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ORIGINAL ARTICLE

Comparative study of single cultures and a consortium of white rot fungi for polychlorinated biphenyls treatment

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Keywords

consortium, detoxification, fungal biodegradation, mycoremediation, *Pleurotus pulmonarius*, polychlorinated biphenyls, *Trametes sanguinea*.

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Abstract

Aims: To evaluate the mycoremediation of polychlorinated biphenyls (PCBs) by either single cultures or binary consortia of *Pleurotus pulmonarius* LBM 105 and *Trametes sanguinea* LBM 023.

Methods and Results: PCBs tolerance, removal capacity, toxicity reduction and ligninolytic enzyme expression were assessed when growing single culture and binary consortium of fungus in 217 mg l^{-1} of a technical mixture of Aroclor 1242, 1254 and 1260 in transformer oil. A decrease in tolerance and variation in ligninolytic enzyme secretion were observed in PCB-amended solid media. *Pleurotus pulmonarius* LBM 105 mono-culture was able to remove up to 95·4% of PCBs, whereas binary consortium and *T. sanguinea* LBM 023 could biodegrade about 55% after 24 days. Significant detoxification levels were detected in all treatments by biosorption mechanism.

Conclusions: *Pleurotus pulmonarius* LBM 105 in single culture had the best performance regarding PCBs biodegradation and toxicity reduction. Ligninolytic enzyme secretion changed in co-culture.

Significance and Impact of the Study: The evaluation of PCBs bioremediation effectiveness of basidiomycetes consortium in terms of PCB removal, toxicity and ligninolytic enzyme production to unravel the differences between using individual cultures or consortium has not been reported. The results from this study enable the selection of *P. pulmonarius* LBM 105 mono-culture to bioremediate PCBs as it showed higher efficiency compared to binary consortium with *T. sanguinea* LBM 023 for potential decontamination of PCB-contaminated transformer oil.

Introduction

Polychlorinated biphenyls (PCBs) are a group of chlorinated organic pollutants that have been used in a wide range of products in the 20th century (Agulló *et al.* 2019). Although their production and usage were banned in the late 1970s, increasing attention has been paid to the elimination of PCBs because of their high persistence, bioaccumulation and toxicity linked to diseases of the immune, reproductive, nervous and endocrine systems (Fan *et al.* 2016; Agulló *et al.* 2019).

Compared to physical–chemical methods, bioremediation is considered an efficient, environmentally friendly and cost-effective technology for the elimination of PCBs using organisms capable of degrading and/or removing toxic compounds (Horváthová *et al.* 2018). Although bacterial PCB degradation is well documented (Borja *et al.* 2005; Chun *et al.* 2019), the metabolism of high chlorinated congeners can lead to the accumulation of toxic dead-end metabolites (e.g. chlorobenzoic acids) (Passatore *et al.* 2014). In this regard, mycoremediation presents several advantages such as the degradation of main metabolites (including chlorobenzoic acids) simultaneously with PCBs and an extracellular enzymatic system that can compensate for the low bioavailability of the compound (Čvančarová *et al.* 2012; Passatore *et al.*

2014). Pollutant bioremediation by fungi can occur via two main mechanisms: enzymatic biodegradation and biosorption through cell walls (Elangovan *et al.* 2019; Tomasini and León-Santiesteban 2019). Particularly, white rot fungi (WRF) exhibit features that make them excellent candidates for remediation technology (Čvančarová *et al.* 2012; Vishwakarma 2019). These ligninolytic fungi with their extracellular, low substrate specificity enzymes have been proved capable of degrading a wide range of organic pollutants, including PCBs (Tomasini and León-Santiesteban 2019; Vishwakarma 2019). The ligninolytic system consists of three major peroxidases: lignin peroxidase (LiP, -EC 1·11·1.14-), manganese peroxidase (MnP, -EC 1·11·1.13-), versatile peroxidases (VP, -EC 1·11·1.16-); and laccase (Lac, -EC 1·10·3.2-), this last one belonging to phenol oxidases (Gao *et al.* 2010; Čvančarová *et al.* 2012; Falade *et al.* 2016). The capacity of genetically susceptible fungi to survive the toxic effects of pollutants plays a critical and underappreciated role in the bioremediation process and constitutes the first step to the selection of capable strains. In this sense, predictive mycology, which consists of using mathematical models to describe fungal growth, is a tool that contributes to enhance the identification of strains with the characteristics of interest (Bevilacqua *et al.* 2017; Sadañoski *et al.* 2018). In addition, during PCBs degradation, toxic metabolites can be generated (Passatore *et al.* 2014; Nair and Abraham 2019). These secondary metabolites can become new bioremediation targets and undergo further degradation (Nair and Abraham 2019). Therefore, metabolic versatility can be a key attribute to reach higher detoxification levels (Gangola *et al.* 2019).

Efforts have been directed to develop strategies that focus on the synergic action of degrading organism (Passatore *et al.* 2014). One approach for the treatment of recalcitrant compounds is the use of consortia (Jiménez *et al.* 2019). Authors suggest that the use of consortia may be a viable strategy for bioremediation systems, causing variation of the enzymatic machinery and enhanced rates of degradation of the compound and toxic metabolites (Kuhar *et al.* 2015; Ijoma and Tekere 2017). It is known that the particular species combination, their mode of interaction and the micro-environmental or nutritional conditions in substrate under colonization are determining factors for successful mixed culture (Gutierrez-Correa *et al.* 1999). Another reason for a synergistic effect on degradation by consortia may be due to cooperative catabolism, where the individual degrader strains transform the pollutant to products which are then used by the second organism, being metabolically active at different parts of the degradation pathway (Ellegaard-Jensen *et al.* 2014). Although there are studies which demonstrate that WRF species can grow together (Chi *et al.* 2007; Qi-he *et al.* 2011; Kumari and Naraian 2016), the effects of co-culture on the tolerance, removal and the residual by-products of the biodegradation are not yet well understood. In this sense, it is necessary to perform studies on these aspects in WRF consortium applied in bioremediation treatments.

