

# Screening pentachlorophenol degradation ability by environmental fungal strains belonging to the phyla Ascomycota and Zygomycota

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**Abstract** Pentachlorophenol (PCP) bioremediation by the fungal strains amongst the cork-colonising community has not yet been analysed. In this paper, the co- and direct metabolism of PCP by each of the 17 fungal species selected from this community were studied. Using hierarchical data analysis, the isolates were ranked by their PCP bioremediation potential. Fifteen isolates were able to degrade PCP under co-metabolic conditions, and surprisingly *Chrysonilia sitophila*, *Trichoderma longibrachiatum*, *Mucor plumbeus*, *Penicillium janczewskii* and *P. glandicola* were able to directly metabolise PCP, leading to its complete depletion from media. PCP degradation intermediates are preliminarily discussed. Data emphasise the significance of these fungi to have an interesting potential to be used in PCP bioremediation processes.

**Keywords** Pentachlorophenol (PCP) · Bioremediation · Ascomycetes · Zygomycetes · Cork

## Abbreviations

PCP Pentachlorophenol  
HQ Hydroquinone  
CHQ Chlorohydroquinone  
TeCHQ Tetrachlorohydroquinone  
DCBQ 2,6-Dichloro-1,4-benzoquinone  
TeCBQ Tetrachloro-1,4-benzoquinone  
PCA Pentachloroanisole

## Introduction

Pentachlorophenol (PCP) was first produced in the 1930s as a wood preservative. Subsequently, it has been used worldwide as a herbicide, biocide and pesticide, resulting in significant contamination of terrestrial and aquatic ecosystems [13]. Due to health and environmental concerns, PCP use is now widely restricted, but it is still authorised, e.g., as a wood preservative. Importantly, man-made PCP has the potential for long-range atmospheric transport, representing an indirect source for the contamination of human foods and drinking-water (for revision see, e.g., [7, 15]). PCP is considered a priority hazardous substance and is believed to be an endocrine disruptor, reinforcing the concerns of the consequences of long-term exposure to humans and wildlife [7]. Recently, Taylor et al. [33] evaluated PCP for its ability to affect natural human killer cell function and demonstrated that 10 µM PCP for 1 h caused a progressive loss (>80%) of lytic function within 6 days of exposure.

Currently available approaches to pollution mitigation, such as incineration and storage, are expensive, inefficient and lead to additional environmental problems through the

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introduction of hazardous by-products. The most promising approach is bioremediation exploiting the catabolic capability of microorganisms. Numerous reports have shown that PCP is degraded in the environment by microorganisms (e.g., [25, 31, 35]), further emphasising the potential of bioremediation for its efficient elimination. Filamentous fungi are unique microorganisms, ensuring the degradation of the lignocellulose plant polymers: the most important degradation event in the carbon cycle of earth. There are about 72,000 named species, and new species are being added at the rate of about 700–1,500 each year [4].

PCP bioremediation by basidiomycetes and ascomycetes (and zygomycetes) fungi has not been fully assessed, as the latter are less studied. The best-studied basidiomycete, the white-rot fungus *Phanerochaete chrysosporium*, is known to mineralize several chlorinated phenols, including PCP [20]. The pathway for the degradation of PCP has been elucidated, identifying as the main metabolites: tetrachlorobenzoquinone (TeCBQ) and tetrachlorodihydroquinone (TeCHQ). The pathways, presumably, lead to CO<sub>2</sub> mainly through reductive dechlorination reactions. However, PCP degradation is not an exclusive feature of white rot fungi; neither can it be correlated solely with ligninolytic strains [19]. For example, the brown rot species *Gloeophyllum striatum* and *G. trabeum* efficiently degraded PCP in the absence of ligninolytic activities [9]. Additionally, PCP disappearance is also efficiently mediated by non-producing and producing phenoloxidases ascomycetes [19]. That is the case for the nonligninolytic ascomycete *Trichoderma harzianum* that can actively degrade PCP [36].

Cork, a material of vegetable origin, is the bark of the cork oak (*Quercus suber*), whose main component is suberin, a complex aromatic biopolymer structurally related to lignin and, accordingly, highly resistant to degradation. Several soft rot species, amongst the cork-colonising consortia, were able to completely perforate the cork cell wall, suggesting their biodegradation ability [29]. Cork-colonising fungi isolates were also observed to degrade some chlorophenols, leading to the formation of chloroanisoles as a minor [28] or major intermediate [1].

In this study, the co- and direct metabolism of PCP by each of the 17 fungal strains selected from the cork-colonising community were studied. Data ranked the isolates by their PCP bioremediation potential, and surprisingly 15 of the fungal strains degraded PCP up to a certain level.

## Materials and methods

### Biological

All fungal strains used were environmental isolates previously isolated from cork samples purchased from several

Portuguese cork industries [8, 17, 30] and are part of the Instituto de Biologia Experimental e Tecnológica (IBET) culture collection. They were: *Chrysonilia sitophila* (DSM 16514), *Mucor plumbeus* Bonord (DSM 16513), *Trichoderma longibrachiatum* Rifai (DSM 16517), *Cladosporium herbarum*, *Penicillium glabrum* (Wehmer) Westling (DSM 16516), *P. olsonii* Bainier and Sartory (DSM 16515), *Eupenicillium hirayamae* D.B. Scott and Stolk (anamorph state of *P. hirayamae*), *P. brevicompactum* Dierckx, *P. glandicola* (Oudem) Seifert and Samson, *P. variabile* Sopp, *P. diversum* Raper and Fennell, *P. decumbens* Thom, *P. cf. janczewskii* K.M. Zalesky, *P. corylophilum* Dierckx, *P. adamezii* Zaleski, *P. fennelliae* Stolk and *P. restrictum* J.C. Gilman and E.V. Abbot.

