

## Article

# Widespread Ability of Ligninolytic Fungi to Degrade Hazardous Organic Pollutants as the Basis for the Self-Purification Ability of Natural Ecosystems and for Mycoremediation Technologies

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**Featured Application:** The potential application of the results is in the field of mycoremediation of contaminated sites.

**Abstract:** The ability of sixteen wood- and soil-inhabiting basidiomycete strains and four ascomycete strains to degrade the most hazardous, widespread, and persistent pollutants (polycyclic aromatic hydrocarbons, oxyethylated nonylphenol, alkylphenol, anthraquinone-type synthetic dyes, and oil) was found. The disappearance of the pollutants, their main metabolites, and some adaptive properties (activities of ligninolytic enzymes, the production of emulsifying compounds and exopolysaccharides) were evaluated. The toxicity of polycyclic aromatic hydrocarbons decreased during degradation. New data were obtained regarding (1) the dependence of the completeness of polycyclic aromatic hydrocarbon degradation on the composition of the ligninolytic enzyme complex; (2) the degradation of neonol AF9-12 by higher fungi (different accessibilities of the oxyethyl chain and the aromatic ring of the molecules to different fungal genera); and (3) the production of an emulsifying agent in response to the presence in the cultivation medium of hydrophobic pollutants as the common property of wood- and soil-inhabiting basidiomycetes and ascomycetes. Promise for use in mycoremediation was shown in the wood-inhabiting basidiomycetes *Pleurotus ostreatus* f. Florida, *Schizophyllum commune*, *Trametes versicolor* MUT 3403, and *Trametes versicolor* DSM11372; the litter-decomposing basidiomycete *Stropharia rugosoannulata*; and the ascomycete *Cladosporium herbarum*. These fungi degrade a wide range of pollutants without accumulation of toxic metabolites and produce ligninolytic enzymes and emulsifying compounds.

**Keywords:** fungi; biodegradation; hazardous pollutants; ligninolytic enzymes; emulsifying activity; mycoremediation

## 1. Introduction

Environmental contamination by hazardous pollutants is a serious and challenging problem of the present time. The range of pollutants, which enter the environment as a result of human economic activities and natural processes, is very broad and includes

several class of compounds (polycyclic aromatic hydrocarbons (PAHs), synthetic dyes, detergents, chlorinated compounds, pharmaceutical compounds, dioxins, plastics, etc.) and resulting mixtures (creosote, oil, etc.). The discharge and accidental entry of pollutants into the environment is a serious problem, especially when the biodegradation activity of the natural microbiota is insufficient for their removal or neutralization.

Natural ecosystems have great detoxification potential, which is ensured by the degradative activity of different organisms. These include basidio- and ascomycetes, some of the most numerous groups of the kingdom of Fungi. The ability of fungi to degrade a wide range of natural compounds and xenobiotics is an important part of the self-purification ability of ecosystems and can be used for the development of ecological biotechnologies [1].

Currently, an active search for technologies of bioremediation of contaminated soils and water is in progress. The fungal degradation or transformation of hazardous organic compounds is called mycoremediation [2–6]. This simple and relatively inexpensive biotechnology could be applied to the remediation of soil and water polluted by industrial and agricultural waste, oil, and its byproducts, and a wide range of xenobiotics [2–6]. Fungi fulfill various functions in ecosystems and have great degradation potential; therefore, they are attractive candidates for use in mycoremediation technologies. However, detailed studies of the degradative properties of fungi and their adaptation to pollution are necessary before they are used for mycoremediation.

Ligninolytic fungi are an eco-physiological group of wood- and soil-inhabiting basidio- and ascomycetes, which are uniquely able to depolymerize and mineralize lignin in nature. The following properties make these fungi attractive for use in mycoremediation: (1) the ability to decompose and mineralize a wide range of persistent aromatic compounds that are structurally similar to the compounds formed during biosynthesis or biodegradation of lignin; (2) the wide distribution in nature; (3) the ability to grow on cheap natural substrates which can easily be introduced to polluted soil; (4) growth in the form of hyphae, allowing them to reach pollutants that are inaccessible to bacteria; (5) the production of a powerful extracellular nonspecific oxidative enzyme system; (6) the presence of low-molecular-weight mediators that may increase the susceptibility of pollutants to enzymatic attack; (7) the production of constitutive forms of ligninolytic enzymes regardless of the presence of pollutants, which prevent the adaptation of the organism to contamination conditions [1,2,7].

The polymeric and hydrophobic structure of the macromolecule of lignin determines the properties of the degrading enzyme system, which is extracellular, nonspecific, and nonhydrolytic. Ligninolytic fungi produce four main groups of lignin-modifying enzymes: lignin peroxidases (EC 1.11.1.14), Mn-dependent peroxidases (Mn-peroxidases, EC 1.11.1.13), versatile peroxidases (EC 1.11.1.16), and laccases (EC 1.10.3.2) [7].

This enzymatic system allows fungi to transform and mineralize, in addition to lignin, pesticides, polychlorinated biphenyls, halogenated aromatic compounds, nitro- and amino-substituted phenols, synthetic dyes, PAHs, etc. [7–9]. Only a few studies have reported the use of ligninolytic fungi for the mycoremediation of sites contaminated by complex mixtures of pollutants, such as creosote and oil [10–14].

Many persistent environmental pollutants are represented by hydrophobic compounds. Their bioavailability can limit the fungal attack [15]. Stimulation of their desorption from soil by various surfactants can increase their bioavailability [16,17]. However, some surfactants, such as Triton X-100, are very difficult to degrade; as a result, stable and toxic products form that can accumulate in the environment [18,19].

Many well-known bacterial degraders are known to synthesize biosurfactants [20]. However, only a few reports have described the production of biosurfactants during the degradation of hydrophobic pollutants by ligninolytic fungi [14,21–24].

The focus of this study is the assessment of the degradative activity towards persistent pollutants (PAHs, nonionic surfactants, anthraquinone-type synthetic dyes, and oil) and of the adaptive potential of natural fungi belonging to different divisions of the

kingdom Fungi (basidio- and ascomycetes) and ecological–physiological groups (wood- and soil-inhabiting fungi). Research on the fungal species combining the ability to degrade a wide range of environmental pollutants with the production of biosurfactants is one possible key to the explanation of the self-purification ability of contaminated ecosystems and the successful implementation of mycoremediation strategies.

## 2. Materials and Methods

### 2.1. Fungi and Cultivation Conditions

*Schizophyllum commune* IBPPM541, *Pleurotus ostreatus* f. Florida IBPPM540, *Pleurotus ostreatus* 336, *Pleurotus ostreatus* D1 (Jacquin) P. Kummer, *Lecanicillium aphanocladii* Zare & W. Gams IBPPM542, and *Fusarium oxysporum* IBPPM543 were from the Collection of Rhizosphere Microorganisms (IBPPM RAS, Russia); *Trametes hirsuta* LE-BIN072, *Trametes maxima* LE-BIN0275, *Trametes ochracea* LE-BIN093, *Trametes gibbosa* LE-BIN1911, *Lenzites betulina* LE-BIN2047, *Pleurotus ostreatus* LE-BIN0432, and *Steccherinum murashkinskyi* LE-BIN1963 were from the RAS Federal Research Centre “Fundamentals of Biotechnology” (Moscow, Russia); *Trametes versicolor* MUT3403, *Pleurotus ostreatus* MUT2977, *Bjerkandera adusta* MUT3398, *Geotrichum candidum* MUT4803, and *Cladosporium herbarum* MUT3238 were from Mycotheca Universitatis Taurinensis (Turin, Italy); *Trametes versicolor* DSM11269 and *Stropharia rugosoannulata* DSM11372 were from Helmholtz Centre for Environmental Research – UFZ (Leipzig, Germany) (Table S1).

The fungi were grown in a basidiomycetes-rich medium with our modifications (g/L) [25]:  $\text{NH}_4\text{NO}_3$ , 0.724;  $\text{KH}_2\text{PO}_4$ , 1.0;  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 1.0; KCl, 0.5; yeast extract, 0.5;  $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ , 0.001;  $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ , 0.0028;  $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ , 0.033; D-glucose, 10.0; peptone, 10.0. Alternatively, the fungi were grown in Kirk’s medium with our modifications (g/L) [26]:  $\text{KH}_2\text{PO}_4$ , 0.2;  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 0.05; CaCl<sub>2</sub>, 0.01; thiamine, 0.0025;  $\text{NH}_4\text{NO}_3$ , 0.724. The pH of Kirk’s medium was maintained with 25 mM phosphate buffer (pH 6.0), and maltose (1%) served as the carbon and energy source. Kirk’s medium was supplemented with 0.1% Tween-80 to increase ligninolytic enzyme production. Cultivation was performed at 24–26 °C with shaking (130 rpm).

Pollutants were added on day 2 of growth to final concentrations of 50 mg/L for PAHs (anthracene, phenanthrene, or fluorene) and anthraquinone dyes (Acid Blue 62 and Reactive Blue), 200 mg/L for a nonionic surfactant (neonol AF9-12) and isononylphenol (isomeric mixture), and 5 g/L for oil. PAHs and isononylphenol were added as a chloroform stock solution: 5 mg/100 µL and 20 mg/200 µL, respectively. Control treatments contained 100 µL of chloroform, which was added on day 2 of fungal growth. After 14 days, residual PAHs, neonol AF9-12, isononylphenol, and oil were extracted from the bulk of flasks (without separation of the cultivation medium and mycelia) with chloroform (5 mL, three times). The resulting extracts were evaporated and were analyzed as described below. The mycelium and the cultivation medium were separated by filtration. The mycelium increment (mg of dry biomass) was determined by weighing.

### 2.2. Chromatography

The PAHs and their metabolites were analyzed by gas chromatography (GC) (Shimadzu 2010) with a flame photometric detector. Compounds were separated by using an HP5 column (Agilent), with helium as the carrier gas. The column temperature was kept at 200 °C for 3 min, then programmed to increase to 270 °C at 15 °C min<sup>−1</sup>, and finally kept at 270 °C for 2 min. Before GC analysis, 2,2'-diphenic and phthalic acids were methylated with  $\text{CH}_3\text{COCl}$ . Commercial anthracene (RT—4.15 min), 9,10-anthraquinone (RT—5.39 min), phenanthrene (RT—4.08 min), phenanthrene-9,10-quinone (RT—6.86 min), fluorene (RT 5.57 min), 9-fluorenone (RT—4.33 min), 2-carboxybenzaldehyde (RT—7.31 min), 2,2'-diphenic acid (RT—6.7 min), and phthalic acid (RT—8.99 min) were used as the marker compounds to identify the PAH oxidation products. GC analysis was carried out at the “Simbioz” Centre for the Collective Use of Research Equipment in the Field of

Physical–Chemical Biology and Nanobiotechnology (Institute of Biochemistry and Physiology of Plants and Microorganisms, Saratov Scientific Centre of the Russian Academy of Sciences (IBPPM RAS)).

