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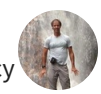

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Field Evaluation of the Lignin-Degrading Fungus *Phanerochaete sordida* to Treat Creosote-Contaminated Soil

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A field study to determine the ability of selected lignin-degrading fungi to remediate soil contaminated with creosote was performed at a wood-treating facility in south central Mississippi in the autumn of 1991. The effects of solid-phase bioremediation with *Phanerochaete sordida* and of two control treatments on soil concentrations of 14 priority pollutant polycyclic aromatic hydrocarbon (PAH) components of creosote were followed for 56 days. PAH analytes containing ≥ 5 rings persisted at their original concentrations in all treatments. However, depletion of 3-ring (85-95%) and 4-ring (24-72%) analytes after 56 days was greater in the fungal treatment than in control treatments in all cases. This finding demonstrates the potential of lignin-degrading fungi in the solid-phase bioremediation of creosote-contaminated soils. However, the persistence of the larger analytes represents a significant challenge to this developing technology.

Introduction

Wood-preserving facilities annually generate *ca.* 1000 dry metric tons of K001 sludge *(1), i.e.,* bottom sediment sludge from the treatment of wastewaters from wood-preserving processes that use creosote and/or pentachlorophenol (PCP) *(2).* Many wood treatment plants have been in operation for several decades. Due to the historic use of materials handling procedures only recently recognized as unacceptable, many wood treatment facilities have extensive contamination. Fifty-five wood treatment plants have been identified in the 1991 U.S. Environmental Protection Agency (EPA) National Priority List. Estimates put the average cost of reclamation of a Superfund site at $25000000. Clearly, true costs will depend on the remediation technology employed.

Bioremediation, in which hazardous waste products are degraded or detoxified by microorganisms, is a potential cost-effective technology for cleanup of creosote-or PCP-contaminated soils. Our research has focused on the degradation of PCP in soil by lignin-degrading fungi. In nature, these fungi cause the "white-rot" of wood, as well as litter decomposition, by breaking down lignin. Lignin is a persistent natural material with a heterogeneous polyaromatic structure *(3).* Degradation of lignin by these fungi requires extracellular enzymes with nonspecific degradative abilities *(3-5).* Laboratory studies have yielded an extensive list of xenobiotics susceptible to degradation by lignin-degrading fungi, including PCP and polyaromtic hydrocarbon (PAH) analytes *(6, 7).* These degradative abilities make these organisms excellent candidates for the remediation of soils contaminated with such compounds.

Degradation of xenobiotics under aqueous conditions, both by fungal cultures and by enzyme preparations *in vitro,* suggests that bioremediation using lignin-degrading fungi has potential. However, the realization of this potential in the solid-phase remediation of soils requires that fungal biotransformation processes be active in soil matrices. Pollutants bound to soil particles are much less available to enzymatic processes and are therefore much more resistant to degradation. Laboratory studies have demonstrated the depletion of *ca.* 98% of the PCP spiked into three soils at a level of 50 μg g^{-1} by *Phanerochaete chrysosporium (7).* The effectiveness of fungal treatment in a field setting was demonstrated at a site contaminated with PCP (250-400 μg g^{-1}) and hydrocarbon solvents *(8).* Despite soil temperatures substantially below the optimal ranges for fungal growth, *ca.* 90% of the PCP in this sandy, alkaline soil was depleted after 6.5 weeks of treatment with either *P. chrysosporium* or *P. sordida,* Depletion of PAH analytes in soil by solid-phase treatment with lignin-degrading fungi has also been reported *(9, 10).*

The objective of the present study was to evaluate the ability of *P. sordida* to decrease the concentration of PAH analytes in soil contaminated with K001 sludge. This study was part of a larger study in which the effects of seven fungal treatments and three controls on soil PCP concentrations were evaluated *(11).*

Experimental Methods

Fungi. *Phanerochaete sordida* (Karst.) Eriksss. and Ryv. was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, WI. Fungal inoculum was prepared by the Lambert Spawn Co., Inc. (Coatsville, PA). *P. sordida was* cultured on a proprietary nutrient-fortified sawdust-based sterile substrate, hereafter referred to as "standard substrate", in autoclavable bags containing microporous filters to allow adequate air exchange. Each bag contained *ca.* 3 kg of inoculum. Noncolonized standard substrate, also packaged in 3-kg bags, was also provided by Lambert for use as a control treatment. Fungal inoculum and standard substrate were delivered to the test site in a refrigerated truck.

