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Title: Bioremediation of long-term PCB-contaminated soil by white-rot fungi

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Dear Editorial Board,
I am taking the liberty to send you a revised manuscript for publication in Journal of Hazardous Materials.
With compliments
Tomas Cajthaml.

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Bioremediation of long-term PCB-contaminated soil by white-rot fungi

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***Response to Reviewers**

Reviewers' comments:

Reviewer #1: The authors revised the manuscript carefully. I think it can be accepted for publication after the authors checked the references carefully.

The references have been checked and corrected.

***Highlights (for review)**

- *Pleurotus ostreatus* efficiently degraded PCBs in real contaminated soil
- The best results, represented by 50% degradation, were observed in rhizosphere soil
- *P. ostreatus* decomposes the aromatic moiety of PCBs in soil *via* chlorobenzoic acids
- The fungus effectively colonized the soil and suppressed other fungi
- Community analysis showed stimulation of bacteria encompassing putative PCB degraders

ABSTRACT

The objective of this work was to test the PCB-degrading abilities of two white-rot fungi, namely *Pleurotus ostreatus* and *Irpex lacteus*, in real contaminated soils with different chemical properties and autochthonous microflora. In addition to the efficiency in PCB removal, attention was given to other important parameters, such as changes in the toxicity and formation of PCB transformation products. Moreover, structural shifts and dynamics of both bacterial and fungal communities were monitored using next-generation sequencing and phospholipid fatty acid analysis. The best results were obtained with *P. ostreatus*, which resulted in PCB removals of 18.5, 41.3 and 50.5% from the bulk, top (surface) and rhizosphere, respectively, of dumpsite soils after 12 weeks of treatment. Numerous transformation products were detected (hydoxylated and methoxylated PCBs, chlorobenzoates and chlorobenzyl alcohols), which indicates that both fungi were able to oxidize and decompose the aromatic moiety of PCBs in the soils. Microbial community analysis revealed that *P. ostreatus* efficiently colonized the soil samples and suppressed other fungal genera. However, the same fungus substantially stimulated bacterial taxa that encompass putative PCB degraders. The results of this study finally demonstrated the feasibility of using this fungus for possible scaled-up bioremediation applications.

Bioremediation of long-term PCB-contaminated soil by white-rot fungi

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The objective of this work was to test the PCB-degrading abilities of two white-rot fungi, namely *Pleurotus ostreatus* and *Irpex lacteus*, in real contaminated soils with different chemical properties and autochthonous microflora. In addition to the efficiency in PCB removal, attention was given to other important parameters, such as changes in the toxicity and formation of PCB transformation products. Moreover, structural shifts and dynamics of both bacterial and fungal communities were monitored using next-generation sequencing and phospholipid fatty acid analysis. The best results were obtained with *P. ostreatus*, which resulted in PCB removals of 18.5, 41.3 and 50.5% from the bulk, top (surface) and rhizosphere, respectively, of dumpsite soils after 12 weeks of treatment. Numerous transformation products were detected (hydoxylated and methoxylated PCBs, chlorobenzoates and chlorobenzyl alcohols), which indicates that both fungi were able to oxidize and decompose the aromatic moiety of PCBs in the soils. Microbial community analysis revealed that *P. ostreatus* efficiently colonized the soil samples and suppressed other fungal genera. However, the same fungus substantially stimulated bacterial taxa that encompass putative PCB degraders. The results of this study finally demonstrated the feasibility of using this fungus for possible scaled-up bioremediation applications.

Keywords

Pleurotus ostreatus, ligninolytic fungi, polychlorinated biphenyls, metabolites, microbial community

1. INTRODUCTION

Polychlorinated biphenyls (PCBs) are xenobiotic compounds that have been used for a number of industrial applications due to their thermal and chemical stability, flame resistance and dielectric properties. Due to their inertness, PCB contamination is still widespread in all types of ecosystems and affects both natural environments and wildlife. Consequently, the clean-up of PCB-contaminated sites has become a priority of great relevance due to the teratogenic, carcinogenic and endocrine-disrupting features of these xenobiotics [1,2]. Until now, the removal of PCBs from contaminated environmental matrices has been primarily performed by incineration at high temperatures. Because this procedure may entail additional risks and represents economically extremely demanding processes, scientific researchers have been focusing on alternative biological PCB clean-up options. Among them, the use of filamentous fungi has been shown to be particularly promising [3,4].

In this respect, filamentous fungi exhibit features that make them excellent candidates for designing effective remediation technology. Their hyphal structures, defined as "fungal highways", allow them to easily penetrate across environmental matrices and to act as dispersion vectors of both pollutant-degrading bacteria [5,6] and pollutants [7]. Another important peculiarity of an ecological subgroup of filamentous fungi, often referred to as "white-rot fungi", is the secretion of oxidative enzymes that are characterized by a low substrate specificity that can degrade a wide range of aromatic organopollutants [8,9]. This nonspecific radical-based system is active in the extracellular environment, and thus, fungi can easily penetrate a contaminated matrix and reach even poorly bioaccessible pollutants.

Several white-rot fungi were tested for their ability to decompose PCBs under model laboratory-scale conditions in liquid systems [10]. The most well-known degrading strains include *Phanerochaete chrysosporium* [11], *Trametes versicolor* [12], *Lentinus edodes* [13], *Phlebia brevispora* [14], *Irpex lacteus*, *Bjerkandera adusta*, *Pycnoporus cinnabarinus*, *Phanerochaete magnoliae* [15] and particularly *Pleurotus* ostreatus [15-17].

Nevertheless, fungi have not yet been widely exploited for their metabolic capabilities [18]. Surprisingly, only a relatively small number of white-rot species have been tested on real PCB-contaminated soil [19,20], although *P. ostreatus* is, thus far, likely the most efficient known PCB-degrading organism [15].

Thus, the objective of this study is to assess the feasibility of different mycoremediation treatments of historically PCB-contaminated soil. Prior to application of the selected remedial techniques, the

physico-chemical properties of the soil and the PCB bioaccessibility were assessed. Thereafter, the degradation capabilities of the white-rot fungi *P. ostreatus* and *Irpex lacteus* and the role of autochthonous microbial communities in the PCB biotransformation process were investigated. Specific attention was given to identifying PCB degradation products and to the residual toxicity at the end of each treatment. Furthermore, to gain new insights into the microbiota composition throughout the remediation processes, the microbial biomass was quantified by phospholipid fatty acid (PLFA) analysis and the diversity and dynamics of both the bacterial and fungal communities were determined *via* the high-throughput 454-pyrosequencing method.

2. EXPERIMENTAL

2.1 Soil samples

Long-term PCB-contaminated soil was collected from the Lhenice dumpsite (South Bohemia, Czech Republic). The contamination was mostly caused by the presence of a Delor 103 PCB mixture (analogous to Aroclor 1242), as reported elsewhere [21]. Three soil samples, which differed in their PCB contents, were selected for microcosm preparation: bulk soil (B soil; total PCB concentration of 705.95 μg g^{-1}), topsoil (T soil; total PCB concentration of 375.81 μg g^{-1}) and rhizosphere soil (R soil; total PCB concentration of 169.36 μg g^{-1}). The soil texture, cation exchange capacity, water-holding capacity as well as the organic carbon, fulvic and humic acids, sulphur, nitrogen and heavy metal (i.e., Cd, Cr, Ni, Pb, etc.) contents are provided in the supplementary material (Table S1). Each soil sample was sieved (< 2 mm) and homogenized by repeated mixing prior to the bioremediation trials.

2.2. Microorganisms and inoculum preparation

P. ostreatus 3004 CCBAS 278 and *I. lacteus* 617/93 were obtained from the Culture Collection of Basidiomycetes of the Academy of Science of the Czech Republic (Prague, CZ). The fungal strains were maintained at 4 °C on Malt Extract Agar plates (MEA) and subcultured every month. Five mycelial plugs (0.7 mm Ø) from 7-d-old MEA cultures were used to inoculate 500-mL Erlenmeyer flasks containing wheat straw-based pellets (8 g), which had been previously sterilized in an autoclave (121 °C, 30 min). The moisture content of the lignocellulosic substrate (LS) was adjusted to 75% (w/w) with sterile deionized water. Inoculated and non-inoculated LS, which were used for the bioaugmentation and biostimulation treatments, respectively, were incubated at 25 °C for 10 days.