The oil disappearance was measured by adsorption chromatography with gravimetric termination. Oil was extracted three times by 5 mL of chloroform; the extracts were evaporated and applied to a column of Al<sub>2</sub>O<sub>3</sub> (10 mL). The oil was eluted by chloroform and dried to complete evaporation of the solvent and the amount of oil was determined by the gravimetric method. The changes in the fractional composition of the oil were determined by adsorption chromatography on an Al<sub>2</sub>O<sub>3</sub> column, followed by polarimetric and gravimetric analysis. The paraffins (Nd = 1.41 – 1.43) were eluted with 40 mL of hexane; naphthenes (Nd = 1.45 – 1.49), with 60 mL of hexane; low-molecular-weight aromatic hydrocarbons (Nd = 1.49 – 1.53), 100 mL of a mixture of 15% benzene in hexane; high-molecular-weight aromatic hydrocarbons (Nd > 1, 59), 100 mL benzene; and tars (refractive index not determined), 80 mL of ethanol/benzene (1:1). The fractions were dried to complete evaporation of the solvent and analyzed by gravimetric method [27]. The oil used was composed as follows (%): paraffins—51.7, naphthenes—17.0, low-molecular-weight aromatic hydrocarbons—10.3, high-molecular-weight aromatic hydrocarbons—7.3, and tars—13.5.

### 2.3. Spectrophotometric Analysis

The nonionic surfactant was determined by spectrophotometric analysis with phosphomolybdic acid [28]. The degradation of the aromatic ring of neonol AF9-12 and isononylphenol was determined spectrophotometrically at 277 nm [29].

Owing to the optical opacity of the basidiomycetes-rich medium, decolorization of dyes was studied on Kirk's medium at 6.0. Aliquots (2 mL) were taken from the flasks at intervals, and the dye disappearance was tested spectrophotometrically by the change in the absorption spectra at 590 nm [30].

### 2.4. Infrared Spectrometry

Infrared spectra of neonol AF9-12 and isononylphenol were recorded on a Nicolet 6700 IR-Fourier spectrometer (Thermo Scientific, USA) in KBr tablets (FT-IR grade, ≥99%, Sigma-Aldrich) in the transmission mode in the range 4000–400 cm<sup>-1</sup>. The spectral resolution was 4 cm<sup>-1</sup>, and the number of scans of the sample and the reference sample was 32.

### 2.5. Enzymatic Analysis

Laccase activity was measured by the oxidation rate for 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) at 436 nm ( $\epsilon = 29300 \text{ M}^{-1}\text{cm}^{-1}$ ), according to Niku-Paavola et al. [31]. Mn-peroxidase was measured by the oxidation rate for 2,6-dimethoxyphenol (DMP) with H<sub>2</sub>O<sub>2</sub> and Mn<sup>2+</sup> at 468 nm ( $\epsilon = 14800 \text{ M}^{-1}\text{cm}^{-1}$ ), according to Heinfling et al. [32]. Peroxidase activity was calculated as the difference between the values for DMP oxidation with and without H<sub>2</sub>O<sub>2</sub>. Lignin peroxidase was measured by the formation of the oxidation product of veratryl alcohol at 310 nm ( $\epsilon = 9300 \text{ M}^{-1}\text{cm}^{-1}$ ) [33]. One unit of enzyme activity (U/mL) is defined as the amount of enzyme that oxidizes 1  $\mu\text{M}$  of substrate per min.

### 2.6. Emulsifying Activity Measurements

The emulsifying activity of the culture medium was tested by Cooper's method [34]. The culture medium after fungal growth both with and without pollutants was mixed 2:3 with kerosene, shaken for 20 min, and left to stand at room temperature for 48 h. Emulsifying activity (E<sub>48</sub>) was calculated as the ratio of emulsion volume to total liquid volume and expressed in percentage.

### 2.7. Polysaccharide Concentration Measurements

Concentrations of free reducing sugars in sample aliquots were determined using the dinitrosalicylic acid (DNSA) method according to Bailey [35]. After acidic hydrolysis, the total sugars in sample aliquots were measured photometrically on the basis of the phenol-sulfuric acid method [36]. For both methods, calibration curves were established from equal amounts of mixed *D*-glucose and *D*-fructose. Polysaccharide concentrations were calculated as the difference between total and free reducing sugar concentrations.

### 2.8. Phytotoxicity Estimation

The test object was sorghum (*Sorghum bicolor* L. Moench). Seeds had been calibrated and surface-sterilized with a mixture of ethanol and hydrogen peroxide (1:1). Germinating capacity was determined as the percentage of germinated seeds relative to the total seeds tested. The tested compounds were dissolved in chloroform at a concentration of 0.25 mg/mL; 1 mL of the solution was applied to sterile paper filters placed in sterile glass Petri dishes 10 cm in diameter. Filters treated with the same amount of chloroform were used as the control treatment. The phytotoxicity of PAHs and their derivatives that are potential fungal metabolites was estimated. Among these compounds, there were PAHs: phenanthrene, anthracene, and fluorene; quinones: phenanthrene-9,10-quinone and 9,10-anthraquinone; ketone 9-fluorenone; acids: 2-formylbenzoic (2-carboxybenzaldehyde), 2,2'-diphenic, and phthalic. On the third day after complete evaporation of the solvent, the filters were moistened with 5 mL of sterile water, and 20 seeds were placed on each of them. Three replicates were used for each tested compound. In 3-day-old seedlings, the length of roots and shoots was measured. Means were calculated and expressed as the percentage of the control values.

### 2.9. Chemicals

Anthracene, fluorene, phenanthrene, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,6-dimethoxyphenol (DMP), veratryl alcohol, 2-carboxybenzaldehyde, 2,2'-diphenic and phthalic acids, Acid Blue 62, and Reactive Blue were from Fluka (Switzerland); all the other pollutants were from Reachim Co. (Russia).

All experiments were run in triplicate, each having been repeated at least three times. Data were statistically processed; the means were calculated and compared by the index of least significant difference at  $p < 0.05$  by using Fisher's exact test. Calculations were performed using Statistica software v.7 (StatSoft, Russia) and Microsoft Excel 2003.

## 3. Results and Discussion

We used 20 strains of fungi belonging to two divisions of the kingdom of Fungi (Basidiomycota and Ascomycota): 16 strains of ligninolytic basidiomycetes (15 strains of wood-inhabiting and 1 strain of soil-inhabiting, litter-decomposing, fungi) and 4 strains of ascomycetes.

Fungi of the genera *Pleurotus* and *Trametes*, and *Bjerkandera adusta* are common objects of biodegradation studies [14,24,37,38]. In our experiments, five strains of *P. ostreatus* were used for the detection of strain-specific differences in degradative properties. Six members of genus *Trametes*, including two strains of *T. versicolor*, were used also. In this case, the differences in the degradative properties of different species of *Trametes* were found. Some other wood-inhabiting basidiomycetes, including *Lenzites betulina*, *Schizophyllum commune*, and *Steccherinum murashkinskyi*, were used. *Pleurotus ostreatus* f. Florida and *Schizophyllum commune* were isolated from creosote-contaminated railway sleeper wood and identified using a polyphasic approach, combining morphophysiological observation of the colonies with molecular tools (data not presented).

Litter-decomposing fungi are another ecophysiological group of basidiomycetes, which can cause the so-called white rot of humus. Some genera of this group are prom-

ising organisms for use in soil remediation. The agaric basidiomycetes *Stropharia rugosoannulata* and *Stropharia coronilla* are efficient PAH degraders, and bisphenol A [39,40]. *S. rugosoannulata* strain DSM11372 was used in our studies.

The participation of ascomycetes in the degradation of pollutants is known. *Fusarium oxysporum* is able to significantly reduce the concentration of oil and to degrade and mineralize anthracene, phenanthrene, and pyrene [41,42]. *Geotrichum candidum* decolorized 21 types of reactive dyes, including azo and anthraquinone dyes [43]. Among the *Cladosporium* species, known to degrade hydrocarbons, *C. resinae* has the name the “kerosene” or “creosote” fungus, because it occurs in aviation fuel and causes damage by clogging filters and corroding pumps and tanks. *C. herbarum*, isolated from polluted sites, is able to degrade PAHs in a liquid medium [44]. The degradative properties of the entomopathogenic *Lecanicillium* species have been poorly studied. For example, *L. saksenae* is an active decomposer of the pesticide pendimethalin, and *V. lecanii* is a decomposer of 2,4-dichlorophenol and 2,4-dichlorophenoxyacetic acid and anthracene [45–47].

On the basis of these literature data, four species of ascomycetes (*C. herbarum*, *F. oxysporum* Schltdl., *G. candidum*, and *L. aphanocladii* Zare & W. Gams) were used in our experiments. *F. oxysporum* Schltdl. and *L. aphanocladii* Zare & W. Gams were isolated from creosote-contaminated samples. The fungi were identified by a polyphasic approach, combining morphophysiological observation of the colonies with molecular tools. The accession numbers of the sequences deposited in GenBank are *F. oxysporum*—MG593980; *L. aphanocladii*—MG593981 [48].

### 3.1. Pollutant Disappearance Experiments

#### 3.1.1. Polycyclic Aromatic Hydrocarbons (PAHs)

The considerable interest in biodegradation mechanisms and environmental fate of PAHs is due to their wide distribution, resistance to degradation, accumulation in soils and sediments, and toxic, mutagenic, and carcinogenic properties [15]. Many reviews on the biodegradation of this class of pollutant are available. The corresponding quinones (e.g., 9,10-anthraquinone, phenanthrene-9,10-quinone, and benzo[a]anthracene-7,12-quinone) are discussed as the main metabolites [38,49].

The three-ring PAHs used in our experiments (phenanthrene, anthracene, and fluorene) are not mutagenic or carcinogenic to humans [15]. However, they are often used as model compounds for the study of the biodegradation of PAHs. They are found in PAH-containing samples, and many PAH derivatives are carcinogenic.

In our experiments, all fungi actively removed PAHs, as evidenced by the decrease in their concentration after 14 days of cultivation (Table 1). Previous experiments showed that PAH sorption by living fungal mats was significantly lower than that by heat-killed ones. This finding agrees with the data of Gramss and Mascher [50]. According to the data obtained, we used two consistent extraction procedures: (1) extraction from full contents of the flasks; (2) the cultivation medium was separated from mycelia, and the mycelium was extracted again. Insignificant sorption of the studied PAHs to mycelia (5–7%) was shown.

