Mycobiology 38(4) : 238-248 (2010)
DOI:10.4489/MYCO.2010.38.4.238

Environmental Factors and Bioremediation of Xenobiotics Using White Rot Fungi

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(Received October 12, 2010. Accepted November 24, 2010)

This review provides background information on the importance of bioremediation approaches. It describes the roles of fungi, specifically white rot fungi, and their extracellular enzymes, laccases, ligninases, and peroxidises, in the degradation of xenobiotic compounds such as single and mixtures of pesticides. We discuss the importance of abiotic factors such as water potential, temperature, and pH stress when considering an environmental screening approach, and examples are provided of the differential effect of white rot fungi on the degradation of single and mixtures of pesticides using fungi such as *Trametes versicolor* and *Phanerochaete chrysosporium*. We also explore the formulation and delivery of fungal bioremedial inoculants to terrestrial ecosystems as well as the use of spent mushroom compost as an approach. Future areas for research and potential exploitation of new techniques are also considered.

KEYWORDS : Environmental factors, Extracellular enzymes, Fungi, Inoculants, Soil, Xenobiotic mixture

There is growing public concern over the wide range of xenobiotic compounds being introduced inadvertently or deliberately into soil. Such contamination can be long-term and have a significant impact on both decomposition processes and thus nutrient cycling. For example, the widespread incorporation of herbicides into soil every year constitutes a major concern since they can potentially pose a threat to our health as well as to the quality of soil, surface water, and groundwater resources [1-4].

Many xenobiotic compounds have medium to long-term stability in soil, and their persistence results in significant impact on the soil ecosystem. For example, chlorinated aromatic herbicides such as triazines are heavily used worldwide for the control of broad-leaved weeds in an agricultural setting [5-8] as well as in urban and recreational areas [9]. Fig. 1 compares the persistence of several xenobiotic compounds in soil, including those that receive attention later in this review. Some groups, such as triazines, are moderately persistent in soil [10] with reported half-life values of up to 50~100 days, depending largely on soil environmental conditions [11]. Microbial metabolism has long been regarded as the most important mechanism of degradation of such compounds in soil [12, 13]. Nevertheless, under conditions of low moisture and nutrient contents, microbial metabolism becomes compromised, and triazines and other xenobiotic compound persistence may increase as a result [14]. Thus, the relationships between soil type, moisture, pH, organic matter, and clay content all affect the binding of individual and mixtures of pesticides in soil. This in turn influences the effectiveness of bioremediation strategies. This review considers several important aspects of bioremediation systems using fungi, including (a) bioremediation approaches; (b) white rot fungi and evidence for their enzyme-mediated remediation; (c) white rot fungi and environmental screening of single and mixtures of pesticides; (d) inoculant production and delivery for soil incorporation; (e) use of spent mushroom composts; and (f) conclusions and future strategies.

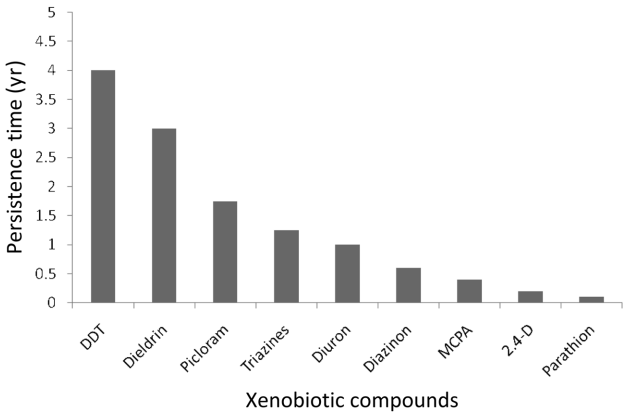

Fig. 1. Summary of the relative persistence levels of different xenobiotic compounds in soil.

Bioremediation Approaches

Microbial metabolism is regarded as the most important mechanism of pesticide degradation in soil [12, 13], and it constitutes the basis for all bioremediation and bioaugmentation strategies. Therefore, conditions that favor microbial growth and activity in soil, such as temperature,

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moisture, nutrient status, pH, and aeration, will also generally promote metabolic degradation of pesticides [3].

As suggested by Gadd [9] amongst others, the composition and size of soil microbial populations, as well as the status of metabolic activity, are the determining factors as to whether or not biodegradation is feasible as a remediation option. Biodegradation refers to the metabolic ability of microorganisms to transform organic contaminants into less harmful compounds [15]. According to McFarland *et al*. [16], bioremediation techniques aim to accelerate the naturally occurring biodegradation process by optimizing the conditions under which it occurs.

In many contaminated areas, even though suitable microbial populations may be available for biodegradation of a given contaminant, environmental conditions may limit or even inhibit this process [15]. In such cases, biostimulation of the degrading potential of native microbial populations and/or the addition of selected degrading microorganisms to contaminated soil (bioaugmentation) have been effective at enhancing pesticide metabolism [2, 17].

Biostimulation typically involves the addition of limited nutrients (e.g., carbon and nitrogen sources, O_2), acid or bases for pH optimization, or water or specific substrates to stimulate specific enzymes. It is an effective bioremediation strategy [15, 16], although it may have poor reproducibility and be dependent on the characteristics of microbial populations [9].