Chemicals. [^{14}C]Phenanthrene (13.1 mCi/mmol, purity > 99%) was purchased from Sigma Chemical Co. (St Louis, MO), and [^{14}C]benzo[*a*]pyrene (9.65 mCi/mmol, purity > 98%) was purchased from California Bionuclear Corp. (Los Angeles, CA). Targeted PAHs used as analytical standards (purity > 99%, except chrysene had purity > 95%) were purchased from Accu-Standsrd (New Haven, CT). Granular anhydrous sodium sulfate (reagent grade) was purchased from Mallinckrodt Specialty Chemicals Co. (Paris, KY). Acetone, obtained from Baxter Healthcare Corp. (Muskegon, MI), and acetonitrile, obtained from Aldrich Chemical Co. (Milwaukee, WI), were chromato-

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graphic grade solvents. Methylene chloride (purity > 99.5%) was purchased from Spectrum Chemical Manufacturing Corp. (Gardena, CA). Water was purified with a Mini-Q Water System (Millipore Corp., Bedford, MA). Poly-Fluorliquid scintillation cocktail was purchased from Packard (Meriden, CT).

Site Description and Soil Characterization. The Escambia Treating Co. (originally Mississippi Wood Preserving Co.) operated a wood-preserving facility from 1946 to 1986 on a 27.5-ha tract just north of Brookhaven, MS. In 1986, soil in the area of two unlined surface impoundments which had been used to dispose of K001 sludge was excavated to a depth of 5 m and deposited on a portion of the property identified as a hazardous waste management unit. Soil from this waste sludge pile was used in the field treatability study. A composite sample consisting of soil samples taken to a depth of 30 cm was analyzed by the USDA Forest Service Soil and Plant Analysis Lab, Berea, KY, for soil chemical characteristics.

Plot Construction and Treatment Application. Design and construction of the test plots and leachate collection system are described in a companion paper *(11)*. Briefly, test plots of dimensions 3 m × 3 m were constructed of no. 14 galvanized sheet metal onto a clay pad designed to channel leachate to centrally placed drainage pipes in each plot and then to a holding tank. Plots were lined with 4-mil polyethylene sheeting, onto which the 3.8-cm perforated PVC drainage pipes were plumbed. The pipes were covered with gravel and then overlaid with a layer of sand to form a level base *ca.* 45 cm below the tops of the plot borders.

Soil was excavated from the waste sludge pile to a depth of *ca.* 40 cm, screened through a 1.9-cm mesh screen, mixed to homogenize the contaminants, and loaded into the test plots to a depth of *ca.* 25 cm 2 days prior to soil inoculation. Based on a bulk density of 0.94 kg L^{-1}, each plot contained 2.2 t (dry weight) of soil. One day later, aspen chips *(ca.* 1.5 × 0.5 × 0.25 cm) which had been sterilized by fumigation with methyl bromide were tilled into the soil to a depth of *ca.* 20 cm. Fungal inoculum was applied and tilled into the soil to a comparable depth on the following day. Control treatment receiving standard substrate or no amendments were tilled as well. Tilling equipment was washed thoroughly between application of different treatments.

Experimental Design. This study was part of a larger study that was designed to evaluate the effects of several fungal and control treatments on soil PCP concentration *(11)*. Described herein are the effects of treatment with *P. sordida* at 10% (inoculum:soil, w:w, dry weight basis) and two control treatments, soil amended with standard substrate at 10% and nonamended soil (no aspen chips or standard substrate). Both amended soils received chips at 2.5% (chips: soil, w:w, dry weight basis) in addition to inoculum or standard substrate. Aeration, irrigation, and sampling protocols, described below, were identical for the fungal and control treatments.

These treatments were assessed in three 3 m × 3 m plots using a completely random design (CRD). Sampling and plot maintenance activities were performed to maintain the integrity of plot quadrants (1.5 m × 1.5 m sections), which were treated as replicates. The finding that the variabilities in PCP and PAH concentrations among CRD sections were statistically indistinguishable from those among true replicates in a concurrently running experiment containing an identical experimental treatment (data not shown) justified this assignment of replication. Treatments were initiated on September 18, 1991, and assessed for 56 days thereafter.

