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Degradation of selected agrochemicals by the white rot fungus *Trametes versicolor*

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Abstract

Use of agrochemicals is a worldwide practice that exerts an important effect on the environment; therefore the search of approaches for the elimination of such pollutants should be encouraged. The degradation of the insecticides imiprothrin (IP) and cypermethrin (CP), the insecticide/nematicide carbofuran (CBF) and the antibiotic of agricultural use oxytetracycline (OTC) were assayed with the white rot fungus *Trametes versicolor*. Experiments with fungal pellets demonstrated extensive degradation of the four tested agrochemicals, at rates that followed the pattern IP > OTC > CP > CBF. *In vitro* assays with laccase-mediator systems showed that this extracellular enzyme participates in the transformation of IP but not in the cases of CBF and OTC. On the other hand, *in vivo* studies with inhibitors of cytochrome P450 revealed that this intracellular system plays an important role in the degradation of IP, OTC and CBF, but not for CP. The compounds 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA) and 3-phenoxybenzoic acid (PBA) were detected as transformation products of CP, as a result of the breakdown of the molecule. Meanwhile, 3-hydroxycarbofuran was detected as a transformation product of CBF; this metabolite tended to accumulate during the process, nonetheless, the toxicity of the system was effectively reduced. Simultaneous degradation of CBF and OTC showed a reduction in toxicity; similarly, when successive additions of OTC were done during the slower degradation of CBF, the fungal pellets were able to degrade both compounds. The

simultaneous degradation of the four compounds successfully took place with minimal inhibition of fungal activity and resulted in the reduction of the global toxicity, thus supporting the potential use of *T. versicolor* for the treatment of diverse agrochemicals.

Highlights

- *Trametes versicolor* efficiently degrades four different agrochemicals simultaneously.
- Order of degradation rates is:
imiprothrin > oxytetracycline > cypermethrin > carbofuran.
- Degradation process usually decreases the initial toxicity of the agrochemicals.
- Cytochrome P450 is involved in the degradation, except for cypermethrin.
- Laccase plays a role in the transformation of imiprothrin.

Keywords

Agrochemicals; Pesticides; Antibiotics; Degradation; Fungi

1. Introduction

The term agrochemical is given to various chemical products widely used in agriculture as prophylactic treatment to maintain high agricultural production ([Sequinatto, 2013](#)). Among the organic pollutants, agrochemicals stand out as their application in most cases is linked to direct contact with the environment, which results in high risk of exposure to living beings. Many agrochemicals may affect non-target organisms and contaminate soil, water sources and air.

Insecticides are widely used in pest control in households as well as in agricultural systems. In particular, pyrethroids account for 25% of the worldwide insecticide market ([Zhang et al., 2010](#)). With the restriction on the use of organophosphates and carbamates, pyrethroids like imiprothrin (IP) and cypermethrin (CP) have generally been regarded as the replacements, because they have higher insect-to-mammalian toxicity ratio ([Hintzen et al., 2009](#)). Despite being less harmful to humans than organophosphates and carbamates, there is an increasing concern as they present reproductive and neurological toxicity, endocrine disruptive effects, and high acute toxicity to fish and aquatic invertebrates ([Abdallah et al., 2010](#) and [Shafer et al., 2005](#)). In addition, long-term exposure to pyrethroids may lead to chronic diseases ([Aksakal et al., 2010](#)) and some of them are considered as possible human carcinogens by the Environmental Protection Agency (EPA).

Carbofuran (CBF) is an agrochemical mainly employed as an insecticide/nematicide. CBF belongs to the family of carbamates, pesticides that act through the inhibition of acetylcholinesterase, an enzyme that degrades the neurotransmitter acetylcholine in

synapses from vertebrates and invertebrates. It is highly toxic to mammals and reportedly embryotoxic and teratogenic ([Gupta, 1994](#)). CBF has been banned in agricultural practice in the United States and the European Union, but it is still widely used in other latitudes.