Alternatively, bioaugmentation is an attractive option. Indeed, the major advantage offered by bioaugmentation is the ability to choose the introduced species based on the goals of the process and the conditions of the matrix. In this case, the success of bioremediation is mainly dependent on the competition/proliferation capability of the introduced species and the bioavailability of the xenobiotic compounds [3]. Bioavailability here refers to the acquisition and subsequent transformation/degradation of the compound [9] and is closely related to its chemical properties, as well as to a wide range of soil physical and chemical parameters.

Complete biodegradation ultimately results in the mineralization of xenobiotic compounds to CO_2 and water [2]. In addition to mineralization, which implies the use of xenobiotics as a carbon source, microorganisms can also co-metabolize pesticides, e.g., transforming them into metabolites while growing and obtaining energy from other substrates found in soil [3, 9]. Many of the reactions involved in co-metabolism of pesticides, including oxidation-reduction, de-halogenation, ring-cleavage, and hydrolysis, occur simultaneously [9]. Transformation can lead to complete detoxification, breakdown of products, which may be further attacked by other microbial groups, or in some cases, to more toxic metabolites [3]. Triazines, among other halogenated aromatic compounds, are often co-metabolized into more toxic metabolites [9, 10]. For example, microbial enzyme-mediated methylation reactions usually increase herbicide lipophilicity and thus the potential for bioaccumulation in the food chain [2]. Generally, biodegradation and co-metabolism occur in combination in soil.

The use of bioremediation to remove pollutants is typically less expensive than equivalent physical-chemical methods. This technology offers the potential to treat contaminated soil and groundwater on site without the need for excavation [3, 18], requires relatively little energy input, and preserves the soil structure [19]. Perhaps the most attractive feature of bioremediation is its reduced impact on natural ecosystems, which should be well received by the public [20]. For fungal systems, bioremediation requires the soil to be aerobic with the provision of enough oxygen to enable effective colonization to occur.

For any xenobiotic compound, the threshold concentration above which remediation becomes necessary is referred to as the so-called "remediation trigger level." However, for many pesticides as well as xenobiotic compounds, the threshold concentration has not yet been established. There is also a need to establish a target concentration once remediation is achieved. Generally, the target concentration is assumed to be in the 1 ppm (mg/L) range, but in practice it can vary from site to site and region to region [3, 10].

Very often, urban application of pesticides is carried out at an excessively high concentration, resulting in pesticide waste characterized by prolonged persistence [3]. When applied at normal agricultural rates, which can be between 1~4.5 kg/ha, pesticide degradation in soil may be around 99% over the course of a growing season. However, Khadrani *et al*. [3] suggested that even at these concentrations, top-soil residues have been found to last for several years, ranging from 0.5 to 2.5 ppm. Unfortunately, even when present in soil at the ppb level (μg/L), many recalcitrant compounds often migrate through leaching and reach groundwater [21].

At present, bioremediation conducted on a commercial scale predominantly utilizes prokaryotes, with comparatively few recent attempts using white rot fungi. Bacteria are very sensitive to fluctuating environmental conditions in soil since their growth requires films of water to form in soil pores. However, filamentous fungi offer major advantages over bacteria regarding the diversity of compounds they are able to oxidize [10]. In addition, they are robust organisms that are generally more tolerant to high concentrations of polluting chemicals than bacteria [22]. Filamentous fungi are also more tolerant of environmental stress and can produce copious amounts of extracellular enzymes during hyphal colonization of soil, resulting in enhanced rates of bioremediation [22, 23]. Therefore, white rot fungi potentially represent a powerful tool for soil bioremediation, with some species already patented [24]. Interestingly, only a few companies have incorpo-

rated ligninolytic fungi for soil remediation into their program, e.g., "EarthFax Development Corp." in USA or "Gebruder Huber Bodenrecycling" in Germany. However, many research studies have been carried out to examine the efficacy of bioremediation fungi in degrading single xenobiotic compounds.

White Rot Fungi: Evidence of Enzyme-mediated Degradation of Xenobiotic Compounds

The application of fungi for the cleanup of contaminated soil first came to attention in the mid-1980s when the white rot fungus *Phanerochaete chrysosporium* was shown to metabolize a range of organic environmental contaminants [25, 26]. Later, this ability was demonstrated for other white rot fungi, including *Trametes versicolor* and *Pleurotus ostreatus* [17]. White rot fungi are the most widely studied and understood ligninolytic fungi to date [2, 3, 27-29]. In nature, these fungi colonize and degrade lignocellulosic materials (normally tree wood) and are responsible for causing white rot of wood [27].

Lignin is a three-dimensional, naturally occurring polymer present in woody plants, and it constitutes one of the most structurally complex and therefore resistant materials to microbial degradation [9, 30]. The ability of white rot fungi to mineralize lignin is generally attributed to the secretion of extracellular ligninolytic enzymes, mostly laccase (LAC), lignin peroxidase (LiP), and manganese peroxidase (MnP) [9, 27, 29]. It is accepted that both of these peroxidases catalyze the oxidation of (endogenously-produced) low-molecular weight mediators using H_2O_2 as an oxidant. These powerful mediators then oxidize lignin, leaving it partially modified and open to further attack by other enzymes such as LAC [9, 10].

