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Bound Residue Formation in PAH Contaminated Soil Composting Using *Phanerochaete Chrysosporium*

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ABSTRACT

The degradation rate of benzo[a]pyrene (B[a]P), a 5-ring PAH compound, was significantly enhanced in fungal (*Phanerochaete chrysosporium*) enriched soil composting microcosm reactors over that observed in unamended soil systems. The maximum B[a]P removal rate was 1.1 µg/g-soil-day with fungal inoculation compared to 0.24 µg/g-soil-day without fungal inoculation for a silt loam Kidman soil. Mass balance considerations suggested that the enhanced removal of B[a]P resulted in the formation of bound contaminant carbon residues in soil. A maximum bound residue formation rate of 0.37 µg/g-soil-day was estimated in fungal inoculated microcosms. This was significantly different from the zero rate under natural soil conditions. The observed B[a]P mineralization rates were less affected by fungal activity than was B[a]P removal. Degradation of B[a]P resulted in humification (*i.e.* polymerization) of most of contaminant carbon rather than conversion to CO_2. The fraction of contaminant carbon that was humified compared to that which was mineralized was dependent on soil type.
A multi-compartment structural activity model has been developed to illustrate the overall degradation of B[a]P during soil composting.

INTRODUCTION

Composting is a potentially viable alternative for the biological treatment of soils contaminated with polynuclear aromatic hydrocarbons (PAHs). Biodegradation of the lower molecular weight compounds (2 and 3 ring PAHs) is well known (1). The heavier molecular weight PAHs (4 or more rings) are more resistant to degradation, especially when adsorbed onto soil solids. In a conventional composting system, biodegradation of the lower molecular weight 2- to 3-ring PAHs were enhanced while the higher molecular weight 4- to 7-ring PAHs appear to remain biologically unavailable due to their low solubility (2). Various methods to enhance the biodegradation of these heavier compounds through the addition of nutrients, inoculum, and growth inducers have met with only limited success (3). Although the enhancements appear to improve the degradation of the lower ring compounds, the heavier PAHs are not significantly affected (3).

Fungal co-metabolism is a biological process known to enhance PAH biodegradation (4). The white rot fungus, *P. chrysosporium*, mediates PAH oxidation by a ligninase catalyzed enzymatic reaction in which veratryl alcohol cation radical is the reactive intermediate (5,6,7). The mobile, low molecular weight cation may react with a PAH compound that is part of an insoluble complex. The interaction of the radical cation with B[a]P results in the formation of B[a]P cations, through which the subsequent derivatives can polymerize with soil organic material to form bound contaminant carbon residue (i.e., humification).

The goal of the present study was to monitor the effects of *P.chrysosporium* on the biotransformation of benzo[a]pyrene in a composting system. Specific research objectives included: 1) monitoring the fraction of B[a]P mineralized to CO_2 due to presence of fungi, 2) estimating the rate of humification of contaminant carbon with and without fungal growth, 3) comparing rates of B[a]P transformation in two different soil types, 4) developing a structural activity model to describe B[a]P transformation in fungal compost bioreactor.

BACKGROUND

B[a]P Mineralization by *P.chrysosporium*

Haemmerli (6) proposed a pathway of initial co-metabolic transformation of B[a]P to quinones by *P.chrysosporium*. Ligninase catalyzes the initial step of the chain reaction, resulting in the formation of a cation radical of veratryl alcohol as a redox mediator (5,6,7). Palmer (8) proposed that a perhydroxy radical formed through veratryl alcohol free radical has a high redox potential and should therefore oxidize lignin or PAHs to the radical cation, thus initiating an additional bond cleavage and further oxygen activation. The perhydroxy radical would then be reduced to hydrogen peroxide, which could initiate a second cycle of peroxidase activity. A redox mediator of low molecular mass would be mobile and could lead to the extensive auto-oxidation of B[a]P taking place at significant distances from the fungal hyphae. Figure 1 illustrates the proposed mechanism of B[a]P degradation by *P.chrysosporium*.

Bound Contaminant Carbon Residue Formation

Humic substances are formed by random polymerization of phenolic compounds. Phenols, originating from higher plants or microbial products, are the major building blocks for humic substances. Therefore, it is not surprising that aromatic intermediates that resemble naturally occurring chemicals and are formed during the transformation of PAHs can also be incorporated into bound soil residue (e.g., humus) through enzymatic or abiotic chemical oxidative coupling reactions. Bollag (9) reported that with fungal enzyme and a catalyst extracted from soil, xenobiotics, such as phenols and substituted anilines, could be covalently cross-coupled to humic constituents and form stable chemical linkages.

