solvent. The ethyl acetate extract was then evaporated, and partitioning of radioactivity in the residue between hexane and water determined. Figure 3 shows the average results obtained under methanogenic conditions for active cultures and controls. At the start of incubation, 80 percent of expected counts could be recovered in the ethyl acetate fraction, 19 percent was left in the culture supernatant. Of radioactivity in the ethyl acetate fraction, only 4 percent was soluble in hexane, the remainder was in the aqueous fraction. After incubation for 28 days at 37°C, 71 percent of expected counts could be recovered by ethyl acetate extraction of the cultures. However, 60 percent of radioactivity in the ethyl acetate fraction was now soluble in hexane; the remaining radioactivity was still soluble in water. In contrast, killed controls showed no change in the phase distribution of label. Similar results were obtained with enrichment cultures established under sulfate, nitrate, or iron-reducing conditions, as well as with ^{14}C-chlorobiphenylpolyglycols incubated in undiluted sludge. These results indicated that in this anaerobic sludge, bacteria were capable of converting chlorobiphenylpolyglycols into a water-insoluble form. A possible mechanism is hydrolysis of the PEG moiety from the aromatic ring, leaving a relatively insoluble polychlorobiphenylol. An analogous reaction has been documented with nonoxynol (Triton N), a PEG-based nonionic surfactant (8). Biodegradation in sewage sludge resulted in cleavage of the PEG moiety from nonoxynol and liberation of the recalcitrant, hydrophobic compound, nonylphenol.

Biodegradation of the labeled arylpolyglycols was also tested in aerobic primary enrichment cultures. Metabolism of the compounds did occur; however, unlike anaerobic cultures, the products remained water soluble. Aerobic primary enrichments were established in triplicate sealed 160 mL vials which were periodically replenished with oxygen. Each culture received 0.1 mM labeled arylpolygycol and 12 mM PEG. Duplicate enrichment cultures were also established with autoclaved inocula as controls. Results are illustrated in Figure

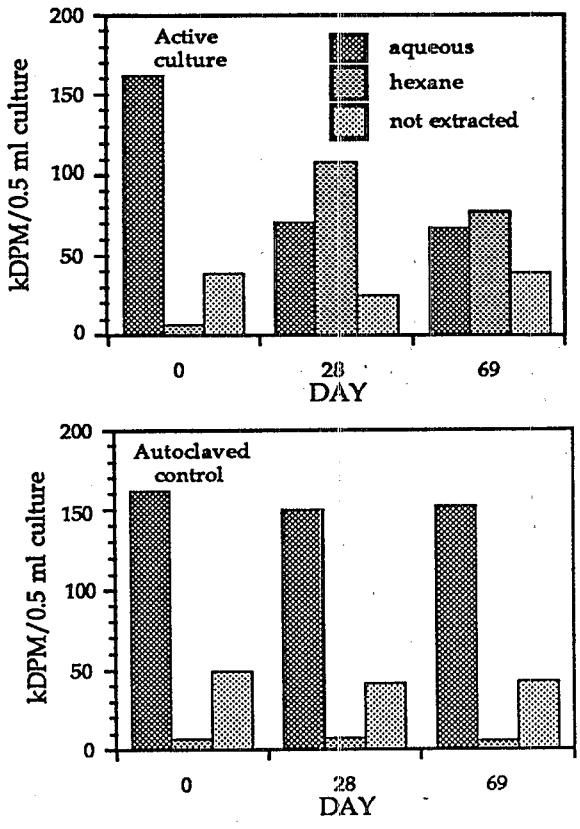

Figure 3. Phase transfer of ^{14}C-HCBP/KPEG reaction products during incubation in methanogenic enrichment cultures. The partitioning of extracted radioactivity from 0.5 mL of culture between hexane and water is shown. The amount of radioactivity not extracted from the culture by ethyl acetate is also indicated.

4. At the start of incubation, an average of 66 percent of total radioactivity added to active cultures could be recovered by ethyl acetate extraction, while 16 percent was not extractable and remained in the culture supernatant. In the ethyl acetate fraction, 90 percent of radioactivity was water soluble. After 41 days of incubation, only 19 percent of radioactivity added to the culture could be recovered by ethyl acetate extraction, while 57 percent was not removed from the cultures after repeated extraction with ethyl acetate. The amount of radioactivity that was soluble in both ethyl acetate and hexane did not increase. This indicated that aerobic biodegradation of the labeled arylpolyglycols had resulted in products that were soluble in water but no longer soluble in ethyl acetate.

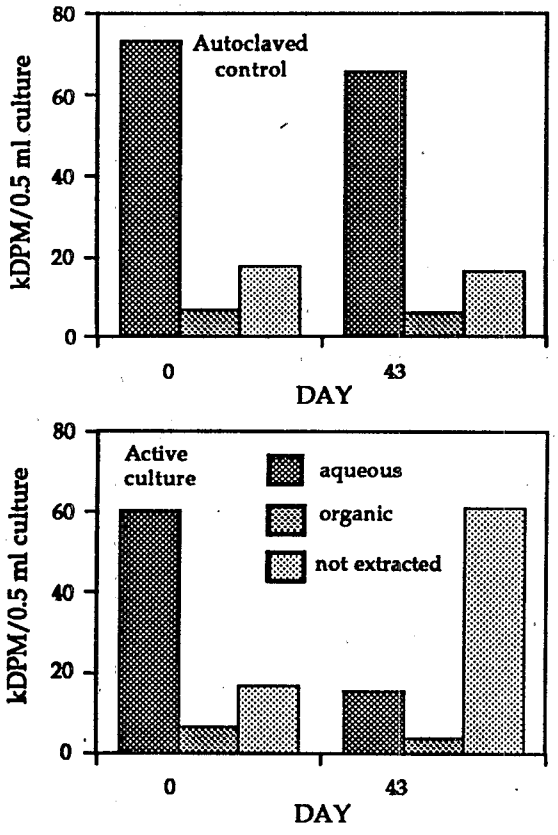

Figure 4. Phase partitioning between hexane and water of ^{14}C-HCBP/KPEG reaction products extractable by ethyl acetate after incubation for 43 days in aerobic enrichment cultures and killed controls. "Not extracted" refers to radioactivity left in the culture sample after four sequential 1 mL extractions with ethyl acetate.

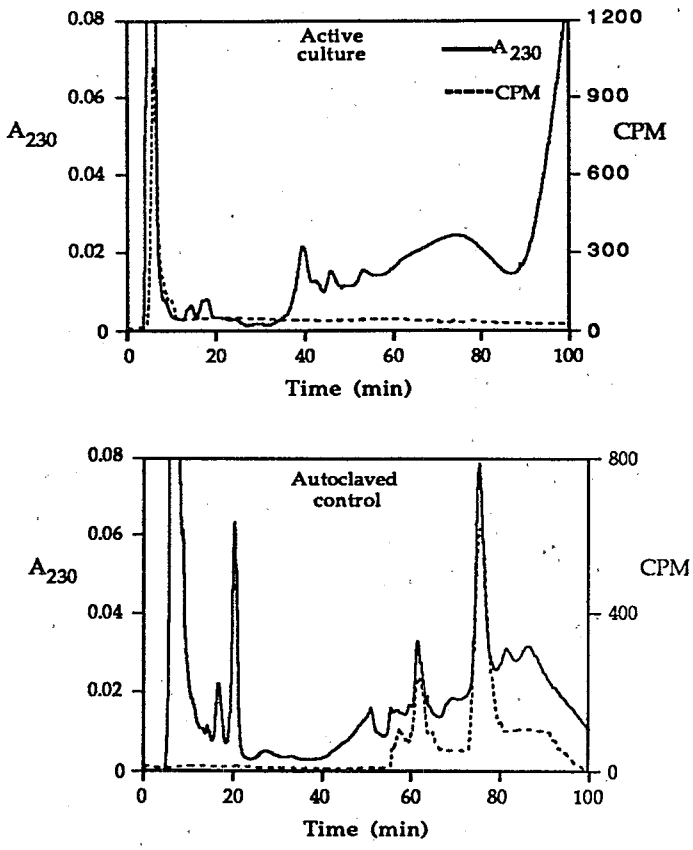

Figure 5. Reverse phase HPLC of aerobic culture supernatants after 41 days of incubation. The column was eluted with a 30 to 50 percent isopropanol gradient at 0.25 mL/min.

Reverse-phase HPLC analysis of the aqueous supernatant of cultures (Figure 5) confirmed that metabolism of the products occurred. Killed controls had radioactive HPLC elution profiles similar to those of the starting material; however, the supernatant of active cultures no longer contained any of the KPEG/^{14}C-HCBP reaction products. Instead, nearly all the radioactivity present in the sample eluted immediately from the column. Using a different HPLC methodology, we have identified two major radioactive peaks with absorbance at 230 nm, indicating the breakdown products still contain an aromatic ring. Currently, we are determining if these compounds are end products or transient intermediates. They may represent products in which a PEG-substituted aromatic ring has been cleaved following hydrolysis of the PEG moiety from the ring, resulting in a chlorinated aromatic carboxylic acid.

In summary, we have examined both aerobic and anaerobic degradation of the arylpolyglycols produced by KPEG reaction with 2,2',4,4',5,5' hexachlorobiphenyl in enrichment cultures established with inocula from a municipal waste treatment plant. Under anaerobic conditions, the PEG moiety was apparently cleaved from the chloroarylpolyglycols, resulting in hydrophobic products. In contrast, aerobic enrichments carried out degradation of the ethyl acetate and water- soluble chloroarylpolyglycols into two water- soluble, but ethyl acetate insoluble, aromatic compounds; possibly chlorinated arylcarboxylic acids. Our data support the possibility of biodegradation of PCB/KPEG products as part of a combined chemical/biological treatment of PCBs. However, the process may be feasible under only aerobic, and not anaerobic, conditions.

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ONSITE BIOLOGICAL PRETREATMENT FOLLOWED BY POTW TREATMENT OF CERCLA LEACHATES

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The objective of this research is to assess the effectiveness of fixed-film anaerobic biological processes in treating and decontaminating leachates containing synthetic organic chemicals (SOCs) that may be regulated under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA). This is an attractive proposition because anaerobic processes result in less air stripping of volatile organic compounds compared to aerobic processes due to their lower gas production rates. Anaerobic pretreatment processes are also expected to reduce problems associated with incomplete degradation of chlorinated compounds as well as pass-through of semivolatile organic compounds, which can occur when CERCLA leachates are discharged without any pretreatment to publicly owned treatment works (POTWs). Two types of anaerobic pretreatment processes are being evaluated in this study: an upflow anaerobic filter reactor and a granular activated carbon (GAC) anaerobic fluidized-bed reactor. The tests are being conducted at U.S. EPA's Test and Evaluation (T&E) Facility in Cincinnati, Ohio.

Leachate Characteristics

The leachate for the experiments is obtained from a large commercial municipal landfill in Georgetown, Ohio. The leachate is highly variable in composition (chemical oxygen demand [COD] levels ranging from 300 to 2,500 mg/L) with low to moderate levels of biodegradability. Sulfate concentrations in the leachate range from 3 to 300 mg/L. Due to its relatively low biodegradable content during the first 6 months of the project, the leachate was supplemented with a mixture of acetic,

propionic, and butyric acids to increase its total COD to approximately 1,600 mg/L. Later, the total COD of the leachate increased significantly to a maximum of 2,500 mg/L, at which time volatile acids addition was discontinued. The leachate was fed without any addition of volatile acids during the remainder of the project. During most of the latter part of the project, the total COD of the raw leachate remained relatively stable, ranging from 400 to 1,000 mg/L. The leachate is rendered hazardous by supplementing it with a mixture of 10 volatile and 4 semivolatile organic compounds, shown with their corresponding target concentrations in Table 1. Chloroform was not added to the leachate until the later stages of the project because of its potential toxicity.

Treatment Systems

The treatability of the leachate is being evaluated in three parallel trains. One train consists of leachate pretreatment in a bench-scale upflow anaerobic filter reactor (6-in. diameter x 48-in. high) packed with 1-in. Pall rings, followed by mixing with raw municipal wastewater in a ratio of 95 percent wastewater to 5 percent leachate and treatment in a bench-scale activated sludge POTW unit. The second train is similar in scale to the first, with the exception that a fluidized-bed reactor (4-in. diameter x 42-in. high) filled with 16 x 20 U.S. mesh GAC is used for leachate pretreatment instead of the upflow filter reactor. The third treatment train consists of a pilot-scale anaerobic filter reactor (4.25-ft diameter x 7.5-ft high) followed by a pilot-scale activated sludge POTW unit. The objective of this process train is to evaluate the scale-up of anaerobic filters and to observe potential problem areas such as bed plugging and wall effects. The anaerobic pretreatment systems are operated at 35°C. The empty bed contact time in the GAC fluidized-bed reactor is maintained between 6 and 8 hours, while a longer detention time of 48 to 96 hours is employed in the anaerobic filter reactors.