**Table 1.** PAHs disappearance (%) by the selected fungi.

№	Fungi	PAH Disappearance (%)		
		ANT	PHEN	FLU
1	<i>Bjerkandera adusta</i> MUT 3398	61.6 ± 3.0	82.8 ± 4.8	96.2 ± 7.2
2	<i>Cladosporium herbarum</i> MUT 3238	94.1 ± 1.0	95.4 ± 2.5	98.0 ± 2.4
3	<i>Fusarium oxysporum</i> IBPPM 543	0 ± 2.3	20.8 ± 1.2	41.9 ± 3.5
4	<i>Geotrichum candidum</i> MUT4803	0 ± 3.0	17.7 ± 1.3	16.8 ± 7.1
5	<i>Lecanicillium aphanocladii</i> IBPPM 542	39.8 ± 12.1	62.8 ± 2.8	81.3 ± 3.0
6	<i>Lenzites betulina</i> LE-BIN 2047	89.3 ± 8.6	74.4 ± 18.8	99.2 ± 5.7

7	<i>Pleurotus ostreatus</i> f. Florida IBPPM 540	50.9 ± 5.7	70.8 ± 2.1	93.9 ± 5.4
8	<i>Pleurotus ostreatus</i> MUT2977	92.0 ± 3.2	71.2 ± 13.7	93.0 ± 1.3
9	<i>Pleurotus ostreatus</i> 336	56.7 ± 1.8	71.3 ± 1.6	86.6 ± 2.9
10	<i>Pleurotus ostreatus</i> D1	85.3 ± 4.2	100 ± 6.8	100 ± 5.3
11	<i>Pleurotus ostreatus</i> LE-BIN0432	36.7 ± 11.8	79.3 ± 15.5	94.8 ± 18.9
12	<i>Schizophyllum commune</i> IBPPM 541	49.5 ± 2.6	39.8 ± 8.5	74.2 ± 9.4
13	<i>Steccherinum murashkinskyi</i> LE-BIN 1963	71.8 ± 2.6	76.1 ± 7.2	91.5 ± 6.5
14	<i>Stropharia rugosoannulata</i> DSM11372	49.5 ± 3.1	55.2 ± 4.9	72.2 ± 7.0
15	<i>Trametes gibbosa</i> LE-BIN 1911	17.0 ± 6.3	45.3 ± 7.2	51.8 ± 18.8
16	<i>Trametes hirsuta</i> LE-BIN 072	37.8 ± 2.6	59.6 ± 4.4	90.4 ± 2.5
17	<i>Trametes maxima</i> LE-BIN 0275	84.6 ± 2.1	78.8 ± 15.6	95.4 ± 22.9
18	<i>Trametes ochracea</i> LE-BIN 093	16.2 ± 5.6	48.5 ± 19.8	85.2 ± 22.1
19	<i>Trametes versicolor</i> DSM11372	57.1 ± 7.8	79.8 ± 2.7	96.8 ± 7.1
20	<i>Trametes versicolor</i> MUT3403	86.4 ± 13.2	60.9 ± 8.6	87.8 ± 2.8

ANT—anthracene; PHEN—phenanthrene; FLU—fluorene.

In general, the basidiomycetes removed these compounds more actively than did the ascomycetes (Table 1). Anthracene was the least available to all fungi. The disappearance of this compound varied from 16 to 91% with the basidiomycetes. Anthracene was inaccessible to the ascomycetes *F. oxysporum* and *G. candidum*. Phenanthrene and fluorene were removed by both basidio- and ascomycetes (17.7–100 and 16.8–100%, respectively) (Table 1).

The degradative activity towards PAHs showed both strain and species differences. Within the species *Pleurotus ostreatus*, the most active was *P. ostreatus* D1 (the disappearance of phenanthrene and fluorene reached 100%). Within the genus *Trametes*, both *T. versicolor* and *T. maxima* strains were active degraders, whereas *T. gibbosa* was not very active.

One of the most active degraders was the ascomycete *C. herbarum* which showed a removal efficiency of about 100% with all PAHs used. The literature has a single report on the degradation of PAHs by another member of this genus, *C. sphaerospermum*. However, in that study, the disappearance of three-ring PAHs did not exceed 35% [44].

GC analysis revealed the presence of additional peaks of products of degradation of anthracene, phenanthrene, and fluorene by the studied fungi. These products were identified as 9,10-anthraquinone (RT = 4.51 min), phenanthrene-9,10-quinone (RT = 5.04 min), and 9-fluorenone (RT = 4.33 min) in the case of anthracene, phenanthrene, and fluorene oxidation, respectively. The products of deeper PAH degradation, such as 2-carboxybenzaldehyde, 2,2'-diphenic and phthalic acids, were identified with all studied members of the genera *Trametes* and *Pleurotus*, and with *S. commune*. When the growth time was extended, these compounds disappeared, which suggests their involvement in the basal metabolism [51]. It should be noted that the revealed metabolites of three-ring PAH degradation by the basidio- and ascomycetes were identical.

The most active degraders of three- and four-ring PAHs were the basidiomycetes *B. adusta*, *P. ostreatus* (all strains used), *S. commune*, *T. maxima*, and *T. versicolor* (both strains used), and two ascomycetes *C. herbarum* and *L. aphanocladii* (Table 1). Although *S. murashkinskyi* revealed a high degradative activity towards PAHs, it was a slowly growing fungus that can complicate its following practical use. However, despite its average degradative activity, the litter-decomposing *S. rugosoannulata* can be promising for further studies, because soil is the natural habitat of this fungus.

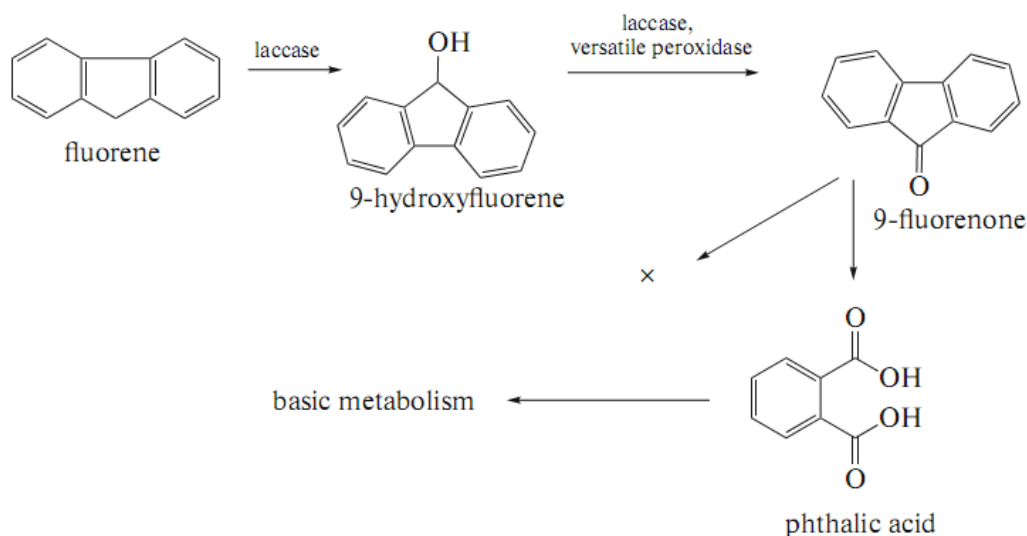
According to the availability to the fungal cells, the studied PAHs can be arranged in the order anthracene → phenanthrene → fluorene. Comparison of the obtained data with the physicochemical properties of these substances suggests that in this case, a coupling of two factors is essential: the solubility and ionization potentials of PAHs. Although anthracene has the lowest ionization potential (7.43 eV), the low solubility of this sub-

stance (0.07 mg/L) makes its molecules poorly available for the fungal attack. Yet, the well-soluble phenanthrene (1.8 mg/L) has a sufficiently high ionization potential (8.03 eV), and as a consequence, its middle availability for the studied fungi. Finally, a “successful” combination of low ionization potential (7.88 eV) and high solubility (1.98 mg/L) makes fluorene the most accessible among the PAHs studied.

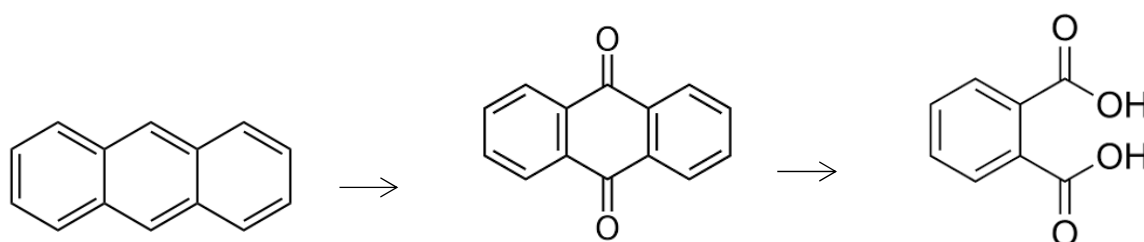
Now, many reviews on the biodegradation of this class of pollutants have already been published. The corresponding PAH-based quinones (e.g., 9,10-anthraquinone, phenanthrene-9,10-quinone, benzo[a]anthracene-7,12-quinone) are discussed as the main metabolites [38,49]. Two possibilities for the formed quinones are known. One is the subsequent utilization of quinone metabolites, as it was shown for *P. ostreatus*, *P. eringii*, *Phanerochaete laevis*, and *Polyporus* sp. S133. In this case, phthalic and 2,2'-diphenic acids resulted from “deep” degradation of fluorene, phenanthrene, and anthracene. The involvement of these metabolites to basic metabolism and their subsequent mineralization to CO<sub>2</sub> are possible, and another possibility is that they can be the end metabolites of PAH degradation; their accumulation was found in *Phanerochaete chrysosporium*, *B. adusta*, and *Pycnoporus sanguineus*.

In our experiments, some differences were also found. The end metabolites of the degradation of PAHs by *S. rugosoannulata* were quinone derivatives: 9,10-anthraquinone, phenanthrene-9,10-quinone, and 9-fluorenone. During PAH degradation by both *Trametes* strains, the traces of the corresponding quinones were found. Similar metabolites were detected during cultivation of *P. ostreatus* strains, *S. commune*, and *B. adusta*. They disappeared when the cultivation time was extended. This indicated that they were utilized further. Phthalic acid was the metabolite resulting from “deep” degradation of anthracene and fluorene by *P. ostreatus* strains. The 2,2'-diphenic and phthalic acids were produced by “deep” phenanthrene degradation by these fungi. These metabolites can be included in the basal metabolism of the fungi. The proposed metabolic pathways of three-ring PAH degradation by studied fungi are shown in Figure 1.