### Fungal cultures

Each fungal strain was conserved as a suspension of spores in glycerol (10%, v/v) at –80°C. The fungal spore suspensions were prepared as previously described [28]. Briefly, the spores were harvested from fungal colonies after ageing for 15 days at 27°C on malt extract agar (Merck) or dichloranglycerol agar (Oxoid) with a saline solution (0.085 g/l NaCl), filtered through glass wool and washed by repeated centrifugation at 4°C (20,000g, 20 min). The spore suspension was allowed to thaw slowly at 4°C and washed in minimal medium (see below) before being used. The optical density (scatter) of the suspension was measured at 600 nm and calibrated by counting the number of spores in a haemocytometer. The initial fungal spores' density in the cultures was standardised to 10<sup>5</sup> spores/ml.

Fungal cultures were grown in media containing sodium nitrate as the sole source of nitrogen. The minimal culture media contained, per liter of water, 1 g K<sub>2</sub>HPO<sub>4</sub>; 3 g NaNO<sub>3</sub>; 10 mg ZnSO<sub>4</sub> · 7H<sub>2</sub>O; 5 mg CuSO<sub>4</sub> · 5H<sub>2</sub>O; 0.5 g MgSO<sub>4</sub> · 7H<sub>2</sub>O; 10 mg FeSO<sub>4</sub> · 7H<sub>2</sub>O; 0.5 g KCl and glucose: 0 and 10 g for minimal media (MM) and glucose minimal media (GMM), respectively. For the PCP degradation experiments the toxic was added to the culture media from a concentrated stock solution (7.5 g/l) in ethanol. The final PCP concentrations in the media ranged from 0 to 20 mg/l, thus guaranteeing that ethanol was not affecting fungal growth (data not shown).

### PCP degradation experiments

The inhibitory effect of PCP on fungal spore germination was inferred by measuring the optical density of the culture media at 600 nm during the incubation period. The method used was adapted from a method for the determination of the broth microdilution minimal inhibitory concentration (MIC) [10]. Briefly, 250 µl of the culture media (MM or GMM) containing a final PCP concentration in the range

1–20 mg/l was spread onto 96-well microtitre plates. The fungal spore suspensions were used to inoculate the culture media, and the plates were incubated in an incubation chamber (Aralab Clima Plus) at 27°C and 70% relative humidity, in the dark, for a maximum period of 60 days. The experiments were performed in quadruplicate, including controls without PCP and controls for PCP abiotic decay.

After incubation a chloramphenicol solution (Sigma) in ethanol (for volume normalisation) was added to the wells. The culture media were centrifuged at 4°C for removal of cell debris and solids (20,000g, 20 min). Samples were stored at –20°C until further analysis.

#### PCP quantification by HPLC

PCP in samples was analysed by high performance liquid chromatography (HPLC), consisting of a Waters 510 Pump, Waters 715 plus Autosampler, Waters Temperature Control Module and Waters 486 Absorbance detector (Waters Chromatography, Milford, MA). Data acquisition was accomplished with the Millennium32 version 3.05.01, 1998 system (Waters Chromatography). PCP and other chlorinated compounds were detected at 230 nm. The chromatographic separation was carried out using a Symmetry C18 column (4.6 × 250 cm), 5-µm particle size (Waters Chromatography) and set at 35°C. The mobile phase, at a flow rate of 0.8 ml/min, consisted of a solution of 0.1% phosphoric acid (solvent A) and acetonitrile (solvent B). The gradient program was implemented as follows: 90–1.0% of A in 30 min, 2 min to return to the initial conditions and 10 min to re-equilibrate the column to the initial conditions. The external standard method was used for PCP quantification (retention time: 30.3), within the limits of 0.1–15 mg/l.

Pure standards of the following chlorinated compounds were also analysed: hydroquinone (HQ) (Aldrich), chlorohydroquinone (CHQ) (Aldrich), tetrachlorohydroquinone (TeCHQ) (Sigma), 2,6-dichloro-1,4-benzoquinone (DCBQ) (Aldrich), tetrachloro-1,4-benzoquinone (TeCBQ) (Riedel-de Haën) and pentachloroanisole (PCA) (Sigma) (retention times: 6.3, 12.6, 21.4, 19.9, 22.1 and 35.6; respectively). All HPLC solvents used were of the highest analytical grade, and water was obtained from a Milli-Q system (Millipore).

#### Glucose quantification

Glucose analysis was undertaken using the HPLC system described above, connected to a LKB 2142 Differential Refractometer (Bromma) detector. Data acquisition was as previously described and chromatographic separation undertaken using an Aminex HPX-87H (300 × 7.8 mm), 9-µm particle size (Bio-Rad) and set at 60°C. Elution was carried out isocratically, at a flow rate of 0.5 ml/min, with

0.005 N of H<sub>2</sub>SO<sub>4</sub>. For the quantification the external standard method was also used (quantification limits: 0.125–50 g/l).

