

Spent oyster mushroom substrate performs better than many mushroom mycelia in removing the biocide pentachlorophenol

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Chlorophenols have been commonly used as disinfectants and preservatives but their recalcitrant nature, persistence and toxicities make them priority pollutants for treatment. The ability of various fungi (*Armillaria gallica*, *A. mellea*, *Ganoderma lucidum*, *Lentinula edodes*, *Phanerochaete chrysosporium*, *Pleurotus pulmonarius*, a *Polyporus* sp., *Coprinus cinereus* and *Volvariella volvacea*), and the spent mushroom substrate of *P. pulmonarius* (SMS) to remove pentachlorophenol (PCP) was compared using a batch cultivation system. The PCP content was monitored by reversed phase HPLC, and the breakdown products were determined by GC-MS. Possession of ligninolytic ability was determined by ability to decolourize the dye Poly-R478 at two N levels. Not all the fungi tested decolourized the dye, and for those that did, not all showed N-modulation response on dye decolourization. All these fungi showed active breakdown in addition to biosorption as their PCP removal mechanisms. The tolerance level of the fungus towards PCP did not correlate with its degradative capacity, nor to its ability to decolourize Poly-R478. The *A. mellea* strain showed the highest degradative capacity (13 mg PCP g⁻¹ mycelium; d.w.) while the *Polyporus* possessed the greatest biosorption capacity (31 mg PCP g⁻¹ mycelium, d.w.). In comparison, *Pleurotus* SMS harbouring both bacteria and fungi functioned over a wide range of initial PCP concentrations and reached a higher degradative capacity (19 mg PCP g⁻¹) in only 3 d. GC-MS chromatograms revealed only residual PCP peaks in SMS extracts, a contrast with the mycelial incubations in which a variety of breakdown products were detectable. Use of SMS for bioremediation of biocide-contaminated sites seems promising.

Pentachlorophenol (PCP) has been the most heavily used pesticide throughout the world. It has been used in large quantities; for example, in the United States during the 1980s, approximately 23 million kg y⁻¹ was used, mainly as a wood preservative (Hattemer-Frey & Travis, 1989). Since the 1960s large amounts of PCP (approx. 5.5 million kg y⁻¹) have been sprayed over vast areas of central China as a molluscicide to control schistosomiasis (Schechter *et al.*, 1996), and this study aims at identifying a cost-effective bioremediation programme to counter widespread PCP contamination.

Pentachlorophenol uncouples oxidative phosphorylation by making cell membranes permeable to protons, resulting in dissipation of transmembrane proton gradients and consequential electrical potentials (McAllister, Lee & Trevors, 1996). PCP has been classified as a group B2 teratogen, probable human carcinogen, extremely toxic to humans with a reference dose of 0.03 mg kg⁻¹ d⁻¹ (see web site: <http://www.epa.gov>) and is highly embryotoxic (Beynon *et al.*, 1981). Use of PCP has been banned or restricted in North America, Europe and some Asian countries, and is on the European and U.S. lists of priority pollutants for remediation treatment.

Widespread use, toxicity to all living organisms (Apajalahti & Salkinoja-Salonen, 1984), long persistence (in terms of

years; Hattemer-Frey & Travis, 1989) cause serious and common problems in soil contamination with this pesticide (Beynon *et al.*, 1981). Direct application (and accidental spillage) followed by leaching, cause water and air pollution as well (Beynon *et al.*, 1981). Reasons for slow or lack of biodegradation in the natural environment (Head, 1998) may include: an insufficient number of PCP degrading organisms in the ecosystem (Miethling & Karlson, 1996), their inhibition by toxic concentrations of PCP and/or its breakdown products (Alleman, Logan & Gilbertson, 1992; Kahru *et al.*, 1996; Jardim, Moraes & Takiyama, 1997), unfavourable conditions in contaminated sites for production of degradative enzymes (Valo, Apajalahti & Salkinoja-Salonen, 1985; McCarthy, Claude & Copley, 1997) and reduced availability of PCP for chemical transformation after binding to the soil matrix, but providing a sink effect (Dec & Bollag, 1990; Ruttimann-Johnson & Lamar, 1997).

The conventional strategy for remediating PCP-contaminated soils involves excavation and incineration or landfilling. Such methods are expensive, obviously destructive to the environment and ineffective for anything other than 'point source pollution'. Bioremediation is a technological process whereby biological systems are manipulated to effect environmental clean-up (Head, 1998). Micro-organisms are

the main decomposers in natural ecosystems and possess a diverse panel of enzymes to degrade many complex organic compounds, and are widely employed in bioremediation programmes in consequence (Reddy, 1995; McAllister *et al.*, 1996; Wunch, Feibelman & Bennett, 1997). Many studies of PCP degradation by bacteria and fungi have been done, especially with *Sphingomonas* and the white rot fungus *Phanerochaete chrysosporium* (Kang & Stevens, 1994; Reddy, 1995; Miethling & Karlson, 1996; Leung *et al.*, 1997; Head, 1998), and degradation pathways have been established (McAllister *et al.*, 1996; McCarthy *et al.*, 1997; Hofrichter *et al.*, 1998). As well as pure cultures, mixed and complex systems have been examined for removal of PCP (Laine & Jørgensen, 1996; Miethling & Karlson, 1996; Ederer *et al.*, 1997; Jaspers *et al.*, 1997; Leung *et al.*, 1997).

