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Comparison of Three Bioremediation Agents for Mineralization and Transformation of Pentachlorophenol in Soil

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Bacteria and fungi have been proposed as bioremediation agents for soil polluted with pentachlorophenol (PCP), and have been studied separately under various conditions (McAllister et al., 1996). Extent of PCP degradation by particular organisms in soil is affected by such factors as temperature, soil moisture, oxygen availability, soil organic matter, and nitrogen availability (Leštan et al., 1996; Okeke et al., 1996; Seech et al., 1991). Under some but not all soil conditions, white-rot fungi may produce transformation products from PCP, including pentachloroanisole (PCA) (Leštan et al., 1996), and non-extractable materials that may be products of polymerization with humic acids (Ruttimann-Johnson and Lamar, 1996). Bacterial bioremediation agents generally mineralize a much greater proportion of PCP than do fungal agents (McAllister et al., 1996). And, although radiotracer studies have indicated that PCP mineralization by bacteria in soil is not complete (Seech et al., 1991), bacterial production of non-extractable products from PCP in soil has not been documented. Because rate and extent of PCP degradation, as well as formation of transformation products, can differ among PCP-metabolizing microbes and can be influenced by soil conditions, the study described below compares the activity under similar soil conditions of two bacteria (strains of *Pseudomonas* and *Flavobacterium)* and a fungus *(Phanerochaete sordida)* with respect to laboratory-scale bioremediation of a sandy, low-organic matter soil that was amended with known quantities of radiolabelled PCP.

MATERIALS AND METHODS

Analytical grade PCP (> 98% pure, dioxin-free) was from Aldrich Chemical Co. (Milwaukee, WI). Uniformly ^{14}C-labeled PCP (specific activity, 7.9 mCi/mmol; purity 98%) was from Sigma Chemical Co. (St. Louis, MO) as a toluene solution. All other chemicals were reagent- or HPLC-grade.

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Gardone soil (sandy, mixed, frigid Aridic haploxeroll), containing 68.1% sand, 29.4% silt, 2.5% clay and 1.3% organic matter with a pH of 6.2, was used. Thirty g soil (dry weight equivalent) was placed into each microcosm bottle (see below) and amended with PCP 1 wk prior to experiments. For most treatments, the soil was amended to 175 ppm (wt PCP/wt dry soil) by adding 5.25 mg PCP in 0.82 mL 65% ethanol to the soil in each bottle. The PCP solution was spiked with 2.77 µCi of ^{14}C-PCP (0.0082 mL in toluene). Because preliminary experiments suggested that the *Flavobacterium* strain was sensitive to 175 ppm PCP, soil for this treatment and its control was amended at 125 ppm PCP, spiked with 1.98 µCi ^{14}C-PCP per 30 g soil. Bottles were then capped for 6 days with daily opening for mixing and solvent evaporation. Subsamples (5 g) taken separately from each bottle after mixing showed little variation in PCP level, indicating that the test compound was homogeneously mixed into the soil.

Individual microcosms were assembled from borosilicate glass bottles (150 mL) wrapped in foil to minimize PCP photodegradation. Each bottle had a Teflon-lined screw cap with two 12-gauge stainless steel needles connected to Teflon-lined tubing for periodic aeration. At 7-day intervals, microcosms were aerated with moist air drawn through the tubing by vacuum pump. Out-going air was passed first through a series of two traps containing Tenax (Chromatography Research Supplies, Addison, IL) to collect ^{14}C-labeled volatile organic compounds, then through a series of two glass vials containing 2:l (v/v) monoethanolamine-ethylene glycol to collect CO_2. Except during aeration, the tubing was clamped to prevent passage of volatiles.

Our objective was to compare examples of organisms that had been demonstrated to degrade PCP relatively quickly. Accordingly we chose two bacterial strains and a fungus species that had shown good degradation of PCP in soil or soil-associated systems. The bacterial bioremediation agents tested were *Pseudomonas* strain SR3 and *Flavobacterium* ATCC53874. A recent study by Karlson et al (1995) proposes that these two bacterial strains be transferred to a species in the genus *Sphingomonas* or *Rhizomonas. Pseudomonas* SR3 (Resnick and Chapman, 1994) was cultured in minimal salts broth with yeast extract (1.0 g/L) and glucose (0.2 g/L) at 30°C with shaking (150 rpm). At mid-log phase (20 hr) it was induced by adding 25 ppm PCP (in 0.5 N NaOH). After 6 hr an additional 50 ppm PCP was added and the culture was grown for 14-16 hr until the PCP was reduced to a non-detectable level as determined by HPLC. lnoculum was added to the soil at 5 X 10^6 cfu/g soil (dry weight equivalent) in an aqueous sodium glutamate solution (4 g/L). Final soil moisture was 28% (wt water/wt dry soil) (water potential = -5 KPa).

