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# Evaluation of bioremediation strategies for treating recalcitrant halo-organic pollutants in soil environments

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ABSTRACT

The aim of this study was to investigate the bioremediation potential of polychlorinated biphenyls (PCBs) in soil, mimicking three strategies: (a) mycoaugmentation: by the addition of *Trametes sanguinea* and *Pleurotus sajor-caju* co-cultures immobilized on sugarcane bagasse; (b) biostimulation: by supplementation of sugarcane bagasse; and (c) natural attenuation: no amendments. The experiments were done in microcosms using Ultisol soil. Remediation effectiveness was assessed based on pollutants content, soil characteristics, and ecotoxicological tests. Biostimulation and mycoaugmentation demonstrated the highest PCBs-removal (approx. 90%) with a significant toxicity reduction at 90 d. The studied strains were able to survive during the incubation period in non-sterilized soil. Laccase, manganese-peroxidase and endoxylanase activities increased significantly in co-cultures after 60 d. Sugarcane bagasse demonstrated to be not only a suitable support for fungal immobilization but also an efficient substrate for fungal colonization of PCBs-contaminated soils. Mycoaugmentation and biostimulation with sugarcane bagasse improved oxidable organic matter and phosphorous contents as well as dehydrogenase activity in soil. Therefore, biostimulation with sugarcane bagasse and mycoaugmentation applying dual white-rot fungal cultures constitute two efficient bioremediation alternatives to restore PCBs-contaminated soils.

## 1. Introduction

Halogenated organic compounds are persistent in natural and built environments. Regardless of restrictions on the production and utilization of many of these compounds in several countries through the Stockholm Convention on Persistent Organic Pollutants (POPs), various "legacy" compounds, including polychlorinated biphenyls (PCBs), are routinely detected in soils and sediments and they accumulate in the food chain, mainly in animals' fatty tissues (Pointing, 2001; Ondarza et al., 2014). PCBs are organic compounds, which contain a biphenyl molecule with multiple chlorides that form 209 different congeners. Their commercial production began in 1929 and they were introduced under a number of trade names and sold in many different countries. Arochlor was the most common trade name for PCBs in the United States, being the mixtures 1254 and 1260 historically used in Argentina (Bright et al., 1995). Their production was banned, but Arochlors still enter the environment due to accidentals spills and leaks from containing material or redistribution processes linked to their physicochemical properties.

Biological, physical, and chemical technologies were employed for PCBs removal (Gomes et al., 2013). Natural attenuation processes which include biological degradation by on-site bacteria, plants and fungi, as well as abiotic processes such as volatilization, dilution, and sorption of contaminants onto organic matter and clay minerals in soil, can be

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applied to treat PCBs-polluted soils as well (Declercq et al., 2012; Kaya et al., 2019; Zhang et al., 2019). Additionally, among biological techniques, mycoaugmentation with white-rot fungi constitutes an interesting strategy for treating contaminated soils. Their bioremediation potential is based on fungal growth via hyphal extension, allowing them to penetrate across environmental matrices and act as dispersion vectors of both pollutant-degrading bacteria and pollutants, and the secretion of low substrate specificity oxidative lignin-modifying enzymes that can degrade a wide range of organic pollutants (Sharma et al., 2018). *Coriolus versicolor* (Cloete and Celliers, 1999), *Phlebia brevispora* (Kamei et al., 2006), *Pleurotus ostreatus* (Gayosso-Canales et al., 2011; Čvančarová et al., 2012), *Irpex lacteus*, *Bjerkandera adusta*, *Pycnoporus cinnabarinus*, *Phanerochaete magnoliae* (Čvančarová et al., 2012), *Pleurotus sajor-caju* (Sadañoski et al., 2019) and *Trametes sanguinea* (Sadañoski et al., 2020) demonstrated their ability to remove PCBs. Nevertheless, the application of white-rot fungi under nonsterile conditions is still a technical challenge, because native or contaminating bacteria grow faster than the fungi and compete for carbon sources and nutrients (Gao et al., 2010). In order to convert complex PCBs mixtures into less toxic substances, one of the most promising bioremediation strategies is combining strains as co-cultures. Although numerous sediments and soil-based studies have been conducted with monocultures, using potential PCBs-degrading fungi (Borazjani et al., 2005; Federici et al., 2012a; Stella et al., 2017), those based on consortia are limited to bacteria and yeast (Liz et al., 2009; Chen et al., 2015). In addition, the degradation of commercial PCBs mixtures with varying degree of chlorination is difficult, and a single fungal strain or monoculture is not capable of attacking all or even most of the PCBs congeners present in contaminated environments (Field and Sierra-Alvarez, 2008). Thus, the use of dual white-rot fungal cultures could be an interesting strategy that combines the capabilities of different fungi in order to attain complete degradation of the PCBs and their metabolites. A co-culture (alternatively named i.e. mixed culture or dual culture) is a synthetic biology system, in which two or more different populations of cells are cultivated with a certain degree of contact between them (Goers et al., 2014). It was observed that fungal co-cultivation may lead to variations in the enzymatic machinery secreted, synergistic interaction of metabolic pathways, better substrate utilization, increased productivity, adaptability and resistance to contamination (Ijoma and Tekere, 2017).

Sugarcane bagasse is the fiber that remains after the sugars have been extracted. It is a by-product of the sugarcane industry composed of cellulose, hemicelluloses, lignin, and small amounts of extractives and mineral salts, constituting an efficient and cost-effective source of nutrients (Pandey et al., 2000). This agro-industrial residue is important in Latin American countries due to its abundant availability (Martín-Lara et al., 2010). Several studies demonstrated that biostimulation with this lignocellulosic material successfully improved soil bioremediation treatment. It can be used as soil amendment to enhance soil hydro, physical-chemical characteristics and support the growth of native microorganisms responsible for pollutants decomposition (Hamzah et al., 2014; Babaei et al., 2020).

The objective of this study was to compare three strategies for the bioremediation of soils contaminated with PCBs: (a) mycoaugmentation with dual white-rot fungal cultures immobilized on sugarcane bagasse; (b) biostimulation with sugarcane bagasse; and (c) natural attenuation. Remediation effectiveness was assessed based on pollutants residual content, soil characteristics, and ecotoxicological tests.