Results

The COD removal efficiency in the bench-scale anaerobic filter and GAC fluidized-bed system averaged 42 and 48 percent, respectively. The average influent COD was about 1,100 mg/L. The COD removal efficiency for the systems was found to increase with increasing influent COD. During the period of the volatile acids addition, the primary COD removal mechanism was methanogenic. After the volatile acids addition was stopped and the COD of the leachate decreased, the COD removal mechanism was due to a combination of methanogenesis and sulfate reduction. The average sulfate reductions in the GAC fluidized-bed and anaerobic filter reactors were 71 and 65 percent, respectively, corresponding to an average influent sulfate concentration of 116 mg/L. In the bench-scale anaerobic filter reactor, most of the organic compounds were removed in excess of 90 percent. Chlorobenzene, ethylbenzene, 1,1-dichloroethane, and trichlorobenzene exhibited gradual degradation, indicating the need for longer acclimation periods. In the bench-scale GAC fluidized-bed reactor, all organic compounds exhibited removal efficiencies exceeding 95 percent with the exception of 1,1-dichloroethane, which required a longer acclimation period. Despite some differences in the design and operation of the pilot-scale anaerobic filter-reactor compared to the bench-scale anaerobic filter reactor, the performance of both systems was similar prior to chloroform addition to the leachate. Within 3 weeks after the addition of chloroform, however, the pilot-scale system showed a decline in the removal of some of the SOCs (including chloroform). SOC removals continued to decline over a period of 4 months, at which time chloroform addition was discontinued. The pilot-scale system is now being monitored to determine whether performance will return to prechloroform addition levels while the SOC consortium (excluding chloroform) continues to be added to the leachate feed.

Table 1. Composition of SOC supplement to the leachates.

Compound		Concentration (g/L)
Volatile Organic Compounds		
	Acetone	10,000
	Methyl Ethyl Ketone	5,000
	Methyl Isobutyl Ketone	1,000
	Trichloroethylene	400
	1,1-Dichloroethane	100
	Methylene Chloride	1,200
	Chloroform	0 to 2,000
	Chlorobenzene	1,100
	Ethylbenzene	600
	Toluene	8,000
Semivolatile Organic Compounds		
	Phenol	2,600
	Nitrobenzene	500
	1,2,4-Trichlorobenzene	200
	Dibutyl Phthalate	200

AEROBIC BIODEGRADATION OF CREOSOTE

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Summary

Performance data on solid-phase (land farming) and slurry-phase bioremediation of pentachlorophenol- (PCP-) and creosote-contaminated subsurface soil (sediment) and surface soil were generated at the bench-scale level (1, 2). Soil samples from slurry reactors and from specially designed land farming chambers were extracted and analyzed by gas chromatography for PCP and 42 monitored creosote constituents to delineate the activity of indigenous microorganisms. Changes in microbial biomass were also recorded. In general, slurry-phase bioremediation resulted in rapid and extensive removal (3 to 5 days to biodegrade >50 percent of targeted compounds) of monitored constituents. However, removal rates from surface soil slurries were slower than those observed with subsurface soil slurries, and removal was generally confined to the more readily biodegradable, lower-molecular-weight compounds. In all cases, solid-phase bioremediation was much less effective. The general order of biodegradation was phenolics>low-molecular-weight PAHs>heterocycles>high-molecular-weight PAHs=PCP. These data suggest that slurry-phase bioremediation strategies can be effectively employed to treat creosote-contaminated, and possibly PCP-contaminated, materials. The efficiency of an integrated, multi-phasic slurry treatment process is currently being evaluated at the pilot-scale level.

Results and Discussion

Surface Soil (SS) Bioremediation. To simplify data presentation, PAHs were arbitrarily divided into three groups: groups 1, 2, and 3 consist of PAHs containing two, three, and four or more fused rings, respectively. In the absence of inorganic nutrient supplements (SS- treatment), biodegradation of phenolic, heterocyclic, and lower-molecular-weight PAH constituents of creosote was most readily apparent (for example, see Figure 1). Biodegradation of the more persistent chemicals was less extensive. Biodegradation of PCP began after a 2-week lag period, ultimately resulting in the removal of approximately 70 percent of this chemical over the course of the study (90 days).

When surface soils were amended on a weekly basis with inorganic nutrients (SS+ treatment), the rate of biodegradation of monitored chemicals was accelerated. As observed in the unamended soils, biodegradation of phenolics, heterocyclics, and low-molecular-weight PAHs was most rapid. Moreover, the extent of biodegradation of the more persistent chemicals (i.e., group 3 PAHs, PCP) was increased.

Biodegradation of all monitored creosote constituents was most rapid and extensive under slurry-phase conditions (2). Within 7 days of slurry incubation, 91, 90, 45, 47, and 30 percent of the

phenolics, group 1 PAHs, heterocyclics, group 2 PAHs, and group 3 PAHs, respectively, were biodegraded. However, PCP was not biodegraded by indigenous microflora established in the reactor. Little change in the total amount of chemical biodegraded was apparent with continued incubation (30 days). This suggests that conditions for biodegradation became limiting (e.g., nutrient limitation, accumulation of bacteriotoxic metabolites, etc.). Alternatively, depletion of the readily biodegradable carbon sources (i.e., group 1 PAHs, phenolics) prevented further catabolism of monitored chemicals.

Subsurface Soil (SBS) Bioremediation. Subsurface soils recovered from a depth of 5 m beneath the highly contaminated, capped solidified sludge material present at the American Creosote Works Superfund site, Pensacola, Florida, contained approximately 7 percent (by weight) unweathered creosote plus PCP (3). Initial microbial population estimates showed that this material was essentially sterile (data not shown). This was presumably due to the high organic loading rate and the presence of fly-ash (added to stabilize the above-lying sludge), which resulted in a soil pH of 10 to 11. Hence, biodegradation of these chemicals during aerobic, solid-phase bioremediation was slow to initiate. After a 1- to 2-week lag phase, however, readily biodegradable chemicals (i.e., phenolics, heterocyclics) were removed, but a majority of the other compounds resisted biological attack. The addition of inorganic, soluble nutrients had little effect on the rate and extent of biodegradation.

As observed with surface soil, biodegradation occurred more rapidly, and was more extensive, during slurry-phase treatment than with solid-phase treatment (2). Within 7 days of slurry incubation, 95, 90, 85, 65, and 50 percent of the group 1 PAHs, phenolic, group 2 PAHs, heterocyclics and group 3 PAHs, respectively, were biodegraded. As before, PCP was not degraded by the microbial community established in the reactor.

Based on these and other data, slurry-phase biotreatment technologies have been integrated into a multi-phasic remediation strategy to ameliorate soil and water contamination by creosote, PCP, and related wastes (4). The ability of this system to remove >90 percent of monitored chemicals from contaminated wastes has been demonstrated at the bench-scale level (3, 5). In association with the Superfund Innovative Technology Evaluation (SITE) Program, pilot-scale performance data are currently being generated at the American Creosote Works Superfund Site, Pensacola, Florida.

Acknowledgments

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Financial support for these studies was provided by the U.S. EPA Superfund Program (Region IV). The ongoing Pilot-Scale Technology Demonstration Project is supported by the U.S. EPA SITE Program, Cincinnati, Ohio.

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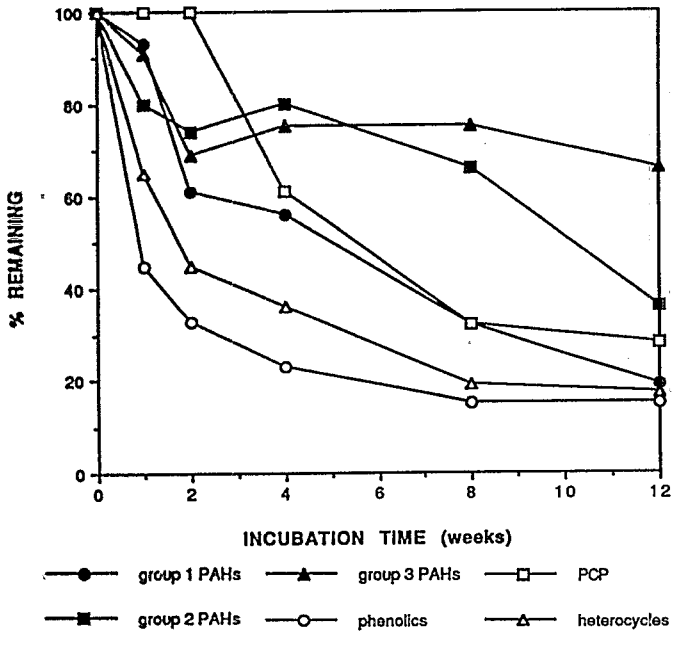

Figure 1. Solid-phase bioremediation, SS.

SECTION FIVE
SEQUENTIAL TREATMENT

Sequential treatment is generally applied to two types of waste: compounds that degrade into stable intermediates, which can be further degraded under different conditions than those used for the parent compound; and complex mixtures of wastes, which are generally degraded in order of their thermodynamic behavior. The classic example of the first type of waste is DDT, which is effectively degraded under alternating anaerobic and aerobic conditions. For waste mixtures, an understanding is needed of the degradation pathways for the waste components and intermediate compounds, the sequence by which complex mixtures are degraded under field conditions, the physical and chemical factors that can influence the degradation pathways, and the availability of organisms or adapted bacterial communities that are able to degrade or transform components of the mixture or intermediate degradation products.

Sequential technologies can vary from the simple coupling of sequential aerobic and anaerobic processes for the degradation of a single compound to the use of sequential reactors containing bacterial cultures adapted for degrading specific compounds or compound classes that are components of a hazardous organic mixture.

A number of recent studies are providing information that may be useful in developing sequential treatment processes. Current research examines an innovative approach to anaerobic/aerobic sequential treatment of hazardous waste leachates in publicly owned treatment works (POTWs). This approach is proposed to address some of the problems in conventional aerobic treatment of these wastes (e.g., pass-through of toxics, air stripping of volatiles, and insufficient anaerobic contact for dechlorination of toxics.) In this process, a GAC-expanded bed is placed between the primary clarifier and the aeration basin. This contact/sorption stage is designed to reduce the pass-through of toxics, retaining them for treatment in an off-line reactor.

Another study examined the methanogenic degradation kinetics of phenolic compounds. To estimate substrate utilization and concomitant bacterial growth, laboratory microcosms containing aquifer material were used to simulate the biotic and abiotic interactions at an abandoned wood preserving plant.

EPA researchers also investigated the bacterial degradation of naphthalene, more complex polychlorinated aromatic hydrocarbons, and structurally related heterocyclic aromatic hydrocarbons. Another study examined anaerobic degradation of highly chlorinated dioxins and dibenzofurans. The results of this study indicated that chlorinated dioxins and dibenzofurans could serve as alternative electron sinks, as is the case for polychlorinated biphenyls (PCBs).

