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# The Role of White-rot Fungi in Herbicide Transformation

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Additional information is available at the end of the chapter

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## Abstract

Understanding herbicide transformation is necessary for pesticide development for their safe and efficient use, as well as for developing pesticide bioremediation strategies for contaminated soil and water. Recent studies persuasively demonstrated the key role of soil white-rot fungi in biotransformation of various anthropogenic environmental contaminants. However, often this common knowledge is not associated with specific metabolic processes of fungi and therefore cannot be transformed into specific recommendations for agricultural practice. The given review offers a systematic collection and analysis of the current knowledge about herbicide transformation by white-rot fungi at the cellular and molecular levels. Special attention is given to the role of oxidative enzymes such as laccases, lignin peroxidases, and manganese peroxidases in the biotransformation processes.

**Keywords:** White-rot fungi, biotransformation, herbicides, oxidases, metabolic fate

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## 1. Introduction

Fungi are unique organisms that colonize all areas of the environment – air, water, and soil. This group lists more than 1.5 million species and is remarkably flexible, occupying all biocenoses from the arctic tundra to the deserts. Biodiversity and specific genetic and molecular organization of fungi provided background for their key role in nature, i.e., maintaining of ecosystems' equilibrium. One of the most important groups playing a key role in the carbon cycle in nature is Wood Degrading Fungi, due to its ability to degrade or even mineralize lignin – widely present and one of the most stable biopolymers. They belong to Basidiomycota and Ascomycota and possess the unique ability to degrade components of xylem cell walls (cellulose, hemicellulose, lignin, and compounds forming these biopolymers). According to Anastasi et al. [1], this group is divided into white-rot fungi (WRF) or white rotters, brown-rot fungi, and soft-rot fungi because of the appearance of rotten wood.

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The ability to degrade lignin and its aromatic compounds is mostly attributed to white-rot fungi [2]. The white-rot decay of wood is performed by the combined action of oxidoreductive metalloenzymes, heme peroxidases, and laccases, encoded by multigene families as well as organic acids, secondary metabolites, and surfactants secreted by WRF [3]. This assembly is considered the Lignin Modifying System (LMS) [4]. It should be mentioned that extracellular ligninolytic enzymes (laccases, lignin peroxidases, manganese peroxidases, and versatile peroxidases) are nonspecific and can act both alone as well as using a redox mediator that enhances the range of potential substrates and provides the possibility of effective oxidation of xenobiotics. The WRF also produces reactive oxygen species (ROS) such as superoxide anion radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radicals ( $OH^\cdot$ ) [5]. WRF, their LMS and ROS are involved in the degradation of lignin and carbohydrate components of wood commonly accomplished by production of carbon dioxide and water. Both WRF and LMS are capable of *in vitro* oxidizing and degrading a broad range of xenobiotics: polycyclic aromatic hydrocarbons (anthracene, benz[a]pyrene, naphthalene, and phenanthrene); polychlorinated phenols (2,4-di-, 2,4,5-, and 2,4,6-tri-, and pentachlorophenols), chlorinated guaiacol and benzoate derivatives, 2,4,6-trichlorophenoxyacetate, and chlorinated biphenyls; stable polymers (polyacrylate, polyacrylamide, polycaprolactam, and polyethylene), 2,4-dichloroaniline, dioxins, explosives nitrates, dyes. The degradation of xenobiotics by WRF as well as enzymatic aspects of these processes is well documented and summarized in several recent reviews [1,6-10]. However, there are contradictory data reported on the role of ligninolytic enzymes in pesticide degradation. There was no relationship between WRF degradation of the dye Poly R-478, a presumptive test for ligninolytic potential, and degradation of the highly available pesticides, diuron, metalaxyl, atrazine or terbuthylazine in liquid culture [11]. Moreover, it was also shown that no degradation of the herbicide picloram by *Ganoderma lucidum* and *Trametes sp.* occurred under liquid stationary conditions in spite of the fact that both extra- and intracellular laccases were produced and, in the case of *Trametes sp.*, the enzyme production level improved especially for secreted laccase [12].

Recent findings have highlighted the molecular aspects of ligninolytic enzymes' functioning [13-15]. Genes-encoding ligninolytic enzymes of the white-rot fungi have been found to undergo differential regulation in response to different environmental signals and stimuli such as carbon and nitrogen concentration in cultural media, presence of xenobiotics and heavy metals, temperature regime, and various lengths of daylight. The analysis of MnP, LiP, and laccase gene promoter regions revealed the presence of xenobiotic response mechanism (XRE – xenobiotic responsive element), suggesting that these enzyme expressions can be similar in the presence of xenobiotics [15]. It was shown that compounds such as paracetic acid, ethanol, sodium arsenite, 2,4-dichlorophenol, and N,N-dimethylformamide enhanced the MnP production [16]. Moreover, a list of available aromatic compounds including xenobiotics (1-hydroxybenzotriazole, 2,5-xylydine, o-toluidine, 3,5-dihydroxytoluene, dimethylphenol, caffeic acid, caffeine, guaiacol, hydroquinone, etc.) that demonstrated the similar effect on laccase production was generated by Piscitelli et al. [17]. The data available confirmed that regulation of the expression of genes-encoded ligninolytic enzymes is a highly complex process. However, the constant progress in molecular and genomic techniques gave new insights on the role of regulating elements in the differential expression of ligninolytic enzymes

in WRF. Further studies will elucidate the mechanisms of ligninolytic enzyme transcriptional regulation and provide deeper understanding of this complicated process.

Thus, the potential of ligninolytic enzymes in degradation of herbicides has not been well characterized yet, especially at the molecular level. Most of the data available correspond to the studies of different herbicide degradation by WRF, their individual ligninolytic enzymes and oxidative enzymes – redox mediator systems that are successful or less successful [6,9,10,18-21]. Few attempts have been made to propose the mechanisms of pesticide degradation (based on pentachlorophenol degradation pathways and ligninolytic enzymes action). The aim of this review is to summarize the data about herbicide degradation by WRF and their ligninolytic enzymes.

## 2. Modern herbicides and common regularities of their transformation

Approximately 2 million tons of pesticides are used worldwide each year [22] and play a significant role in modern agricultural practices. Approximately half of this volume is herbicides that are routinely applied to crops at rates varying from g to kg ha<sup>-1</sup>. In 2010, about 907 million kg of active ingredients of herbicides was applied in the world (FAO data), and this figure continues to grow. 2019 estimates demonstrate that the herbicides market will experience both the highest growth rate as well as the highest volume traded in the next years as compared with other pesticides. The expected annual growth rate of herbicides for the given period is computed to be 6.1% [23].

Although attempts to reduce pesticide use through organic agricultural practices and the use of other technologies continue, direct and indirect exposure to pesticides is still an important health risk factor. About one-third of the agricultural products are produced by using pesticides [24]. Without pesticide application the loss of fruits, vegetables, and cereals from pest injury would reach 78%, 54%, and 32%, respectively [25].

The emergence of herbicide-resistant (HR) genetically engineered crops in 1996 made it possible for farmers to use a broad-spectrum herbicide, glyphosate, in ways that were previously impossible. From 1996 through 2011, 0.55 billion hectares of HR corn, soybeans, and cotton were grown in the USA, and in 2011, an estimated 94% of the soybean area planted, 72% of corn, and 96% of cotton were planted to HR varieties, respectively, which led to a 239 million kg increase in herbicide use [26].

Priority pesticides vary significantly for different regions and crops, and have evolved with time. The era of organic synthetic pesticides started approximately 70 years ago from DDT, 2,4-D, and such compounds as HCH, dieldrin were added to the most actively used compounds at the second wave. The assortment of modern pesticides is highly variable in trademarks and based on relatively wide (but much shorter) row of successfully commercialized active ingredients. However, the bulk of the world market is formed by a very small number of compounds, even taking into consideration their variability for different regions (really – for main crops of these regions). Atrazine, glyphosate, acetochlor, metolachlor,

tefluthrin, cyfluthrin, and, maybe, mesotrione should be considered as priority pesticides for environmental/health risks due to their wide application as protection tools in cereal agriculture. It should be noted, however, that the integral impact of two parameters, i.e., manufacturing volume and toxicity of active compounds, determines danger of different agrochemicals and necessity in their efficient decontamination.

Only a lesser part of applied pesticides reaches the target organism, with the remainder being deposited on the soil and nontarget organisms, as well as moving into the environment [27]. The metabolic fate of pesticides is dependent on their physico-chemical characteristics, field abiotic conditions, and plant and microbial communities. Transformation of pesticides includes abiotic processes (such as photolysis, hydrolysis, oxidation, and rearrangements) and chemical/biological reactions. The variety of biotransformation processes for herbicides should be considered in connection with specific features of the microenvironments in and near target organisms involved into metabolic pathways. So the key stage for determining further biotransformation of herbicides is their adsorption (and adsorption of their intermediate metabolites) to soil and soil colloids. These processes are highly important to regulate the dynamics of action for modern herbicide preparations. The ratio between free and adsorbed forms of herbicides determines the rate of their abiotic transformation. Nevertheless, enzymatic transformation (i.e., biotransformation) is the major driver of detoxification.