Strains belonging to the genera of *Pleurotus* and *Trametes* demonstrated to be promising for mycoremediation of PCBs (Sadañoski *et al.* 2018, 2019, 2020a, 2020b). For instance, in previous studies, PCB contaminated soil treated with *Pleurotus pulmonarius* LBM 105 and *Trametes sanguinea* LBM 023 immobilized on sugarcane bagasse showed higher enzyme activities and detoxification levels when the strains were applied in co-culture (Sadañoski *et al.* 2020b). Owing to this, we consider that the combination of *P. pulmonarius* LBM 105 and *T. sanguinea* LBM 023 could lead to an improvement in the time of treatment and detoxification of PCBs in transformer oil. This is the first study that compares the efficiency of the individual species, *T. sanguinea* LBM 023 and *P. pulmonarius* LBM 105, with their fungal consortium to treat a PCBs mixture in transformer oil in liquid media. The aim of the present work was to evaluate co-culture tolerance, degradation and detoxification ability of high concentrations of PCBs in transformer oil, as well as, ligninolytic enzyme secretion.

Materials and Methods

Fungal strains, inoculum preparation and chemicals

Pleurotus pulmonarius LBM 105 and *T. sanguinea* LBM 023 were isolated in Misiones (Argentina). These WRF species are deposited in the culture collection of the Instituto de Biotecnología Misiones 'Dra. María Ebe Reca', Universidad Nacional de Misiones, Argentina. Stock cultures were maintained by monthly sub-culturing on Petri dishes in malt extract 12·7 g l^{-1} and agar 17 g l^{-1} (MEA, Biokar Diagnostics®) at 4°C. Inoculum was obtained from active MEA plates incubated at 28 ± 1°C for 7 days in darkness.

The PCBs in transformer oil were obtained from KIOSHI S.A. (Buenos Aires, Argentina) which contained a mixture of Aroclors 1242, 1254 and 1260. A stock solution (1000 mg ml^{-1}) of the PCBs mixture was prepared in acetone (Biopack®) and used for spiking culture media. All chemicals used were of analytical grade or trace analysis quality.

Consortium tolerance towards PCBs

Fungal consortium tolerance towards PCBs was analysed in Petri dishes (85 mm ø) containing 12 ml of MEA

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supplemented with 85 mg l^{-1} of PCBs, pH 4·8 (Sadañoski *et al.* 2018). A 5-mm ø plug cut with a cork-borer from the margin of a 7-day-old culture of WRF in MEA was inoculated on the edge of a Petri dish opposite to another 5-mm ø agar plug of WRF (40 mm apart). Cultures were incubated in the dark at 28 ± 1°C. Petri dishes inoculated with single fungal species and binary consortium without PCBs were used as controls. The radial growth was measured daily until complete coverage of the plate. Fungal growth was modelled using a logistic equation (Sadañoski *et al.* 2018):

$$D = \frac{D_{\max}}{1 + e^{k(\tau - t)}} \qquad (1)$$

where D was the diameter of the fungal colony, with $D_{\max}$ being the maximum diameter (set to 42·5 mm, corresponding to half the maximum diameter of the Petri dishes); k was the rate of fungal growth (m day^{-1}); τ was the time needed to attain half of $D_{\max}$ (d) and t was the time (d). Fitting was performed through the software InfoStat 2018p using a least squares approach with non-linear regression (Di Rienzo *et al.* 2016).

Fungal growth τ was standardized as $\Delta\tau = \tau$PCBs–τC, where τPCBs were the values from medium supplemented with PCBs and τC were the values from control culture without PCBs to ignore the effect of nutrient components. A positive value of $\Delta\tau$ proved fungal growth inhibition in response to PCBs and/or toxic metabolites.

Degradation of PCBs

A nitrogen-limited mineral medium (GA) was used, containing glucose, 10 g; asparagine monohydrate, 0·5 g; $MgSO_4 \cdot 7H_2O$, 0·5 g; H_2KPO_4, 0·5 g; HK_2PO_4, 0·6 g; $CuSO_4 \cdot 5H_2O$, 0·4 mg; $MnCl_2 \cdot 4H_2O$, 0·1 mg; H_3BO_3, 0·1 mg; $CaCl_2$, 0·1 g; $Na_2MoO_4 \cdot 2H_2O$, 0·02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3·5 mg; thiamine hydrochloride, 0·1 mg; Tween 80, 1·7 mmol; distilled water up to 1 l, pH was adjusted to 4·6 with citrate-phosphate buffer 500 mol l^{-1} which is the fungal optimal growth condition (Haglund *et al.* 2002). Tween 80 was added to enhance PCBs' bioavailability (Wu *et al.* 2016). Erlenmeyer flasks (250 ml) with 20 ml of medium were autoclaved (105 °C, 20 min) and spiked with 4000 μg of PCBs in acetone reaching an initial concentration of 217 mg l^{-1} per flask. Then, one agar plug (5 mm ø) of the fungus grown for 7 days on MEA plates was inoculated. Unspiked and abiotic cultures were used as controls. Heat-killed controls were obtained by the addition of PCBs to autoclaved 7-day-old fungal cultures. All cultivations were carried out at 28 ± 1 °C under static conditions for 24 days. The samples were harvested every 8 days and the mycelium was separated from the supernatant by centrifugation (4700 g, 10 min). All the compounds were purchased from Biopack®.