Oxytetracycline (OTC) is a broad-spectrum antibiotic included in the group of tetracyclines. OTC interferes in bacterial protein synthesis. It is one of only two antimicrobials (as streptomycin) to be registered by the EPA for use in plant agriculture as a prophylactic treatment ([Vidaver, 2002](#)). Environmental risk of release of antibiotics is related to the growing resistance of pathogenic bacteria and the potential dissemination of antibiotic resistance, which is considered as an ecological problem ([Kümmerer, 2004](#)). Moreover, antibiotics have the potential to affect microbial communities and therefore unbalance natural processes that take place in the environment like organic matter degradation ([Kümmerer, 2009](#)).

Besides reducing the use of these pesticides and finding a less harmful replacement, polluted sites must be decontaminated. Therefore, remediation techniques must be developed and implemented to remove these residues from the environment. White rot fungi (WRF) have the potential to transform and sometimes mineralize a broad spectrum of xenobiotics such as textile dyes ([Borchert and Libra, 2001](#) and [Swamy and Ramsay, 1999](#)), chlorinated solvents ([Mendoza-Cantu et al., 2000](#)), polycyclic aromatic hydrocarbons (PAHs) ([Canet et al., 2001](#) and [Zheng and Obbard, 2001](#)), brominated flame retardants ([Zhou et al., 2007](#)), pharmaceuticals ([Hata et al., 2010](#) and [Marco-Urrea et al., 2009](#)) and UV filters ([Gago-Ferrero et al., 2012](#)). They use a nonspecific enzymatic system that includes extracellular lignin modifying enzymes (mainly laccase, lignin peroxidase, manganese peroxidase and versatile peroxidase) and intracellular enzymes such as the cytochrome P450 system, among others ([Asgher et al., 2008](#)).

The purpose of the present study was to evaluate the capacity of the WRF *Trametes versicolor* to degrade selected agrochemicals with different structures and action mechanisms. Toxicological aspects were considered to assay the pertinence of the process, and transformation products were detected for CBF and CP.

2. Materials and methods

2.1. Chemicals and fungal strain

CBF (98%), OTC (> 95%), IP (33442, PESTANAL®) and CP (36128, PESTANAL®) analytical standards were purchased from Sigma–Aldrich (Barcelona, Spain). For degradation experiments commercial insecticide Baygon® (imiprothrin 0.1 g/100 g, cypermethrin 0.1 g/100 g; Johnson's Wax Spain, Madrid) was used. Purified laccase (> 20 U mg⁻¹) from *T. versicolor* was acquired from Sigma Aldrich (Barcelona, Spain). Malt extract was purchased from Scharlau (Barcelona, Spain). The laccase mediator 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) (> 98%) and the cytochrome P450 inhibitor 1-aminobenzotriazole (ABT) (> 98%) were

purchased from Sigma–Aldrich (Barcelona, Spain). Laccase mediators 1-hydroxybenzotriazole hydrate (HOBT) (> 97%) and violuric acid (VA) (> 97%) were acquired from Fluka (Barcelona, Spain). Other chemicals were of the highest available purity and were purchased from Sigma–Aldrich (Barcelona, Spain).

T. versicolor (ATCC#42530) was maintained by subculturing on 2% malt extract agar slants (pH 4.5) at 25 °C. Subcultures were routinely made every 30 d. A mycelial suspension was prepared as described elsewhere ([Font et al., 2003](#)). Pellets of *T. versicolor* were produced by inoculating 1 mL of this mycelial suspension in a 1 L Erlenmeyer flask containing 250 mL of malt extract broth (20 g L⁻¹) with shaking (130 rpm, radius = 25 mm) at 25 °C for 5 d.

For the analytical determination of pyrethroid metabolites 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA), d₆-cis-DCCA and 3-phenoxybenzoic acid (PBA) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). ¹³C-PBA was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Solvents with quality for organic residue analysis were purchased from J.T. Baker (Deventer, The Netherlands). For the determination of transformation products from CBF, 3-hydroxycarbofuran (99.5%) and 3-ketocarbofuran (99.5%) were obtained from Chemservice (West Chester, Pennsylvania, USA).

