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BINDING OF PENTACHLOROPHENOL TO HUMIC SUBSTANCES IN SOIL BY THE ACTION OF WHITE ROT FUNGI

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Summary—The fate of ^{14}C-pentachlorophenol (^{14}C-PCP) in soil inoculated with each of four wood degrading fungi was studied. After 9 wk of incubation with the fungi, the majority of the ^{14}C (between 34 and 65%) was found bound to humic (HA), fulvic acid (FA) and humin, with lesser amounts found in the organic-extractable fraction or mineralized. The highest amounts of PCP were bound to HA (20-36% , compared to 7.5-8.3% in the uninoculated controls). Binding to FA and to humin was lower, ranging between 8.7 and 17.5% and 4.8 and 11.1% , respectively, in the fungal cultures. In control experiments around 5% of the radioactivity was found associated to FA and between 2 and 4% to the humin. The highest binding of PCP to the humic materials was obtained with *Pleurotus ostreatus*, followed by *Irpex lacteus, Trametes versicolor* and *Bjerkandera adusta*. Mineralization of PCP from soil cultures by the fungi was low. The fungus that mineralized the most PCP was *T. versicolor*, converting 8.8% of ^{14}C-PCP into $^{14}CO_2$, compared to 0.6% mineralized in the uninoculated controls. Methylation of PCP to pentachloroanisole by the fungi was low, ranging between 0 and 6.8% after 9 wk. © 1997 Elsevier Science Ltd

INTRODUCTION

Humic substances are amorphous, mostly dark-colored, partly aromatic polymeric materials that are formed in soil by condensation reactions involving polyphenols and quinones. Polyphenols derived from lignin or synthesized by microorganisms are enzymatically converted to quinones, which undergo self-condensation or combine with peptides, amino sugar compounds, fatty acids, and possibly other types of molecules, to give rise to complex polymers (Stevenson, 1982). Microbial transformations occurring in the soil, such as β -oxidation of side chains with release of acetic acid, decarboxylation, demethoxylation and the formation of additional hydroxyl groups, give rise to a large variety of phenolic compounds, which are primary precursors in the formation of humic acid polymers (Flaig *et al.*, 1975). The more reactive phenolic compounds such as ortho-dihydroxy and trihydroxy phenols readily autooxidize to form polymers at pH ≥ 6 (Haider *et al.*, 1974). However, these and less reactive compounds such as syringic acid, ferulic acid, coniferyl alcohol, vanillic acid and orcinol are readily oxidized and linked into humic polymers by microbial phenol oxidases and peroxidases that are associated with soil humic colloids or are synthesized *de novo* by soil microbes, including lignin-degrading fungi (Martin *et al.*, 1975; Ladd and Butler, 1975; Sulfita and Bollag, 1981). Peroxidases and oxidases produced by fungi are able to oxidize numerous aromatic xenobiotics, including polychlorinated phenols (Hammel and Tardone, 1988; Joshi and Gold, 1993). Subsequent to the first oxidation steps, aromatic xenobiotics could either be further degraded or cross-linked to other humic matter precursors to form hybrid humic compounds. Therefore, phenolic degradation products of xenobiotics such as PCP could be considered part of the pool of precursor molecules for humic acid synthesis.

White rot fungi have been shown to cause a considerable depletion of PCP from soil in laboratory-scale experiments (Lamar *et al.*, 1990a,b) and under field conditions (Lamar and Dietrich, 1990; Lamar *et al.*, 1993). Only CO_2 and pentachloroanisole (PCA) have been identified as transformation products. These products only account for a minor part of the starting material. A large part of the PCP is transformed into nonextractable products as yet not identified. Our objective was to determine if PCP is bound to soil humic materials by white rot fungi. This type of binding is important if soil bioremediation purposes are envisaged for white rot fungi, because it might decrease or eliminate the toxicity of the PCP by limiting its availability.