The classic concepts of pesticide metabolism [28,29] divide their transformation into three phases. In the first phase, the parent compounds are transformed through oxidation, reduction, or hydrolysis to more water-soluble and usually less toxic products. As a whole, oxidation (hydroxylation, dealkylation, and deamination), hydrolysis (esters, amides, and nitriles) and reduction reactions are considered as main factors for this phase. The main process of the second phase is the conjugation of the obtained derivatives to a sugar (typically glucose), glutathione, or amino acid with further increased water solubility, reduced toxicity, and support of internal transport of the metabolite for final transformation. The third phase provides further conjugation and results in nontoxic final products of metabolic pathways of the pesticides.

An important factor in the transformation of pesticides in soil is a complementary action of plants and microorganisms on them. The roles of plants may be simply characterized as reduction of toxicity, whereas microorganisms are responsible for deep destruction and mineralization. The line of enzymes and catalyzed reactions for microorganisms is much wider as compared with plants. Several processes, such as dehalogenation or C-P bond cleavage, are associated mainly with microbial metabolism of pesticides. (On the other hand, however, glutathione conjugation is a typical tool for plant transformation of pesticides.) The common opinion in modern remediation biotechnology is that the tasks of detoxification cannot be solved at the plant level alone and should be based on the detailed analysis of the most efficient microbial participants of this process. A significant additional factor of interest concerning microbial detoxification is the lower cost of such technologies as compared with the alternative ones [30].

Pathways of pesticide destruction have been described in many works, both at the levels of the species responsible and the enzymes involved. Currently, the existing information is

systematized in several sources, and Biocatalysis/Biodegradation Database of the University of Minnesota (EAWAG-BBD; <http://eawag-bbd.ethz.ch>) seems the most informative available tool. This database contains information on microbial biocatalytic reactions and biodegradation pathways for primarily xenobiotics. This permanently maintained and updated system collects data about hundreds of pathways, enzymes, and microorganisms, thousands of reactions and compounds of environmental interests. In addition, this database contains two supporting tools. The Pathway Prediction System predicts microbial catabolic reactions using substructure searching, a rule-base, and atom-to-atom mapping. The biotransformation rules are based on reactions found in the EAWAG-BBD or in the scientific literature. The Biochemical Periodic Table provides an overview of microbial interactions with different chemical elements. Individual element pages contain a summary of published data about microbial interactions with the selected element.

It should be noted that efficient recommendations for microbial remediation require integral knowledge about potential of individual enzymatic reactions and specific features of their interactions for different microbial species. Current information about genetic regulation of coupled reactions may significantly improve bioremediation technologies as well as contribute to empiric data about multistep detoxification with the use of different microorganisms. That is why the further consideration of fungal destruction of herbicides will provide data on integrated potential of multienzyme systems from different detoxifiers rather than data about elementary catalytic reactions, but first of all about.

### 3. Effects of herbicides on soil fungi

There are two main techniques of herbicide application in the field. The first one is foliar spray, and the second is soil application. In case with soil application, the herbicide is introduced directly into the soil and so can affect soil microorganisms. However, even in case with foliar application, significant amounts of these chemicals reach the soil. Therefore, although herbicides are very useful in farming, under certain circumstances they may turn into pollutants, affecting soil microflora and deteriorating the quality of soil if there are sensitive organisms and/or if the degradation products are toxic. Among various indicators used in monitoring soil biological activity, microbial community structure seems to be the most preferred due to its sensitivity to the environmental changes. To address these concerns, the impacts of herbicides on soil microbial communities are widely studied and discussed.

In general, the recommended field rate of herbicide had no major effects on soil microorganisms, but excessive doses retard the reproduction rate of some groups of microflora and may reduce enzyme activity and populations of various microorganisms in soil, including fungi (Table 1) [31-35]. No significant changes in soil microflora were detected using phospholipid fatty acid (PLFA) profiles' analysis after atrazine, bentazon, or glyphosate application by Banks and coauthors [35]. Crouzet and coauthors [33] tested the herbicide mesotrione in chernozem soil at the rates from 0.45 to 45 mg/kg and recorded only small genetic structural shifts in the bacterial and fungal communities. Maximum dissimilarity of the bacterial and fungal genetic

structures between control and herbicide-treated soil did not exceed 12% and 28%, respectively. Martinez et al. [36] did not demonstrate any significant changes in the multiplication of bacteria and fungi following an application of sulfentrazone. Allievi and Gigliotti demonstrated no significant differences in number of aerobic bacteria in soil attributable to cinosulfuron treatment at the field rate 0.42  $\mu\text{g}/\text{kg}$  after 1 and 4 weeks of incubation under laboratory conditions [31]. Possible effects of the herbicide on the specific group of microorganisms of the microbial community resulting in eventual counterbalance by the development of another group were further tested. To execute this, the individual microbial strains were isolated and their sensitivity in relation to cinosulfuron was tested. Among eighteen studied strains of aerobic bacteria from uncultivated soil, a fourth of the tested strains underwent some growth inhibition in the presence of the herbicide, and for one strain total and permanent inhibition was observed. In the case of fungi, however, only two of seventeen fungi strains underwent temporary growth inhibition. In the case of isolates from agricultural soil, neither bacterial nor fungal isolates were sensitive to the studied herbicide. The herbicide cinosulfuron was concluded to negatively affect only a few aspects of the microbial community in soil ecosystems, even at concentrations higher than those currently in use. Baćmaga and coauthors [37] also reported on the absence of adverse effects of the herbicide metazachlor at the recommended dose (0.3 mg/kg) on soil microorganisms including oligotrophic bacteria, *Azotobacter* spp. bacteria, organotrophic bacteria, actinobacteria, and fungi. When applied at excessive doses, metazachlor inhibited significantly the reproduction of all analyzed microorganisms, including fungi.

Herbicide	Effect at field rate	Effect at excessive rates	Ref.
<b>Auxin growth regulators</b>			
2,4-D	No effect	Stimulation	[38]
<b>Amino acid biosynthesis inhibitors</b>			
<i>EPSP synthase inhibitors</i>			
	No effect	nd	[35]
Glyphosate	Stimulation	nd	[39]
	No effect	No effect	[40]
<i>ALS inhibitors</i>			
Cinosulfuron	No effect	Temporary growth inhibition or no effect	[31]
Imazethapyr	No effect	Inhibition	[38]
Metsulfuron-methyl	Stimulation	nd	[41]
Nicosulfuron	Inhibition	Inhibition at intermediate doses, no effect at high doses	[42]
Sulfosulfuron	Stimulation	Inhibition	[43]
	No effect	nd	[44]
<i>Glutamine synthetase inhibitors</i>			
Glufosinate	Inhibition	Inhibition	[45]
<b>Photosynthesis inhibitors</b>			

Herbicide	Effect at field rate	Effect at excessive rates	Ref.
<i>Systemic herbicides inhibiting PSII</i>			
Atrazine	No effect	nd	[35]
Isoproturon	Inhibition	nd	[46]
	Inhibition	nd	[44]
Linuron	No effect	No effect	[47]
Metribuzin	No effect	nd	[44]
<i>Contact herbicides inhibiting PSII</i>			
Bentazon	No effect	nd	[35]
<i>Contact herbicides inhibiting PPO</i>			
Brominal	Inhibition	Inhibition	[32]
	Temporary inhibition	Temporary inhibition	[48]
Sulfentrazone	No effect	nd	[36]
<b>Lipid biosynthesis disrupters (ACC inhibitors)</b>			
Clodinafop	No effect	nd	[44]
<b>Pigments biosynthesis inhibitors (HPPD inhibitors)</b>			
Mesotrione	No effect	Slightly modified the fungal genetic structures	[33]
<b>Seedling growth inhibitors</b>			
Alachlor	No effect	Inhibition	[49]
Butachlor	Stimulation	Inhibition	[50]
Metazachlor	No effect	Inhibition	[37]
Napropamide	Inhibition followed by stimulation	nd	[51]

ACC – acetyl-CoA carboxylase; ALS – acetolactate synthase; EPSP – 5-enolpyruvylshikimate-3-phosphate; HPPD – p-hydroxyphenylpyruvate dioxygenase; PPO – protoporphyrinogen oxidase; PSII – photosystem II  
 nd – no data

**Table 1.** Influence of different herbicides on soil fungi

The negative influence of the herbicides on fungi was also reported by Kucharski and Wyszowska [43], who tested herbicide Apyros 75 WG (a.i. sulfosulfuron), and by Zhang and coauthors [38], who studied the effect of imazethapyr in two agricultural soils. The ratio of fungi/bacteria in the imazethapyr-treated soil tended to decrease in the initial 15 d incubation period when compared to the control, and then recovered after 30 d of incubation. Stimulation of bacterial and suppression of fungal population due to isoproturon application was reported by Nowak et al. [46]. Omar and Abdel-Sater studied the effect of soil treatment with brominal on population counts of bacteria, actinobacteria, and cellulolytic fungi in soil and found out that the herbicide significantly decreased the total number of cellulolytic fungi and most fungal species while bacterial populations in soil treated with the herbicide was promoted at field application rates and inhibited only at higher levels [32]. Pampulha et al. demonstrated a significant decrease of soil bacteria, fungi, and actinobacteria populations 40 days after glufosinate application [45].