#### Detection of PCP degradation intermediates

The HPLC software (Millennium32 version 3.05.01, 1998, Waters Chromatography) was used to retrieve the full peak list of each 230-nm chromatographic analysis. Integration parameters (equal to those used to integrate the PCP peak) were set to: peak width = 15.0, threshold = 30.0, minimum area = 0 and minimum height = 300. Only the peaks detected between 6.5 and 35 min were considered. PCP and chloramphenicol peaks were used to align the chromatograms. The peak lists were initially compared using Microsoft Excel tools (pivot tables) to select data containing solely the peaks that were PCP dependent. The retention times of the peaks in the resulting matrix were compared to those of the pure standards, which provided preliminary identity.

#### Computational interpretation of data

The numerical data for PCP decay—PCP<sub>d</sub> (calculated as the percentage of PCP removed from the media relatively to its initial concentration) and residual glucose—G<sub>r</sub> (calculated as the percentage of glucose in the media relatively to its initial concentration) were analysed by hierarchical cluster analysis using the complete linkage between the groups calculated by the squared Euclidian distance method (SPSS v. 16.0). The distance between two clusters was calculated as the greatest distance between the members of the relevant clusters.

## Results

#### Fungal growth behaviour in the presence of PCP

The inhibitory effect of PCP on germination ability of the fungal spores under co-metabolic conditions (GMM) was inferred measuring the optical density of the submerged culture media at 600 nm during the incubation period. Increase of absorbance was taken as an indication of growth. Spore formation (gauged by eye) and/or the absorbance approaching a constant value indicated that the culture had entered a stationary growth phase. The fungal isolates can be divided into four main groups according to their PCP tolerance level (mg/l): 5 ≤ [*C. sitophila*, *P. diversum*; *P. fennelliae*, *P. restrictum*] < 10; 10 ≤ [*C. herbarum*, *M. plumbeus*, *P. variabile*] < 15; 15 ≤ [*P. adametzii*, *P. corylophilum*, *P. decumbens*, *P. glandicola*] < 20; 20 ≤ [*P. brevicompactum*, *E. hirayamae*, *P. janczewskii*, *P. olsonii*, *P. glabrum*, *T. longibrachiatum*] (Table 1). For

**Table 1** Mycelium growth in the cultures and PCP decay (%) under co-metabolic conditions (GMM) after 40–60 days of incubation

Fungal species	PCP initial concentrations (mg/L)							
	5		10		15		20	
	PCP <sub>d</sub>	G <sub>r</sub>	PCP <sub>d</sub>	G <sub>r</sub>	PCP <sub>d</sub>	G <sub>r</sub>	PCP <sub>d</sub>	G <sub>r</sub>
<i>C. herbarum</i>	45	0	43	33	–	82	–	99
<i>C. sitophila</i>	79	0	–	96	–	100	–	100
<i>M. plumbeus</i>	100	0	100	0	–	86	–	100
<i>P. adametzii</i>	48	0	10	0	7	71	–	100
<i>P. brevicompactum</i>	77	0	58	0	61	0	59	0
<i>P. corylophilum</i>	45	0	43	30	37	74	–	99
<i>P. decumbens</i>	72	0	40	0	35	0	–	100
<i>P. fennelliae</i>	88	0	–	88	–	96	–	100
<i>P. glandicola</i>	100	0	81	6	27	18	25	93
<i>P. glabrum</i>	75	0	45	0	48	0	39	0
<i>E. hirayamae</i>	74	0	57	0	40	0	24	0
<i>P. janczewskii</i>	73	0	61	0	62	0	56	0
<i>P. olsonii</i>	68	0	52	0	63	0	59	0
<i>P. variabile</i>	74	0	22	59	–	93	–	100
<i>T. longibrachiatum</i>	100	0	100	6	77	20	39	76

Residual glucose concentrations are also shown. PCP decay (%) and residual glucose were calculated relative to their initial concentration in GMM. Values maximum ST = ±14.5%. PCP abiotic decay in media after incubation was 33 ± 10%

PCP<sub>d</sub> PCP decay (%); G<sub>r</sub> residual glucose in the culture media (%); – inhibition of spores' germination

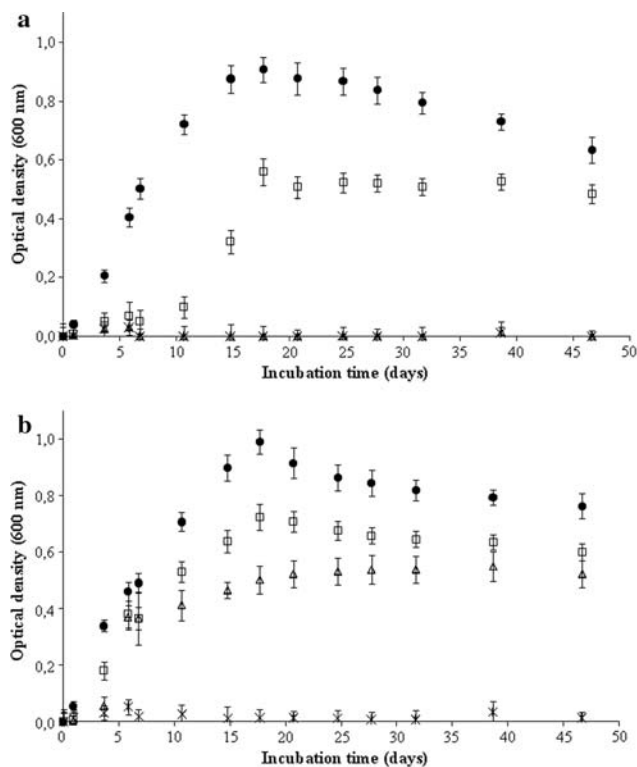
each fungal strain, the growth curve in GMM containing PCP can provide additional information on its toxicity (example shown in Fig. 1). In fact, for example in the growing cultures of *C. sitophila*, the lag phase of growth increases (5 mg/l PCP), but in *M. plumbeus* the growth curves are either rather similar (5 mg/l PCP) or suggest a lower production of fungal biomass (10 mg/l PCP) (see Fig. 1).