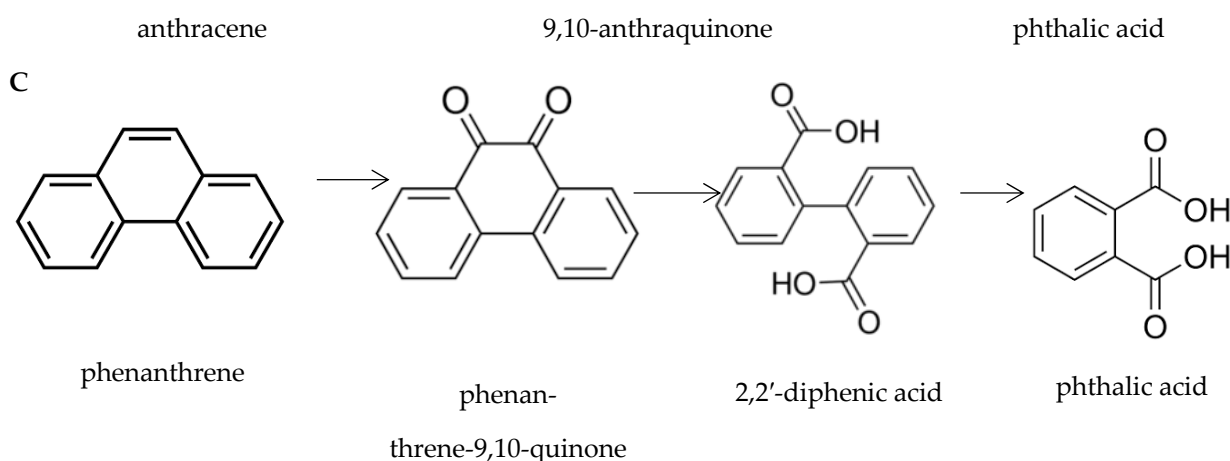
A



B





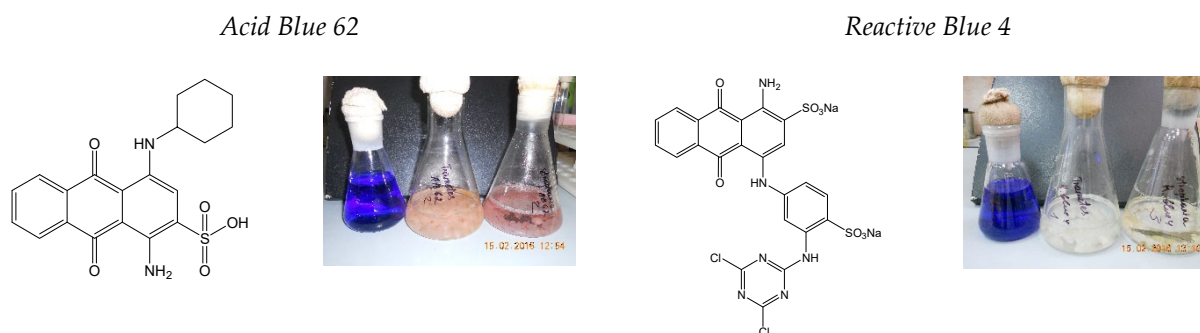


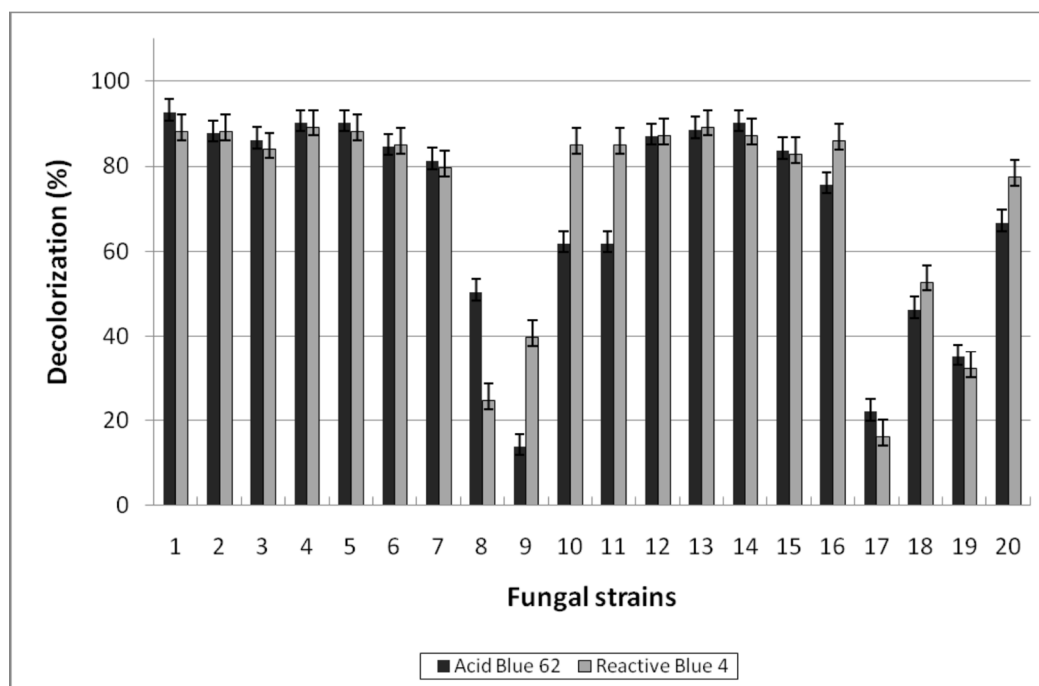
**Figure 1.** The proposed metabolic pathways of three-ring PAH degradation—fluorene (A), anthracene (B), and phenanthrene (C) degradation.

### 3.1.2. Decolorization of Anthraquinone-Type Dyes

The three-ring structure of anthraquinone (the metabolite of the fungal degradation of anthracene), occupies the central part of a range of dyes, the so-called anthraquinone-type dyes. These dyes are considered to be the most stable among the different classes of dyes currently used [52,53]. Their entry to the environment with waste water is a serious problem. Many industrial dyes are toxic mutagenic and carcinogenic [54,55]. Although anthraquinone dyes are a large group of synthetic dyes, PolyR-478 and Remazol Brilliant Blue R (RBBR) are most often used as models of similar dyes in biodegradation studies [56].

In our study, two other dyes of this type (Acid Blue 62 and Reactive Blue 4), which differed by substituents at the anthraquinone structure, were chosen. All the studied fungi decolorized anthraquinone dyes, and both the chromophore part of the molecule and the aromatic ring were available for the fungal degradation. Figure 2 (top) shows the chemical structures of these dyes. This evidenced by a decrease in absorption at the UV and visible regions of the spectra of Acid Blue 62 and Reactive Blue 4. Most strains of basidiomycetes decolorized both dyes after 6 days (Figure 2). An exception was *S. commune*, which decolorized Reactive Blue 4 only by 60%.





**Figure 2.** Anthraquinone dyes decolorization after 2 days' cultivation of the studied fungi: 1—*P. ostreatus* f. Florida, 2—*P. ostreatus* MUT2977, 3—*P. ostreatus* 336, 4—*P. ostreatus* D1, 5—*P. ostreatus* LE-BIN0432, 6—*B. adusta*, 7—*Len. betulina*, 8—*St. murashkinskyi*, 9—*Sch. commune*, 10—*Str. rugosoannulata*, 11—*T. versicolor* DSM11372, 12—*T. versicolor* MUT3403, 13—*T. hirsuta*, 14—*T. maxima*, 15—*T. ochracea*, 16—*T. gibbosa*, 17—*Lec. aphanocladii*, 18—*F. oxysporum*, 19—*G. candidum*, 20—*C. herbarum*.

The decolorization of Acid Blue 62 resulted in the change of color from dark blue to light reddish-brown. This destruction of the molecule was accompanied by the formation of precipitate, which may indicate the polymerization of the products formed, similar to data obtained by Sugano et al. [54]. The degradation of Reactive Blue 4 was more complete, as can be judged by the complete decolorization of the cultivation medium (Figure 2, photo).

Both dyes were less accessible to the ascomycetes. Decolorization varied from 40% for *G. candidum* to 70% for *L. aphanocladii*. An exception was *C. herbarum*, which decolorized both studied dyes completely, without marked sorption by mycelia.

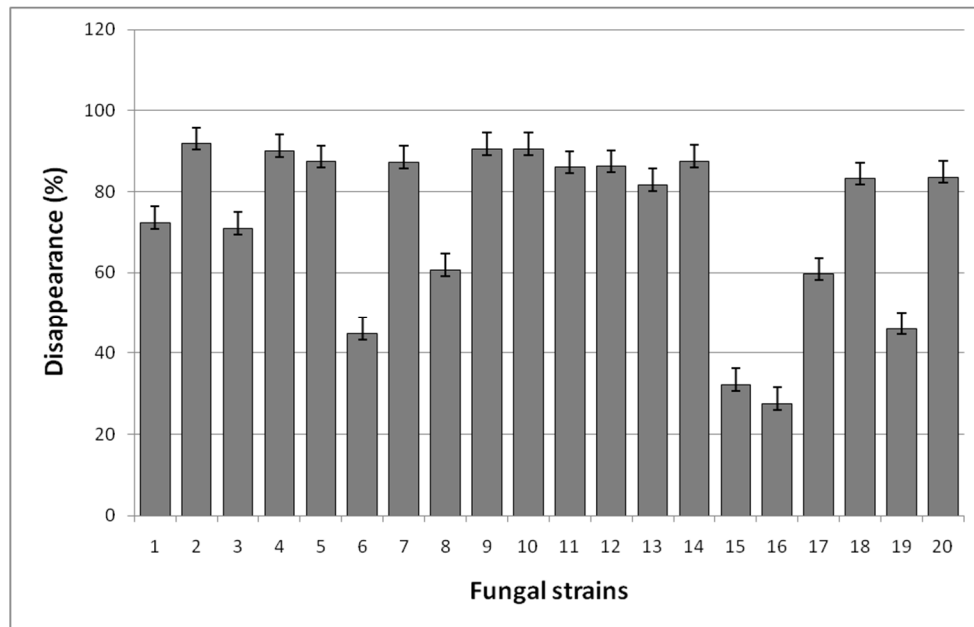
As seen from Figure 2, the ligninolytic fungi (*P. ostreatus* strains, *Trametes* species, *B. adusta*, *L. betulina*, and *Str. rugosoannulata*) and ascomycetes (*F. oxysporum* and *C. herbarum*) actively decolorized anthraquinone-type dyes. This property can be indirect evidence of production of ligninolytic enzymes (laccase and Mn-peroxidase) by these fungi under studied conditions.

### 3.1.3. Oil Disappearance

Creosote and oil are complex pollutants that cause serious environmental problems [11,12,15,57]. The use of the ligninolytic fungi for the remediation of the soils contaminated by such mixtures has been discussed [10,11]. We studied the ability of the fungi to metabolize oil to assess their mycoremediation potential. Most wood-inhabiting basidiomycetes actively utilized oil; the disappearance was 72 to 92% during 14 days of cultivation (Figure 3A). The exceptions were *B. adusta*, *S. murashkinskyi*, and two members of the genus *Trametes* (*T. ochracea* and *T. gibbosa*). Litter-decomposing *S. rugosoannulata* removed up to 90% of oil. The studied ascomycetes remove oil actively, too. The disappearance varied from 60 to 84% with the exception of *G. candidum* (the decrease did not

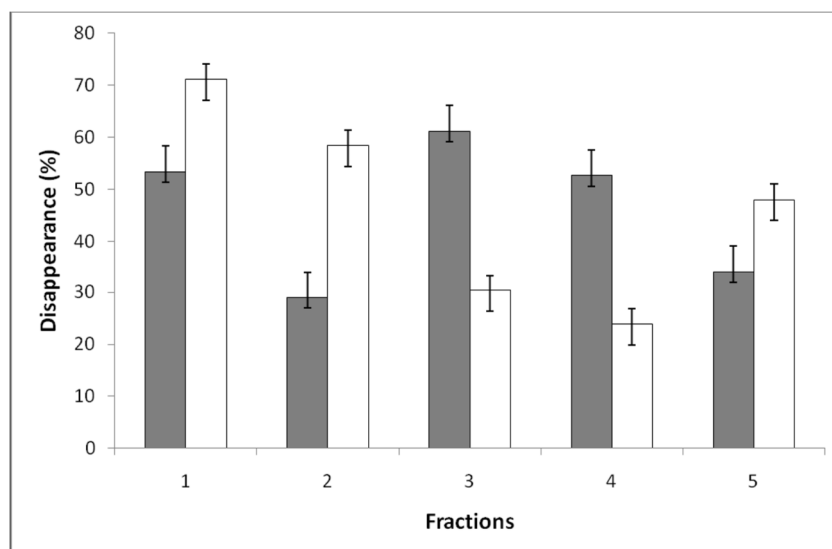
exceed 46%). Weak activity of different strains of *G. candidum* towards oil hydrocarbons has been shown before [58].