The evidences of no effect or positive effect of the herbicides on fungi growth were also numerously demonstrated. Araújo et al. [39] proved that soil pollution with glyphosate increased populations of fungi and actinobacteria while depressing counts of the other bacteria. Kucharski and Wyszowska [43] demonstrated a stimulating effect of sulfosulfuron on fungi in the objects treated with the recommended dose of the herbicide 8.9 µg/kg. Treatment of soil with 2,4-D butyl ester at the extremely high dose of 1000 mg/g caused a decline in culturable microbial counts, with the exception of fungal numbers, which increased over the incubation time [34]. At that, when herbicide concentration increased, the Gram-negative/Gram-positive bacteria ratio decreased dramatically in the studied soils. Soil treatment with linuron at the dosages of 4–400 mg/kg did not change the fungal numbers significantly in two agricultural soils as compared to the corresponding controls [47]. Sørensen et al. explained the observed phenomenon by the presence of linuron-degrading fungi, including different species of *Cunninghamella*, *Mortierella*, *Talaromyces*, *Rhizopus*, *Rhizoctonia*, and *Aspergillus* [52]. Along with linuron-degrading fungi, there are some soil bacteria which are able to use herbicide as a source of C and N, resulting in a significant increase in bacterial counts [53]. The latter is confirmed by increased bacterial numbers in soils treated with the high dosage of linuron [47].

He et al. studied the effects of metsulfuron-methyl on soil microorganisms by the method of microbial inoculation culture and found an inhibiting effect of the herbicide on the aerobic heterotrophic bacteria, whereas the number of tolerant fungi increased greatly in the rhizosphere after the application of metsulfuron-methyl [41]. Impact of another sulfonyleurea herbicide, nicosulfuron, on the structure, abundance, and function of the soil microbial community using standardized methodologies (PLFAs, taxa-specific qPCR, and enzyme activities) was investigated by [42]. Soil concentrations of nicosulfuron exceeding 1 µg/g resulted in significant reduction of the total PLFAs, although significant reductions of the bacterial PLFAs were observed only at nicosulfuron concentration levels above 10 µg/g. A different picture was evident for fungal PLFAs with significant reductions observed only at intermediate herbicide concentration levels (1–10 µg/g) compared to the control. Besides, qPCR analysis demonstrated that fungi showed the highest sensitivity to nicosulfuron and their abundance was reduced even at the lowest concentration levels of the herbicide (0.25–1 µg/g). Finally, field experiments showed that nicosulfuron applied to the field at dose rates ×1, ×2, and ×5 of the recommended did not significantly affect either the soil microbial biomass or the abundance of fungi and bacteria or enzymatic activity. No significant changes in fungal numbers due to clodinafop introduction into the soil were observed by [43]. Wardle and Parkinson [40] reported that bacterial propagules were temporarily enhanced while actinobacteria and fungal propagule numbers were unaffected by glyphosate. Min et al. [50] reported the influences of the herbicide butachlor on microbial populations, respiration, nitrogen fixation, and nitrification and on the activities of dehydrogenase and hydrogen peroxidase in paddy soil. The results showed that the number of actinobacteria declined significantly after the application of butachlor at different concentrations ranging from 5.5 to 22 mg/kg, while that of the other bacteria and fungi increased. However, at higher butachlor concentrations the growth of fungi was retarded, and the growth of anaerobic hydrolytic fermentative bacteria, sulfate-reducing bacteria, and denitrifying bacteria was stimulated. Treatment of soil with another acetanilide herbicide, napropamide, resulted in decrease of populations of

bacteria, while the populations of fungi displayed the decreasing, recovering, and increasing trend [51].

Detailed examination of the observed effects of the herbicides on soil fungi associated with the mode of action of herbicides (Table 1) does not reveal any interrelationships between herbicide identity and their toxicity to fungi. Though herbicides inhibiting amino acid synthesis (ALS and glutamine synthetase inhibitors), contact herbicides inhibiting PPO, and seedling growth inhibitors are seemingly the most toxic, a detailed systematic study needs to be conducted to prove or disprove this observation. Moreover, currently, no general pattern of soil microbiota responses has been inferred regarding herbicide doses applied, exposure time, soil type, or other environmental factors [40,54]. The latter results very likely from the fact that up to now most studies dealing with pesticide soil microbial toxicity were performed using methods that were not well standardized, which did not allow their comparative meta-analysis, and focused on the independent assessment of effects on population, diversity, or functional endpoints, which did not provide a comprehensive view of the toxicity of the pesticide [42]. Standardization of the advanced methodologies available in soil microbial ecology is a necessary step toward harmonization of datasets and is a prerequisite for their integration in the regulatory framework of pesticide soil microbial toxicity assessment [55]. Standards for a number of methods have been already developed and others are under development at the International Standard Organisation (ISO) by TC190/SC4/WG4 and can be found elsewhere [42]. These include:

- Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates (ISO/TS 22939)
- Determination of soil microbial diversity. Part 1: method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis (ISO/TS 22843 part 1)
- Determination of soil microbial diversity. Part 2: method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method (ISO/TS 22843 part 2)
- Method to directly extract DNA from soil samples (ISO11063)
- Estimation of abundance of selected microbial gene sequences by quantitative real-time PCR from DNA directly extracted from soil (ISO/DIS 17601)

Therefore, there is a global need for more complex investigations of the functional diversity responses and degrading activity of soil microbial communities in order to provide deeper insight for herbicide risk assessment. The combined utilization of the above standardized molecular and biochemical methods that provide data of different resolution levels guarantee an accurate estimation of pesticide-driven effects on soil microbes [42].

#### **4. Transformation of the herbicides by white-rot-fungi**

It is well documented that a wide range of pollutants including pesticides are transformed and degraded by WRF: pentachlorophenols, isoproturon, derivative of isoxaflutole, atrazine,

simazine, propazine, lindane, atrazine, diuron, terbuthylazine, metalaxyl, DDT, dieldrin, aldrin, heptachlor, chlordane, etc. [11,56-65]. This list may be expanded given the strong evidence for WRF degradation potential toward different classes of pollutants. The data on herbicide degradation by WRF are summarized partly in Table 2. It should be mentioned that a large number of works were performed using stationary conditions on liquid media and solid system fermentation conditions. However, there are contradictory data about level of herbicide degradation, role of ligninolytic enzymes in this procedure, and mechanism of degradation as well.

Fungus	Herbicide	Cultivation		Disappearance, %	References
		Type	Days		
<i>Agrocybe semiorbicularis</i>	Atrazine		42	40	
	Diuron	Stat	42	70	[11]
	Terbuthylazine		42	60	
<i>Auricularia auricula</i>	Atrazine		42	16	
	Diuron	Stat	42	10	[11]
	Terbuthylazine		42	37	
<i>Cerrena maxima</i>	Atrazine	Sub	40	83	[66]
<i>Cerrena maxima</i> & <i>Coriolus hirsutus</i>	Atrazine	Sub	40	78	[66]
<i>Coriopsis fulvocinerea</i>	Atrazine	Sub	40	88	[66]
<i>Coriolus hirsutus</i>	Atrazine	Sub	40	91	[66]
<i>Coriolus versicolor</i>	Atrazine		42	86	[11]
	Chloronitrofen		12	30	[67]
	Diuron	Stat	42	99	[11]
	Nitrofen		12	80	[67]
	Terbuthylazine		42	63	[11]
<i>Dichotomitus squalens</i>	Atrazine		42	25	
	Diuron	Stat	42	21	[11]
	Terbuthylazine		42	52	
<i>Flammulina velupites</i>	Diuron	Stat	42	6	[11]
	Terbuthylazine		42	30	
<i>Ganoderma lucidum</i>	Bentazon (5 mM)	Stat	10	88	[68]
	Bentazon (20 mM)		10	55	[69]
	Bentazon (50 mM)	Sol	10	90	[68]
	Diuron (30 $\mu$ M)	Stat	10	55	[69]
	Picloram		10	0	[12]
<i>Hypholoma fasciculare</i>	Atrazine		42	57	
	Diuron	Stat	42	71	[11]
	Terbuthylazine		42	97	
<i>Phanerochaete chrysosporium</i>		Stat	14	0	[57]
	Atrazine	Stat	10	60	[70]

Fungus	Herbicide	Cultivation		Disappearance, %	References
		Type	Days		
			42	20	[11]
	Bentazon	Sol	33	55	[71]
			20	65	[72]
	Diketonitrile (derivative of isoxaflutole)	Stat	15	42	[74]
	Diuron	Stat	10	94	[75]
			42	3	[11]
	Isoproturon	Bio-beds	28	78	[76]
			100	>99	[76]
	MCPA	Sol	20	75	[73]
	Propazine		8	45	[70]
	Simazine		8	5	[70]
	Terbuthylazine		42	53	[11]
			8	95	[70]
	Atrazine		42	15	
<i>Pleurotus ostreatus</i>	Diuron	Stat	42	12	[11]
	Terbuthylazine		42	30	
	Atrazine		42	57	
<i>Stereum hirsutum</i>	Diuron	Stat	42	80	[11]
	Terbuthylazine		42	88	
<i>Trametes</i> sp.	Picloram	Stat	10	0	[12]
<i>Trametes versicolor</i>	Diketonitrile (derivative of isoxaflutole)	Stat	15	34	[74]

Stat – Stationary conditions on liquid media  
 Sub – Submerged cultivation on liquid media  
 Sol – Solid-state cultivation

**Table 2.** Degradation of herbicides by white-rot fungi

Several fungi, such as *Agrocybe semiorbicularis*, *Auricularia auricula*, *Coriolus versicolor*, *Dichomitus squalens*, *Flammulina velupites*, *Hypholoma fasciculare*, *Pleurotus ostreatus*, *Phanerochaete velutina*, and *Stereum hirsutum* have shown the ability to degrade various herbicides like atrazine, diuron, and terbuthylazine with different efficiencies [72]. *Coriolus versicolor*, *Hypholoma fasciculare*, and *Stereum hirsutum* degraded more than 86% of diuron, atrazine, and terbuthylazine in 6 weeks. They were also the most active in ligninolytic enzymes' production. However, the ability of WRF to degrade aromatic herbicides, diuron, atrazine, and terbuthylazine, did not correlate with their ligninolytic activity determined in the Poly R-478 decoloration test (which is used as an indicator of ligninolytic activity). The possible explanation of these results was the difference in LME patterns produced by fungi in liquid cultures. Interesting that under field trials the most effective strain *S. hirsutum* was inactive in herbicide

degradation and the other strains *C. versicolor* and *H. fasciculare* demonstrated 30% of chloropyriphos degradation in 6 weeks [72].