2.3 Microcosm preparation

The three soil samples (B, T and R), whose moisture contents were previously adjusted to 60% of their respective water holding capacities (w/w), were mixed with either a *P. ostreatus* or *I. lacteus* colonized substrate for bioaugmentation treatments to reach a final soil:LS mass ratio of 5:1 (w/w). Soil samples mixed only with non-inoculated LS were prepared for biostimulation treatments in the same ratios as described above. Non-amended and non-inoculated soil samples were prepared and are referred to as incubation controls (initial soil). All the experiments were performed in triplicate under non-sterile conditions. All the microcosms were incubated in a ventilated room at approximately 25 °C for 12 weeks, and their moisture contents were kept constant by periodical addition of sterile deionized water. Four harvests were done after 0, 2, 6 and 12 weeks of incubation. Based on the scheduled harvests, the three replicates for each treatment were wholly sacrificed for the analyses. The soil samples were air dried for the chemical analysis, lyophilized for the PLFA analysis and stored at -80 °C for high-throughput sequencing.

2.4 Extraction and determination of PCB concentration and bioaccessibility

PCBs were extracted by the Dionex 200 Accelerated Solvent Extraction (ASE) system (Palaiseau, France), purified and then analyzed using gas chromatography-mass spectrometry (GC-MS; 450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA) as reported elsewhere [21] . The most relevant PCB congeners in the Delor 103 technical mixture and the commonly monitored congeners were quantified [22].

The bioaccessible fraction of PCBs was estimated using sequential supercritical fluid extraction (SFE) and an empirical two-site model, as reported elsewhere [21,23].

2.5 Extraction and identification of PCB degradation intermediates

PCB metabolites were extracted by the ASE system according to a previously developed method [24]. The organic extracts were purified by gel permeation chromatography as reported elsewhere [25]. The samples were analyzed by GC-MS either directly without derivatization or after methylation with diazomethane. The PCB metabolites were separated and identified using two different splitless (1 min, 60 °C) methods: (*i*) oven heated to 100 °C (25 °C min^{-1}) and additionally heated to 280 °C (7.5 °C min^{-1}); (*ii*) oven heated to 120 °C (25 °C min^{-1}) and additionally heated to 280 °C (2.5 °C min^{-1}). The mass spectra were recorded as reported elsewhere [15].

2.6 Ecotoxicology test with *Vibrio fischeri*

The inhibitory effect of soil extracts on the light emission by *V. fischeri* was measured (30 min of exposure) at the end of each treatment using the Lumino-M90a (ZD Dolní Újezd, Czech Republic) luminometer according to the normalized ISO 11348-3:2007 protocol, where aliquots of ethyl acetate extracts (0.5 mL) were re-dissolved in 6% DMSO; luminescence inhibition (I) was determined as described elsewhere [26].

2.7 Extraction and analysis of phospholipid fatty acids

Extraction and analysis of phospholipid fatty acids were performed as described by [27]. Phospholipids were extracted using a chloroform:methanol:phosphate buffer (1:2:0.8, v/v/v) and then separated by solid-phase extraction cartridges (LiChrolut Si 60, Merck). Samples then underwent mild alkaline methanolysis and the methylated PLFAs were analyzed by GC-MS. fungal biomass was quantified based on the 18:2ω6,9 content; the bacterial biomass was quantified as the sum of i14:0, i15:0, a15:0, 16:1ω5, 16:1ω7, 16:1ω9, 10Me-16:0, i16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 18:1ω7, 10Me-18:0, cy19:0 (actinomycetes 10Me-16:0, 10Me-17:0, 10Me-18:0; Gram+ i14:0, i15:0, a15:0, i16:0, i17:0, a17:0; Gram− 16:1ω7, 16:1ω9, 18:1ω7, cy17:0, cy19:0) as reported elsewhere [21].

2.8 DNA extraction, amplification and pyrosequencing analysis

Genomic DNA was extracted from microcosm samples (three replicates) using the modified Miller method described by [28]. The V4–V6 hypervariable regions of the bacterial 16S rRNA gene and the fungal internal transcribed spacer ITS1 and ITS4 regions were amplified as described by [21]. The PCR products of each sample were pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The concentration of purified DNA was quantified using ND1000 (Nano-Drop, Wilmington, DE, USA). In the second amplification step, fusion primers were tailored for tag-encoded 454-Titanium pyrosequencing: different barcode sequences were added at the 5’ end of the forward primer separated by a trinucleotide spacer as reported by [29]. Titanium A adaptors were also used (Roche, Basel, Switzerland). Purification of the PCR products was performed using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany), and the DNA concentration was quantified by the ND1000 (Nano-Drop, Wilmington, DE, USA) and Quant-iT Picogreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Purified amplicons were used for the subsequent emulsion PCR (emPCR Kit Lib-L, Roche), the products of which were sequenced on the 454 GS Junior platform (Roche) according to the manufacturer’s instructions (Roche, Basel, Switzerland).

2.9 Pyrosequencing data analysis

Bacterial and fungal sequences were processed with the QIIME 1.6.0 software package. Quality filtering steps were performed to trim off barcodes and primers from the raw sequences and to remove sequences (*i*) < 200 nt in length, (*ii*) with homopolymers longer than 6 nt and (*iii*) with a quality score < 25 and < 20 for bacteria and fungi, respectively. Denoising was performed as described by [30]. QIIME's implementation of OTUPipe script was employed for chimera checking and OTU picking [31,32]. The 16S Microbial blast database and UNITE database were used as reference databases for bacterial and fungal chimeric sequence detection, respectively. The resulting chimera-free reads were clustered into OTUs based on their sequence similarity at 97%. Representative sequences of each OTU were aligned using MUSCLE [33] and employed for taxonomy assignment. The Ribosomal Database Project (RDP) classifier and UNITE database were used to taxonomically classify the bacterial and fungal sequences, respectively. Alpha-rarefaction and Alpha-diversity analyses were performed using QIIME 1.6.0, and the Beta-diversity was assessed by principal component analysis (PCA) using Minitab 16 version 2.2.0 (Minitab, Inc., PA, USA).

3. RESULTS AND DISCUSSION

3.1 Bioaccessibility and biodegradation of PCBs

The estimation of PCB bioaccessibility is crucial for predicting the efficiency of the biodegradation process. Thus, sequential supercritical carbon dioxide extraction (SFE) was employed to quantify the bioaccessible fraction (F) of PCBs in initial soil B, T and R and to estimate the efficiency of selected remediation approaches for these contaminated matrices [34,35]. The bioaccessible fraction of total PCBs was 94, 62 and 64% (R^2=0.993, 0.997 and 0.977) in the B, T and R soils, respectively. The higher bioaccessibility of PCBs in the bulk soil could be due to the lower amount of organic matter in this soil, as has been reported previously [21].

The time courses of PCB degradation in both mycoaugmented and biostimulated microcosms are shown in Figure 1. For the most contaminated soil (B soil), no significant decrease in PCB concentration was observed within the first 6 weeks of incubation regardless of the treatment employed. Moderate removals (18.5 and 19.3%) were observed only after 12 weeks in *P. ostreatus* and *I. lacteus*-augmented microcosms, respectively (t-test P values: 0.011 and 0.006). Conversely, in the same time interval (12 weeks), the PCB removal from the topsoil (T soil) was 41.3 and 39.4% in the presence of *P. ostreatus* and *I. lacteus*, respectively. Despite the similar degradation performances of the two fungi at the end of the treatment, the removal of PCBs in the T soil was more rapid with *I. lacteus* than that with *P. ostreatus* within the first 2 weeks (4.55 *vs.* 2.50 ppm per

day). On the contrary, *P. ostreatus* was more efficient than *I. lacteus* during the following 4 weeks of incubation (2.84 *vs.* 2.02 ppm per day). In the rhizosphere soil (R soil), the greatest PCB depletion (50.5%) was achieved in the *P. ostreatus*-augmented soil, whilst *I. lacteus* led to a degradation of 30.3% of the original amount. However, comparing the amounts of removed PCBs, *P. ostreatus* was more effective in the B and T soils than in the R soil, which corresponded to the removal of 130.5, 155.2 and 85.5 μg g^{-1}, respectively. The fact that the degradation was slower in the case of the R soil can be explained by the congener ratios in the soils, where the R soil contained slightly shifted to higher chlorinated, i.e., more recalcitrant congeners [21]. The greater degradation ability of *P. ostreatus* compared with the other fungal strains has been previously demonstrated in both liquid and artificially contaminated solid systems [15,36].

Regardless of the soil typology, the degree of PCB removal in biostimulation microcosms was invariably lower than that achieved in bioaugmented microcosms after 12 weeks of incubation. Generally, even though modest removal of PCBs was observed in the topsoil (22.9%), the stimulation of resident microorganisms *via* the addition of a lignocellulosic substrate was not as successful as the bioaugmentation strategy. Similar results were also observed in previous work with *Lentinus tigrinus* [20].