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MATERIALS AND METHODS

Fungi and inoculum preparation

Pleurotus ostreatus (Jacq.:Fr.) Kummer FP-102311 sp., *Irpex lacteus* (Fr.:Fr.) Fr. Mad-517, *Bjerkandera adusta* (Wild.:Fr.) Karst FP-135160-sp. and *Trametes versicolor* (L.:Fr.) Pilat MD-277 were obtained from the culture collection at the Center for Forest Mycology Research at the Forest Products Laboratory, Madison, WI. They were maintained in malt agar slants at 4°C. Inocula were prepared by growing each fungus separately on a nutrient-fortified grain-sawdust mixture (proprietary formulation of L.F. Lambert Spawn Co., Inc., Coatesville, PA, referred to hereafter as "spawn"). The spawn was autoclaved three times for 1 h on consecutive days, brought to 60% moisture with sterile deionized, distilled water, inoculated with slants of each fungus and incubated at 30°C for 1 wk.

Soil

Marshan sandy loam (fine-silty, mixed, mesic, Mollic-Hapludalf) was collected from the A-horizon, air dried, sieved and stored in plastic bags at 4°C. Characteristics of this soil were reported by Lamar *et al.* (1990a). Prior to culture preparation the soil was sterilized by fumigation with methylbromide (Lamar *et al.*, 1990a).

Soil cultures

Soil cultures were prepared using Marshan soil contaminated with a mixture of ^{14}C labeled- and unlabeled-PCP. Transformation of PCP by *I. lacteus, T. versicolor* and *B. adusta* was evaluated in one experiment. Transformation by *P. ostreatus* was evaluated in a second experiment. The conditions of the second experiment differed slightly, as noted below, from the conditions used for the rest of the fungi. Pentachlorophenol was added to the soil in an acetone solution, under sterile conditions. The soil was mixed thoroughly and the acetone was allowed to evaporate. The final concentration of PCP was 75 μg g^{-1} soil (dry weight basis) for all cultures, except for *P. ostreatus,* for which the final concentration was 100 μg g^{-1}. For *I. lacteus, B. adusta* and *T. versicolor* cultures the moisture content of the soil was adjusted to 48% with sterile, deionzed, distilled H_2O. For *P. ostreatus* cultures the soil was adjusted to 39% moisture. Cultures were prepared in 125 ml Erlenmeyer flasks containing 30g (wet wt) of contaminated soil. ^{14}C-Pentachlorophenol (58127 dis min^{-1} per flask in *P. ostreatus* cultures and 431545 per flask for the rest of the fungi) was added to each flask in an acetone solution and mixed well with the soil. After allowing the acetone to evaporate, the soil in each flask was inoculated by mixing in spawn-supporting growth of *I. lacteus, B. adusta, T. versicolor* or *P. ostreatus* at a rate of 10% (dry wt of spawn to dry wt of soil). Sterile spawn was added at the same rate to prepare control cultures. All experiments were prepared in triplicate. Finally, the flasks were fitted with inlet–outlet gas ports to allow for aeration and for flushing and trapping of $^{14}CO_2$. Cultures were incubated at 30°C and flushed every 2d with air that had been bubbled through water. The evolved $^{14}CO_2$ was trapped in ethanolamine containing scintillation fluid (Keyser *et al.*, 1978) and counted in a 1214 Rackbeta scintillation counter (LKB Wallac, San Francisco, CA). It has been shown that trapping of $^{14}CO_2$ by this method is quantitative (Kirk *et al.*, 1975). *Irpex lacteus, B. adusta* and *T. versicolor* cultures were incubated for 9 wk and *P. ostreatus* cultures for 4 wk.

