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Research Paper

Fungal bioremediation of agricultural wastewater in a long-term treatment: biomass stabilization by immobilization strategy

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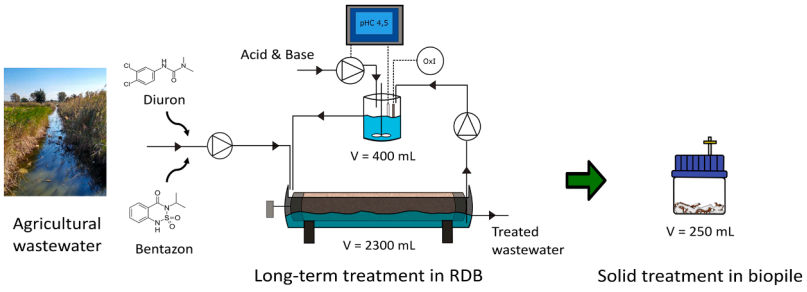
HIGHLIGHTS

- A fungal bioremediation treatment was successfully operated for more than 7 months.
- Using the suitable substrate and operating conditions improved biomass retention.
- Inoculation of *T. versicolor* led to the establishment of a stable fungal consortium.
- Fungal bioremediation technology is ready for full-scale application.

GRAPHICAL ABSTRACT



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ABSTRACT

Fungal bioremediation emerges as an effective technology for pesticide treatment, but its successful implementation depends on overcoming the problem of microbial contamination. In this regard, fungal immobilization on wood seems to be a promising strategy, but there are two main drawbacks: the predominant removal of pesticides by sorption and fungal detachment. In this study, agricultural wastewater with pesticides was treated by *Trametes versicolor* immobilized on wood chips in a rotary drum bioreactor (RDB) for 225 days, achieving fungal consolidation and high pesticide biodegradation through two main improvements: the use of a more favorable substrate and the modification of operating conditions. Fungal community dynamic was assessed by denaturing gradient gel electrophoresis (DGGE) analysis and subsequent prominent band sequencing, showing a quite stable community in the RDB, mainly attributed to the presence of *T. versicolor*. Pesticide removals were up to 54 % diuron and 48 % bentazon throughout the treatment. Afterwards, pesticide-contaminated wood chips were treated by *T. versicolor* in a solid biopile-like system. Hence, these results demonstrate that the microbial contamination constraint has definitely been overcome, and fungal bioremediation technology is ready to be implemented on a larger scale.

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1. Introduction

Pesticides are a broad group of chemical compounds used to improve agricultural production. However, pesticides have a high potential to drift into aquatic environments after application through storm-water discharges, surface runoff and leaching from agricultural areas (Vryzas, 2018). Thus, water bodies located near intensive agricultural fields are more vulnerable to pesticide contamination (Deknock et al., 2019). Furthermore, most pesticides are considered persistent compounds that, depending on their solubility, can remain dissolved in water or bioaccumulate in biota (Andrade et al., 2021). Long-term exposure to pesticides can lead to undesirable and harmful effects on the environment and human health. Even at low concentrations, chronic exposure to pesticides can pose a serious health threat not only to agricultural workers but also to the general population (Leong et al., 2020).

In this scenario, the development of effective treatments for the removal of pesticides in agricultural wastewater (AW) is mandatory. Physical-chemical methods have the advantage of being well-established, fast and effective processes against pesticides. However, these processes generate by-products (such as in adsorption) or toxic transformation products (such as in single ozonation) and they are considered relatively expensive (Ahmed et al., 2017). In contrast, bioremediation has emerged as a cost-effective, sustainable, and efficient alternative, although it typically requires longer treatment times and involves more complications related to biomass maintenance (Azubuike et al., 2020).

Among other microorganisms, white-rot fungi (WRF) have attracted attention for their unique and versatile metabolism, which is capable of degrading a wide range of xenobiotics through the activity of their intracellular and extracellular enzyme systems (Zhuo and Fan, 2021). In addition, WRF have shown excellent resistance to highly toxic waters without any prior acclimatisation process, unlike other biological treatments such as conventional activated sludge (Mir-Tutusaus et al., 2017). Nevertheless, fungal treatment still presents major challenges: nutrient addition, foaming, biomass ageing and microbial contamination (Mir-Tutusaus et al., 2018). In this regard, immobilization on lignocellulosic materials has been proposed as a promising strategy to address some of these operational limitations. Immobilization on supports prevents mycelial dispersion, which frequently causes foaming and blocking, and allows separation between hydraulic retention time (HRT) and cellular retention time. In addition, lignocellulosic materials, such as wood, provide a specific source of nutrients for fungi that facilitates operation under non-sterile conditions (Torán et al., 2017).

A previous scientific paper used *T. versicolor* immobilized on wood to treat agricultural wastewater in a rotating drum bioreactor (RDB) (Beltrán-Flores et al., 2020). This reactor was operated for a short period of time to evaluate the feasibility of the treatment, but some critical drawbacks, such as the loss of immobilized biomass, were detected. However, these problems may be solved by implementing favorable operational strategies to consolidate biomass immobilization, thus enhancing pesticide degradation, in a long-term continuous treatment. Therefore, the aim of the present study was to set up a RDB using *Trametes versicolor* immobilized on *Quercus ilex* wood chips to remove diuron and bentazon from AW over a long-term period. Throughout the treatment, rotation frequency and HRT were modified to analyse their effect on the bioreactor performance, including biomass maintenance and removal efficiency. A comprehensive study of the microbial populations was also conducted to evaluate the relative abundance of *T. versicolor* as well as other prominent members of the fungal community throughout the treatment, until a stable community with degrading capability was achieved.

2. Materials and methods

2.1. Fungal strain and culture conditions

T. versicolor ATCC 42530 was purchased from the American Type Culture Collection. *T. versicolor* was maintained on malt extract (2 % w/v) at 25 °C and routinely subcultured every 30 days. A mycelial suspension of *T. versicolor* was prepared as previously described elsewhere (Blánquez et al., 2004). *T. versicolor* immobilized on *Q. ilex* wood chips was prepared as described by Beltrán-Flores et al. (2021).

2.2. Agricultural wastewater

Wastewater was collected from an agricultural drainage channel in the Llobregat River Basin in Gavà (Catalonia, Spain) in July (AW I), October (AW II) and December (AW III) 2020, and subsequently stored at 4 ºC. In the case of the first water collection, a coagulation-flocculation pre-treatment was required to erase algae. Pretreated AW I was treated in Period I (P-I), while AW II & III in Period II (P-II) and III (P-III), respectively. Coagulant ferric chloride (40 mg L^{-1}) and flocculant PROSEDIM CS 209 (2 mg L^{-1}) were added according to the following process: 2 min coagulation at 200 rpm, 15 min flocculation at 20 rpm and 30 s sedimentation. The physicochemical characteristics of the AW are summarized in Table S1 (Supplementary material). The wastewater was adjusted to pH 4.5 and spiked with 10 ppm diuron and bentazon to allow detection by HPLC-UV. Stock solutions of diuron and bentazon were previously prepared in acetonitrile of high purity grade at 10 mg·mL^{-1}.

2.3. Chemicals and reagents

Diuron was purchased from Sigma Aldrich (Barcelona, Spain) and KAOS-B (48 % bentazon) was acquired from SAPEC AGRO (Barcelona, Spain). Acetonitrile and formic acid were supplied by Merck (Darmstadt, Germany). These chemicals and others used for *T. versicolor* subculture were of high purity grade.