A main feature of LAC is its highly non-specific nature with regard to the breakdown of substrates [30]. Further, xenobiotics share at least one of many sub-structures (e.g., combination of functional groups) present in the lignin molecule [9]. This explains the ability of white rot fungi to tolerate and degrade such a wide range of environmental organic pollutants, even when at high concentrations [3, 9]. It has been shown that both LAC and peroxidases co-metabolize these compounds with lignin through similar oxidative mechanisms [2, 9, 30], although with no net energy gain. In fact, oxidation of lignin is performed in order for these fungi to have access to wood polysaccharides, which is their main energy source and which other microorganisms cannot access [10]. This implies that the presence of lignocellulosic substrates is a requirement for the degradation of xenobiotic compounds [29].

Although LAC activity is involved in lignin degradation, it is also associated with microbial growth [24] or specific interactions between microorganisms, particularly with regard to *T. versicolor* [30]. Determining the activity of LAC in soil inoculated with white rot species provides a measure of the colonizing ability of the fungus and can be used to monitor the bioremediation of numerous soil contaminants, among them triazine pesticides [28, 30, 31]. Other applications of LAC activity were reported by Schmidt *et al*. [27], who studied the impact of fungal inoculum properties on *T. versicolor* growth and activity in soil. Novotný *et al.* [32] measured LAC activity to demonstrate the correlation between its production and the degradation of polycyclic aromatic hydrocarbons (PAHs) by several strains of white rot fungi in both liquid culture and soil.

Paszczynski and Crawford [33], Gadd [9], and Šašek *et al*. [24] reviewed a series of applications involving white rot fungi, among them *T. versicolor*, for environmental remediation of pesticides, disinfectants (e.g., pentachlorophenol) synthetic dyes, benzene derivatives (e.g., petrol and diesel), PAHs, explosives (trinitrotoluene, TNT derivatives), and industrial solvents (e.g., polychlorinated biphenyls). They also offered descriptions on the use of these fungi for the biotransformation of coal, treatment of effluents from paper and olive-processing plants, and the degradation of synthetic polymers (e.g., plastics) and other materials (e.g., nylon). All of these applications were found to be related to the production of LACs, Mn-peroxidase or (less frequently) ligninperoxidases, both alone or in combination, which has been corroborated by other studies [28, 30, 31, 34].

However, most studies performed thus far have focused on the screening of white rot fungi for the bioremediation of xenobiotic contaminants in liquid culture media [35], bioreactors [33], sterile soil [36] or soil extract broth [28]. In non-sterile soil, where pesticide degradation may be influenced by other factors other than the fungus, our knowledge is more limited [37]. Recently, Fragoeiro and Magan [28] reported the successful application of three white rot species, including *T. versicolor*, for the bioremediation of mixtures of pesticides in non-sterile sandy loam soil under low water potential conditions. This demonstrated that differential breakdown of mixtures of pesticides occurred possibly due to environmental factors.

LAC activity in the biodegradation of xenobiotic compounds with lignin-like structures has already attracted considerable interest [38], and its biodegradative effects on different contaminants have been exhaustively studied. Specifically, LAC enzyme is a copper-containing phenoloxidase involved in the degradation of lignin [10], and it oxidizes phenol and phenolic lignin sub-structures [39]. The catabolic role of fungal LAC in lignin biodegradation is not well understood [38, 40], but there have been some successful instances of this enzyme performing decontamination. For example, dye decoloration by *Trametes hispida* [35], degradation of azo-dyes by *Pyricularia oryzae* [41], and textile effluent degradation by *T. versi-*

color have all been attributed to LAC activity. Esposito *et al.* [42] also reported that LAC from *Cerrena unicolor* results in complete transformation of 2,4 DCP in soil colloids. Additionally, the various roles of LAC have recently been comprehensively reviewed [11].

Demir [43] examined the biological degradation of benzene and toluene by *T. versicolor* as well as biomass determinations. Complete removal of benzene and toluene was observed after 4 hr when the initial toluene concentration was 50 mg/L, whereas it took 36 hr for complete degradation at an initial concentration of 300 mg/L. Regarding benzene, biodegradation was completed after 4 hr at an initial concentration of 50 mg/L, whereas it took 42 hr to completely remove benzene at 300 mg/L. Addition of veratryl alcohol, a LAC inducer, to the basic feed medium enhanced the performance of the enzyme system and shortened the overall time period of biodegradation completed in a shorter time period.

Han *et al.* [2] studied the degradation of phenanthrene by *T. versicolor* and purified its LAC. After 36 hr, about 46 and 65% of the compound added at initial concentrations of 100 mg/L to shaken and static fungal cultures were removed, respectively. Although the removal percentage was highest (76.7%) at 10 mg/L, the transformation rate was maximal (0.82 mg/hr) at 100 mg/L of phenanthrene in the fungal culture. However, when purified LAC of *T. versicolor* was reacted with phenanthrene, the compound was not transformed. Another interesting example of contaminant degradation and enzyme activity was seen in the study described by Barr and Aust [44]. They found cyanide to be quite toxic to spores of *P. chrysosporium* (50% inhibition of glucose metabolism at 2.6 mg/L) due to the absence of LiPs, which can rapidly metabolize cyanide, as ligninolytic 6-day-old cultures were able to tolerate considerably higher cyanide concentrations (50% inhibition of glucose metabolism at 182 mg/L). Valli *et al.* [39] demonstrated the mineralization of 2,7-dichlorobenzenop-dioxin by *P. chrysosporium*. Their results showed that purified LiPs and MnPs were capable of mineralization in a multistep pathway. Esposito *et al.* [42] showed that different actinomycetes were able to degrade diuron in soil using MnPs.