PCBs quantification

For PCBs quantification, fungal cultures from the degradation PCBs assays were harvested after 24 days of incubation with the chemical. Supernatants were first homogenized and the culture homogenates were acidified by the addition of 200 μl of H_2SO_4 1 mol l^{-1}. The PCBs were extracted from the supernatant by washing three times with 20 ml of hexane with agitation (250 rev min^{-1}, 25 ± 1°C) for 30 min (Mouhamadou *et al.* 2013). The chemicals were purchased from Anedra®.

Quantitative analyses of PCBs were performed using a gas chromatograph (Agilent 6890) equipped with a HP1 capillary column (Length: 30 m, ID: 0·23 mm, Film: 0·25 μm) and an electron capture detector (μECD). The abiotic losses were determined from cell-free controls incubated in parallel with fungal cultures. The reference material was 50 μg g^{-1} of AccuStandard Aroclor 1242, 1254 and 1260. The recovery of PCBs was obtained by:

$$\text{Recovery of PCBs}\,(\%) = \frac{A \times 100}{B} \qquad (2)$$

PCBs removal was calculated as follows:

$$\text{PCBs removal}\,(\%) = \left(1 - \frac{A}{B}\right) \times 100 \qquad (3)$$

where A was PCBs concentration in the supernatant after the mycoremediation treatment, and B was the concentration of PCBs in the supernatant of the heat-killed controls.

Toxicity assay

The toxicity test of samples after degradation in liquid media was performed with *Ganoderma* sp. LBM 001. This strain was selected due to its high sensitivity towards PCBs in previous studies (Sadañoski *et al.* 2018, 2019). Microbial toxicity was evaluated in Petri dishes containing 12 ml of supplemented sugarcane bagasse culture media (Sadañoski *et al.* 2019). Aliquots (100 μl) of supernatants of treatments and controls of 24 days were spread on the surface of the plates using a Drigalski spatula and one agar plug (5 mm ø) of *Ganoderma* sp. LBM 001 was inoculated. Radial growth was measured every 48 h and fungal growth was modelled using a logistic model (Equation 1) setting $D_{\max}$ to 50 mm which corresponds to the diameter of the Petri dishes. The cultures without inocula and spiked with PCBs were used as abiotic controls. Heat-killed controls were obtained by the addition of PCBs to autoclaved 7-day-old fungal cultures. The

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time needed to attain half of D_{max} (τ) was determined to compare the effectiveness of the detoxification process for every treatment.

Enzymatic activity

Ligninolytic activity in solid media was determined by adding specific substrates to MEA with PCBs to identify enzyme secretion. MEA plates were supplemented with following: 2,6-dimethoxyphenol (DMP) (1 mmol l^{-1}) to detect Lac activity (Fonseca *et al.* 2010); $MnCl_2.4H_2O$ (1 mmol l^{-1}) to estimate MnP (Jarosz-Wilkolazka *et al.* 2002) and Azure B (50 μmol l^{-1}) to assess LiP (Archibald 1992). The Lac activity was confirmed when an orange–yellow staining zone appeared corresponding to the oxidation of DMP to 2,2′,6,6′-tetramethoxydibenzo-1,1′-diquinone (Beck *et al.* 2018). MnP activity was observed as a brown staining zone due to MnO_2 precipitation (Perez and Jeffries 1992). In the case of LiP activity, a decolourized zone appeared when the dye was degraded by the enzyme. Radial extension of the mycelium and the halos resulting from enzyme activity were measured every 24 h until complete coverage of the Petri dish. The chemicals were purchased from Sigma-Aldrich®.

Lac activity was determined at 8, 16 and 24 days of incubation from supernatants of GA cultures with PCBs. Mycelium was separated from the supernatant by centrifugation at 4700 ***g*** for 10 min. Lac activity from liquid culture supernatant was measured using DMP 5 mmol l^{-1} as substrate in sodium acetate buffer 0·1 mol l^{-1} (pH 3·6) at 30°C. The change in absorbance was monitored at 469 nm (ϵ469 = 27·5 $mmol^{-1}$ l cm^{-1}) (Fonseca *et al.* 2010). The enzyme activities were expressed as international units (U), defined as the amount of enzyme needed to produce 1 μmol product min^{-1} at 30°C. The activity was expressed as U l^{-1}.

To establish the enzymatic profile and the molecular weight of Lac isoenzymes, the supernatants were subjected to SDS-PAGE (12% w/v) followed by renaturation and detection with DMP (Murugesan *et al.* 2006; Fonseca *et al.* 2015). The profile was compared to a molecular weight marker (Amersham, ECL Rainbow Marker-Full Range, GE Healthcare).

Statistical analysis

All assays were performed in triplicates. The data were processed using Microsoft Excel and the statistical analysis was performed using Statgraphics Centurion XVI.I software. The Anderson-Darling test was used to test normality and the Bartlett test was used to test the homogeneity of variances. Data that assumed a normal distribution were submitted to the one-way ANOVA test followed by Tukey tests for analyses with more than two groups. Results were presented as the mean ± SD.

Results

Consortium tolerance towards PCBs

Tolerance was determined using predictive mycology. There was no statistically significant effect of treatment with mono-culture and co-culture to k and τ ($P > 0·05$). Nevertheless, the strain and the presence of PCBs had a statistically significant effect on k and τ values ($P < 0·05$) (Table 1).

To assess consortium tolerance to PCBs, $\Delta\tau$ values were analysed. It was observed a statistically significant effect of fungi and treatment for mono-cultures and co-cultures ($P < 0·05$). In general, it was detected a decrease in PCBs tolerance to co-culture compared to mono-culture ($P < 0·05$) (Table 2). Figure 1 shows the fungal strains growth and the fitting corresponding to Equation 1, where it is possible to identify clearly the sensitivity of *P. pulmonarius* LBM 105.