## 2. Materials and methods

### 2.1. PCBs, sugarcane bagasse and soil

Transformer oil containing a mixture of Arochlor 1242, 1254 and 1260, obtained from Kioshi S.A. (Buenos Aires, Argentina) was used in this work. All the solvents were purchased from Merck or Cicarelli (Santa Fe, Argentina) and were of p.a. quality, trace analysis quality or gradient grade. Sugarcane bagasse was a by-product of the 2017 harvest from the Sugar Mill San Javier (Misiones, Argentina). It was air-dried, grinded in an electric mill, sieved and homogenized to obtain 0.4 mm particle sizes. Non-polluted soil from the upper 5–15 cm was collected from an experimental site near the city of Posadas, Misiones, Argentina where soils are deep, red colored, belonging to the order Ultisol, composed mainly by clay and well-structured due to the presence of aluminum and iron oxides (Moscatelli, 1990). Samples were dried at 35 °C, sieved (1.7 mm sieve), homogenized and stored in darkness at 10–15 °C until use. The main properties of the soil were pH, 5.81; organic carbon, 2.66 ± 0.32%; oxidable organic matter, 4.58 ± 0.55%; available phosphorus, 15.11 ± 4.62 mg $Kg^{-1}$; aluminum, 0.02 ± 0.01 mEq 100 $g^{-1}$ and soil dehydrogenase activity (DHA), 0.19 ± 0.01 mIU $g^{-1}$.

### 2.2. Bioremediation assay

#### 2.2.1. Microorganisms and inoculum preparation for mycoaugmentation treatments

*T. sanguinea* LBM 023 and *P. sajor-caju* LBM 105 were isolated in Misiones (Argentina). These white-rot fungal species were selected because of their high efficiency in PCBs removal demonstrated in previous investigations in liquid media (Sadañoski et al., 2018, 2019) and are deposited in the culture collection of the Instituto de Biotecnología Misiones 'María Ebe Reca', Universidad Nacional de Misiones, Argentina.

Inoculum was prepared in 500 mL Erlenmeyer flasks containing 8 g of sugarcane bagasse sterilized at 121 ± 1 °C 30 min, and adjusted to 75% (w $w^{-1}$) moisture content with sterile deionized water. Six mycelial plugs (0.5 mm Ø) from 5-7-d-old MEA (malt extract, 12.7 g $L^{-1}$; agar, 20 g $L^{-1}$) colonized by *T. sanguinea* LBM 023 or *P. sajor-caju* LBM 105 were inoculated in sugarcane bagasse for monoculture treatments. Co-cultures were prepared by seeding three mycelial plugs from each strain in sugarcane bagasse. Inoculated and non-inoculated sugarcane bagasse, used respectively for mycoaugmentation and biostimulation treatments, were incubated at 25 ± 1 °C during 10 d (Stella et al., 2017).

#### 2.2.2. Experimental design

Biotreatments were carried out in glass pots with non-sterile soil adjusted to 60% (w $w^{-1}$), moisture content. Soil samples (48 g) were spiked with transformer oil contaminated with PCBs. The PCBs congeners concentration are shown in Table 1 and it was selected because it was in the range of previously reported soil PCB levels (Dudášová et al., 2016; Stella et al., 2017; Cappelletti et al.,2018; Horváthová et al., 2018). Three test microcosms were operated: (i) the mycoaugmentation set was prepared by mixing polluted soil with either *T. sanguinea* LBM 023, *P. sajor-caju* LBM 105 or the co-culture colonized substrate, to reach a final soil: sugarcane bagasse mass ratio of 3:1 (w $w^{-1}$). (ii) The biostimulation set included soil samples mixed with non-inoculated sugarcane bagasse prepared in the same ratios as described before. (iii) Non-amended soil (containing only distilled water and PCBs) was used to verify natural attenuation. All the experiments were performed in triplicate under non-sterile conditions and incubated 90 d in darkness at 25 ± 1 °C, deionized water was added periodically to keep 75% (w $w^{-1}$) moisture content constant. Three replicates of each treatment were harvested after 0, 14, 42, 60 and 90 d of incubation. A soil sample (<1 g) was used for the viability assay and the rest was air dried and kept at -20 °C for toxicity analysis and PCBs determination or at room temperature until enzymatic quantification and chemical soil characterization.

### 2.3. Fungal survival

In order to verify fungal survival, strains inoculated in the microcosms at the beginning of each treatment was isolated by direct plating method (Kazerooni et al., 2017). Briefly, soil samples were placed on MEA supplemented with sugarcane bagasse, 1 g $L^{-1}$; ampicillin, 0.01 g $L^{-1}$; and carbendazim 50, 0.01 g $L^{-1}$, in order to control bacterial and

**Table 1**
Concentrations of PCBs congeners in soil and percentile representation of each congener in Arochlor 1242, 1254 and 1260 mixtures. Values are expressed as means ± standard deviation.

| Congener Number | IUPAC Name | Concentration in soil (μg $g^{-1}$) | Representation in the mixture (% w $w^{-1}$) | | |
|---|---|---|---|---|---|
| | | | Arochlor 1242 | Arochlor 1254 | Arochlor 1260 |
| 18 | 2,2′,5-Trichlorobiphenyl | 0.863 ± 0.096 | 1.242 ± 0.242 | | |
| 52 | 2,2′,5,5′-Tetrachlorobiphenyl | 1.349 ± 0.060 | 0.975 ± 0.154 | 0.975 ± 0.154 | |
| 66 | 2,3′,4,4′-Tetrachlorobiphenyl | 4.412 ± 0.701 | 3.137 ± 0.074 | 3.137 ± 0.074 | |
| 87 | 2,2′,3,4,5′-Pentachlorobiphenyl | 3.103 ± 0.131 | | 4.476 ± 0.407 | |
| 101 | 2,2′,4,5,5′-Pentachlorobiphenyl | 7.531 ± 0.209 | | 10.876 ± 1.111 | |
| 110 | 2,3,3′,4′,6-Pentachlorobiphenyl | 9.628 ± 6.839 | | 13.128 ± 7.059 | |
| 153 | 2,2′,4,4′,5,5′-Hexachlorobiphenyl | 13.157 ± 0.634 | | | 19.145 ± 1.703 |
| 180 | 2,2′,3,4,4′,5,5′-Heptachlorobiphenyl | 17.469 ± 1.182 | | | 25.414 ± 2.147 |
| 187 | 2,2′,3,4′,5,5′,6-Heptachlorobiphenyl | 12.035 ± 0.900 | | | 17.496 ± 1.345 |
| Sum | | 69.547 ± 9.799 | | | |