In the soil environment, such an initial co-metabolic transformation may result in subsequent attack by other indigenous organisms. Furthermore, the site and mechanism of initial biotransformation of PAHs will depend on molecular structure, enzymatic capabilities of the microorganisms involved, the redox potential, pH, and ionic environment. These factors will not only modify the rate, but also the pathway and the ultimate products of the degradation (10).

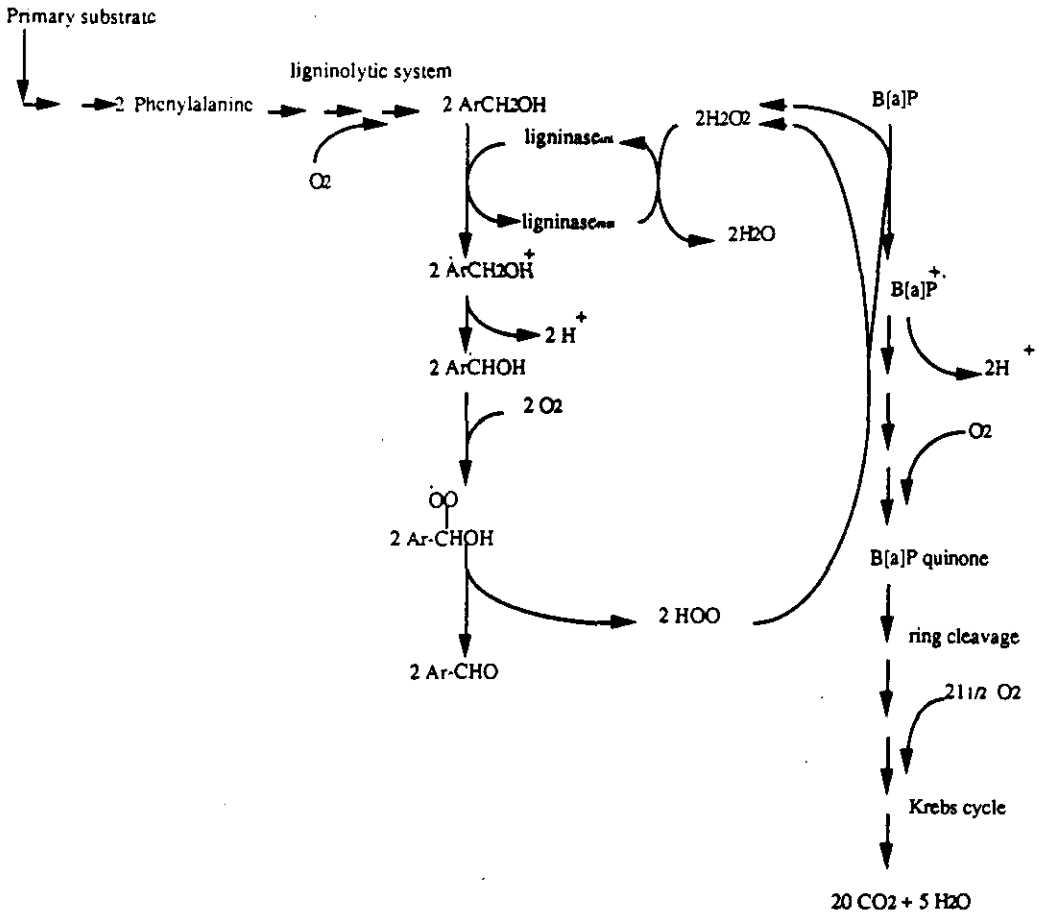

Figure 1
A Hypothetical Mechanism of the Overall Process of B[a]P Degradation.

ArCH2OH = veratryl alcohol; Ar $^{\cdot}CH2OH^{+}$ = veratryl alcohol cation radical; Ar $^{\cdot}CH2OH$ = veratryl alcohol free radical; Ar $^{\cdot}OOCHOH$ = veratryl alcohol peroxy radical; Ar-CHO = veratraldehyde; HOO $^{\cdot}$ = perhydroxy radical. ligninase int = intermediate state ligninase; ligninase rest = rest state ligninase.

