United States
Environmental Protection
Agency

Municipal Environmental Research
Laboratory
Cincinnati OH 45268

Research and Development

EPA-600/S2-83-117 Dec. 1983

EPA

Project Summary

Microbial Degradation of Selected Hazardous Materials: Pentachlorophenol, Hexachlorocyclopentadiene, and Methyl Parathion

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This program was a limited feasibility study in which a number of pure microbial cultures were evaluated for potential in biodegradation of pentachlorophenol (PCP), hexachlorocyclopentadiene (HCCP), and methyl parathion (MP) in an aqueous medium under aerobic conditions. Following the initial screening and selection process, pure culture organisms identified as having potential for biodegradation of the selected chemicals were subjected to further testing and evaluation. Although no completely conclusive evidence for biodegradation of these substances was obtained, data indicate that a number of fungi have potential for the disposal of PCP, HCCP, and MP. One bacterial culture demonstrated tolerance to PCP at 200 ppm in soil and reduced the PCP concentration in an aqueous medium when dextrose was provided. This bacterial isolate, as well as a fungal isolate, may have potential for removal of PCP from spill-contaminated areas.

In the testing performed, the percent-disappearance of the challenging hazardous material was corrected for bioaccumulation and settling out; electron capture gas chromatographic (GC-EC) analysis of extracts from several pentachlorophenol tests did not indicate the presence of chlorinated byproducts or of metabolites. Additional tests are recommended. Improvements on existing high performance liquid chromatography (HPLC) and GC-EC methods were achieved.

The limited scope of this project did not allow for sufficient adaptation of cultures for complete biological removal of the selected chemicals. Time constraints and budgetary requirements precluded the use of C-14-labeled chemicals and the extensive analyses required for the isolation, identification, and quantification of potential metabolites or the byproducts of biodegradation or biotransformation of the selected chemicals.

This Project Summary was developed by EPA's Municipal Environmental Research Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Large volumes of hazardous substances and wastes are transported each year throughout the United States via highways, railroads, and waterways. In spite of precautions taken in the transport and transfer of these substances, accidents resulting in spills of the materials do occur. In addition to accidental spills of hazardous materials, improper or intentional disposal or dumping of chemicals—both in containers and directly—has contaminated much land. Spills pose serious threats to the environment and, indeed, to human life. In many instances, the technology for effective cleanup of a hazardous materials spill or release does not exist.

Biological degradation is recognized as an effective process for disposing of or detoxifying many spilled organic mate-

rials. Biodegradation may be used either as a controlled method in biological reaction systems or as a natural method acting through indigenous microbial populations. Biodegradation of many of the chemical substances designated as hazardous materials has been demonstrated. As research further elucidates the metabolic capabilities of microorganisms, other substances may be found to biodegrade under a given set of conditions. Some chemicals are considered biorefractory simply because microorganisms found in conventional biological treatment systems, or in the soil or water receiving a spill, are unable to biodegrade the material. In such cases, the seeding with microorganisms endogenous to the site, the use of microbes that have been adapted *in situ* or through culturing, or the alteration of environmental or nutrient conditions may result in biodegradation of hazardous materials that resisted biological destruction or detoxification because of structure, composition, concentration, or ambient conditions.

Results and Discussion

General

The reported studies were done aerobically in a basic salts medium that commonly had the composition:

K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
NH_4NO_3	1.0 g
$CaCl_2$ (0.027 g/ml)	1.0 ml
$MgSO_4$ (0.022 g/ml)	5.0 ml
$FeCl_3$ (0.00025 g/ml)	1.0 ml
distilled water (to make)	1.0 liter

Depending on the desired test conditions, the pH was adjusted between 6.5 and 8.5 by altering the level of potassium dihydrogen phosphate or by adding sodium hydroxide. Most tests included a carbon food source; dextrose at the 0.1% or 0.2% level was most commonly used, but tests were also run with yeast extract and with peptones.

A first attempt was always made to challenge microorganisms with high levels of hazardous materials (HM) as aqueous pollutants, namely, 2 g/liter of PCP, 1 g/liter HCCP, and 1 g/liter of MP (as Monsanto Methyl Parathion 500* with 47% active ingredient).