fast fungal growth respectively, and were incubated at 28 ± 1 °C. Colonies with macroscopic and microscopic basidiomycetes characteristics were re-isolated and identified by amplifying, cloning and further sequencing fragments corresponding to the internal transcribed spacer (ITS) region in their total genomic DNA, using the primer sequences described by White et al. (1990). PCR was carried out in 20 μL reactions containing Taq buffer, 1 X; $MgCl_2$, 3 Mm; dNTPs, 0.2 mM; primers, 10 pmol; Taq polymerase, 1 U; and DNA 60 ng. The PCR program was as follows: 4 min at 94 °C; 40 rounds of 30 s, 94 °C; 30 s, 55 °C; and 30 s, 72 °C; and final extension for 10 min at 72 °C. All fragments were then submitted to a sequencing service (Macrogen, Korea). The reference databases of the referring ITS1–ITS4 fragment were retrieved from the NCBI website (https://www.ncbi.nlm.nih.gov). A homology search was performed by using the BLAST software tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare nucleotide acid sequence similarities against the GenBank database. The sequencing results of PCR products were edited, aligned, and analyzed, with the respective sequences in the reference database, using BioEdit Sequence Alignment Editor and Clustal W. Two dendrograms were constructed (for *Trametes* and *Pleurotus*) with MEGA 7 (Kumar et al., 2016) by the Neighbor-join (NJ) method with a 1000 repetition boostrap. *Trichoderma* sp. was used as outgroup in both dendrograms.

### 2.4. Soil analysis

#### 2.4.1. Soil chemical characterization

The soil was chemically analyzed following the techniques detailed by Marban and Ratto (2005). Soil pH was measured in a solution with a soil-water ratio of 1:2.5 using a pH meter HANNA Hi2221. The moisture content of the soil was gravimetrically measured by drying 1 g of soil sample at 105 °C for 24 h. Total organic carbon (OC) was determined by the method of Walkey and Black (1934). Available phosphorus was extracted with sodium bicarbonate 0.5 M pH 8.5 in a 1:10 ratio and quantified by reaction with Molybdenum Blue (Mo), and subsequent reduction of the Mo complex with ascorbic acid. The resulting color was measured at 880 nm. Aluminum was extracted with potassium chloride 1 M and quantified spectrophotometrically at 395 nm.

#### 2.4.2. Soil enzymes

Soil samples were extracted with sodium phosphate buffer (25 mM, pH 7.0) (Anasonye et al., 2014). Laccase (Lac) activity was measured at 30 ± 1 °C using 5 mM 2,6-dimethoxyphenol (DMP) as substrate in sodium acetate buffer 0.1 M (pH 3.6), at 469 nm ($\varepsilon 469 = 27.5\ mM^{-1}\ cm^{-1}$) (Saparrat et al., 2002). Mn-peroxidase (MnP) activity was determined at 30 ± 1 °C by monitoring the oxidation of DMP 5 mM in sodium acetate buffer 0.1 M (pH 3.6), $MnSO_4$ 0.7 mM, and $H_2O_2$ 0.1 mM at 469 nm (Heinzkill et al., 1998). Endoxylanase (EX) and endoglucanase (EG) activities were measured by the release of reducing sugars at 50 °C using beech wood xylan 2% (w $v^{-1}$); or carboxymethylcellulose in sodium acetate buffer, 50 mM (pH 4.8); respectively as substrates. Reducing sugars were determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959).

To assess Lac isoenzymes molecular weight, an electrophoretic separation by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (12% w $v^{-1}$) was performed, followed by a subsequent renaturalization with Triton X-100, 0.2% (v $v^{-1}$). After protein separation, the gel was incubated in sodium acetate buffer, 0.1 M (pH 3.6), containing DMP 5 mM, for Lac activity detection (Fonseca et al., 2010). After 10 min of incubation, the solution was discarded, and the gel was immediately scanned with a Scanner HP Deskjet F300. Lac enzyme bands were compared with a molecular weight marker (Prestained Kaleidoscope SDS-PAGE standard broad range, BioRad). The electrophoretic run was performed at 120 V for 120 min in Tris-Glycine buffer 1.5 M (pH 8.3).

Additionally, DHA, frequently used as overall soil microbial activity indicator (Quilchano and Marañón, 2002; Wolinska and Stepniewsk, 2012), was evaluated following Trevors et al. (1982) method, modified by García et al. (1997). Briefly, 0.5 g of dried soil adjusted to 60% of field capacity was incubated for 24 h at 20 ± 1 °C in darkness with the addition of 2-piodophenyl-3 p-nitrophenyl- 5 tetrazolium chloride (INT) 0.4% (w $v^{-1}$), as substrate. The product formed: iodonitrotetrazolium formazan (INTF) was extracted with methanol and spectrophotometrically measured at 490 nm. Soils without substrate were used as controls and INTF concentration calculated by using a calibration curve.

The enzyme activities were expressed in milli-International Units (mIU), defined as the amount of enzyme required to produce 1 nmol of product per minute under the conditions tested (Federici et al., 2012a). These mIU were expressed per gram of wet soil (mIU $g^{-1}$) (Yanto et al., 2017). MnP activity was always corrected for Lac activity.

#### 2.4.3. Phytotoxicity test

Soil phytotoxicity after the different treatments (considering the possible presence of PCBs-mixture residues and degradation products), was determined measuring lettuce *(Lactuca sativa)* seeds germination, root elongation and hypocotyl growth. Twenty seeds were placed in sterile Petri plates containing 30 g of soil samples subjected to either biostimulation, mycoaugmentation or natural attenuation. Soils samples without PCBs were used as controls. Petri plates were sealed and incubated at 23 ± 1 °C in darkness, during 120 h. At the end of the incubation period, the number of germinated seeds was registered, and the length of roots and hypocotyls was measured (Aparicio et al., 2015).

#### 2.4.4. PCBs extraction and quantification from substrates

PCBs were extracted from the substrates according to Metcalfe and Metcalfe (1997). Subsamples of 3 g of dried substrate of every treatment were homogenized with anhydrous sodium sulfate (previously dried at 400 ± 1 °C), spiked with PCB #103 as internal standard and Soxhlet extracted with n-hexane: dichloromethane (50:50) for 6 h.

PCBs fraction was further purified using activated silica gel columns and extracts were concentrated and stored in 1 mL vials at - 20 °C prior

to Gas Chromatography (GC) analyses. PCBs (IUPAC 8, 18, 28, 52, 66, 87, 101, 110, 118, 138, 153, 156, 180, 187) were identified and quantified using a Shimadzu QP-2010 chromatograph with a mass detector (GC-MS), equipped with a ZB-5Msi capillary column (30 m, 0.25 mm i.d., 0.25 mm film thickness). One μL was injected in spit less mode at 300 °C. The oven temperature was programmed for an increase of 20 °C $min^{-1}$ to 160 °C, rise of 1.5 °C $min^{-1}$ to 240 °C, and then 30 °C $min^{-1}$ to 300 °C, held for 2 min. Ultra-high purity helium was used as carrier gas (21.7 mL $min^{-1}$) and nitrogen as make-up gas. Each congener was identified and confirmed by its retention time, in comparison with PCB 103 and by its majority ions (one for quantification and two for confirmation) considering a deviation of ±10%. Removal percentage was calculated as:

$$Removal(\%) = \left(1 - \frac{A}{B}\right) \times 100$$

Where A is the residual fraction of PCBs congeners after remediation and B is the total amount of PCBs congeners before remediation.