In many countries, the spent mushroom substrates (SMS) remaining after harvesting of mushroom crops are often discarded as wastes. Usage tends to have been limited to soil conditioning and fertilizing (Chong & Rinker, 1994; Gerrits, 1994; Landschoot & McNitt, 1994; Maher, 1994). Yet peroxidases, phenol oxidase and cellobiosidase have been recovered from mushroom composts (Wood & Goodenough, 1977; Mishra & Leatham, 1990; Ball & Jackson, 1995) and are of the sort known to degrade organopollutants (Okeke *et al.*, 1994b; Masaphy, Henis & Levanon, 1996; Ruttimann-Johnson & Lamar, 1997; Hofrichter *et al.*, 1998). Indeed, mushroom composts and their microorganisms have been demonstrated to breakdown phenolic pollutants including pesticides (Kuo & Regan, 1992, 1998; Okeke *et al.*, 1993, 1996; Regan, 1994).

Pleurotus pulmonarius (syn. *P. sajor-caju*) is commonly cultivated in subtropical and tropical developing countries, and its spent substrate is widely available. In this study we compare the efficiency of pentachlorophenol removal by the SMS of *P. pulmonarius* with the removal ability of a variety of fungi. *Phanerochaete chrysosporium* was included because a lot of work has been done with it. Other organisms were the soil fungi *Armillaria gallica* and *A. mellea* and *Ganoderma lucidum*, wood rot fungi *Lentinula edodes*, *Polyporus* sp., the ink-cap mushroom *Coprinus cinereus* (a common weed in mushroom cultivations) and the straw mushroom *Volvariella volvacea*. Disappearance of PCP is a simple assay to reflect removal but the breakdown intermediates might be more toxic (Makinen *et al.*, 1993; Ho & Bolton, 1998). The quality of the transformation products was therefore analysed by gas chromatography-mass spectrometry (GC-MS) (Okeke *et al.*, 1993, 1994b; Aiken & Logan, 1996; Laine & Jørgensen, 1996; Miethling & Karlson, 1996; Semple & Cain, 1996; Gremaud & Turesky, 1997; McCarthy *et al.*, 1997). As far as we are aware, the abilities of *Armillaria* spp., *Ganoderma*, *P. pulmonarius* and *Volvariella* to break down PCP have not previously been tested.

MATERIALS AND METHODS

Culture maintenance

Table 1 shows the list of fungi used. Cultures were maintained at 25 °C on mushroom complete medium (CM, Raper & Miles, 1958) consisting of (g l⁻¹): glucose (20), yeast extract

Table 1. List of fungi tested

	Strain	Source
<i>Armillaria gallica</i> Merxm. & Romagn.	1039, 1057, AC1	Michigan, U.S.A. and Toronto, Canada
<i>A. mellea</i> (Vahl: Fr.) P. Kumm.	M51	Germany
<i>Coprinus cinereus</i> (Schaeff.: Fr.) Gray	Meathop	Cumbria, U.K.
<i>Ganoderma lucidum</i> (Curtis: Fr.) P. Karst.	HK-1	Hong Kong
<i>Lentinula edodes</i> (Berk.) Pegler	L54*, L65, L67, L68	Fujian Province, China
<i>L. edodes</i>	HL2-17, HL8-1, HL10-6, HL11-1, HL11-2, HL11-4, HLE, HLF	Hubei Province, China
<i>Phanerochaete</i> <i>chrysosporium</i> Burds.	M1	Singapore
<i>Pleurotus pulmonarius</i> (Fr.) Quél.	PL-27*	India
<i>Polyporus</i> sp.	Cv-1	Hong Kong
<i>Volvariella volvacea</i> (Bull.: Fr.) Singer	V34*	Thailand

* Cultivated strain. Others are field isolates.

(2), KH₂PO₄ (0.46), K₂HPO₄ (1), MgSO₄ · 5H₂O (0.5) and agar (15).

Preparation of spent oyster mushroom substrate (SMS)

The production compost for mushrooms of *Pleurotus pulmonarius* was prefermented for 2 d and consisted of paddy straw, wheat bran and lime mixed in 44:5:1 ratio by weight, and then combined with 1.5 vol. of water (Chiu *et al.*, 1998). A fully grown CM plate culture was used as an inoculum. The solid compost material (SMS) left behind after the harvest of two flushes of mushroom crops was collected, air-dried, and manually chopped into pieces 3 cm long. This *Pleurotus* SMS was stored at room temperature in a sealed plastic bag which was then placed in a dry and dark container containing silica gel. Table 2 lists the physical and chemical characteristics of this spent oyster mushroom compost (Ching, 1997).

Tolerance test

Inocula (3 mm diam.) were cut with a cork borer from fully-grown CM cultures. Single inocula were transferred into the wells of six-well plates (Costar no. 3506, Cambridge, MA). Each well was supplemented with 0, 5, 10, 15, 20 or 25 mg PCP l⁻¹ in a basal medium (BM) composed of (g l⁻¹): asparagine (2); glucose (20), MgSO₄ · 5H₂O (0.5), KH₂PO₄ (0.46), KH₂PO₄ (1) and agar (15). All the tests were incubated at 25 °C in darkness until the mycelia in the control grew and reached the edge of the well (35 mm diam.). Three replicates were performed for each strain.

Dye decolourization test

Poly-R478 (Sigma P-1900), a lignin model compound, was added at 0.02% (w/w) to BM which contained 0.1, 1 or 2 g asparagine l⁻¹ (1.3, 13 and 26 mM, respectively). A 90 mm

Table 2. Physical and chemical characteristics of the spent oyster mushroom compost used

pH	7–8
Ash content (%)	72.92 ± 0.09
Salinity, (% by weight)	0.1
Electrical conductivity, μs	830 ± 1
Carbon (%)	23.6 ± 0.9
Hydrogen (%)	4.06 ± 0.23
Nitrogen (%)	5.99 ± 3.29
Mg (mg g^{-1})	7.72 ± 2.22
Ca (mg g^{-1})	30.13 ± 5.83
Na (mg g^{-1})	1.32 ± 0.28
K (mg g^{-1})	4.47 ± 0.58
Mn (mg g^{-1})	2.20 ± 0.45
Ni (mg g^{-1})	Nil
Zn ($\mu\text{g g}^{-1}$)	8.4 ± 8.3
Fe (mg g^{-1})	2.34 ± 0.64
Cr (mg g^{-1})	Nil
Cd (mg g^{-1})	Nil
Orthophosphate (mg g^{-1})	0.652 ± 0.031