*Mention of trade names or commercial products does not constitute endorsement or recommendation for use

In practice, the PCP solubility was strongly dependent on pH, so that most work was done at pH 8.5 at ca. 200 ppm. Further, HCCP was very difficult to solubilize. Conventional techniques were used to achieve dispersion; nonetheless, settling out remained a major problem so that probably no more than 500 ppm actually dissolved or remained suspended in the medium. Even this concentration (500 ppm) is suspected of being higher than the actual concentration; a more realistic level is less than 50 ppm. Because of the difficulties encountered in solubilizing HCCP, the work with that HM was abandoned; without reasonably high concentrations of HM in the medium, analytical data on percent-removal are too unreliable. In the work with MP, the initial concentration was 250 ppm. Some experiments were done on PCP+oil mixed with soil (this was a simulant for an actual spill site), where the concentration of PCP+oil in soil ranged up to 15% (v:w) with the PCP concentration having a maximum of 4500 ppm (in sandy soil).

Normally, each HM was challenged by 24 pure culture microorganisms at room temperature on rotary shaking tables; there were two controls. The cultures were obtained in four ways: (1) American Type Culture Collection (ATCC) (pure, identified, on slants); (2) Quartermaster Corps at Natick, MA (same at ATCC); (3) contractor cultures and subcultures of lower purity (not always well-identified by genus and species and obtained from soil, sludge, newspapers, bagasse, and other sources); and (4) subcultures (not identified by genus and species but characterized by morphology, color, etc.) that were obtained by serial subculturing from screening tests in which microorganisms appeared to have mutated and were achieving biodegradation.

Not only in the screening tests but in other work, samples were usually centrifuged and the centrifugate and sediment were analyzed separately. In some cases (because of time/funding constraints), only the supernatant was analyzed; in a few cases during pilot plant runs, the samples were not centrifuged but the entire sample was extracted.

PCP was originally analyzed by applying HPLC to processed hexane extracts. Subsequently, GC-EC procedure was applied to an acetate derivative of PCP. Similarly, a hexane extract of HCCP was analyzed both by HPLC (using acetonitrile) and by GC-EC. In the case of MP, the commercial product was first diluted with methanol. For analysis, a 1-to-1 medium-to-methanol extract was fed to the HPLC. Reproducibility of data was poor. Further, there was only about 50% recovery (of MP by HPLC analyses) for known standards. In screening experiments, for example, 166 ppm of MP was added to the controls but only 96 ppm was recovered. Additional work is required to develop an analytical method that will reliably reflect MP levels in the medium or in the biomass (bioaccumulated or precipitated). Note, however, that the analytical protocols were based on published data available in 1977; analytical procedures of improved reliability are undoubtedly available now.

PCP Screening Experiments

Following incubation at room temperature on a rotary shaking table, the contents of each flask were centrifuged. Supernatants and sediments were extracted and analyzed by HPLC. These studies indicated that fungal isolates were the most likely candidate organisms for removal of PCP from the medium. Subsequent tests included evaluation of three bacterial isolates obtained from PCP-contaminated media and a fungal contaminant of a HCCP medium. Data from tests in which the basic salts medium was maintained at a pH of 8.5 (addition of NaOH) indicated that bacterial isolate 041 was a candidate organism for further study. (Organism 041 was a gram-positive coccus isolated from PCP-contaminated medium. Colonies of this microorganism were rough, off-white, and irregular when subcultured on a basic salts agar plus yeast extract.) The final screening studies on biodegradation of PCP used the basic salts medium containing 0.1% dextrose for evaluation of the degradation potential of bacterial isolate 041 and fungus 044. (The fungus was isolated from an HCCP-contaminated medium.) The bacterium (041) reduced the concentration of PCP in the medium by 20% to 42%, whereas the fungus reduced the concentration by 30% to 39% in the screening experiments. Based on these results, it appeared that bacterial isolate 041 and fungus 044 were candidate organisms for evaluation of PCP removal in pilot-scale experiments.