2.4. AW treatment

Two rotating drum bioreactors (RDB) were operated in parallel, one of the reactors was assembled with wood initially colonized by *T. versicolor*, which is the experimental reactor (E-RDB), while the other reactor was set up only with wood as a control (C-RDB). Each RDB was constructed with a methacrylate tube (length: 45.0 cm x diameter: 8.4 cm) supported on a polyvinylchloride gutter, which contained 2.3 L of agricultural wastewater. The remaining RDB characteristics have been described in a recent study (Beltrán-Flores et al., 2020). In this case, the inner tube rotated one and a half turns every either 4 or 24 h (depending on the experiment) to ensure aerobic conditions. An external recirculation loop (4.7 L·day^{-1}) was required for pH adjustment and dissolved oxygen measurement, which were performed in a recirculation tank (≈ 0.4 L). The pH was automatically controlled at 4.5 by adding either 1 M HCl or NaOH. The dissolved oxygen (DO) level was monitored using a CyberScan 600 Series Waterproof Handheld (Eutech Instruments). The DO level remained above 30 % in the recirculation tank throughout the treatment.

Liquid samples were withdrawn from the reactor effluent for analysis of pesticide concentration, laccase, color, chemical oxygen demand (COD), heterotrophic plate count (HPC), and microbial community analysis. Solid samples were taken at the beginning and end of each period for ergosterol, fiber and microbial population characterizations. The reactors were successfully operated in continuous mode during 225 days. However, the treatment can be divided into three stages according to the different operational conditions implemented in each period, as shown in Table 1.

Table 1
Values of the main operating variables in each treatment period.

Period	Time (days)	Rotation frequency	Wood amount (g)	HRT (d)
P-I	0–93	1.5 turns every 4 h	315	3
P-II	93–186	1.5 turns every 24 h	210 (old) + 335 (fresh) = 545	3
P-III	186–225	1.5 turns every 24 h	545 (old)	5

P-I, P-II and P-III are periods I, II and III, respectively.

2.5. Pesticide analysis in liquid phase

Residual concentrations of diuron and bentazon were quantified by high-performance liquid chromatography (HPLC), with a lower limit of detection of 0.5 ppm. Liquid samples were initially filtered through Millipore Millex-GV PVDF filters (0.22 μm). Analyses were performed using a Dionex Ultimate 3000 HPLC system equipped with a UV detector operating at 254 nm. The separation was conducted in a C18 reversed-phase column (Phenomenex®, Kinetex® EVO C18 100 Å, 5 μm, 4.6 mm × 150 mm) at 30 °C with a mobile phase consisting of 0.01 % formic acid solution and acetonitrile. The organic gradient for chromatographic separation was described elsewhere (García-Vara et al., 2021). The flow rate and sample injection volume were 0.9 $mL{\cdot}min^{-1}$ and 40 μL, respectively.

2.6. DNA extraction and PCR-DGGE of the fungal community

Effluent and lignocellulosic support samples from the bioreactors were externally collected at regular intervals, immediately stored at 4°C and delivered within a few hours. Liquid samples were then filtered through 0.22 μm filters (Durapore®: PDVF membrane, 47 mm diameter), using a vacuum filtration pump (Millipore). After that, dry filters were stored at − 80°C until DNA extraction. Solid samples, instead, were processed to separate the fungal mycelium from the wood chips by mechanical force (sterile forceps), followed by agitation (30 min, 200 RPM), and sonication (7 min) in phosphate buffer (0.1 M, pH 7.0).

Total DNA extraction was then performed on filters using DNeasy® PowerWater DNA extraction kit (Qiagen), and on solid samples using DNeasy® PowerSoil DNA extraction kit (Qiagen) always following manufacturer instructions. After extraction, all DNA samples were stored at − 20°C until further processing. These samples were subsequently amplified by nested PCR to reduce non-specific products and increase sensitivity, using two sets of specific primers targeting fungal ITS (Internal Transcribed Spacer) region. The reaction mix was prepared on a final volume of 50 μL, containing 10–50 ng of DNA template, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 200 μM each of dATP, dCTP, dGTP and dTTP premixed, 12.5 pmoles of each primer and 0.5 U of Taq DNA polymerase enzyme (Invitrogen, Waltham, Massachusetts, USA).

The primer pair used for the first round of the nested PCR was EF4 and ITS4 (Gardes and Bruns, 1993), while primers ITS1F and ITS2R (White et al., 1990) were utilized for the second round. A GC-clamp (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C −3′) was attached to the 5′ end of the primer ITS1F to prevent complete denaturation for DGGE profiling. All PCR reactions were carried out in a Bio-Rad Thermocycler (model S1000) programmed for 35 cycles, each of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, an initial denaturation step of 94 °C for 5 min, and a final extension step at 72 °C for 7 min. The specific amplification products were analysed using agarose gel (1.5 %) in 1x TBE buffer (Trisborate–EDTA) stained with 0.5 μg/mL ethidium bromide.

Afterwards, all amplification products were further studied by denaturing gradient gel electrophoresis (DGGE) to assess fungal genetic variations throughout the experiment and provide an estimate of richness and abundance of prominent members of the fungal assemblage.

All DGGEs were performed in a "Dcode Universal Mutation Detection System" (Bio-Rad). Desired PCR products were loaded into 6 % (wt/vol) polyacrylamide gels with a 15–55 % chemical denaturing gradient (100 % denaturant contained 7 M urea and 40 % (v/v) deionized formamide) and submerged in 1x TAE (40 mM Tris acetate at pH 7.4, 20 mM sodium acetate, 1 mM EDTA).

Electrophoresis was performed in all cases at a constant 75 V and a temperature of 60 °C for 16 h using a Bio-Rad Power Pac 1000 power supply. The gels were inspected under UV illumination using a BioRad Universal Hood II Gel Doc™ XR System (BioRad) and photographed after staining them with ethidium bromide.

After visualization of DGGE profiles, prominent bands were cut, recovered, and stored at − 20°C in a buffer solution. Later, all excised bands were reamplified and sequenced at external facilities by Macrogen, Inc. (South Korea). The resulting sequences were compared with those in the Gen-Bank nucleotide database by using the Basic Local Alignment Search Tool (BLAST) to identify the closest known relatives (Altschul et al., 1990). Sequences determined in this study are available at the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers OL684714 through OL684779. InfoQuest™ FP (v4.5) software (Bio-rad, USA) was used to analyse the DGGE profiles and perform hierarchical cluster analysis by an unweighted pair group with mathematical averages (UPGMA) based on Euclidean distances. Subsequent analyses and statistics were performed using R version 4.1.2.

2.7. Fiber content analysis

Cellulose, hemicellulose and lignin contents were analysed by the method of Van Soest et al. (1991). The fiber content study was performed using an Ankom200 Fiber Analyzer incubator (Ankom Technology, USA) by continuously introducing sulfuric acid (96 %), an acid detergent solution (consisting of sulfuric acid and cetyltrimethylammonium bromide) and a neutral detergent solution (containing SDS, disodium EDTA dihydrate, 10-hydrated sodium tetraborate, anhydrous dibasic sodium phosphate and triethylene glycol, pH 6.9–7.1). Ash was obtained after at least 3.5 h incineration in a muffle at 550 °C. Fiber components were calculated as follows:

$$\text{Hemicellulose (\%)} = \text{NDF} - \text{ADF} \quad (1)$$

$$\text{Cellulose (\%)} = \text{ADF} - \text{ADL} \quad (2)$$

$$\text{Lignin (\%)} = \text{ADL} - \text{Ash} \quad (3)$$

where NDF represents the natural detergent fiber, ADF corresponds to acid detergent fiber and ADL means acid detergent lignin.