PCP extractions and quantification

After incubation, the soil from each flask was transferred to a polypropylene 200 ml centrifuge bottle. Unreacted PCP and other extractable transformation products were extracted twice for 1 h in a rotating tumbler shaker with 100 ml of a mixture of hexane-acetone (1/1) acidified to pH 2 with H_2SO_4. Extractions were done under N_2. The residual soil was left in the centrifuge bottles for subsequent extraction steps (described below). Aliquots (1 ml) of the hexane-acetone extract were counted in a scintillation counter in 10 ml of polyfluor scintillation fluid. The amounts of PCP and pentachloroanisole (PCA) present in the extracts were evaluated by gas chromatography (GC). Forty millilitre aliquots of the hexace extracts were dried by passing them through Na_2SO_4 columns. The columns were washed with 5.0 ml hexane before and after passing the extracts. For each sample the hexace extract and the second wash were combined and evaporated under N_2 in a TurboVap LV (Zymarck Corporation) to approximately 5.0 ml. The residual extracts were filtered through Milex SR 0.5 μm filters (Millipore, Bedford, MA) into 10 ml volumetric flasks and the volume adjusted to 10.0 ml with hexane. All glassware and the Na_2SO_4 were muffled for 4 h at 450°C before use. Pentachlorophenol was analyzed by GC as its trimethylsilyl derivative and PCA was analyzed without derivatization as described by Lamar *et al.* (1993). Both compounds were quantitated using authentic standards. In the case of PCP the standard was derivatized with N,O-*bis* (trimethylsilyl) acetamide (Sulpeco). Analyses were performed on a Hewlett Packard Model 5890A GC equipped with a 63Ni e.c.d. and a 30 m × 0.321 mm DB-5 fused silica capillary column. All conditions were as described by Lamar *et al.* (1993).

Humic material extractions

Fulvic acid (FA) and humic acid (HA) were extracted from the organic-extracted soil. Fulvic

acid was extracted using the procedure described by Gregor and Powell (1986). Ninety millilitres of sodium pyrophosphate (0.1 M, PH 2.0) were added to centrifuge bottles containing the soil. The bottles were shaken under N_2 in a rotating tumbler shaker for 24 h. The FA extract was separated from the residual soil by centrifugation at 8K rev min^{-1} in a GSA rotor for 30 min using a Sorvall centrifuge. The residual soil was further extracted with NaOH 0.5 M (90 ml per bottle) under N_2 by shaking in a rotating tumbler shaker for 24 h. The HA extract was separated by centrifugation as described for the FA. Fulvic acid and HA were obtained by freeze drying of the corresponding solutions and weighed. The residual soil containing the humin was dried, mixed well and kept for combustion.

Combustions

The radioactivity present in the HA, FA, and that associated with the humin in the residual soil fraction was estimated by combustion. Approximately 0.2 g samples of these fractions were combusted. Combustion were done in duplicate using a Harvey Biological Oxidizer model OX 600 (R.J. Harvey Instrument Co., Hillside, NJ). The efficiency of the oxidations was determined by assessing recovery of ^{14}C from combustions of equivalent amounts of unlabeled FA or HA that were spiked with a standard solution of known radioactivity. The efficiencies for humin oxidations were determined by combusting an equivalent amount of Batavia soil (Lamar *et al.*, 1990a), instead of unlabeled humin, spiked with a known amount of radioactivity. Results were corrected for combustion efficiency.

RESULTS

Mineralization of PCP in soil cultures

Mineralization of PCP from all fungal cultures was significantly greater than that from control cultures, except for cultures inoculated with *B. adusta* (Table 1). *Trametes versicolor* and *I. lacteus* mineralized the greatest amount of PCP, converting 7.2 and 8.8% of the PCP to CO_2 after 9 wk. respectively. Although significantly greater than the control, mineralization of PCP by *P. ostreatus* was low (Table 1). Mineralization rates were greatest between days 10 and 25 (Fig. 1). After this time mineralization continued, but at a slower rate.