2.2. Experimental procedures

2.2.1. *In vivo* degradation experiments

All *in vivo* experiments were carried out using *T. versicolor* pellets. Degradation experiments for IP and CP were performed in 125 mL amber bottles containing 0.8 g of wet mycelial pellets (equivalent to 0.03 g DW) in a total volume of 10 mL of a chemically defined medium. Defined medium contained per liter: 8 g glucose, 0.05 g ammonium tartrate, 10 and 100 mL respectively, of a micro and macronutrient solution ([Kirk et al., 1978](#)) and 1.168 g 2,2-dimethylsuccinate. Each unitary experiment was conducted in triplicate and included uninoculated controls and heat-killed controls (autoclaved cultures cultivated under identical conditions to those of experimental cultures) to evaluate adsorption. Commercial IP and CP emulsion mixture was added into the bottles at a final concentration of 10 mg L⁻¹ each. After insecticides addition, amber bottles were incubated under shaken conditions (130 rpm) at 25 °C in the dark, to obviate the possible influence of light on CP and IP stabilities. At each sample point unitary triplicates were sacrificed and extracted as described in [Section 2.3.1](#), and analyzed as in [Section 2.3.2](#). Experiments for degradation of CBF and OTC were done in 250 mL flasks containing 10 g wet fungal pellets in a total volume of 100 mL defined medium. Samples were withdrawn from triplicate cultures at each sampling point and included analogous controls as described above. When indicated, a final concentration of 5 mM of ABT, the cytochrome P450 inhibitor, was added. Abiotic degradation of agrochemicals in time-course experiments was calculated by comparing the initial and final concentrations of the uninoculated controls; adsorption of the pesticides was

determined from the difference in the agrochemical concentrations between uninoculated and heat-killed controls; minimum biodegradation is the difference between the final concentration of pesticides of the experimental flasks and that of the heat-killed control; total elimination is the sum of abiotic degradation, adsorption and minimum biodegradation.

2.2.2. Assays with laccase and laccase-mediator systems

Laccase and laccase-mediator degradation experiments were performed per triplicate in 250 mL Erlenmeyer flasks containing 100 mL of a purified laccase solution at 1000 U L^{-1} (pH 4.5). Effect of laccase mediators was evaluated by the addition of HOBt, ABTS and VA at a final concentration of 1 mM. All flasks contained 10 mg L^{-1} of CBF, OTC or the IP and CP mixture and were shaken (130 rpm) at $25 \text{ }^{\circ}\text{C}$. For IP and CP, 5 mL samples were taken at 0, 3, 8 and 24 h, and extracted and analyzed as described in [2.3.1](#) and [2.3.2](#), respectively. In experiments with CBF and OTC, 1 mL samples at designated times (from 0 h to 96 h) were withdrawn, and 100 μL acetic acid was added to stop the reaction ([García-Galán et al., 2011](#) and [Rodríguez-Rodríguez et al., 2012b](#)) prior analysis as indicated in 2.3.2.

2.3. Analytical procedures

2.3.1. Sample processing

Extraction of IP and CP was performed *in situ* inside the experimental bottles. An equal volume of chloroform was added to the medium contained in the bottles (1:1 v/v) and extracted by 5 min sonication at $30 \text{ }^{\circ}\text{C}$ in a sonication bath (Branson 3510 Ultrasonic Cleaner, Branson Ultrasonics Corporation, Danbury, US) and vigorously shaken manually for 1 min. The emulsion was centrifuged for 2 min at 3500 rpm, and the organic phase was transferred to a vial. The chloroform was then evaporated under a nitrogen stream, and the concentrated extracts were reconstituted in 1 mL of methanol. The extraction yield was $63 \pm 10 \%$. Samples for CBF and OTC analyses were obtained by withdrawing 1.5 mL broth from the culture flasks, and subsequently centrifuging at 3000 rpm for 5 min; supernatant was directly injected in the HPLC; recoveries for OTC and CBF were $> 90\%$.