In mineral soils, most humic material occurs in association with clay, as a clay-metal-SOM (soil organic matter) complex. Bonding mechanisms for the retention of organic chemicals by humic substances in soil include ion exchange, hydrogen bonding, *van der Waal's* forces, hydrophobic binding, charge transfer, and ligand exchange (11). Chemical alteration and subsequent covalent bonding usually produce more persistent complexes which may constitute a part of the so-called "bound residues" in soil. Partitioning into hydrophobic media has been proposed as a mechanism for retention of nonpolar organic molecules, particularly PAHs, by soil organic matter.

The interaction between B[a]P and humic substances is a physical reaction (12) with binding being rapid and completely reversible (13,14). B[a]P derivatives in fungal enzymatic reactions can be quinones and various phenolic compounds. Unlike B[a]P, the B[a]P derivatives could be polar and hydrophilic. The potential binding could involve several mechanisms including covalent bonding, ion

exchange, hydrogen bonding, hydrophobic binding etc.. These reactions may be irreversible and constitute "bound residues" in soil.

A Conceptual Structural Activity Model for Multi-Compartment Analysis in Soil Composting System

In a soil composting system, soil organic matter content can be increased by adding amendment and fungal inoculum. Since the affinity of B[a]P for binding to soil organic matter (SOM) is high, binding of B[a]P to SOM may affect the B[a]P availability, hence reducing the extent of B[a]P mineralization. Moreover, the 'bound residue' formation of B[a]P derivatives will also reduce the potential of B[a]P mineralization. Mineralization is thermodynamically more favorable than 'bound residue' formation the ultimate product CO_2 is thermodynamically more stable than the 'bound residues' (15). However, the 'bound residue' formation may be kinetically favorable due to the lower activation energy achieved through specific enzymatic catalysis. For higher clay content soil, such as silt loam Kidman soil, the clay-metal-SOM complex formation could be extensive and more favorable to bound residue formation. To interpret the interaction of multi-compartment reaction activities a conceptual model is hypothesized in Figure 2.

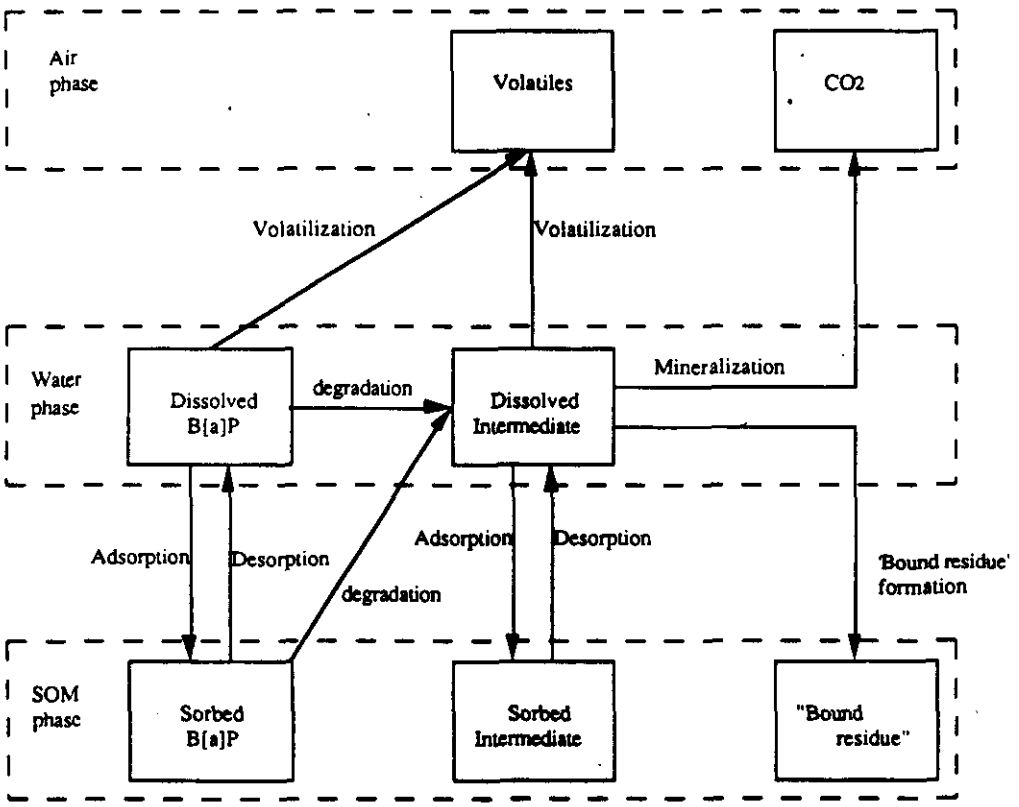

Figure 2
Conceptual Model Used for Multi-compartment Analysis.