HCCP Screening Experiments

The initial HCCP screening experiments used methanol as a solvent for addition of the HCCP to the basic salts medium containing 0.1% dextrose. Twenty-four organisms were separately incubated with HCCP at an original concentration of 1000 ppm. Following incubation at room

temperature on a rotary shaking table, the entire contents (medium and biomass with settled HCCP) of each flask were extracted separately and analyzed for HCCP. The HCCP concentration was reduced in seven of the flasks when compared to the controls. In the second screening experiment, the seven most effective organisms were tested when HCCP was added to the medium without the solubilizing agent (methanol). Results from this comparison indicated that, in 14-day tests, the presence of the solubilizer enhanced the reduction in HCCP concentration in three of the seven flasks, but that the other four flasks outperformed with no solubilizer. In the third test, HCCP removal was followed in flasks containing the seven organisms and 1000 ppm HCCP (in methanol) added to a basic salts solution with dextrose. The entire contents of each flask were extracted and analyzed for HCCP following 6 days of incubation on a rotary shaking table at room temperature. The HCCP concentration was reduced in all test flasks (the range was 10% to 60%). A bacterial culture (006), a yeast (369), and two fungal cultures (022 and 123 (QM-9123)) were identified as the organisms with greatest potential for HCCP degradation. Bacterium 006 is a gram-negative rod isolated from septic tank seed. Organism 369 (ATTC-1369) is a yeast, *Candida tropicalis*. The putative fungus (022) was isolated from soil and produced white, stringy colonies on Sabouraud's agar. Gram strains showed huge, oblong, rectangular gram-positive rods with connecting branches. Organism 123 was *Trichoderma viride*.

Maintenance of HCCP in suspension was recognized as a major problem since the distribution of HCCP between the medium and the biomass (including settled HCCP) indicated extensive loss of HCCP from solution. A number of emulsifying agents and techniques were tried with no marked success. In view of more promising results in the PCP area and because of the difficulty of solubilizing (emulsifying) HCCP, for example, in soil that had been contaminated by a spill, work on HCCP was halted. HCCP biodegradation can occur; however, the material is not irrevocably biorefractory.

MP Screening Experiments

Seven series of experiments were undertaken to evaluate the potential of pure cultures for the degradation of MP. Again, the initial screening test evaluated 24 organisms. Preliminary experiments indicated that disappearance of MP from the medium was increased when dextrose was provided as a nutrient source. Four microbial cultures were selected in further screening studies and monitored for removal of MP following 6 days of incubation at room temperature. Data from these studies confirm that the presence of dextrose in the medium increases the reduction of MP concentration. A bacterium (003) and a fungus (021) were identified as candidate organisms with potential for biodegradation of MP. The 003 culture was isolated from septic tank seed and gram strains showed gram-positive cocci in clusters. Fungus culture 021 was tentatively identified as *Rhizopus* sp.

With dextrose present, the percent-disappearance of MP with organisms 003 and 021 ranged between 30% and 50%. A few organisms (010, a fungus isolated from cotton seed; 016, a soil fungus; 020, a soil fungus; 123, a fungus, *Trichoderma viride*; 645, a fungus, *Gliocladium virens*) showed high (40% to 65% removal) over the first few days but then lost biodegradation capability. Time did not permit serial subculturing to determine whether there was: die out, a latency period during buildup of mutants that could improve degradation, loss of the organism by competition (biological selection), or some other occurrence. As with the HCCP work, a decision was made to spend the remaining effort on PCP; further work on MP was halted.

Batch Tests

Only a limited number of batch tests were conducted. The majority of these tests was designed to determine the best level of a bacteriostatic agent to control the growth of competing bacteria in the degradation of PCP by a fungus (organism 044). Sodium azide (NaN_3) is known to adversely affect the cell wall of bacteria (and thus lead to lysis and lack of growth) but was not known to affect the growth of fungi. The batch tests were run in aerated 40-liter tanks containing basic salts (pH 8.0), 0.1% dextrose, 100 ppm PCP, and NaN_3 ranging from 0.1% to 0.002%. No azide was added to the controls. About 0.5 g of PCP was added to each tank daily, along with dextrose and medium, as required. The data showed that 0.002% NaN_3 was an appropriate concentration of bacteriostatic agent for the pilot-scale PCP studies where the biodegrading organism was the fungus 044.