Extract of 1 gm substrate in 100 ml ultra pure water. Except for pH, data are presented as mean ± S.D. of three replicates.

diam. Petri plate was used. Otherwise, inoculation and incubation followed the procedure detailed for the tolerance test above. A decolorized zone appeared when the fungus degraded the dye (Glenn & Gold, 1983; Andersson & Henrysson, 1996; Wunch *et al.*, 1997). Daily measurement of the colony and decolorized zone (if any), in three replicates, was performed for each strain.

Removal of PCP

Batch system. A fungus was grown to its log phase in liquid CM at 26° with shaking at 160 rpm until sufficient biomass was obtained. Samples of mycelium (1 g f.w. but blotted dry with six sheets of sterilized filter paper) or 0.25 g of spent mushroom compost (5–7 pieces of the 3 cm SMS) was added aseptically to 48.75 ml of sterile minimal medium (glucose (10 g l⁻¹), (NH₄)₂SO₄ (0.48 g l⁻¹), KH₂PO₄ (0.2 g l⁻¹), MgSO₄·7H₂O (0.05 g l⁻¹), thiamine HCl (0.1 mg l⁻¹)). The suspension was then incubated as above. After incubation for one day, 1.25 ml of PCP (Sigma P-9441) stock (1 mg ml⁻¹ methanol) was added (final concentration = 25 mg l⁻¹), and incubation was resumed (Alleman *et al.*, 1992; Lievreumont *et al.*, 1996). For the screening test, a fixed period of 7 d incubation was used for comparison. Five replicates were performed with each strain or batch of SMS.

Extraction of PCP. At the end of the experiment, SMS or fungal biomass was separated from the incubation medium by filtration using a nickel sieve (pore size 1 mm diam.). Microbial mass, if still present in the filtrate, was harvested by centrifugation, and the resultant pellet was pooled with the residue from the filtration. Filtrate and residue were extracted for PCP separately (Okeke *et al.*, 1993, 1996; Liang & McFarland, 1994; Aiken & Logan, 1996; Laine & Jørgensen, 1996). The residue biomass was first oven-dried at 70° and weighed. For extraction, 10 ml of hexane was added, and the

suspension mixed vigorously for 2 h. The extraction was repeated once. The two hexane extracts were pooled, and the solvent was removed by rotary evaporation at 50°. The residue was then re-dissolved in 1 ml of HPLC grade absolute methanol and stored in darkness at 4° until further analysis. In parallel, mycelia and SMS grown in normal broth without PCP were extracted and served as reference. All samples were filtered through 0.45 μm filters (Acrodisc syringe filters 4CR PTFE) before analysis. For these and all other procedures the chemicals and solvents used were of the highest purity available.

Quantification of PCP

A series of PCP standards (HPLC grade, Sigma P-1045) was prepared from 0.1 to 1 mg ml⁻¹ and used to construct a standard curve for quantification. Reverse-phase HPLC was used to quantify PCP with a SymmetryTM c18 (5 μm particle) column (4.6 mm by 250 mm) (Okeke *et al.*, 1993). Isocratic elution was made by acetonitrile:water:acetic acid (75:24:875:0.125; by volume) at 1 ml min⁻¹ using an HPLC system (Aiken & Logan, 1996) which comprised a liquid chromatography pumping system (Waters 600), automatic sampler (Waters 717 Plus), a variable wavelength photodiode array detector (Waters 996) and an integrator (Waters model 600). The effluent was monitored at 215 nm (the maximum absorption wavelength determined by the HPLC).

Removal is expressed in the following two terms:

$$\text{Removal efficiency (\%)} = \frac{\text{amount of PCP removed}}{\text{amount of PCP added}} \times 100\%$$

$$\text{Removal capacity (mg PCP g}^{-1}\text{; d.w.)} = \frac{\text{the amount of PCP removed}}{\text{mass harvested}}$$

Removal is mediated by the following mechanisms: biosorption removal refers to the amount of PCP bound in and to the harvested mass; i.e. it includes adsorption and internalization of PCP. Biodegradation removal is calculated by subtracting the total residual PCP measured (PCP in filtrate + PCP bound by the mycelial mass) from the initial PCP added. Thus:

$$\text{Removal capacity by biosorption (mg PCP g}^{-1}\text{)} = \frac{\text{amount of PCP bound by the cell mass}}{\text{biomass}}$$

$$\text{Removal capacity by biodegradation (mg PCP g}^{-1}\text{)} = \frac{\left[\text{initial PCP} \right] - \left[\text{PCP in filtrate} \right] - \left[\text{PCP bound by cell wall mass} \right]}{\text{biomass}}$$

Analysis of transformation products by GC-MS

One μl of sample was injected in splitless mode using an HP7673 automatic injector into a Hewlett Packard 6890 series gas chromatograph (Atlanta, GA) connected to a model 5973 mass selective detector at 62.5 kN m⁻². A HP-5 MS column of 5% phenyl methyl silicone (30 m × 0.25 mm, 0.25 μm film thickness) was used. Carrier gas was helium with a constant

flow rate of 28 cm s⁻¹ and the total flow was 45 ml min⁻¹. The oven program was set at 80° for 1 min to 200° at 25° min⁻¹, resting for 20 min. Injection port temperature was set at 200°. The mass selective detector was operated at -70 eV (Okeke *et al.*, 1994b; Laine & Jørgensen, 1996; Miethling & Karlson, 1996; Gremaud & Turesky, 1997; McCarthy *et al.*, 1997). The resolved GC peaks were identified and matched with the fragmentation profiles in the Wiley spectral library.