3.2 *Vibrio fischeri* toxicity assay

The validity of selected bioremediation methods must be corroborated by the efficiency in the degradation of target pollutants as well as in the toxicity reduction of contaminated matrices. Therefore, a toxicological assay using *V. fischeri* was performed to assess the acute toxicity of the soil extracts before and after the treatments (Figure 2). For bulk soil B, the slight PCB removal in both myco-augmented and biostimulated microcosms did not substantially change the toxicity during the incubation period, whereas an increase in luminescence inhibition was observed after treatment of the topsoil in all cases. Specifically, for the T soil, higher toxicity values were obtained in *P. ostreatus* and *I. lacteus* treated soil (37.0 and 30.4%, respectively, compared to the initial percentage of inhibition) than in the biostimulated microcosms, despite the ability of these two fungal strains to achieve PCB removals of 41.3 and 39.4%, respectively. These outcomes suggested that the fungal transformation of PCBs in the T soil led to the formation of metabolite amounts (e.g., chlorobenzoates, see below), which were more acutely toxic than the parent compounds [15,37,38]. Conversely, *P. ostreatus* and *I. lacteus* substantially reduced the toxicity (63.9 and 55.3%, respectively) in R soil after 12 weeks of treatment.

3.2 Detection and identification of PCB degradation intermediates

An extensive set of qualitative GC-MS analyses was undertaken to identify PCB metabolites and to gain insights into the fungal PCB degradation pathways in the soil systems. Several intermediates were detected throughout the mycoremediation treatments, and their structures were suggested by comparing them with the data in the NIST 08 library and independently by interpreting the fragmentation patterns (Table 1). Product ion scan (MS/MS) was used when necessary to clarify the fragmentation sequence, and all the molecular weights were estimated according to the chemical ionization technique [15].

Derivatization with diazomethane enabled detection of 3 and 5 isomers of hydroxylated tri- and tetra-chlorobiphenyls, respectively, in both bioaugmented and biostimulated microcosms, either in the early phases or at the end of the treatments. Dihydroxylated tri- and tetrachlorobiphenyls were also found in the B and R soils after treatment with *I. lacteus*. The formation of the latter compounds can be attributed either to the intracellular cytochrome P450 monooxygenases system (CYP450) of *Irpex lacteus* or to the dioxygenase activity of autochthonous bacteria [39,40].

Moreover, methoxy-substituted-derivatives of tetrachlorobiphenyl were detected without derivatization in *P. ostreatus* microcosms at the end of the incubation. The fungal transformation of PCBs to their corresponding hydroxylated and methoxylated forms has already been demonstrated in model liquid systems [41,15]. In particular, both studies proposed a pathway for the biotransformation of PCBs, where methoxylated PCBs are formed *via* the initial hydroxylation reaction mediated by the CYP450. Furthermore, the formation of hydroxylated compounds when biostimulation treatment was employed suggested that indigenous microorganisms took part in the transformation process of PCBs. In this connection, an early study of PCB microbial degradation reported the ability of soil fungus *Rhizopus japonicus* to convert 4-chlorobiphenyl and 4,4´-dichlorobiphenyl to their corresponding hydroxylated forms [41].

In addition, trichlorobenzoic acids, dichloro benzylalcohols and trichlorocresol were identified in *P. ostreatus* microcosms with B and T soils after 12 weeks of incubation. The formation of chlorobenzoates (CBAs) from hydroxylated PCBs and their further transformation *via* a reductive pathway has already been proposed [15,11]. In particular, the concomitant presence of reduced forms of chlorobenzoic acids (dichloro benzylalcohols and trichlorocresol) confirmed the hypothesis that a reductive mechanism operates on the carboxyl group of CBAs, which is in agreement with other studies [15,37,42]. Once CYP450 oxidized the aromatic structure of PCBs, the ring fission reaction can be mediated by a different enzymatic system (*i.e.*, ligninolytic system), as suggested by [43]. Thereafter, side-chain reductive reactions of CBAs might proceed *via* a two-

step process catalyzed by NADPH-dependent aryl-aldehyde dehydrogenase and aryl-alcohol dehydrogenase [42].

All of the detected metabolites were found at trace levels with the exception of 2,3,6-trichlorobenzoic acid. The accumulation of this intermediate is probably due to its recalcitrance, as demonstrated elsewhere [37,42]. Indeed, the double *ortho*-chlorine substitution and the electron-withdrawing effect of these chlorine atoms adjacent to the carboxyl group could prevent enzymatic attack towards CBAs.

3.4 PLFA analysis

PLFAs are widely used as taxonomic and primarily phylogenetic biomarkers to describe the structure and the size of the microbial community. In this way, PLFA analysis was performed to evaluate changes occurring in both bacterial and fungal communities during the mycoremediation treatments (Figure 3). The highest concentrations of the fungal (eukaryotic) PLFA marker were detected in 6-week-old *P. ostreatus*-augmented microcosms with B (4.02 μg g^{-1}), T (14.36 μg g^{-1}) and R (11.38 μg g^{-1}) soils; in these microcosms, the presence of *P. ostreatus* appeared to stimulate the growth of bacteria with the highest concentrations of bacterial biomass being attained in T soil (4.20 μg g^{-1}) and R (1.66 μg g^{-1}) soils. For *I. lacteus* augmentation treatment, the maximum amount of fungal biomass was measured in the topsoil after 6 weeks of incubation. Despite the fact that the values cannot be directly compared among different species, this value was much lower than that found in *P. ostreatus* microcosms (1.34 *vs* 14.35 μg g^{-1}). As opposed to *P. ostreatus*, in *I. lacteus* microcosms, no stimulation of bacterial growth was evident; conversely, in the B soil, bacterial biomass was lower than that detected in biostimulated microcosms regardless of the treatment time. Lastly, the addition of the non-inoculated lignocellulosic substrate (biostimulation treatments) positively affected the autochthonous microorganisms: the highest bacterial concentration was reached in the B, T and R soils (1.34. 1.86 and 1.48 μg g^{-1}, respectively) after 6 weeks of incubation.

3.5. Tag-encoded 454-pyrosequencing

Undoubtedly, high-throughput next-generation sequencing methods represent a powerful alternative to conventional approaches (mostly based on cloning and Sanger sequencing), which enables detailed identification and relative quantification of microbial populations from environmental samples. In this study, 454 tag-encoded pyrosequencing was selected to gain insights into the dynamic shifts in both bacterial and fungal communities throughout the mycoremediation treatments.

The pyrosequencing procedure yielded approximately 186.000 and 163.000 bacterial and fungal sequences, respectively. Upon data processing with the QIIME software, 24 and 47% of the obtained sequences were omitted because they did not meet the stringent quality control criteria. The remaining sequences were aligned and subjected to cluster analysis to ascertain their phylogenetic affiliations. Sequences that could not be assigned to any phylum were assigned as ND (not determined).

In the bacterial community, a considerable decline of *Actinobacteria* and *Chloroflexi* occurred in all cases, whereas a distinct increase was observed in the relative abundance of *Firmicutes* when soil B and R were treated with *P. ostreatus* (Figure 4). This phylum accounted for 94.0 and 64.4% of the total reads in soil B and R, respectively, after 6 weeks of incubation. A similar increase was also found for the *Proteobacteria* phylum in all the soil samples augmented with *I. lacteus*. After 6 weeks of *I. lacteus* treatment, *Proteobacteria* were the most abundant in the B, T and R soils, making up 90.1, 82.3 and 92.3%, respectively, of the entire bacterial population. At the end of incubation, this phylum was still the predominant one in soil B, whereas an increase in *Bacteroidetes* was evident in the T (37.5 %) and R (48.2%) soils. In the biostimulated microcosms, the addition of the lignocellulosic substrate promoted primarily the growth of *Proteobacteria,* the relative abundance of which amounted to 71.1, 64.6 and 60.8% of the total reads in the B, T and R soils, respectively, after 6 weeks and 66.7, 55.0 and 62.7% after 12 weeks. The other phyla (*Acidobacteria, Gemmatimonadetes* and *Verrucomicrobia*) represented a significantly smaller fraction of bacterial consortia in all microcosms at the end of the incubation period (sum of the relative abundances < 10.0% in all cases). The presence of sequences belonging to the *Chloroflexi* phylum at the beginning of the mycoremediation treatments suggested that a certain microflora specialized in PCB degradation adapted over time [21]. With this regard, anaerobic bacteria belonging to the *Chloroflexi* phylum play the primary roles in the reductive dehalogenation of higher chlorinated biphenyls [44]. These microorganisms can use the chlorine atoms of PCBs as final electron acceptors during the dehalorespiration process, leading to the formation of lower chlorinated compounds. Moreover, the hypothesis of the occurrence of adaptation processes was also supported by the initial presence of microorganisms belonging to the *Actinobacteria*, *Proteobacteria* and *Firmicutes* phyla. As a matter of fact, these phyla encompass well-known genera with reported PCB-degrading ability, such as *Rhodococcus*, *Bacillus*, *Pseudomonas*, *Achromobacter* and *Burkholderia* [44,45]. In addition to the remarkable shift in bacterial population, augmentation with *P. ostreatus* and *I. lacteus* led to a decrease in both the Chao1 and Shannon diversity indexes with the exception of the Shannon index when soil B was treated with *P.*

ostreatus (supplementary material – Table S2). Conversely, no appreciable changes in bacterial diversity were observed in biostimulated microcosms.