The B[a]P and the derivatives in the composting system is viewed as being distributed among three compartments: soil air phase, soil water phase, and soil organic matter phase.

MATERIALS AND METHODS

The microcosm was designed to simulate biopile/compost treatment facilities including fungal inoculation, aeration, amendments and moisture addition. Radio-labeled Benzo[a]pyrene (B[a]P) was chosen as the model PAH compound typically found in petroleum and creosote wastes. Radiolabeled ^{14}C benzo[a]pyrene spikes (Chemsyn Laboratories, Lexana, Kansas) were added at 40.32 µCi per kilogram of dry soil which, together with non-labeled benzo[a]pyrene amounted to a total compound loading of 100 mg B[a]P per kilogram of soil. Compost reactors were placed in constant temperature rooms (20°C) and incubated in the dark to minimize any photo-induced degradation experiments. Kidman soil and McLauren soil were used as typical silt loam and sandy loam soils. A description of soil properties is given in Table 1. Moistened corn cobs were chosen as the primary growth and energy source for the fungal culture. The fungal inoculation was 10^5 - 10^6 fungal spores / gram of soil. Loss of B[a]P and the labeled ^{14}C in soil microcosms were monitored over a 120 days period. Sequential sacrifice of soil composting microcosms were used to assess the effectiveness of *P. chrysosporium* to remediate B[a]P contaminated soils. Microcosms were 125 ml Erlenmeyer flasks equipped with rubber stoppers and gas exchange valves (Figure 3). Each microcosm received 2.5 grams of coarse corncobs (particle diameter ≥ 2 mm) to which ten grams of B[a]P spiked soil were added. Samples were run in triplicate with two noncontaminated (B[a]P free) microcosms serving as controls. Another group of microcosms receiving neither fungal inoculum nor corn cob amendments were incubated to compare fungal treatment to that achievable in the natural soil environment.

Seven sets of microcosms were prepared for each soil evaluated. At each measurement event, compost microcosms were sacrificed and soil mixture samples were air dried. One gram of soil and 0.5 gram of corn cobs were then subsampled from each microcosm for combustion.

Table 1
Soil Characteristics

Soil		Kidman	McLauren
Texture		Silt loam	Sandy loam
Moisture (@1/3 bar)	(%)	20	12.4
pH		7.2	4.7
ECE	(mmhos/cm)	1.1	0.4
Organic Carbon	(%)	0.51	0.94
CEC	(meq/100gm soil)	11.7	6.35
Chelatable:			
Fe	(mg/kg)	4.5	8.7
Cu	(mg/kg)	1.6	0.1
Mn	(mg/kg)	6.3	3.5
Zn	(mg/kg)	0.6	0.1
Acid Digestion:			
Fe	(mg/kg)	14130	5346
Cu	(mg/kg)	14.3	<0.1
Mn	(mg/kg)	351.5	85.3
Zn	(mg/kg)	45.7	11.5
Bacteria	(c.f.u./gm soil)	6.7×10^6	6.3×10^5
Fungi(unamended)	(c.f.u./gm soil)	1.9×10^4	7.5×10^4