Statistical validation

The data obtained were treated with one-way ANOVA, and if any significant difference was observed among the treatment groups, further ranking of the groups was performed with the Student–Keuls test. Significance of treatment effect was confirmed at 5% level of error.

RESULTS

PCP tolerance test

Table 3 summarizes the results. Most *L. edodes* strains, together with the Meathop strain of *C. cinereus*, formed the most sensitive group towards PCP. The *Armillaria* strains, *Pleurotus pulmonarius* and *Phanerochaete chrysosporium* were most tolerant. Further, when the inocula of *L. edodes* strains HL8-1, HL11-2 and HL11-4 from the plates with different PCP concentrations were transferred to normal medium without PCP, only the inocula of HL11-4 at 5 mg l⁻¹ resumed growth. Evidently, PCP was toxic to these cultures and showed a dose-dependent lethal effect. In liquid batch cultures incubated for 7 d, 25 mg PCP l⁻¹ caused reduction in growth of the biomass by 74% in *Volvariella volvacea* V34; 77% in *A. gallica* strain 1057; 30% in *A. mellea* strain M51; 28% in *Ganoderma lucidum* strain HK-1 and 17% in *P. pulmonarius* strain PL-27.

Dye decolourization test

Dye decolourization by *A. gallica* strains 1039 and 1057 and *P. chrysosporium* were induced at low N concentration (13 mM).

Table 3. Tolerance level towards PCP shown by the fungi tested

The highest PCP concentration tolerated (mg l ⁻¹)	Fungal strain
< 5	<i>Coprinus cinereus</i> <i>Lentinula edodes</i> L65, HL2-17, HL8-1, HL10-6, HL11-1, HL11-2, HL11-4, HLE, HLF
5	<i>Lentinula edodes</i> L54, L67 and L68
10	<i>Volvariella volvacea</i> <i>Ganoderma lucidum</i> <i>Polyporus</i> sp.
25	<i>Armillaria gallica</i> all strains <i>A. mellea</i> <i>Phanerochaete chrysosporium</i> <i>Pleurotus pulmonarius</i>

The plates were incubated at 25° in darkness for 2 wk. None of these strains could tolerate 100 mg l⁻¹ PCP in the plate test.

Table 4. Ability to decolourize plate-cultures incorporating the lignin-model substrate poly-R478 and the effect of nitrogen concentration

Fungal strain	Growth category	Decolourized zone produced		
		Asparagine concentration (mM)		Time taken for appearance (d)
		1.3	26	
<i>Armillaria gallica</i> AC1	Slow	Diffuse	None	25
<i>A. gallica</i> 1039 & 1057	Slow	Diffuse	None	> 33
<i>A. mellea</i>	Slow	Central	Central	20
<i>Coprinus cinereus</i>	Fast	None	None	—
<i>Ganoderma lucidum</i>	Fast	Diffuse	Diffuse	1
<i>Lentinula edodes</i> L65 and HL11-2	Medium	Central	None	13
<i>L. edodes</i> HLE, L54 and L67	Medium	Central	Central	> 4
<i>L. edodes</i> L68	Medium	None*	None*	—
<i>Phanerochaete chrysosporium</i>	Fast	Diffuse	None	1
<i>Pleurotus pulmonarius</i>	Fast	Central	Central	1
<i>Polyporus</i> sp.	Fast	None	None	—
<i>Volvariella volvacea</i>	Fast	None	None	—

The plates were incubated at 25° in darkness for 7 d to 1 mo, depending on the growth rate of the fungi tested. Growth rate categories: slow growers took 36–54 d to fully cover a 9 cm plate; medium growers, 19–28 d; and fast growers, 4–10 d. When produced, decolourized zones either originated and radiated from the central inoculum during incubation or were produced as scattered decolourized areas (= diffuse).
* Red crystals appeared in the agar medium.

Further reduction of N content to 1.3 mM triggered dye decolourization by *A. gallica* strain AC1 and *L. edodes* strains HL11-2 and L65 but not *V. volvacea*, *L. edodes* strain L68, *Polyporus* sp. and *C. cinereus* (Table 4). Unlike the others, strain L68 produced red precipitates on the plates. *Lentinula edodes* strains HLE, L54 and L67 were similar to *A. mellea*, *G. lucidum* and *P. pulmonarius* in showing a constitutive ability to decolourize the dye. Two patterns of decolourized zone were observed: a central decolourized zone and scattered decolourized areas. In all cases, all the decolourized zones were smaller than the diameters of the corresponding colonies, consistent with decolourization being a secondary metabolic activity of the older mycelium. *Ganoderma lucidum*, *P. chrysosporium* and *P. pulmonarius* were able to decolourize the dye in only 1 d after inoculation. There was no correlation between the extent of growth inhibition by PCP (referred to in the previous section) and the possession of ligninolytic enzymes as demonstrated by the dye decolourization test.

Removal of PCP by fungi

Pentachlorophenol samples were stable over a 22 d incubation period as determined by HPLC and GC-MS.