In the consortium without PCBs, both *T. sanguinea* LMB 023 and *P. pulmonarius* LBM 105 had similar growth rate (Table 1), meaning that, in the Petri dish, each species could occupy half of the whole space. It is important to point out that *T. sanguinea* LBM 023 and *P. pulmonarius* LBM 105 had a similar growth behaviour, as observed in the Fig. 1a, where growth curves are

Table 1 Fungal τ (time to attain a half of the maximum diameter) (days) and k of velocity of growth (diameter of growth/time) on solid media for *Trametes sanguinea* LBM 023 and *Pleurotus pulmonarius* LBM 105 for mono-culture and co-culture in presence (with) and absence (without) PCBs

Fungi	PCBs	Treatment	k	T
Trametes sanguinea	With	Co-culture	$1·22 \pm 0·09^{b}$	$1·85 \pm 0·06^{b}$
Trametes sanguinea	With	Mono-culture	$1·35 \pm 0·11^{bc}$	$1·69 \pm 0·06^{ab}$
Trametes sanguinea	Without	Co-culture	$1·29 \pm 0·06^{bc}$	$1·72 \pm 0·04^{ab}$
Trametes sanguinea	Without	Mono-culture	$1·43 \pm 0·08^{c}$	$1·63 \pm 0·04^{a}$
Pleurotus pulmonarius	With	Co-culture	$1·00 \pm 0·03^{a}$	$2·15 \pm 0·03^{c}$
Pleurotus pulmonarius	With	Mono-culture	$1·00 \pm 0·03^{a}$	$2·10 \pm 0·03^{c}$
Pleurotus pulmonarius	Without	Co-culture	$1·32 \pm 0·07^{bc}$	$1·78 \pm 0·04^{ab}$
Pleurotus pulmonarius	Without	Mono-culture	$1·43 \pm 0·06^{bc}$	$1·63 \pm 0·04^{b}$

The data are the mean ± SD. Means with different letters are significantly different ($P < 0·05$).

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Table 2 Fungal $\Delta\tau$ on solid media for *Trametes sanguinea* LBM 023 and *Pleurotus pulmonarius* LBM 105 for mono-culture and co-culture. The data are the mean ± standard deviation. Means with different letters are significantly different ($P < 0.05$)

Fungi	Treatment	$\Delta\tau$
Trametes sanguinea	Mono-culture	0·06 ± 0·01[a]
Trametes sanguinea	Co-culture	0·14 ± 0·06[b]
Pleurotus pulmonarius	Mono-culture	0·28 ± 0·01[c]
Pleurotus pulmonarius	Co-culture	0·37 ± 0·01[d]

almost overlapped. However, the mycelial growth decreased for *P. pulmonarius* LBM 105 in PCBs presence in mono-culture and co-culture owing to *T. sanguinea* LBM 023 keeping the same growth rate (Fig. 1b). For this reason, *T. sanguinea* LBM 023 could colonize most of the medium as observed in Fig. 1b.

In the interspecific interactions, mycelial morphology changed following contact in both presence and absence of PCBs. A mycelial barrage was observed at the interaction zone between *T. sanguinea* LBM 023 and *P. pulmonarius* LBM 105. A bright yellow–orange pigment was present in the agar in the interaction zone just after contact (Fig. 1).

Mycoremediation of PCBs

Pleurotus pulmonarius LBM 105 was capable of removing 95·39 ± 0·42% of PCBs in GA media after 24 days in mono-culture. There were no significant differences between the removal percentages of PCBs by *T. sanguinea* LBM 023 (59·86 ± 11·35%) and the binary consortium (47·13 ± 2·22%) ($P > 0.05$). PCBs removal is displayed as the sums of Aroclors compared to heat-killed controls.