#### PCP abiotic degradation

Analyses of the abiotic degradation of PCP in the non-inoculated media (MM and GMM) were made to evaluate the contribution of physical-chemical events in the overall decay. Abiotic decay of PCP in the GMM and in the MM were 33 ± 10% and 7.5 ± 1.5%, respectively. Independent tests on PCP absorption to the plates, the fungal spores and the mycelium showed an insignificant contribution to the overall abiotic decay (data not shown).

#### PCP biotic degradation in co-metabolic conditions (GMM)

PCP<sub>d</sub> and the percentage of G<sub>r</sub> measured in the 17 fungal cultures in co-metabolic conditions (GMM) are presented in Table 1. The fungus micro-cultures were treated as a



**Fig. 1** Analysis of fungal growth profiles in GMM containing PCP. The growth curves of *C. sitophila* (a) and *M. plumbeus* (b) strains are shown as an example. Fungal growth was monitored along the incubation period measuring the media scatter at 600 nm. Initial PCP concentration in media was in mg/l: 0 (filled circle); 5 (open square); 10 (open triangle) and 15 (multiplication)

single sample, selecting only the individual wells that reported the same growth behaviour. Independent runs indicated the cultures behaviour to be consistent (data not shown), and replicates of the same fungus showed a consistent standard deviation (<10%) for PCP decay. The chloramphenicol concentration measured chromatographically was used to infer the cultures' final volume, allowing to calculate accurately PCP decay in media (thus eliminating errors introduced by evaporation).

PCP decay observed in the cultures fully inhibited by the toxic was 29 ± 10%, agreeing with the abiotic decay determined in the non-inoculated GMM controls. In these cultures glucose was not consumed, or only minor consumption was detected (≤20%), confirming the growth inhibition data. Active fungal growth results in glucose exhaustion from the media (Table 1). In view of that, overall decay of PCP in the active growing cultures has only a minor contribution from abiotic decay of PCP (≥7.5 ± 1.5%). Glucose quantification for different replicates showed a consistently low standard deviation (≤2%). Amongst the isolates tested, those able to degrade PCP in all concentrations in order of efficiency were: *P. brevicompactum* and *P. olsonii* > *P. janczewskii* > *P. glabrum* >

*E. hirayamae*  $\gg$  *T. longibrachiatum*. Only three isolates completely consumed the PCP from the media: *T. longibrachiatum* and *M. plumbeus* > *P. glandicola*, up to PCP initial concentration of 10 and 5 mg/l, respectively. The moderate (~50–75%) and the weakest (<45%) PCP consumers were *P. decumbens* > *P. variabile* > *P. fennelliae* and *C. sitophila* (PCP up to 15, 10 and 5 mg/L) and *P. adametzii* and *P. corylophilum* > *C. herbarum* (PCP up to 15 and 10, respectively). *P. restrictum* and *P. diversum* were unable to degrade PCP, although they can still grow in the presence of 5 mg/l of PCP.

Cluster analysis of PCP biotic degradation in co-metabolic conditions (GMM)

The relationships between the capacities of the isolates to tolerate and degrade PCP co-metabolically were established by using a hierarchical cluster analysis. In the resulting tree (depicted in Fig. 2), the branch lengths reflect the degree of dissimilarity between the fungal strains. There are six main clusters (cutoff rescaled distance of six) that separate the isolates by their PCP bioremediation potential (accounting for, equally, the species tolerance level and PCP degradation capacity): high (I and II), moderate (III, IV), low (V) and null (VI). In fact, cluster VI can be regarded as the outlier group due to the apparent inability of the isolates, *P. restrictum* and *P. diversum*, to degrade PCP. The fungal species with the strongest bioremediation potential are the most tolerant ones: *P. brevicompactum*, *E. hirayamae*, *P. janczewskii*, *P. olsonii* and *P. glabrum*, with the exception of *T. longibrachiatum*. The latter has

**Table 2** PCP decay in the fungal cultures under metabolic conditions (MM) after 50–60 days of incubation

Fungal species	PCP initial concentrations (mg/l)		
	PCP <sub>d</sub>		
	1	2.5	5
<i>C. sitophila</i>	100	100	–
<i>M. plumbeus</i>	100	100	85
<i>P. glandicola</i>	100	100	67
<i>P. janczewskii</i>	100	41	34
<i>T. longibrachiatum</i>	100	28	–

PCP decay (%) was calculated relative to its initial concentration in MM. PCP abiotic decay in media after incubation was 7.5 ± 1.5%