Removal efficiencies. In a 7 d incubation, the maximum total removal efficiency (75–78%) was obtained with mycelium of *G. lucidum* (Fig. 1), *P. chrysosporium* and *Polyporus* sp. Minimum removal occurred with two *A. gallica* strains (45–49%). In all cases, both biosorption to the fungal mass and degradation had taken place though the relative proportions of these

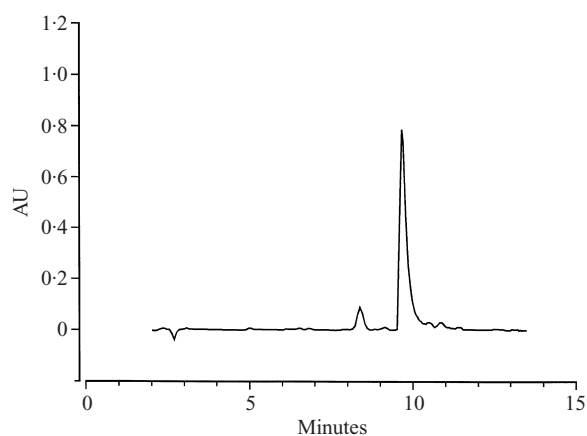


Fig. 1. HPLC chromatogram of the fungal mass extract of *Ganoderma lucidum*. The major peak is residual PCP.

Table 5. Removal efficiencies of PCP (% \pm s.d.) by various fungi tested in batch culture

	Strain	Biosorption	Degradation
<i>Armillaria gallica</i>	I039	16.1 \pm 1.7	29.2 \pm 5.6
	I057	24.7 \pm 0.5	23.2 \pm 3.3
<i>A. mellea</i>	M51	8.5 \pm 6.3	59.0 \pm 10.5
<i>Ganoderma lucidum</i>	HK-1	23.1 \pm 2.5	55.0 \pm 3.5
<i>Lentinula edodes</i>	L54	41.6 \pm 8.3	37.0 \pm 15.0
	L67	28.6 \pm 1.4	37.4 \pm 2.0
	L68	62.3 \pm 3.6	11.0 \pm 11.9
<i>Phanerochaete chrysosporium</i>	M1	36.4 \pm 5.7	47.4 \pm 4.7
<i>Pleurotus pulmonarius</i>	PL-27	15.3 \pm 7.4	49.6 \pm 10.8
<i>Polyporus</i> sp.	Cv-1	76.0 \pm 5.0	1.0 \pm 1.0
<i>Volvariella volvacea</i>	V34	36.5 \pm 3.5	29.0 \pm 4.6

Table 6. Absolute PCP biosorption and degradation capacities of fungal mycelia in batch cultures

	Strain	PCP removal capacity (mg g ⁻¹ d.w.; mean \pm s.d.)	
		Biosorption	Degradation
<i>Armillaria gallica</i>	I039	3.5 \pm 0.9	6.0 \pm 0.5
	I057	4.1 \pm 0.1	3.9 \pm 0.5
<i>A. mellea</i>	M51	2.1 \pm 1.6	13.4 \pm 2.1
<i>Ganoderma lucidum</i>	HK-1	3.9 \pm 0.4	9.3 \pm 0.6
<i>Lentinula edodes</i>	L54	9.1 \pm 4.7	4.1 \pm 3.8
	L67	3.4 \pm 0.1	4.4 \pm 0.3
	L68	14.9 \pm 4.1	3.8 \pm 3.8
<i>Phanerochaete chrysosporium</i>	M1	5.3 \pm 0.9	6.8 \pm 0.4
<i>Pleurotus pulmonarius</i>	PL-27	3.2 \pm 1.7	10.0 \pm 2.2
<i>Polyporus</i> sp.	Cv-1	31.1 \pm 3.4	1.5 \pm 1.6
<i>Volvariella volvacea</i>	V34	5.8 \pm 0.8	4.5 \pm 0.6

mechanisms for removal of PCP differed between fungi (Table 5).

Removal capacities. Table 6 shows removal capacities of the fungi tested. *Polyporus* sp. showed the highest biosorption capacity (31.1 \pm 3.4 mg PCP g⁻¹ biomass d.w.), but *A. mellea* possessed the highest degradative ability towards PCP

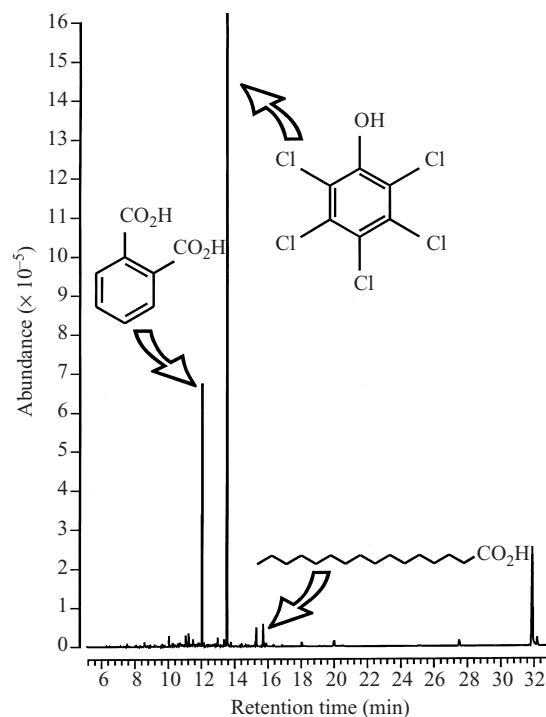


Fig. 2. GC-MS spectrum of the extract of the fungal biomass of *Pleurotus pulmonarius* after 2 d incubation in a medium containing 25 mg l⁻¹ PCP. The most prominent peak (retention time 13.53 min) is PCP, the next most prominent peak at 12.03 min is 1,2-dicarboxybenzoic acid. Trace amounts of hexadecanoic acid (15.73 min) and some of its derivatives (31.96 and 32.25 min) are also evident. The traces at 15.31 and 27.58 min remain unidentified.

(13.4 \pm 2.1 mg PCP g⁻¹ biomass d.w.), though this was closely followed by *P. pulmonarius* and *G. lucidum*.