Binding of PCP to humic material

Substantial ^{14}C was found in the humic material fractions extracted from the soil of the fungal cultures (Table 1). In contrast, most of the recovered ^{14}C in control cultures was found in the organic extract. In fungal cultures, most of the radioactive label was found in the HA fraction. Between 20.3 and 36.0% of the PCP was bound to this fraction by the fungal strains, compared to only 7.5-8.3% in the uninoculated controls. Binding of PCP to FA was lower than to HA and ranged between 8.7 *(B. adusta)* and 17.5% *(P. ostreatus)*, compared to 4.9-5.2% in the controls. The higher percentage of label associated with HA does not simply reflect a higher amount of HA than of FA in the soil. The total amount of FA extracted from the soil was 0.119 g g^{-1} dry wt soil compared to 0.148 g g^{-1} dry wt soil for HA. The HA-to-FA ratio of the soil was therefore 1.24. The ratio of ^{14}C bound to these fractions by the fungi tested varied between 1.96 and 2.33. Thus, PCP was preferentially bound to HA. The fraction of soil remaining after the extraction of FA and HA contains inorganic components (e.g. clay) and nonextractable organic matter, called humin. Humin is similar in structure to HA, but it is strongly chelated to metals and bound to clay, becoming insoluble (Schnitzer, 1991). The amount of PCP bound to this fraction by the fungal strains was low (between 5 and 11% of the label). The proportion of label bound to humin by *B. adusta* was not significantly different to the uninoculated control. The highest binding of PCP to all three humic matter fractions was obtained with *P. ostreatus*, followed by *I. lacteus* and *T. versicolor*. The results of ^{14}C associated with the different fractions and mineralized for the latter two fungi were never significantly different.

Table 1. Percentage recoveries of ^{14}C as $^{14}CO_2$, in a hexane–acetone extract, and bound to humic material fractions after fungal growth

	Percentage ^{14}C in:					
Fungus	CO_2	Hexane extract	FA	HA	Humin	Total
Control	0.6 (0.3) a	59.1 (6.9) a	5.2 (1.0) a	8.3 (1.4) a	3.9 (0.8) a	77.1 (4.2) a
I. lacteus	7.2 (±1.6) b	16.1 (1.1) b	13.6 (1.2) b	26.2 (0.2) b	5.7 (0.4) b	68.8 (3.1) a
B. adusta	3.3 (0.2) a,b	31.4 (3.6) c	8.7 (0.2) c	20.3 (1.4) c	4.8 (0.3) a,b	68.5 (2.0) a
T.versicolor	8.8 (4.1) b	8.5 (1.7) d	11.8 (0.6) b	23.2 (0.1) b,c	6.1 (0.5) b	58.4 (8.2) a
Control*	0.2 (0.1) a	77.9 (6.0) a	4.9 (2.0) a	7.5 (0.8) a	2.2 (0.4) a	92.7 (5.2) a
*P. ostreatus**	0.9 (0.3) b	7.4 (0.4) b	17.5 (1.2) b	36.0 (0.7) b	11.1 (0.5) b	72.9 (1.2) b

*The last two rows represent a separate experiment. *Irpex lacteus*, *B. adusta* and *T. versicolor* cultures were incubated for 9 wk. *Pleurotus ostreatus* cultures were incubated for 4 wk.

Values are means (SD) of three replicate determinations. For each experiment, means within columns followed by the same letter are not significantly different, as determined by the Fisher protected least-significant difference multiple range test ($\alpha = 0.05$).