ANAEROBIC/AEROBIC SEQUENTIAL TREATMENT OF CERCLA LEACHATES IN POTWS: AN INNOVATIVE TREATMENT APPROACH

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The presented innovative approach to anaerobic/aerobic sequential treatment of hazardous waste leachates in POTWs is proposed to mitigate some of the problems with conventional aerobic treatment of these wastes, i.e., pass-through of toxics, air stripping of volatiles, and lack of sufficient anaerobic contact for dechlorination of toxics. Figure 1 is a conceptual schematic of the process. A contact/sorption state consisting of a granular activated carbon (GAC) expanded bed, which would be placed between the primary clarifier and the aeration basin of the POTW, is designed to reduce pass-through of

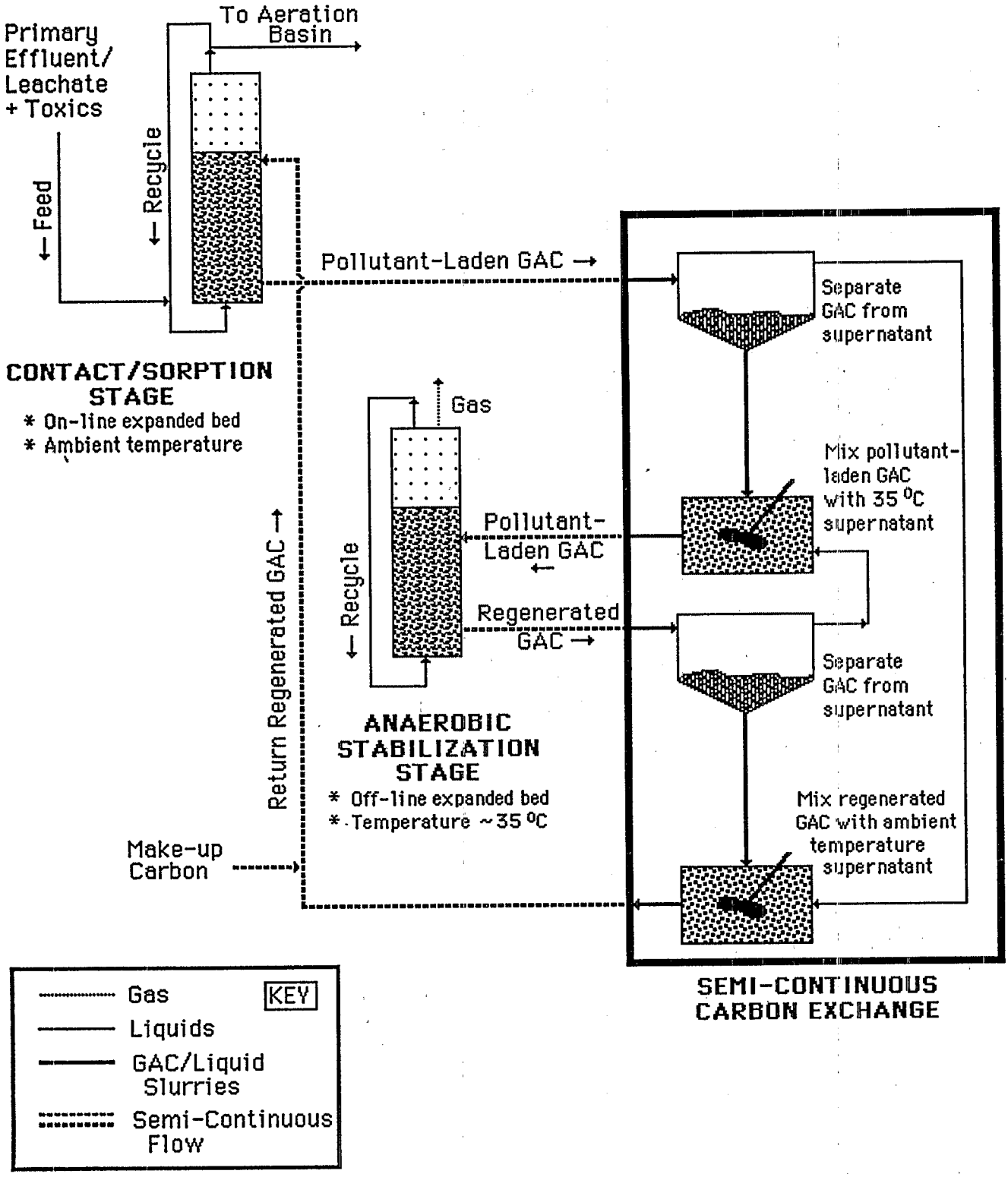

Figure 1. Conceptual schematic of proposed system.

toxics, retaining them for treatment in an off-line reactor. The exhausted GAC from the contact/sorption stage is exchanged with "bioregenerated" GAC from an off-line anaerobic stabilization stage, conserving the GAC in the system. In order to reduce heat losses incurred during the exchange of GAC between the heated stabilization stage and the ambient temperature contact/sorption stage, the respective GAC streams are separated from their supernatants, and the supernatants are recycled. This minimizes the loss of heated supernatant in the second stage. In addition, methane gas, a recoverable source of energy, is generated in the second stage. The long overall retention time of the biomass and GAC in this system encourages dechlorination and destruction of recalcitrant toxics trapped and removed in the first stage.

Two 87-L/day bench-scale systems are in operation for proof-of-concept studies. Each system has two stages. The first stage is operated as a contact/sorption unit and the second as a stabilization unit. The carbon retention time (CRT) in the first stage is 2 days, with a hydraulic retention time (HRT) of 30 minutes. The target CRT in the second stage is 15 days. One system (control) treats primary effluent only and one system (test) treats primary effluent spiked with 5 percent landfill leachate and a mixture of nine volatile and five semivolatile hazardous organic compounds. Concentrations of the spiked organics, as well as conventional wastewater treatment parameters such as chemical oxygen demand (COD), are routinely monitored. Results to date indicate little or no pass-through to the aeration basin of most of the spiked organics, and average COD removals in the 40 to 50 percent range.

METHANOGENIC DEGRADATION KINETICS OF PHENOLIC COMPOUNDS

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Introduction

Microbiologists have come to appreciate the power of the quantitative approach in their research. It is no longer enough to simply describe the organisms that occupy a given habitat; the rates at which they carry out metabolic functions of ecological importance must be estimated. Only when quantitative information about metabolic activities is coupled with knowledge of organismal types can our understanding of the concerted actions of the members of a community be considered complete.

The quantitative approach in microbial ecology involves the estimation of parameters in equations chosen to represent the process under study, such as substrate depletion and concomitant growth. A major factor affecting activity and growth of microorganisms in many environments (e.g., aquifer sediment) is related to the presence of solid surfaces in those environments. Surfaces may alter the availability of organic chemicals, change the levels of various organic and inorganic nutrients, and/or retain microorganisms. Bacterial cells that are attached to subsurface materials may have physiological activities quite different from cells that are in suspension. Most of these phenomena have not been investigated for subsurface environments.

The generally accepted equations describing substrate utilization and concomitant bacterial growth without decay are the ones proposed by Monod (1):

$$\frac{ds}{dt} = \frac{\mu_m X S}{Y(K_s+S)} \qquad \frac{dX}{dt} = \frac{\mu_m X S}{K_s+S}$$

where:

μ_m = maximum specific growth rate, 1/day

K_s = half-saturation constant, mg/L — numerically equal to that substrate concentration that yields a growth rate equal to one-half μ_m

Y = yield coefficient, mg cells/mg substrate utilized

S = substrate concentration at time t, mg/L

X = biomass at time t, mg/L

The above relationships were developed from experiments using pure cultures of bacteria utilizing single organic compounds. It remains to be determined if these expressions describe the degradation in the subsurface environment by a complex mixed microbial population of single compounds in a complex mixture of compounds.

In this study, we present evidence that the Monod equations adequately describe both the utilization of phenolic compounds at very low environmental concentrations (oligotrophic) and the concomitant bacterial growth.

Materials and Methods

The study site is located in Escambia County within the city of Pensacola, Florida, adjacent to an abandoned wood preserving plant (2). The wood preserving process consisted of steam pressure treatment of pine poles with creosote and/or pentachlorophenol (PCP). Large but unknown quantities of wastewaters, consisting of extracted moisture from the poles, cellular debris, creosote, PCP, and diesel fuel were discharged to surface impoundments. The impoundments were unlined and hydraulically in direct contact with the sand-and-gravel aquifer. The aquifer consists of deltaic deposits of fine-to-coarse graveled quartz interbedded with discontinuous silts and clay intervals.

Microcosms used for the study were prepared in 4-L sample bottles and contained approximately 3 kg of aquifer material collected from a depth of 5 to 6 m at a site 30 m downgradient from the contamination source. Phenolic compounds were added to 2.5 L of mineral salts solution (3) at concentrations similar to the aquifer concentrations, 20 to 40 mg/L. Amorphous FeS was used as a reducing agent (4) to ensure methanogenic conditions. The microcosms were prepared, stored, and sampled in an anaerobic glove box containing an O_2-free argon atmosphere at 22°C.

Samples for substrate utilization were removed from the microcosms at approximately 3-day intervals after gentle mixing. Analyses were done by reverse-phase gradient-elution HPLC using a UV detector set at a wavelength of 280 nm.

Nonlinear Parameter Estimation

Substrate depletion and bacterial growth curves were fitted to the Monod equations using nonlinear regression analysis. The method of Marquardt (5) was used for the estimation of parameter values that minimized the sum of the squared residuals. Because the Monod equations do not have explicit analytical solutions for substrate and biomass concentrations as a function of time, a simultaneous solution of both equations was accomplished using a fourth-order Runge-Kutta numerical procedure. The statistical basis for these analyses is presented by Robinson (6), and requires that the sensitivity of the independent variable to changes in each of the parameters be calculable. The partial derivatives of the substrate (S) with respect to μ_m, K_s, and Y satisfy this requirement. These expressions are derived from the integrated Monod equation by implicit differentiation. Unique estimates of the parameters can be obtained when the initial substrate concentration (S_o) is in the mixed-order region and proceeds through the first-order region during the course of the experiment.

Parameter estimates with the 95 percent confidence intervals are given in Table 1. The time interval before the onset of rapid methanogenesis varied from 28 days for 3-methylphenol to 119 days for 2-methylphenol, even though the inoculum history suggests that all of the microorganisms in the microcosms had been exposed to all of the phenolic compounds for a considerable length of time (~ 80 years).

Discussion and Conclusions

Laboratory microcosms containing aquifer material simulate the same biotic and abiotic interactions that occur at the Pensacola site. An important consideration in determining the ultimate environmental fate of contaminants is the adsorption of both the substrate and the biomass to the aquifer sediment. Studies in the laboratory and at the research site have shown that substrate adsorption to aquifer sediments of the four phenolic compounds tested was insignificant (Retardation Factors ranged from 1.01 for phenol to 1.10 for 4-methylphenol), and that greater than 99 percent of the biomass was associated with the aquifer sediment. However, for modeling purposes, the biomass on the sediment may be treated as if it were uniformly distributed throughout the liquid volume. These considerations justify the modeling of substrate utilization in the microcosms as batch reactions.

The bacterial substrate utilization and growth data for all of the compounds tested could be modeled successfully using the Monod equations. The long apparent lag times for the phenolic compounds could be attributed to extremely low initial biomass concentration in the microcosms. The kinetic constants for all of the compounds are very similar, and given that the inocula were acclimated to all of the phenolic compounds, it is unclear why the range of onset times was so great. There also appears to be a lack of correlation between the onset times and the model parameters.

Although we know of no other kinetic studies conducted under similar conditions, it appears that the values of the parameters obtained are reasonable and consistent with values expected of organisms from oligotrophic environments. The extremely low Y values for the phenolic compounds suggest that these organisms have adapted to this environment by utilizing 99+ percent of the available energy for maintaining cellular integrity, or that they are very inefficient at capturing the free energy available. This phenomenon is currently under investigation.

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Table 1. Results.

Compound	μ_m, 1/day	K_s, mg/L	Y, mg/mg
Phenol	0.104 ± 0.022	2.00 ± 6.10	0.003 ± 0.003
2-Methylphenol	0.040 ± 0.010	0.27 ± 0.35	0.003 ± 0.004
3-Methylphenol	0.122 ± 0.068	0.40 ± 1.11	0.002 ± 0.004
4-Methylphenol	0.095 ± 0.045	1.90 ± 10.1	0.052 ± 0.139

DEGRADATION OF NAPHTHALENE, PAHS, AND HETEROCYCLICS

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Naphthalene is the simplest fused polycyclic aromatic hydrocarbon. Information obtained from studies of its bacterial degradation may be used in understanding and predicting the pathways used in the metabolism of more complex polycyclic aromatic hydrocarbons and structurally related heterocyclic aromatic compounds.

In spite of the relative simplicity of naphthalene, much about its bacterial metabolism has remained unclear, particularly the steps in the metabolic pathway by which 1,2-dihydroxynaphthalene (DHN, Figure 1, I) is metabolized to salicylaldehyde. This is primarily because of the chemical instability of various chemical intermediates implicated or identified in this pathway. Thus DHN is rapidly and spontaneously oxidized in water to 1,2-naphthoquinone, and the potential ring-cleavage products *cis-o*-hydroxybenzalpyruvate (Figure 1, IV), *trans-o*-hydroxybenzalpyruvate (Figure 1, III), and 2-hydroxychromene-2-carboxylate (Figure 1, V) all undergo isomerizations in water.