2.8. Fungal treatment in a biopile system

A total of 105 g of polluted wood chips and colonized wood chips were withdrawn from the control and experimental RDBs, respectively, after 93 days of treatment. Afterwards, 30 g of contaminated wood was treated by *T. versicolor* in a biopile system per duplicate using three different strategies: inoculation on wood, and re-inoculation and non-re-inoculation on wood containing pre-grown fungus. For this purpose, 7 mL of the mycelial suspension was inoculated onto the wood in Scott glass bottles (Duran, Inc; 250 mL, 95 ×105 mm) equipped with one port screw cap opened with a passive air intake through a filter (0.45 μm). The culture was maintained at a constant temperature of 25 °C under static and non-sterile conditions for up to 48 days. Solid samples were withdrawn after 27 and 48 days.

2.9. Other analyses

Both pesticides, diuron and bentazon, were extracted from wood following a modified method of Köck-Schulmeyer et al. (2013) as previously done in another study (Beltrán-Flores et al., 2021). Ergosterol content was monitored to quantify fungal biomass. The ergosterol analysis was conducted by homogenization and extraction of colonized wood in triplicate as described elsewhere (Rodríguez-Rodríguez et al., 2010a). Laccase activity was determined through the absorbance changes induced by the oxidation of 2,6-dymetoxyphenol (DMP) and expressed in activity units per litre ($UA{\cdot}L^{-1}$), as described elsewhere (Wariishi et al., 1992).

Conductivity was determined using a CRISON MicroCM 2100 conductometer. Color was measured as absorbance at a wavelength of 650 nm by an UNICAM 8625 UV/VIS. COD and ammonia concentrations were measured with the commercial kits LCK114 or LCK314m and LCH303, respectively (Hach Lange, Germany). Total suspended solids (TSSs) and volatile suspended solids (VSSs) were measured according to the standard methods 2540 D and 2540 E, respectively, and HPC were determined following the standard method 9215 (Baird et al., 2017). Chloride, nitrite and sulfate concentrations were quantified by ion chromatography using a Dionex ICS-2000.

Surface growth of *T. versicolor* on wood was assessed by scanning electron microscopy (SEM). Solid samples were fixed on a 2.5 % glutaraldehyde solution with 0.1 M phosphate buffer (PB). Afterwards, post-fixation was performed using 1 % osmium vapor containing 0.8 % ferrocyanide in 0.1 M PB overnight. Afterwards, the samples were washed with ultrapure water and dehydrated in increasing concentrations of ethanol (50 %, 70 %, 90 %, 96 % and 100 %). Finally, the samples were subjected to the critical point drying method (Baltec CPD030) and metallized with AuPd.

3. Results and discussion

3.1. Colonization of wood chips

Q. ilex wood chips presented the following characteristics: apparent and real densities of 0.27 $g{\cdot}mL^{-1}$ ± 0.01 and 0.89 $g{\cdot}mL^{-1}$ ± 0.02, respectively, and a porosity of 69.3 % ± 0.5 % (Beltrán-Flores et al., 2021). Afterwards, *T. versicolor* was inoculated on *Q. ilex* wood in a sterile box, reaching 0.26 $mg{\cdot}g^{-1}$ DW of ergosterol content after 17 days of culture. This value was considerably higher than that obtained in a screening assay by Torán et al. (2017), in which up to 0.031 $mg{\cdot}g^{-1}$ DW were reported using pine bark as immobilization support after 9 days of culture. These results can be explained by the fact that, in nature, *T. versicolor* is more frequently found on hardwood species, such as *Q. ilex*, than in conifers (Cotter, 2014).

3.2. Reactor performance

3.2.1. Biomass content

The initial treatment conditions are those indicated as P-I in Table 1, corresponding to the first 93 days of treatment. The main objective of P-I was to evaluate the adaptability of the fungus to the real environmental conditions found in an RDB. In this regard, an RDB had been previously used for the treatment of AW by *T. versicolor* pre-grown on lignocellulosic substrate (Beltrán-Flores et al., 2020). However, the fungus apparently detached from the wood surface throughout the treatment. In that case, the loss of biomass was mainly attributed to the application of an excessive continuous rotation speed (6 rpm). High rotation speeds can cause undesirable damage to the mycelial structure of fungi (Zhong, 2010). Consequently, in P-I of the present study, the rotation frequency was considerably reduced from 6 rpm to 1.5 turns every 4 h. The time-course of ergosterol content was monitored to quantify the presence of fungal biomass over the studied period.

Despite this adjustment, a significant decrease in fungal biomass was observed after 78 days of treatment, when only 20 % of the initial fungal content remained immobilized on the wood (see Table 2). P-I was maintained until 93 days to give to the fungus a chance to recolonize the wood, as occurred in a previous study after significant decreases in biomass (Rodríguez-Rodríguez et al., 2010b), but the ergosterol level was not recovered.

The substantial loss of biomass in P-I was compensated by replacing part of the polluted wood with fresh inoculated wood chips to the E-RDB. The same procedure was performed in the C-RDB, but in this case replacing the contaminated wood by fresh wood chips. Subsequently, the rotation frequency was reduced to 1.5 turns every 24 h (6 times less than in P-I) during the next 93 days of P-II (186 days in total). As shown in Table 2, the ergosterol level was successfully maintained during the first 78 days of P-II. This result showed evidence that static conditions favored the preservation and growth of the fungal biomass. By reducing the turning frequency to 24 h, the non-submerged biomass fraction probably had a longer recovery period from micropollutant exposure and submergence, which probably benefited fungal immobilization and growth.

In the 186-day sample, a significant drop in ergosterol level was detected. However, this decrease was attributed to an incident in the control system on day 181, which caused the pH of the water to drop from 4.5 to 3.3 for 24 h. After this period, this technical failure was solved and the pH reset to 4.5. It is well known that a too acidic pH inhibits the enzymatic production and fungal growth (Mir-Tutusaus et al., 2018). In P-III, the HRT was increased from 3 to 5 days, thus keeping the water longer in the reactor. In this case, a remarkable fungal colonization of the wood was achieved after only 44 days of P-III (225 days in total). This rapid colonization is probably a result of the consolidation of the established fungal community (see section 3.2.2), and an enhanced reduction in pesticide concentration (see section 3.2.4).

Variations in the fungal biomass content throughout the treatment detected via ergosterol analysis were also visualized by SEM. As shown in Fig. 1(a) and (b), wood chips were densely colonized by *T. versicolor* mycelial biomass under sterile conditions. Afterwards, the colonized wood was used to set up a RDB under non-sterile environment in the operational conditions of P-I. As demonstrated by the ergosterol results, the fungal mycelial density was considerably reduced after 93 days of treatment (see Fig. 1(c)). However, the same extent of colonization was recovered after implementing the modifications of the operational conditions in P-II and P-III (see Fig. 1(d)).

3.3. Fungal community assemblage

In order to assess the fungal diversity of both reactors and the behavior and persistence of *T. versicolor* after being inoculated, a DGGE analysis was performed. In Fig. S1 (Supplementary materials), the DGGE

Table 2
Fungal biomass presented on the wood chips, in absolute and relative terms, throughout the treatment.

Period	Time (days)	Ergosterol amount ± SD (mg·g DW $wood^{-1}$)	Remaining biomass ± SD (%)
I	0	0.26 ± 0.02	100
I	48	0.12 ± 0.02	44 ± 6
I	78	0.05 ± 0.01	20 ± 2
I	93	0.05 ± 0.01	21 ± 1
II	93	0.15 ± 0.01	56 ± 4
II	123	0.12 ± 0.01	45 ± 2
II	141	0.13 ± 0.02	50 ± 8
II	171	0.15 ± 0.01	59 ± 0
II	186	0.09 ± 0.01	36 ± 1
III	186	0.09 ± 0.01	36 ± 1
III	225	0.25 ± 0.03	97 ± 13

*Results are shown as mean values and corresponding standard deviations for triplicate measurements.