Concerning fungal diversity, the majority of the detected fungal sequences belonged to the *Basidiomycota*, *Ascomycota* and *Zygomicota* phyla (Figure 5). The *Basidiomycota* phylum was predominant in all *P. ostreatus*-augmented microcosms during the entire treatment period, whereas a general decline was observed in *I. lacteus*-augmented soil samples B, T and R, which reached 69, 21 and 1%, respectively, of the total fungi at the end of incubation. Furthermore, genus level analysis established that the detected *Basidiomycota* sequences were represented by *P. ostreatus* and *I. lacteus* in their respective microcosms. These results confirmed the ability of the two selected white-rot fungi to compete with the autochthonous mycobiota*:* the use of wheat straw-based pellets as lignocellulosic substrate supported the growth of both fungi and the preparation of fully colonized LS because the inoculum formulation prior to being mixed with the soil increased their antagonistic potential, as reported in a previous study [34]. Nonetheless, the *I. lacteus* sequence abundance drastically decreased during the last 6 weeks of incubation in soils T and R with a concomitant increase in unidentified fungal sequences. It is noteworthy that the soil does not represent a natural habitat for these fungi; therefore, it is not unexpected that one of the strains was unable to compete with autochthonous soil microflora.

Interestingly, sequences representative of the phylum *Basidiomycota* were also found in biostimulated samples of soils T and R after 12 weeks: the genus-level taxonomy assignment demonstrated that the *Agrocybe* and *Sphaerobolus* genera were the most abundant *Basidiomycetes* in soil T (45.5 and 49.7%, respectively), whereas *Pluteus* and *Cryptococcus* predominated in soil R (27.5 and 7.3%, respectively).

Regarding the *Ascomycota* phylum, analysis of the fungal sequences in the biostimulated microcosms showed the highest abundance in R soil (54.4 %), whereas a lower amount of the relevant sequences was found in soils B and T (19.3 and 17.4% of the total fungal sequences, respectively) at the beginning of the treatments. An increase from 19.3 to 36% was observed in soil B during incubation, which was primarily due to the growth of *Penicillium* and *Stachybotrys*. In contrast, a descending profile in the relative abundance of *Ascomycota* sequences was observed in soils T (from 17.4 to 0.1%) and R (from 54.4 to 31.4%) throughout the entire incubation. The ability of the *Penicillium* species to remove chlorinated biphenyls is well known. Indeed, several species belonging to this genus (*P. chrysogenum*, *P. purpurescens*, *P. digitatum*, *P. aurantiogriseum*) were isolated from PCB-contaminated soils and tested for their ability to degrade PCBs in liquid systems [46-48]: the studies confirmed that these fungi can co-metabolize PCBs when a readily available carbon source is provided.

In all of the soil samples, the *Zygomycota* phylum was dominated by members of the *Mucor* genus in all of the biostimulation treatments; the highest relative abundance of Zygomycetes was reached after 6 weeks of incubation in soils B and R (12.4 and 19.6%, respectively).

Based on the abundance of various taxa within each community, Alpha diversity was also assessed for fungi exhibiting a highly variable Chao1 index in all the treatments (Table S3). On the other hand, the Shannon index indicated that the biodiversity tended to decrease in *P. ostreatus* microcosms in all cases with a temporary increase observed only in soil B. Moreover, a similar increase was also observed when the same polluted matrix underwent *I. lacteus* and biostimulation treatment.

Lastly, PCA was performed to provide an overview of the similarities in the microbial communities among treatments, soil matrix and time of incubation (supplementary material – Figure S1 A, B and C). When the abundances of both fungal and bacterial phyla were analyzed, the percentage variance explained by the first and second components amounted to 31.1 and 17.2%, respectively. Great similarities were observed in augmented-microcosms at the beginning, which are grouped together in the lower right-hand quadrant ((P0A, P0B, P0C, I0A, I0B and I0C; Figure S1 B). Analogies among *P. ostreatus* treatments in the later phase of incubation with soils B and R (P6A, P12A, P6C and P12C) appeared to be positively correlated with the *Basidiomycota* and *Firmicutes* phyla, as indicated by the common position of the scores of these treatments with loadings of the aforementioned two variables in the lower left-hand quadrant of the biplot (Figure S1A). In contrast, *I. lacteus* treatments in the middle phase of incubation (I6A, I6B and I6C) segregated along the second component from their respective microcosms at the end of incubation with the exception of soil B (I12A). Indeed, as illustrated above, the *Proteobacteria* phylum was the dominant phylum in *I. lacteus*-augmented microcosms after 6 weeks of incubation and remained the most abundant phylum at the end of incubation only in soil B, whereas an increase of *Bacteroidetes* appeared in soils T and R. Moreover, PCA analysis revealed that the remaining two microcosms (I12B and I12C) grouped with the 6-week-old biostimulated-treatments of soil B, T and R (B6A, B6B and B6C, respectively).

4. CONCLUSION

In this study, among the tested remediation approaches, fungal bioaugmentation was found to be substantially more efficient than biostimulation in the degradation of PCBs with the *P. ostreatus* treatment being the best in terms of both PCB removal and acute toxicity reduction, particularly for rhizosphere soil. The identification of hydroxylated and methoxylated PCB derivatives as well as the presence of degradation intermediates, such as chlorobenzoates, chlorobenzaldehydes,

chlorobenzyl alcohols and chlorocresols, confirmed that the process of degradation of PCBs involved both fungal intracellular and extracellular enzymes. Moreover, the detection of mono-hydroxylated-PCB derivatives in biostimulated microcosms also suggested that the resident microbiota could take part in the biotransformation of PCBs. Furthermore, phospholipid fatty acid analysis showed that the fungal biomass of *P. ostrateus* was probably much higher than that of *I. lacteus* during the entire incubation period and that the introduction of this allochthonous fungus significantly stimulated the bacterial growth. In particular, *P. ostreatus* augmentation clearly increased the relative abundance of *Firmicutes* when the *Proteobacteria* phylum was the second most abundant. A similar increase was observed for the *Proteobacteria* phylum in all the soil samples treated with *I. lacteus*. It is noteworthy that these bacterial phyla comprise genera with a reported PCB-degrading ability. In addition, fungal community analysis showed that the sequences belonging to *P. ostreatus* in its respective microcosms were strikingly abundant for the entire incubation period. This latter finding proved the remarkable ability of this fungus to compete with the autochthonous fungal microbiota and thus, to efficiently grow in PCB-contaminated soils under non-sterile conditions.

In conclusion, the extensive PCB degradation performance in a relatively short time period and the outstanding competitiveness of *P. ostreatus* make its application in the remediation of PCB contaminated soil potentially transferable to a larger scale.