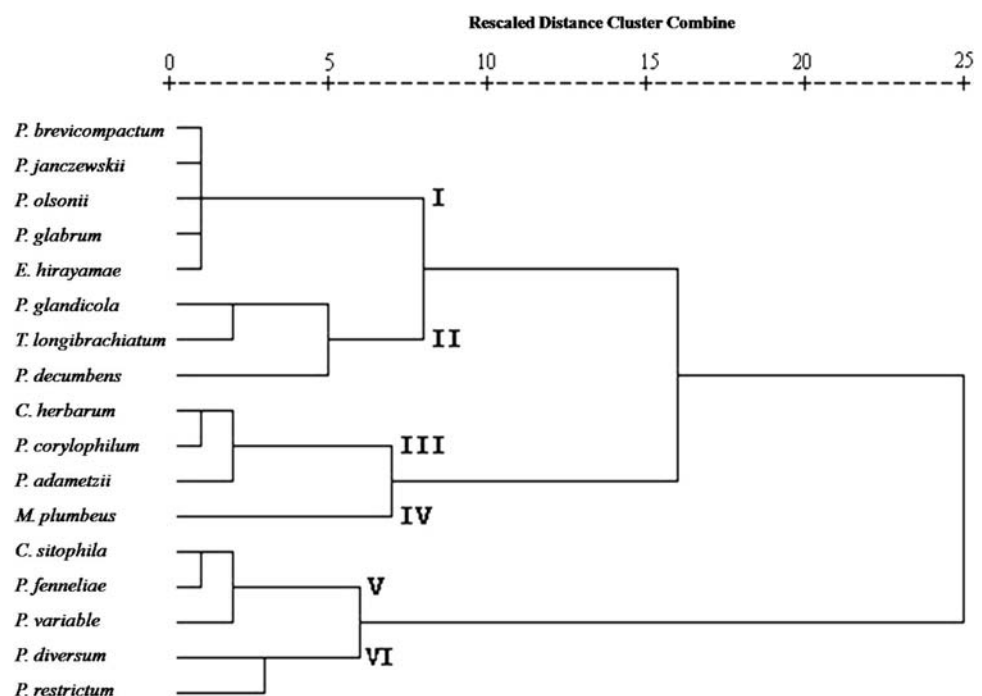
PCP<sub>d</sub> PCP decay (%), – inhibition of spores' germination

only scarcely grown in the presence of 20 mg/l of PCP, as shown by the high G<sub>r</sub> content.

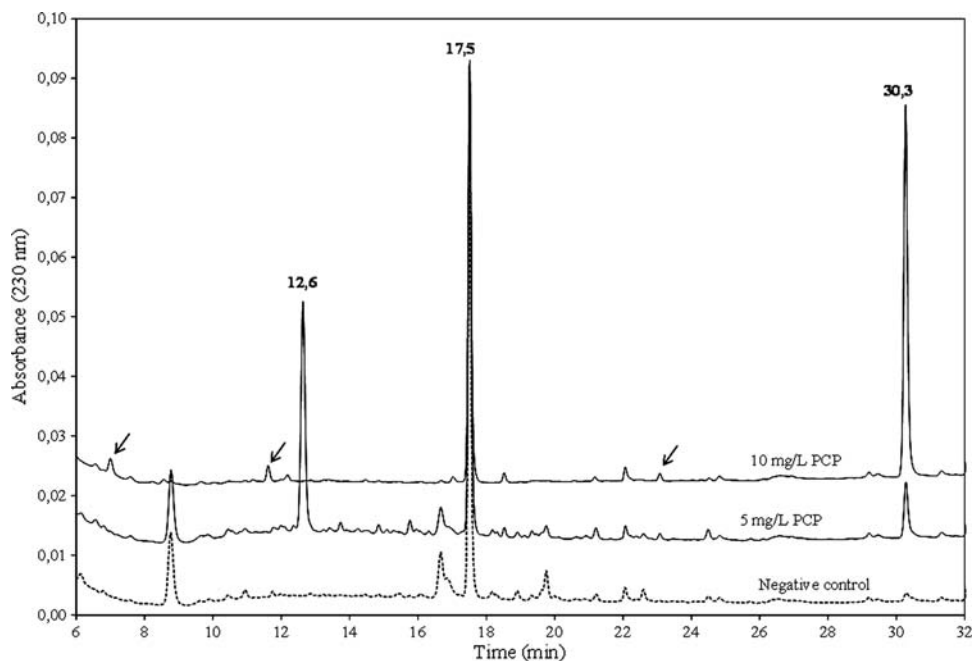
PCP biotic degradation by fungi in metabolic conditions

PCP metabolism by some of the tested fungal isolates was observed in MM containing PCP up to initial levels of 5 mg/l. Interestingly, amongst the 17 isolates tested only *M. plumbeus*, *P. glandicola*, *C. sitophila*, *P. janczewskii* and *T. longibrachiatum* were able to consume PCP and have tolerated the toxic in a distinctive way (Table 2). From these, only *C. sitophila* and *T. longibrachiatum* failed to grow in the presence of 5 mg/l, yet completely metabolised PCP at an initial concentration of 2.5 and 1 mg/l, respectively.

**Fig. 2** Cluster analysis of the growth and bioremediation behaviour of the cork-colonising fungal strains during co-metabolic degradation of PCP. Data were analysed by hierarchical cluster, using the complete linkage between the groups calculated by the squared Euclidian distance method. The resulting six clusters of strains are labelled



**Fig. 3** Chromatographic profiles (between 6 and 32 min) of *P. variable* culture extracts after growth in GMM (negative control) and in GMM containing PCP (initial concentrations of 5 and 10 mg/l). Some of the newly formed peaks are labelled with an arrow, and the peak eluting at 12.6 min was putatively identified as CHQ. PCP decay in media, under co-metabolic conditions, was 74 and 22% for PCP initial concentrations of 5 and 10 mg/l, respectively. The peaks eluting at 30.3 and 17.5 min correspond to PCP and chloramphenicol, respectively