Flavobacterium sp. (ATCC #53874, a replacement deposit for the original ATCC #39723) (Saber and Crawford, 1985) was microencapsulated in calcium alginate (Crawford et al, 1990) before adding to soil, because preliminary experiments indicated that it might survive poorly when added directly to PCP-contaminated soil. One mL of the microbead slurry was added to 30 g soil for each *Flavobacterium* treatment replicate, producing a cell density of 5×10^6 cfu/g soil. Soil moisture content was adjusted to 28%. *P. sordida* was obtained as mycelial spawn from L. F. Lambert Spawn Co. (Coatesville, PA) and stored at 4°C. The strain had originally been supplied to Lambert by USDA Forest Products Laboratory, Madison, WI. The medium for fungus growth was alder sawdust and ground millet seed (4:1, w/w) milled to pass a #50 screen, moistened with water (3 mL water/g dry weight), and autoclaved two times 24 hr apart. The culture was allowed to grow undisturbed for 7 days at 23°C in the dark prior to adding it to the soil at a rate of 5% (dry wt inoculum/dry wt soil).

Seven experimental treatments included three bioremediation treatments and four controls: *Pseudomonas* strain SR3 and its sodium glutamate control; *Flavobacterium* and its calcium alginate microbead control: *P. sordida* and its control of alder sawdust and millet; and a control amended with water only. Microcosm bottles were incubated with aeration (described previously), and mixed once at 14 days of incubation, during the first sampling (see below). Otherwise, the soil was not disturbed. Three replicate microcosms per treatment were arranged in a completely randomized design in a covered water bath at 28°C. Bacterial treatments were incubated for 42 days; the *P. sordida* treatment and sawdust check were incubated for 56 days. Prior reports (Lamar et al, 1993; Seech et al, 1991) showed that these times were adequate in each case for the organism to degrade most of the PCP present.

For PCP extraction, wet soil (equivalent to 5 g dry weight) was placed in a scintillation vial with 15 mL dichloromethane (DCM), mixed thoroughly and kept at 4°C for 1 wk. The DCM layer was removed and the partially-extracted soil was transferred into cellulose thimbles (10 X 50 mm; Whatman) for soxhlett extraction (4 hr at 75°C) in 4:1 (v/v) acidified (pH = 2) methanol:hexane. Extracts were treated with K_2SO_4 and then concentrated and exchanged into 5.0 mL methanol using modified Kuderna-Danish glassware.

Analysis was done on a Spectra-Physics 8800 HPLC system with a spectral array UV detector, an EG&G/Berthold LB-507-A radioactivity detector, and FOCUS software. The isocratic mobile phase was 60:40

(v/v) CH_3CN:1% H_3PO_4 at a flow rate of 0.5 mL/min used with an EnviroSep-PP column (175 mm x 3.2 mm, Phenomonex), PCP and PCA were monitored by UV absorption at 220, 245 and 280 nm to allow for peak purity determinations. The radioisotope detector enabled detection of PCP degradation products (if any) in chromatogram peaks.

^{14}C-PCP-contaminated soil (0.5 g dry weight equivalent), sandwiched between two combusto-pads (Packard) in a paper thimble, was combusted in a Packard Model DO306 Tri-carb sample oxidizer modified to sustain a higher burn temperature. Radioactivity trapped in Carbosorb + Permafluor scintillation cocktails (Packard) was quantified by liquid scintillation counting (LSC). PCP mineralized to CO_2 was quantitated by adding 500 µL of each CO_2-trapping solution (see above) to 15 mL of Permafluor scintillation cocktail and analyzing by LSC. Tenax traps for capturing volatile organic compounds were eluted first with methanol, then with toluene; eluates were added to Permafluor scintillation cocktail for LSC. Organic extracts awaiting HPLC analysis were stored at -2O°C, extracted soils awaiting oxidation were kept at 4°C and pre-extraction soil samples were stored at -80°C. Data were analyzed for homogeneous groups and analysis of variance using Statistix 3.1 (NH Analytical Software, Roseville, MN) with a rejection level of 0.05. The entire experiment was performed twice with similar results.