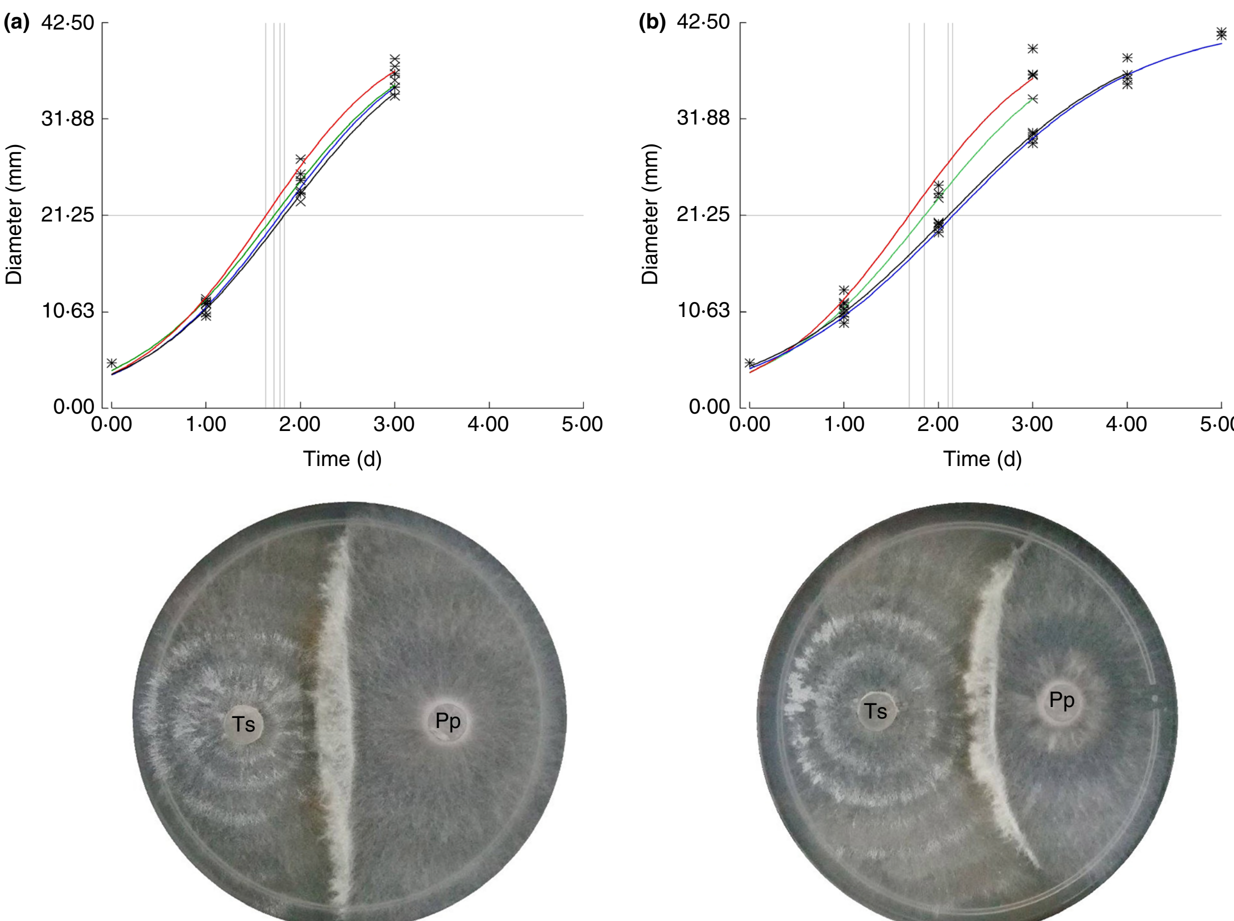

Figure 1 Fungal growth modelling of *Trametes sanguinea* LBM 023 (Ts) and *Pleurotus pulmonarius* LBM 105 (Pp) co-culture in solid media. (a) Co-culture in MEA plate without PCBs. (b) Co-culture in MEA plate with PCBs. (●) *T. sanguinea* LBM 023 in mono-culture. (●) *T. sanguinea* LBM 023 in co-culture. (●) *P. pulmonarius* LBM 105 in mono-culture. (●) *P. pulmonarius* LBM 105 in co-culture. [Colour figure can be viewed at wileyonlinelibrary.com]

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Culture media spiked with acetone proved that this solvent did not show signs of toxicity towards the strains.

Microbial toxicity revealed that mycoremediation with mono-cultures and binary consortium reduced the toxic effect of PCBs, mainly through mycelia adsorption considering that lower toxicity levels were detected for heat-killed cultures (Fig. 2).

Enzymatic activity

The activity of Lac, MnP and LiP enzymes was monitored in solid culture media to observe a possible relation between degradation of the PCBs mixture and activity of these ligninolytic enzymes (Fig. 3). Regarding Lac activity, the yellow stained zone characteristic of DMP oxidation was noticeable since the first hours of incubation in cultures of *P. pulmonarius* LBM 105. The enzyme activity expanded radially from the edge of the colony following its growth. The diameter, time of appearance and colour intensity of the halos formed by the oxidation of DMP remained the same under all conditions. In the dual cultures, the colour intensity was higher in the contact zone of mycelia. As for MnP, a brown halo corresponding to the precipitation of MnO_2 was observed for *P. pulmonarius* LBM 105. The staining zone appeared and expanded radially from the centre of the colony without reaching the edge. MnP activity was identified sooner in co-cultures (3rd day) than in the single culture (4th day). Colour intensity of the halos was unaltered throughout the different cultures. MnP activity was not registered in *T. sanguinea* LBM 023 cultures. LiP activity was detected in cultures of *P. pulmonarius* LBM 105. In the mono-cultures, decolourization started on the edge of the colony, whereas for co-cultures, the Azure B degradation began in the mycelia contact zone. In the case of *T. sanguinea* LBM 023, LiP activity was detected in co-cultures with and without PCBs. Complete decolourization of the plates was only achieved in co-culture. Lac extracellular activity was noticeable for all cultures from 16 d in liquid culture media. *T. sanguinea* LBM 023, *P. pulmonarius* LBM 105 and co-culture reached peak Lac activity with $548{\cdot}4 \pm 35{\cdot}5$, $425{\cdot}7 \pm 23{\cdot}2$ and $167 \pm 27{\cdot}3$ U l^{-1}, respectively, at 24 days (Fig. 4). The two-way ANOVA outcome showed enzyme activity to be significantly influenced by the treatment (df = 2; $F = 215{\cdot}73$; $P < 0{\cdot}05$), time (df = 2; $F = 587{\cdot}31$; $P < 0{\cdot}05$) and their interaction (df = 4; $F = 148{\cdot}79$; $P < 0{\cdot}05$).

SDS-PAGE analyses with DMP from supernatants revealed that Lac enzymatic patterns varied with incubation period (Fig. 4). A Lac of high electrophoretic mobility (35 kDa) was distinguished after 8 days of incubation of *P. pulmonarius* LBM 105. This fungus secreted three isozymes of 78, 53 and 35 kDa at 16 and 24 days of culture. Both *T. sanguinea* LBM 023 and co-culture secreted a Lac of 47 kDa at 24 days of incubation. The pattern observed for Lac activity on the polyacrylamide gel correlated with the activity determined by spectrophotometer.