#### Analysis of PCP metabolic intermediates

Each fungal culture produced a PCP-specific chromatographic footprint (UV 230 nm), which was highly consistent between replicates (see “Detection of PCP degradation intermediates” above). They can be regarded qualitatively as the metabolic fingerprinting of the extracellular metabolites of the fungus, which can be computed so as to select solely the peaks, species specific, which are PCP dependent. The putative identity of PCP degradation intermediates (in MM and GMM) was made through analysis of the similarity of the chromatographic peaks and retention times ( $T_r$ ) with that of the standard compounds. Only in co-metabolic conditions could the newly formed peaks be associated to the standards, as depicted in Fig. 3, e.g., *P. variable*. The putative identification of CHQ was possible for the cultures of *C. sitophila*, *P. corylophilum*, *P. glabrum*, *P. glandicola*, *P. janczewskii* and *P. variable*; 2,6-dichloro-1,4-benzoquinone (DCBQ) in the cultures of *C. herbarum*, *P. decumbens* and *T. longibrachiatum*; and tetrachloro-1,4-benzoquinone (TeCBQ) in the cultures of *P. adametzii*.

#### Discussion

The species diversity amongst the cork-colonising fungal communities is largely influenced by the origin of the cork, explaining some of the discrepancy found in the strains reported by different authors [1, 2, 8, 11, 17, 26–29]. In this study, the fungal strains used were isolated from cork bark samples, which were collected during plank preparation for

the manufacture of stoppers. Within this community *Penicillium* spp. is the dominant genus, agreeing with the previous reports on cork microbiology [26]. From the 17 fungal strains tested, only 1 belongs to the Zygomycota phylum (*Mucor* sp.), while the remaining 16 belong to the Ascomycota phylum (13 *Penicillium* spp., one *Trichoderma* sp., one *Cladosporium* sp. and one *Chrysonilia* sp.).

The capacity of ascomycetes and zygomycetes fungi to tolerate and degrade PCP in a submerged culture has seldom been studied. Our aim was to evaluate individually the significance of these strains for PCP degradation. The maximum concentration of PCP used in the cultures was 20 mg/l providing the PCP in a bio-available fraction (PCP solubility in water is ~18 and 20 mg/l at 20 and 25°C, respectively). The degree of abiotic degradation in the non-inoculated controls (after incubation in the dark) was higher than that previously reported by other authors, 8% after 12 days [24] or null after 8 days [34], probably due to the high incubation periods used here (40–60 days). Also, the abiotic decay was found to be higher in the GMM (~30%) than in the MM media (7.5%). PCP photolytical degradation in water has been reported to produce lower chlorinated phenols and chlorinated dihydroxybenzenes, which can be ultimately mineralised to CO<sub>2</sub> [37]. The 230-nm chromatographic peak lists of the abiotic decay controls were not altered after incubation, suggesting PCP volatilisation or transformation to compounds not absorbing at 230 nm. This was apparently favoured in the GMM, relative to the MM.

From the 17 strains tested, all were observed to tolerate PCP co-metabolically (Table 1). PCP is known to strongly affect spore germination, even in cultures of the highly

efficient PCP degrading white rot fungus *P. chrysosporium*, which has been reported to stop germination in the presence of 4 mg/L of PCP, with this effect only being circumvented when the toxic was added to the growing mycelium [16]. Surprisingly, in this work, the spores of all the strains tested were able to germinate in the presence of 5 mg/L of PCP (GMM) (Table 1). Below the species toxic MICs, increasing PCP concentration in media resulted in unchanged or apparently reduced biomass (data not shown). The latter is demonstrated in Fig. 1 for *C. sitophila* (5 mg/l) and *M. plumbeus* (5 and 10 mg/l) and has been previously observed in cultures of the basidiomycetes *P. sordida* and *P. chrysosporium*, where high concentrations of PCP have greatly decreased mycelial extension rates [12].

The phylum Ascomycota is taxonomically a heterogeneous group, consequently reflecting a rather diverse behaviour in the degradation capacity of PCP [23, 24]. In fact, while analysing their PCP bioremediation potential under co-metabolic conditions, the 16 tested ascomycetes have clustered in 6 different groups (Fig. 2) with the strains *P. diversum* and *P. restrictum* as the outlier group. The latter were able to germinate in 5 mg/l PCP, but completely failed to degrade it. Other environmental strains of the phylum Ascomycota have previously been reported to efficiently degrade PCP co-metabolically (e.g., *Trichoderma* sp. [5, 6], some *Penicillium* spp. [24] and *Cladosporium* sp. [3]).

*Mucor plumbeus*, the only zygomycete strain in this cork-colonising community, completely removed PCP in the GMM (up to 10 mg/l). The phylum Zygomycota is, relative to the Ascomycota, taxonomically a more homogeneous group and, although devoid of ligninolytic activities, has been previously reported to extensively deplete PCP [22]. For example in the case of *Rhizopus nigricans*, while failing to sporulate in PCP liquid medium, 12.5 mg/l PCP could be efficiently co-metabolised [34]. Furthermore, the zygomycete *M. ramosissimus* was also observed to efficiently degrade PCP [32].

Very efficient PCP degradation was observed in the cultures of four ascomycetes, *P. glandicola*, *C. sitophila*, *P. janczewskii* and *T. longibrachiatum* and the zygomycete *M. plumbeus*, under metabolic conditions (MM) (Table 2). These strains tolerated the toxic in a distinctive way, but all could completely remove PCP from the media at an initial concentration of 1 mg/l. This has been reported in cultures of the brown rot basidiomycete *Gloeophyllum striatum*, which is devoid of ligninolytic activities, but in a submerged culture degraded 1.3 mg/l PCP more efficiently in metabolic than co-metabolic conditions [9].