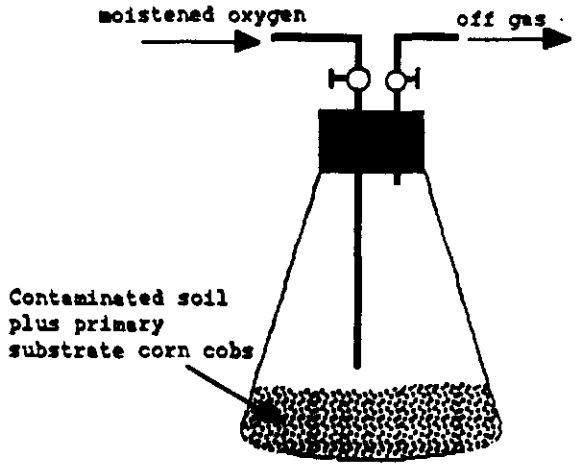

Figure 3
Compost Microcosm

The remaining fraction was extracted for 24 hours using Soxhlet extraction with 200 ml of a 1:1 methylene chloride/acetone mixed solvent (SW-846, Method 3540) (16). After evaporation of the solvent, the residual material was dissolved in acetonitrile and brought to a volume of 10 ml for B[a]P and organic soluble compounds measurement. The concentration of B[a]P was determined by high performance liquid chromatography (HPLC) (SW-846, ,Method 8310)(16) on a Shimadzu HPLC SCL-6B, SIL-6A, LC-6A, and SPD-6A system. The total residue of ^{14}C labeled was determined by combustion of soil and corncob subsamples (Harvy Biological Oxidizer - Model OX 400) followed by scintillation counting (Beckman LS 1701 Liquid Scintillation System). Extractable ^{14}C was quantified in the organic extract by liquid scintillation.

Composting microcosms were open to air and incubated at 20°C for the first 75 days. The moisture content was maintained at 40 - 60% by conducting weekly soil weighing followed by addition of distilled deionized water when necessary. After 75 days, microcosms were exposed to pure oxygen to evaluate the effect of oxygen intensity on B[a]P bound residue formation and mineralization. Moistened oxygen was used to evacuate microcosm headspace. Aeration of microcosms was conducted once every three days. This procedure involved flushing the gas (at a flow rate of 150 ml/min) through each microcosm for five minutes.

RESULTS

Treatment efficiency was evaluated in terms of the removal rate of B[a]P, total ^{14}C and extractable ^{14}C. In all control units, no radioactivity nor benzo[a]pyrene was detected. Differences between the total residue of ^{14}C and the extractable ^{14}C provided an estimate of the irreversibly bound or humified contaminant carbon. The sum of mineralization and volatilization was estimated by the loss of total ^{14}C residue in soil mixture. The change in B[a]P concentration, total ^{14}C removal, and the cumulative bound residue formation over time are shown graphically in Figures 4 to 8. Table 2 shows the results of statistical analysis of the zero order reaction rates.

DISCUSSION

Figures 4 and 5 indicate that well aerated fungal treatment

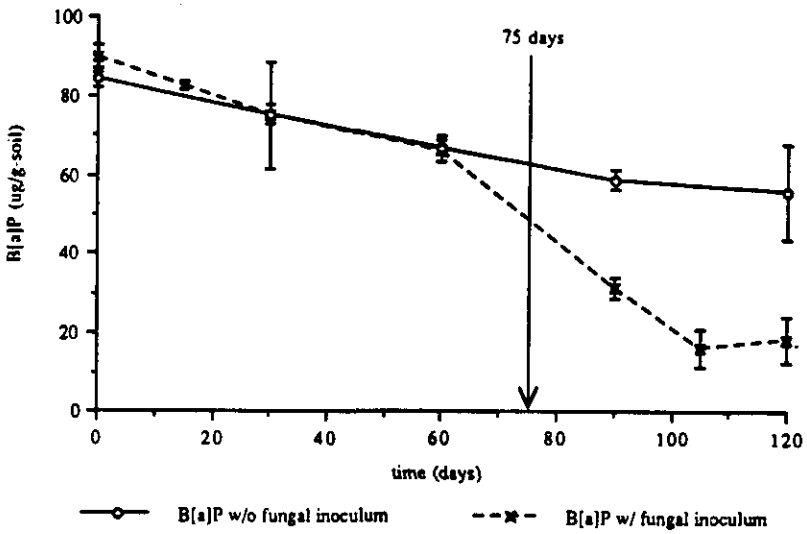

Figure 4
B[a]P Removal in Silt Loam Kidman Soil

significantly enhanced B[a]P degradation in Kidman silt loam soil mixture. Before 75 days, high soil moisture level may have resulted in limited oxygen transfer causing reduced fungal activity. After 75 days, microcosms were sealed and moistened oxygen gas was introduced to the remaining microcosms to maintain aerobic conditions and suitable moisture content. During oxygen supplement conditions, B[a]P removal rate was 1.1 µg/g-soil/day with fungal inoculum versus 0.24 µg/g-soil/day without fungal inoculum. The total ^{14}C removal rate was 0.27 µg/g-soil/day and the bound residue formation rate was 0.37 µg/g-soil/day with fungal inoculum. Statistical analysis indicated that zero reaction rates can not be rejected for these two