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Table

Table 1. Degradation intermediates of PCBs in ethyl acetate extracts not derivatized (---) or derivatized with diazomethane (Met) with retention times (t_R), molecular weights (MW), fragmentation patterns. The columns treatment (Treat.), Soil and Time specify where the intermediates were detected. The structures labelled with one asterisk (*) and two asterisk (**) were identified by method (*i*) and (*ii*), respectively (see *chapter 2.5*).

t_R (min)	MW according to CI	*m/z* of fragment ions (relative abundance)	Possible structure	Treat.	Soil	Time	Deriv.
18.471	176	178 (13.0), 176 (20.9), 143 (18.0), 141 (44.5), 139 (16.3), 115 (24.5), 113 (41.8), 111 (31.5), 77 (100), 75 (41.6)	?,?-dichlorobenzyl alcohol*	*P. ost.*	B	12w	---
21.600	238	242 (7.1), 240 (19.1), 238 (20.2), 211(30.1), 209 (96.4), 207 (100), 183 (7.1), 181 (13.4), 179(16.2), 143 (16.2), 109 (13.1), 74 (13.7)	2,3,6-trichlorobenzoic acid*	*P. ost.*	B-T	12w	Met
22.585	210	212 (28.4), 210 (39.8), 177 (61.4), 175 (80.3), 173 (25.2), 147 (76.9), 145 (36.4), 111 (100), 109 (177.6)	?,?,?-trichlorocresol*	*P. ost.*	B	6w	Met
31.325	320	322 (83.1), 320 (69.7), 307 (10.1), 305 (44.3), 281 (44.1), 279 (100), 277 (23.4), 207 (65.4)	?,?,?,?-tetrachloro-?-methoxybiphenyl*	*P. ost.*	B	12w	---
31.394	320	322 (82.2), 320 (68.9), 307 (10.5), 305 (44.5), 281 (43.8), 279 (98.5), 277 (22.4), 207 (65.9)	?,?,?,?-tetrachloro-?-methoxybiphenyl*	*P. ost.*	T	12w	---
30.091	286	290 (65.7), 288 (88.3), 286 (100), 273 (61.2), 271 (60.3), 245 (58.1), 243 (67.6), 173 (70.2)	?,?,?-trichloro-?-hydroxybiphenyl*	*I.lac.*	B	2w	Met
31.420	286	290 (64.6), 288 (89.9), 286 (100), 273 (51.3), 271 (50.3), 245 (47.1), 243 (44.6), 173 (65.2)	?,?,?-trichloro-?-hydroxybiphenyl*	*Bio*	B	6w	Met
				I.lac.	B	2w	Met
39.586	286	290 (66.6), 288 (87.9), 286 (100), 273 (58.3), 271 (61.4), 245 (46.2), 243 (42.5), 173 (67.7)	?,?,?-trichloro-?-hydroxybiphenyl**	*Bio*	T	12w	Met

				I.lac.	B	2w	Met
				P.ost.	B	12w	Met
32.302	320	322 (81.1), 320 (67.4), 307 (11.2), 305 (42.1), 281 (42.2), 279 (100), 277 (20.4), 207 (64.4)	?,?,?,?-tetrachloro-?-hydroxybiphenyl*	*Bio*	R	12w	Met
33.037	320	322 (80.3), 320 (66.4), 307 (10.3), 305 (41.1), 281 (44.5), 279 (100), 277 (21.3), 207 (65.0)	?,?,?,?-tetrachloro-?-hydroxybiphenyl*	*Bio*	R	12w	Met
33.349	320	322 (77.1), 320 (73.8), 307 (9.2), 305 (48.8), 281 (48.9), 279 (100), 277 (18.4), 207 (54.8)	?,?,?,?-tetrachloro-?-hydroxybiphenyl*	*P. ost.*	R	6w	Met
33.787	320	322 (69.2), 320 (87.8), 307 (13.4), 305 (50.8), 281 (49.6), 279 (100), 277 (19.9), 207 (58.1)	?,?,?,?-tetrachloro-?-hydroxybiphenyl*	*Bio*	R	12w	Met
45.016	320	322 (72.1), 320 (74.5), 307 (12.2), 305 (49.6), 281 (45.9), 279 (100), 277 (20.3), 207 (55.8)	?,?,?,?-tetrachloro-?-hydroxybiphenyl**	*P. ost.*	B	12w	Met
31.433	316	320 (36.6), 318(100), 316 (95.1), 305(27.0), 303 (63.3), 301 (57.2), 275 (42.8), 273 (72.3), 260 (39.7), 258 (48.6), 240 (30.4), 238 (58.7)	?,?,?-trichloro-?,?-dihydroxybiphenyl*	*I.lac.*	B	2w	Met
39.592	316	320 (38.4), 318(99.5), 316 (85.1), 305(25.6), 303 (61.2), 301 (53.2), 275 (44.8), 273 (78.9), 260 (36.3), 258 (51.2), 240 (34.3), 238 (56.2)	?,?,?-trichloro-?,?-dihydroxybiphenyl**	*I.lac*	R	6w	Met
41.206	352	354 (48.5), 352 (100), 350 (66.3), 339 (17.8), 337 (40.2), 335 (28.4), 294 (24.2), 292 (29.2), 290 (22.7), 240 (10.2), 238 (13.3), 236 (34.5)	?,?,?,?-tetrachloro-?,?-dihydroxybiphenyl**	*I.lac*	R	6w	Met
41.601	352	354 (49.2), 352 (98.5), 350 (64.2), 339 (18.6), 337 (42.5), 335 (19.7), 294 (26.3), 292 (25.4), 290 (20.1), 240 (12.5), 238 (14.7), 236 (32.5)	?,?,?,?-tetrachloro-?,?-dihydroxybiphenyl**	*I.lac*	B	6-12w	Met

Figure

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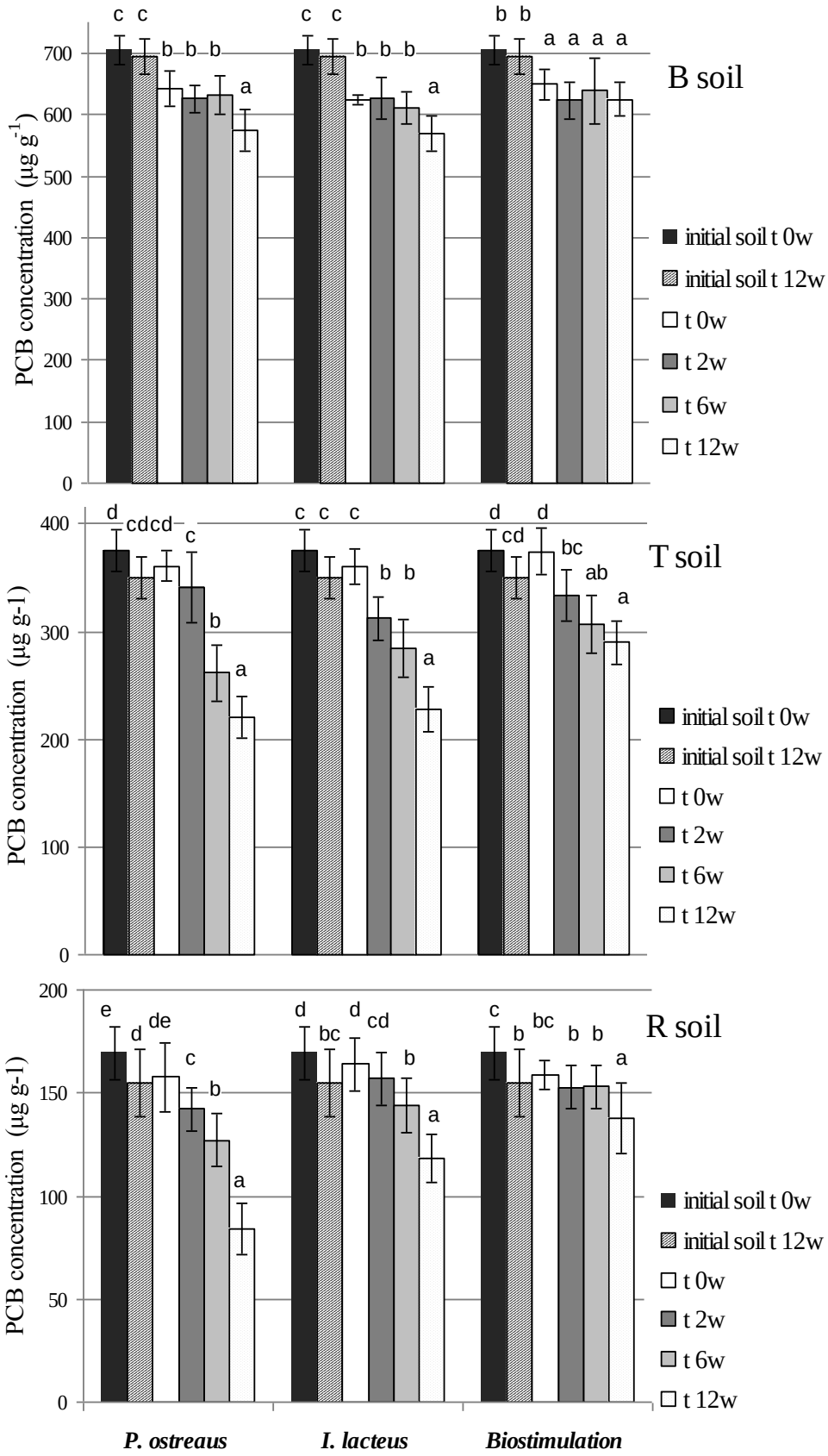


Figure 1 Time course (w stands for weeks) of PCB degradation in mycoaugmented (*P. ostreatus* and *I. lacteus*) and biostimulated (*Biostimulation*) microcosms with bulk soil (B soil), topsoil (T soil) and rhizosphere soil (R soil). The data are the mean ± standard deviation of three replicates.