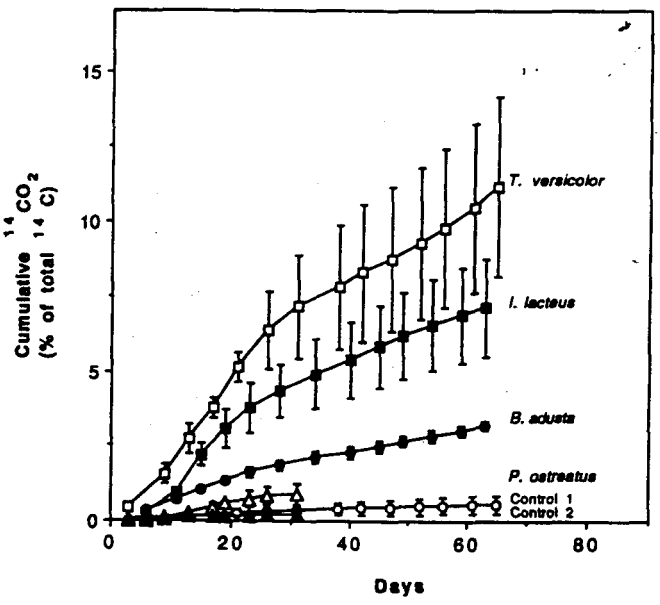

Fig. 1. Time-course of ^{14}C-PCP mineralization in soil cultures. Mineralization by *I. lacteus*, *B. adusta*, *T. versicolor* and an uninoculated control were obtained from one experiment. Mineralization by *P. ostreatus* and an uninoculated control were obtained from a separate experiment. Error bars represent the SD of three determinations.

The total amount of radioactivity recovered from the cultures was usually between 60 and 70% (with the exception of one set of control cultures, where 98% of the label was recovered). These low recoveries may have been due to volatilization, binding of PCP to the glassware, or inefficiency in the combustion of the organic matter.

PCA production

The radioactivity present in the hexace extract (Table 1) consisted of unreacted PCP and extractable PCP transformation products. Analysis by GC of this fraction (Table 2) revealed that in cultures of *I. lacteus*, *T. versicolor* and *P. ostreatus* there was very little PCP remaining in the soil (around 5%). *Bjerkandera adusta* cultures were less efficient in transforming PCP, with 29% of the PCP remaining as such after 9 wk incubation. Other white rot fungi, in particular *P. chrysosporium*, transform a high proportion of PCP into its methoxylated analog, PCA, in soil culture (Lamar and Dietrich, 1990; Lamar *et al.*, 1993). The fungi used in our study produced very low amounts of PCA (Table 2). *Trametes versicolor* produced the highest amount, methylating 6.8% of the PCP originally present in the culture. No PCA could be detected in extracts from *P. ostreatus* cultures. The production of other extractable transformation products from PCP was not investigated.

Table 2. Percentage of PCP and PCA recovered from soil fungal cultures in hexane–acetone extracts

Fungus	Residual PCP (%)	PCA (%)
Control	65.1 (9.0)	1.3 (0.1)
I. lacteus	4.9 (0.8)	2.5 (0.4)
B. adusta	29.2 (3.9)	4.4 (0.3)
T. versicolor	5.2 (1.2)	6.8 (2.3)
Control[a]	71.5 (4.1)	Not detected
P. ostreatus[a]	5.6 (0.2)	Not detected

[a]The last two rows represent a separate experiment. *Irpex lacteus*, *B. adusta* and *T. versicolor* cultures were incubated for 9 wk. *Pleurotus ostreatus* cultures were incubated for 4 wk.
Values are means (SD) of three replicate determinations.

DISCUSSION

Numerous publications have shown that white rot fungi can efficiently deplete PCP in contaminated soil, but the question of the fate of the portion of the PCP that was converted to nonextractable products remains unanswered (Lamar *et al.*, 1990a,b, 1993; Lamar and Dietrich, 1990). Our work has demonstrated that a large part of the PCP present in contaminated soil is bound to the soil organic matter by the action of white rot fungi. *Pluerotus ostreatus* was the most efficient in this process, binding as much as 65% of the PCP to the three fractions of humic materials that were separated in this study. Between 34 and 46% of the xenobiotic was bound to the soil organic matter by the rest of the fungi. Thus, the most important action of the fungi on the PCP was binding to soil humic components. Complete metabolism (mineralization) and transformation to PCA were comparatively much less significant. Covalent binding of pollutants to soil fractions is important because it may reduce their bioavailability and therefore their toxicity. It remains to be established