GC-MS analyses revealed that in the PCP suspensions: (i) chloroanisoles as PCP breakdown intermediates were only detected in *L. edodes* strain L68, *Polyporus* sp., *C. cinereus* and *P. chrysosporium* (confirmed as 1-chloro-3-methoxy-benzene for the first three and pentachloro-methoxybenzene for *P. chrysosporium* by comparison with authentic commercial samples); (ii) other phenolic derivatives were also detected: accumulation of butylated hydroxytoluene or 3,5-di-tert-butyl phenol and 1,2-dicarboxybenzoic acid and its esters in most strains; appearance of: 2-methyl-1,3 benzenediol in strains HK-1, M51, I057 and HL11-2, benzenediol in V34, and 3,3-dimethyl-cyclohexanol in M51; (iii) the novel appearance of 1-chloro-octadecane with accumulation of hexadecanoic acid, octadecanoic acid [CH₃(CH₂)₁₅COOCH₃] and their methyl esters was detected. These fatty acids and their esters were only in trace amounts in comparison to the residual PCP peak except in the cases of strains *Pleurotus* (Fig. 2), *A. mellea* and *Ganoderma*; (iv) *L. edodes* HLE was unique in producing 1-butyl-, 1-octyl-benzene and 6-phenyl-dodecane, with trace amounts of other aromatic compounds.

Removal of PCP by spent mushroom compost

Kinetics. After the first 10 min incubation, 25% of the added PCP could be extracted from the solid mass of SMS indicating

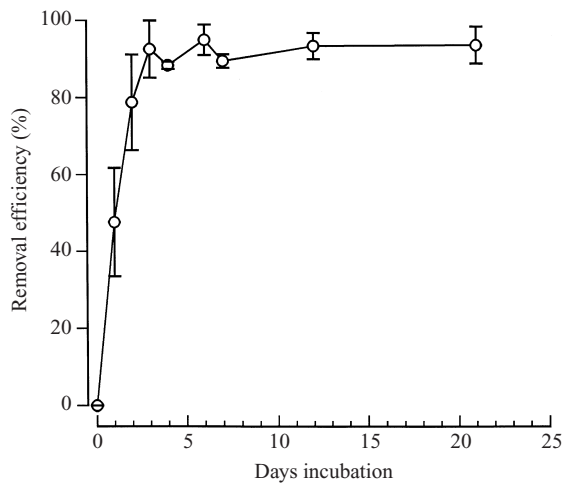


Fig. 3. Removal efficiency of PCP by spent oyster mushroom substrate.

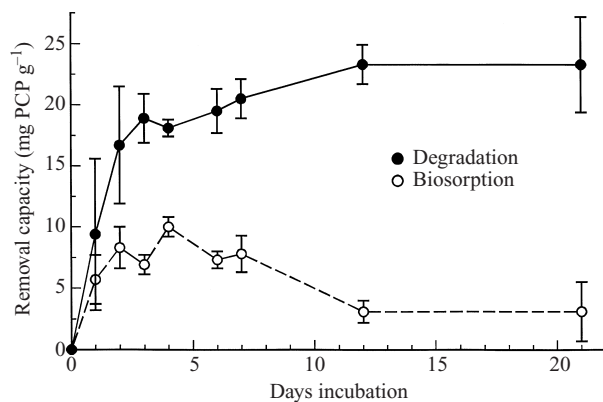


Fig. 4. Absolute removal capacity of PCP by spent oyster mushroom substrate in terms of biosorption and biodegradation.

Table 7. Effect of initial PCP concentration on PCP removal capacity of *Pleurotus* spent mushroom substrate after 7 d incubation

Initial PCP concentration (mg PCP added g ⁻¹ SMS)	Removal capacity (mg PCP removed g ⁻¹ SMS ± s.d.)	
	Biosorption	Degradation
4.7	0.7 ± 0.1	3.9 ± 0.1
23.7	6.5 ± 0.6	14.6 ± 0.8
28.2	7.3 ± 0.7	19.5 ± 1.8
31.7	7.9 ± 1.5	20.5 ± 1.6

rapid biosorption. The maximum removal efficiency by SMS ($95.1 \pm 4\%$) was attained at day 3 (Fig. 3) but then declined while the degradation capacity reached a high level by day 3 and continued to increase (Fig. 4). Continued decline in PCP content as incubation time increased (until < 10% of the PCP was left in the suspension) showed that PCP degradation was occurring alongside biosorption (Figs 3, 4).

Effect of initial PCP concentration. Both biosorption and degradation showed evidence of saturation kinetics (Table 7),

although biosorption capacity reached a plateau at the initial concentration of 24 mg PCP g^{-1} SMS whereas the maximum degradative capacity was reached at the higher initial doses tested ($28\text{--}32 \text{ mg PCP g}^{-1}$ SMS).

Biotransformation of PCP as determined by GC-MS. Both the HPLC chromatograms and GC-MS spectra for the solid mass extract and the filtrate extract of the SMS suspensions revealed only PCP peaks from two days incubation onwards.

DISCUSSION

PCP tolerance

Tolerance levels observed differed between tests carried out on agar plates and those done in broth. A plausible reason for this is that the actual dose delivered differed because of different extents of contact in the two cultivation methods (Alleman *et al.*, 1992). Most of the fungi tested here are known to produce exopolysaccharides which have been explored for medicinal use (Sanchez-Hernandez, Garcia-Mendoza & Novaes-Ledieu, 1993; Zhang, Zhang & Wang, 1995; Gutierrez, Prieto & Martinez, 1996). Water-insoluble polysaccharides have been observed in cultures of *Armillaria* spp. and *Pleurotus pulmonarius* in this study. These may form a diffusion barrier and binding matrix for PCP and so enable the fungi to show higher tolerance to the presence of PCP.