The chromatographic profiles of the fungal cultures, where the recovered PCP was significantly reduced, revealed the appearance of a variable number of new peaks indicating their active biotransformation (ca. 10 and 3 novel

peaks per degrading culture in co- and metabolic conditions, respectively). However, from our results, it cannot be excluded that another process (such as bioaccumulation) also may be involved. At this stage, efforts to identify the intermediate products of PCP degradation were made solely by comparing the chromatographic features of the peaks with that of pure compounds. Under co-metabolic conditions some PCP sub-products were putatively indentified: TeCBQ (*P. adametzii*), DCBQ (*C. herbarum*, *P. decumbens* and *T. longibrachiatum*) and CHQ (*C. sitophila*, *P. corylophilum*, *P. glabrum*, *P. glandicola*, *P. janczewskii* and *P. variabile*) (Fig. 3), thus suggesting a PCP degradation pathway similar to that previously described for *P. chrysosporium* [20]. On the other hand, under metabolic conditions, none of the newly formed peaks could be associated to the standard compounds.

The potential of cork-colonising fungi to degrade chlorophenols has previously been analysed (e.g., [1, 14, 18, 28]). In one study, the majority of the fungal strains tested have transformed 2,4,6-trichlorophenol (TCP) (1 mg/l) in the corresponding anisole [1]. On the contrary, in an earlier study, some ascomycetes strains, including *C. sitophila*, degraded the chlorophenol in a rather heterogeneous fashion, but its methylation was not the preferential pathway [28]. This discrepancy is due to the different experimental conditions used, namely the nitrogen source used. A similar effect was reported in different studies on *P. chrysosporium* that degraded efficiently both chlorophenols: TCP [21] and PCP [12, 20], forming the corresponding anisoles as vestigial or major intermediates under high-carbon-low-nitrogen conditions and high-carbon-high-nitrogen conditions, respectively. Interestingly, in the study reported here, pentachloroanisole was not detected in any of the culture extracts, neither in GMM or MM, with both media containing nitrate as the sole nitrogen source.

## Conclusion

The purpose of this study was to assess the behaviour of each of the 17 fungal species tested from the cork-colonising community during co- and direct metabolism of PCP. Our data further emphasise the significance of fungi belonging to a phylum other than Basidiomycota to have an interesting potential to be used in PCP biodegradation processes. In fact, 15 of the 17 strains tested could degrade PCP to a certain extent. Additionally, PCP in media was completely degraded co-metabolically by *T. longibrachiatum*, *P. glandicola* and *M. plumbeus* and metabolically by those and *P. janczewskii* and *C. sitophila*. Additional studies will be carried out to investigate in the most efficient fungal strains the identity of PCP degradation intermediates, aiming to better understand its degradation pathways.