### 2.5. Statistical analysis

The Shapiro Wilk test and Bartlett's test were applied to ensure the normality and the homogeneity of variances assumptions, respectively (Shapiro and Wilk, 1965; Snedecor and Cochran, 1989). Data that assumed a normal distribution were submitted to the one-way ANOVA test, followed by Fisher's least significant difference (LSD) tests for analyses with more than two groups. The data that assumed a non-parametric distribution were analyzed by the Kruskal-Wallis test. The analyses were performed using the software InfoStat 2016 (Di Rienzo et al., 2016), considering 95% as a confidence interval ($p \leq 0.05$).

## 3. Results

### 3.1. Fungal survival evaluation

The studied strains were immobilized in sugarcane bagasse and mixed in contaminated soil. Both strains were capable of colonizing the soil and the agro-industrial residue during the incubation period. The ITS alignment was used to identify the fungal species recovered from direct plating technique at 90 d, with macroscopic and microscopic basidiomycetes characteristics. The sequence alignment obtained for the mycoaugmentation treatment with *T. sanguinea* LBM 023 and the co-culture with the database available at the NCBI, showed 99% of identity with *T. sanguinea* (KF850158, e-value:0). The results of the sequence alignment obtained for the mycoaugmentation treatment with *P. sajor-caju* LBM 105, with the database available at the NCBI, showed 99% of identity with *P. sajor-caju* (KY962450.1; e -value: 0) for ITS1-5.8S. Both sequences were aligned with sequences we previously obtained, registering 100% of identity. *P. sajor-caju* LBM 105 could not be isolated from the microcosm mycoaugmented with the co-culture.

### 3.2. Enzyme activities detected and soil chemical properties

Fig. 1 illustrates the enzyme kinetics of mycoaugmented microcosms. Lac (827.27 ± 177.35 mU $g^{-1}$), MnP (353.36 ± 175.95 mU $g^{-1}$) and EX (2776.34 ± 711.15 mU $g^{-1}$) activities exhibited similar response, with a peak at 60 d during co-cultivation ($p < 0.05$). Increased Lac titers were detected in dual cultures at 60 d (2.5 and 9-fold higher than the ones measured in monocultures of *T. sanguinea* LBM 023 and *P. sajor-caju*

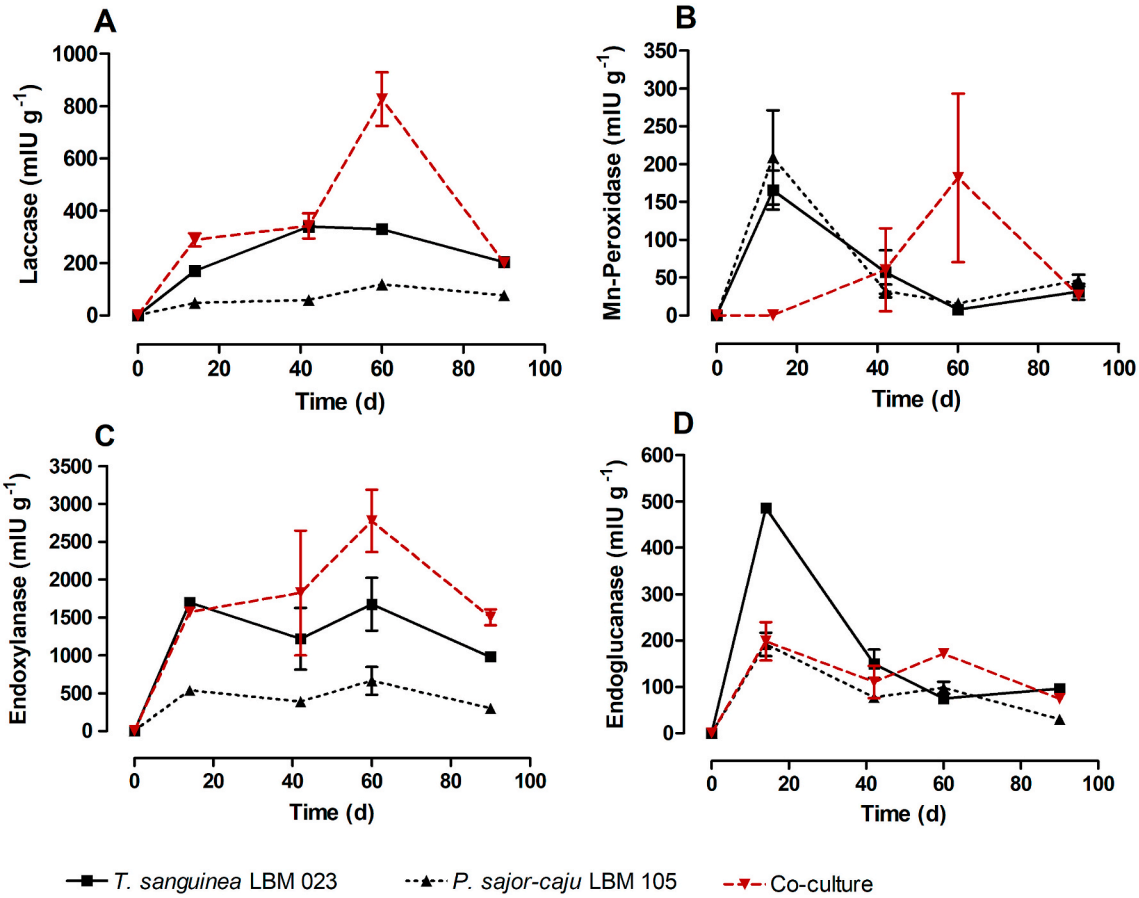


**Fig. 1.** Enzyme activities detected in mycoaugmented soils. Lac (Fig. 1 A), MnP (Fig. 1 B), EX (Fig. 1 C), EG (Fig. 1 D). Data are means ± standard deviations.