Plot Maintenance and Sampling. Precipitation levels were recorded, and soil was monitored for water content on five days during each week of the study. Soil and air temperature measurements at 08:00,12:00, and 16:00 were recorded on the same days. Soil water contents were determined gravimetrically. Soil water contents were kept above 20% moisture (dry weight basis) by irrigation with tap water. Plots were tilled to a depth of*ca.* 20 cm on a weekly basis to provide aeration. The tilling equipment was washed thoroughly between aeration of different treatments. To maintain the integrity of CRD sections, each section was tilled separately.

Soil samples were taken at 1,7,14,28, and 56 days after treatment application. Samples were taken to a depth of *ca.* 20 cm from three random locations within each CRD section (12 samples per plot) with a soil core sampler. Cores were homogenized, chips were removed, and soil subsamples were taken to fill 40-mL amber glass sample containers. A composite chip sample was also obtained from the three samples taken from each section. Samples were stored at -20 °C.

Analytical Procedures. Soil samples were analyzed in duplicate (24 measurements per treatment per sample time) for concentrations of 14 of the 16 PAH analytes listed as priority pollutants by the EPA. These were acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*] fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene, benzo[*g,h,i*]perylene, and indeno[1,2,3-*cd*] pyrene. Naphthalene was not included in this study due to its unreliable recovery by use of the described method, and acenaphthylene was not detected.

Samples were extracted using a modification of EPA method 3550 *(12)*. Use of the nonmodified method recovered only 73% of a [^{14}C]phenanthrene surrogate from soil amended with fungal inoculum or standard substrate. Thus, *ca.* 2-g samples were mixed with 6 g of anhydrous sodium sulfate in 25 × 150 mm culture tubes and stored capped with Teflon-lined caps at 25 °C for 18 h. Each sample was sonicated in 20 mL of acetone: methylene chloride (1:1, v:v) for 2 min at power setting 5 using a Heat Systems (Farmingdale, NY) XL2020 sonicator with a microtip probe. The soil was allowed to settle for *ca.* 2 h, the solvents were gently decanted through G6 glass fiber filters (Fisher Scientific Co., Pittsburgh, PA) using vacuum filtration, and the soil was extracted identically a second time. The second extract and consecutive rinses of the soil remaining on the filters with 5 mL of methylene chloride and 5 mL of acetone were pooled with the first extract. Extracts were stored at 25 °C for 18 h and then evaporated to dryness under N_2 at 40 °C using a Zymark (Hopkinton, MA) TurboVap ZW700 evaporator. Samples were solubilized by adding 5 or 10 mL of acetonitrile to the resulting tarry residue, immediately sealing the tubes with parafilm, and incubating at 25 °C for 1 h. The tubes were then gently vortexed, the acetonitrile extracts were decanted to 12-mL amber screw-cap vials, and the vials were sealed with Teflon-lined caps. Aspen chip samples were ground in a Miracle Mill (Markson Scientific, Phoenix, AZ) prior to extraction of *ca.* l-g subsamples as

described above. Moisture contents were determined gravimetrically after drying subsamples at 80 °C for 18 h.

[^{14}C]Phenanthrene and [^{14}C]benzo[*a*]pyrene were added to a selected subset of soil samples as surrogate analytes *ca.* 1 h prior to the addition of sodium sulfate. Surrogate analyte recoveries were determined from the radioactivities of acetonitrile extracts, as determined with a 1214 Rack-Beta liquid scintillation counter (Pharmacia Wallac Oy, Turku, Finland). Surrogate phenanthrene recoveries averaged 95 ± 4% (n = 58) and ranged from 93 to 97% from the different treatment matrices and from 92 to 99% for different extraction runs. Surrogate benzo[*a*]pyrene recoveries averaged 98 ± 4% (n = 26) and ranged from 97 to 98% from the different treatment matrices and from 95 to 102% for different extraction runs.