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## References

- Alvarez-Rodriguez ML, Lopez-Ocana L, Lopez-Coronado JM, Rodriguez E, Martinez MJ, Larriba G, Coque JJR (2002) Cork taint of wines: role of the filamentous fungi isolated from cork in the formation of 2,4,6-trichloroanisole by O-methylation of 2,4,6-trichlorophenol. *Appl Environ Microbiol* 68(12):5860–5869
- Basílio MC, Gaspar R, Silva Pereira C, San Romão MV (2006) *Penicillium glabrum* cork colonising isolates—preliminary analysis of their genomic similarity. *Rev Iberoam Micol* 23(3):151–154
- Borazjani H, Ferguson B, Hendrix F, McFarland L, McGinnis G, Pope D, Strobel D, Wagner J (1989) *Cladosporium* sp., a potential fungus for bioremediation of wood-treating wastes. *Abs Papers Am Chem Soc* 197:80-ENVR
- Carlile M, Watkinson S, Gooday G (2001) *The fungi*. Elsevier Academic Press, Amsterdam, p 588
- Cserjesi AJ (1967) Adaptation of fungi to pentachlorophenol and its biodegradation. *Can J Microbiol* 13(9):1243–1245
- Cserjesi AJ, Johnson EL (1972) Methylation of pentachlorophenol by *Trichoderma virgatum*. *Can J Microbiol* 18(1):45–47
- Czaplicka M (2004) Sources and transformations of chlorophenols in the natural environment. *Sci Total Environ* 322(1–3):21–39
- Danesh P, Velez Caldas F, Figueiredo Marques J, San Romão M (1997) Mycobiota in Portuguese ‘normal’ and ‘green’ cork throughout the manufacturing process of stoppers. *J Appl Microbiol* 82(6):689–694
- Fahr K, Wetzstein HG, Grey R, Schlosser D (1999) Degradation of 2,4-dichlorophenol and pentachlorophenol by two brown rot fungi. *FEMS Microbiol Lett* 175(1):127–132
- Johnson ME, Szekely A, Warnock DW (1998) In vitro activity of voriconazole, itraconazole and amphotericin B against filamentous fungi. *J Antimicrob Chemother* 42:741–745
- Lacey J (1973) The air spora of a Portuguese cork factory. *Ann Occup Hyg* 16(3):223–230
- Lamar RT, Larsen MJ, Kirk TK (1990) Sensitivity to and degradation of pentachlorophenol by *Phanerochaete* spp. *Appl Environ Microbiol* 56(11):3519–3526
- Lyytikäinen M, Sormunen A, Peräniemi S, Kukkonen J (2001) Environmental fate and bioavailability of wood preservatives in freshwater sediments near an old sawmill site. *Chemosphere* 44(3):341–350
- Maggi L, Mazzoleni V, Fumi MD, Salinas MR (2008) Transformation ability of fungi isolated from cork and grape to produce 2,4,6-trichloroanisole from 2,4,6-trichlorophenol. *Food Addit Contam* 25(3):265–269
- McLellan L, Carvalho M, Silva Pereira C, Hursthouse A, Morrison C, Tatner P, Martins I, Romão MVS, Leitão M (2007) The environmental behaviour of polychlorinated phenols and its relevance to cork forest ecosystems: a review. *J Environ Monitor* 9:1055–1063
- Mileski GJ, Bumpus JA, Jurek MA, Aust SD (1988) Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium*. *Appl Environ Microbiol* 54(12):2885–2889
- Oliveira AC, Peres CM, Correia Pires JM, Silva Pereira C, Vitorino S, Figueiredo Marques JJ, Barreto Crespo MT, San Romão MV (2003) Cork stoppers industry: defining appropriate mould colonization. *Microbiol Res* 158(2):117–124
- Prak S, Gunata Z, Guiraud JP, Schorr-Galindo S (2007) Fungal strains isolated from cork stoppers and the formation of 2,4,6-trichloroanisole involved in the cork taint of wine. *Food Microbiol* 24:271–280
- Rabinovich ML, Bolobova AV, Vasil’chenko LG (2004) Fungal decomposition of natural aromatic structures and xenobiotics: a review. *Appl Biochem Microbiol* 40(1):1–17
- Reddy GVB, Gold MH (2000) Degradation of pentachlorophenol by *Phanerochaete chrysosporium*: intermediates and reactions involved. *Microbiology* 146:405–413
- Reddy GVB, Gelpke MDS, Gold MH (1998) Degradation of 2,4,6-trichlorophenol by *Phanerochaete chrysosporium*: involvement of reductive dechlorination. *J Bacteriol* 180(19):5159–5164
- Seigle-Murandi F, Guiraud P, Steiman R, Benoitguyod JL (1992) Phenoxidase production and vanillic acid metabolism by Zygomycetes. *Microbiologica* 15(2):157–165
- Seigle-Murandi F, Steiman R, Benoitguyod JL, Guiraud P (1993) Fungal degradation of pentachlorophenol by micromycetes. *J Biotechnol* 30(1):27–35
- Seigle-Murandi F, Toe A, Benoitguyod JL, Steiman R, Kadri M (1995) Depletion of pentachlorophenol by Deuteromycetes isolated from soil. *Chemosphere* 31(2):2677–2686
- Semple KT, Reid BJ, Fermor TR (2001) Impact of composting strategies on the treatment of soils contaminated with organic pollutants. *Environ Pollut* 26:9–283
- Serra R, Peterson S, Venancio A (2008) Multilocus sequence identification of *Penicillium* species in cork bark during plank preparation for the manufacture of stoppers. *Res Microbiol* 159(3):178–186
- Silva Pereira C, Marques JJJ, San Romão MV (2000) Cork taint in wine: scientific knowledge and public perception—a critical review. *Crit Rev Microbiol* 26(3):147–162
- Silva Pereira C, Pires A, Valle MJ, Vilas-Boas L, Figueiredo Marques JJ, San Romão MV (2000) Role of *Chrysonilia sitophila* on the quality for cork stoppers for sealing wine bottle. *J Ind Microbiol Biotech* 24:256–261
- Silva Pereira C, Soares GAM, Oliveira AC, Rosa ME, Pereira H, Moreno N, Romão MVS (2006) Effect of fungal colonization on mechanical performance of cork. *Int Biodeter Biodegr* 57(4):244–250
- Soares GAM, Basílio MC, Tenreiro R, San Romão MV (2003) Diversity of *Penicillium* spp. colonising cork slabs: a classical and molecular approach. In: Lima N, Smith D (eds) *Biological resource centres and the use of microbes*. Micoteca da Universidade do Minho, Braga, pp 161–170
- Solyanikova IP, Golovleva LA (2004) Bacterial degradation of chlorophenols: pathways, biochemical and genetic aspects. *J Environ Sci Health Part B* 39(3):333–351
- Szewczyk R, Bernat P, Milczarek K, Długonski J (2003) Application of microscopic fungi isolated from polluted industrial areas for polycyclic aromatic hydrocarbons and pentachlorophenol reduction. *Biodegradation* 14(1):1–8
- Taylor TR, Tucker T, Whalen MM (2005) Persistent inhibition of human natural killer cell function by ziram and pentachlorophenol. *Environ Toxicol* 20(4):418–424
- Tomasini A, Flores V, Cortes D, Barrios-Gonzalez J (2001) An isolate of *Rhizopus nigricans* capable of tolerating and removing pentachlorophenol. *World J Microbiol Biotechnol* 17(2):201–205
- Tortella GR, Diez MC, Duran N (2005) Fungal diversity and use in decomposition of environmental pollutants. *Crit Rev Microbiol* 31(4):197–212
- van Leeuwen JA, Nicholson BC, Hayes KP, Mulcahy DE (1997) Degradation of chlorophenolic compounds by *Trichoderma harzianum* isolated from Lake Bonney, South-Eastern South Australia. *Environ Toxicol Water Qual* 12(4):335–342
- Wong AS, Crosby DG (1981) Photo-decomposition of pentachlorophenol in water. *J Agric Food Chem* 29(1):125–130