Discussion

The tolerance method applied in this study, as a strategy to identify tolerant fungal consortiums to PCBs mixtures, opened the possibility of identifying the combination of strains which could be suitable to be applied in contaminated matrices. This innovative method has been used successfully in other works as a part of a low time-consuming and health security model, complemented with conventional microbiological selection techniques based

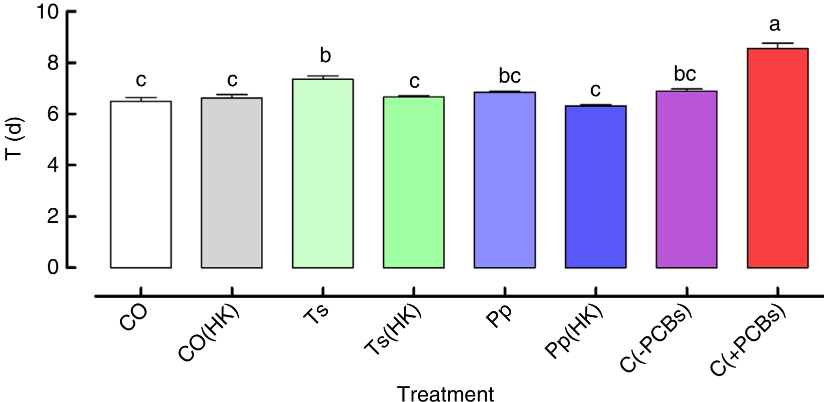

Figure 2 Toxicity after mycoremediation treatments using τ (*d*) values of *Ganoderma* sp. LBM 001 as indicator. Binary consortium (CO), *Trametes sanguinea* LBM 023 in mono-culture (Ts), *Pleurotus pulmonarius* LBM 105 in mono-culture (Pp) after 24 days of incubation with 217 mg l^{-1} of PCBs. CO(HK), Ts(HK) and Pp(HK) correspond to heat-killed controls, C(+PCBs) and C(-PCBs) are the abiotic controls with and without PCBs, respectively. The results are the means of the replicates and the error bars represent the standard deviations. [Colour figure can be viewed at wileyonlinelibrary.com]

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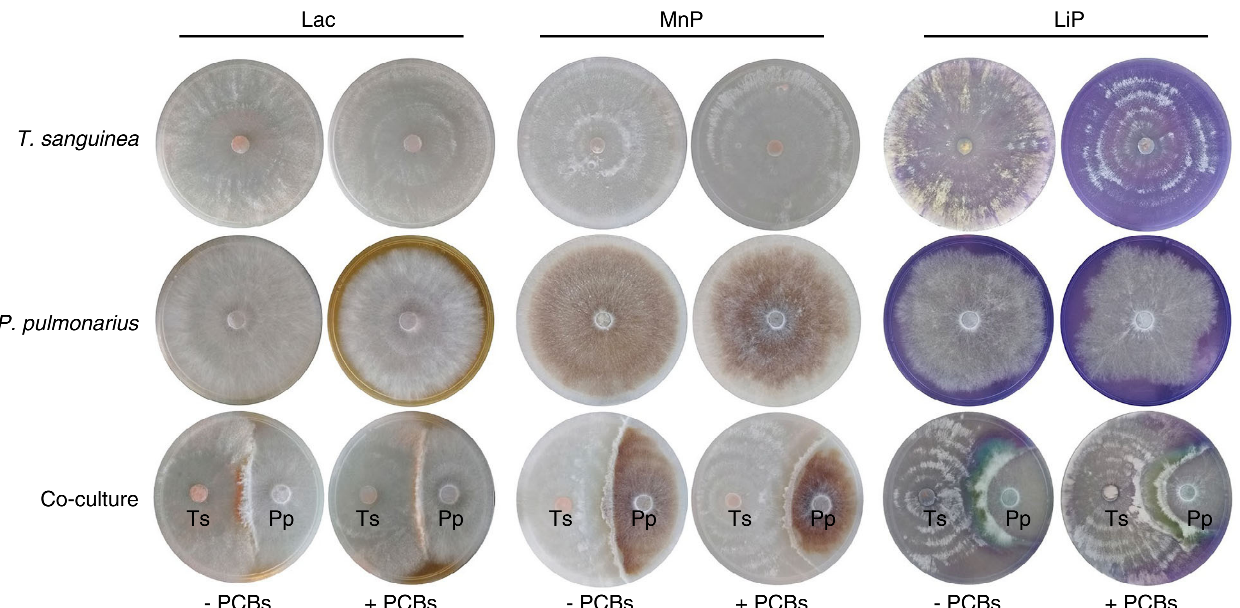

Figure 3 Ligninolytic enzyme secretion of *Trametes sanguinea* LBM 023 (Ts), *Pleurotus pulmonarius* LBM 105 (Pp) and their consortium in solid culture media with (+ PCBs) and without (− PCBs) PCBs. [Colour figure can be viewed at wileyonlinelibrary.com]

on the use of predictive mycology to mono-cultures (Bevilacqua *et al.* 2017; Sadañoski *et al.* 2018; Fonseca *et al.* 2020; Velázquez *et al.* 2021). However, to our knowledge, this is the first study that introduces the ratio of the coefficient of growth rate (k) as a better approach to predict the inhibition effect based on the fungal halo to select fungal consortiums.