**A**



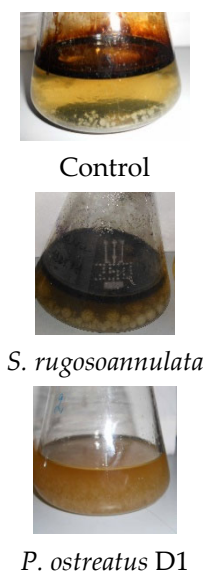
1—*P. ostreatus* f. Florida, 2—*P. ostreatus* MUT2977, 3—*P. ostreatus* 336, 4—*P. ostreatus* D1, 5—*P. ostreatus* LE-BIN0432, 6—*B. adusta*, 7—*Len. betulina*, 8—*St. murashkinskyi*, 9—*Sch. commune*, 10—*Str. rugosoannulata*, 11—*T. versicolor* DSM11372, 12—*T. versicolor* MUT3403, 13—*T. hirsuta*, 14—*T. maxima*, 15—*T. ochracea*, 16—*T. gibbosa*, 17—*Lec. aphano-cladii*, 18—*F. oxysporum*, 19—*G. candidum*, 20—*C. herbarum*.

**B**



1—alkanes, 2—naphthenes, 3—low-molecular weight aromatic hydrocarbons, 4—high-molecular weight aromatic hydrocarbons, 5—tars.

**C**



**Figure 3.** Oil disappearance by studied fungi (A), disappearance of fractions of oil degradation during growth of *P. ostreatus* D1 (■) and *Str. rugosoannulata* (□) in basidiomycetes-rich medium (B), and the fungal growth (C).

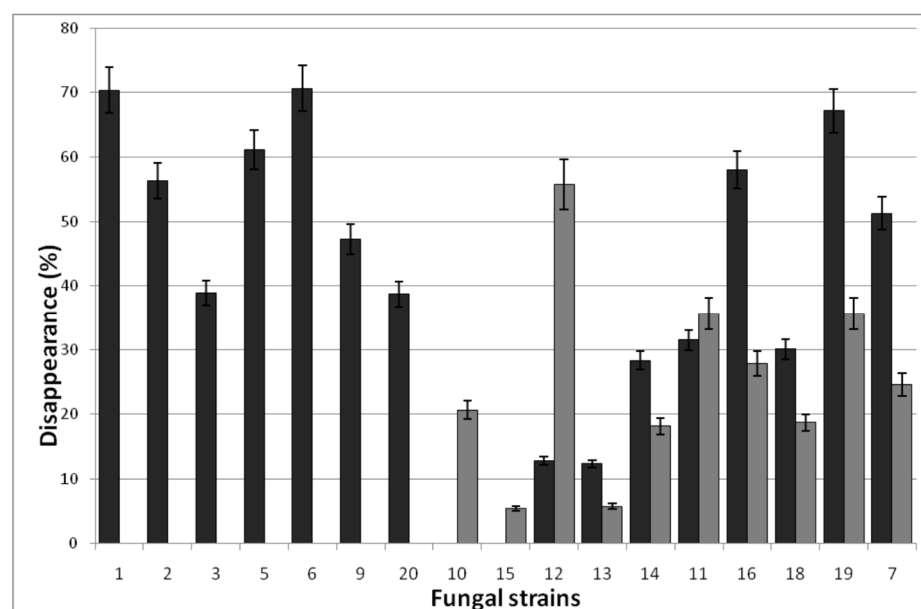
An increase of biomass of the fungi was observed in all treatments; the mycelium grew in the form of pellets of various sizes. Figure 3C shows the growth of the fungi *P. ostreatus* D1 and *S. rugosoannulata* in the basidiomycetes-rich environment with oil after 14 days.

The study of changes of fraction composition of oil revealed some differences among the fungi used. Wood-inhabiting basidiomycete *P. ostreatus* D1 utilized all fractions of oil with the exception of naphthenes. Their disappearance varied from 52 to 66% (Figure 3B). Whereas soil-inhabiting *S. rugosoannulata* more actively degrades alkanes and naphthenes, fractions of low- and high-molecular-weight aromatic hydrocarbons were less accessible to this fungus (Figure 3B). This can result from the low activities of ligninolytic enzymes produced by *S. rugosoannulata* in comparison with *P. ostreatus*.

### 3.1.4. Surfactant Degradation

The use of different surfactants can stimulate desorption of hydrophobic pollutants from soil and increase their bioavailability [16,17]. Often Tween-40, Tween-80, and Triton X-100 are used. For example, PAH degradation in polluted soil by *P. ostreatus* can be increased with Tween-40, Tween-80, and Triton X-100 [59,60]. Tweens do not affect the growth of fungi and are catabolized by them [61]. Significant range of the surfactants (such as oxyethylated alkylphenols) often more effectively increases the solubility of hydrophobic compounds. However, some surfactants themselves are very difficult to degrade, resulting in the formation of stable and toxic end products and their accumulation in the environment [18,19]. The possibility of practical use of these compounds in bioremediation processes can be limited by their resistance to degradation. In addition, a result of their degradation can be the formation of stable and toxic alkylphenols as final products. Bacterial degradation of this class of compounds has been studied quite well, but the information on their degradation by fungi is limited [62].

We used neonol AF9-12 as an example of oxyethylated alkylphenols. Although it was not toxic for all the fungi studied, only 17 fungi metabolized this compound, and the degree of degradation and the site of the attack of its molecule are different (Figure 4).



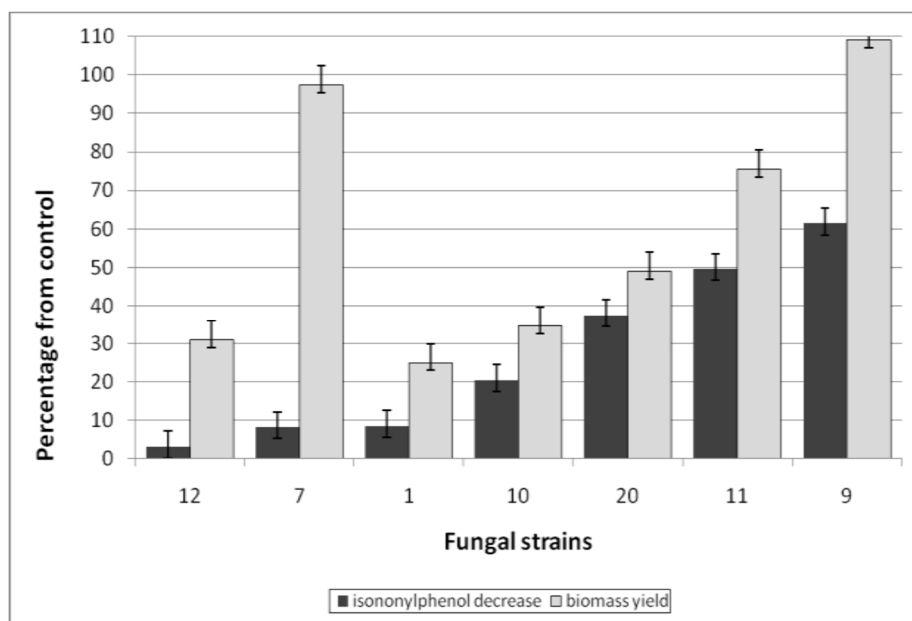
**Figure 4.** The disappearance of neonol AF9-12 by studied fungi: 1—*P. ostreatus* f. Florida, 2—*P. ostreatus* MUT2977, 3—*P. ostreatus* 336, 5—*P. ostreatus* LE-BIN0432, 6—*B. adusta*, 7—*Len. betulina*, 9—*Sch. commune*, 10—*Str. rugosoannulata*, 11—*T. versicolor* DSM11372, 12—*T. versicolor* MUT3403, 13—*T. hirsuta*, 14—*T. maxima*, 15—*T. ochracea*, 16—*T. gibbosa*, 18—*F. oxysporum*, 19—*G. candidum*, 20—*C. herbarum*; ■—oxyethyl chain, ■—aromatic ring.

The use of spectrophotometric methods [28,29] revealed that basidiomycetes *P. ostreatus* f. Florida, *P. ostreatus* MUT, *P. ostreatus* 336, *P. ostreatus* LE-BINB, *B. adusta*, *S. commune*, and ascomycete *C. herbarum* attacked the oxyethyl chain, while the aromatic ring remained intact. *S. rugosoannulata* and *T. ochracea* degraded the aromatic ring.

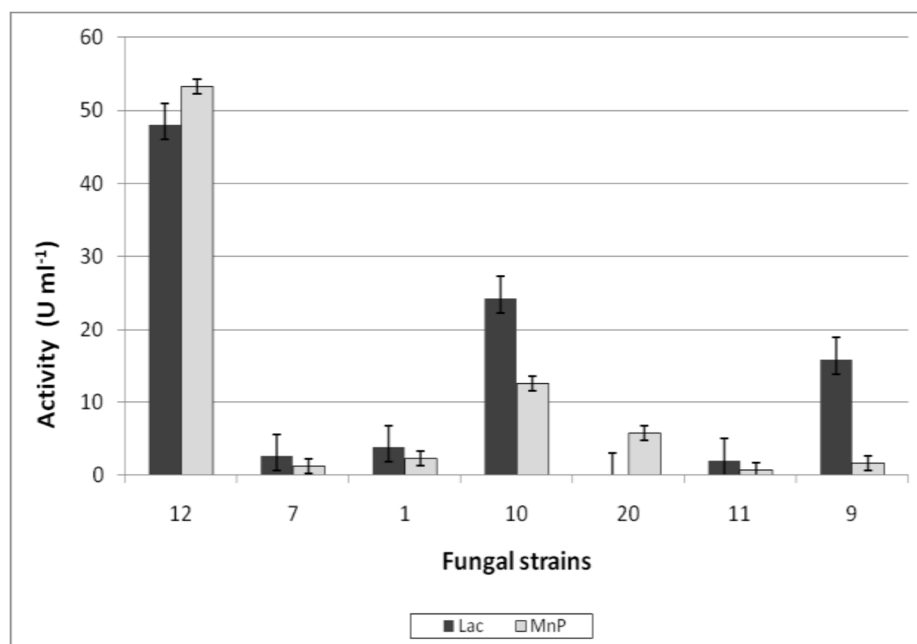
Finally, another nine species of the fungi, including basidiomycetes *L. betulina*, *T. versicolor* DSM, *T. versicolor* MUT, *T. hirsuta*, *T. maxima*, and *T. gibbosa*, and ascomycetes *L. aphanocladii*, *F. oxysporum*, and *G. candidum*, destroyed both parts of the neonol molecule (Figure 4).

The cleavage of the oxyethyl side chain leads to the formation of the alkylphenols. In our work we used isononylphenol as model of alkylphenols to study the activity of fungi. Isononylphenol decreased the growth of the most studied fungi, as evidenced by the significant decrease of the amount of biomass compared to the control treatment (Figure 5A). The exceptions were fungi *S. murashkinskyi*, *T. hirsuta*, *T. ochracea*, *F. oxysporum*, and *G. candidum*. The amounts of the mycelium of these fungi in the control treatments and in the presence of isononylphenol were approximately the same. An increase (by 1.5 times) of biomass of *F. oxysporum* in the presence of this pollutant was found.