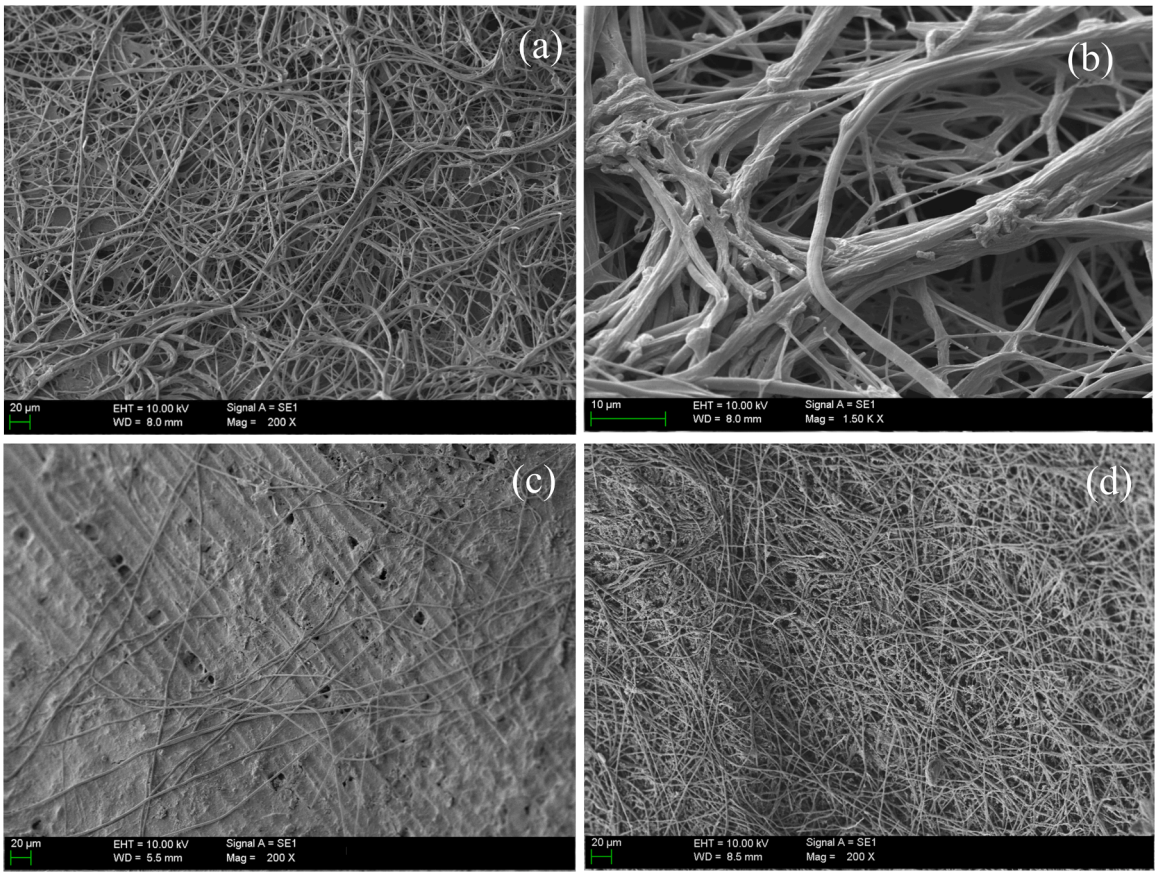

Fig. 1. SEM images of initial colonized wood for magnifications of 200 X (a) and 1500 X (b), after 93 days for 200 X (c) and 230 days for 200 X (d).

band profiles and UPGMA cluster analysis of the fungal populations from the lignocellulosic support of the reactors can be observed. These samples were taken at four different times: at the start of the experiment ("t_0"), at the end of period I ("t_{93}"), at the end of period II ("t_{186}") and finally, near the end of the experiment ("t_{216}"). This figure shows remarkable differences between samples from both reactors, control (C) and experimental (E), as they are clearly separated in the cluster analysis, while replicates of the same sample show a high degree of homogeneity.

It can also be noted that the fungal profiles corresponding to the C-RDB exhibit a higher degree of complexity than those belonging to the E-RDB, thus revealing a higher fungal diversity in the former in comparison with the latter. This suggests that inoculation of *T. versicolor* in the E-RDB contributes to a more stable, less diverse community of fungal species, as it dominates the competition for space and nutrients.

To fully comprehend fungal dynamics in the system, effluent of the reactors was also analysed along the experiment. The analysis of these samples provides data about those fungi that were washed away from the lignocellulosic support. This, coupled with the previous analysis of the lignocellulosic support, allows for a better understanding of how fungal populations evolve inside the system throughout the treatment. Fig. S2 (Supplementary materials) shows the DGGE band profiles and cluster analysis corresponding to the fungal community from the effluent. Unlike in the lignocellulosic matrix study, in this case both RDBs presented a common dominant phylotype throughout the entire experiment. Interestingly, samples taken from the same time tend to cluster together regardless of which reactor they come from. This can be explained by shifts in the composition of the influent fungal communities that could affect survival and adherence of different species inside both reactors. However, in the same way as in the lignocellulosic support analysis, diversity in the C-RDB tends to be higher than in the E-RDB, further supporting the hypothesis that inoculated *T. versicolor* has a stabilizing effect on the community, reducing diversity as it consolidates.

In order to assess the presence of *T. versicolor* throughout the experiment prominent bands of the gels were sequenced, allowing the calculation of the relative abundance of each phylotypes present in solid and liquid samples from both reactors (Figs. 2 and 3 respectively). In the wooden substrate from the E-RDB (Fig. 2), it could be observed how relative abundance of *T. versicolor* decreased over time from 90 % of the total fungal diversity, down to a 30 % at the end of stage II, and from this point to the end of the experiment it remained constant. This suggests that the reactor reached a stable state where *Trametes* coexisted with two more evenly distributed fungal phylotypes: *Dipodascus* sp. and *Rhodotorula* sp. (note that Evenness index is close to 1). *Dipodascus* sp. is found in the wastewater used as the influent (Fig. 3) and *Rhodotorula* sp. was already present in the wood inoculated with *T. versicolor* (Fig. 2). Both genera have been described in biodegradation studies (De Hoog and Smith, 2011; Dusengemungu et al., 2020), and *Rhodotorula* sp. was also detected in a reactor inoculated with *T. versicolor* (del Álamo et al., 2022). Regarding the C-RDB, a clear dominance of *Penicillium* is detected in the early stages, (Fig. 2). An increase of fungal diversity was observed over time, so that at the end of the stage III, 8 different phylotypes were detected, with no evidence of having reached a fungal community stability, as opposed to the E-RDB.