RESULTS AND DISCUSSION

Over 65% of the available ^{14}C-PCP was recovered as ^{14}C-CO_2 from soil treated with *Pseudomonas* SR3 or *Flavobacterium,* and most of the mineralization occurred in the first 4 days (Fig. 1). The respective control treatments yielded only 0.2% of the initial ^{14}C-PCP as ^{14}C-CO_2 Percent recovery of ^{14}C-PCP as ^{14}C-CO_2 from the *P. sordida* treatment was 5.1% by the end of the experiment (56 days) (Fig. 11, significantly higher ($p = 0.02$) than the 0.3% mineralization observed in the sawdust control treatment.

No extractable transformation products were recovered from the *Pseudomonas* or *Flavobacterium* treatments, as determined by radioisotope detection in the HPLC procedure. Remaining extractable PCP was 1-3% of initial levels. In each of these treatments 12-14% of the initial ^{14}C-PCP was converted to non-extractable material in the soil (Fig. 2A, C), as determined by combustion of post-extraction soil samples. *P. sordida* transformed much of the labeled PCP to PCA (Fig. 2E) under our experimental conditions. Recovery of the ^{14}C-PCA was 52% of the initial PCP by day 14 and 62% by day 56. The sawdust

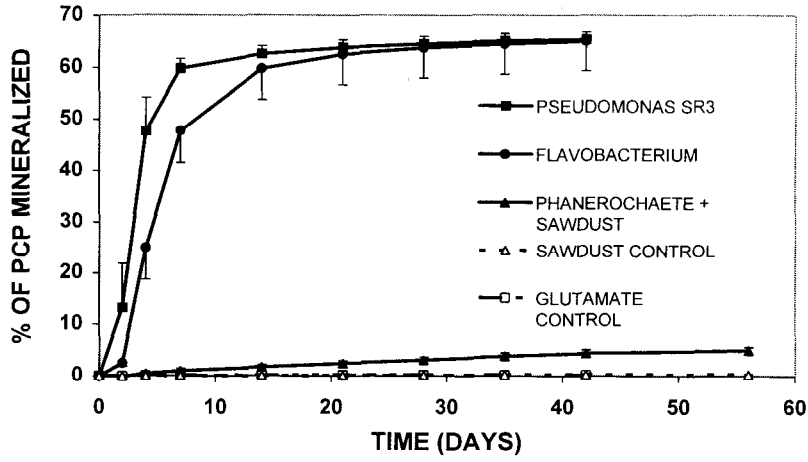

Figure 1. Time course of PCP mineralization (percent of initial ^{14}C-PCP recovered as ^{14}C-CO_2) in microcosms treated with bioremediation microbes. Initial levels of PCP in soil were 175 ppm for *Pseudomonas* and *Phanerochaete (P. sordida),* 125 ppm for *Flavobacterium.* Error bars are + or - one standard error of the mean.

control treatment transformed 0.5% of the initial radiolabel to PCA by the end of the experiment. As in the bacterial treatments, some of the initial radiolabel in the *P. sordida* treatment was present as non-extractable material in the soil at the end of the experiment. Statistical analysis by linear contrast showed the 17% nonextractable proportion in the *P. sordida* treatment to be significantly ($p = 0.04$) greater than the average non-extractable proportion in the bacterial treatments (12% and 14% for *Flavobacterium* and *Pseudomonas,* respectively).

Very little of the radiolabel was recovered from the Tenax resin traps (0.01 % of the initial PCP dose from control treatments, 0.002% from bacterial treatments, and 0.15% from the fungal treatment), indicating that volatilization of PCP or any of its organic metabolic products from soil was low under our conditions. Similarly, less than 1% of the radiolabel was recovered from glassware and tubing by extraction with methanol. Approximately 80% of the ^{14}C in the original ^{14}C-PCP was recovered from each of the soil treatments which had been inoculated with bacteria, 87% from the *P. sordida*-inoculated treatment, and 100 ± 6% from the control treatments.