Despite many reports that the degradation of xenobiotics by white rot fungi is mediated by enzymes involved in lignin degradation, some authors have presented contradictory evidence. For example, Jackson *et al.* [6] reported the degradation of TNT by non-ligninolytic strains of *P. chrysosporium*. Bending *et al.* [45] showed > 86% degradation of atrazine and terbuthylazine by white rot fungi in liquid culture and found no relationship between degradation rate and ligninolytic activity. Other studies featuring *P. chrysosporium* in liquid culture have reported biotransformation of the insecticide lindane independently of the production of ligninolytic enzymes [46]. These researchers ruled out the involvement of peroxidases in lindane biotransformation and mineralization, and they assessed the activity of cytochrome P450 monooxygenase, an enzymatic system used by many organisms for detoxification. They found that the P450 inactivator 1-aminobenzotriazole drastically reduced pesticide metabolism, whereas phobarbital, a P450 inducer, did not increase lindane breakdown.

Whether the degradation of pesticides is carried out by lignin-degrading enzymes or other enzymatic systems, or both, the use of fungi in bioremediation is clearly very promising, and further studies should be conducted to understand which enzymes are involved. Such information could be very useful in establishing the best conditions for enzyme production followed by fungal bioremediation *in situ*. It is also necessary to assess the production of these enzymes in soil, since it is where bioremediation occurs under field conditions and since there are considerably more studies on enzyme production in liquid cultures. Additionally, there is little information on the degradation of mixtures of xenobiotics, which is more common in nature than single ones.

Recent work on the *P. chrysosporium* genome has shown that cytochrome P450 monooxygenases constitute the largest and most important group of P450 genes in any fungal species and that they are differentially expressed depending on xenobiotic type and nutrition [47, 48]. This has resulted in the development of powerful tools useful in understanding the roles of key enzymes produced by *P. chrysosporium* in the bioremediation of different xenobiotic compounds under various environmental conditions. However, these tools have seldom been applied to the examination of how environmental stress factors such as water potential and temperature effects on the expression of P450 gene clusters involved in enzyme production both *in vitro* and *in situ*.

The *in vitro* environmentally relevant screening of white rot species using both single and mixtures of pesticides can be used to identify potential candidates when followed by *in situ* microcosm studies using different inoculant formulations. This can be instructive and useful in understanding the approaches and strategies needed for the development of effective bioremediation systems using white rot fungi.

Screening of White Rot Fungi for Tolerance to Single and Mixtures of Xenobiotic Compounds

Tolerance and growth of white rot fungi in the presence of individual and mixtures of xenobiotic compounds may vary depending upon the type and concentration of the xenobiotic as well as the nutritional and environmental conditions. It is important to use realistic nutritional media such as soil extract-based systems, in which the water potential, pH, and temperature can be modified, since

Table 1. Concentrations (mg/L) of eight test isolates of simazine, trifluralin, and dieldrin, both individually and as a mixture, that cause 50% reduction in fungal growth (EC_{50}) when grown on soil extract agar at 15°C

Isolates		Simazine	Trifluralin	Dieldrin	Mixture
	Ψ (MPa)	EC_{50} (mg/L)	EC_{50} (mg/L)	EC_{50} (mg/L)	EC_{50} (mg/L)
Pleurotus cystidious	−0.7	46.0	33.9	21.2	28.3
	−2.8	27.4	8.6	13.3	70.5
Pleurotus sajor-caju	−0.7	70.7	20.0	15.0	17.6
	−2.8	11.2	23.3	N.G.	10.8
Trametes socotrana	−0.7	N.I.	38.3	44.4	33.1
	−2.8	25.5	15.3	31.2	38.0
Polystictus sanguineaus	−0.7	N.I.	47.1	17.4	22.4
	−2.8	14.7	17.6	10.8	11.9
Trametes versicolor	−0.7	314.0	213.0	22.6	55.8
	−2.8	N.I.	30.9	1,207.0	13.6
T. versicolor	−0.7	N.I.	24.6	115.0	32.3
	−2.8	26.2	50.6	24.6	25.0
Phanerochaete chrysosporium	−0.7	N.G.	14.7	14.4	2.8
	−2.8	N.G.	N.G.	N.G.	N.G.
Pleurotus ostreatus	−0.7	45.0	33.6	19.9	14.1
	−2.8	19.9	27.5	12.2	19.8

N.G., no growth; N.I., no inhibition.

these conditions can have a significant impact on the relative tolerance of a potential fungal bioremediation agent. Table 1 shows an example of the concentrations of single and mixtures of pesticides (effective concentration; EC_{50} values) required to control a range of white rot fungal isolates in response to changes in water potential. The results highlight the importance of examining such factors when determining the relative tolerance/sensitivity to single and mixtures of pesticides as well as when choosing appropriate isolates for subsequent use *in situ*. In this case, −0.7 and −2.8 MPa water potential were used to represent conditions under which water was available and plants would grow, with the latter being twice the wilting point of plants. This result can be compared with previous *in vitro* modifications of soil extract media in which the water potential was modified matrically using PEG8000 as a solute. We previously showed that many *Trametes* and related species are more sensitive to matric than solute stress [46, 49, 50]. Table 1 shows an example of the relative growth levels of isolates of *T. versicolor* and *P. ostreatus* in response to individual and mixtures of pesticides. The results show that under matric stress conditions, these fungi are able to grow at the wilting point of plants but not at twice this level. This approach is important in determining whether or not the candidate isolates may be effective *in situ*.