LBM 105, respectively) (Fig. 1 A). Moreover, MnP measured in co-culture was twice the registered in monocultures at 60 d (Fig. 1 B). EX activity increased as well in dual cultures (Fig. 1 C). Maximal EG activity was produced by *T. sanguinea* LBM 023 (486.04 ± 11.65 mU $g^{-1}$), approximately 2.5-fold higher than those measured in *P. sajor-caju* LBM 105 cultures and the co-culture on 14 d (Fig. 1 D). None of the enzymes evaluated were detected in the soil treated by natural attenuation.

To study the effect of co-culture on Lac protein secretion, PAGE profiles were obtained for all cultures (Fig. 2). A single Lac band of around 50 kDa was detected in cultures of *T. sanguinea* LBM 023 and in dual cultures, but despite distinguishing enzyme activity in soil, Lac bands were not revealed in zymograms of *P. sajor-caju* LBM 105.

Fig. 3 and Table 2 depict soil chemical modifications due to bioremediation treatments. A decrease in pH was observed in mycoaugmented soils, while those subjected to natural attenuation and biostimulation displayed similar pH values (Fig. 3). Biostimulation and mycoaugmentation treatments increased organic carbon and oxidable organic matter percentages ($p < 0.05$). A slight rise in phosphorous concentration in mycoaugmented soils and a decrease in biostimulated ones with respect to controls was detected as well ($p < 0.05$). Mycoaugmentation rise aluminum concentration. Highest DHA was observed in soil treated with *P. sajor-caju* LBM 105 but this activity increased in biostimulation and bioaugmentation assays in comparison with those exposed to natural attenuation ($p < 0.05$).

### 3.3. PCBs removal

The treatments evaluated decreased significantly the concentration of Arochlors (mainly 1242 and 1254) in contaminated soils after 90 d ($p < 0.05$) (Fig. 4). Dual culture mycoaugmentation removed 91% of the PCBs. While applying *P. sajor-caju* and *T. sanguinea* monocultures 90 and 88% PCBs-removal was detected, respectively. By biostimulation a decreased of 88% was also observed. No significant abiotic losses of PCBs were recorded during the 90 d of the assay. Most of PCBs congeners decreased over 80% in soil treated by biostimulation and mycoaugmentation. Conversely, after 90 d of natural attenuation still high concentrations of PCBs were detected (Fig. 4B).

### 3.4. Phytotoxicity

Changes in soil toxicity were evaluated during bioremediation assays using the *L. sativa* toxicity test (Fig. 5). Radicle and hypocotyl lengths as well as germination percentages were similar in control soils and those subjected to co-culture bioaugmentation and biostimulation treatments. Results showed that 73% of the seeds germinated in control soil (without PCBs). Seedling germination was not observed in soil subjected to natural attenuation.

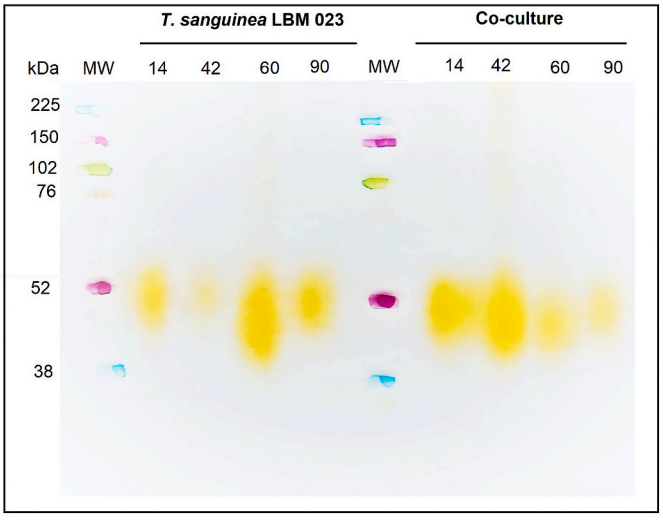


**Fig. 2.** Lac isoenzymatic profile. Extracts obtained from PCBs-contaminated soil bioremediated with *T. sanguinea* and fungal co-cultures of *T. sanguinea* and *P. sajor-caju* after 14, 42, 60 and 90 d of incubation (SDS-PAGE, 12% w $v^{-1}$, revealed with DMP as substrate).

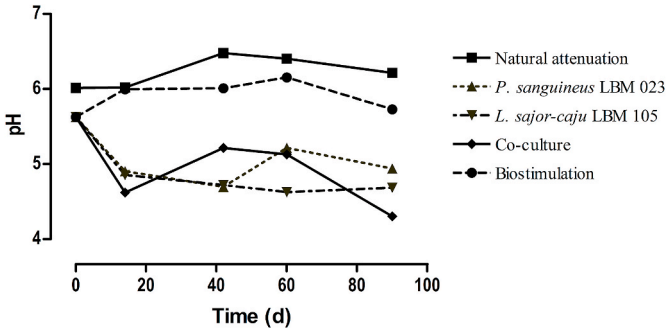


**Fig. 3.** pH of soil subjected to different remediation treatments after 14, 42, 60 and 90 d. Data are means ± standard deviations.

## 4. Discussion

In this work, two white-rot fungi (*T. sanguinea* and *P. sajor-caju*) were immobilized separately or in dual cultures on sugarcane bagasse in order to improve their ability to compete with the resident microbiota of soil and stimulate enzyme secretion. Other authors also used sugarcane bagasse as lignocellulosic substrate for white-rot fungal growth (Fernández-Sánchez et al., 2001; Pérez-Armendáriz et al., 2004; Dzul-Puc et al., 2005; Cortés-Espinosa et al., 2006). Fungal strains such as *Pleurotus* sp. and *Dichomitus squalens* also demonstrated highly competitive with the soil microbiota in a previous study (Lang et al., 1997). Federici et al. (2012a) monitored the survival of *Lentinus tigrinus* by determining ergosterol and demonstrated that the fungus remained throughout all the incubation period. Similarly, Stella et al. (2017) proved that *P. ostreatus* colonized efficiently the soil samples until the end of the treatment. The inoculum formulation, using sugarcane bagasse, may be a determinant for the efficacious colonization of *T. sanguinea* LBM 023 and *P. sajor-caju* LBM 105. In addition, the appropriate concentration of fungal inoculum may confer them an initial competitive advantage over resident microbiota (Schmidt et al., 2005) and, at the same time, lead to an enhancement of the biodiversity of the resident microbiota (Federici et al., 2012a) or stimulate bacterial growth with PCBs biodegradation capacity (Stella et al., 2017). While both strains were isolated from the polluted soil at 90 d in monocultures, only *T. sanguinea* LBM 023 could be successfully isolated after co-cultivation. Direct plating technique and ITS alignment were employed for fungal recovery and identification respectively, which contrary to PCR-based methods, allows the discrimination between viable and dead cells (Kazerooni et al., 2017). The scant growth of *P. sajor-caju* LBM 105 might have hampered its detection. ITS region of nuclear rRNA gene is considered the most appropriate candidate for barcoding the fungal kingdom (Rossman, 2007).