Relative abundance of fungal populations in influent and effluent samples was also calculated at different stages of the experiment (Fig. 3). Expectedly, *T. versicolor* was detected at all stages of the E-RDB, but not in the C-RDB. However, it can be observed that *T. versicolor* relative abundance decreased in the effluent as the experiment progressed. This, coupled with the results obtained from the wooden matrix analysis, suggests that the inoculated fungus consolidated over time in the E-RDB,

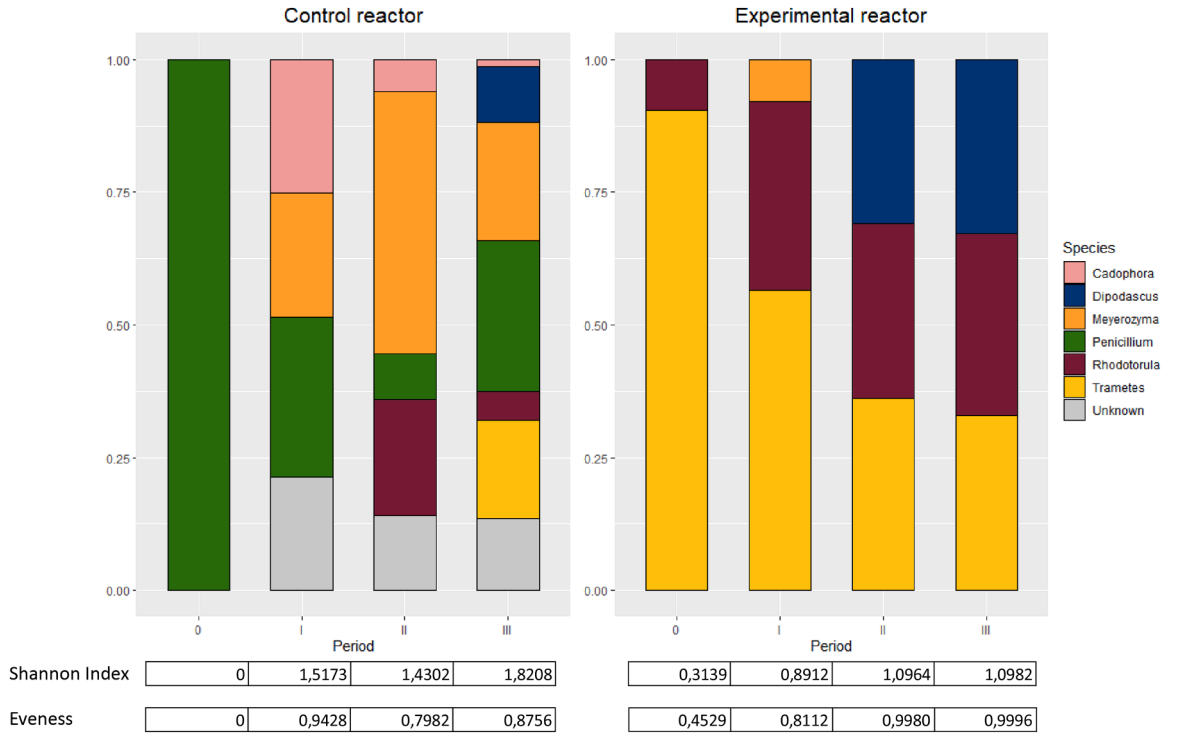

Fig. 2. Relative abundance of fungal populations in the control reactor (left), and in the experimental reactor (right), corresponding to the lignocellulosic support samples from the stages studied (0, initial; I, t93; II, t186; III, t216). Values for Shannon Diversity and Evenness Indices are shown at the bottom of each sample.

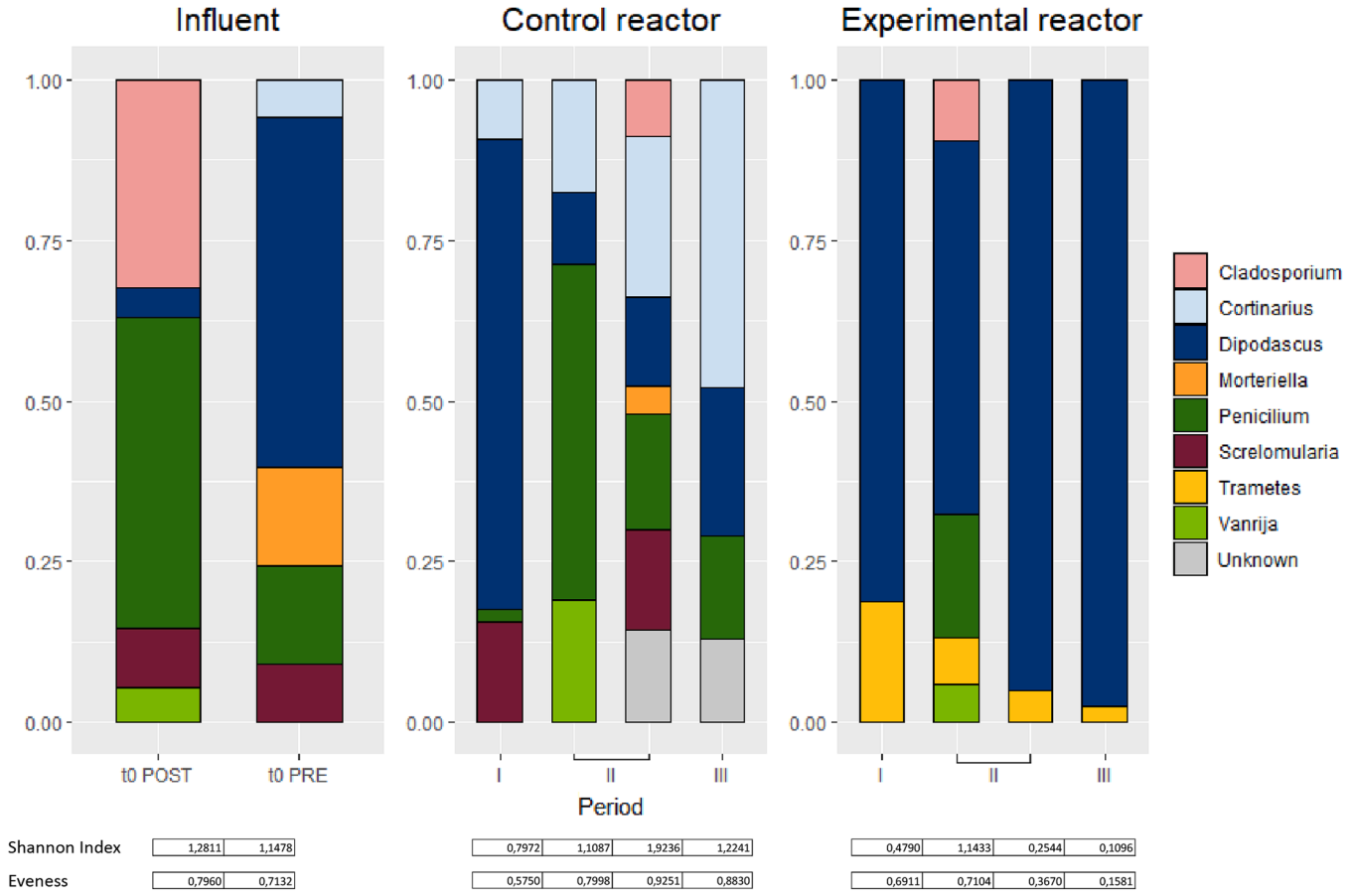

Fig. 3. Relative abundance of fungal populations in the control reactor (left), and in the experimental reactor (right), corresponding to the effluent samples from the stages studied (0, initial; I, t60; II, t120 and t186; III, t216). Values for Shannon Diversity and Evenness Indices are shown at the bottom of each sample.

keeping attached to the lignocellulosic matrix and therefore reducing its washout from the system. As the experiment progresses, a clearly dominant phylotype appears in the effluent of both reactors: *Dipodascus* (De Hoog and Smith, 2011; Dusengemungu et al., 2020; Kawai and Hu, 2009). This fungal genus, ends up representing a 40 % of the fungal diversity in the C-RDB, and more than 90 % in the E-RDB. *Dipodascus* was, in fact, the dominant genus detected in the original AW used as influent for this experiment before pre-treatment was performed, when its presence decreased in favor of the ubiquitous *Penicillium*. This common fungal genus colonizes the lignocellulosic substrate in the C-RDB, and it seems that *Dipodascus* is not able to remain attached to it, and so it is washed from the system. Interestingly, in the E-RDB *Penicillium* is not part of the dominant population, probably displaced by the inoculated *Trametes*.