White-rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* converted up to 35–40% of diketonitrile (a soil transformation product of the herbicide isoxaflutol) to inactive benzoic acid analogue after 15 days under stationary conditions on liquid media [74]. The level of ligninolytic enzymes, such as laccases, produced during fermentation seemed to be correlated with herbicide degradation, confirming the role of these enzymes in degradation processes. However, the authors underlined that induction of laccase production sixfold via addition of 2,5-xylydine did not lead to any significant diketonitrile cleavage increase.

It was shown that *Coriolus hirsutus*, *Cerrena maxima*, *Corioloopsis fulvocinerea*, and co-cultured *Coriolus hirsutus*/*Cerrena maxima* [66] can degrade atrazine under submerged cultivation; the herbicide removal was 77–91% after 40 days' cultivation. It is interesting to mention that negligible amounts of atrazine were found to be absorbed on mycelium. The activity of laccase was rather high, allowing the proposal of laccase participation in atrazine degradation by these fungi. This hypothesis was supported by the study of atrazine degradation in the presence of laccase inducers (guaiacol and syringaldehyde) under submerged cultivation [77]. The efficiency of herbicide degradation was higher in induced cultures by 78–98% and the highest level of atrazine removal was achieved for *Corioloopsis fulvocinerea* using guaiacol as an inducer.

Hiratsuka et al. [67] reported that *Coriolus versicolor* IFO 30340 degraded 30% of chloronitrofen (CNP) and 80% of nitrofen (NIP) after 12 days' cultivation under stationary conditions on liquid media. The herbicide degradation rate depended on the nitrogen concentration in the media and was higher under low nitrogen conditions, suggesting that the lignin degradative system was responsible for the herbicide degradation. However, LiP, MnP, and laccase as well as culture filtrate did not oxidize herbicides. Neither chloronitrofen nor nitrofen were oxidized by the laccase – redox-mediator system using HBT, which is a well-known laccase redox-mediator. These results draw the conclusion that extracellular ligninolytic enzymes were not involved in the initial step of CNP or NIP degradation by *Coriolus versicolor* IFO 30340. The sequential identification of products formed during the metabolism of CNP and its intermediates by *C. versicolor* enabled the authors to propose four different pathways for the degradation of CNP: aromatic hydroxylation, oxidative dechlorination, reductive dechlorination, and the reduction of the nitro group to amine. The aromatic hydroxylation to form 2,4,6-trichloro-3-hydroxy-4'-nitrodiphenyl ether and the oxidative dechlorination to form 2,4-dichloro-6-hydroxy-4'-nitrodiphenyl ether were assumed to catalyze by cytochrome P450-type enzyme(s) because these paths were efficiently shut off by the exogenous addition of piperonyl butoxide, a P450 inhibitor. The conversion of CNP to NIP by *Coriolus versicolor* IFO 30340 should be reductive dechlorination. Reductive dechlorination reactions were involved in the degradation of pentachlorophenol by *P. chrysosporium* [60]. CNP was also converted to 2,4,6-trichloro-4'-aminodiphenyl ether by *C. versicolor*. The reductive dechlorination and nitro-reduction reactions were also found as initial reactions in CNP degradation, which were enhanced upon the addition of the cytochrome P450 inhibitor. Aromatic hydroxylation and oxidative dechlorination were also observed during the fungal conversion of NIP; however, the products formed were not identified – they were assumed to be either 2, 4-dichloro-3-

hydroxy-4'-nitrodiphenyl ether or 2, 4-dichloro-6-hydroxy-4'-nitrodiphenyl ether and 2-chloro-4-hydroxy-4'-nitrodiphenyl ether or 2-hydroxy-4-chloro-4'-nitrodiphenyl ether, respectively. The fungal conversion of NIP was also effectively inhibited by piperonyl butoxide.

Based on the result obtained, the authors assumed that cytochrome P450 played an important role in lowering the ionization potential of environmentally persistent aromatics and in providing suitable substrates for ligninolytic one-electron oxidizing enzymes for effective degradation. When diphenyl ether, 4-chlorodiphenyl ether, and 4-nitrodiphenyl ether were added to the fungal culture, 4-hydroxydiphenyl ether, 4-chloro-4'-hydroxydiphenyl ether, and 4-nitro-4'-hydroxydiphenyl ether were identified as the major products, respectively. 4-chlorophenol and 4-nitrophenol were detected in trace amounts from 4-chlorodiphenyl ether and 4-nitrodiphenyl ether, respectively, but the counterpart hydroquinone was not observed. These data suggest that the formation of phenolic products from either the A or B ring of CNP might be derived via a different pathway, and that the direct ether cleavage might not have occurred. These findings gave evidence that fungi degraded herbicides via different pathways using their multiple metabolic systems.

*Ganoderma lucidum* was shown to be resistant to the herbicides diuron and bentazon [69]: the upper limits were 80  $\mu$ M and 20 mM, respectively. This finding can be explained by higher toxicity of the metabolites formed during diuron transformation. It was reported previously that some of the metabolites resulting from fungal transformation of diuron may be even more toxic than the parent compound [78]. *G. lucidum* was able to efficiently remove 55% of diuron and 88% of bentazon after 10 days' cultivation in liquid cultures. Both bentazon and diuron strongly improved the production of laccase by the fungus inducing one of the two laccase isoforms. Native PAGE analysis of the extracellular enzymes revealed that the improvement in the laccase activity in response to the herbicides was not due to the expression of a new laccase, but that it was due to the overproduction of an already existing isoform in the noninduced cultures. Similar results were obtained with *Trametes versicolor* and *Abortiporus biennis* [79], where their constitutive laccases were overproduced in the presence of paraquat, a quaternary nitrogen herbicide. The electrophoretic analysis of extracellular enzymes from *G. lucidum* showed that laccase1 was the dominant enzyme under noninduced conditions. Interestingly, the herbicides induced only the laccase2 isoform while the laccase1 was suppressed in these cultures. Such results suggest that laccase2 is, probably, the isoform more intensely involved in the defense system of the fungus, considering that both herbicides strongly inhibited the fungus growth. These observations show that these types of enzymes have, at least in part, an important role in the degradation of pollutants under in vivo conditions.

The comparative study of herbicide bentazon degradation by *Ganoderma lucidum* in liquid and solid-state cultures using corn cob as substrate has been performed [68]. The fungus was more resistant to herbicide and more efficient in its degradation in solid-state cultures in comparison with liquid cultures: 50 mM against 20 mM and 90% against 55%, respectively. The authors proposed two, not mutually exclusive, possible explanations: a lower availability of herbicide due to its adsorption to the insoluble substrate corn cob for this observation and the higher

activities of both laccase and Mn peroxidase in solid-state cultures compared to the liquid cultures, where the high laccase activity was detected. However, no metabolite products were found in the combined aqueous and methanolic extracts. The *G. lucidum* crude filtrates containing laccase and Mn peroxidase were shown to degrade bentazon in vitro. The experiments with addition of  $Mn^{2+}$ , ABTS, Tween 80, and  $H_2O_2$  to crude filtrates demonstrated synergisms in bentazon degradation, suggesting that both laccase and Mn peroxidase were involved in its degradation. It is well known that ABTS mediates the oxidation of non-phenolic compounds of lignin [80] and the presence of unsaturated fatty acids (Tween 80) improves the oxidation process catalyzed by Mn peroxidases and laccases due to the production of lipid peroxy or alkoxy radicals [81]. The hypothetical mechanism of bentazon degradation may be the following Mn peroxidase and laccase generated lipid peroxy or alkoxy radicals; in the presence of these radicals Mn peroxidase oxidizes  $Mn^{2+}$  to  $Mn^{3+}$ , which in turn oxidizes bentazon, whereas laccase uses ABTS as redox-mediator for bentazon oxidation. However, no degradation of picloram *G. lucidum* and *Trametes sp.* were observed in liquid cultures, maybe due to its high substitution of the aromatic ring [12]. This herbicide enhanced the production of laccase by *Trametes sp.*, whereas the enzyme production by *G. lucidum* was suppressed. The authors assumed that enzyme production inhibition could be occurring at the mRNA level after picloram has entered the cell or by enzyme modification before or after secretion [12]. The exposition of *G. lucidum* and *Trametes sp.* to picloram revealed a peculiar mechanism of transitory bioaccumulation of herbicide by both fungi.