A



B



**Figure 5.** The isononylphenol disappearance and biomass yield (A) and ligninolytic enzymes production (B) during growth of studied fungi at the presence of isononylphenol: 1—*P. ostreatus* f. Florida, 7—*L. betulina*, 9—*S. commune*, 10—*S. rugosoannulata*, 11—*T. versicolor* DSM11372, 12—*T. versicolor* MUT3403, 20—*C. herbarum*.

Among 20 studied fungi, only six species of basidiomycetes (*T. versicolor* MUT, *L. betulina*, *P. ostreatus* f. Florida, *S. rugosoannulata*, *S. commune*) and one ascomycete (*C. herbarum*) could degrade isononylphenol. The most active degrader of the isononylphenol was *S. commune*; the disappearance was 61% after 14 days of cultivation at the start concentration of 200 mg/L (Figure 5A).

Infrared spectroscopy is widely used to study the fine structural changes in molecules of various chemical compounds and materials. This method is widely used in studying the ways of degradation of various alkylphenols [63–65]. We used fungi to study changes in surfactant structure during fungal degradation: *P. ostreatus* Florida (attacked the oxyethyl chain), *S. rugosoannulata* (degraded the aromatic ring), *T. versicolor* DSM (both parts of the neonol molecule), and *S. commune* (the most active degrader of isononylphenol).

In the IR spectra of samples, the degradation of neonol *P. ostreatus* Florida (Figure S1) mutually altered the intensity of the absorption bands in the 2958 and 2926 cm<sup>-1</sup> region, corresponding to the antisymmetric and symmetric CH vibrations of the alkyl chain, and 2874 and 2858 cm<sup>-1</sup>, corresponding to antisymmetric and symmetric CH aromatic rings. It indicates an increase in the fraction of absorption of the latter. This fact is also supported by an increase in the intensity of deformation bands at 1453 cm<sup>-1</sup> and skeletal vibrations at 1252 cm<sup>-1</sup> alkyl fragments. Some authors associate an increase in absorption at 1252 cm<sup>-1</sup> with the formation of aromatic esters during the degradation of alkylphenols. In addition, there is a significant increase in band absorption at 1402 cm<sup>-1</sup>, which is associated with the deformation vibrations of the OH group, and the increase in intensity usually indirectly indicates the formation of hydrogen bonds, which indicates the appearance in the medium of polar components originating from the original neon by shortening the hydroxyethyl fragment.

The notable changes in the IR spectra of samples obtained after neonol degradation by *S. rugosoannulata* (Figure S2) were revealed. The wide absorption band of OH groups

at 3365  $\text{cm}^{-1}$  shows a shift of about 80  $\text{cm}^{-1}$  toward higher frequencies. It indicates the degradation of some hydrogen bonds and a certain increase of the proportion of hydrophobic components. The absorption bands of antisymmetric and symmetric vibrations of alkyl fragments at 2974 and 2927  $\text{cm}^{-1}$ , respectively, become more pronounced compared to the spectra of control treatment. Two bands in the region of symmetric and antisymmetric vibrations of C–H in aromatic rings degenerate into one, broadened with a maximum at 2883  $\text{cm}^{-1}$  after degradation. It is also characteristic of stretching vibrations of C–H-saturated hydrocarbons. The intense absorption at 881  $\text{cm}^{-1}$ , which is characteristic of 1,4-disubstituted benzenes, completely disappears, while the intensity of the stretching vibrations of the C–OH end groups of polyoxyethyl substituents at 1049 and 1089  $\text{cm}^{-1}$  decreases significantly. In addition, there is a pronounced absorption at 1115  $\text{cm}^{-1}$ , characteristic of acetals. These data can show the destruction of aromatic rings during the degradation of neonol.

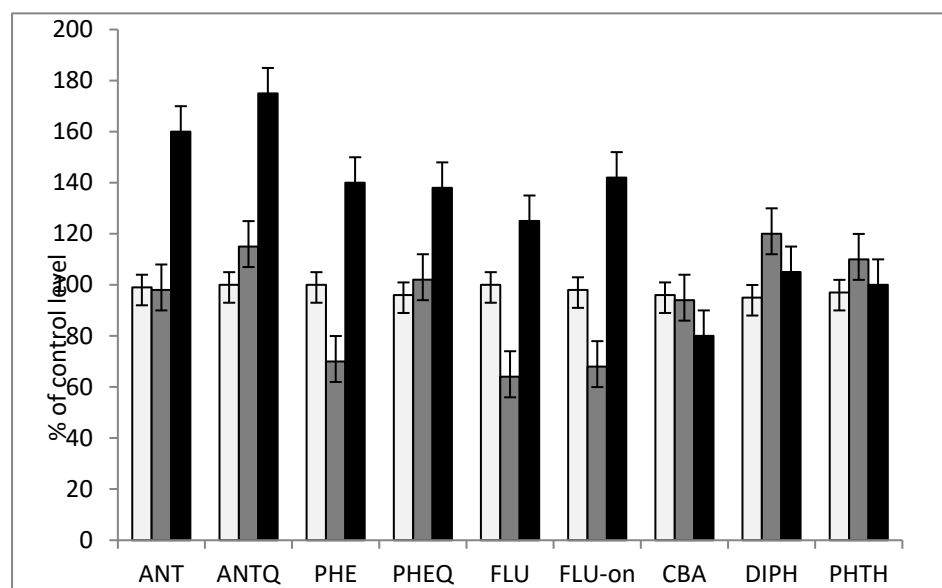
In the IR spectra of samples after the degradation of neonol *T. versicolor* DSM (Figure S3), the changes analogous to those described above are observed. Thus, the absorption bands of antisymmetric and symmetric vibrations of alkyl fragments at 2957 and 2925  $\text{cm}^{-1}$ , respectively, as well as the corresponding bands of antisymmetric and symmetric vibrations of CH of aromatic rings at 2874 and 2860  $\text{cm}^{-1}$ , become less intense compared to the control spectra. There is also a decrease in the intensity of the bands attributable to the vibrations of the C–O–C groups of the hydroxyethyl fragment at 1037 and 1106  $\text{cm}^{-1}$ , as well as the absorption bands characteristic of the deformation vibrations of the CH groups of alkyl chains. The appearance of an absorption band at 1294  $\text{cm}^{-1}$ , which is associated with dimers of carboxylic acids, is noteworthy. The appearance of carboxylic acids can be explained by oxidative degradation of aromatic rings of alkylphenols. Thus, based on the spectral data, it is possible to draw a conclusion about the most probable destruction of some of the neonol molecules affecting both aromatic rings and polyoxyethyl fragments.

Isononylphenol is a product of incomplete degradation of oxyethylated alkylphenol. Since the structure of isononylphenol does not include polyoxyethyl substituents and has free phenolic hydroxyl, IR spectra of the isononylphenol and neonol differ significantly due to the manifestation of the isononyl substituent bands. There is an intense absorption in the isononylphenol spectrum in the form of a somewhat broadened peak at 1456  $\text{cm}^{-1}$ , which refers to the deformation vibrations of methylene units, and intensive absorption at 1379  $\text{cm}^{-1}$ , related to the vibrations of the isopropyl fragment. The vibrations of the aromatic ring are more vivid and intensive at 1596 and 1513  $\text{cm}^{-1}$ . Analysis of IR spectra of samples after degradation of isononylphenol by *S. commune* (Figure S4) reveals significant changes. The intensity of the bands characteristic for deformation vibrations of alkyl fragments at 1379–1456  $\text{cm}^{-1}$ , as well as vibrations of aromatic rings close to 1500, 1600  $\text{cm}^{-1}$ , is significantly reduced. The intensive absorption band at 830  $\text{cm}^{-1}$ , which is attributed to the vibrations of disubstituted benzene rings, completely disappears. Simultaneously, there is a significant decrease in the intensity of antisymmetric and symmetric vibrations of both aliphatic and aromatic CH fragments in the region of 2960–2853  $\text{cm}^{-1}$ . All this indicates the destruction, to a large extent, of the structure of both aromatic rings and alkyl substituents of isononylphenol. Oxidative destruction of aromatic rings of isononylphenol is confirmed by the appearance of weak absorption bands at 1727–1739  $\text{cm}^{-1}$ , characteristic of the carbonyl groups of the carboxyl fragment.

Subsequent use of GC revealed three metabolites of neonol degradation by the fungus *T. versicolor* DSM with retention times of 5.50 to 5.76 min. Metabolite with retention time of 5.73 min was detected after degradation of isononylphenol by *S. commune*. The presence of products with similar retention times suggests that the degradation of neonol *T. versicolor* DSM and isononylphenol *S. commune* at a certain stage can pass along a similar path.

### 3.2. The Study of the Phytotoxicity of Parent Compounds and Metabolites Revealed

Phytotesting as a fast toxicological examination of the media is widely used now. The main parameters taken into account are usually germinating capacity and germinating power, average length of the root, average length of the shoot, development of root hairs, and root orientation. The most informative characteristics are root development (length) and germinating power. In our experiments the effects of the parent PAHs and its fungal metabolites on sorghum germination, length of shoots, and length of roots were checked (Figure 6). Control levels represent the corresponding values (germination of seeds, length of shoots, length of roots) in control treatments (without pollutants).



**Figure 6.** Effect of PAHs and their fungal metabolites on germination of seeds (□), length of shoots (■), and length of roots (■) of sorghum: ANT—anthracene, ANTQ—9,10-anthraquinone, PHE—phenanthrene, PHEQ—phenanthrene-9,10-quinone, FLU—fluorene, FLU-on—9-fluorenone, CBA—2-carboxybenzaldehyde, DIPH—2,2'-diphenic acid, PHTH—phthalic acid.

Phytotests showed that the effects of PAHs and their derivatives agree with aqueous solubility of the tested compounds. PAHs, as poorly soluble compounds, were less phytotoxic than their oxidized derivatives. Oxidized PAH metabolites presented by quinones, ketones, and acids are more soluble in water than PAH; therefore, their phytotoxicity can be greater. Being more hydrophilic and electrophilic, these compounds are more capable of forming covalent bonds with numerous cell structures [66,67] and producing a pronounced toxic effect on plants. In nature, oxidized derivatives of PAH can arise rather intensely under the effect of physicochemical factors [68] and as a result of microbial degradation of hydrocarbons [69,70]. Greater toxicity of contaminated soil may depend exactly on more toxic microbial metabolites arising as a result of oil decomposition [71]. At the same time, the toxic effect produced by oxidized metabolites of PAH and possible fate of these compounds in soil biocenoses should be taken into account. It should be noted that oxidized derivatives of PAH are not only more easily available to soil microflora [72] but also are capable of impairing enzyme systems in plants [73].

### 3.3. Study of Adaptive Properties of Fungi

Three different aspects were evaluated in our experiments: (1) the ligninolytic enzymes, which play a key role in the degradation of aromatic compounds by ligninolytic fungi; (2) emulsifying compounds production, which can increase solubility of hydrophobic compounds and promote the catalytic action of ligninolytic enzymes; (3) extracellular polysaccharides production, which can be depot for ligninolytic enzymes.