Finally, it can be observed how Shannon indexes corresponding to the E-RDB are usually lower than in the C-RDB for both lignocellulosic support and liquid samples (Shannon Diversity Index data is shown in Figs. 2 and 3). This is consistent with the previous hypothesis that inoculation of *T. versicolor* in the E-RDB contributes to a more stable and less diverse fungal community, as it consolidates in the wooden matrix.

Therefore, the present study has demonstrated the ability of *T. versicolor* immobilized on wood to survive under non-sterile conditions during long-term treatments. Although the relative abundance of this fungus is reduced, a stable consortium is established that succeeds in maintaining its degrading capability, as shown in Section 3.2.4.

3.3.1. Evolution of bacterial counts

The HPC results are presented as the logarithm of colony-forming units (CFU) per mL in Fig. S3 (Supplementary materials). Bacterial counts remained at a lower level in the E-RDB compared to the C-RDB throughout the entire treatment, especially during P-II and P-III. Therefore, bacterial contamination was successfully controlled in the E-RDB, indicating that the immobilization on *Q. ilex* wood can be an appropriate strategy to limit bacterial growth. In other systems where glucose is used instead of wood for fungal maintenance, high bacterial growths have been frequently reported (Hu et al., 2021; Mir-Tutusaus et al., 2017).

3.3.2. Pesticide removal

Time-course profiles of diuron and bentazon concentrations are presented in Fig. S4 (Supplementary material). However, to facilitate interpretation of the results, pesticide concentrations were converted into relative removals and grouped into 15-days periods in Fig. 4. As can be seen, the elimination efficiencies achieved in the RDB-E were clearly higher than those in the RDB-C for both pesticides throughout the treatment, demonstrating the favorable contribution of *T. versicolor* to the removal capacity of the system, reaching removal peaks of 54 % diuron and 48 % bentazon.

Comparing pesticides, the diuron removal was clearly higher to that of bentazon, even in the case of the C-RDB. This phenomenon was partly related to the different sorption capacity of *Q. ilex* for each pesticide. Beltrán-Flores et al. (2020) showed that diuron had higher affinity for *Q. ilex* than bentazon, which was related to the different hydrophobic properties of the compounds. Thus, wood not only serves as an immobilization support and a substrate for fungus, but can also play a significant role in pesticide removal by sorption process. However, the removal in the C-RDB cannot be attributed exclusively to sorption, as other fungi detected in the microbial community (such as *Dipodascus* sp. and *Rhodotorula* sp.) have been described in bioremediation studies (De Hoog and Smith, 2011; Dusengemungu et al., 2020).

A partial renovation of the colonized wood was required to recover the biomass washed out of the reactor and the treatment capacity of the system from 93 days onwards (see Section 2.2.1). In this regard, the highest removal efficiencies were achieved at the beginning of P-I and P-II, when fresh biomass was added to the system. Changes in operating conditions enhanced biomass retention, and as a result, the E-RDB

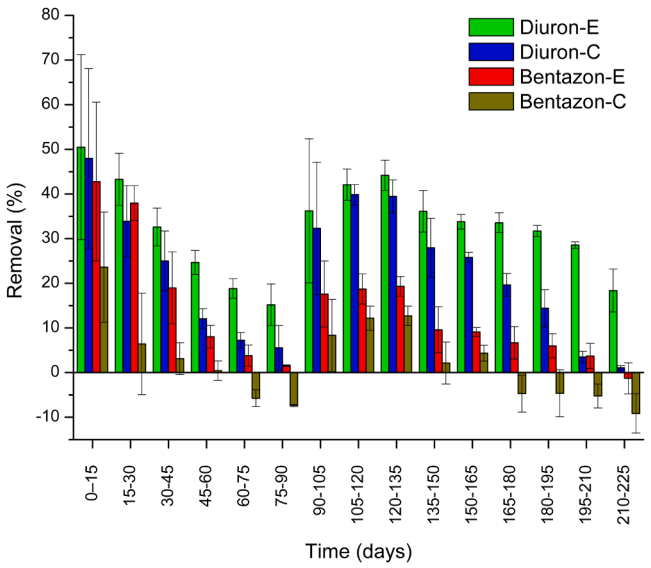

Fig. 4. Time-course profiles of diuron and bentazon removals grouped in 15-days periods obtained in the E-RDB and the C-RDB. The error bars represent the standard deviations for the specified periods.

maintained its treatment capacity for a longer period of time (225 days). In contrast, the C-RDB even showed bentazon from 60 to 75 days onwards in P-I, and from 165 to 180 days onwards in P-II. Therefore, inoculation of T. versicolor improved treatment efficiency while extending the useful operational period of the system.

Of particular interest is the maintenance of diuron removal capacity by the E-RDB in P-II and P-III, being considerably more stable over time than in P-I. In contrast to P-I, where E-RDB and C-RDB removals seemed to follow similar trends, a divergence was observed in the last two periods. This phenomenon was mainly attributed to a higher amount of fungal biomass immobilized on the wood in the E-RDB as a consequence of the reduction of the reactor rotation frequency in P-II and the increase of the HRT from 3 to 5 days in P-III. In P-I, the E-RDB achieved up to 12.6 % more removal than the C-RDB (days 45–60). In P-II, biomass maintenance was consolidated and increased by up to 13.9 % (days 165–180). Finally, increasing HRT prolonged treatment time, resulting in an improvement of up to 25.1 % in the E-RDB compared to the C-RDB.

In the case of bentazon, its removal was probably influenced by the presence of laccase, which was only detected at the beginning of P-I and P-II, when fresh biomass was introduced into the reactor (see Fig. S5 of Supplementary material). Recently, laccase has been reported to be involved in bentazon biodegradation (García-Vara et al., 2021), while there is no clear consensus for diuron (Coelho-Moreira et al., 2018; Hu et al., 2020b). Nevertheless, the lack of laccase does not necessarily entail fungal inactivation, as *T. versicolor* possesses an intracellular enzyme system capable of degrading this type of compounds (Mir-Tutusaus et al., 2017).