PCP Pilot-Scale Tests

The screening studies on PCP, HCCP, and MP revealed that PCP was the most acceptable choice for pilot studies. Further, there was interest in determining to what extent a biodegradation process might be applicable to an actual release/spill situation involving PCP and fuel oil in soil.

The pilot scale unit (Figures 1, 2, and 3) was fabricated from methyl methacrylate sheets and divided into four chambers that serially overflowed from the first to the second, etc. The unit was tightly sealed, as was the feedstock tank. Each chamber contained 24 liters (6 gal) of medium and was equipped with wands for introducing filtered air for aeration and mixing. Peristaltic pumps were used to meter the feed and to proportion the sludge return. Typical pumping rates were 0.25 gal/hr of feed and 0.1 gal/hr of sludge return. The chambers were connected by weirs; the overflow (exit) pipe extended to the top of the fourth chamber. Depending on the design of each test and on changes in protocol during the tests (which were conducted over a period of 2 weeks each), the residence time in the system ranged from 40 to 100 hours. The chambers were routinely monitored for temperature, DO, and pH.

The pilot-scale system was first checked by filling the storage chamber and tanks with basic salts and 0.1% dextrose and then inoculating the chambers with bacterium 016 (800, 500, 300, and 300 ml inoculum in chambers 1 through 4, respectively). [Organism 016 forms "puff balls" that are easily detected (Figure 3).] The air flow and fluid movement patterns were observed and some modifications in design were made. In the second run, the system was cleaned, refilled, and again inoculated with organism 016. Percent BOD_5 reduction was measured in each of the four chambers. Note that this is a flow-through system and processed about 54 gal/day. The initial DO (feed) was about 2 ppm, but decreased to about 0.5 ppm in chambers 1 and 2 until rising to 5 to 6 ppm in chambers 3 and 4. BOD of the feed was about 2000 ppm. Equilibrium BOD_5 reduction in the chambers was 34%, 70%, 92%, and 93%, respectively (cumulative percents).

Four pilot-scale tests were performed with PCP. The medium containing dissolved and possibly suspended PCP, as well as PCP in the biomass, was sampled. Extracts were processed and derivatized for quantitation by GC-EC. Separate analyses were performed to correct

percent-removed for loss by settling or by incorporation in the biomass.

In pilot test no. 1, the feed contained 100 ppm of PCP at a pH of 8.5. The inoculum was the fungus 044. An anti-foaming agent was added. As the degradation proceeded, the pH dropped and the rate of degradation decreased. (The solubility of PCP is much greater in basic solutions.) The maximum percent-removal (corrected for bioaccumulated PCP) was over 90%. Attempts to regain the high degradation rate were not successful.

In the second test, similar conditions were used and data obtained. Acid metabolites lowered the pH and precipitated quantities of PCP, such that the total PCP level in the sludge sometimes approached 500 ppm. Note that the pilot tests were performed in continuous, flow-through reactors and that biomass (sludge) had to be partly voided and partly reintroduced.

In the third test, 0.002% NaN_3 (bacteriostat) was added. The system malfunctioned and, after the problem was corrected, reinoculation (on days 5 and 10 of the 14-day test) resulted in 70% disappearance.

In test no. 4, organism 041 (bacterium) was evaluated. Unfortunately, the feed tank developed overwhelming bacterial contamination. A new feed system was developed. Changes were also made in the nutrient (yeast extract was added to the dextrose) and in the basic salts medium (NH_4NO_3 was eliminated and replaced by yeast extract). The recycling of biomass was halted. The analysis showed unacceptable variability (the biomass, for example, formed an emulsion with the hexane extractant). The system may have achieved greater than 70% disappearance of the PCP. The pilot work showed definite promise, but had to be abandoned (limited funding).