Extracted PAHs were quantified by high-performance liquid chromatography (HPLC) essentially as described in EPA method 8310 *(12)*. Generally, quantitation by HPLC was performed immediately; however, concentrations of the PAHs measured in this study did not vary after storage of acetonitrile extracts for > 12 weeks. Peaks were identified based on their coelution and identical UV absorption spectra when compared with pure standards. For peak identification, the spectra of sample peaks were compared to reference spectra obtained from pure standards by use of a 1090L Series II HPLC equipped with a diode-array detector (Hewlett Packard, Little Falls, DE). Routine quantitation was performed by monitoring column eluant for absorbance at 295 nm with a Holochrome variable wavelength detector (Gilson Medical Electronics, Inc., Middleton, WI) with the use of six-point external standard calibration curves.

The reliability and precision of the detection system (HPLC, UV absorbance, and integration) was assessed by the variation among different analytical runs exhibited by analyses of a quality-control soil extract obtained from a composite sample of the study soil. Coefficients of variation of the concentrations of targeted PAHs averaged 4.5% (n = 12) and ranged from 2.4% (phenanthrene and benzo[*a*]anthracene) to 8.5% (anthracene). The reliability and precision of the analytical method (extraction and detection) was assessed by the variation among different extractions of subsamples from a composite sample of the study soil. Coefficients of variation of the concentrations of the targeted analytes averaged 10.5% (n = 10) and ranged from 7.9% (fluoranthene and benzo[*g,h.i*]perylene) to 14.8% (anthracene).

Statistical Analysis. Because initial levels of contaminant varied somewhat among plots, treatment effects were evaluated in terms of the percentage of the contaminant remaining after 56 days of treatment. To obviate any potential matrix effects on analytical procedures, concentrations at the first day after treatment application were considered initial levels. In order to reduce nonnormality and the possible influence of outliers, the percentage contaminant remaining in the samples from each CRD section were averaged to give a mean value for each section. Evaluation of the depletion of each compound at each sample time was performed by analysis of variance (ANOVA). If the ANOVA showed the treatment means to be significantly different, a Tukey multiple comparison was used to determine which treatments were significantly different *(13)*.

In analyzing depletion of PAH analytes as a function of the number of rings (3-, 4-, 5-, or 6-ring compounds), each of the 24 analytical determinations of each treatment at each sampling time was considered to be a separate measurement. The percentage changes in the concentration of the 14 targeted PAH analytes were first contrasted using a Hotelling T^2 statistic *(13)*. If this test identified some contrast significance, the multivariate extension of the Scheffe technique was used to give simultaneous confidence intervals for contraste comparing the average percent reductions of analytes in each ring class *(13)*.

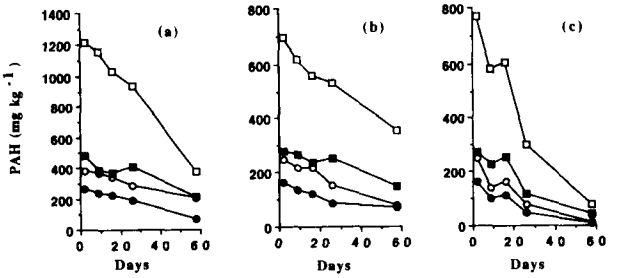

Figure 1. Concentration of 3-fing PAH analytes (mg kg^{-1}) in nonamended soil (a) and in soil amended with standard substrate (b) or with *P. sordida* (c). The analytes were acenaphthene (O), fluarene (●), phenanthrene (□), and anthracene (■).

Results

Soil Characteristics. The sludge pile soil was a clay with the following chemical characteristics: CEC 8.87 mequiv 100 g^{-1}, base saturation 54.8%, PH = 3.8, 0.04% total nitrogen, and 2.17% total carbon. Laboratory studies (data not shown) indicated that soil sterilization was not necessary for survival of *P. sordida* in this soil.

Soil Moisture and Temperature. Irrigation was needed to maintain the water content of nonamended soil between 15 and 24%. Natural precipitation maintained the water contents of soil amended with *P. sordida* or sterile substrate between 33% and 68% without supplemental irrigation, due to the greater water-holding capacity conferred by the amendments. Soil temperatures of the amended soils during the first 45 days of the study ranged from 21 to 38 °C. After 45 days, soil temperatures dropped precipitously all subsequent readings fell below 15 °C.