The most studied WRF is *P. chrysosporium*, which was shown to degrade a wide range of herbicides under different conditions. MCPA and bentazon were degraded by *P. chrysosporium* at 65% and 75%, respectively, in 20 days [73]. *P. chrysosporium* degraded isoproturon belonging to phenylurea groups [73,76], atrazine [70], and also diuron [82]. However, according to [57], no atrazine degradation was observed by this fungus in liquid cultures. The degradation efficiency of *P. chrysosporium* was higher in solid-state cultures in comparison with liquid ones [71,73]. Two mechanisms of herbicides degradation were proposed: the action of ligninolytic enzymes and the action of intracellular enzymes in particular cytochrome P450. In [75], the degradation of diuron by *P. chrysosporium* was studied including the identification of products formed and the evaluation of cytochrome P450's role. Two findings were of great importance: the considerable amounts of diuron, DCPMU [1-(3,4-dichlorophenyl)-3-methylurea], and DCPU [1-(3,4-dichlorophenyl)urea] found in fresh mycelia and the inhibition of diuron degradation by ABT (1-aminobenzotriazole), a cytochrome P450 inhibitor. These results confirmed the intracellular mechanism of this herbicide degradation resulting in N-demethylation. However, after 5 days concentrations of DCPMU and DCPU were higher in cultural filtrates than in mycelia extracts suggesting possible involvement of lignolytic enzymes in degradation of these metabolites. According to da Silva Coelho-Moreira et al. [75], enzymatic crude extracts supplied with combinations of veratryl alcohol  $H_2O_2$  and  $Mn^{2+}$  did not degrade the herbicide, it is possible that DCPMU and DCPU can be further transformed by MnP.

*P. chrysosporium* is also able to transform atrazine, its transformation product and other s-triazine herbicides [70]. The first and main step in the chlorinated-s-triazine degradation

pathway by the fungus was mono-N-dealkylation. Hydroxyatrazine was the main degradation product found in soils treated with atrazine and in liquid cultures. *P. chrysosporium* actively transformed hydroxyatrazine to an unknown compound that accumulated in the culture medium. It was established that the presence of both alkyl groups and chlorine at the 2-position are necessary for the mono N-dealkylation of atrazine by *P. chrysosporium*. Consequently, formation of desethylhydroxyatrazine in liquid cultures should result from hydrolysis of deethylatrazine. Experiments with terbuthylazine, atrazine, and simazine also show that the removal of the ethyl side chain is the preferential reaction, and might depend on the mass of the second alkyl group. In other words, compounds with a high-mass group linked to one amino substituent are expected to undergo a higher N-dealkylation affecting the other chain. The symmetric compounds propazine and simazine were also degraded at a slower rate than atrazine. Neither LiPs nor MnPs transformed atrazine and its N-dealkylated metabolites. It was shown that atrazine N-dealkylation decreased in the presence of cytochrome P450 inhibitor. Moreover, herbicide degradation was supported by mycelium. Therefore, the cytochrome P450 involvement in atrazine degradation was assumed. These data are in line with previously published study of atrazine degradation by *Pleurotus pulmonarius*, which involved such enzymes as lipoxygenase, peroxidase, and cytochrome P-450 [83].  $Mn^{2+}$ , which activates these enzymes, stimulated atrazine transformation to N-dealkylated and propylhydroxylated metabolites whereas antioxidants and inhibitors of lipoxygenase and peroxidase (nordihydroguaiaretic acid) as well as cytochrome P-450 (piperonyl butoxide) suppressed its degradation.

To analyze data presented in Table 2, rate of herbicide disappearance was calculated as the ratio of disappearance (%) to the duration of degradation (days), followed by an average value calculation for every herbicide (Fig. 1). Taking into consideration the effect of cultivation conditions on herbicide degradation by fungi, only data on stationary conditions on liquid media were treated this way.

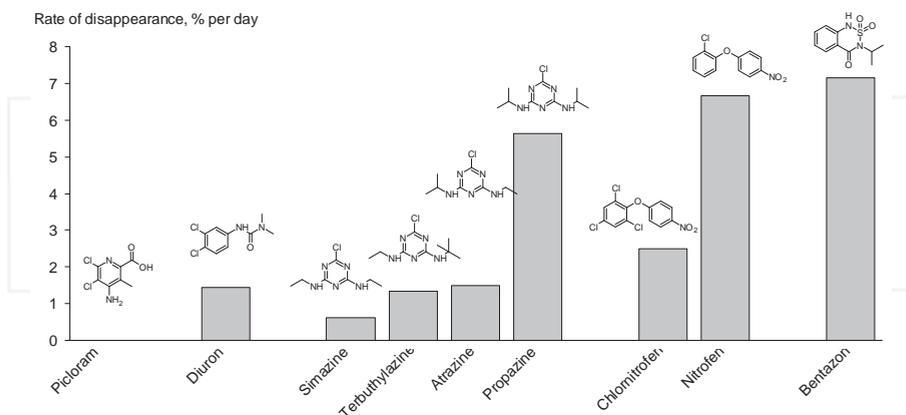


Figure 1. Relationship between rate of disappearance of herbicides and their structure.

Obtained results correspond well to the study [70], where it was established that the presence of alkyl groups is necessary for the degradation of s-triazine herbicides by *P. chrysosporium* via mono N-dealkylation. Moreover, ability of WRF fungi to degrade s-triazines seems to enhance along with increase in the amount of exactly branched alkyl groups. However, detailed quantitative structure–degradation activity studies should be conducted to prove or disprove this preliminary observation. Another important conclusion is a marked negative influence of chlorine in the herbicide molecule on the degradation rate, which can be seen from the comparison of degradation rate of nitrofen (one atom of chlorine) and clornitrofen (three atoms of chlorine) and the highest degradation rate of bentazon, which is the only chlorine-less herbicide in the presented range (Fig. 1). Therefore, data presented in Fig. 1 demonstrate clearly the barest necessity of further QSAR studies. Together with knowledge on main enzymatic pathways of herbicide degradation, the latter will improve significantly the preliminary assessment of degradation ability of WRF in relation to the herbicide of known structure.

The contradictory data about participation of ligninolytic enzymes in the herbicide degradation and transformation did not allow establishing their precise role in these processes [18,75, 81,84,85,86]. We summarized the data about efficiency of individual ligninolytic enzymes, their mixtures, and enzymes – redox-mediator systems in herbicide degradation in Table 3. As can be seen, no degradation of diketonitrile, diuron, atrazine, chloronitrofen, nitrofen, glyphosate was observed for MnP and LiP crude extracts and purified enzymes from *P. chrysosporium*, *Trametes versicolor*, and *Coriolus versicolor* even in the presence of redox-mediators [67,69,70,74,75,81]. However, MnP from *P. chrysosporium* degraded Irgarol 1081 up to 37% after 24 h [87] and LiP from *P. chrysosporium* degraded bentazon up to 100% after 4 h [71]. Moreover, bentazon was effectively transformed by laccase with catechol, laccase, and MnP crude extracts with redox-mediator ABTS, recombinant MnP [69,71,88]. Analysis of the data summarized in Table 3 draw to the conclusion that MnP, laccase, and laccase – redox-mediator systems are the most efficient tools for degradation of a wide range of herbicides – diketonitrile, glyphosate, Pesticide Mix 34, chloroxuron, atrazine, and dymron [74,81,86,89], however, with few exceptions, namely, choronitrofen and nitrofen [67]. It should be underlined that efficiency of laccase – redox-mediator systems toward different herbicides strongly depends on the redox-mediator used, which in turn depends on mechanisms of the mediators' oxidation by enzyme and the reactivity of the mediators' intermediates.