Under the best conditions during limited experiments with PCP in a flow-through, pilot-scale test chamber, the percent-disappearance (corrected for assimilation and precipitation) was about 95%. The residence time in these experiments ranged from 40 to 100 hr.

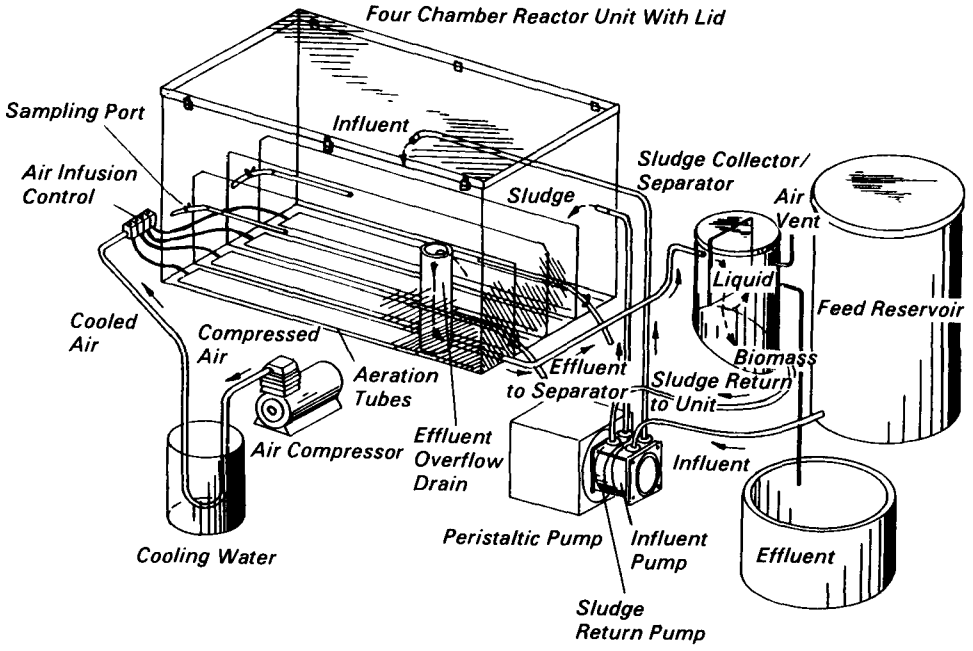

Figure 1. *Schematic diagram of continuous pilot-scale reactor.*

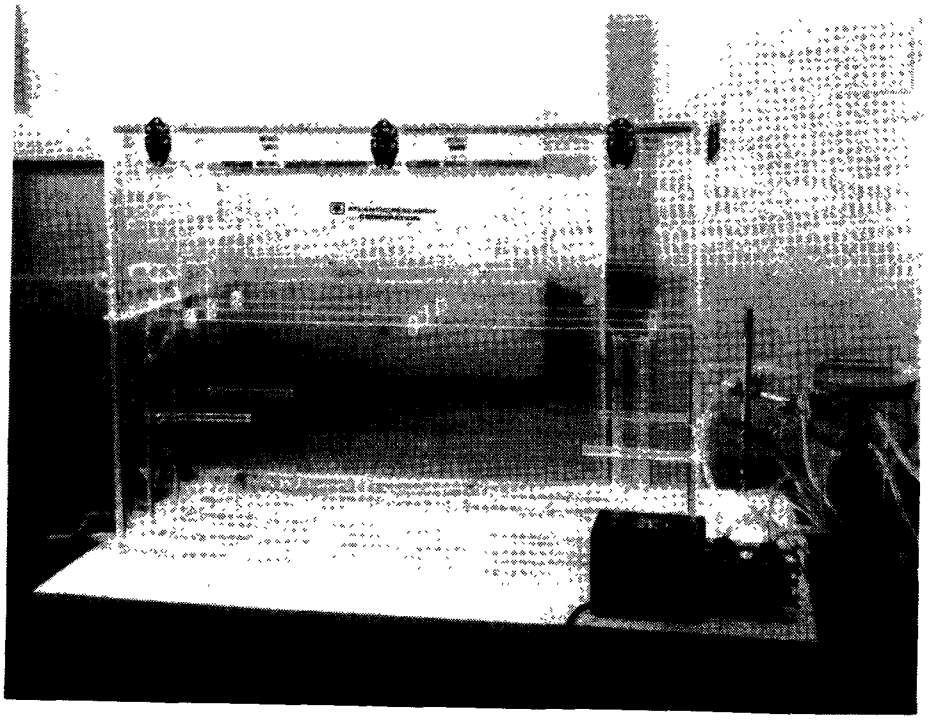

Figure 2. *Continuous pilot plant unit.*

PCP-Spill-Contaminated Soil Tests

Over a period of years more than a decade ago, a spent mixture of PCP+fuel oil, used as a wood preservative, was routinely pumped into a 25-ft-deep well at a plant in Haverford, PA. The material migrated through the soil, entered an unused, deeply buried drainage pipe, and polluted a small stream at a housing development about one-half mile away.