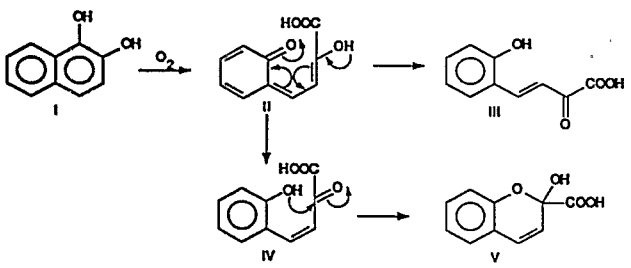

Figure 1. 1,2-Dihydroxynaphthalene diroxygenase (nah C product).

Davies and Evans (2) identified a product of the oxidation of DHN by cell extracts of a naphthalene-grown *Pseudomonas* strain as *o*-hydroxybenzalpyruvate (HBPA), initially isolated as its perchlorate, and suggested that it was probably the *cis*- isomer based on its chemical properties. Cell extracts metabolized both *cis*- and *trans*- isomers to salicylaldehyde. The *cis*- isomer of HBPA was spontaneously converted at neutral pH to its hemiketal, 2-hydroxychromene-2-carboxylate (HCCA), which was not metabolized by cells or cell extracts and was considered to be an artifact.

Barnsley (1) subsequently demonstrated that, instead of being an artifact, HCCA was the initial product of the enzymatic ring cleavage of DHN. He did this by incubating cell extracts, and he also purified dihydroxynaphthalene dioxygenase (4) with 1,2-dihydroxynaphthalene at pH 5.5. (At this pH, the autoxidation of DHN to 1,2-naphthoquinone is somewhat reduced.) After 2 minutes, the incubation mixture was applied to and eluted from a column of Sephadex G-25 in order to separate the large protein components of the cell extracts from lower -molecular- weight reaction products which were collected and freeze-dried. In this way, a small amount of a single chemical, 2-hydroxychromene-2-carboxylate, was obtained, and it was proposed that this was the initial ring-cleavage product. An HCCA-metabolizing enzyme (isomerase), which catalyzed the conversion of that compound to *trans*-HBPA at pH 10 (Km = 0.2 mM), was also evident.

Both of these studies were hampered by their use of 1,2-dihydroxynaphthalene as substrate. Incubations required large amounts of enzyme, and yielded product only in quantities that were insufficient to identify rigorously.

Accordingly, a way to identify the DHN ring cleavage product was adopted that involved cloning the genes encoding the first three enzymes of the pathway away from genes encoding enzymes catalyzing subsequent steps. Bacteria carrying these genes would be able to transform the stable substrate, naphthalene, to the ring-cleavage products, which could then be prepared on a large scale and identified. Such clones would also be useful for the preparation of analogous pathway intermediates from other hydrocarbons and heterocyclics.

The NAH7 plasmid from *Pseudomonas putida* G7 carries genes encoding the complete degradation of naphthalene. These genes are grouped in two operons (5, 6). The operon encoding the metabolism of naphthalene to salicylate has been cloned in the plasmid vector pMMB277 on an 11 kb *Eco*RI-*Hin*dIII fragment (Figure 2). Several deletions and subclones have been obtained that eliminate DNA between *nahC* (the 1,2-dihydroxynaphthalene dioxygenase gene located 2.15 to 3.05 kb from the *Hin*dIII site [3]) and the *Hin*dIII site and which inactivate the gene encoding the enzyme that degrades the product of 1,2-dihydroxynaphthalene cleavage. One of these subclones is a 10 kb *Eco*RI-*Cla*I fragment inserted in pMMB277. *Pseudomonas aeruginosa* PAO1 carrying this plasmid transforms naphthalene to the ring-cleavage products which accumulate. These products have been separated by chromatography on Sephadex G-25 and identified as *trans-o*-hydroxybenzalpyruvate and 2-hydroxychromene-2-carboxylate (the hemiketal of *cis-o*-hydroxybenzalpyruvate) by NMR and mass spectrometry.

Both *cis-* and *trans-*isomers are probably formed by the spontaneous rearomatization of an unstable ring-cleavage product (Figure 1, II). They are both rapidly degraded to salicylaldehyde by cell extracts of strain PAO1 carrying the cloned *Eco*RI-*Hin*dIII fragment. On addition of NAD^+, salicylaldehyde is oxidized to salicylate.

2-Hydroxychromene-2-carboxylate could have been rapidly formed from the accumulating *cis-* isomer by nucleophilic attack of the *ortho*-hydroxyl oxygen on the carbonyl carbon. This hemiketal is not metabolized at neutral pH by cell extracts of PAO1 carrying the cloned *Eco*RI-*Hin*dIII fragment. In wild-type naphthalene-degrading bacteria, the *cis-* isomer is probably metabolized before the formation of the hemiketal can occur.

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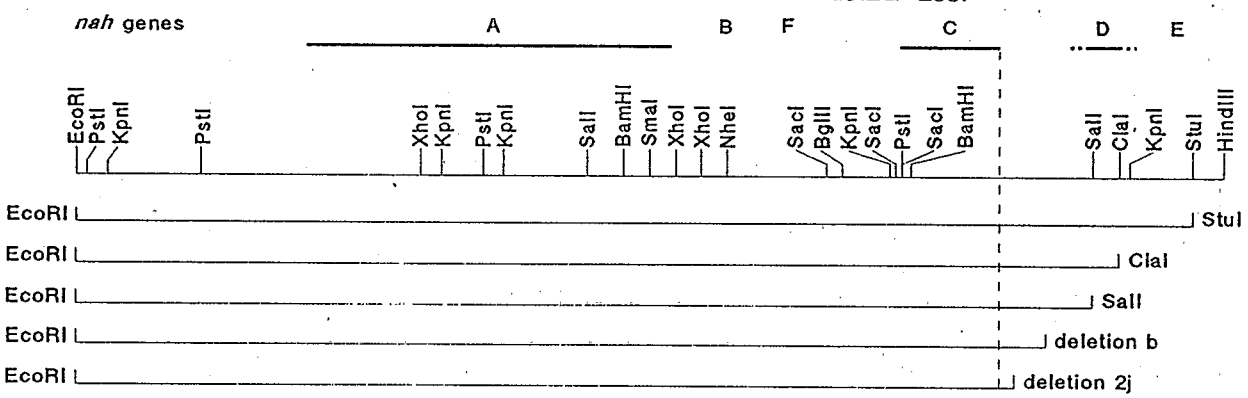

Figure 2. 11 kb NAH7 plasmid fragment encoding the metabolism of naphthalene to salicylate.

ANAEROBIC DEGRADATION OF HIGHLY CHLORINATED DIOXINS AND DIBENZOFURANS

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Summary

Araclor 1242-contaminated Hudson River sediments (HR), creosote-contaminated Pensacola soil (PS), and chlorophenol adapted Cherokee Pond sediments (ACP) were anaerobically incubated with 50 µg/L of the following dioxin (dibenzo-p-dioxin) and dibenzofuran congeners: 1,2,3,4,6,9-hexa (HexaCDD), 1,2,4,6,8,9/1,2,4,6,7,9-hexa isomer (HexaCDDi), and 1,2,3,4,6,7,9-hepta (HeptaCDD) chlorinated dioxins, and 1,2,4,6,8-penta (PentaCDF) and 1,2,3,4,6,7,8-hepta (HeptaCDF) chlorinated dibenzofurans. The initial analytical data from the first 8 weeks of incubation suggest that HR sediments exhibit a more extensive activity towards the congeners than the PS inoculum. No data are available yet for ACP sediments. Substrate disappearance was observed for PentaCDF (35 percent), HexaCDDi (<10 percent), and HeptaCDD (10 percent) in one or more replicates of HR incubations. No detectable levels of intermediates have been observed, except for a small amount (3 to 4 µg/L) of HexaCDD obtained from the incubation with HeptaCDD. This intermediate was identified by GC/MS; however, isomer assignment was impossible due to the extremely low concentration levels. Although the initial results do not show extensive activity against the congeners tested, the presence of a lower chlorinated metabolite may be the first indication that highly chlorinated dioxin and dibenzofuran isomers could serve as alternative electron sinks, as is observed for polychlorinated biphenyls (PCBs).

Experimental Procedures

Experimental Setup and Sample Preparation. Three replicates of microcosms (50 mL total liquid volume, 50 ± 2 g solids) were spiked with 100 µL of the respective dioxin and dibenzofuran from 50 mg/L (except for HexaCDDi, 5 mg/L) nonane stock solutions to give a final concentration of 50 µg/L (5 µg/L for HexaCDDi). In addition, duplicate killed (autoclaved) biological controls, live biological controls without PCDD or PCDF, and chemical controls without inocula, have been established and were monitored along with the cultures.

All bottles were manually shaken, decapped, and sampled (5 mL) with a 10-mL glass syringe to contain both sediment or soil and aqueous phase, under a continuous stream of nitrogen in the headspace. Two volumes of hexane/acetone (9:1) and 0.5 µg of octachloronaphthalene (as an internal standard) were added to each sample, which was then shaken overnight on a wrist-action shaker. The extraction solvent was decanted from the soil, sediment, or aqueous phase, and extracted with 2 mL of concentrated H_2SO_4. The extract was then back-extracted with 2 mL of a 2 percent $CaCl_2$ solution (in distilled water), and dried over Na_2SO_4. The resulting extract was eluted over a Pasteur pipette packed with Florisil (60 mesh)/Cu-powder (40 mesh) (1:4 ratio), to remove excess sulfate. The sample was then concentrated to 1 mL under a constant stream of N_2. To this fraction, 100 µL of dodecane was added. The sample was then further concentrated to 100µL under a gentle stream of N_2 and used for GC/MS and GC-ECD analyses.

Analytical Procedures. The samples were analyzed both on a Triple Stage Quadrupole TSQ 70 Finnigan MAT GC/MS, and on a 5890A Hewlett-Packard Gas Chromatograph equipped with an electron capture detector (ECD).

GC/MS operating conditions: Column: DB-5, 60 m, 0.32 µm I.D., 0.25µm film thickness; column head pressure: 25 kPa; injection: on-column; injector temperature: 60°C; initial temp.: 90°C, hold 5 min; rate 1: 25°C min^{-1}; temp. 2: 200°C, hold 15 min; rate 2: 4°C min^{-1}; temp. 3: 250°C, hold 15 min.

Gas chromatographic conditions: Column: DB-5, 30 m, 0.32 µm I.D., 0.25 µm film thickness; carrier gas: helium (linear flow velocity: 25 cm s^{-1}); make-up gas: argon/methane; column head pressure: 14 kPa; injection: split/splitless (10:1 ratio); injector temperature: 250°C; detector temperature: 275°C; initial temperature: 250°C, hold 5 min; rate 1: 2°C min^{-1}; final temperature: 300°C, hold 5 min. The high initial temperature caused the Araclor in samples from bottles containing Hudson River

sediments to elute in the first 10 min, without interfering with the dioxin (25.8 min) and dibenzofuran isomers (penta: 20 min, hepta: 24 min). The internal standard (octachloronaphthalene) eluted after 19 minutes.

Results and Discussion

Table 1 represents the time zero samples, and Table 2 shows the results from samples taken after 2 months. The recovery efficiencies of all isomers from the chemical controls always exceeded 92 percent, while they varied between 20 and 45 percent from the samples containing inoculum. The low value obtained for the killed control of penta CDF incubations (Table 1) cannot be ascribed to low recovery efficiencies, as the data are corrected for sorption with the internal standard. Hence, they have to be explained either by incorrect spiking with the furan, or a volatilization loss when these controls were autoclaved twice. To prevent these kinds of losses, killed controls of the Cherokee Pond incubations were spiked with 0.5 mL of concentrated H_2SO_4, instead of twice autoclaved.

The recoveries of penta CDF in the live HR incubations and killed controls, after normalization, were only 60 and 87 percent, respectively (Table 2). Further analysis of the 8-month incubation samples might yield more information with respect to this phenomenon. In the case of HR incubations with HeptaCDD, however, the third replicate yielded a much lower concentration than both other replicates. Although no discernible peak could be observed during full scan GC/MS analysis of this sample between masses 100 and 480, single ion monitoring (SIM) between masses 380 and 410 indicated the presence of a chlorinated compound (Figure 1). Scans for the base peak M^+ (m/z 390), $M+2^+$ (m/z 392) and $M-2^+$ (m/z 388) ion abundances in the molecular ion cluster agreed well with those of a HexaCDD standard (1,2,3,4,6,9-HexaCDD) and published values (1).