2.3.2. Analysis of agrochemicals and transformation products of CP and CBF

The pesticide extracts were filtered through a $0.45 \mu\text{m}$ membrane and placed in amber HPLC vials to avoid photodegradation. The samples were analyzed on a Dionex Ultimate 3000 HPLC system equipped with a UV detector and a GraceSmart RP18 column ($250 \text{ mm} \times 4.6 \text{ mm}$, particle size $5 \mu\text{m}$). Detection of IP and CP was done at 225 nm. A ramp of acetonitrile (B) and MilliQ water (A) was used as the mobile phase at a flow rate of 1.5 mL min^{-1} : the eluent gradient started with 50% B and increased to 100% B from 0 to 20 min; from 20 to 21 min the gradient decreased to 50% B and remained at 50% B for 4 more minutes. The injection volume was 40 μL , and the

temperature of the column was maintained at 30 °C. Detection of CBF was done at 215 nm; chromatographic separation was achieved with a mobile phase of methanol-water (60:40), added isocratically at 1 mL min⁻¹ ([Plangklang and Reungsang, 2010](#)). The injection volume was 10 µL, and column temperature was maintained at 30 °C. Limit of detection for CBF was 0.2 mg L⁻¹. Detection of OTC was performed at 355 nm; separation of 10 µL samples employed a mobile phase of methanol-acetonitrile-oxalic acid (0.01 M) (11:22:67) added isocratically at 1 mL min⁻¹ at 30 °C ([Yuan et al., 2011](#)). Limit of detection for OTC was 0.2 mg L⁻¹.

For the analyses of PBA and DCCA, metabolites of CP, an aliquot of the filtered extracts (180 µL) was analyzed by previous addition of 20 µL of an internal standard solution, consisting of ¹³C-PBA (0.05 ng µL⁻¹) and d₆-*cis*-DCCA (0.1 ng µL⁻¹). Analyses were carried out with an LC system Symbiosis Pico (Spark Holland, Emmen, The Netherlands) using a Purospher® STAR RP-18 end-capped column (125 mm × 2 mm × 5 µm particle size), supplied by Merck (Darmstadt, Germany) preceded by a C18 guard column (2.1 mm × 10 mm) supplied by Waters. The mobile phases were acidified water (0.1% acetic acid) and acetonitrile. The program worked isocratically with 50% of each solvent. Mass spectrometric analysis was performed with a hybrid QqLIT Applied Biosystem MSD Sciex 4000QTRAP (Applied Biosystems, Foster City, CA) using ESI mode. Experiments were carried out in negative ionization mode. Selected transitions for PBA were: 213 → 169 (quantification; declustering potential, DP – 45 V; collision energy, CE – 20 V; collision cell exit potential, CXP – 10 V) and 213 → 93 (confirmation; DP – 40 V; CE – 30 V; CXP – 10 V); selected transitions for DCCA were 207 → 35 (quantification; DP – 25 V; CE – 30 V; CXP – 5 V) and 209 → 35 (confirmation; DP – 25 V; CE – 20 V; CXP – 10 V), following [Olsson et al. \(2004\)](#) indications. Other parameters were: entrance potential – 10 V; temperature 550 °C; ion source gas 50 psi; ionspray voltage – 4400 V; curtain gas 10 psi; CAD gas medium. Limits of detection and quantification were 0.36 µg L⁻¹ and 1.2 µg L⁻¹, respectively for PBA, and 0.27 µg L⁻¹ and 0.9 µg L⁻¹, respectively for DCCA. Recoveries were 70% and 71% for PBA and DCCA, respectively.