The leachate is being collected and processed but the question remains as to whether any pretreatment in the soil itself might be advantageous.

Microbial test cultures were taken from the disposal-well bottom liquid, from a test boring about 300 ft distant, and from the leachate into the stream. The well

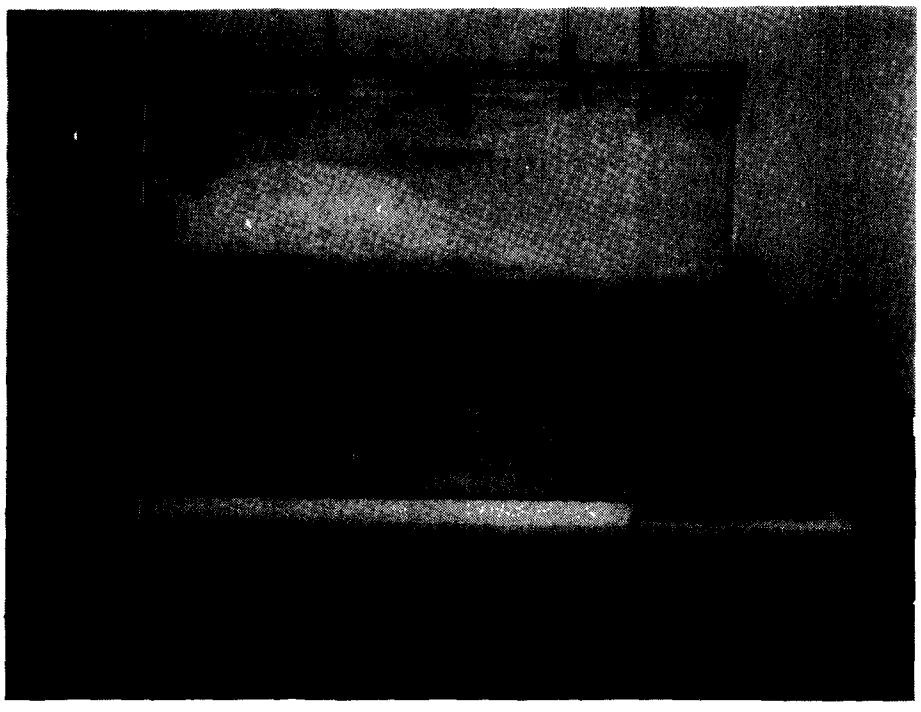

Figure 3. Continuous pilot plant unit on 8th day of operation with organism 016 (fungus) and food (no pollutant).

culture showed only 2 colony-forming units (cfu)/ml, but the other sites showed levels of 8 x 10^4 and 3 x 10^5 cfu/ml, respectively. The first sample was essentially sterile. The others showed high levels of bacteria but almost no fungi or actinomycetes, which commonly account for 50% of the cfu's in (uncontaminated) soil.