Soil chemical modifications contribute to the evaluation of bioremediation treatment effectiveness. Quantification of soil enzyme activities is useful to determine the impact of pollution on soil health and to monitor the decontamination process (Rao et al., 2014; Song et al., 2017). Fungal co-cultivation could generate oxidative stress, accelerating the shift to secondary metabolism and stimulating the secretion of ligninolytic enzymes (Chi et al., 2007). Experimental evidence suggests that competition for space and nutrients may result in increased lignin degradation (Asiegbu et al., 1996) and enhanced production of lignin modifying enzymes, such as Lac (Freitag and Morrell, 1992; Baldrian, 2004). This could explain the significant rise detected in dual cultures in

**Table 2**
Main soil properties after different bioremediation treatments. Values are expressed as means ± standard deviation, mean values within a column superscripted with the same letter were not significantly different ($p < 0.05$).

| Treatment | Time (d) | pH | Organic carbon (%) | Oxidable organic matter (%) | Available phosphorus (mg $Kg^{-1}$) | Aluminium (mEq 100 $g^{-1}$) | DHA (mIU $g^{-1}$) |
|---|---|---|---|---|---|---|---|
| Natural attenuation | 90 | $6.22^{c}$ | $3.38 \pm 0.06^{ab}$ | $5.82 \pm 0.11^{ab}$ | $15.11 \pm 0.91^{abc}$ | $0.00 \pm 0.00^{a}$ | $0.25 \pm 0.10^{a}$ |
| Biostimulation | 0 | $5.63^{abc}$ | $8.28 \pm 0.64^{abc}$ | $14.27 \pm 1.10^{abc}$ | $38.84 \pm 2.05^{a}$ | $0.00 \pm 0.02^{a}$ | $1.78 \pm 0.04^{abc}$ |
| Biostimulation | 90 | $5.73^{abc}$ | $8.96 \pm 0.45^{bc}$ | $15.44 \pm 0.77^{c}$ | $13.63 \pm 0.11^{d}$ | $0.00 \pm 0.00^{a}$ | $2.17 \pm 0.22^{bc}$ |
| *T. sanguinea* LBM 023 | 90 | $4.94^{ab}$ | $8.73 \pm 0.25^{bc}$ | $15.05 \pm 0.44^{bc}$ | $19.89 \pm 3.78^{bcd}$ | $0.09 \pm 0.03^{b}$ | $1.52 \pm 1.22^{abc}$ |
| *P. sajor-caju* LBM 105 | 90 | $4.69^{a}$ | $8.24 \pm 0.06^{abc}$ | $14.20 \pm 0.11^{abc}$ | $23.48 \pm 1.51^{cd}$ | $0.01 \pm 0.01^{ab}$ | $6.40 \pm 0.88^{c}$ |
| Co-culture | 90 | $4.31^{a}$ | $8.88 \pm 0.80^{c}$ | $15.31 \pm 1.38^{bc}$ | $18.83 \pm 1.84^{bcd}$ | $0.06 \pm 0.03^{ab}$ | $1.08 \pm 1.13^{ab}$ |

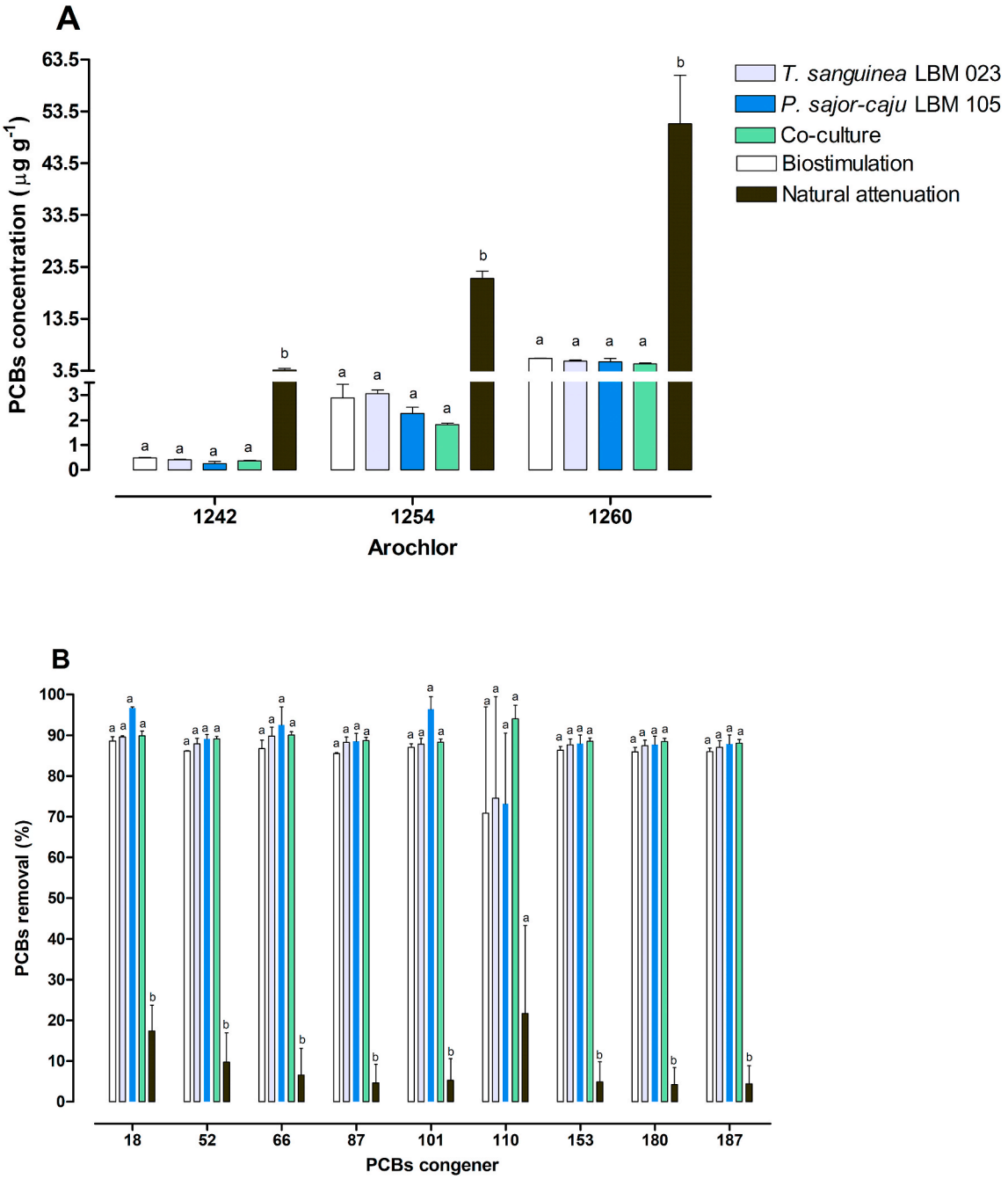


**Fig. 4.** PCBs in soil treated by mycoaugmentation (with monocultures of *T. sanguinea* LBM 023 or *P. sajor-caju* LBM 105 and dual cultures), biostimulation and natural attenuation at 90 d. (A) PCBs concentration (µg) in g OC of soil. (B) Percentages of congener's removal. Means with different letters are significantly different ($p < 0.05$).