Table 1. Time zero analysis of sediment and soil incubations with five dioxin and dibenzofuran isomers. All concentrations [(x ± s) ppb] were normalized with respect to octachloronaphthalene.

Isomer	Inoculum[1]	Live[2]	Killed[2]	Controls[3] Biological	Chemical
HeptaCDF	HR	40.4 ± 0.1	42.2 ± 3.5	0.0	51.8 ± 0.9
	PS	44.9 ± 2.9	45.5 ± 0.5	0.0	51.1 ± 0.5
PentaCDF	HR	41.3 ± 1.4	35.5 ± 2.9	0.0	51.0 ± 6.7
	PS	49.9 ± 4.1	50.7 ± 2.9	0.0	50.2 ± 0.9
HeptaCDD	HR	47.3 ± 3.0	47.6 ± 2.3	0.0	44.3 ± 1.2
	PS	48.9 ± 11.1	47.4 ± 1.6	0.0	46.6 ± 0.1
HexaCDD	HR	50.9 ± 0.9	52.8 ± 2.1	0.0	48.6 ± 1.2
	PS	55.7 ± 6.7	51.8 ± 8.1	0.0	56.2 ± 0.8
HexaCDDi	HR	4.9 ± 0.4	4.6 ± 0.6	0.0	4.8 ± 0.1
	PS	4.7 ± 0.6	4.3 ± 0.8	0.0	4.6 ± 0.4

[1]Abbreviations: HR, Hudson River; PS, Pensacola Soil.
[2]Average of three samples.
[3]All data from the controls are average values from two samples.

Table 2. Analysis of sediment and soil incubations after 2 months. All concentrations [(x ± s) ppb] were normalized with respect to octachloronaphthalene. The number of replicates is the same as in Table 1.

Isomer	Inoculum	Live	Killed	Controls Biological	Chemical
HeptaCDF	HR	42.5 ± 5.0	43.4 ± 1.5	0.0	43.1 ± 1.2
	PS	45.6 ± 2.9	44.1 ± 0.9	0.0	43.8 ± 4.9
PentaCDF	HR	27.4 ± 1.0	38.5 ± 1.9	0.0	44.0 ± 1.3
	PS	50.7 ± 0.2	48.8 ± 0.8	0.0	48.2 ± 2.4
HeptaCDD	HR	41.4 ± 5.8[1]	48.0 ± 3.3	0.0	47.9 ± 2.6
	PS	48.6 ± 1.2	48.3 ± 2.4	0.0	48.2 ± 0.8
HexaCDD	HR	49.9 ± 7.1	52.2 ± 2.3	0.0	49.2 ± 0.7
	PS	48.1 ± 7.7	48.8 ± 3.2	0.0	52.5 ± 0.8
HexaCDDi	HR	4.4 ± 1.1	4.3 ± 0.4	0.0	4.6 ± 0.3
	PS	4.5 ± 0.3	4.3 ± 0.8	0.0	4.3 ± 0.6

[1]The concentration of HeptaCDD in one replicate decreased by ± 10 percent.

Except for the presence of the m/e 327 ion, which was out of scan range, the relative abundance percentages of the three main ions (Figure 2) were well within the U.S. EPA acceptance criteria for isomer identification (2). Since background ion abundance levels interfered strongly with the peak identification, a total extract of this replicate will be analyzed to conclusively identify this ion cluster as a HexaCDD.

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SECTION SIX
METABOLIC PROCESS CHARACTERIZATION

EPA's metabolic processes research generates a better understanding of the processes by which microorganisms degrade chemicals, expanding the range of organisms that can be used in biotreatment technologies. Based on the insights gained from this research, scientists can choose indigenous organisms or enhanced organisms to meet needs in pollution cleanup and control.

Data from one research project suggest that actively nitrifying Nitrosomonas europaea have a potential role in biotreatment of halogenated aliphatic compounds. In another project, researchers are developing sulfate-reducing bacterial cultures that can degrade chlorinated aromatic compounds, and are evaluating sewage sludge and freshwater sediments for sulfidogenic activity that results in microbial transformation of certain of these compounds. Another study is investigating ring fission of polycyclic aromatic hydrocarbons by white rot fungi (Phanaerochaete chrysosporium), a phenomenon previously thought to be unique to certain bacteria. Other researchers are conducting genetic and soil studies to develop strains of the bacteria Pseudomonas with sufficiently high soil survivability for use in biotreatment of polychlorinated biphenyls (PCBs). Researchers are also studying the degradation of trichloroethylene (TCE) by Pseudomonas cepacia. A major limitation of these organisms for TCE bioremediation is their requirement for exogenous aromatic inducers. Current research is focusing on a mutant of G4 that does not require induction.

DEGRADATION OF HALOGENATED ALIPHATIC COMPOUNDS BY THE AMMONIA-OXIDIZING BACTERIUM *NITROSOMONAS EUROPAEA*

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The ubiquitous soil-, marine-, and freshwater-dwelling ammonia-oxidizing nitrifying bacteria are obligate chemolithoautotrophic aerobes. They depend for growth on the activity of the enzyme ammonia monoxygenase (AMO): $2H^+ + 2e^- + O_2 + NH_3 \rightarrow NH_2OH + H_2O$. The two electrons for the AMO reaction originate in the subsequent reaction catalyzed by hydroxylamine oxidoreductase (HAO): $H_2O + NH_2OH \rightarrow 4e^- + 5H^+ + NO_2^-$ (1).

Nitrosomonas, like the methylotrophs (2), is capable of the oxidation of many organic compounds. In addition, our laboratory at Minnesota (3,4) and the laboratory of Arp at Corvallis (5) have observed degradation of many halogenated aliphatic compounds as summarized in Table 1.

In no case is there evidence that oxidation of an organic substrate will support growth. Response to inhibitors of AMO (2-chloro-6-trichloromethyl pyridine ["nitrapyrin"], acetylene or -dipyridyl) and the fact that the organic co-oxidized substrate inhibits ammonia oxidation indicates that degradation is catalyzed by AMO. For all compounds, the concomitant oxidation of ammonia is required for degradation of halogenated hydrocarbons. Depending on the substrate, hydroxylamine and/or hydrazine (a nonbiological substrate for HAO) can also serve as electron donors for degradation in cells. Low levels of degradation are observed for a short time in the absence of added electron-donating co-substrate; it is presumed but not known that an endogenous electron donor is involved.

Clearly *Nitrosomonas* is able to degrade a wide spectrum of halogenated aliphatics. The exceptions are tetrachloroethylene and carbon tetrachloride. The observation that the soluble methane-oxidizing enzyme from methylotrophs will oxidize fluorotrichlorethylene (6) suggests that the absence of a C-H bond need not be limiting.

Table 1. Degradation of halogenated aliphatic compounds.

Substrates[a]	Products
<u>Haloalkanes:</u>	
Bromomethane[b]	Formaldehyde
Chloromethane[c]	Formaldehyde
Dibromomethane	
Dichloromethane	
Trichloromethane	
Tetrachloromethane[4]	
Bromoethane	
Chloroethane[c]	Acetaldehyde, 2-Chloroethanol
Fluoroethane[c]	
Iodoethane[c]	
1,2-Dibromoethane	
1,1,2-Trichloroethane	
Chloropropane	Propionaldehyde, 3-Chloro-1-propanol 1-Chloro-2-propanol
1,2,3-Trichloropropane	
Chlorobutane[c]	Butyraldehyde, 4-Chloro- -butanol
<u>Haloalkenes:</u>	
Chloroethylene	
cis 1,2-Dibromoethylene	
trans 1,2-Dibromoethylene[d]	
1,1-Dichloroethylene	
cis 1,2-Dichloroethylene	
trans 1,2-Dichloroethylene[d]	
Trichloroethylene	
Tetrachloroethylene[d]	
1,3-Dibromopropene	
2,3-Dichloropropene	
1,1,3-Trichloropropene	
Nitrapyrin	

a. All compounds reported in reference 4 except as indicated.
b. See ref. 1 and 2.
c. See ref 5.
d. Tested but not degraded.

The product of oxidation of some substrates by *Nitrosomonas*, possibly an epoxide intermediate, forms an irreversible derivative of AMO and thus inactivates the enzyme. Reaction with acetylene, the best example of this kind of compound, results in derivatization of a specific membrane polypeptide (7). Nevertheless, the oxidation of most halogenated compounds can continue for days provided that the concentration of ammonia remains high enough.

We have recently demonstrated the ammonia-dependent degradation of 1,3-dibromopropene, 1,1,3-trichloropropene, 2-chloro-6-trichloromethyl pyridine nitrapyrin, and 2,3-dichloropropene by *Nitrosomonas*. The rates were 4.0, 1.6, 5.8, and 29 μmoles hr^{-1} wet $weight^{-1}$. Halogenated substrates were measured by electron capture detector after gas chromatography. At a concentration of 30 to 40 μM, the first three compounds completely and irreversibly inhibit oxidation of ammonia. Ring ^{14}C-labeled nitrapyrin derivatizes all membrane proteins equally. Thus the reactive product of oxidation of nitrapyrin appears to be membrane soluble and long lived.

In nature or in pollution treatment, actively nitrifying *Nitrosomonas* would appear to have a potential role in the degradation of halogenated aliphatic compounds.

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DEGRADATION OF CHLORINATED AROMATIC COMPOUNDS UNDER SULFATE-REDUCING CONDITIONS

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Despite the recent progress made in describing microbial transformations that occur under anaerobic conditions, our understanding of the role sulfate-reducing bacteria may play in the remediation of environmental contaminants is still very much in its infancy. The aromatic nucleus is the second most common naturally occurring organic residue in the biosphere and is the basic structural unit of innumerable anthropogenic materials that are produced in enormous quantities all over the world. Yet the first description of an aromatic

compound being used by a sulfate reducer was not published until 1980 (1).

The number of aromatic substrates known to be amenable to microbial transformations under sulfidogenic conditions is still small, but includes a rapidly growing list of nonhalogenated compounds. Some of these transformations are catalyzed by pure cultures of sulfate-reducing bacteria, though none of the metabolic pathways have been elucidated.

The number of halogenated aromatic compounds reportedly susceptible to microbial transformations under sulfate-reducing conditions is as yet limited to the five chlorinated phenols whose structures are shown below. Results from several laboratory studies have suggested that sulfate may inhibit the anaerobic degradation of chloroaromatic compounds by preventing dehalogenation (2,3,4,5,6). The mechanism of this apparent sulfate inhibition of dehalogenation is unclear. Some investigators have suggested that the inhibitory effect of sulfate may be caused by competition for an electron donor between the sulfate-reducing bacteria and the organism(s) responsible for dehalogenation; that is, the sulfidogens are able to outcompete dehalogenators for available hydrogen (2,6,7). It is interesting to note, however, that sulfate does not inhibit dehalogenation by *Desulfomonile tiedjei* (8,9), the only known obligately anaerobic dechlorinating bacterium which also happens to be a sulfidogen.

Based perhaps, in part, on the rather consistently observed disparity in transformation potential between the sulfate-reducing and methanogenic regions within their shallow aquifer study site, Kuhn and his co-workers (6) have speculated that the anaerobic dehalogenation potential might be lower in environments that maintain a higher redox potential (i.e., denitrifying and sulfidogenic), especially for less highly halogenated aromatic compounds. Vogel et al. (10) have proposed a similar explanation for the anaerobic dehalogenation of aliphatic compounds.

Despite the evidence linking sulfate with inhibition of reductive dehalogenation, it is worth noting that several of the reports of this phenomenon are from the same laboratory and used material from the same shallow aquifer (2,3,6), so it is perhaps premature to make any generalizations. Based on results of a recent study in which chlorophenol degradation occurred during sulfidogenesis (4) and another in which haloaromatic degradation was shown to be coupled to sulfate reduction (11), it is possible that sulfate inhibition of dehalogenation may be a site-specific characteristic.

The objectives of this research are to:

1. Develop sulfidogenic cultures or consortia that are able to dehalogenate chlorinated benzenes, phenols, benzoates, anilines, and biphenyls.

2. Determine the activity of the cultures obtained over a range of environmental conditions:

3. Evaluate the substrate specificity of the cultures towards all of the compounds classes.

4. Evaluate in microcosm experiments the ability of these cultures to enhance the degradation of hazardous chlorinated aromatic compounds in contaminated soils and sediments.