Chip and Leachate PAH Concentrations. Aspen chips were included as part of the treatment due to their ability to adsorb soil-bound contaminants *(8)* and as an additional source of nutrients for fungal growth. Chip PAH concentrations 1, 7, and 56 days after treatment applications were 10-5070 of those found in the soil. Thus, adsorption of PAHs by aspen chips added at a loading level of 2.5% could account for the disappearance of only *ca.* 19% of the soil analytes. In addition, EPA analyses (data not shown) of the sand underlying the contaminated soil and of leachate generated during the course of the study revealed negligible losses by these routes.

Soil PAH Concentrations. The bulk of the targeted PAH analytes in this soil consisted of the lower molecular weight species 55,42,2, and 1% were 3-,4-,5-, and 6-ring analytes, respectively. Concentrations of each analyte in soil amended either with fungal inoculum or with sterile substrate were similar; however, higher concentrations were observed in nonamended soil (Figures 1 and 2).

No significant decrease of PAH analytes with ≥ 5 rings occurred in any of the treatments tested. Therefore, the

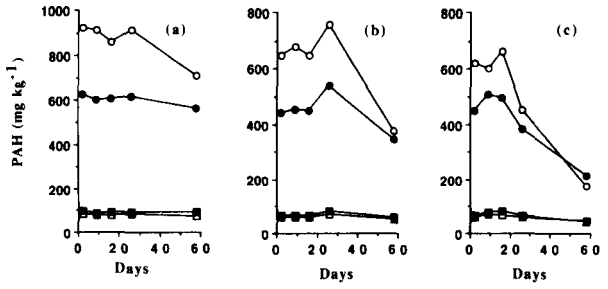

Figure 2. Concentration of 4-ring PAH anelytes (mg kg^{-1}) in nonamended soil (a) and In soil amended with standard substrate (b) or with *P. sordida* (c). The analytes were fluoranthene (O), pyrene (●), benz-[a]anthracene (□), and chrysene (■).

Table I. Effect of Fungal and Control Treatments on the Percentage of PAH Analytes Remaining in Soil after 56 Days

	PAH remaining (%)[b]		
compd[a]	*P. sordida*	standard substrate	nonamended
acenaphthene	5b	32a	53a
fluorene	5b	43a	25ab
phenanthrene	10b	51a	31ab
anthracene	15b	52a	43ab
fluoranthene	28b	58a	77a
pyrene	48b	78a	90a
benz[*a*]anthracene	76a	86a	89a
chrysene	67b	86ab	94a

[a] Compounds are listed in order of elution during reverse-phase HPLC. [b] Values within rows followed by the same letter are not significantly different, as determined by Tukey's multiple comparison test ($\alpha = 0.05$).

discussion which follows addresses the depletion of 3- and 4-ring analytes.

Three-ring analytes were more rapidly depleted in soil treated with *P. sordida* than in soil subjected to either control treatment (Figure 1). After 28 days, percent depletion of acenaphthene, phenanthrene, and anthracene was significantly greater in fungal-treated soil than in either control soil. Percent depletion in fungal-treated soil was still substantially greater after 56 days (Table I). However, due to the extreme variability of the data, only acenaphthene was depleted to a significantly greater extent in fungal-treated soil than in both control soils at this time. A decrease in depletion rates in fungal-treated soil at low concentrations, coupled with continuing losses of the more concentrated contaminants in control soils (Figure 1), may also help to explain this loss of significance.

Four-ring analytes were also depleted more rapidly in *P. sordida*-treated soil than in control soils (Figure 2). After 56 days, depletion of fluoranthene and pyrene in fungal-treated soil significantly exceeded losses from control soils (Table I). Greater depletion of benz[*a*]-anthracene and chrysene in fungal-treated soil was also observed, although these differences were not in general significant.

Rates of depletion of the targeted PAH analytes in soil treated with *P. sordida* could be grouped into three statistically distinct classes, defined by the number of rings in the analyte. Three-ring analytes were rapidly and extensively depleted by this fungal treatment. Concentrations of 3-ring analytes were decreased an average of 31% after 7 days and an average of 91% after 56 days. Four-ring analytes were more persistent: losses first became apparent between 14 and 28 days of treatment, and an average of 45% was depleted after 56 days. Five- and 6-ring analytes were the most recalcitrant species, persisting at original levels throughout the course of the study.