The lower tolerance of binary consortium respect of mono-cultures could be associated with competition. This type of interaction is common between WRF (Boddy 2000). The pigmented mycelial barrage formed at the interaction zone of co-cultures with and without PCBs could be related to direct combative interactions between mycelia to defend or compete for resources. These changes in mycelial morphology are always accompanied by the secretion of extracellular metabolites such as alcohols, aldehydes, ketones, terpenes and aromatic compounds (Evans *et al.* 2008). Luo *et al.* (2017) revealed that metabolic pathways of carnitine, lipid, ethylene and trehalose are all involved in defensive response to abiotic and/or biotic stressful conditions among WRF interactions. These common metabolic reactions in WRF in response to stressful conditions in the interaction zone probably affect the cell wall synthesis, osmolyte production and carbon/energy regulation in the interaction zones. Another extracellular signalling and metabolic pathway which is induced during the interspecific interaction are some oxidative enzymes (as laccases and peroxidases), to mediate the oxidative stress by removing reactive oxygen species (Ferreira *et al.* 2006). With respect to the domination by *T. sanguinea* LBM 023 in binary consortium with PCBs, it could be applied the Hutchinson niche concept where PCBs mixtures act as stressors and contribute to shape fungal populations, selecting for traits that allow individuals to survive and compete under specific conditions (Hutchinson 1957). In addition, there are genetic capacity for stress tolerance from every fungal phyla and subphyla (Treseder *et al.* 2014). Particularly, the genera *Trametes* sp. and *Pleurotus* sp., belong to the phylum Basidiomycota and subphylum Agaricomycotina which are reported as organic pollutant degraders. In general, these fungi are saprotrophs, ectomycorrhizal symbionts, pathogens of plants and animals, and parasites of other fungi (Harms *et al.* 2011). These wood-inhabiting WRF such as *Pleurotus* spp. (in the order Agaricales) and *Trametes* spp. (in the order Polyporales) secrete oxidative exoenzymes used in lignin and lignocellulose decomposition to mineralize numerous organic chemicals co-metabolically as PAHs and PCP (Tuomela *et al.* 1998; Sekan *et al.* 2019). Fungi of *Pleurotus* genus have been proved to be able to degrade PCBs successfully in liquid culture and contaminated soils in laboratory scale, indicating the feasibility of using these fungi for bioremediation applications (Stela *et al.* 2017; Sadañoski *et al.* 2019; Šrédlová *et al.* 2020). Fungi belonging to *Trametes* genus also demonstrated their capacity of biodegradation of PCBs (Sadañoski *et al.* 2020a, 2020b).

With respect to the bioremediation using microbial consortiums, different outcomes are reported in the

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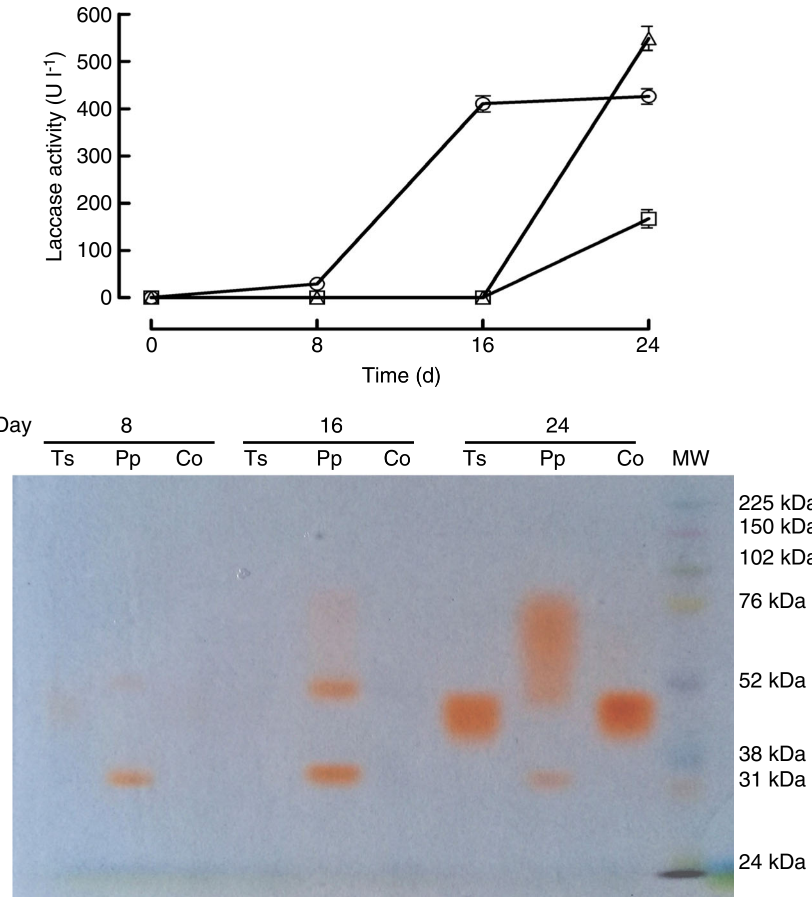

Figure 4 Lac enzyme secretion during PCBs treatment in liquid culture media. *Trametes sanguinea* LBM 023 (△), *Pleurotus pulmonarius* LBM 105 (○), binary consortium (□). The data are the mean ± standard deviation. Below, the isoenzyme profile obtained by SDS-PAGE for *T. sanguinea* LBM 023 (Ts). *Pleurotus pulmonarius* LBM 105 (Pp) and the binary consortium (Co). MW, molecular weight marker. [Colour figure can be viewed at wileyonlinelibrary.com]

literature. Kuhar *et al.* (2015) observed an improvement of malachite green decolourization and detoxification when applying a co-culture of *Trametes versicolor* and *Ganoderma lucidum* in solid state fermentation. Comparable results were obtained by Kumari and Naraian (2016) where the co-culture of *Rhizoctonia solani* and *Pleurotus florida* was more efficient for the decolourization of brilliant green in liquid culture. Conversely, Correa *et al.* (2018) determined that the degradation efficiency decreased when using WRF co-cultures for the removal of a binary mixture of dyes, owing to competition for space and nutrients between the species. Similar findings were reported by Jimenez *et al.* (2019); they hypothesized that low-molecular-weight mediators necessary for the catalytic activity were missing in co-culture or that inhibitors of the degradation reaction were synthesized. Therefore, it is feasible to assume that the more efficient bioremediation of PCBs by individual species could be explained by some factors that affect the behaviour of the individual species and the consortium. Furthermore, these results show that co-culture bioremediation effectiveness depends on the particular species kept in co-culture and the cultivation conditions (Chi *et al.* 2007; Avelino *et al.* 2020).