Multiple pair-wise comparisons were performed by the Tukey's test ($P<0.05$). Same lowercase letters above bars indicate that differences among the harvests (time) within the same treatment were not significant.

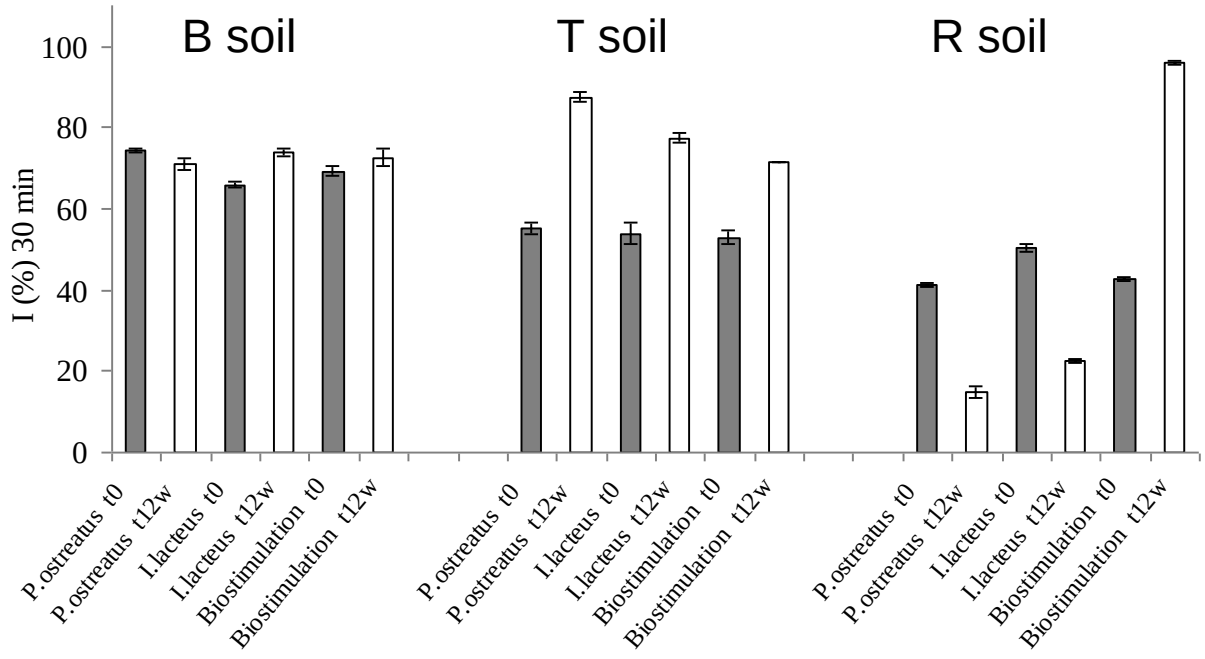


Figure 2 Percentage of luminescence inhibition in mycoaugmented (*P. ostreatus* and *I. lacteus*) and biostimulated microcosms with soils B, T and R at the beginning (t0) and after 12 weeks (t12w) of each treatment.

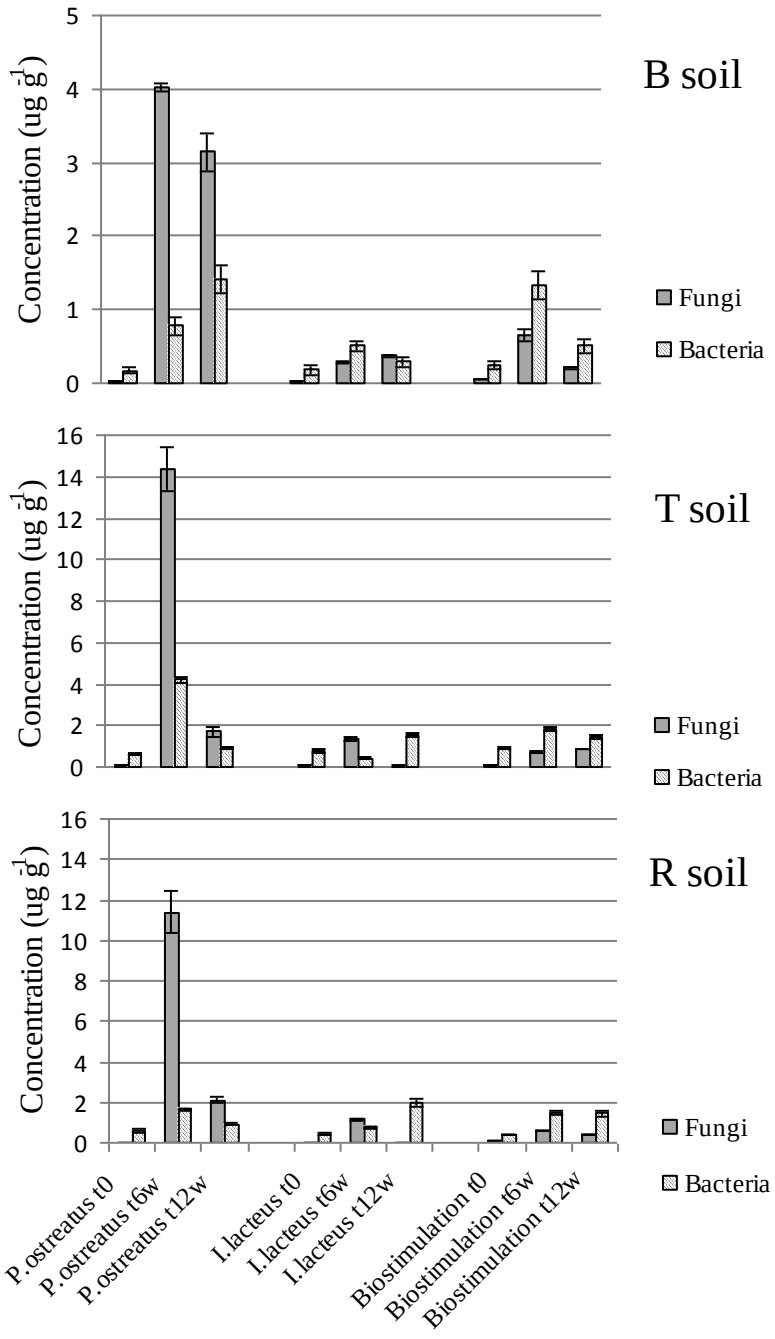


Figure 3 Phospholipid fatty acid concentrations (μg g^{-1}) in myco-augmented (*P. ostreatus* and *I. lacteus*) and biostimulated (Biostimulation) microcosms with bulk soil (B soil), topsoil (T soil) and rhizosphere soil (R soil) at the beginning (t0), and after 6 and 12 weeks (t6w and t12w, respectively). The data are the means ± standard deviation of five replicates.