The tested wild isolates of *Lentinula edodes* are generally sensitive to PCP. Although this contrasts with results for the three cultivated strains examined by Okeke *et al.* (1994a), the strains tested here do differ in their abilities to decolourize poly R-478. Production of ligninolytic enzymes has been correlated with the decolourization of polymeric dyes such as poly R-478 (Glenn & Gold, 1983; Ollikka *et al.*, 1993). Yet decolourization can also be caused by biosorption (Beaudette *et al.*, 1998) as with *L. edodes* L68. A fungus without poly-R478 dye decolourizing ability could also tolerate PCP (compare Tables 3 and 4). Metabolism of chlorinated hydrocarbons is not unusual in fungi. Many white rot fungi and polypores, such as *Ganoderma lucidum* and *Polyporus* studied here, can synthesize, and some can release, large quantities of organic chlorides, including halomethanes, which are thought to be utilized as substrate cofactors for ligninolytic pathways (Benyon *et al.*, 1981; Oberg, Brunberg & Hjelm, 1997; Teunissen & Field, 1998; Watling & Harper, 1998). Significantly, though no ligninolytic enzymes were produced by the straw mushroom *Volvariella volvacea* (Table 4; Chang, 1992), biodegradation of PCP was observed (Tables 5, 6) which implies the involvement of other enzymatic systems for transforming PCP.

Enzyme systems for transformation of PCP

The bacterial biodegradation of PCP can occur by three processes: hydroxylation, oxygenolysis and reductive dechlorination (McAllister *et al.*, 1996) involving the following enzymes: PCP-4-monooxygenase, tetrachlorohydroquinone reductive dehalogenase (TCHQ dehalogenase) and

hydroxylase (McAllister *et al.*, 1996; Miethling & Karlson, 1996; Ederer *et al.*, 1997; Leung *et al.*, 1997; McCarthy *et al.*, 1997). For fungal systems, dechlorination, O-methylation and oxidation are involved in degrading PCP (Beynon *et al.*, 1981; McAllister *et al.*, 1996) with the following enzymes: phenol oxidases including horseradish-like peroxidase (Tatsumi, Wada & Ichikawa, 1996), lignin peroxidase (LiP, EC 1.11.1.14; Hammel & Tardone, 1988; Teunissen & Field, 1998), manganese peroxidase (MnP, EC 1.11.1.13; Hofrichter *et al.*, 1998) and laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2; Roy-Arcand & Archibald, 1991; Ricotta, Unz & Bollag, 1996). A variety of fungal ligninolytic systems have been described: (i) *Phanerochaete chrysosporium* and *A. mellea* possess all three enzymes (LiP, MnP and laccase) (Robene-Soustrade *et al.*, 1992; Pelaez *et al.*, 1995; Curir *et al.*, 1997; Ruttimann-Johnson & Lamar, 1997); (ii) *Lentinula edodes* and *P. pulmonarius* produce MnP and laccase (Okeke *et al.*, 1994a; Grabski *et al.*, 1995; Camarero *et al.*, 1996; Dannibale *et al.*, 1996; Martinez *et al.*, 1996; Munoz *et al.*, 1997); (iii) *Ganoderma lucidum* produces LiP and MnP (Pelaez, Martinez & Martinez, 1995; D'Souza, Boominathan & Reddy, 1996). Tolerance cannot be assured by particular enzyme activities, however, and must be confirmed experimentally. For example, although *C. cinereus* possesses a horseradish-like peroxidase (Morita *et al.*, 1988), it was killed at 5 mg PCP l⁻¹ (Table 3), and *L. edodes* strain HLE, which was a constitutive poly-R478 dye decolourizer (Table 4), was also killed at 5 mg PCP l⁻¹ (Table 3).

The appearance of novel products (e.g. 1-chloro-octadecane and chloroanisoles) in GC-MS chromatograms in comparison to those of control extracts confirm that all tested fungi degrade PCP, whilst HPLC quantification shows they do this to different extents (Tables 5 and 6). The GC-MS analyses also confirm that *P. chrysosporium* carries out O-methylation for transforming PCP before dechlorination and ring cleavage (Mileski *et al.*, 1988). All tested fungi carry out dechlorination. Similar O-methylation was only observed with *L. edodes* L68, *Polyporus* sp. and *C. cinereus*. Methylation and hydroxylation are commonly employed for transforming PCP with the enhanced production of fatty acids and dicarboxylic acids by most of the tested fungi, suggesting the possible involvement of a cytochrome P450 system (van den Brink *et al.*, 1998). Ring cleavage seems to be the limiting step in complete transformation of PCP. The PCP-tolerant and constitutive poly-R478 dye decolourizers, *A. mellea*, *P. pulmonarius* and *G. lucidum*, showed the higher degradation capacities (Table 6) with less residual PCP and more breakdown products (1,2-dicarboxybenzoic acid and derivatives, hexadecanoic acid and derivatives, etc.), presumably owing to their production of ligninolytic enzymes. The PCP-sensitive strains, *C. cinereus* Meathop, and *L. edodes* HLE and HL11-2 (Table 3), dechlorinated PCP too but were presumably killed before production of the relevant enzymes for further degradation. The poor PCP-degraders which were also incapable of poly-R478 dye decolourization (Table 4), *V. volvacea*, *L. edodes* L68 and *Polyporus* sp., just accumulated the dechlorinated phenolic derivatives.