Enzyme	Herbicide	Redox mediator	Reaction conditions	Duration h	Disappearance, %	Fungus	Ref.
Laccase	Atrazine	No	25°C, pH 4.5	240	0	<i>Coriolopsis fulvocinerea</i>	Koroleva & Gorbatova (unpublished data)
		[Ru(bpy) <sub>2</sub> Cl <sub>2</sub> ]			0		
		[Ru(phpy)(phen) <sub>2</sub> ]PF <sub>6</sub>			0		
		HBT			70		
	Syringaldezine	0					
Bentazon	Catechol	25°C, pH 4.0	0.5	100	<i>Polyporus pinsitus</i>	[88]	
Chloronitrofen	No				0	<i>Coriolus versicolor</i>	[67]
	HBT				0		

Enzyme	Herbicide	Redox mediator	Reaction conditions	Duration h	Disappearance, %	Fungus	Ref.	
	Diketoneitrile (derivative of isoxaflutole)	ABTS	pH 3.0		0.3–0.4 nmol/(h unit)	<i>Trametes versicolor</i>	[74]	
		No	37°C	24	0			
	Dymron	ABTS				>90		
		HBA	60°C	24		90	<i>Trametes versicolor</i>	[89]
		MeHBA				90		
Glyphosate	No		pH 6.0, Mn <sup>2+</sup> + H <sub>2</sub> O <sub>2</sub> + Tween 80	24	90	<i>Trametes versicolor</i>	[81]	
	No		pH 6.0, Mn <sup>2+</sup> + Tween 80		90			
Nitrofen	No				0	<i>Coriolus versicolor</i>	[67]	
	HBT				0			
Laccase, immobilized	Chloroxuron ed	No			0.5	80		
		3-HAA	30°C, pH 4.5		0.5	80	<i>Trametes versicolor</i>	[86]
		HBT			0.3	100		
		Syrinaldehyde			0.5	80		
LiP	Atrazine	No	30°C, pH 5, veratryl alcohol + Mn <sup>2+</sup> + H <sub>2</sub> O <sub>2</sub>	1	0	<i>Phanerochaete chrysosporium</i>	[70]	
	Bentazon	No	pH 3.5, veratryl alcohol + H <sub>2</sub> O <sub>2</sub>	4	~100	<i>Phanerochaete chrysosporium</i>	[71]	
		No				0	<i>Coriolus versicolor</i>	
	Chloronitrofen	No				0	<i>Phanerochaete chrysosporium</i>	[67]
		No				0		
	Glyphosate	No		pH 3.0, veratryl alcohol + Mn <sup>2+</sup> + H <sub>2</sub> O <sub>2</sub> + Tween 80	24	0	<i>Trametes versicolor</i>	[81]
		No				0	<i>Coriolus versicolor</i>	
	Nitrofen	No				0	<i>Phanerochaete chrysosporium</i>	[67]
		No				0		
	MnP	Atrazine	No	30°C, pH 5, veratryl alcohol + Mn <sup>2+</sup> + H <sub>2</sub> O <sub>2</sub>	1	0	<i>Phanerochaete chrysosporium</i>	[70]
Bentazon		No	pH 4.5, Mn <sup>2+</sup> + Tween 80	168	~700	<i>Aspergillus oryzae</i>	[71]	
Chloronitrofen		No			0	<i>Coriolus versicolor</i>	[67]	
Glyphosate		No	pH 4.5, Mn <sup>2+</sup> + H <sub>2</sub> O <sub>2</sub> + Tween 80	24	100	<i>Nematoloma frowardii</i>	[81]	

Enzyme	Herbicide	Redox mediator	Reaction conditions	Duration h	Disappearance, %	Fungus	Ref.
		No	pH 4.5, Mn <sup>2+</sup> + Tween 80		100		
	Irgarol 1051	No	30°C, Mn <sup>2+</sup> + glucose + glucose oxidase	24	37	<i>Phanerochaete chrysosporium</i>	[87]
	Nitrofen	No			0	<i>Coriolus versicolor</i>	[67]
	Pesticide Mix 34	No	35°C, pH 4.5, Mn <sup>2+</sup> + H <sub>2</sub> O <sub>2</sub> + Tween 80	144	20-100	<i>Nematoloma frowardii</i>	[81]
Lac+MnP	Bentazon	ABTS	Mn <sup>2+</sup> + H <sub>2</sub> O <sub>2</sub> + Tween 80	24	98	<i>Ganoderma lucidum</i>	[69]
		No	39°C, veratryl alcohol + Mn <sup>2+</sup> + H <sub>2</sub> O <sub>2</sub>	24	0		[57]
	Atrazine					<i>Phanerochaete chrysosporium</i>	
		No	30°C, pH 5, veratryl alcohol + Mn <sup>2+</sup> + H <sub>2</sub> O <sub>2</sub>	1	0		[70]
LiP+MnP		No			0		
	Diketoneitrile (derivative of isoxaflutole)	1-HBT	30°C, pH 3 or 5, H <sub>2</sub> O <sub>2</sub>	12	0	<i>Phanerochaete chrysosporium</i>	[74]
		3-HAA			0		
		ABTS			0		
	Diuron	No	pH 3.0, veratryl alcohol + Mn <sup>2+</sup> + H <sub>2</sub> O <sub>2</sub>	24	0	<i>Phanerochaete chrysosporium</i>	[75]

Irgarol 1051 – derivative of s-triazine herbicide

3-HAA – 3-hydroxy-antranilic acid

1-HBT – 3-hydroxybenzotriazole

HBA – 4-hydroxybenzoic acid

MeHBA – methyl-4-hydroxybenzoic acid

NNDS – 1-nitroso-2-naphthol-3,6-disulfonic acid

Laccase immobilized – Laccase immobilized on an electrospun zein polyurethane nanofiber via cross-linking with glutaraldehyde

**Table 3.** Degradation of herbicides by ligninolytic enzymes produced by white-rot fungi

In the study of atrazine degradation with purified laccase from *Coriolopsis fulvocinerea*, no herbicide degradation was observed (Koroleva & Gorbatova, unpublished data). The screening of redox mediators (syringaldehyde, [Ru(phpy)(phen)<sub>2</sub>]PF<sub>6</sub>, [Ru(bpy)<sub>2</sub>Cl<sub>2</sub>], HBT) revealed that only HBT caused the decrease in atrazine concentration in the system laccase–atrazine–redox-mediator. A more detailed study of components of the model system “atrazine/laccase/HBT” showed that HBT itself reacted with atrazine and other chlorine-containing atrazine derivatives directly, without laccase involvement, and did not interact with the atrazine

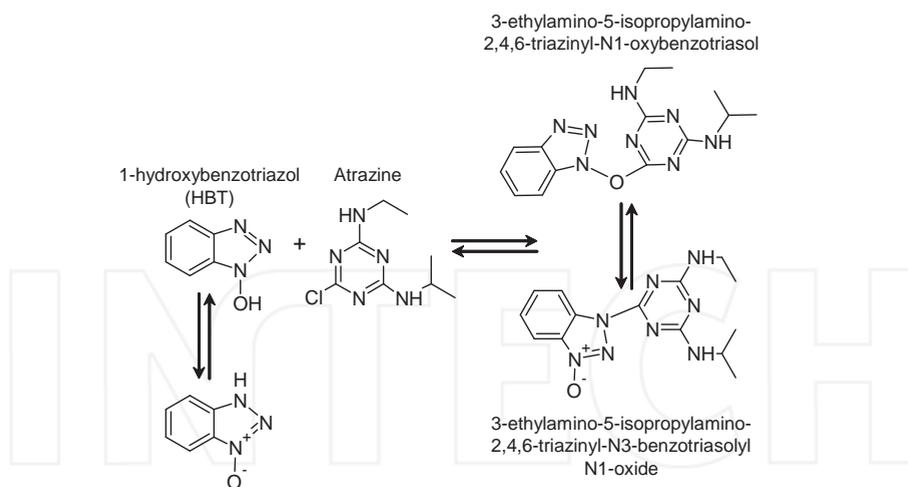
hydroxy derivatives. It is known that HBT in aqueous solution can pass into ionic form. Therefore, it has been suggested that two products can form, both consisting of HBT and atrazine, with the formation of (-N-O-C-) bonds in position (2) of atrazine. Addition of laccase to a solution of HBT/Atr resulted in the formation of several products, one of them having a retention time matching that of HBT-Atr compound. In enzymatic reactions, two other products formed with retention times of 15.3 min and 19.4 min, which were identified as deethylatrazine (DEA) and the compound formed by the interaction of DEA and HBT. Thus, the addition of enzyme resulted in the formation of new products different from that formed in the reaction of HBT with atrazine. The model system "atrazine/laccase/HBT" was studied at different molar ratios of atrazine/mediator (9:1 to 1:9) and at two different concentrations of enzyme (0.02  $\mu\text{M}$  and 1.0  $\mu\text{M}$ ). The deepest atrazine conversion – up to 70% in 10 days – was observed at HBT/Atr ratio of 9/1 and enzyme concentration of 0.02  $\mu\text{M}$ . Proton nuclear magnetic resonance ( $^1\text{H-NMR}$ ) and HPLC-MS/MS allowed confirming the product identification in the model systems "Atr/HBT" and "Atr/HBT/laccase": the formation of Atr-HBT in the "Atr/HBT" system, and DEA and DEA-HBT in the "Atr/HBT/laccase" system. Atr-HBT existed in two forms: protonated (M.W. 315 g/mol) and diprotonated (M.W. 316 g/mol). In the reaction "Atr/HBT/laccase" DEA is formed, as well as protonated (M.W. 287 g/mol) and diprotonated (M.W. 288 g/mol) forms of the product DEA-HBT. Based on the data obtained for the five established structures of the products, we have proposed the atrazine oxidation scheme by the "laccase/HBT" system (Fig. 2), which includes nonenzymatic and enzymatic stages (Fig. 3).

During the nonenzymatic stage, a product consisting of atrazine and HBT is formed. As the substrates and the products in the "Atr/HBT" system are in equilibrium, the addition of laccase to the reaction causes the oxidation of HBT and the formation of HBT radical. The HBT radical reacts with the Atr-HBT compound and triggers the dissociation of the (-NH-CH-) bonds, resulting in the formation of DEA-HBT and ethyl alcohol. In turn, DEA-HBT decomposes to form two products: DEA and HBT. The ability of HBT to form tautomeric forms and to directly react with atrazine suggested that HBT would degrade in the reaction mixture. However, under the proposed scheme, during the hydrolysis of DEA-HBT, DEA and HBT formed. This may be one of the reasons for the effectiveness of HBT as a redox-mediator in laccase – redox-mediator system.