The same trend of increasing stability with an increasing number of rings was evident in control treatments as well. Three-ring analytes were depleted to significantly greater extents than were compounds with ≥ 5 rings in both controls. However, the depletion of 4-ring analytes was not significantly different from that of the higher molecular weight analytes in either control treatment.

Discussion

Solid-phase bioremediation with *P. sordida* under field conditions increased the rates of disappearance of 3- and 4-ring priority pollutant PAH component of creosote in this contaminated soil over that observed in control soil treatments. Because depletion rates of 3- and 4-ring analytes in soil amended with standard substrate were lower than those in soil amended with *P. sordida*, the observed rate increases in the latter case were due to fungal activity rather than to the stimulation of indigenous biota by added nutrients.

Average decreases in 3- and 4-ring analytes of 91 and 45%, respectively, were observed after treatment with *P. sordida* for 56 days. Detectable depletion of 5- and 6-ring analytes did not occur. However, because only 3% of the total weight of the measured analytes contaminating this soil contained ≥ 5 rings, total PAH mass was depleted by 75% after 8 weeks of fungal treatment.

Volatilization of PAHs containing more than 2 rings from soil has been reported to be negligible *(14)*, Abiotic processes leading to the depletion of PAH analytes containing more than 2 rings from soil have been found to be minor *(14)* or absent *(15)*. Losses of contaminants must therefore be attributed to biotransformation.

Persistence of PAH analytes in nontreated soils would be expected to differ as a function of soil type, microbial population, pollutant concentration, and presence and concentrations of co-contaminants *(15)*. Indeed, a wide range of half-lives for these compounds in untreated soils have been reported *(16)*. However, analytes with ≥ 4 rings have been reported to be more recalcitrant in the soil environment than those containing ≤ 3 rings *(14, 17-19)*. In this light, the consistent greater depletion of 4-ring analytes by treatment with *P. sordida* compared to that in control treatments is an encouraging result.

Five- and 6-ring analytes were not decreased by fungal treatment in this study. Degradation of these compounds by lignin-degrading fungi does occur under aqueous conditions *(20-22)*. The persistence of these compounds in soil maybe due to their low bioavailability when bound to soil particles. It is also possible that biotransformation of these lower-level contaminants may have been competitively inhibited by the abundance of more readily available substrates. High concentrations of readily available and oxidizable compounds would be expected to scavenge nonspecific oxidants, the agents whereby fungal degradative activity occurs.

However, depletion of PAHs containing ≥ 5 rings in soil by lignin-degrading fungi has been reported, under conditions providing a higher fungus:contaminant ratio than that used here *(9, 10)*. Thus, the failure of *P. sordida* to

transform this class of compounds in the present study may represent an engineering challenge rather than a fundamental limitation of the technology.

Other solid-phase technologies that have been assessed in field bioremediation studies at sites contaminated with PAHs include biostimulation, or landfarming *(23)*, and bacterial inoculation *(24)*. Depletion rates of 3- and 4-ring analytes in soil treated with *P. sordida* compare favorably to those described in field reports of either technology *(23, 24)*. Landfarming has been widely studied in the laboratory, and results indicate that PAH analytes containing ≥ 5 rings are not depleted by this approach *(25, 26)*. In fact, depletion of 4-ring analytes by landfarming techniques is unpredictable *(19, 25)*, even in cases where the presence of soil microbes capable of degrading the targeted contaminants have been demonstrated *(27)*.

In conclusion, this field treatability study demonstrate the potential and current limitations of the use of lignin-degrading fungi in the solid-phase bioremediation of a recalcitrant class of hazardous organic compounds. Depletion of 3- and 4-ring PAH analytes was stimulated by *P. sordida* treatment. However, higher molecular weight analytes were not affected by this treatment.

The technology described herein has been shown to successfully treat PCP under diverse conditions in laboratory and field studies *(7, 8, 11)*. Thus, these fungi are robust enough for practical application under a variety of conditions. However, the persistence of highly toxic PAHs containing ≥ 5 rings represents a significant challenge which must be overcome before the technology can be considered a viable treatment option for creosote-contaminated sites. Finally, it should be cautioned that an understanding of the nature and toxicity of the transformation products of any remediation treatment is necessary before that treatment can be considered an acceptable reclamation method. This question is the topic of ongoing research in our laboratory.

Acknowledgments

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