Analyses of transformation products from CBF (3-hydroxycarbofuran and 3-ketocarbofuran) were performed using an HPLC system (1200 series, Agilent Technologies, CA, USA) coupled to a 6130 quadrupole mass spectrometer with a G1978B multimode ion source (ESI and atmospheric pressure chemical ionization, APCI). Chromatographic separation was achieved with an Agilent Eclipse XDB-C18 column (150 mm × 4.6 mm i.d., particle size 5 µm; Agilent Technologies, CA, USA) and two mobile phases: water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B). Gradient employed was as follows: 60% B for 3 min, a 22 min linear gradient to 100% B, 5 min at 100% B and 0.1 min gradient back to 60% B, followed by 5.9 min at initial conditions. The mobile phase flow rate was 0.4 mL min⁻¹, and the injection volume was 25 µL. The mass spectrometer was operated in the selected-ion monitoring (SIM) positive mode electrospray ionization and was set up to monitor the ions *m/z* 260 and *m/z* 163 (3-hydroxycarbofuran), and *m/z* 236 and *m/z* 179

(3-ketocarbofuran). The mass spectrometry detector was set up using the following conditions: scanning range, 100 – 400; drying gas (N₂) flow, 5 L/min; nebulizer pressure, 60 psi; dry gas temperature, 350 °C; vaporizer temperature, 200 °C; capillary voltage, 2000 V; and fragmentor voltage, 90 V. Recovery of the method was 75%. Limit of detection and limit of quantification were 10 µg L⁻¹ and 21 µg L⁻¹ for CFN and 3-hydroxycarbofuran; 88 µg L⁻¹ and 164 µg L⁻¹ for 3-ketocarbofuran.

2.3.3. *Vibrio fischeri* bioluminescence inhibition test (Microtox Test)

A Microtox acute toxicity bioassay kit from Microbics was used in toxicity tests. *V. fischeri* is a marine bioluminescent bacterium that emits in the range of the visible light (maximum intensity at 490 nm). Toxicity data were based on the decrease of bacterial bioluminescence after 5 min and 15 min of exposure to dilutions of the samples (pH 7) at 25 °C. Toxicity was expressed as toxicity units (TU). The experimental samples tested were collected from time-course degradation experiments described in [Section 2.2.1](#) at initial time and selected time-points.

2.3.4. Other analysis

Laccase activity was measured using a modified version of the method for manganese peroxidase determination ([Wariishi, 1992](#)); the reaction mixture consisted of 200 µL sodium malonate (250 mM, pH 4.5), 50 µL 2,6-dimethoxyphenol (DMP, 20 mM) and 600 µL sample. DMP is oxidized by laccase even in the absence of a cofactor. Changes in the absorbance at 468 nm were monitored for 2 min at 30 °C. Results were expressed as U per liter. One U was defined as the number of micromoles of DMP oxidized per min. The DMP extinction coefficient was 24,800 M⁻¹ cm⁻¹. Glucose concentration was measured with a biochemical analyzer YSI 2700 SELECT (Yellow Spring Instruments) in the concentration range 0–20 ± 0.04 g L⁻¹. Biomass amount (dry weight) was determined as the constant weight at 100 °C.

3. Results and discussion

3.1. Degradation of pyrethroids (IP/CP)

Time-course *in vivo* experiments with IP and CP at 10 mg L⁻¹ showed a steep drop in concentration of IP but a less abrupt decrease in CP concentration ([Fig. 1A](#) and [B](#), respectively) in experimental flasks. Elimination values were higher than 90%, achieved after 2 d for IP and 15 d for CP. These differences in the degradation efficiencies could be dependent on the molecular structure of the pyrethroids, as it has been reported elsewhere ([Chen et al., 2012](#), [Guo et al., 2009](#) and [Wang et al., 2011](#)). No differences were found in pyrethroid concentrations at the end of the treatment between the uninoculated and the heat-killed controls, which indicates that the role of adsorption of the pollutants to biomass is negligible. Nonetheless, the concentrations of IP and CP were reduced by 19% and 23%, respectively, finding that could be explained by abiotic losses. Minimum biodegradation was calculated by subtracting the abiotic removal

value to the 90% degradation value, which resulted in 71% and 67% for IP and CP, respectively. Separate quantification of CP isomers (*cis*-CP and *trans*-CP) revealed that the fungal removal of CP was not dependent on geometric isomerism (data not shown).