The high potential of WRF as well as their ligninolytic enzymes in herbicide transformation is well documented. Nevertheless, the mechanisms of degradation and degradation pathways for many herbicides are still not explored. Further studies are needed to elucidate the mechanism of herbicide degradation by WRF and ligninolytic enzymes and identify the metabolites formed.

## **5. Bioremediation technologies based on application of white-rot-fungi or their extracellular enzymes**

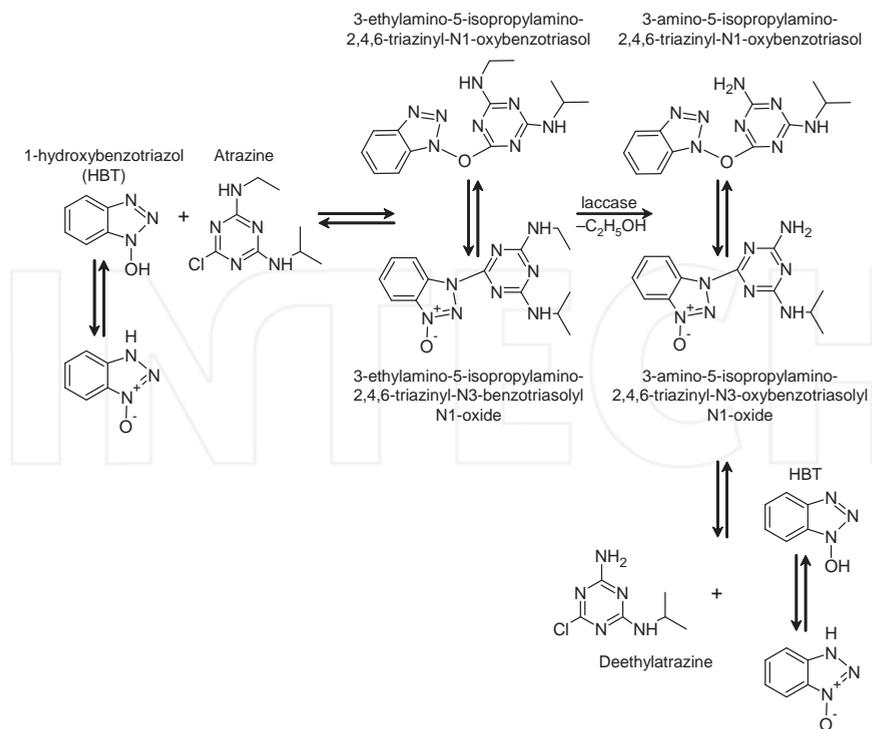
The increasing use of agricultural chemicals including herbicides results in the accumulation of these compounds and their derivatives in soil and water. Many herbicides have medium-



**Figure 2.** Scheme of atrazine oxidation in an “Atr/HBT” system.

to long-term stability in soil and so their persistence has a significant impact on the functioning of soil ecosystems. Biological decomposition of herbicides is the most important and effective way to remove these compounds. Therefore, bioremediation is now regarded as a promising strategy for the rehabilitation of polluted environments because of its cost efficiency and environmental friendliness. A detailed examination of the advantages and the disadvantages of bioremediation as well as comparison of bacteria and white-rot fungi in terms of their usage for bioremediation can be found in [9,90]. Filamentous fungi in general and white-rot fungi in particular are generally more tolerant to high concentrations of organic and inorganic toxicants as compared to bacteria [8,91]. On the other hand, white-rot fungi possess great powers of endurance under environmental stresses [91-93]. Finally, white-rot fungi are unique among eukaryotic or prokaryotic microorganisms, because they possess a very powerful extracellular oxidative lignin-modifying enzyme system, which has broad substrate specificity and is able to oxidize a fair amount of organic pollutants [91]. So, white-rot fungi are likely to be powerful prospective agents in soil bioremediation technologies [90,91]. Table 2 gives some examples of white-rot fungi that have been demonstrated to be able to degrade herbicides effectively.

Currently, more than ten species of white-rot fungi can be considered as the effective degraders of different herbicides (Table 2). Among them, *Ph. chrysosporium* and *T. versicolor* have become the most commonly used indicators in herbicide biodegradation studies due to their good degrading capacity, fast growth, and easy handling in culture [19]. Achieved efficiency of the herbicide degradation by white-rot fungi is usually very high; *Ph. chrysosporium* has been shown to decrease 2  $\mu\text{M}$  atrazine in growth medium by 48% within the first 4 days of incubation [58]. Koroleva and coauthors reported that *Cer. maxima*, *Coriopsis fulvocenerea*, and *C. hirsutus* consumed up to 50% atrazine in 5-day cultivation in the presence of the xenobiotic and at least 80–92% in 40 days [66]. According to the data presented by Bending and coauthors, maximum degradation of herbicides by *T. versicolor*, *H. fasciculare*, and *S. hirsutum* after 42 days



**Figure 3.** General scheme of atrazine oxidation in an “Atr/HBT/laccase” system.

of cultivation was above 86% for diuron, atrazine, and terbuthylazine and about 44% for metalaxyl [72]. However, the degradation efficiency depends greatly on the initial concentration of the herbicides. After 10-day cultivation of *G. Lucidum*, residual concentration of diuron was 48% when initial concentration of the herbicide was 30  $\mu\text{M}$  and increased to 81% when initial concentration of the herbicide was 80  $\mu\text{M}$ . Corresponding values for bentazon initial concentrations 5 and 20  $\mu\text{M}$  were 61% and 85%, respectively [68]. The observed phenomenon most likely results from fungi inhibition at the excessive rates of herbicide application.

In spite of high degradation potential of white-rot fungi demonstrated in lab settings, fungi are rarely agents of choice for environmental biotechnology. The most important problem is that many research studies examine only destruction of single xenobiotic, whereas in reality mixtures of xenobiotics differing in their structure and mode are subjects for detoxification in the environment [90]. The latter can be toxic for the fungi, resulting in significant inhibition of their growth and, in turn, in the target herbicide degradation. For example, Maceil and coauthors studied effects caused by picloram on the white-rot fungi *G. lucidum* and *Trametes* sp. They found oxidative stress in the fungi induced by the herbicide and inability of the studied fungal strain to degrade picloram effectively [12]. Taking into consideration real contamination of soil with mixtures of xenobiotic compounds, studies of such multitarget

degradation have to be specially addressed [94,95]. Additionally, low bioavailability of xenobiotic and preferential use of carbon compounds other than the contaminant of interest is often among potential reasons for the general lack of success of bioremediation strategies [94]. Thus, researches are needed to develop and engineer bioremediation technologies that are appropriate for sites with complex contaminants [90].

Bending et al. [11] studied degradation of the herbicides diuron, atrazine, and terbuthylazine in the so-called biobeds inoculated with white-rot fungi. Biobeds are on-farm pesticide bioremediation constructions developed in Sweden to retain pesticide spills occurring during filling the spraying equipment and facilitate natural attenuation and are currently being evaluated in a number of other European countries [96]. Biobed matrix was prepared by mixing together barley straw, topsoil, and compost [97]. When *Cor. versicolor*, *H. fasciculare*, and *S. hirsutum* were grown in biobed matrix, they were all able to degrade the herbicides, although there were differences in the relative degrading capacities of the fungi in liquid and biobed media. Wirén-Lehr et al. studied the degradation of isoproturon in biobeds with and without inoculation with *Ph. chrysosporium*. They determined that after 28 days in biobeds inoculated with the fungus, total extractable isoproturon decreased by 78%, and after 100 days by >99%, i.e., the herbicide had disappeared in the biobeds, while in noninoculated biobeds that value after 100 days was 76% [76].

To confine white-rot fungi within the toxic environment, a new methodology, which uses growing on potato dextrose agar only or dextrose agar enriched with adsorbent materials, was explored for the removal of xenobiotics from wastewaters [98-100]. This methodology, assuming combined adsorption of organic toxicants followed by their removal, debarred mycelium entrance in the contaminated medium, and excreted fungal enzymes could degrade only the contaminants that entered the medium. The advantage of this methodology is that it avoids additional contamination of the environment with fungal hyphae and exudates, scarce aeration for fungal activity, the continuous contaminant supplying for fungal activity, and the fungus can be easily removed with the agar medium. The developed methodology was successfully employed for simultaneous removal of five coexisting xenobiotics including herbicide linuron from wastewaters, using isolates of *T. versicolor* and *S. hirsutum* as biodegradation agents [101]. Treatments with *T. versicolor* removed linuron from wastewater completely or almost completely, with removal percentages varying from 95% to 100%, depending on adsorbent material used. *S. hirsutum* did not show a great potential to degrade linuron, although after 20 days, the amount of compound removed by this fungus was statistically greater than the control in some cases. Of special importance was that the wastewater used in the study was a real leachate collected from a municipal landfill. Loffredo et al. [100] demonstrated also degradation of linuron from a similar municipal landfill leachate by the described approach, using the fungus *P. ostreatus*.