### 3.3.1. Ligninolytic Enzymes Production

As mentioned above, the key stages of the degradation of aromatic pollutants, including PAHs, can catalyze ligninolytic enzymes. We have shown that the degradation of PAHs by the studied basidiomycetes was accompanied by the production of ligninolytic enzymes (Table 2). All representatives of the genera *Pleurotus*, *Trametes*, as well as *L. betulina*, *S. murashkinskyi*, and *S. commune*, produced laccases and Mn-peroxidases. Lignin peroxidase and Mn-peroxidase activities were revealed in *B. adusta*, whereas *S. rugosoannulata* had only laccase. It was first found that the PAH degradation by ascomycetes *L. aphanocladii*, *F. oxysporum*, and *C. herbarum* was accompanied by the production of peroxidases. In *G. candidum*, the activities of these enzymes were not revealed. Perhaps the low degradative activity of this fungus towards PAHs is the result of this.

**Table 2.** Activities of extracellular enzymes (U/mL) after 14 days of the cultivation of the studied fungi both with and without pollutants.

Fungal Strains	Activity, U/mL								
	Control (without Pollutant)	Pollutants							
		ANT	PHEN	FLU	Neonol	INP	AB62	RB4	Oil
<i>B. adusta</i> : LiP	0	42.3	9.5	37.2	49.9	94.4	0	0	46.8
MnP	0	3.2	3.4	21.5	318.2	135.0	8.0	15.0	58.3
<i>C. herbarum</i> : PerOx	0	12.3	10.4	12.2	13.1	5.8	4.7	2.0	11.2
<i>F. oxysporum</i> : PerOx	0	0.9	3.8	0.5	7.4	0.2	82.4	18.1	4.8
<i>G. candidum</i> : PerOx	0	0	0	0	0	0	0	0	0
<i>L. aphanocladii</i> : PerOx	0	12.4	7.6	12.2	7.8	8.0	8.0	3.8	6.2
<i>L. betulina</i> : Lac	4.8	5.3	1.0	2.4	3.1	2.9	2.2	1.0	15.4
MnP	3.7	21.2	22.5	21.4	7.5	1.3	12.6	4.8	14.4
<i>P. ostreatus</i> Florida: Lac	49.9	7.3	46.2	16.9	336.4	3.8	18.8	8.4	86.4
MnP	242.5	38.4	78.8	14.2	741.0	2.3	5.1	5.0	136.8
<i>P. ostreatus</i> MUT2977:									
Lac	40.1	24.9	288.5	158.2	33.3	1.4	23.4	3.6	24.0
MnP	392.6	118.5	494.9	291.7	167.0	4.6	5.4	1.8	44.0
<i>P. ostreatus</i> 336: Lac									
Lac	98.9	33.2	3.0	0.6	3.5	1.1	73.6	7.1	56.7
MnP	145.6	25.9	7.1	12.2	11.5	1.2	5.1	3.6	217.9
<i>P. ostreatus</i> D1: Lac									
Lac	49.1	30.1	40.1	18.0	52.3	6.8	55.2	7.2	29.5
MnP	403.0	112.1	456.9	355.8	283.0	17.5	68.8	10.0	139.4
<i>P. ostreatus</i> LE-BIN0432:									
Lac	64.0	16.9	2.7	1.5	34.0	3.0	88.0	5.8	18.4
MnP	450.1	57.0	26.2	24.0	8.2	3.0	5.1	2.7	2.5
<i>S. commune</i> : Lac									
Lac	26.8	11.1	13.2	15.9	15.4	5.0	8.8	7.9	14.2
MnP	86.3	3.0	27.5	3.4	1.6	0.4	20.8	12.3	1.0
LiP						12.5			
<i>S. murashkinskyi</i> : Lac									
Lac	10.1	13.6	44.0	18.0	215.1	31.8	12.2	15.6	24.0
MnP	12.8	14.2	16.6	22.3	249.7	35.7	18.3	18.8	29.1
<i>S. rugosoannulata</i> : Lac									
Lac	2.1	5.1	4.8	2.7	34.7	11.7	15.5	13.2	8.7
MnP	7.2	1.3	2.5	1.0	104.0	43.7	17.3	14.7	23.6
LiP					58.0				
<i>T. gibbosa</i> : Lac									
Lac	23.3	26.4	26.5	24.8	39.0	3.1	20.0	22.3	99.5
MnP	48.4	61.5	70.7	41.2	7.5	0.6	14.9	9.6	17.5
<i>T. hirsuta</i> : Lac									
Lac	32.2	18.5	13.7	26.3	174.8	9.8	39.0	4.0	98.5
MnP	56.6	49.0	110.8	199.3	202.0	14.6	7.5	0.6	17.7
<i>T. maxima</i> : Lac									
Lac	15.5	13.7	3.1	6.6	308.9	26.6	13.9	4.2	12.2

MnP	16.1	14.4	6.5	31.5	72.3	6.9	42.5	14.2	18.6
<i>T. ochracea</i> : Lac	9.3	7.2	7.1	6.0	394.1	31.3	9.4	10.5	4.8
MnP	50.1	166.3	48.3	32.2	50.4	6.3	5.5	2.8	3.2
<i>T. versicolor</i> DSM11372:									
Lac	3.4	126.0	141.6	73.7	1.9	2.0	35.5	4.0	8.2
MnP	24.1	9.6	15.3	10.4	17.5	0.8	17.5	1.2	18.3
LiP					16.6				23.7
<i>T. versicolor</i> MUT3403:									
Lac	76.7	103.4	17.6	164.0	32.0	48.0	32.1	48.1	49.5
MnP	47.9	128.5	64.1	86.4	149.5	53.2	14.9	5.3	13.2

ANT—anthracene; PHEN—phenanthrene; FLU—fluorene; neonol—neonol AF9-12; INP—isononylphenol; AB62—Acid Blue 62; RB4—Reactive Blue 4; Lac—laccase; MnP—Mn-peroxidase; LiP—lignin peroxidase.

A study of the production of ligninolytic enzymes during the decolorization of dyes showed that all basidiomycetes produced laccases and Mn-peroxidases, whereas ascomycetes showed only peroxidase activity (Table 2). We have previously shown that both investigated dyes are available for both ligninolytic peroxidases and laccases [30]. Apparently, the activity of only peroxidase (in the case of ascomycetes) is not enough for complete decolorization of these dyes. The decolorization activity of *G. candidum* suggested the production of peroxidase. This enzyme oxidized 2,6-dimethoxyphenol and anthraquinone-type dyes [74].

To study the effect of oil on the production of ligninolytic enzymes, we used the basidiomycetes-rich environment, which supports the production laccase and peroxidases. As in the case of other pollutants, the process of the oil degradation by basidiomycetes was accompanied by the production of ligninolytic enzymes. All representatives of the species *P. ostreatus*, *L. betulina* produced laccase and Mn-peroxidase, which is a characteristic property of these fungi. The presence of lignin peroxidase was shown in all representatives of the genus *Trametes* and in the litter-decomposing fungus *S. rugosoannulata* (Table 2). The activity of this enzyme was absent in the control treatments (without pollutant) and in the presence of PAHs. Perhaps oil can contain any compounds which can act as inducers of this enzyme. Oil degradation by three ascomycetes, as well as the degradation of the other studied pollutants, was accompanied by the production of the single ligninolytic enzyme, peroxidase. The activity of ligninolytic enzymes in *G. candidum* was not revealed.

The degradation of neonol AF9-12 was accompanied by the production of laccases and Mn-peroxidases by basidiomycetes and only peroxidases in ascomycetes (Table 2). Many authors discuss the Mn-peroxidases, laccases, and cyt P450 as the enzymes which can be involved in alkylphenols degradation. Commonly, ligninolytic fungi can polymerize the alkylphenols or degrade the primary structure of these compounds [75]. The degradation of isononylphenol was accompanied by the production of laccase and Mn-peroxidase in all the mentioned basidiomycetes and peroxidase in ascomycete *C. herbarum* (Table 2, Figure 5B). In addition, *T. versicolor* MUT, as a response to the presence of isononylphenol in the cultivation medium, produced lignin peroxidase, in contrast to the control treatment (without pollutant).

Different studies have shown that extracellular ligninolytic enzymes of these fungi are responsible for the initial oxidation of different pollutants, including PAHs. Ligninolytic fungi were found to polymerize alkylphenols as a result of their initial attack by extracellular lignin-modifying enzymes, and to degrade the primary structure of these compounds [14,24,37,38]. In our experiments, the study of the activity of the main enzymes of the ligninolytic complex in the cultivation medium with or without pollutants revealed the production of laccase, Mn-peroxidase, and lignin peroxidase. Differences in the activity and composition of the ligninolytic complexes of individual species were

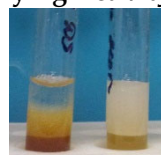
noted: *P. ostreatus* strains, *T. versicolor* strains, and *S. commune* produced laccase and Mn-peroxidase; *S. rugosoannulata* produced only laccase under the experimental conditions, and *B. adusta* produced lignin peroxidase and Mn-peroxidase. Finally, the main active producers of ligninolytic enzymes were fungi belonging to *Pleurotus*. The degradation of pollutants was accompanied by the production of laccases and Mn-peroxidases in basidiomycetes, whereas the ascomycetes produced only peroxidases. Three from studied basidiomycetes (*Sch. commune*, *Str. rugosoannulata*, and *T. versicolor*) can produce a third ligninolytic enzyme—lignin peroxidase—as response to the presence of neonol AF9-12 or isononylphenol. Thus, among the studied fungi, laccase and Mn-peroxidase are the most common ligninolytic enzymes. Earlier, we showed that the presence of these enzymes is a necessary condition for the complete degradation of PAH [76–78]. The production of ligninolytic laccases and peroxidases in the presence of pollutants can be seen as one of the mechanisms of adaptation of fungi.

### 3.3.2. Emulsifying Compounds Production

One of the strategies of adaptation of microorganisms to contamination by hydrophobic compounds is the production of emulsifying compounds (biosurfactants), which increase the availability of the pollutant for its utilization. Now, separate reports on the production of biosurfactants by ligninolytic fungi are available. We found that the process of degradation of hydrophobic compounds (PAHs, isononylphenol, and oil) by most of the studied fungi was accompanied by the formation of foam, which may serve as an indirect sign of the production of substances with emulsifying activity. For all the studied fungi, we determined the emulsifying activity of the culture liquid after 24 and 48 h of cultivation (Table 3).

**Table 3.** Emulsifying activities of cultivation medium after growth of studied fungi in the presence of hydrophobic pollutants ( $E_{48}$ , %).