The applicability of fungi to the bioremediation of soil contaminated with pesticides depends on the capacity of fungi to grow in the presence of such compounds as well as their ability to produce degradative enzymes. Additionally, complementary information on the capacity and ability of fungi to produce the key extracellular enzymes required for degradation of individual and mixtures of xenobiotic compounds is required. A significant amount of research on white rot fungi has been conducted in liquid and/or synthetic media, but less is known about its bioremediation capabilities in soil, especially under different environmental conditions. Tekere *et al.* [51] and Hestbjerg *et al.* [40] reported that field conditions do not always enable white rot fungi such as *P. chrysosporium* to achieve optimum activity, and therefore it is not a good competitor in a soil environment [37, 40]. This latter finding was reinforced by Radtke *et al.* [10], who reported that bacteria from polluted and agricultural soil antagonize the growth of *P. chrysosporium* on solid media. Nevertheless, some studies have described the successful application of *P. chrysosporium* as a bioremediation agent in soil. For example, McFarland *et al.* [16] described complete alachlor transformation by this fungus within 56 days of treatment. Reddy and Mathew [7] also showed that this species was able to degrade DDT, lindane, and atrazine.

Recently, we demonstrated that under different osmotic stress regimes, white rot fungi are able to differentially degrade mixtures of pesticides in soil extract broth (Table 2) [31]. We also demonstrated an increase in the range of hydrolytic enzyme production, including ligninases and cellulases, even under water stress conditions (Table 3). Although it is accepted that extracellular ligninolytic enzymes are at least in part responsible for the critical initial reactions of pollutant transformation, the production and activity of these enzymes in contaminated soil under different field conditions have not been examined in detail, although they are critical for successful degradation [36, 52].

Table 2. Mean fungal growth rates (± SD) for *Trametes versicolor* (R101) and *Pleurotus ostreatus* in soil extract supplemented with three pesticides, both individually and as a mixture, under different matric potentials at 15°C

		T. versicolor R101			*P. ostreatus*		
Ψ (MPa)		−0.5	−1.5	−2.8	−0.5	−1.5	−2.8
Simazine (mg/L)	0	0.6 ± 0.07	0.5 ± 0.04	0	1.7 ± 0.03	0	
	5	0.7 ± 0.07	0.4 ± 0.03	0	0.6 ± 0.09	0	0
	10	0.6 ± 0.18	0.5 ± 0.01	0	0.2 ± 0.36	0	0
Trifluralin (mg/L)	5	0.6 ± 0.03	0.4 ± 0.02	0	1.0 ± 0.09	0	0
	10	0.4 ± 0.01	0.5 ± 0.06	0.1 ± 0.01	0.4 ± 0.07	0	0
Dieldrin (mg/L)	5	0.7 ± 0.03	0.3 ± 0.01	0	0.2 ± 0.10	0	0
	10	0.7 ± 0.10	0.5 ± 0.03	0.1 ± 0.01	1.0 ± 0.00	0	0
Mixture (mg/L)	5	0.7 ± 0.02	0.4 ± 0.01	0	1.5 ± 0.00	0	0
	10	0.8 ± 0.05	0.4 ± 0.04	0	0.1 ± 0.00	0	0

Table 3. Effects of simazine, trifluralin, and dieldrin (0, 5, and 10 mg/L), both individually and as a mixture, on ligninolytic activities of *Trametes versicolor* (R101) and *Pleurotus ostreatus* at 15°C in response to solute water potential (expressed as radius of enzymatic clearing zone ± SD of the mean, n = 3)

		T. versicolor (R101)		*P. ostreatus*	
Ψ (MPa)		−0.7	−2.8	−0.7	−2.8
Simazine (mg/L)	0	21 ± 0.6	11 ± 1.0	40 ± 0.0	27 ± 0.7
	5	21 ± 0.6	10 ± 1.0	21 ± 0.4	13 ± 0.4
	10	16 ± 0.6	13 ± 1.0	21 ± 1.2	10 ± 0.1
Trifluralin (mg/L)	5	14 ± 0.6	12 ± 0.5	35 ± 0.6	25 ± 0.6
	10	11 ± 0.6	8 ± 0.6	28 ± 0.6	19 ± 0.5
Dieldrin (mg/L)	5	15 ± 1.0	10 ± 1.5	36 ± 1.5	13 ± 1.9
	10	11 ± 1.0	6 ± 1.0	25 ± 0.2	15 ± 0.6
Mixture (mg/L)	5	15 ± 0.6	8 ± 0.6	25 ± 0.6	22 ± 1.5
	10	21 ± 0.6	9 ± 0.6	17 ± 0.6	18 ± 1.0

Inoculant Production for Soil Incorporation of Bioremedial Fungi

There are many studies on how to optimize the biodegradation potential of white rot fungi in contaminated soil [16, 35, 53, 54]. If it is accepted that extracellular ligninolytic enzymes are at least in part responsible for the critical initial reactions of pollutant transformation, then the production and activity of these enzymes in contaminated soil under field conditions are both prerequisites for the successful application of white rot fungi in soil bioremediation [36].