Evidently, many fungi should be able to breakdown PCP. Among the candidate bioremediation organisms examined

here, *A. mellea* is the best PCP degrader (Table 6) and has the advantage of forming rhizomorphs to ramify through soil and solid wastes. Unfortunately, its role as a plant pathogen and slow grow rate (Table 4) would greatly limit its practical application. *Ganoderma lucidum* is a weak pathogen of plant roots and would be similarly restricted in usefulness. *Phanerochaete chrysosporium*, *A. gallica* strains, and some *L. edodes* strains showed nutrient modulated production of dye decolourization abilities (Table 4), presumably representing ligninolytic enzymes. Soils and sediments which can be carbon-limited environments for growth might limit the potential value of such strains for application in field conditions (Reddy, 1995). Although *P. chrysosporium* is a fast grower (Table 4), this study confirms that many fungi showed either higher removal of, or greater tolerance to, organic aromatic pollutants (Alleman *et al.*, 1992; Lamar & Evans, 1993; Okeke *et al.*, 1996; Wunch *et al.*, 1997; Beaudette *et al.*, 1998), so it is difficult to justify the singular popularity of this organism in such studies. In contrast, *P. pulmonarius* is an edible crop, is able to survive in soil and compete with the indigenous microbiota (In der Wiesche, Martens & Zadrazil, 1996) and has been the subject of studies attempting to utilize *Pleurotus* species to breakdown pesticides in the laboratory and field (In der Wiesche, Martens & Zadrazil, 1996; Masaphy *et al.*, 1996; Hofrichter *et al.*, 1998). Its complete degradation pathway for PCP, however, needs further elucidation. Further application would also require growth, survival and continuity, as well as readiness of enzyme production by such a bioremediating agent, to be established in a soil system (Lang, Eller & Zadrazil, 1997; Head, 1998).

PCP removal by biosorption

Fungal cultures and spent mushroom substrate both showed evidence of biosorption and degradation as removal mechanisms. In the SMS system, the absence of further increase in removal capacity with lengthened incubation may be caused by nutrient exhaustion, adverse change in the cultivation medium (e.g. accumulation of chloride ions) or immobilization of PCP by biosorption. The advantages of biosorption include the rapidity of biosorption kinetics (Faust & Aly, 1987) and the fact that a good adsorbent could concentrate PCP so it can be transported away from the contaminated site. This allows more cost-effective strategies for fast physical, chemical or enzymatic mineralization of concentrated pollutants to be used (Jardim *et al.*, 1997; Ho & Bolton, 1998). The experiments show that the adsorbing biomass might be killed by the toxicity of PCP or its metabolites. This possibility was evidenced by the failure to resume growth by the inocula of *L. edodes* from PCP-containing plate cultures, and the great retardation in growth in non-ligninolytic *V. volvacea* (Jardim *et al.*, 1997). Viability may not be important for biosorption, however, as it is presumably mostly dependent on the wall and extracellular matrix. Among the mycelia tested, *Polyporus* sp. was the best sorbent for adsorptive removal of PCP, possibly because of its unique compact hydrophobic colonial growth form (unpublished observation).

Advantages of using spent mushroom substrate in removal of PCP

Composts used for commercial cultivation of mushrooms harbour diverse microorganisms which might contribute to degradation of PCP. Spent sawdust compost from *L. edodes* cultivation and *Agaricus* SMS (which is comprised of casing soil and wheat straw-based compost) have been shown to degrade PCP in soil (Okeke *et al.*, 1993; Semple & Fermor, 1995). The *Lentinula* compost removed about 6 mg PCP g⁻¹ compost (Okeke *et al.*, 1993), whereas the SMS of oyster mushroom used in our studies removed 19 mg PCP g⁻¹ compost. This oyster mushroom SMS functioned at a wide range of PCP concentrations (Table 7) and required only 3 d to reach its high removal capacity (Fig. 3).

Oyster mushroom SMS offers the advantages of an integrated approach to PCP removal, expressed as rapid physical biosorption accompanied by degradation by a consortium of enzymes from diverse microorganisms (Kuo & Regan, 1992; Regan, 1994; Semple & Fermor, 1995). Bacterial components in this *Pleurotus* SMS identified so far are the Gram-positive *Cellulomonas cellulans* and the Gram-negative *Burkholderia gladioli* (syn. *Pseudomonas gladioli*) (Ching, 1997). *Trichoderma harzianum* was also isolated from SMS incubated in PCP (Ching, 1997). *Pleurotus* SMS also recommends itself as a potential bioremediating agent for PCP-contaminated soil as it is relatively nutrient rich (Table 2) and would encourage the growth of microorganisms and be free of problems of enzyme suppression by nutrient limitation and also provide manganese as cofactor for MnP (Masaphy *et al.*, 1996). Mycelium of *P. pulmonarius* is demonstrated here to degrade PCP itself (Tables 5, 6), and *Trichoderma* (Ching, 1997), a common weed fungus of mushroom compost (Grogan *et al.*, 1997), has been reported to methylate PCP to pentachloroanisole (Beynon *et al.*, 1981; McAllister *et al.*, 1996), while *Pseudomonas* and *Burkholderia* could break down chlorobenzene (Leung *et al.*, 1997; Hübner *et al.*, 1998). The material presumably harbours a range of microorganisms able to contribute to biosorption (Semple & Fermor, 1995) as well as enzymic biodegradation (Mishra & Leatham, 1990; Ball & Jackson, 1995; Reddy, 1995; McAllister *et al.*, 1996). As GC-MS failed to detect any chlorinated compounds other than PCP from day 2 onwards, PCP once degraded is presumably completely mineralized by oyster mushroom SMS.

Mushroom cultivation is a common practice all over the world and is a major income source in China and other developing countries which also suffer from serious pollution. In 1993, about 3 million tonnes of SMS were produced in 12 EU countries and about 0.6 million tonne in China (Luo, 1992; Gerrits, 1994). With the increasing trend of mushroom production, a conservative estimate is that over 5 million tonnes of SMS are generated as solid waste annually. This study identifies an alternative use for spent mushroom substrate for bioremediation of biocide-contaminated sites which is amenable to both industrial and smallholder-scale application. Commercial practicalities and performance under realistic conditions in field trials must be established. Further investigation is also needed to identify the complete biodegradative pathway as well as the micro-organisms involved.

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