Fungi	Emulsifying Activity ( $E_{48}$ , %)				
	ANT	PHEN	FLU	INP	oil
<i>B. adusta</i> MUT 3398	21.8 ± 3.4	28.2 ± 3.2	34.0 ± 3.1	30.4 ± 13.0	7.6 ± 1.3
<i>C. herbarum</i> MUT 3238	34.0 ± 5.5	54.6 ± 13.	24.2 ± 1.3	13.2 ± 3.3	32.3 ± 3.6
<i>F. oxysporum</i> IBPPM 543	47.1 ± 2.3	0 ± 0	0 ± 0	0 ± 0	27.0 ± 3.4
<i>G. candidum</i> MUT4803	0 ± 0	34.3 ± 5.7	0 ± 0	54.2 ± 7.7	6.5 ± 3.8
<i>Lec. aphanocladii</i> IBPPM 542	19.3 ± 5.0	12.5 ± 3.9	6.2 ± 2.8	0 ± 0	18.2 ± 3.7
<i>Len. betulina</i> LE-BIN 2047	69.8 ± 12.3	21.9 ± 1.3	38.2 ± 4.9	47.5 ± 8.4	60 ± 5.5
<i>P. ostreatus</i> f. Florida IBPPM 540	65.7 ± 6.9	38.1 ± 3.4	0.7 ± 0.2	26.7 ± 3.6	30.1 ± 6.8
<i>P. ostreatus</i> MUT 2977	3.0 ± 5.2	0 ± 0	5.0 ± 8.7	12.3 ± 4.9	23.2 ± 3.0
<i>P. ostreatus</i> 336	21.6 ± 3.7	21.1 ± 2.5	22.4 ± 4.3	18.6 ± 2.9	27.1 ± 3.3
<i>P. ostreatus</i> D1	3.5 ± 1.0	2.0 ± 0.4	2.5 ± 0.2	26.1 ± 1.8	38.2 ± 5.6
<i>P. ostreatus</i> LE-BIN 0432	21.3 ± 3.0	27.7 ± 2.5	32.4 ± 1.3	28.1 ± 1.9	37.1 ± 3.8
<i>Sch. commune</i> IBPPM 541	12.4 ± 3.4	3.3 ± 0.3	23.3 ± 1.4	28.4 ± 1.9	29.1 ± 2.3
<i>St. murashkinskyi</i> LE-BIN 1963	42.0 ± 1.0	27.0 ± 3.4	38.7 ± 4.2	19.6 ± 5.1	21.4 ± 4.1
<i>Str. rugosoannulata</i> DSM11372	41.8 ± 1.9	38.7 ± 11.9	26.9 ± 3.8	9.8 ± 3.8	44.3 ± 12.7
<i>T. gibbosa</i> LE-BIN 1911	17.8 ± 6.7	6.3 ± 3.8	2.0 ± 3.4	0 ± 0	52.3 ± 12.1
<i>T. hirsuta</i> LE-BIN 072	34.2 ± 1.6	59.4 ± 4.4	21.2 ± 1.1	60.7 ± 0.6	35.2 ± 3.6
<i>T. maxima</i> LE-BIN 0275	38.1 ± 2.3	49.3 ± 5.0	35.8 ± 1.9	40.1 ± 0.9	42.2 ± 1.8
<i>T. ochracea</i> LE-BIN 093	22.4 ± 1.5	6.3 ± 2.8	0.4 ± 0.4	0 ± 0	52.6 ± 9.3



<i>T. versicolor</i> DSM11269	12.0 ± 1.9	9.2 ± 2.2	15.4 ± 7.5	39.4 ± 2.3	21.7 ± 2.8
<i>T. versicolor</i> MUT 3403	1.4 ± 2.5	11.6 ± 2.3	2.6 ± 2.3	19.5 ± 3.8	29.7 ± 3.6

ANT—anthracene, PHEN—phenanthrene, FLU—fluorene; INP—isononylphenol.

In the control treatments (without pollutants), only a trace formation of foam was found; the  $E_{24}$  value does not exceed 1%, and  $E_{48}$  was about 0%. It was found that all studied fungi produced biosurfactants as a response to the presence of hydrophobic pollutants in the culture medium. The emulsifying activity increased from 2 to 70 times. It is shown that the production of emulsifying compounds depends not only on the nature of the pollutant, but also on the strain and species used. For example, from the five studied strains, the highest emulsifying activity was detected in *P. ostreatus* f. Florida, while the lowest was in *P. ostreatus* MUT. Among the representatives of the genus *Trametes*, the most active surfactant producer was *T. hirsuta*, and the least active was *T. gibbosa*.

Now, separate reports on the production of biosurfactants by ligninolytic fungi are available. For example, the production of a biosurfactant that increases the solubility of hydrophobic compounds has been found in *Coriolus versicolor*, *Pleurotus ostreatus* [22,23,79], *Phellinus* sp., and *Polyporus sulphureus* [23], and this biosurfactant was a multicomponent substance consisting of a protein portion and a polysaccharide [22].

The formation of emulsifiers in response to the presence of pollutants in the medium was also found in ascomycetes, among which *L. aphanocladii* and *C. herbarum* were the most active. Although the production of biosurfactants by ascomycetes is well-known [80], that as a response to the presence of hydrophobic compounds by fungi of genera *Fusarium*, *Cladosporium*, and *Lecanicillium* has not been described previously.

Finally, in our study, the production of emulsifying compounds as a response to the presence in the cultivation medium of hydrophobic pollutants was the common property of wood- and soil-inhabiting basidiomycetes and ascomycetes. Perhaps during the degradation of these substrates, fungus produces substances with emulsifying activity that can make them more accessible. In some cases, the presence of emulsifying activity is associated with the production of EPS, or with “nonpolysaccharide” fungal products, such as glycoproteins [81].

### 3.3.3. Extracellular Polysaccharides Production

The third strategy of adaptation toward the pollutant contamination of the environment can be extracellular polymeric substances such as exopolysaccharides (EPS) production. EPS are produced by both bacteria and fungi. EPS are compounds consisting of a large number of monosaccharides linked glycosidically and can be homo- or heteropolymers of monosaccharide residues and can be linear or branched [82]. For a long time, it has been known that laccases and other lignin-modifying enzymes are embedded within EPS sheets around the hyphae of wood-rotting basidiomycetes, and such structures have been proposed to be involved in recognition mechanisms in fungal cell–wood surface interactions and to play specific roles in the biodeterioration of wood [83]. However, direct effects of fungal EPS on the mode of action of lignin-modifying enzymes have not yet been described in the literature.

In our experiments, EPS concentrations varied from 0.15 to 1.5 µg/mL for all studied fungi. *G. candidum*, which produced about 3.3 µg/mL of EPS in control treatment (without pollutants), was one exclusion. The decrease in EPS concentration in the presence of all studied pollutants was found for all studied fungi. Its concentration decreased significantly, and in some cases was nearly zero. Simultaneous decrease in EPS concentration and increase of emulsifying activity of cultivation medium in the presence of pollutants should be noted. Perhaps any emulsifying compounds, which fungi produce as a response to the presence of pollutants, can decrease the surface tension of the cultivation medium. This can result in disruptions of the production/synthesis EPS or affect their migration from cell surface to environment. This phenomenon will be studied further.

#### 4. Conclusions

Finally, the high degradative activities of the studied fungi toward hazardous pollutants were shown. Table 4 summarizes the properties which we found in the studied fungi. PAHs, oil, and anthraquinone dyes were the most accessible for fungal degradation. The studied surfactants were available for a limited number of fungi. The presence of activities of ligninolytic enzymes correlates with the ability of fungi to degrade pollutants. In addition, most of the studied fungi were able to produce emulsifying compounds, which, apparently, allows them to adapt to the conditions of pollution with hydrophobic pollutants.

**Table 4.** The main properties of studied fungi.

Fungi	Properties						Emulsifying Compounds
	Degradation			INP	Oil	Lac/MnP/LiP	
	PAHs	Dyes	Neonol				
<i>B. adusta</i> MUT 3398	+	+	+				+
<i>C. herbarum</i> MUT 3238	+	+			+	+	+
<i>F. oxysporum</i> IBPPM 543		+			+		
<i>G. candidum</i> MUT4803			+				
<i>Lec. aphanocladii</i> IBPPM 542	+				+	+	
<i>Len. betulina</i> LE-BIN 2047		+	+		+		+
<i>P. ostreatus</i> f. Florida IBPPM 540	+	+	+		+	+	+
<i>P. ostreatus</i> MUT2977	+	+			+	+	
<i>P. ostreatus</i> 336	+	+			+		+
<i>P. ostreatus</i> D1	+	+			+	+	
<i>P. ostreatus</i> LE-BIN0432	+	+			+		+
<i>Sch. commune</i> IBPPM 541	+			+	+	+	+
<i>St. murashkinskyi</i> LE-BIN 1963	+				+		+
<i>Str. rugosoannulata</i> DSM11372	+	+			+	+	+
<i>T. gibbosa</i> LE-BIN 1911		+	+				
<i>T. hirsuta</i> LE-BIN 072		+			+		+
<i>T. maxima</i> LE-BIN 0275	+	+			+		+
<i>T. ochracea</i> LE-BIN 093		+					
<i>T. versicolor</i> DSM11372	+	+		+	+	+	+
<i>T. versicolor</i> MUT3403	+	+	+		+	+	

“+” — the presence of property.

The revealed differences depended on the pollutant, the ecological and taxonomic accessory of the fungus, and, in some cases, the fungal strain. The results suggest that fungi with potential development for mycoremediation should be assessed for the following properties: the degradation of the wide range of pollutants without accumulation of the toxic metabolites; the production of emulsifying compounds; the production of ligninolytic enzymes (at least laccase and Mn-peroxidase), which can a necessary condition for the complete degradation of PAH. On the basis of the screening data, such as the degradation of wide range of pollutants without metabolites accumulation, active production of ligninolytic enzymes, and emulsifying compounds, the follow fungi can be chosen as promising for the development of mycoremediation technologies (Table 4): wood-inhabiting basidiomycetes *Pleurotus ostreatus* f. Florida, *Schizophyllum commune*, *Trametes versicolor* MUT 3403, and *Trametes versicolor* DSM11372; the litter-decomposing basidiomycete *Stropharia rugosoannulata*, and the ascomycete *Cladosporium herbarum*.

The most active degraders of this compound include the representatives of the genus *Pleurotus*, *B. adusta*, *T. versicolor* MUT3403, *T. gibbosa*, and ascomycete *G. candidum*. *P.*

*ostreatus* f. Florida and *B. adusta* more actively removed the oxyethyl chain, and *T. versicolor* MUT3403 more actively degraded the aromatic ring. Perhaps the fungi, which actively destroyed both parts of the neonol molecule, can be used for more perspective for following studies and practical use.

**Supplementary Materials:** The following supporting information can be downloaded at: [www.mdpi.com/article/10.3390/app12042164/s1](http://www.mdpi.com/article/10.3390/app12042164/s1), Table S1: List of fungi screened; Figure S1: IR-spectra neonol AF9-12: control treatment (blue line) and after *P. ostreatus* Florida degradation (red line); Figure S2: IR-spectra neonol AF9-12: control treatment (blue line) and after *S. rugosoannulata* degradation (red line); Figure S3: IR-spectra neonol AF9-12: control treatment (blue line) and after *T. versicolor* DSM degradation (red line); Figure S4: IR-spectra Isononylphenol: control treatment (green line) and after *Sch. commune* degradation (1 week – blue and 2 weeks – red lines)

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**Conflicts of Interest:** The authors declare that they have no conflicts of interest.

**Ethical Approval:** This article does not contain any studies with human participants or animals performed by any of the authors.

## Abbreviations

PAHs	polycyclic aromatic hydrocarbons
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt
DMP	2,6-dimethoxyphenol

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