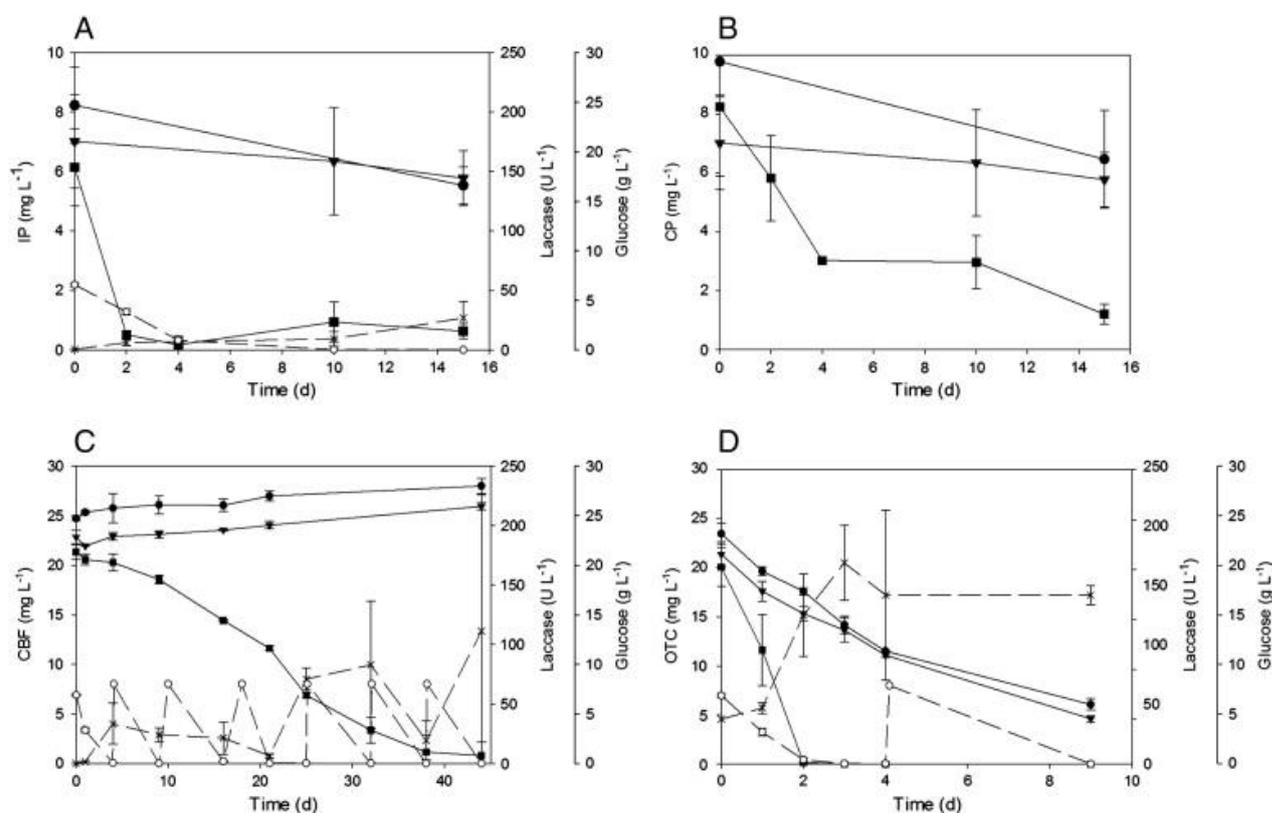


Fig. 1. Degradation profiles of IP (A), CP (B), CBF (C) and OTC (D) by *T. versicolor*. Symbols: uninoculated controls (●), heat-killed controls (▼), reaction cultures (■), glucose (○) and laccase activity (×). Values plotted are the means \pm standard deviation (SD) for triplicate cultures. Laccase and glucose consumption profiles in CP are the same as in IP. Values plotted are the means \pm SD for triplicate cultures.