A wide range of saprophytic microorganisms exists in natural soil. Introduction of white rot fungi requires effective growth and competition with these native populations. Additionally, bioremedial fungi should be able to secrete the necessary enzymes into the soil matrix in order to enhance the degradation of pesticide molecules that would otherwise be unable to be incorporated across cell walls [25].

Most protocols for delivering inoculum of wood rot fungi for soil bioremediation have been adopted from mushroom growers, who have perfected the art of producing fungal spawn on lignocellulosic waste. Species used in mushroom production have been formulated on inexpensive substrates, including corncob, sawdust, wood chips, peat or wheat straw. When used in bioremediation, these substrates are impregnated with mycelium and mixed with contaminated soil [7, 33]. There is little information available on the survival of white rot fungi in soil, especially those fungi not consumed by humans. Several groups are investigating methods of improving the survival of wood rot fungi in polluted soils [9, 35, 53]. Certainly, better fungal growth could help introduced fungi overcome competition from indigenous microorganisms as well as enhance bioremediation. This is important as native soil microorganisms may occupy the lignocellulosic substrate, which restrains the growth and activity of white rot fungi, inhibits fungal lignino-cellulose decomposition, and reduces enzyme release [52].

The introduction ratio of white rot fungi has an important impact on its economics of practical application. For example, in studies by Fragoeiro and Magan [28], a ratio of 5 g of inoculant to 95 g of soil was used. The effect of using this approach on the differential breakdown of mixtures of xenobiotic compounds under different environmental regimes is shown in Table 4. Other authors have used very different ratios. For example, Novotny *et al.* [21] reported the degradation of dye in soil using a 50 : 50 soil : straw-based inoculant of *Irpex lacteus*; Canet *et al.*

Table 4. Comparison of the effects of woodchips and fungal inoculants on percentages of pesticide (%) (simazine, trifluralin, and dieldrin, 10 mg/kg) degraded after 6 and 12 wk at water potentials of −7.0 and −2.8 MPa in soil microcosms at 15°C

Incubation (wk)	W. potential (−MPa)	Treatment	Percentage pesticide degraded		
			Simazine	Trifluralin	Dieldrin
6	0.7	Woodchips	41.4* (2.5)	56.0 (58.4)	71.2 (23.7)
		T. versicolor	89.9*	77.7	48.2*
		P. chrysosporium	63.8*	74.7	87.3
6	2.8	Woodchips	13.8 (21.2)	75.2 (57.1)	61.8 (40.0)
		T. versicolor	57.1*	81.7*	70.7*
		P. chrysosporium	64.4*	85.5*	69.9*
12	0.7	Woodchips	46.6* (27.5)	67.5 (62.4)	79.4 (53.8)
		T. versicolor	73.5*	76.5	52.7
		P. chrysopsporium	75.6*	57.3	100.0*
12	2.8	Woodchips	75.7* (29.9)	92.1* (64.2)	61.6 (40.2)
		T. versicolor	57.3	80.9*	51.0
		P. chrysopsporium	64.3*	93.7*	79.7*

Figures in parentheses are for comparison with degradation in natural soil.
*Significantly different from the controls based on actual concentration using high-performance liquid chromatography ($p = 0.05$).

[25] used straw-based inoculum with an incorporation rate of 40% incorporation; Ryan and Bumpus [35] used a 25% straw-based inoculum; Meysami and Baheri [54] used 10% straw-based inoculum; and Morgan *et al.* [53] used 4 g of ground maize cobs to 1 g of soil. We believe that some of these formulations are very unrealistic for the bioremediation of xenobiotics in contaminated soils from a practical and economic point of view. Furthermore, few, if any, have examined the effect of water potential on mixtures of pesticides. Novotný *et al.* [55] used the same species as the present study along with *Pleurotus ostreatus* and found that the latter was better than both *P. chrysosporium* and *T. versicolor*. However, they used sterile soil only, which is devoid of all natural microbial communities otherwise present.

Boyle [56] reported increases in growth and carbon dioxide production in natural soil supplemented with carbon. He observed that mineralization of [^{14}C] pentachlorophenol (degradation to $^{14}CO_2$) was much faster in soil that had been amended with alfalfa and benomyl and inoculated with *T. versicolor*. Another study showed that the addition of straw increases the hyphal length of white rot fungi in soil [53]. Besides strong growth, it is important that the inoculation conditions promote enzyme production. Moredo *et al.* [57] investigated ligninolytic enzyme production by the white rot fungi *P. chrysosporium* and *T. versicolor* after pre-cultivation on various insoluble lignocellulosic materials, including grape seeds, barley bran, and wood shavings. Cultures of *P. chrysosporium* pregrown on grape seeds and barley bran showed maximum LiP and MnP activities (1,000 and 1,232 units/L, respectively). On the other hand, *T. versicolor* pre-cultivated with the same lignocellulosic residues showed maximum LAC activity (approx. 250 units/L). *In vitro* decoloration of the polymeric dye Poly R-478 using the extracellular liquid obtained in the above-mentioned cultures was carried out in order to determine the respective capabilities of LAC, LiP, and MnP. It is noteworthy that the degrading capability of LiP upon pre-cultivation of *P. chrysosporium* with barley bran gives a percentage of decoloration of about 80% after 100 sec [57]. Utilization of these solid substrates in soil may also be advantageous as a means of evenly distributing fungal inoculum in large volumes of soil [58], and according to Singleton [59], growth amendments could also exert beneficial effects by adsorbing pollutants and hence decreasing the bioavailability of toxic pollutants.