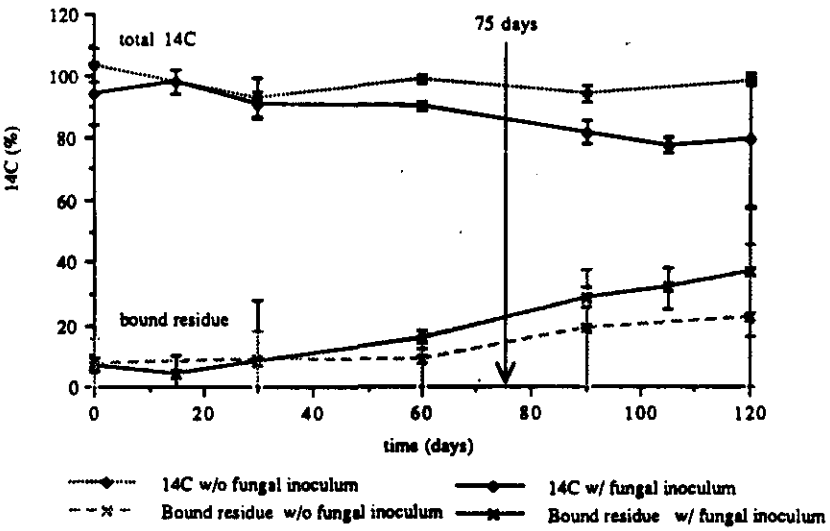

Figure 5
Total ^{14}C Removal and Bound Residue Formation
in Silt Loam Kidman Soil

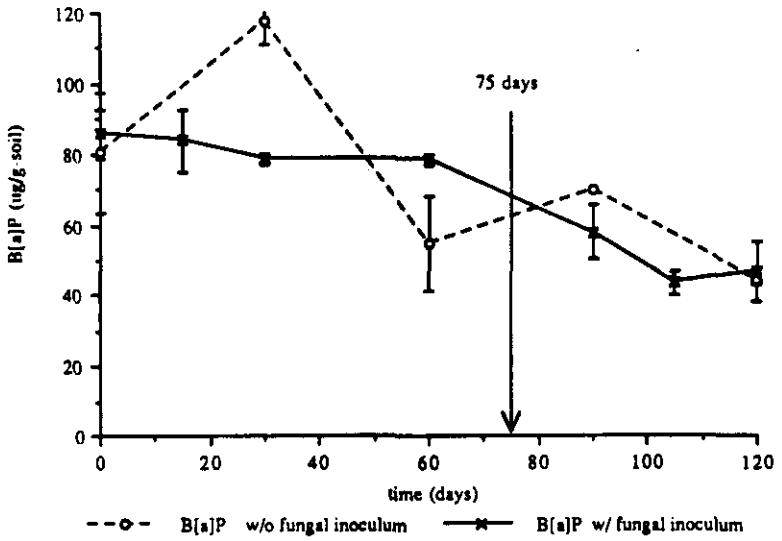

Figure 6
B[a]P Removal in Sandy Loam McLauren Soil

control tests (Table 1). However, before 75 days incubation, neither B[a]P nor ^{14}C removal rate were significantly different from the control tests. After 105 days incubation (after 45 days of fungal activity) both B[a]P and ^{14}C removal ceased. Figure 5 indicates that bound residue formation seemed to be the major mechanism for B[a]P removal in Kidman silt loam soil. Approximately 37% of the ^{14}C-B[a]P derivatives were bound as solvent nonextractable residues at the end of experiment, while less than 20% were mineralized and/or volatilized. Figure 8 shows that the bound residue formation in Kidman soil was higher than in McLauren soil under all conditions. This may be due to the higher clay content of Kidman soil.