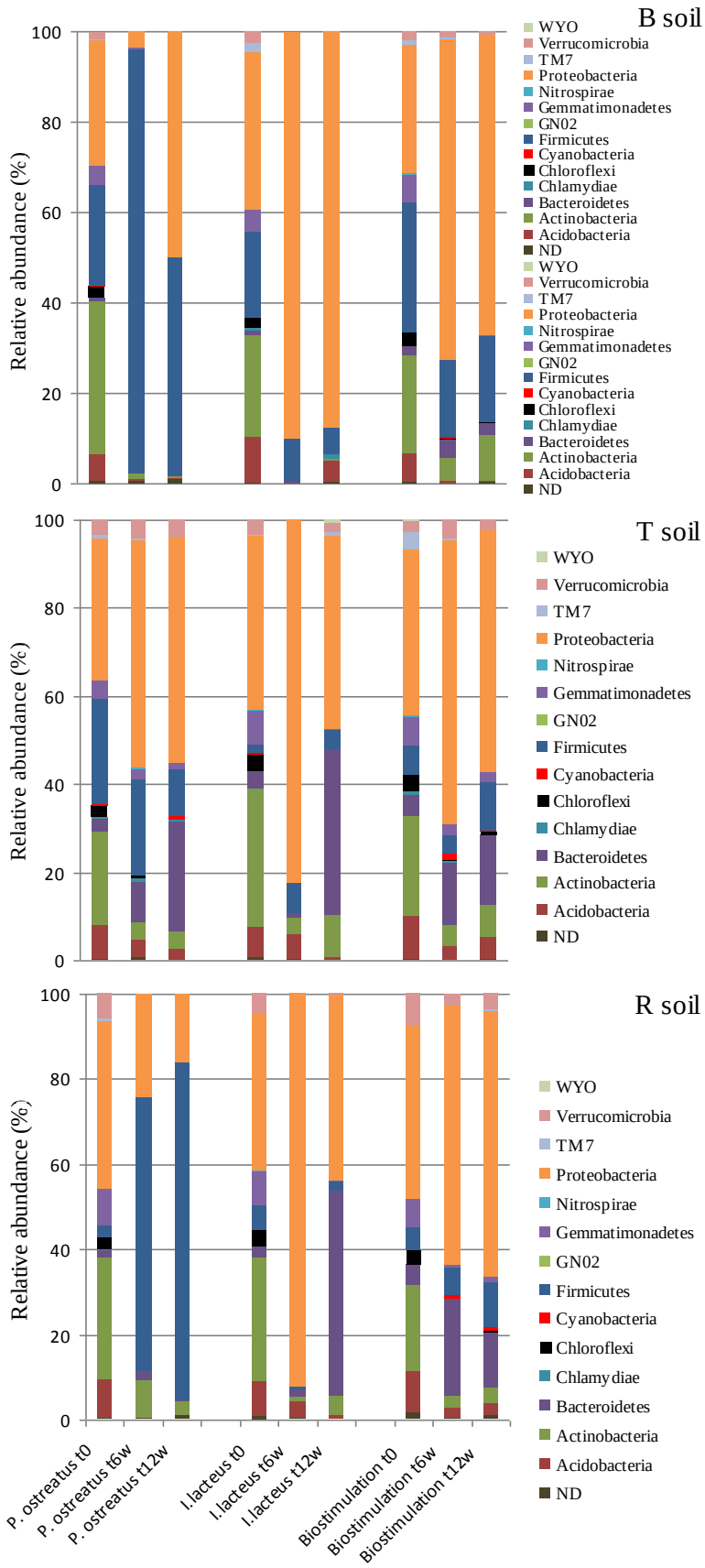


Figure 4 Taxonomic profiles of the bacterial community at the phylum level in myco-augmented (*P. ost* and *I. lac*) and biostimulated (Bio) microcosms with soil B, T and R. The segments composing each bar represent the relative abundances of each phylum, determined by pyrosequencing 16S rDNA after 0, 6 and 12 weeks (t0, t6w and t12w) of treatment. Sequences that could not be assigned to any phylum were referred to as ND (not determined).

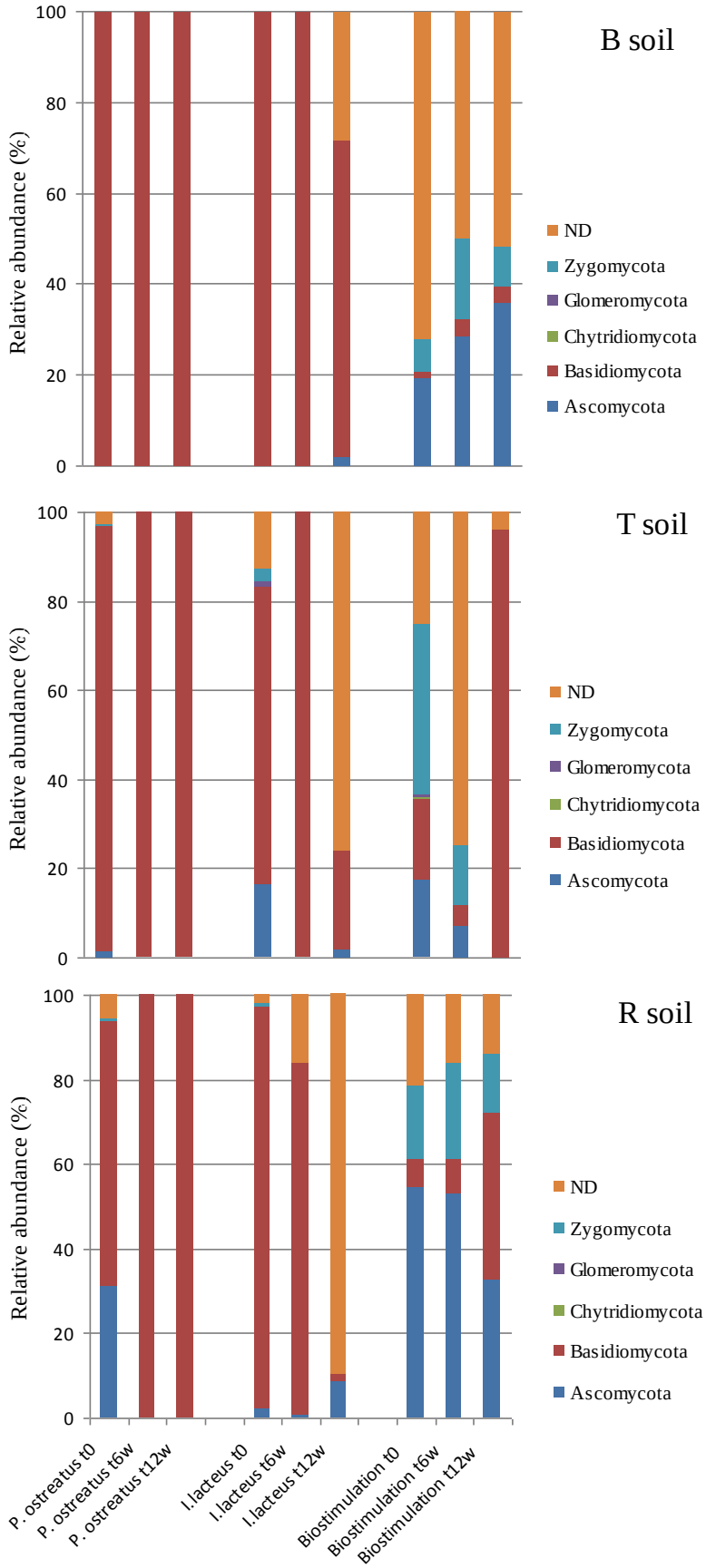


Figure 5 Taxonomic profiles of the fungal community at the phylum level in myco-augmented (*P. ost*. and *I. lac*) and biostimulated (Bio) microcosms with soils B, T and R. The segments composing each bar represent the relative abundance of each phylum, determined by pyrosequencing of the internal transcribed spacer (ITS) after 0, 6 and 12 weeks (t0, t6w and t12w) of treatment. Sequences that could not be classified into any phylum were assigned as ND (not determined).

STable 1. Physico-chemical properties of the bulk soil (B soil), topsoil (T soil) and rhizosphere soil (R soil). Adapted from Stella et al., 2015