3.3.3. COD and color

The initial COD of the AW was considerably increased from 144 and 44 mg $O_2 \cdot L^{-1}$ in P-I and P-II&III, respectively, to approximately 3000 mg $O_2 \cdot L^{-1}$ upon addition of the stock solutions of diuron and bentazon (10 mg·mL^{-1}). This increase was mainly attributed to the use of acetonitrile as solvent for the stock solutions of each pesticide (1 mg·mL^{-1}), since its theoretical contribution to the COD of the AW (2451 mg $O_2 \cdot L^{-1}$) is much higher than that of diuron (13 mg $O_2 \cdot L^{-1}$) and bentazon (15 mg $O_2 \cdot L^{-1}$). However, as shown in Fig. 5, COD was rapidly reduced by about half in the first days of treatment in both reactors, indicating that acetonitrile was easily removed. However, after this drastic decrease, the COD values of both reactors were still considerably higher than the COD of the original wastewater. This

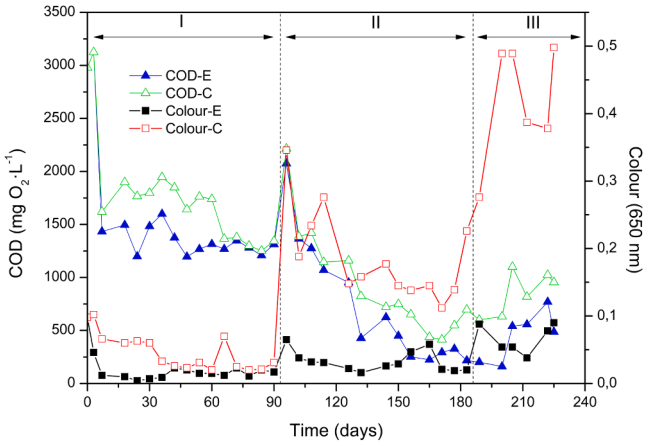

Fig. 5. COD and color profiles of the AW during the continuous treatment. Blue and green triangles are the COD concentrations of the effluent streams in the E-RDB and C-RDB, respectively, while the black and red squares are the color levels of the effluent streams in the E-RDB and C-RDB, respectively. Vertical dashed lines divide each operating period (I: Period I, II: Period II and III: Period III).

difference was associated with the extraction of some soluble compounds from the wood: initially, a large amount of these extracts was released, but as the treatment progressed, their contribution to COD became less important. In P-III, COD level rose slightly as a consequence of the increase in HRT from 3 to 5 days.

Contradictory results have been previously reported regarding the COD reduction capacity of *T. versicolor* (Cruz-Morató et al., 2013; Souza et al., 2014). In the present study, a better performance was observed in the E-RDB compared to the C-RDB, suggesting that *T. versicolor* along with the microbial consortium contributed to reduce COD generated by the extraction of organic compounds from wood. In this regard, although COD remained above the original COD throughout the treatment, the E-RDB showed considerably lower levels than the C-RDB, with average relative removal of 14 %, 23 % and 51 % in P-I, P-II and P-III, respectively.

Similar results were obtained with respect to color analysis. A considerable increase in color was detected when the wastewater came in contact with the wood owing to the extraction of some soluble compounds that give the characteristic dark color to *Q. ilex* wood. In P-II and P-III, considerable colouration increases were shown in the C-RDB as a result of the introduction of fresh wood and the increased HRT, respectively. However, the E-RDB showed higher decolorization over time, reaching average eliminations of 62 % in P-I, 82 % in P-II and 84 % in P-III compared to the C-RDB. As shown in Fig. 5, the addition of fresh wood in P-II and the increase of HRT in P-III leaded to more intense colors in the C-RDB, while remaining approximately constant in the E-RDB. Similar results were obtained by Torán et al. (2017) using *T. versicolor* immobilized on pine wood in a trickle-bed reactor, in which approximately constant colouration levels were reported during the treatment. Therefore, the E-RDB proved to be efficient in terms of COD and color removal.

3.3.4. Variation of fiber content in wood

A fiber analysis was conducted to assess the wood consumption by the fungal biomass. The chemical composition of each fiber type is given in Table 3. For a better interpretation of the results, the inert ash fraction was considered inert and used as a basis to calculate the relative content of each fiber referred to the initial wood content (t = 0), and thus obtain the percentage reduction of each component.

As can be seen, the degradation capacity of the fungal consortium changes depending on the fiber type. Interestingly, the most highly degraded compound was lignin. Unlike cellulose and hemicellulose, which are relatively easily biodegradable polysaccharides used as substrates in the primary metabolism of multiple fungi and other microorganisms, lignin is an extremely recalcitrant compound and only seems to be biodegradable through secondary metabolism by the ligninolytic system of WRF. Since lignin oxidation results in no energy gain, the WRF main objective in secreting lignocellulosic enzymes (primally lignin peroxidase, manganese peroxidase and laccase) is to access the internal polysaccharides of the wood (Pointing, 2001). The fact that the highest wood consumption seems to be lignin indicates that *T. versicolor*, as the only WRF detected in the E-RDB, was probably the main microorganism consuming wood components.

Table 3
Comparison of fiber composition between the wood chips from the E-RDB after 225 days of treatment and the original wood.

Components	Composition (%·DW^{-1})		Reduction (%)
	E-RDB (t = 0 days)	E-RDB (t = 225 days)	
NDF (%)	84.34 ± 0.25	83.60 ± 0.01	–
ADF (%)	60.51 ± 0.03	60.62 ± 0.11	–
ADL (%)	15.53 ± 0.31	13.13 ± 0.08	–
Hemicellulose	23.83 ± 0.28	22.98 ± 0.12	8.55 ± 0.10
Cellulose	44.98 ± 0.34	47.49 ± 0.19	-0.12 ± 0.01
Lignin	11.68 ± 0.32	9.07 ± 0.09	26.36 ± 0.72
Ash	3.85 ± 0.01	4.06 ± 0.01	0
Wood mass	–	–	5.17 ± 0.01

*Results are shown as mean values and corresponding standard deviations for triplicate measurements.

This analysis also showed a slight loss of wood mass during the treatment. This result supports the hypothesis that the increase in COD was caused by the release of soluble organic matter from wood. However, since wood decomposition was relatively low, substrate replacement was found to be unnecessary to ensure survival of *T. versicolor*. Therefore, fresh wood would only be required to restore the adsorption capacity of the system.

3.4. Solid-phase treatment in a biopile system

In treatments involving sorption, not only the removal performance in the liquid phase, but also the fate of the polluted sorbent should be studied. Thus, additional remediation approaches should be explored to reduce the pesticide content in the solid by-products. Accordingly, a solid-phase treatment was performed in a biopile-like system by inoculating *T. versicolor* onto the by-products. Samples were withdrawn from the E-RDB (inoculated wood) and C-RDB (non-inoculated wood) after 93 days of treatment (at the end of P-I), to be subsequently treated in the biopile for up to 48 days. In this regard, three different strategies were assessed: wood inoculation with *T. versicolor* (C-I), no re-inoculation (E-N) and re-inoculation of colonized wood (E-R).

Although a similar experiment was performed in a recent study (Beltrán-Flores et al., 2021), the by-products had been subjected to a treatment period of only 20 days and the biopile treatment period had been limited to 27 days. The use of more realistic operational periods both in the RDB (93 days), which in turn change the characteristics of the wood and therefore the post-treatment performance, and in the biopile (48 days), can provide more reliable results and useful information for further full-scale application.

Fig. 6 shows the results of the biopile experiment after 27 and 48 days of treatment. As expected, higher residual concentrations were detected for diuron, which as a hydrophobic compound tends to be sorbed by wood. Interestingly, higher initial concentrations were found in the colonized wood than in the wood without fungus. This fact evidenced a slight sorption contribution of the fungal biofilm to the overall removal, which has been previously reported (Hu et al., 2020a).