how stable the PCP-humic matter hybrid products are. It is theoretically possible that residual chlorinated organics may subsequently be released from these products. Humic materials, however, are recalcitrant and are recycled in nature in an extremely slow fashion. The decomposition rate of old organic matter (humic material) is estimated to be 2.5×10^5 times lower than the decomposition rate for easily decomposable organic matter (e.g. sugars and amino acids) and 1×10^5 times lower than that of slowly decomposable organic matter (e.g. hemicelluloses) (Paul and Clark, 1989). There is evidence that xenobiotic-humic acid complexes are degraded slowly as well. Work done on the mineralization of chloroaniline–humic acid complexes in soil has shown that these hybrid products have a low turnover rate (3–4% mineralization in 100 d of incubation, Saxena and Bartha, 1983). Humic acid was mineralized at a similar rate by this system. Haider and Martin (1988) showed that liquid cultures of *P. chrysosporium* mineralized between 17 and 30% of different chlorophenols bound to humic acid in an 18 d incubation, which was lower than the degradation rate of humic acid by this organism. Chloroaniline–lignin complexes were mineralized by *P. chrysosporium* in a liquid culture at the same rate as nonxenobiotic control lignin (Arjmand and Sandermann, 1985). Free chloroanilines were mineralized to a lesser extent. Slow degradation of PCP-humic materials complexes would most probably give rise to other degradation products, not to PCP itself. The toxicity of such degradation products is not known.

The binding of PCP to humic materials was most probably catalyzed by oxidative enzymes produced by the fungi. Studies done *in vitro* have shown that peroxidases and laccases are able to bind xenobiotics to HA and FA (reviewed by Bollag, 1991; Bol1ag *et al.*, 1992). Horseradish peroxidase (HRP) catalyzed the binding of 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol to humic acid (Hatcher *et al.*, 1993; Lassen *et al.*, 1994) and HRP, lactoperoxidase and chloroperoxidase have been shown to incorporate phenol in the humic acid structure (Lassen *et al.*, 1991). Similarly, 2,4-dichlorophenol was coupled to FA by tyrosinase, laccase and peroxidase (Sarkar *et al.*, 1988). Oxidative enzymes such as laccase, HRP and tyrosinase have also been shown to cross-link chlorinated phenols to themselves and chlorinated anilines to guaiacol and ferulic acid to yield humic-like oligomers (Bollag *et al.*, 1977; Tatsumi *et al.*, 1992, 1994; Dec and Bollag, 1994).

The white rot fungi used in our study are known to produce these types of oxidative enzymes. Laccase is produced by *T. versicolor* (Vyas *et al.*, 1994; Peláez *et al.*, 1995) and *P. ostreatus* (Peláez *et al.*, 1995; Tuor *et al.*, 1995), but not by *B. adusta* (Peláez *et al.*, 1995). Lignin peroxidase (LiP) and manganese peroxidase (MnP) have been detected in cultures of *T. versicolor* (Waldner *et al.*, 1988; Johansson *et al.*, 1993), *B. adusta* and *P. ostreatus* (Peláez *et al.*, 1995; Tuor *et al.*, 1995; Youn *et al.*, 1995). Ligninolytic enzymes produced by *I. lacteus* have not been described.

The fungal strains used in our study may be useful for bioremediation of PCP-contaminated soil, because they rapidly and effectively decrease the concentration of PCP with little conversion to PCA. It has been shown in field and laboratory studies with *P. chrysosporium* and *P. sordida* that a large proportion of the PCP is converted to PCA in the first 2 wk of exposure. Pentachlroanisole, which is also toxic, is accumulated by these fungi during this period, after which it decreases slowly (Lamar and Dietrich, 1990; Lamar *et al.*, 1990b, 1993). Whether this decrease is due to binding to humic material or to mineralization is not known. The use of strains that do not produce significant amounts of PCA would be advantageous for bioremediation purposes.

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