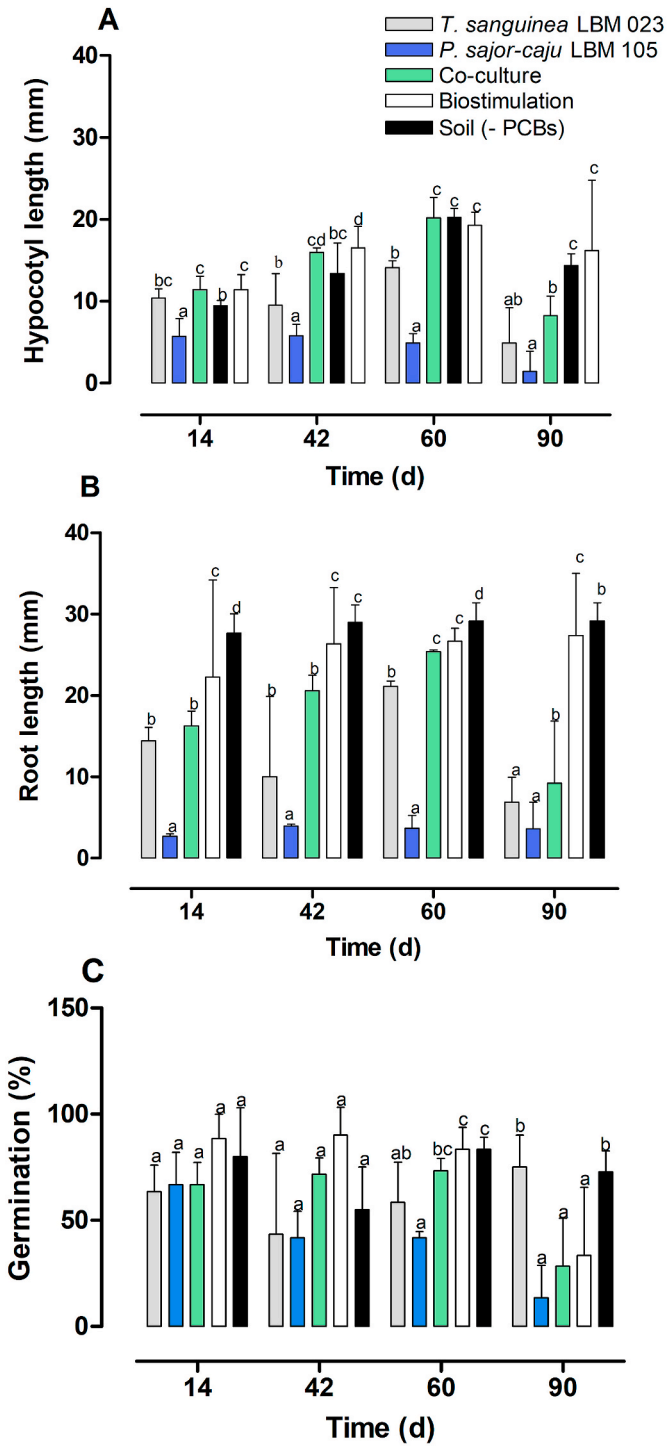


**Fig. 5.** Soil phytotoxicity after the different remediation treatments was determined measuring lettuce seedlings hypocotyl length (A), root elongation (B) and seed germination percentage (C). Data are means ± standard deviations. Means with different letters are significantly different ($p < 0.05$).

Lac and MnP activities at 60 d compared to monocultures. Similar outcomes were obtained by Verma and Madamwar (2002); Baldrian (2004); Chi et al. (2007); Qi-He et al. (2011) and Kuhar et al. (2015).

EX activity detected in co-culture, was significantly higher than the secreted by monocultures but EG production was remarkably lower. Metreveli et al. (2017) reported a significant increase in cellulases and xylanases production in submerged fermentation trials with co-cultures of *I. lacteus* and *Schizophylum commune*. On the contrary, Ma and Ruan (2015) informed lower enzyme production during co-cultivation than in monocultures, while Carabajal et al. (2012) did not detect changes in cellulases and xylanases secretion in co-cultures of *P. ostreatus* and *Pleurotus citrinopileatus* with respect to monocultures.

Lac isoenzymatic pattern differed between monocultures and co-cultures of *Ganoderma lucidum/Trametes versicolor*, a new Lac band was observed in dual cultures (Kuhar et al., 2015). Nevertheless, in this work a single Lac band of around 50 kDa was detected in cultures of *T. sanguinea* LBM 023 and in dual cultures. In general, on SDS-.PAGE, most laccases show mobilities corresponding to molecular weights of 60–80 kDa, but differences may be attributed to enzyme glycosylation (Xiao et al., 2006).