This report will summarize some of the work in progress aimed at evaluating sewage sludge and several freshwater sediments for sulfidogenic activity that results in microbial transformations of a selected number of chlorinated aromatic compounds.

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RING-FISSION OF POLYCYCLIC AROMATIC HYDROCARBONS BY WHITE ROT FUNGI

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Polycyclic aromatic hydrocarbons (PAHs) are major pollutants of both anthropogenic and natural pyrolytic origin, occurring in soils, sediments, and airborne particulates. The crucial step in their biodegradation is oxidative fission of the fused aromatic ring system, an event previously thought unique to certain bacteria. Recent evidence necessitates a revision of this view: the lignin-degrading fungi that cause white rot of wood have also been shown to mineralize a wide variety of aromatic pollutants, including certain PAHs, under culture conditions that promote the expression of ligninolytic metabolism. A key component of the fungal lignonolytic system is thought to consist of extracellular lignin peroxidases (LiPs), which have been shown to catalyze the one-electron oxidation of various lignin-related substrates. LiPs have also been shown to oxidize certain PAHs and other aromatic pollutants *in vitro*, and it has been proposed that these enzymes play an important role in fungal xenobiotic metabolism. However, it has never been demonstrated that any PAH is oxidized by LiPs *in vivo*, or that the products of such a reaction are subsequently cleaved to smaller, monocyclic compounds. To address these questions, we have examined the fate of one PAH, anthracene, in cultures of the ligninolytic basidiomycete *Phanaerochaete chrysosporium*.

Anthracene (AC) is the simplest PAH to be a LiP substrate. We found that it was oxidized to a

single end product by both crude and purified preparations of the enzyme, and that this product was indistinguishable from 9,10-anthraquinone (AQ) when subjected to thin-layer chromatography (TLC) on silica or gas chromatography/electron impact mass spectrometry. Since other PAHs that have been examined in detail give mixtures of products when oxidized by LiP, we concluded that AC was the PAH most likely to yield diagnostic metabolites in fungal cultures, and selected it for further studies. In fungal cultures, [$^{14}C_{1\text{-}4,4a,9a}$]AC and [$^{14}C_{phenyl}$]AQ were mineralized to the same extent, with 13.4 ± 3.9 percent of AQ, and 12.9 ± 1.3 percent of AC, oxidized to CO_2 in 14 days. Moreover, the cultures rapidly oxidized AC to AQ. The quinone was the predominant neutral AC metabolite found when the culture medium was analyzed by reverse-phase high performance liquid chromatography (HPLC) or TLC on silica, and an isotope dilution experiment done on the extracellular medium and mycelium showed that AQ accounted for 38 percent of the AC originally added after 48 hours in culture. The abiotic oxidation of AC in uninoculated cultures gave only 1 percent conversion to AQ in this time. These results support a role for LiP in AC oxidation by *Phanerochaete*, and show that the pathway AC→AQ →CO_2 is quantitatively important in AC metabolism by the fungus.

Analysis of the acidic metabolites formed from AC and AQ by *P. chrysosporium* showed that both compounds were cleaved to phthalic acid. The identification of the ring-fission metabolite as phthalate was based on three findings: 1) it was indistinguishable from authentic phthalic acid by ion exclusion HPLC, 2) it recrystallized with authentic phthalic acid to constant ^{14}C specific activity in an isotope dilution experiment, and 3) after treatment with diazomethane, it was indistinguishable from authentic dimethyl phthalate by TLC on silica. The isotope dilution experiment showed that both AC and AQ were accumulated as phthalic acid in 12 percent yield after 12 days in culture. Phthalic acid was not a dead-end metabolite in fungal cultures: it also was mineralized, but at only about one-third the rate that AC and AQ were. The relative persistence of phthalate in the cultures probably explains our success in identifying it as an intermediary metabolite, and the bulk of AC/AQ mineralization is presumably due to further degradation of the moiety that is cleaved from AQ to give phthalate. Our results show that the pathway AC→ AQ→ phthalate is a major one in AC ring-fission by *Phanerochaete*. This fungal pathway clearly differs from the classical bacterial one, which proceeds AC →AC-*cis*-1,2-dihydrodiol→salicylate. It is noteworthy that the principal oxidized products to accumulate from AC, namely AQ and phthalic acid, can both be degraded by *Phanaerochaete* if they are given to freshly ligninolytic cultures. This result shows that the cessation of organopollutant mineralization activity after 2 to 4 weeks that is generally observed in *P. chrysosporium* cultures is not due in every case to the accumulation of recalcitrant products that the fungus cannot further metabolize. This result is rather an artifact of laboratory culture conditions. A principal direction for future work, accordingly, should be the development of methods to prolong biodegradative capability in fungal cultures.

AEROBIC BIODEGRADATION OF POLYCHLORINATED BIPHENYLS: GENETIC AND SOIL STUDIES

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A practical process for aerobic bioremediation of PCB-containing soil is dependent upon isolating or developing suitable organisms. Two of the most important characteristics for such organisms are 1) high levels of degradative activity against many PCB congeners, and 2) the ability to survive on the soil long enough for significant PCB degradation to occur. Recombinant DNA technology is currently being used to develop bacterial strains with these and other desirable properties and to study the genes involved in PCB degradation from a strain of *Pseudomonas* designated LB400.

Pseudomonas sp. strain LB400 is able to degrade a wide variety of PCBs but has many characteristics which make it unattractive for use in a bioremediation process. One serious shortcoming is that the organism has been reported to lose viability very rapidly on soil. This would make it neces-

sary to add organisms frequently to a site or reactor, thus making a process less feasible. Previous studies have shown that *Escherichia coli* strain FM4560 (a genetically modified organism containing the LB400 *bph A, B,* and *C* genes) degrades PCBs nearly as well as LB400 without exhibiting many of its undesirable traits. For instance, FM4560 had better survivability than LB400 in laboratory media containing PCBs and did not require growth on biphenyl for high levels of degradative activity.

The abilities of FM4560 to survive and to degrade PCBs on soil were compared with those of LB400 to determine if the recombinant strain is potentially more useful than the naturally occurring organism. Survivability on soil was examined using PCB-contaminated material from a site in Glens Falls, New York (dragstrip soil). This material contained approximately 550 parts per million of highly evaporated Aroclor 1242, and therefore appeared similar in composition to Aroclor 1248. A series of 2-dram vials containing dragstrip soil were inoculated with either FM4560 or LB400, sealed, and incubated at 23° C without shaking. Colony formation on selective media was used to measure cell survival. The presence of active *bph* genes in LB400 was determined by growth on biphenyl, while *bph* gene activity in FM4560 colonies was demonstrated by their ability to produce yellow *meta*-cleavage product when sprayed with an ether solution of biphenyl or 2,3-dihydroxybiphenyl.

Similar survival curves were obtained for both FM4560 and LB400. Early time points showed significant increases in cell number, presumably resulting from growth on stored intracellular nutrients. After 72 hours, cell numbers returned to their original level and continued to decrease such that by 8 days approximately 20 percent of the cells remained culturable. After 28 days of incubation, the number of culturable cells was 2 percent of the initial value.

The presence of active *bph* genes in the surviving cells was examined at each time point. For LB400 the number of viable cells unable to grow using biphenyl remained relatively stable at approximately 2.5 percent for the first 8 days and then increased to 4.2 percent for the remainder of the experiment. All of the FM4560 colonies contained active *bph* genes.

The ability of *E. coli* strain FM4560 to degrade PCBs on soil from the Glens Falls site was compared with that of *Pseudomonas* sp. strain LB400. All experiments were conducted in sealed, sterile, 2-dram glass vials containing 0.1 gram of nonsterile soil and 1.0 mL of bacterial culture. Under most of the experimental conditions, PCB degradation by strain FM4560 was significantly greater than that by LB400.

Analysis of the LB400 *bphA* Gene

Biphenyl/PCB dioxygenase (encoded by the *bphA* gene) is the enzyme primarily responsible for PCB degradation. Knowledge of the structure, function, and regulation of *bphA* is therefore crucial for the development of genetically modified bacteria with superior PCB-degrading abilities. The nucleotide sequence of the gene(s) for biphenyl/PCB dioxygenase from strain LB400 has been obtained. This sequence was compared with that of toluene dioxygenase from *Pseudomonas putida* strain F1, a multi-component enzyme made up of 4 distinct subunits. The genes for these subunits are co-transcribed and arranged in the order *todC1* (ISP_{TOL} large subunit), *todC2* (ISP_{TOL} small subunit), *todB* ($Ferrodoxin_{TOL}$), and *todA* ($Reductase_{TOL}$). Computer analysis of the *bphA* nucleotide sequence identified 4 open reading frames whose DNA and protein sequences were similar to those encoding the 4 subunits of toluene dioxygenase. For example, 67.5 percent identity in the nucleotide sequence and 65.5 percent identity in the protein sequence between *todC1* and the putative ISP large subunit of biphenyl/PCB dioxygenase were obtained. Sequences upstream of the identified homologous coding region showed no significant homology. These data suggest a relationship between the dioxygenase responsible for biphenyl/PCB degradation and those used to degrade a variety of other aromatic hydrocarbons, including toluene and benzene.

Transcription of the *bphA* region is being examined using the technique of S1 nuclease mapping. Two mRNA 5' ends are visible that map to the area immediately before the first *bphA* coding region. An additional transcript has been detected that maps at least 1,500 base pairs before this coding region. The start site of this transcript has not yet been determined. RNA 5' ends can arise from either transcription initiation or an RNA-processing event.

To differentiate between these two possibilities, DNA fragments corresponding to the putative promoter regions were cloned into a promoter detection vector containing the promoter-less galactokinase gene. Several small fragments (< 300 base pairs) that span the start of the *bphA* coding region demonstrated promoter activity, strongly suggesting that transcription of biphenyl dioxygenase originates from within this region. Studies are currently under way to examine the effect of biphenyl on promoter activity, since it is known that PCB degradation by LB 400 is enhanced when the organism is grown using biphenyl.

MANIPULATION OF TCE-DEGRADATIVE GENES OF *PSEUDOMONAS CEPACIA*

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Summary

Pseudomonas cepacia G4, like several other toluene-utilizing bacteria, can degrade trichloroethylene (TCE) only when the requisite oxygenase is expressed in response to aromatic inducers. A major limitation to the application of these organisms for TCE bioremediation is their requirement for exogenous inducers. A mutant of G4 (G4 Phe1) was selected for its ability to degrade TCE, 1,1 dichloroethylene, and *cis*-and *trans*-1,2-dichloroethylene without requiring induction. Experiments to determine the genetic basis for this altered regulation indicated that enzymes involved in the conversions of cresols to ring cleavage products were constitutively expressed. Genetic stability of the strain was assessed following 100 generations of nonselective growth. The constitutive phenotype was completely stable under these conditions. G4 Phe1 was anticipated to be highly sensitive to chlorinated aromatics as a result of the constitutive aromatic *meta*-fission pathway. This anticipated problem was partially ameliorated through the introduction of a derivative of the pJP4 2 ,4D-degradative plasmid, pRO101 (Tn1721:pJP4), which encodes an *ortho*-fission chlorocatechol pathway.

Results and Discussion

Pseudomonas cepacia strain G4 possesses a novel pathway of toluene catabolism (1). Mutants unable to hydroxylate toluene, *o*-cresol, *m*-cresol, and phenol were also shown to be incapable of the degradation of TCE, suggesting that an *ortho*-acting toluene monooxygenase may be responsible for its degradation (2). Pursuant to a more complete genetic description several mutant classes were analyzed. A variant was produced from one class lacking detectable toluene monooxygenase activity that constitutively degraded TCE, 1,1-dichloroethylene, and *cis*- and *trans*-1,2-dichloroethylene (Table 1). G4 and its constitutive derivative G4 Phe1 were examined for their ability to convert 3-trifluoromethyl phenol (TFMP) to 2-hydroxy-7,7,7-tri- fluoro-heptadienoic acid (TFHA) during growth on lactate. This chromogenic reaction requires two enzymes: a toluene/cresol monooxygenase and a catechol-2,3-dioxygenase (2) (Figure 1). G4 clearly exhibited an inducible response in its rate of production of TFHA (measured by absorbance at 385 nm). G4 Phe1, however, lacked any detectable inductive response, demonstrating instead a fairly consistent level of activity throughout growth. The high specific activity of the second enzyme of TFMP metabolism, catechol-2,3-dioxygenase (C23O), was determined under both induced and noninduced conditions. Table 2 clearly indicates that C23O was constitutive in G4 Phe1. In addition, the evidence suggests that C23O in G4 is under the control of more than one promoter/operator.