The rate of PCP degradation in soil, and production and persistence of transformation products, by bioremediation organisms varies with

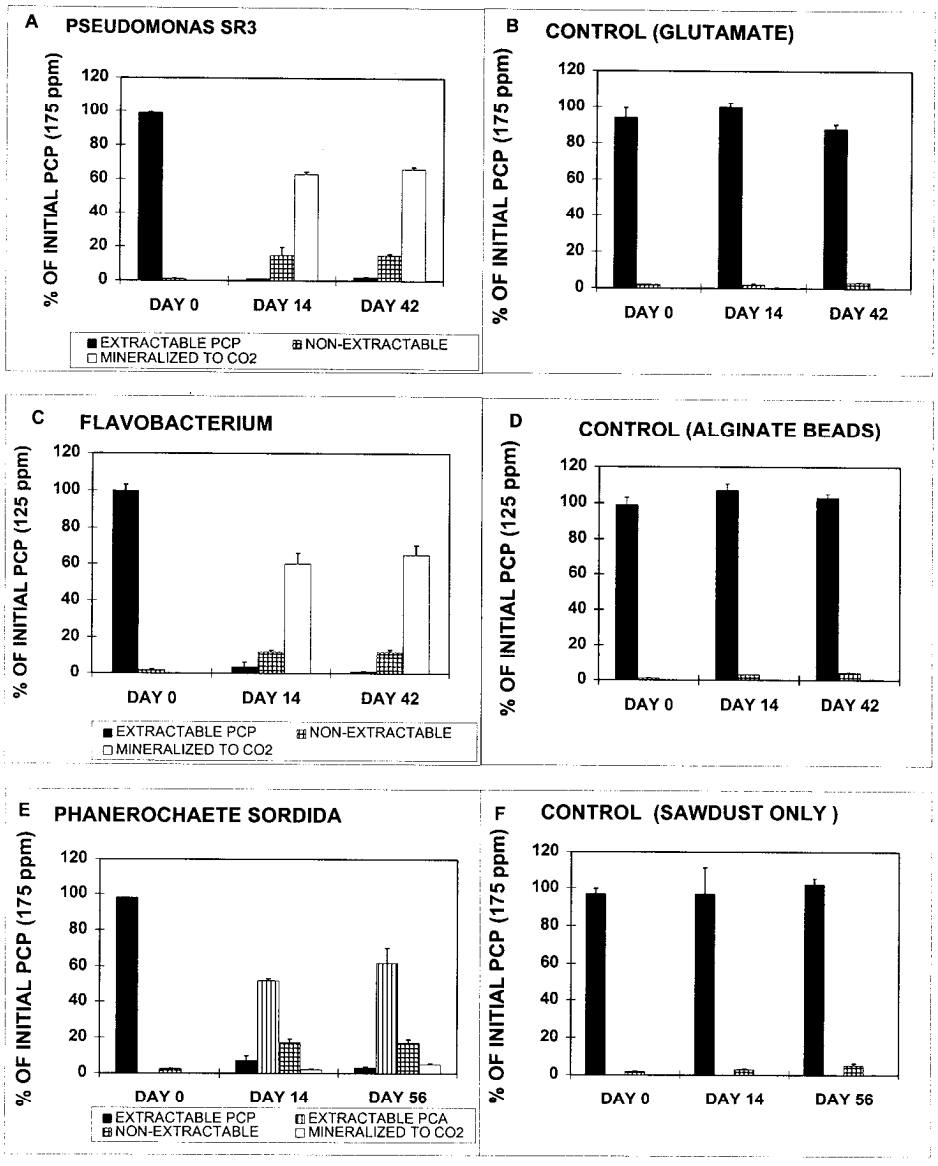

Figure 2. Fate of PCP in microcosms inoculated with bioremediation organisms (A,C,E) and the respective controls (B,D,F). Components measured were: PCP recoverable by soxhlett extraction, PCA recoverable by soxhlett extraction, non-extractable radiolabel measured as total ^{14}C recovered from combustion of post-extraction soil, and ^{14}C-CO_2 recovered from microcosm headspace. Volatile organic component (not shown) was less than 1% of initial radiolabel in all cases.

physical and chemical aspects of the soil environment (McAllister et al., 1996). Under our experimental conditions (sandy soil, adequate moisture, moderately warm temperatures, atmospheric oxygen level) the two bioremediation bacteria acted quickly to reduce the pentachlorophenol in the soil. They mineralized approximately 65% of the initial PCP leaving little PCP in the soil after 42 days. These bacteria left 12-14% of the initial radiolabel from PCP as non-extractable products in the soil. There has been no prior experimental verification or quantification of non-extractable PCP metabolites in soil exposed to PCP-degrading bacteria. Our results with respect to percent mineralization of PCP by *Flavobacterium* in soil are in agreement with a previously published report (Seech et al., 1991); although *Pseudomonas* strain SR3 is known to degrade PCP in liquid culture (Resnick and Chapman, 1994) there are no previous reports of PCP mineralization by SR3 in soil.