Mycoagumentation and biostimulation treatments reduced significantly PCBs concentrations in soil at the end of the experiment. The highest removal efficiency was attained with dual cultures, which was also the unique treatment capable of improving the degradation potential of PCB # 110. Bacterial consortia also proved their efficiency for PCBs-removal in soil (Horváthová et al., 2018). Fungal dual cultures were assayed in the degradation of mycotoxins and dyes (Das et al., 2015; Kuhar et al., 2015). Highest degradation of Aflatoxin B1 was recorded with co-cultures of *Aspergillus flavus* and *P. ostreatus* (Das et al., 2015). The time for achieving complete decolorization of malachite green by co-culturing *G. lucidum* and *T. versicolor* was markedly shorter than that observed in monocultures (Kuhar et al., 2015). But as far as we know, white rot fungal co-cultures were not previously assayed for PCBs removal, although several studies proved the potential of single strains such as *P. ostreatus* (Kubátová et al., 2001; Čvančarová et al., 2012) and *L. tigrinus* (Federici et al., 2012a) for the removal of this contaminant. A mycoaugmentation treatment with *P. ostreatus* diminished up to 50% PCBs concentration in an historically contaminated soil (Stella et al., 2017). The differences among the patterns of PCBs congeners' biodegradation could be related as well to key contaminant properties such as water-solubility, hydrophobicity (log P), binding affinity to organic soil colloids (log KOC) and molecular weight (MW) of congeners, as demonstrated by Federici et al. (2012b). Since the biodegradability of a PCBs molecule depends upon its degree of chlorination, Arochlors mixtures with higher content of highly chlorinated PCBs such as Arochlors 1260 and 1254, would be less biodegradable than those with a predominance of lower chlorinated compounds congeners (Bokvajova et al., 1994). This pattern was also observed by Kubátová et al. (2001) in four strains of *P. ostreatus*, which removed about 40% of Delor 103 and more competently the compounds with a lower degree of chlorination. In contrast, in our work removal efficiency kept constant while increasing the percentage of highly chlorinated congeners in Arochlors composition (Fig. 4B). In this study, PCBs removal under natural attenuation conditions was significantly low. Earlier studies demonstrated that the efficiency of natural attenuation depends on the autochthonous microbial communities present in soil (Zhang et al., 2019). In addition, phase transfer processes such as sorption and volatilization could be responsible for reducing microorganisms' exposure to the pollutant and can even cause an increase in contaminant reactivity (Declercq et al., 2012).

The addition of sugarcane bagasse to the contaminated soil resulted in a significant decrease in PCBs concentration. This observed effect may be due to a reduction and stabilization of the contaminant (Song et al., 2017). Sugarcane bagasse supplementation could affect the movement and bioavailability of PCBs due to the change in the content of organic matter (especially humic acids) (Ye et al., 2017). In contrast with our results, lower percentages of PCBs removal were detected when applying biostimulation approaches than with white-rot fungal augmentation, by Federici et al. (2012a) and Stella et al. (2017).

The decrease in pH observed in mycoaugmentation treatments might be associated with the production of organic acids, chelators and extracellular enzymes (Milagres et al., 2002; Isitua and Ibeh, 2010). Organic acids contribute to the mobilization of insoluble soil phosphorous to water soluble fractions that play important roles in soil nutrient

availability and productivity (Dotaniya et al., 2016), as it was observed in the bioremediation treatments (Table 2). Moreover, the interaction between soil components (inorganic mineral particles, organic matter) and highly hydrophobic contaminants such as the organochlorine pesticides dichloro diphenyl trichloroethanes (DDTs) and hexachlorocyolohexanes (HCHs), could be modulated by organic acids. The effect is associated with locking and unlocking mechanisms that depend on the chemical structure of the contaminant and the organic acids, leading to higher water solubility or the association with soil dissolved organic matter, and therefore modifying the contaminant bioavailability (White et al., 2003; Zhenhua et al., 2011; Mitton et al., 2012; Gonzalez et al., 2019).

Some remediation methods intentionally reduce the pH in order to facilitate the migration of some contaminants, mainly heavy metals and, thus, facilitate phytoextraction (Amoakwah et al., 2014). In this sense, mycoremediation could be an effective strategy in combination with phytoremediation in order to facilitate the treatment of soils co-contaminated with PCBs or other organic contaminants and heavy metals.

The significant rise in organic matter due to biostimulation and mycoaugmentation treatments may contribute to increasing the reserve of nutrients, cation exchange, water retention, and stimulate soil microbial processes (Battelle & NFESC, 1996; Dotaniya et al., 2016). High percentages of organic carbon in soil demonstrated to favor the growth of most fungi (Kazerooni et al., 2017). This increase in the content of organic matter can be attributed mainly to the addition of sugarcane bagasse. The application of sugarcane industry by-products reduced the recommended dose of inorganic chemical fertilizers and improved soil organic matter content during crop production (Dotaniya et al., 2016).

DHA is considered as a biomarker of microbial oxidative activity and soil detoxification (Dawson et al., 2007; Alvarenga et al., 2018). In all bioremediation treatments, DHA increased at 90 d. The higher values of DHA activity in soil mycoaugmented with *P. sajor-caju* LBM 105 compared to controls may be explained by enhanced PCBs removal, contributing in decreasing the prejudicial pollutant effects on soil microbiota, with a consequent improvement in soil biochemical parameters (Baćmaga et al., 2017). Also, the addition of sugarcane bagasse could stimulate enzyme secretion by the autochthonous microbiota and/or inoculated strains (Raimondo et al., 2020). Reduced enzyme activity in controls soils compared to treated ones could indicate toxic effects on soil microorganisms or enzyme inhibition exerted by PCBs. A similar effect was reported by Raimondo et al. (2019) in lindane contaminated soil.

*L. sativa* toxicity test proved to be an appropriate monitor of PCBs remediation. The sensitivity of this technique was also demonstrated in previous works (Aparicio et al., 2015; Rede et al., 2016). Biostimulation and mycoaugmentation treatments decreased significantly PCBs toxicity at 90 d. Nevertheless, in mycoaugmented soils seed germination percentage slightly diminished throughout the incubation period. This suggests that PCBs fungal transformation could led to the formation of toxic intermediate compounds, which affected seed germination. Stella et al. (2017) attributed the increase in toxicity registered in PCBs-contaminated soil treated with *P. ostreatus* and *I. lacteus* to the formation of these toxic metabolites. Low concentrations of these soluble compounds could not inhibit germination but affected root or hypocotyl elongation (Sobrero and Ronco, 2004). The production of free radicals during the biodegradation process might contribute to the increase in phytotoxicity as well (Song et al., 2017).

## 5. Conclusion

Sugarcane bagasse demonstrated to be not only a suitable support for fungal immobilization but also an efficient substrate for fungal colonization of PCBs-contaminated soils. Biostimulation as well as mycoaugmentation applying dual cultures led to efficient PCBs-removal (approx. 90%) and a remarkable toxicity reduction at 90 d. Moreover, both treatments improved soil characteristics. Therefore, they constitute efficient bioremediation approaches to restore PCBs-contaminated soils. In addition, the use of a local agro-industrial residue to treat polluted soil contribute to circular agro-waste management strategies. The bioremediation techniques assayed in this work based on fungi and agro industrial biodegradable wastes could be suitable applied as well in the remediation of other halo-organic pollutants with high hydrophobicity and recalcitrance, as white-rot fungi are capable of degrading a wide variety of aromatic compounds.

## Author statement

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## Declaration of competing interest

The authors declare no potential competing interests.

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