Stability of the constitutive property of G4 Phe1 was assessed after growth under non-selective conditions (i.e., basal salts medium with sodium lactate at 20 mM as the sole carbon source) and serial dilution (allowing ca. 10 generations per transfer) for 100 generations. All independent isolates examined (>1,000) were found to have maintained constitutivity as determined by their ability to immediately metabolize TFMP to TFHA.

Since chloroaromatics are frequent co-contaminants with TCE at waste sites, the constitutive aromatic monooxygenase of G4 Phe1 might cause serious problems since it can produce toxic chlorocatechols from these chloroaromatics.

G4 Phe1 metabolized chlorobenzene to 2-chlorophenol and then to 3-chlorocatechol (not further metabolized by the C23O of G4). Plasmid pRO101 (pJP4::Tn1721), which encodes a chlorocatechol-1,2-dioxygenase (that accepts both mono-and dichlorinated catechols), was introduced into this strain. G4 Phe1 (pRO101) utilized 2,4-dichlorophenoxyacetic acid as a sole carbon source, was resistant to phenylmercuric acetate, and did not accumulate 3-chlorocatechol. Concentrations of 2-chlorophenol and chlorobenzene that interfered with the degradation of TCE by G4 Phe1 (pRO101) (100μM) were 10-fold higher than those with an inhibitory effect on the plasmid-free strain G4 Phe1 (1-10 μM).

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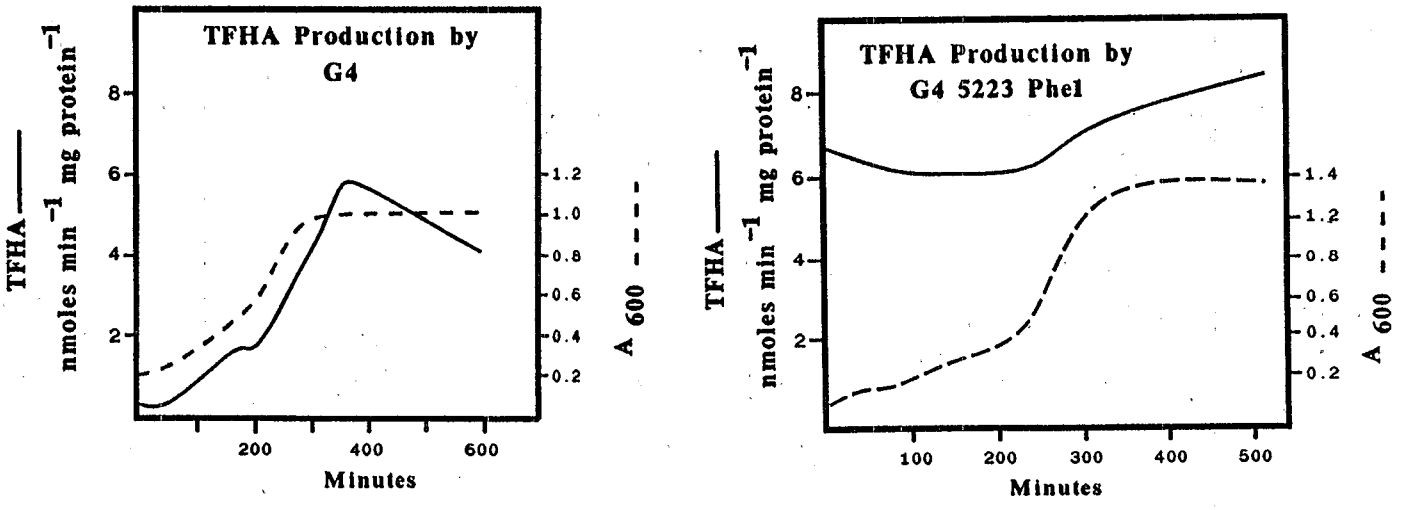

Figure 1. Rates of conversion of TFMP to TFHA during growth.

Table 1. Action of *P. cepacia* G4 Phe(1) on chlorinated ethylenes.

	% Chloroethylene Remaining[a]				
Strain	1,1-DCE	cis-1,2-DCE	trans-1,2-DCE	TCE	PCE
Uninoculated	100 ±2	100 ±4	100 ±9	100 ±3	100 ±7
G4 Uninduced	104 ±3	69 ±19	107 ±5	133 ±5	103 ±7
Phe(1)	50 ±3	12^{12} ±9	0^{M}	2 ±2	104 ±3

[a] Substrate remaining expressed as percentage of that determined in uninoculated controls. Abbreviations: 1,1-DCE, 1,1-Dichloroethylene; *cis*-1,2-DCE, *cis*-1,2-Dichloroethylene, *trans*-1,2-DCE, *trans*-1,2-Dichloroethylene; TCE, Trichloroethylene; PCE, perchloroethylene; ^{M}, Metabolite detected by GC.

Table 2. Differences in specific activity of catechol-2,3-dioxygenase in wild type and mutants of G4.

Catechol-2,3-dioxygenase nmoles min^{-1} $mg\ protein^{-1}$			
Substrate		Cat[a]	3mCat
Strain	Inducer		
G4	none	2.1	3.7
G4	phenol	53.6	62.5
G4 5223	none	0.07	2.2
G45223	phenol	13.1	31.4
Phe(1)	none	156.0	50.4
Phe(1)	phenol	48.0	34.9

[a]Cat, catecol; 3mCat, 3-methylcatechol.

SECTION SEVEN
RISK ASSESSMENT

A number of the high-priority compounds that require disposal are known carcinogens or precarcinogens. Since biodegradation does not necessarily result in total degradation to carbon dioxide and water, researchers need to assess whether ultimate or procarcinogens are created by a given biological treatment. Public health evaluations must be conducted to determine the toxicity of substances at Superfund sites, assess the safety of nonindigenous organisms, and compare bioremediation with other potential technologies.

Work in this research area is designed to develop comparative risk assessment methods to evaluate and contrast mutagenic/carcinogenic products potentially generated by different microbial treatment processes in different environmental settings. Current EPA projects are addressing the use of in vitro bioassays to evaluate the dynamic processes that occur within Superfund sites. Results suggest that using these tests on metabolites from bioremediation, as well as products of other remediation technologies, can be a valuable asset in evaluating treatment alternatives.

GENOTOXICITY ASSAYS OF METABOLITES FROM BIOLOGICAL TREATMENT PROCESS

Larry D. Claxton, Health Effects Research Laboratory, Research Triangle Park, North Carolina.

The present framework for public health evaluations at Superfund sites and for the development of health-based performance goals is provided in the U.S. EPA Superfund Public Health Evaluation Manual (SPHEM) (1). Public health evaluations are one of the driving forces for investigations that determine the need to undertake remedial action, for feasibility studies during the cleanup phase, and for determining the effectiveness of the cleanup procedures.

The health evaluation process as outlined in the SPHEM typically involves five steps. In the first step, indicator compounds are selected from among the list of compounds known to be present at the site. The selection of these indicator compounds is based on known toxicity, physical/chemical factors, and concentration at the site. All evaluations after this step are based upon knowledge about these selected indicator compounds. The second step is using knowledge about the fate and transport of the indicator compounds to estimate exposure concentrations. For the third step, human intake is estimated using "standard assumptions" for daily water and air intake. The fourth step involves a review of the toxicity of the indicator chemicals. The final step is calculating human health risks from the exposure and toxicity information.

These health evaluations are applicable in deciding when a site requires remedial action, to aid in feasibility studies for cleanup alternatives, and to evaluate the effectiveness of any cleanup procedures used for a site. Each of these decision processes, therefore, is dependent upon the identification and quantification of indicator chemicals and the toxicity information for these compounds.

Several weaknesses associated with this process are identified in the SPHEM. The following statements, for example, are made:

"...important chemical data are frequently unavailable."

"...toxicity testing has not kept pace with the need for information on many chemicals...."

"...exposure assessment often requires many assumptions."

"...it would be unrealistic to expect that all data necessary to determine precisely the health risks associated with every site will be available."

Although many of these weaknesses would result in an underestimation of human risks associated with a site, a number of the data and knowledge gaps may cause an overestimation of the human health risks.

For most remediation technologies (not just bioremediation), the products from the remediation efforts are not predicted or monitored. Instead, the process is monitored to understand to what extent the pollutant or indicator chemical is depleted. There are a few exceptions to this statement. For example, incinerators may be monitored for the production of dioxins. The interaction of the known toxic pollutants and other ancillary components and the degrading microorganisms will not always produce nontoxic substances; however, there is currently no provision for examining this potential.

Although present knowledge and procedures provide a usable framework for human risk assessment associated with the remediation of Superfund sites, those risk assessments (applied to all technologies) are highly imprecise because of major data and knowledge gaps.

Public health evaluations for Superfund sites span a continuum of complexity, detail, level of effort, accuracy, and precision. As the complexity of the site increases and/or the detail of information decreases, risk assessments typically become less precise and accurate. There is a need to fill important data and knowledge gaps concerning the toxicology of substances found within Superfund sites, the safety of any nonindigenous microorganisms, the toxicology of bioremediation products, and the making of multimedia assessments for comparing bioremediation with other potential technologies. In addition, substances within Superfund sites have the potential for undergoing natural phototransformation and ecological transformation thus creating additional products that are also potentially toxic. There is a primary need, therefore, to have bioassay monitoring and research capabilities that can address these complex issues in a more direct and reliable manner.

This presentation will illustrate how *in vitro* bioassays that detect mutagens and many genotoxic carcinogens can be used to evaluate the dynamic processes that can occur within Superfund sites. For example, sites containing high levels of the wood preservative pentachlorophenol (PCP) could be expected to have other chlorinated phenols. Testing of other chlorinated phenols demonstrated that some are less mutagenic and others are more mutagenic (2). Next, the presence of one toxicant may alter the toxicity of another pollutant. For example, PCP potentiates the genotoxicity of 2,6-dinitrotoluene (3). During bioremediation, it is also possible for the products of bioremediation to be genotoxic (3). Because of its persistent nature, the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) remains an environmental hazard. 2,4,5-trichlorophenol (2,4,5-TCP), one of the three reported metabolites of 2,4,5-T, is 100-fold more genotoxic than 2,4,5-T (4). However, when one tests the metabolites of 2,4,5-T produced by a bioremediation organism (*Pseudomonas cepacia* AC1100), the genotoxicity of 2,4,5-T is reduced when 2,4,5-T is the sole carbon source (4). Because 2,4,5-TCP did not accummulate to a high enough degree to increase mutagenicity, results would support the use of bioremediation as a viable alternative for the treatment of 2,4,5-T sites (4).

These and other results demonstrate that the use of short-term mutagenicity assays can be a valuable asset in evaluating treatment alternatives including bioremediation.

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SECTION EIGHT
BIOREMEDIATION FIELD INITIATIVE

As part of its overall strategy to increase the use of bioremediation to treat hazardous wastes, EPA implemented the Bioremediation Field Initiative. This program assists the regions and the states in conducting field tests and evaluations of this technology. At more than 140 sites in the United States, treatability studies are being conducted and bioremediation is being planned, is in full-scale operation, or has been completed. Petroleum, creosote, and solvent wastes make up almost three quarters of the waste types undergoing bioremediation.

RESULTS OF THE BIOREMEDIATION FIELD INITIATIVE

Fran Kremer, U.S. Environmental Protection Agency, Cincinnati, OH; John Wilson, U.S. Environmental Protection Agency; and Walter Kovalick and Nancy Dean, U.S. Environmental Protection Agency, Washington, DC.

Background

As we approach the treatment of hazardous wastes, which are increasingly more diverse with respect to the contaminants and the contaminated matrices, we will be more reliant on innovative technologies for improved treatment efficiencies and lower costs. To meet these objectives, the U.S. Environmental Protection Agency's (EPA) Administrator, William Reilly, has sought to develop an agenda for the 1990s to identify strategies for increasing the use of bioremediation for the treatment of hazardous wastes. To develop this agenda, assistance has been received from biotreatment companies, site cleanup contractors, industry, academia, environmental organizations, and other federal agencies, in addition to the various offices within EPA.

One of the initial recommendations from this consortium was the need to expand our field experience using this technology. Even though bioremediation is a viable technology to treat some hazardous wastes, it has not been fully utilized for the many different types of wastes and site conditions requiring remediation. It was recommended that EPA serve as a focal point in fostering field tests, demonstrations, and evaluations of bioremediation, using good test protocols and documentation of results.