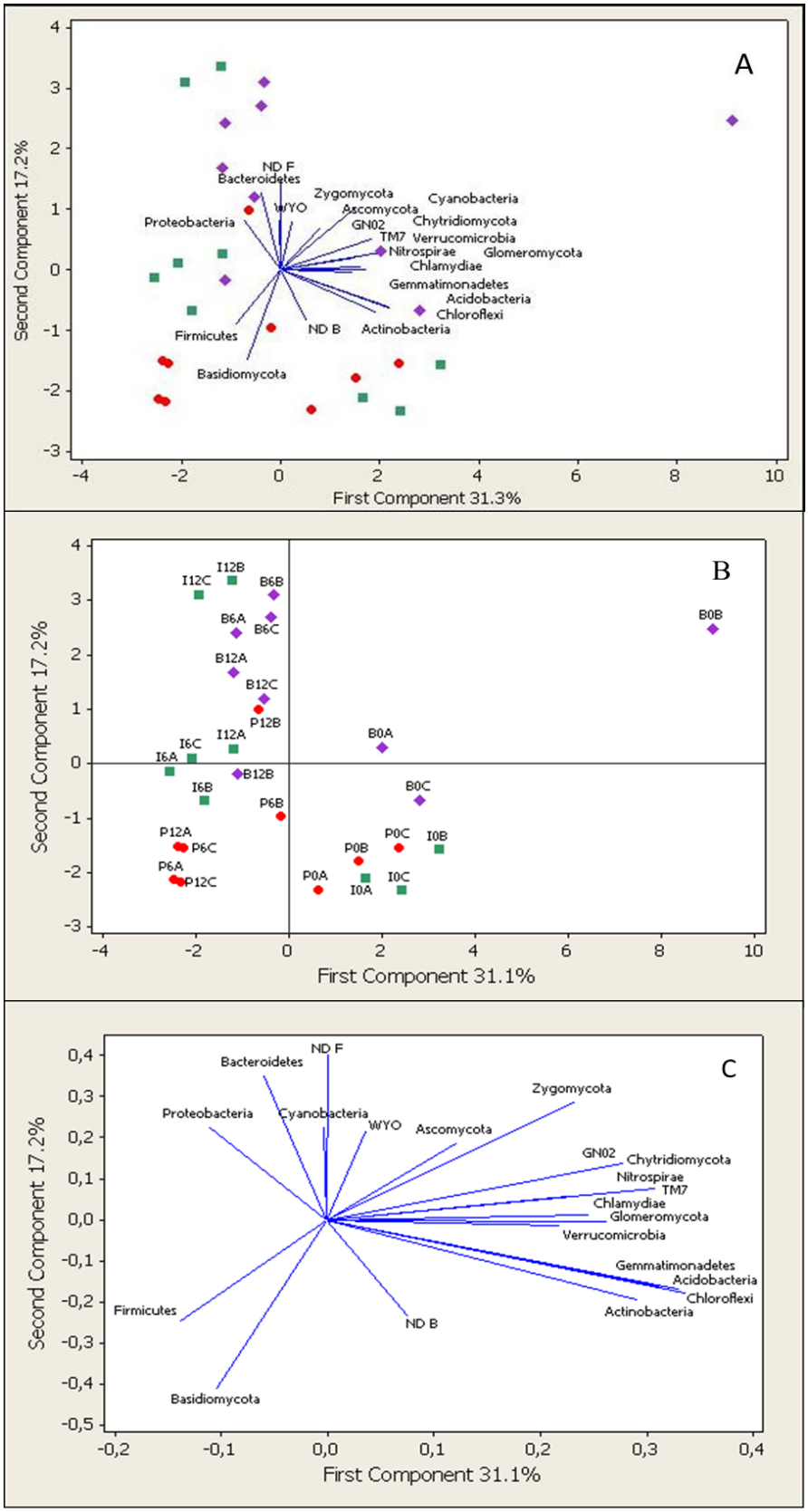
	Units	**B soil**	**T soil**	**R soil**
Combustion loss	%	3.81	6.15	6.46
C (Organic)	%	0.73	2.52	1.76
Fulvic acids	%	0.08	0.57	0.40
Humic acids	%	0.17	0.37	0.24
N total	%	0.07	0.23	0.17
Bulk density	g/cm^3	1.33	1.21	1.17
Accessible Ca	$mg\ kg^{-1}$	1650.00	2649.00	2355.00
Accessible Mg	$mg\ kg^{-1}$	325.00	311.00	266.00
Accessible K	$mg\ kg^{-1}$	102.00	412.00	247.00
Accessible P	$mg\ kg^{-1}$	45.40	111.40	79.10
Water holding capacity (WHC)	% Vol.	31.80	42.40	39.90
pH H_2O	---	6.60	6.7	6.16
pH $CaCl_2$	---	6.37	5.81	5.74
S/SO_4 - $CaCl_2$ leachate	$mg\ kg^{-1}$	5.20	9.40	7.30
Exchangeable H^+	$mmol^+/100\ g$	0.50	2.60	2.80
Exchangeable Ca	$mmol^+/100\ g$	9.24	16.40	12.89
Exchangeable Mg	$mmol^+/100\ g$	2.73	5.56	2.21
Exchangeable K	$mmol^+/100\ g$	0.26	1.25	0.70
Exchangeable Na	$mmol^+/100\ g$	0.05	0.05	0.05
CEC	$mmol^+/100\ g$	12.67	20.12	18.13
Sulphur content	$mmol^+/100\ g$	12.67	17.51	15.33
Degree of saturation	%	100.00	87.00	85.00
ECEC	$mmol^+/100\ g$	12.21	17.81	13.88
Hg AMA	$mg\ kg^{-1}$	0.04	0.05	0.05
As (Aqua regia)	$mg\ kg^{-1}$	43.91	35.86	43.93
Be (Aqua regia)	$mg\ kg^{-1}$	1.62	1.56	1.65
Cd (Aqua regia)	$mg\ kg^{-1}$	0.22	0.34	0.25
Co (Aqua regia)	$mg\ kg^{-1}$	15.67	13.38	17.66
Cr (Aqua regia)	$mg\ kg^{-1}$	160.10	123.00	174.00
Cu (Aqua regia)	$mg\ kg^{-1}$	31.30	20.60	29.00
Mo (Aqua regia)	$mg\ kg^{-1}$	0.05	0.05	0.05
Ni (Aqua regia)	$mg\ kg^{-1}$	54.10	43.70	52.60
Pb (Aqua regia)	$mg\ kg^{-1}$	24.60	22.80	22.80
V (Aqua regia)	$mg\ kg^{-1}$	40.30	41.60	44.40
Zn (Aqua regia)	$mg\ kg^{-1}$	97.00	100.80	100.20
Particles <0.001 mm	%	7.30	7.40	8.80
Particles <0.002 mm	%	9.20	9.80	11.10
Particles < 0.01 mm	%	18.30	19.80	22.80
Particles < 0.05 mm	%	40.40	39.80	46.60
Particles 0.01-0.05 mm	%	22.10	20.00	23.80
Particles 0.05-0.25 mm	%	24.80	26.60	22.20
Particles 0.25-2.0 mm	%	34.80	33.60	31.20

STable 2. Estimated Chao1 and Shannon indeces for bacterial taxa in myco-augmented (*P. ostreatus* and *I. lacteus*) and biostimulated (Bio) microcosms with B, T and R soils at the beginning (t0), and after 6 and 12 weeks (t6w and t12w, respectively).

	B soil		T soil		R soil	
	Chao1	Shannon	Chao1	Shannon	Chao1	Shannon
P. ostreatus t0	233.3	5.75	269.2	5.97	254.6	6.65
P. ostreatus t6w	97.2	3.68	276.8	6.23	143.5	4.69
P. ostreatus t12w	87.0	3.47	221.8	6.13	151.9	4.25
I. lacteus t0	277.8	6.22	275.4	6.80	256.4	6.79
I. lacteus t6w	41.9	2.17	113.4	4.17	82.8	3.92
I. lacteus t12w	56.0	2.91	140.3	5.40	137.8	4.53
Bio t0	273.2	5.63	310.1	6.85	352.4	6.85
Bio t6w	151.2	5.49	255.7	6.33	263.1	6.08
Bio t12w	170.7	5.63	218.0	6.45	327.0	5.94

STable 3. Estimated Chao1 and Shannon indeces for fungal taxa in myco-augmented (*P. ostreatus* and *I. lacteus*) and biostimulated (Bio) microcosms with B, T and R soils at the beginning (t0), and after 6 and 12 weeks (t6w and t12w, respectively).

	B soil		T soil		R soil	
	Chao1	Shannon	Chao1	Shannon	Chao1	Shannon
P. ostreatus t0	6.5	0.66	20.3	0.90	32.3	2.45
P. ostreatus t6w	48.0	3.94	6.7	0.65	45.4	0.56
P. ostreatu. t12w	7.9	0.54	5.2	0.51	6.5	0.54
I. lacteus t0	5.6	0.96	45.5	2.81	30.9	1.47
I. lacteus t6w	7.7	1.08	5.8	0.47	4.3	1.37
I. lacteus t12w	10.3	1.24	23.3	2.17	19.3	1.33
Bio t0	36.7	2.23	47.1	4.24	4.2	0.52
Bio t6w	42.4	3.15	43.9	2.56	60.8	3.93
Bio t12w	49.7	3.16	5.7	0.65	59.3	3.72



SFigure 1. Principal Component Analysis (PCA) biplot (A), scores plot (B) and loadings plot (C) of microbial communities and mycoremediation treatments. In biplot A and scores plot B, red circles, green squares and purple rhombus refer to *Pleurotus ostreatus* augmentation, *Irpex lacteus* augmentation and biostimulation treatments, respectively. In scores plot (B), the first capital letter of the alphanumeric code indicates the treatment type (P=Pleurotus, I=Irpex and B=biostimulation), the Arabic number the weeks of incubation (0, 6 and 12 weeks) and the last capital letter the polluted matrix (A=B soil, B=T soil and C=R soil). The percentage variance explained by each component is shown in X and Y axes captions.