Degradation of CP was confirmed by the detection of two transformation products (Fig. 2) which appear as a result of the breakdown of the molecule: DCCA and PBA. The molar amount of DCCA accumulated at the end of the assay in the heat-killed and abiotic controls approximately corresponds to the molar losses observed for CP (stoichiometry of the hydrolysis reaction is 1:1); however further DCCA elimination by the fungus is suggested, as the molar amount of this transformation product at time 15 d was lower than the molar reduction in CP during the same period. In the case of PBA, detected concentrations were very low compared to those of DCCA (1000-fold) even in the controls, suggesting high instability of the product in the matrix and making it difficult to conclude whether PBA is further eliminated by biological action. Microbial degradation of pyrethroids has been mostly reported for bacterial strains. Nonetheless, a fungal strain from the genus *Cladosporium* isolated from sludge, showed the capacity to remove several pyrethroids, including CP at efficiencies over 90% in only 5 d in liquid cultures (Chen et al., 2011). No reports regarding fungal degradation of IP were found in specialized literature.

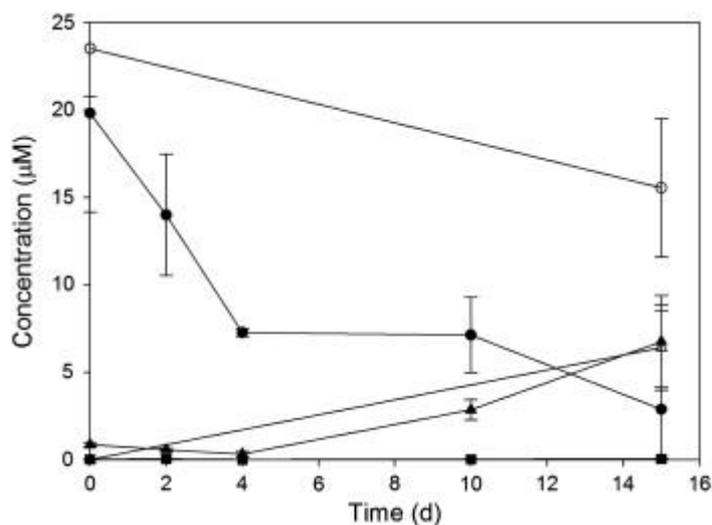


Fig. 2. Time-course evolution of major transformation products in the degradation of CP by *T. versicolor* pellets. Symbols: (●) CP; (■) PBA; (▲) DCCA. Filled symbols correspond to inoculated experiments and empty symbols to uninoculated ones. Values plotted are the means \pm SD for triplicate cultures.

A continuous increase in extracellular laccase activity was detected throughout the experiment (Fig. 1), from the day 10 onwards. As laccase was constitutively produced in the cultures, studies were carried out to define the involvement of laccase and laccase-mediator systems in the degradation of IP and CP. Laccases are multicopper-containing oxidases that catalyze the oxidation of substituted phenols, anilines and aromatic thiols (Cañas and Camarero, 2010). Laccase mediator ABTS can oxidize non-phenolic compounds via an electron transfer route described elsewhere (Baiocco et al., 2003). The other mediators, HOBt and violuric acid, generate a highly reactive nitroxyl radical ($> \text{N-O}^\bullet$) when oxidized by laccase, which subsequently oxidizes the target substrate by a mechanism of hydrogen atom transfer (Fabbrini et al., 2002). Enzymatic assays with purified laccase at 1000 U L^{-1} failed to transform IP and CP. In contrast, when any of the mediators were added to the mixture, 70–100% of the original IP was transformed after 8 h and $> 90\%$ after 24 h (Fig. 3). However, cypermethrin showed a different behavior, and negligible transformation was achieved with the addition of HOBt or VA. Although these mediators are synthetic and are not found in the nature, some lignin-related phenols and unsaturated fatty acids present in the fungal mycelium can act as mediators and promote oxidation of pollutants in the environment (Cañas and Camarero, 2010). Thus, the results suggest that laccase plays a role on the degradation of IP, whereas this role is not clear in the case of CP. Together with laccase, cytochrome P450 system has proved important in the detoxification of some environmental contaminants, and it is present not only in WRF but in bacteria as well (Doddapaneni and Yadav, 2004 and Lewis and Wiseman, 2005). Its role in the transformation of IP and CP was assessed by the addition of the cytochrome P450 inhibitor ABT in *T. versicolor* cultures. In the case of IP (Fig. 4A) the addition of the inhibitor induced a delay in its biotransformation in comparison with the inhibitor-free flasks. Degradation of CP occurred at the same rate in inhibited and inhibitor-free bottles, as the differences were not statistically significant ($p = 0.05$) (Fig. 4B). The results, therefore, suggest that