Based on this recommendation, the Office of Solid Waste and Emergency Response (OSWER) and the Office of Research and Development (ORD) have instituted a Bioremediation Field Program. This program provides assistance to the regions and the states in conducting field tests and carrying out evaluations of site cleanups using bioremediation. Sites considered in this program include Superfund sites, RCRA corrective action sites, and Underground Storage Tank (UST) sites. The program is designed to:

1. More fully assess and document performance of full-scale field applications of bioremediation.

2. Provide technical assistance at various stages of site remediation, from site characterization to full-scale implementation.

3. Regularly provide information on bioremediation projects being undertaken nationally.

As solid, full-scale performance data are needed to assess the capabilities of this technology, evaluations of field operations are being undertaken. Sites considered for evaluation have field biological units for treatment of wastes in situ or ex situ, i.e., treatment of solids or ground water in place or treatment in a reactor or land treatment facility.

Technical assistance is available to the regions and the states on treatability and field pilot studies. This is to ensure adequate site characterizations, proper design of treatability studies, and interpretation of results. In some cases, EPA may conduct the treatability work. This assistance is available through EPA's Technical Support Centers. Assistance is currently being provided on a number of creosote sites and chemical facilities.

Data are being compiled on laboratory-, pilot-, and full-scale projects in order that EPA will have a central repository of treatment information. Treatability data are being collected from the regions, states, other federal agencies, and the private sector. These data will be available through the Risk Reduction Engineering Laboratory's Treatability Database Program (513-569-7503) and also through the Alternative Treatment Technology Information Center (ATTIC) (301-816-9153). The Treatability Database Program provides specific information on the treatability of specific chemicals for a variety of technologies, including bioremediation. ATTIC is an on-line information retrieval network that provides current information on innovative treatment methods for hazardous wastes. The Treatability Database Program and ATTIC are currently available.

To date, over 130 sites have been identified across the country where treatability studies are being conducted and bioremediation is being planned, is under full-scale operation, or has been completed. These sites include CERCLA, RCRA, and UST sites. Petroleum, creosote, and solvent wastes make up almost three quarters of the waste types being biologically treated.

SECTION NINE
OIL SPILL BIOREMEDIATION PROJECT

EPA's Oil Spill Bioremediation Project in Prince William Sound, Alaska, examined whether the addition of nutrients to oil-contaminated beaches would sufficiently enhance oil degradation rates to enhance biodegradation. The success of this project demonstrated that bioremediation should be considered as a key component in any cleanup strategy for future oil spills impacting the shoreline.

OIL SPILL BIOREMEDIATION PROJECT

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Introduction

In the several weeks following the Exxon Valdez oil spill in Prince William Sound, Alaska, several million gallons of Prudhoe Bay crude oil, a well-studied oil with respect to previous cold water biodegradation studies, had contaminated almost 300 miles of rocky coastline in Prince William Sound. This confronted Exxon, the state of Alaska, and the U.S. Coast Guard with the largest cleanup effort in U.S. history. As a variety of cleanup options were assessed and implemented, it became clear to EPA's Office of Research and Development and its scientists that bioremediation was also a reasonable cleanup option despite the complexity of the environmental setting. We reasoned that the oil would become quickly colonized with oil-degrading bacteria but that their ability to degrade oil would be limited by the availability of nitrogen and phosphorus nutrients. Artificially adding these nutrients would therefore enhance biodegradation rates, something that has been observed many times in laboratory studies. Thus, the Alaskan Bioremediation Project was initiated. An approach was developed to determine whether the addition of nitrogen- and phosphorus-containing fertilizers to oil-contaminated beaches would sufficiently enhance oil biodegradation rates to permit consideration of bioremediation as a secondary cleanup tool. A plan was conceived to conduct an initial field demonstration of this approach; if it were successful, recommendations for wider scale application would be made to Exxon. EPA would then provide a followup field study as a definitive indication of the success of the large-scale application.

The Field Demonstration

Field operations were begun in early May 1989. Two sites were selected: Snug Harbor and Passage Cove. These beaches were mainly composed of large cobblestone overlying a mixed sand and gravel base. Both beaches had a thin layer of oil covering the surface of the cobblestone, as well as oil mixed into the sand and gravel under the cobble to varying depths.

Selection of fertilizers was based on application strategies, logistical problems for large-scale application, commercial availability (particularly if large-scale application became reasonable), and their ability to provide nitrogen and phosphorus nutrients to the microbial communities on the surface and the subsurface beach material over sustained periods. Three application strategies were adopted for testing: commercially available slow release formulations, an oleophilic fertilizer, and water-soluble fertilizer applied as a solution.

Commercial slow release fertilizer formulations were screened for the best nutrient release rate characteristics. The strategy was to apply the best product to the beach surface and then allow tidal action to disperse the released nutrients over the contaminated area of the beach. The product had to

remain on the beach for several weeks while still delivering sufficient quantities of nutrients. Oleophilic fertilizers are thought to essentially dissolve the nutrients into the oil when applied directly to the oiled beach material. Nutrients sequestered in the oil phase would presumably facilitate bacterial growth on the surface over sustained periods. The oleophilic fertilizer Inipol EAP 22, produced by Elf Aquataine Company (Artix, France), was selected. Fertilizer granules (about 2 to 3 mm in diameter), produced by Sierra Chemicals (Milpitas, California), were selected as the main slow release fertilizer formulation. These granules (Customblen) have a N:P:K ratio of 28:8:0 and slowly release ammonia, nitrate, and phosphate from inorganic ammonium nitrate and ammonium phosphate encapsulated within a diene-treated vegetable oil coating. The fertilizer granules were broadcast onto the beach surface at a concentration of 90 gm/m^2 using a mechanical seed spreader. Their high specific gravity, propensity to adhere to the oil, and tendency to entrain under rocks and in interstitial spaces ensured that they would remain on most low and moderate energy beaches in Prince William Sound for 2 to 3 weeks.

The third type of fertilizer application involved spray irrigation with an aqueous fertilizer solution. This approach produced the most defined, controlled, and reproducible introduction of nutrients into the oiled beach material, particularly for oil below the beach surface. It was accomplished by dissolving commercially available sources of ammonium nitrate (34-0-0) and triple phosphate (0-45-0) into seawater pumped from below the beach. The resulting fertilizer solution was then sprayed over the beach surface at low tide using a pump and lawn sprinkler heads.

The first application of the oleophilic fertilizer occurred July 8, 1989, at the Snug Harbor site. Approximately 2 to 3 weeks following application, the treated beach showed a visually pronounced reduction in the amount of oil on the surface of the cobblestone. This produced a striking "window" against the oiled beach background. Differences between treated and untreated portions of the beach were dramatic. Close examination of the beach, however, revealed that significant quantities of oil remained under the cobblestone as well as within the beach subsurface. Over the next few weeks, however, even this oil slowly disappeared. This contrasted with the untreated control areas, in which there was little visual change. Subsequent studies in the laboratory verified that Inipol was not a chemical rock washer.

Definitive information on the role of biodegradation in this event was established by extracting oil from surface samples of cobble in the oleophilic fertilizer-treated beach and analyzing the extracts by gas chromatography. The sampling and analysis showed that this visual disappearance of the oil was accompanied by significant decreases in total oil residues (i.e., weight of extractable material) and changes in hydrocarbon composition. This change in hydrocarbon composition was largely due to biodegradation. Thus, it is reasonable to assume that the decreases in oil residue weight on the cobblestone were caused by biodegradation. We suspect that after a certain extent of oil biodegradation was achieved, the physical nature of the oil changed into a less sticky, flaky consistency, and that this innocuous degraded material was then easily scoured from the rock surfaces by tidal action.

Summary and Conclusions

Results from our initial field studies were sufficient for Exxon to consider the use of bioremediation on a large scale as a finishing step for their cleanup effort. We recommended that the oleophilic fertilizer, Inipol, be applied to beaches with only surface oil, and that a combination of Inipol and the fertilizer granules (Customblen) be applied to beaches with both surface and subsurface oil. The granules provided a simple means of releasing nutrients into the beach subsurface by tidal action and thereby potentially enhancing biodegradation of subsurface oil.

Exxon began fertilizer application in early August 1989 to approximately 50 miles of beach in Prince William Sound that had been physically washed. Increasing the biodegradation rate of oil at this point was very important because maximal degradation could be achieved before winter conditions slowed biodegradation processes. In many cases, the results of large-scale fertilizer application were as dramatic as our initial observations at Snug

Harbor; that is, where the oil was spread thinly over the cobble surface (as was the case on many beaches that had been physically washed), the oil disappeared over a 20-day period. Unexpectedly, it also appeared that even oil underneath the cobble had disappeared in a shorter time period than that observed at Snug Harbor. Although it is difficult to prove experimentally, we believe that the physical cleaning process used by Exxon dispersed the oil throughout the beach material to such an extent that the exposed oil surface area was greatly increased, allowing greater bacterial colonization and subsequent biodegradation.

Our Passage Cove study was initiated in late July 1989 as the definitive technical support site for the large-scale application of Inipol and Customblen fertilizers. These fertilizers were applied in combination to a large test beach and samples of beach material were analyzed for changes in oil residue weight and aliphatic hydrocarbon composition. These changes were compared to those observed in an untreated control beach. In addition, a beach treated with a seawater solution of inorganic fertilizer (applied via a sprinkler system) was examined in the same way.

Approximately 2 to 3 weeks following initiation of this study, oil on the cobble surfaces in the Customblen/Inipol- and the fertilizer solution-treated beaches had been degraded to the point of producing visibly cleaner surfaces, much as we had seen in Snug Harbor. Surface oil on the control beach, however, was still very apparent, showing no visual reduction in the amount of oil. Disappearance of oil from the rock surfaces on the beach treated with the fertilizer solution provided definitive proof that biodegradation (and not chemical washing) was responsible for the oil removal, as there was no other reasonable mechanism to explain the effect of nutrient addition.

Despite sampling and interpretation complications resulting from the high variability in oil distribution on the beaches, we have been able to show, statistically, that oil biodegradation (as measured by changes in oil chemistry) was significantly greater on the beach treated with the fertilizer solution than it was on the control beach. Based on this information, we projected that after 45 days approximately 4 to 5 times more oil remained on the control test beach than on the fertilizer solution-treated test beach. This corresponded to an enhanced biodegradation rate of about two to three fold. On the Inipol/Customblen-treated beach, it appeared that accelerated biodegradation (approximately a two- to threefold increase in biodegradation rates) occurred early in the test when nutrient concentrations were highest.

The long-term benefit of fertilizer application was realized during examination of the beaches in Passage Cove in November 1989 and early June 1990. At the later sampling, virtually no oil was observed on either of the treated beaches, at both the surface and the subsurface (8-in. depth). However, the untreated control still showed areas of heavy oil contamination in the subsurface beach material. These observations provided the final definitive demonstration of the long-term success that can be expected from bioremediation of oil-contaminated beaches.

As a result, in spring of 1990, bioremediation became an integral part of a cleanup plan for the remaining oil-contaminated shorelines in Prince William Sound. To follow the success of this treatment, a joint bioremediation monitoring program was conceived and implemented by scientists from Exxon, EPA, ADEC and the University of Alaska (using logistical and resources support from Exxon). The central success of this monitoring program paved the way for multiple reapplication of the fertilizers during summer 1990, a necessary step in many cases because of the large quantities of oil remaining in some areas.

The success of our field demonstration program has now set the stage for the consideration of bioremediation as a key component in any cleanup strategy developed for future oil spills. Its use and effectiveness will depend on the amount of oil present in the contaminated environmental matrix, i.e., a longer time will be required for degradation of high concentrations of oil, and consequently a longer period of fertilizer application will also be required. In addition, location of the oil (in the absence of physical cleanup, subsurface oil may only be treatable by bioremediation) and the acceptability of other cleanup options must be considered. In most

aquatic environments, enrichments of oil-degrading microbial communities occur relatively soon after oil contaminates shorelines. It is unlikely that natural sources of nitrogen and phosphorus will be sufficient to give maximal degradation rates in light of the available degradable organic carbon from the oil. Thus, the application of fertilizers should enhance degradation and eventually remove the oil. Although oxygen may become limiting in certain situations (e.g., fine-grain sandy beaches), the high porosity and large tidal fluxes characteristic of Prince William Sound beaches precluded this as a limitation.

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