Use of Spent Mushroom Composts

Composting matrices and composts are rich sources of xenobiotic-degrading microorganisms, including bacteria, actinomycetes, and ligninolytic fungi, all of which can degrade pollutants to innocuous compounds such as carbon dioxide and water. These microorganisms can also biotransform pollutants into less toxic substances and/or immobilize pollutants within an organic matrix, thereby reducing pollutant bioavailability [60].

Spent mushroom compost (SMC) is a byproduct of mushroom production and is produced in large amounts. For every 200 g of *Pleurotus* spp. produced in Malaysia, about 600 g of spent compost is produced [59], or 5 kg of SMC generated for every 1 kg of edible mushrooms according to Law *et al.* [61]. This resulted in 40 M tonnes of SMC in 1999 alone. Therefore, the disposal of SMC is a major problem for mushroom farmers. Most either discretely burn or discard it [59], and thus its exploitation as a potential bioremediation adjuvant has received significant attention [62].

Mushroom cultivation involves the pure culture of

spawn, composting, pasteurization of the substrate, and careful regulation of growing conditions [63]. The substrates are lignocellulosic residues, such as straw, horse manure, chicken manure, and activators [63]. The purpose of composting the substrate is to exclude microorganisms that may interfere with mushroom growth. Following mushroom harvest, SMC is likely to contain not only a large and diverse group of microorganisms but also a wide range of extracellular enzymes that are active against wheat straw [63]. Singh *et al.* [64] reported the extraction of cellulase, hemicellulose, β-glucosidase, lignin peroxidises, and LAC from SMC. It also contains very high organic content (20%), including cellulose, hemicellulose, and lignin [65], from the unused lignocellulosic substrate [64].

Previous research has provided some interesting findings using this type of compost as a bioremediation adjuvant. Law *et al.* [61] reported that SMC of *Pleurotus pulmonarius* could remove 89.0 +/– 0.4% of 100 mg of PCP/L within 2 days at room temperature predominantly by biodegradation. Kuo and Regan [65] used sterilized SMC as an adsorption medium for the removal of a mixture of pesticides (carbaryl, carbofuran, and aldicarb) with a concentration range of 0~30 mg/L and found that SMC was able to successfully adsorb carbamate pesticides from aqueous solutions, possibly due to increased organic matter content.

With mushroom production being the largest solid-state fermentation industry in the world [66], and with so much waste being produced, it is extremely important to find a use for SMC. Thus, SMC as a soil amendment for the improvement of pesticide bioremediation is an interesting area. Furthermore, there is no information on the effect of SMC addition on soil enzymes, soil respiration, and soil populations, or on how these metabolic parameters are affected by the presence of pesticides and water availability.

We previously examined the addition of a mixture of 5 g of SMC and 95 g of unsterile sandy loam soil treated with 10 mg/kg of soil to a combination of pesticides (simazine, trifluralin, and dieldrin) using a moisture adsorption curve to achieve water potentials of –0.7 and 2.8 MPa [28]. These treatments were stored for 42 and 84 days at 15°C. The amount of CO_2 produced was measured by GC analysis and by determining total ligninolytic activity, whereas high-performance liquid chromatography with UV detection was used to analyze the amount of each pesticide remaining in each of the treatments. Fig. 2 shows the effect of each treatment on CO_2 production. The soil amended with SMC had by far the highest microbial activity regardless of water potential and pesticide treatment. Fig. 3 shows that in all cases, the SMC-amended treatments (– and + pesticides) had the highest ligninolytic activities after 42 and 84 days. Table 5 shows the effect of SMC on the differential breakdown of the

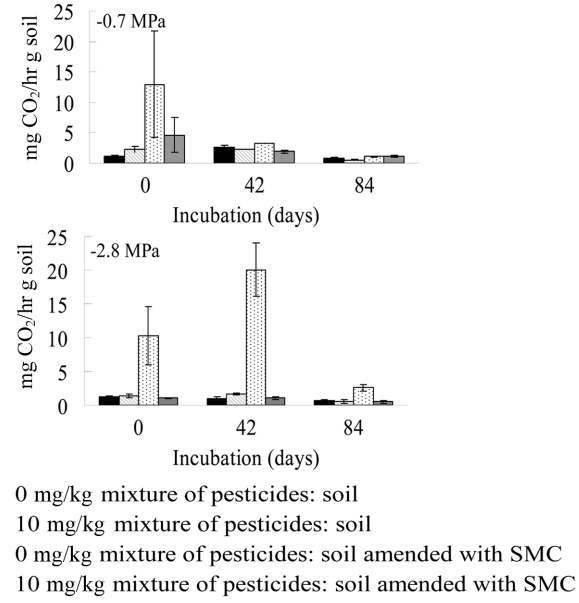

Fig. 2. Changes in carbon dioxide concentrations in soil microcosms, control soil, and soil inoculated with spent mushroom composts (SMC) for up to 84 days at 15°C under two different water potential regimes (–0.7 and –2.8 MPa) and supplemented with a mixture of simazine, trifluralin, and dieldrin (0 and 10 mg/kg soil). Bars represent the standard deviation of the mean (n = 3 microcosms) per treatment.