Lab tests with bacterium 041 and fungus 044 were conducted in beakers on separate sterile samples of sand and of soil that had been contaminated with levels of the PCP-oil mixture (about 1% PCP in oil). The sand or soil samples weighed 200 g and the fraction of PCP+oil added ranged from 0 (control) to 30 ml (15% v:w). Basic salts medium with 0.2% dextrose was added. Both bacterium 041 and fungus 044 grew in the PCP-free control.

At levels of about 1% PCP+oil in the test beakers (100-300 ppm PCP in sand), bacterial growth was strong but fungal growth (044) was somewhat inhibited. Tests with sterile soil, repeated up to the 2% level, showed that growth rate decreased with increasing PCP+oil level. Subcultures (agar and also liquid medium) were taken after 4 days of incubation.

The data showed that the growth of fungus 044 was significantly inhibited at PCP+oil levels above 0.5% (v:w) but that bacterium 041 was more tolerant with severe inhibition, only occurring beyond the 2% level.

Determination of the extent of degradation or of any byproducts or metabolites formed could not be undertaken (funding and time constraints).

It is surmised that bacterium 041 and even fungus 044 could possibly be adapted to consume the PCP+oil contaminant if aerobic growth were initiated at the fringe of the contaminated area at Haverford and if nutrients and possibly some food other than the oil (e.g., dextrose) were provided.

Conclusions

The following conclusions are drawn from this study:

1. The limited solubility of PCP, HCCP, and MP in water makes it difficult to evaluate microbial assimilation, or biodegradation, or both in an aqueous system.
2. Only tentative evidence for biodegradation of PCP, HCCP, or MP by pure culture organisms was obtained in these studies from shaker table tests.
3. Data indicate that a fungal isolate (044) has potential for assimilation of PCP and may cause degradation. A bacterial isolate (041) appeared to reduce the PCP concentration by 20% to 42% when dextrose was provided as a carbon source.
4. A flow-through bioreactor (fungal isolate 044, aerobic, pH 8.5, with nutrients and dextrose added, and having a residence time of ca. 50 hours) achieved 95% disappearance of PCP (100 ppm) corrected for bioaccumulation and precipitation and has putative potential for the degradation of PCP and other organochlorine compounds. In severely limited and incomplete analytical work, no byproducts or partial decomposition products (metabolites) of PCP were detected.
5. The bacterial isolate (041) tolerated higher concentrations of PCP+oil in sandy soil than did the fungal isolate (044). The bacterial isolate may have greater potential than the fungal isolate for removal of PCP from spill-contaminated areas.
6. The amount of HCCP that disappeared from aqueous suspensions was increased by the presence of several of the pure culture organisms when compared to the controls. Based on very limited analyses, no chlorinated byproducts were observed in the aqueous medium assayed for HCCP removal by microorganisms.
7. Methyl parathion studies indicated that two organisms (bacteria 003 and 021) may have potential for removal of this pesticide when a carbon source, such as dextrose, is provided.

Recommendations

Based on the results of the study, the following recommendations are made:

1. Bacterium 041 should be further evaluated for potential use in the decontamination of PCP/oil spill sites.
2. Adaptation of organisms for removal of PCP, HCCP, and MP should be evaluated.
3. Future biodegradation studies in this area should use C-14-labeled compounds or follow other accepted procedure to facilitate isolation and/

or identification of metabolites (and byproducts) and elucidation of mechanisms of biotransformation or biodegradation.

4. Analytical protocols should be developed to provide reproducible, if not 100%, recovery levels of challenging compounds (such as HCCP and PCP) from mixtures with fungi and bacteria. (Such mixtures have a tendency to promote emulsification and to yield poor separations with extractants.)
5. Attempts should be made where practical to identify effective organisms minimally by genus and preferably by species.

The full report was submitted in fulfillment of Contract No. 68-03-2491 by Atlantic Research Corporation under the sponsorship of the U.S. Environmental Protection Agency.

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The complete report, entitled "Microbial Degradation of Selected Hazardous Materials: Pentachlorophenol, Hexachlorocyclopentadiene, and Methyl Parathion," (Order No. PB 84-123 934; Cost: $11.50, subject to change) will be available only from:
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