P. sordida also produced a relatively rapid decrease of PCP in soil under our experimental conditions. The PCP decrease was almost as rapid as that caused by bacteria, but mineralization of PCP by the fungus was minimal. There have been relatively few studies documenting mineralization of PCP by *Phanerochaete* species in soil (McAllister et al., 1996), and most have shown a similarly low extent of mineralization. An exception is the study by Chung and Aust (1995) in which 18% of the PCP in soil was mineralized; however, their microcosms were periodically purged with pure oxygen. Other studies with *Phanerochaete* species have demonstrated that transformation products such as PCA may be produced in some soils and under certain conditions. The production of PCA, and its subsequent mineralization, by *P. chrysosporium* appear to depend in part on such factors as nitrogen availability or temperature (Leštan et al., 1996). The species of *Phanerochaete,* and amount of inoculum per soil weight, also affect PCA metabolism (Lamar et al., 1993). Under our conditions however, PCA was the major product of PCP transformation and was not mineralized during the 56 day incubation period. Except for the study by Chung and Aust (19951, performed with pure oxygen, we found no previous reports that verify and quantify the amount of PCP converted to non-extractable products in the soil by *Phanerochaete* species. We observed that 17% of the initial PCP radiolabel was converted to such products. Non-extractable PCP transformation products were not characterized, because they could not be extracted for analysis. Such products could be in the form of polymers formed with humic acid (Ruttimann-Johnson and Lamar, 1996), as has been observed with *Phanerochaete* species. It is possible that for the bacteria, and perhaps for the fungus, these non-extractable products are organic-insoluble constituents of microbial

biomass produced through metabolism of the PCP. Other studies would be useful to assess the possible bioavailability or ecological hazard, if any, of transformation products from PCP bioremediation in soil.

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REFERENCES

Chung N, Aust SD (1995) Degradation of pentachlorophenol in soil by *Phanerochaete chrysosporium.* J Haz Mat 41:177-183

Crawford R, O'Reilly K, Tao H (1990) Microorganism stabilization for *in situ* degradation of toxic chemicals. In: Kamely, Chakrabarty, Omenn, (eds.) Biotechnology and Biodegradation, Advances in Applied Biotechnology Series Vol. 4. Gulf Publishing, Houston, pp. 203-211

Karlson U, Rojo F, van Elsas J, Moore E (1995) Genetic and serological evidence for the recognition of four pentachlorophenol-degrading bacterial strains as a species of the genus *Sphingomonas.* System Appl Microbiol 18:539-548

Lamar RT, Evans JW, Glaser JA (1993) Solid-phase treatment of a pentachlorophenol-contaminated soil using lignin-degrading fungi. Environ Sci Technol 27:2566-2571

Leštan D, Leštan M, Chapelle JA, Lamar RT (1996) Biological potential of fungal inocula for bioaugmentation of contaminated soils. J Ind Microbiol 16:286-294

McAllister KA, Lee H, Trevors JT (1996) Microbial degradation of pentachlorophenol. Biodegradation 7:1-40

Okeke BC, Smith JE, Paterson A, Watson-Craik IA (1996) Influence of environmental parameters on pentachlorophenol biotransformation in soil by *Lentinula edodes* and *Phanerochaete chrysosporium.* Appl Microbiol Biotechnol 45:263-266

Resnick SM, Chapman PJ (1994) Physiological properties and substrate specificity of a pentachlorophenol-degrading *Pseudomonas* species. Biodegradation 5:47-54

Ruttimann-Johnson C, Lamar RT (1996) Polymerization of pentachlorophenol and ferulic acid by fungal extracellular lignin-degrading enzymes. Appl Environ Microbiol 62:3890-3893

Saber DL, Crawford RL (1985) Isolation and characterization of *Flavobacterium* strains that degrade pentachlorophenol. Appl Environ Microbiol 50:1512-1518

Seech AG, Trevors JT, Bulman TL (1991) Biodegradation of pentachlorophenol in soil: the response to physical, chemical and biological treatments. Can J Microbiol 37:440-444