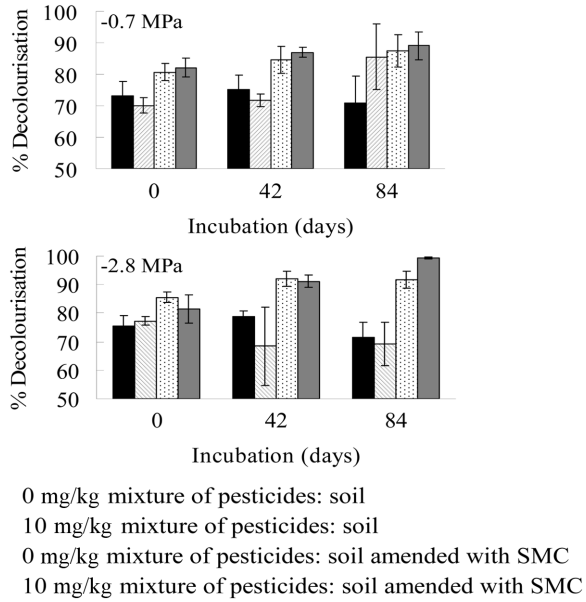

Fig. 3. Total ligninolytic activities (expressed as % decoloration of Poly R478) in soil microcosms, control soil, and soil inoculated with spent mushroom composts (SMC) for up to 84 days at 15°C under two different water potential regimes (–0.7 and –2.8 MPa) and supplemented with a mixture of simazine, trifluralin, and dieldrin (0 and 10 mg/k soil). Bars represent the SD of the mean (n = 3 microcosms) per treatment.

Table 5. Comparison of the percentage pesticide concentrations remaining in soil amended with SMC and supplemented with a pesticide mixture of 10 mg/kg of soil after 42 and 84 days incubation at 15°C under two different water regimes

		(% Remaining)					
Time		Simazine		Trifluralin		Dieldrin	
(days)	Ψ (MPa)	Soil + SMC	Control	Soil + SMC	Control	Soil + SMC	Control
42	−0.7	13.2*	97.5	40.0	24.6	39.5	20.9
	−2.8	37.5*	78.8	27.4	42.9	25.4*	60.0
		L.S.D. = 15.20		L.S.D. = 42.28		L.S.D. = 22.10	
84	−0.7	18.2*	72.5	21.8	37.6	19.9*	46.5
	−2.8	58.1*	70.1	18.4*	35.8	14.5*	59.8
		L.S.D. = 14.17		L.S.D. = 16.28		L.S.D. = 21.76	

SMC, spent mushroom composts; L.S.D., least significant difference.
*Significantly different from the control.

three pesticides under different water potential and time conditions. The results demonstrate that there was significant enhanced breakdown of all three pesticides after 42 and 84 days and under both water potential regimes due to SMC inoculation. This suggests that SMC contains a mixture of ligninolytic enzymes, including LACs, actinomycetes, and in some cases, basidiomycete hyphae. Therefore, this may be an effective approach in bioremediation systems. Advantages include maintenance of an aerobic soil structure, effective rates of moisture retention, and avoidance of anaerobic conditions.

Conclusions and Future Strategies

Application of fungal technology for the cleanup of contaminants has shown promise ever since 1985, when the white rot species *Phanerochaete chrysosporium* was found capable of metabolizing a number of important environmental pollutants and strains were subsequently commercialized for the treatment of contaminated soil-based xenobiotic contaminants. There have been many studies that have shown white rot fungi to be useful for the *in vitro* and *in situ* degradation of predominantly single contaminants, although in mixtures of xenobiotic compounds have been addressed occasionally. In reality, contaminated soil usually involves a mixture of xenobiotic compounds. It is thus surprising that only a few studies have addressed this important aspect, e.g., the differential degradation of xenobiotic compounds in natural soil ecosystems. The second area we have tried to address is the impact of environmental conditions. Most research is carried out under largely optimal conditions for the growth/colonization of fungal inoculants. However, as we have shown, white rot fungi can grow effectively under water stress conditions where no plant growth occurs [23, 67]. This may become very important in the future when we examine the different interacting factors that affect bioremediation by fungi, and indeed all microorganisms, including the impacts of climate change. Thus, elevated CO_2 concentrations and slightly increased temperatures may have subtle but significant effects on the functioning of terrestrial ecosystems as well as any introduced microorganisms from a bioremedial perspective. However, practically no work has been done to address these aspects.

The last area that will hasten the development of remediation approaches is the determination of the genomes of specific white rot fungi. For example, research into the *P. chrysosporium* genome has resulted in the elucidation of specific cytochrome P450 monooxygenases, which may be differentially expressed in the presence of xenobiotic compounds [14, 47]. Knowledge of these gene clusters and their relative expression levels can now be quantified and integrated with data on associated ecological and physiological factors, which will result in a more complete understanding of the mechanisms of action and functioning of fungal bioremediation systems. This could result in a more integrated "Systems" approach for the effective exploitation of bioremedial fungi for treating xenobiotics in terrestrial ecosystems.

Acknowledgements

We are grateful to Mrs. Esther Baxter for her assistance with the preparation of this review.

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