Microbial toxicity employing *Ganoderma* sp. LBM 001 demonstrated to be simple, fast, inexpensive, and only a small quantity of sample was needed to induce physiological changes to evaluate the formation of toxic metabolites by the mycoremediation of PCBs. Treatments with monoculture and binary consortium provided evidence that toxicity of intermediates produced during the biodegradation process was lower than the parental pollutant. Considering that lower toxicity levels were observed for heat-killed cultures, toxicity reduction could be attributed mainly to the biosorption process of the inactivated mycelium. In this sense, fungal cell walls are complex macromolecular structures consisting of chitins, glucans, mannans, proteins and other polysaccharides. This variety of components provides many functional groups that can engage in sorption processes (Gadd 2009; Harms *et al.* 2011). Biosorption influences bioavailability of substances and therefore their distribution, transport and ultimate fate in the environment. Thus, biosorption is considered an advantage when the organic pollutant and/or its metabolic products are extremely recalcitrant to biodegradation (Gadd 2009).

Only a few works evaluating PCBs degradation by WRF performed microbial toxicity assays (Čvančarová

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et al. 2012; Stella *et al.* 2017; Sadañoski *et al.* 2019). Čvančarová *et al.* (2012) investigated acute toxicity after PCBs treatment in liquid culture for several WRF. They observed that only *P. ostreatus* was able to decrease toxicity significantly; they suggest that the other fungal treatments lead to the accumulation of toxic metabolites, such as chlorobenzoic acids.

Fungal co-culture is often associated with improved enzymatic activities (Dwievedi *et al.* 2011; Zhong *et al.* 2018). Authors have suggested that co-culturing could cause oxidative stress accelerating fungal metabolic switch to secondary metabolism, thus stimulating production of ligninolytic enzymes (Ijoma and Tekere 2017). The higher colour intensity in the co-culture contact zone in the agar plates assays could suggest higher Lac activity; similar results were reported by Kuhar *et al.* (2015) when they confronted *G. lucidum* to *T. versicolor*. In the case of peroxidases, not only activity was detected sooner in dual cultures but also LiP activity improved under this condition as evidenced by complete decolourization of the MEA plate supplemented with Azure B. Peroxidases are suitable catalysts for bioremediation, as they exhibit broad substrate specificity and high redox potential (Ali *et al.* 2019). Qi-he *et al.* (2011) evaluated WRF for ligninolytic enzyme production and probed that co-culture of *Pleurotus ostreatus* and *Phlebia radiata* improved MnP and LiP secretion. Authors observed that co-cultures can achieve stable expression of LiP even when single strains cultures showed no capability for LiP production (Ijoma and Tekere 2017). The low specificity of fungal enzymes enables these organisms to co-metabolize structurally distinct compounds belonging to different pollutant classes, including PCBs congeners (Elangovan *et al.* 2019).

Previous results demonstrated that Lac parameters were the main variables which described the variability among WRF tolerant to PCBs (Sadañoski *et al.* 2018). Diverse studies have shown that Lac from WRF degrades PCBs congeners (Dietrich *et al.* 1995; Yadav *et al.* 1995; Novotny *et al.* 1997; Moeder *et al.* 2005; Li *et al.* 2018), although their exact role in the biodegradation process has not yet been fully elucidated (Field and Sierra-Alvarez 2008).

In contrast to Lac secretion in solid media, Lac secretion by the consortium was significantly lower than the mono-cultures in liquid culture with PCBs. This difference emphasizes the complexity of the physiological processes involved in Lac production, which depends on mode of cultivation and medium composition (Kuhar *et al.* 2015; Bentil *et al.* 2018). Moreover, co-culture in solid media can favour mycelia interaction; contact between the growing fungi has been observed to be of importance for Lac enhancement (Kuhar *et al.* 2015). Co-cultivation has usually proven to be efficient in increasing enzyme expression; however, Qi-he *et al.* (2011) and Chi *et al.* (2007) assayed several WRF combinations in liquid culture for Lac production. In both cases, they found mixed results with Lac induction or repression depending on the particular species combination.

The literature establishes that fungal Lac molecular mass generally ranges from 50 to 100 kDa (Mishra *et al.* 2019). For *P. pulmonarius* LBM 105, three isozymes were detected; two of them fall within this range. The third one was an atypical low-molecular-mass Lac (35 kDa). This Lac with high electrophoretic mobility was previously reported by the workgroup and was expressed only in the presence of PCBs at 21 days of culture (Sadañoski *et al.* 2019). To our best knowledge, there are no reports of a small Lac for *P. pulmonarius*. Wang and Ng (2006) purified a 34 kDa Lac from *Pleurotus eryngii*. They proposed that the unexpected low molecular mass was due to the enzyme having two domains instead of the typical three domains, similar to the small Lac found in bacteria (Gabdulkhakov *et al.* 2019). WRF often possess multiple Lac genes that are differentially regulated (Jiménez *et al.* 2019), which may have different biological roles (Nakamura and Go 2005). From this perspective, biodegradation efficiency by Lac enzymes might be related to versatility instead of higher secretion. The similar Lac profile between the culture media of *T. sanguinea* LBM 023 and the binary consortium could be due to the domination by *T. sanguinea* mentioned previously.

Establishing the bioremediation potential of an organism and/or its enzymatic machinery represents the first step for the design of an application strategy. In this regard, the following approach will depend on the subject matrix (e.g. soil, water, sludge) and the abilities and limitations displayed by the selected bioremediation agent (Vishnoi and Dixit 2019).

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Darío Zapata: Supervision, Funding Acquisition. María Isabel Fonseca: Review, Supervision, Project Administration.

Conflict of Interest

No conflict of interest declared.

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