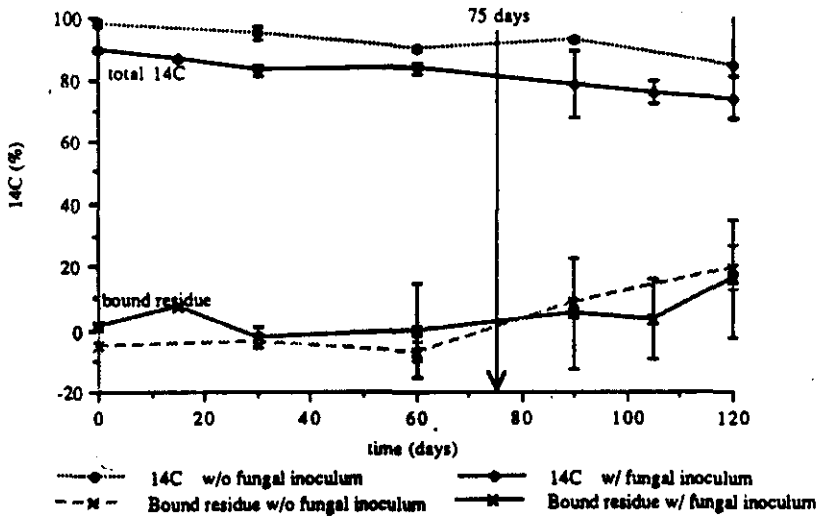

Figure 7
Total ^{14}C Removal and Bound Residue Formation in Sandy Loam McLauren Soil

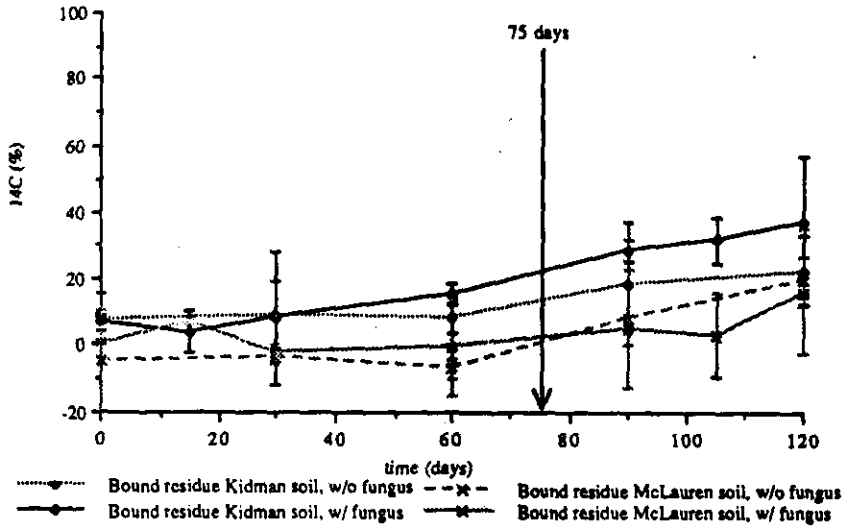

Figure 8
Comparison of Bound Residue Formation:
Kidman Soil vs. McLauren Soil.

Data suggests that the fungal metabolism enhanced both the B[a]P removal and the bound residue formation. The adsorption of B[a]P onto the soil surface had a negligible effect on B[a]P bioavailability, since the higher clay content of Kidman soil did not appear to limit the B[a]P degradation rate. The clay-metal-SOM complex encouraged the B[a]P derivatives bound residue formation, which, in turn, reduced