An approach assuming an introduction preliminary inoculated matrix rather than fungal inoculum itself seems to be very promising with respect to the contaminated soil as well. Recently, some companies have included the use of ligninolytic fungi for soil remediation into their programs, for example, "EarthFax Development Corp." in the USA and "Gebrüder Huber Bodenrecycling" in Germany [90]. EarthFax Engineering, Inc. and its affiliate EarthFax

Development Corp. have demonstrated the degradation of polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzo furans (PCDFs) in soil under pilot-scale conditions through the use of sawdust thoroughly colonized with the white-rot fungus *P. ostreatus*. After 282 days of the experiment, the degradation values of the dioxins varied from 61% to 80%, depending on the PCDD structure [<http://www.earthfax.com>]. The obtained results indicate the clear necessity of further examination of inoculated-matrix-based approach to develop technologies of remediation of the herbicide polluted environments. Overall, future research should be geared toward narrowing the gaps between fungal-based bioremediation in laboratory and environmental applications [20,102].

Although the mechanisms involved in herbicide degradation by white-rot fungi are not clearly understood, most scientists emphasize the role of the extracellular enzymes of LMS in the degradation of the herbicides by WRF [8,9,103,104]. An alternate pathway of detoxification is the use of a cytochrome P450 monooxygenase system, independent of the production of ligninolytic peroxidase enzymes [105]. To date, the latter was clearly proved only for the fungus *Ph. chrysosporium* and so, the capacity of extracellular lignin-modifying enzymes to degrade herbicides has been mainly investigated [18].

The three principal classes of these enzymes, namely lignin peroxidases, manganese peroxidases, and laccase, are likely able to degrade not only phenols, chlorophenols, and aromatic amines but also non-phenolic compounds such as phenylureas, phenylamides, and s-triazines [100], and the presence of redox active mediators can enlarge the range of compounds that could be oxidized by these enzymes [106]. Table 3 gives some examples of herbicide degradation by the above enzymes. Laccase from *Ph. chrysosporium* converts the diketonitrile isoxaflutole to the acid in the presence of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) acting as a redox mediator at pH 3 [74]. LiP and MnP produced by *Ph. chrysosporium* degraded isoproturon in both in vivo and in vitro experiments [73]. MnP from *Ph. chrysosporium* oxidized bentazon in the presence of Mn(II) and Tween 80 [71]. The herbicide glyphosate was degraded by laccase of *N. frowardii* MnP and *T. versicolor* in the presence of ABTS as a mediator [81]. These reports clearly show the potential application of extracellular enzymes of white-rot fungi in the treatment of soil and wastewater contaminated with herbicides [18].

Although application of LiP, MnP, and laccase for degradation many organic pollutants including aromatic compounds, pentachlorophenol, dyes, chlorophenol, urea derivatives, etc., is well known [21,107], only a few papers concerning herbicide degradation specifically are available. Bollag suggested that it is possible to enhance the natural process of xenobiotic binding and incorporation into the humic substances by adding laccase to the soil [108]. Chlorinated phenols and anilines were transformed in soil by oxidative coupling reactions mediated by laccase or peroxidase [109]. The herbicide bentazon was incubated with laccase or peroxidase in the presence of guaiacol, which was used as a model humic monomer. Although bentazon did not react significantly with guaiacol in the presence of the enzymes solely, the reaction of the herbicide with guaiacol was almost complete in 30 min in the presence guaiacol and ferulic acid, which are the electron donor co-substrates in most of the oxidative coupling reactions [88]. Laccase from *C. unicolor* displayed a lower efficiency in oxidizing the herbicides 2,4-D and simazine, but the enzyme oxidized efficiently 2,4-DCP, a derivative of 2,4-D [110]. González Matute and coauthors demonstrated the ability of extracellular enzymes

of *A. blazei* to degrade the herbicide metsulfuron-methyl [111]. Crude enzyme preparation was obtained from spent compost, which was the residual compost waste generated by the fungi cultivation industry. The degradation of the herbicide was confirmed using bioassay experiments with oil rape (*Brassica napus* L.) as the plant indicator. The detoxifying capacity of the preparations containing lignolytic enzymes and products of coal solubilization by *T. hirsuta* and *T. maxima* in respect to the herbicide atrazine was demonstrated by Klein et al. [112].

Pizzul et al. conducted degradation tests using purified MnP from *N. frowardii* and LiP and laccase from *T. versicolor* in combination with different mediators in order to estimate transformation of glyphosate and Pesticide Mix 34. The latter included the herbicides atrazine, chlorotoluron, chloroxuron, diuron, fenuron, isoproturon, linuron, metamitron, metazachlor, metobromuron, metolachlor, metoxuron, metribuzin, monolinuron, prometryn, simazine, terbuthylazine, and terbutryn. Authors demonstrated that MnP and laccase were very efficient in the transformation of glyphosate and led to (aminomethyl) phosphonic acid formation (metabolite of glyphosate) and accumulation. In addition, simultaneous degradation of 22 pesticides in a mixture was obtained by the action of MnP in the presence of Tween 80 and  $\text{MnSO}_4$ , with degradation values varying from 20% to 100% [81].

However, real contaminated environments contain usually a wide number of different chemical species, some of which can inhibit fungal growth and/or reduce enzymatic activity [106]. To preserve the enzyme's activity and stability over time, immobilization of the enzyme can be used. Immobilized enzymes have usually a long-term and operational stability, being very stable toward physical, chemical, and biological denaturing agents. Furthermore, they may be reused and recovered at the end of the process [85,91]. Immobilization of laccase from *T. versicolor* onto a hydrophilic PVDF microfiltration membrane allowed obtaining the membrane grafted with 220U enzyme activity used in a filtration module to transform a phenylurea herbicide derivative 2-HF (*N,N'*-(dimethyl)-*N*-(2-hydroxyphenyl)urea) from waste water. No 2-HF was found in permeate 5 min after the beginning of the experiment [85]. Laccase from *T. versicolor* immobilized on an zein polyurethane nanofiber *via* cross-linking with glutaraldehyde completely degraded the phenylurea herbicide chloroxuron within 30 min in the presence of 1 mM 1-hydroxybenzotriazole [86].

Both LiP, MnP and laccase may behave as powerful catalysts in the biodegradation of herbicides. However, their full-scale application for remediation of polluted environments is still limited. The latter may derive from several drawbacks and disadvantages of the enzymes application such as enzyme instability in the environment and loss of their activity. Immobilization of the enzymes is likely to be a promising way to develop a successful approach for the remediation of the herbicide polluted sites.

## 6. Conclusion

The high potential of WRF as well as their ligninolytic enzymes in herbicide transformation is analyzed in the present review. Analysis of literature data on degradation rate of herbicides by WRF demonstrated enhancing WRF degradation capacity along with increase content of branched alkyl groups in the herbicide molecule. However, detailed quantitative structure-

degradation activity studies should be conducted to prove or disprove this preliminary observation. Therefore, the mechanisms of herbicides degradation by WRF for many herbicides are still not explored and degradation pathways are not established, including the identification of the metabolites formed.

The ligninolytic enzymes MnP and laccase were shown to behave as powerful catalysts in the biodegradation of herbicides. However, their full-scale application for remediation of polluted environments is still limited. The latter may derive from several drawbacks and disadvantages of the enzymes application such as enzyme instability in the environment and loss of their activity. Immobilization of the enzymes is likely to be a promising way to develop a successful approach for the remediation of the herbicide polluted sites.

The potential of ligninolytic enzymes in the degradation of herbicides is beginning to be characterized at the molecular level. The constant progress in molecular and genomic techniques has provided new insights on the role of regulating elements in the differential expression of ligninolytic enzymes in WRF. Further studies will elucidate the mechanisms of ligninolytic enzymes' transcriptional regulation and provide deeper understanding of this complicated process.

It should be noted that efficient recommendations for microbial remediation need integral knowledge about potential of individual enzymatic reactions and specific features of their interactions for different microbial species. Current information about genetic regulation of coupled reactions may improve significantly bioremediation technologies, as well as empiric data regarding multistep detoxification with the use of different microorganisms.

Analysis presented in this review confirms the important role of white-rot fungi as participants in herbicide decontamination in the environment and the prospects of the development of new biotechnological preparations on the basis of fungal enzymes. The most important tasks in the development of bioremediation technologies and recent results of key stakeholders in this field are discussed.

## 7. Abbreviations

ABTS – 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)

ACC – acetyl-CoA carboxylase

ALS – acetolactate synthase

DEA – deethylatrazine

DCPMU – 1-(3,4-dichlorophenyl)-3-methylurea

DCPU – 1-(3,4-dichlorophenyl)urea

<sup>1</sup>H-NMR – proton nuclear magnetic resonance

HPPD – p-hydroxyphenylpyruvate dioxygenase

HR – herbicide-resistant crop

EPSP – 5-enolpyruvylshikimate-3-phosphate

LiP – lignin peroxidase

LMS – lignin modifying system

MnP – Mn peroxidase

PCDD – polychlorinated dibenzo-p-dioxin

PCDF – polychlorinated dibenzo furan

PCR – polymerase chain reaction

PLEL – phospholipid ether lipids

PLFA – phospholipid fatty acid

PPO – protoporphyrinogen oxidase

PSII – photosystem II

ROS – reactive oxygen species

WRF – white-rot fungi

XRE – xenobiotic responsive element

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