A mass balance of the total amount of both pesticides introduced into the reactor showed that the experimental sorbed amount was found to be considerably lower, 0.207 mg·g^{-1} for diuron and 0.010 mg·g^{-1} for

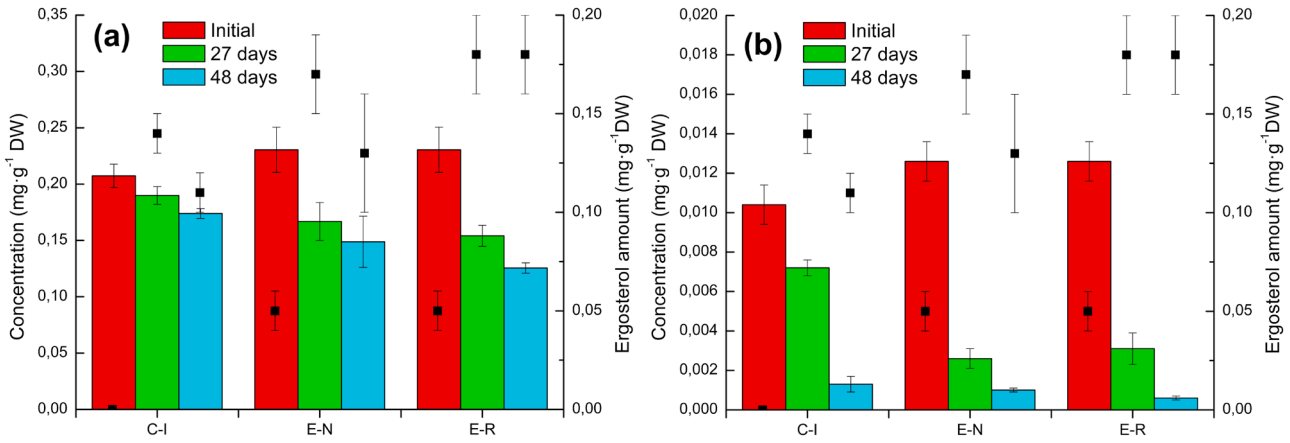

Fig. 6. Evolution of residual concentration of diuron (a) and bentazon (b), and ergosterol content in the biowastes during the biopile treatment. Residual concentrations are represented with bars after 0 (blue), 27 (green) and 48 (yellow) days of treatment (n = 2). Black line is the average ergosterol level for three replicates. Biopile conditions: C-I corresponds with wood inoculated; E-N with biowastes without re-inoculation; E-R with biowastes with re-inoculation. Results are shown as mean values and corresponding standard deviations for duplicate measurements.

bentazon, compared to those reported in the above-mentioned study, being of up to 0.240 mg·g^{-1} for diuron and 0.020 mg·g^{-1} for bentazon (Beltrán-Flores et al., 2021). These results suggest that the wood reduced part of its adsorption capacity when subjected to a long-term treatment, probably as a result of the consumption and degradation of this substrate by the fungal consortium.

Fig. 6 shows that the relative elimination of bentazon is higher than that of diuron throughout the treatment. For example, for E-I and 48 days, relative removals were 95 % for bentazon and 45 % for diuron. However, biodegradation kinetics of both pesticides was also estimated to compare the treatment efficiency with the literature. Table 4 shows the biodegradation kinetic constants and rates for both pesticides, which were found to be clearly higher for bentazon, as shown in Fig. 6(b). It is feasible to assume that bentazon, being a more polar molecule, has a lower diffusion capacity inside the wood, and thereby higher bioavailability to interact with the fungus.

Table 4 also shows that E-R was the most efficient strategy in terms of degradation rate, followed by E-N and C-I. However, the degradation performance of the E-R was only slightly higher than that achieved by the E-N, which questions the viability of reinoculation. In this regard, similar results of diuron degradation rates were reported in another solid-phase biopile treatment with *T. versicolor* (Beltrán-Flores et al., 2021). With regard to bentazon, the removal efficiency was notably higher, but in that case the wood had been previously subjected to a much shorter period of treatment, 20 days instead of 93 days used in the present study. This result may be due to the fact that older wood may constitute a worse substrate for the fungus, thus depressing its metabolic activity in the biopile treatment.

In any case, the application of the E-R was justified considering ergosterol content, being more stable than in the E-N, which showed a significant decrease after 48 days (Fig. 6). The partial decomposition of the wood along with the presence of pre-grown fungus probably benefited the adaptation and growth of the fresh inoculum.

These results suggest that *T. versicolor*-based biopile can be a useful tool for the bioremediation of polluted substrate, thus allowing its reuse as immobilization support. This technology would reduce substrate requirements in full-scale applications, while maintaining remarkable removal efficiencies and biomass growths. Wood could also be acquired as a by-product or waste from timber industries in the case of full-scale application, making the process even more sustainable, especially compared to other materials commonly used for fungal immobilization such as polyurethane foam (Mir-Tutusaus et al., 2018).

4. Conclusions

Agricultural wastewater was successfully treated by *T. versicolor* immobilized on *Q. ilex* wood chips in a rotating drum bioreactor during a long-term process (> 7 months). Inoculation of *T. versicolor* on *Q. ilex* wood chips and adjustment of the operational parameters led to the consolidation of a fungal consortium in the reactor, composed of *T. versicolor*, *Dipodascus* sp. and *Rhodotorula* sp., with enhanced degradative capacity. Decreasing rotation and increasing HRT improved total biomass retention and thus, pesticide removal over a longer operational period. Significant COD and color removals were also obtained compared to the control reactor. Afterwards, solid by-products exposed to the long-term process were treated by *T. versicolor* in a biopile-like system achieving high biodegradation yields. Therefore, unlike previous studies, biomass was maintained, high biodegradation (not only sorption) was achieved, contaminated by-products were successfully treated after a long period of exposure, and in addition, a detailed

Table 4
Biodegradation rates and first-order kinetic constants of diuron and bentazon removals by *T. versicolor* using different inoculation strategies in a solid-phase biopile treatment.

Biopile conditions	Pesticide: diuron				Pesticide: bentazon			
	Degradation rate (mg·g^{-1} day^{-1}) x 10^3	R^2	k x 10^2 (days^{-1})	R^2	Degradation rate (mg·g^{-1} day^{-1}) x 10^3	R^2	k x 10^2 (days^{-1})	R^2
Inoculated (C-I)	0.70 ± 0.03	1.00	0.37 ± 0.03	0.99	0.19 ± 0.05	0.94	4.19 ± 1.92	0.86
Non-re-inoculated (E-N)	1.73 ± 0.42	0.94	0.93 ± 0.18	0.96	0.25 ± 0.08	0.90	5.30 ± 0.37	1.00
Re-inoculated (E-R)	2.22 ± 0.41	0.97	1.28 ± 0.15	0.99	0.26 ± 0.07	0.94	6.29 ± 0.74	0.99

*Results are shown as mean values and corresponding standard deviations for duplicate measurements.

microbial study was included confirming fungal consolidation in the reactor. These results demonstrated that the use of the appropriate substrate, treatment system and operating conditions allow fungal technology to be implemented under non-sterile conditions during long-term processes, thus being ready for full-scale applications.

Environmental implication

In this paper, two of the main drawbacks of fungal bioremediation technology have been overcome: bacterial contamination and high adsorption on the substrate. Thus, agricultural wastewater was successfully treated by *T. versicolor* immobilized on wood for a long-term period of 225 days, which represents an important advance in the use of fungal technology in real applications, and therefore, in the field of persistent organic pollutants removal from the environment. Special emphasis has been placed on the biodegradation of pesticides, so we believe that this article is ideal for publication in Journal of Hazardous Materials.

CRediT authorship contribution statement

Eduardo Beltrán Flores: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Martí Pla Ferriol:** Investigation, Formal analysis, Writing – original draft, Writing – review & editing of fungal community analysis. **Maira Martínez-Alonso:** Supervision, Conceptualization, Resources, Writing – review & editing of fungal community analysis. **Núria Gaju:** Supervision, Conceptualization, Resources, Writing – review & editing of fungal community analysis. **Paqui Blánquez:** Supervision, Conceptualization, Resources, Writing – review & editing. **Montserrat Sarrà:** Supervision, Conceptualization, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Eduardo Beltran Flores reports financial support was provided by Spanish Ministry of Economy and Competitiveness State Research Agency.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.129614.

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