Table 2
Statistical Analysis of Reaction Rates

Sample description	Linear regression & F test description	B[a]P removal (ug/g-soil - day)		Total 14C removal (ug/g-soil - day)		Bound residue formation (ug/g-soil - day)	
		Kidman	McLauren	Kidman	McLauren	Kidman	McLauren
1. Control test: 0-120 days	rate	0.240	0.400	0.038	0.093	0.130	0.190
w/o fungal inoculum	95%lower	0.120	0.008	-0.052	0.003	0.015	0.040
w/o corn cob amendment	95%upper	0.360	0.800	0.130	0.180	0.270	0.350
exposure to oxygen	significant to zero rate:	yes	yes	no	no	no	yes
	significant between two soils:	no		no		yes	
2. Fungal enrichment: 0-75 days	rate	0.390	0.120	0.110	0.093	0.230	-0.064
w/ fungal inoculum	95%lower	0.320	-0.023	-0.064	0.035	0.060	-0.290
w/ corn cob amendment	95%upper	0.460	0.270	0.280	0.150	0.510	1.600
exposure to air	significant to zero rate:	yes	no	no	no	no	no
(soil mixture too wet, not well aerated)	significant to control test:	no	no	no	yes	no	no
	significant between two soils:	yes		yes		no	
3. Fungal enrichment: 75-105 days	rate	1.100	0.760	0.270	0.170	0.370	0.098
w/ fungal inoculum	95%lower	1.000	0.560	0.170	-0.082	0.190	-0.490
w/ corn cob amendment	95%upper	1.300	1.000	0.380	0.430	0.560	0.690
exposure to oxygen	significant to zero rate:	yes	yes	yes	no	yes	no
(soil mixture well aerated)	significant to control test:	yes	no	yes	yes	yes	no
	significant between two soils:	yes		no		yes	
4. Fungal enrichment: 105-120 days	rate	-0.160	0.230	0.120	0.130	0.340	0.860
w/ fungal inoculum	95%lower	-1.000	-1.200	-2.400	-0.710	-2.000	-1.500
w/ corn cob amendment	95%upper	-0.660	0.780	2.200	1.000	2.600	3.200
exposure to oxygen	significant to zero rate:	no	no	no	no	no	no
(soil mixture well aerated)	significant to control test:	yes	no	yes	yes	no	no
	significant between two soils:	yes		no		no	

the extent of mineralization. The apparent mineralization rates seemed less affected by fungal treatment.

The reasons for the declining B[a]P degradation rate during the late stages of treatment are unclear. The authors would suggest the following possibilities: 1) ligninase inhibition, 2) mass transport resistance, and 3) moisture content limitation. Ligninase activity could be inhibited by protease enzymes or pH depression. Dosoretz et al. (17) reported protease activity promotes the decline of ligninase activity in the culture fluid of *P. chrysosporium* grown in submerged batch culture on nitrogen limited media. In addition to the presence of proteases, the soil pH is critical to ligninase activity. It has been reported that ligninase has a pH optimum of 4.5 with substantial suppression of activity below pH 3.5 and above 5.5 (18). *P.chrysosporium* enrichment could have resulted in pH depression through microbial metabolism. Finally mass transport resistance may have resulted in limiting the supply of oxygen to the fungus and/or the mobility of veratryl alcohol cation free radical in the soil system. Mass transfer resistance related to 1) supply of fungal nutrients, 2) mobility of free radical species, or 3) availability of soil moisture could lead to a substantial reduction in the observed B[a]P transformation rate. Since soil has a strong ability to absorb moisture from the environment, the moisture content within the original saturated corncob particles will decrease overtime. It is conceivable that fungal activity may be limited by the low moisture content existing within the primary substrate (i.e., corn cobs).

In contrast to results observed in Kidman soil, B[a]P removal rates in fungal inoculated McLauren sandy loam soil mixture were insignificant to control tests (Figure 6). For McLauren soil, the rate of bound residue formation in control test was 0.19 µg/g-soil/day (Figure 7 and Table 2), which differed significantly from the zero removal rate for Kidman soil control test. However, the rate of bound residue formation in the fungal inoculated McLauren soil mixture was zero compared to 0.37 µg/g-soil/day observed in Kidman soil. Table 1 indicates that McLauren soil has a lower pH (pH = 4.5) and higher indigenous fungal population than Kidman soil (pH = 7.2). The pH of McLauren soil is favorable to native fungal activity which may have contributed to B[a]P degradation. The higher CEC of Kidman soil increases the buffer capacity at microsites within the soil (19), which reduces the impact of protons released as metabolites of *P. chrysosporium* enrichment. Other possible reasons for the low B[a]P removal rates in McLauren soil include the presence of microbial antagonists to *P.chrysosporium* (20) and the presence of inhibitory transition metals, such as Cu^{2+} or Mn^{2+} (21).

CONCLUSION

The preliminary experimental results indicate that the clay-metal SOM content in soil may favor the bound residue formation and limit the ultimate B[a]P mineralization during fungal composting treatment of PAH contaminated soils. Other soil characteristics, such as pH, CEC, trace metal and native microflora, may also influence B[a]P degradation. Future research should be aimed at evaluating the potential of bound residue pollutants to release and migrate away from soil matrix. Mass balance approaches may be applied to evaluate multi-compartment model parameters, which would help in developing methods for further detoxification of PAHs within the composting system either through enhancing mineralization or humification.

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