both laccase and the cytochrome P450 are involved in the transformation of IP; meanwhile the cytochrome P450 seems not involved in CP degradation. On the other hand, the slow but steady decrease of CP concentration in the laccase/mediators experiments suggests that laccase could play a role in CP degradation when used together with mediators, but other enzymatic compounds are likely to act in the process. In this respect, carboxylesterases are regarded as the main enzymes linked to pyrethroid degradation in pyrethroid-degrading bacteria, where ester hydrolysis of the carboxylester linkage is the main degradation pathway ([Guo et al., 2009](#) and [Wang et al., 2011](#)). Similarly, yeasts show pyrethroid hydrolyzing enzymes, and may have broad-spectrum substrate specificity ([Chen et al., 2012](#)).

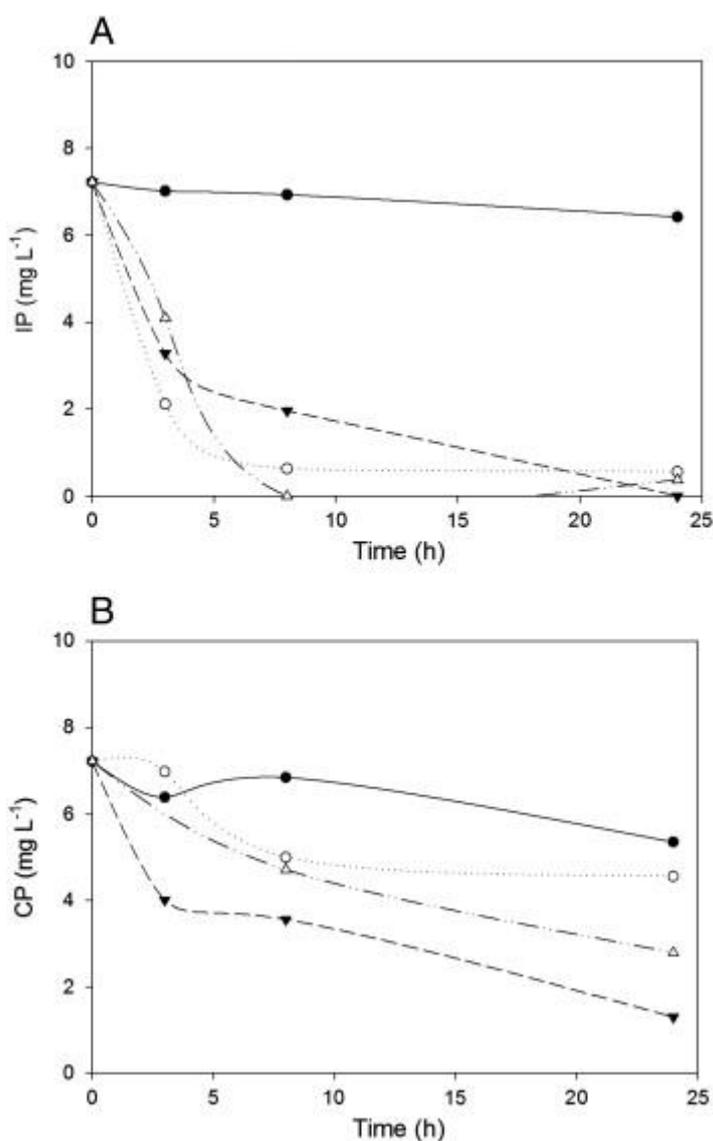


Fig. 3. Degradation profiles of IP (A) and CP (B) by purified laccase. Symbols: laccase without mediators (●), laccase/HOBt (○), laccase/ABTS (▼) and laccase/VA (Δ).

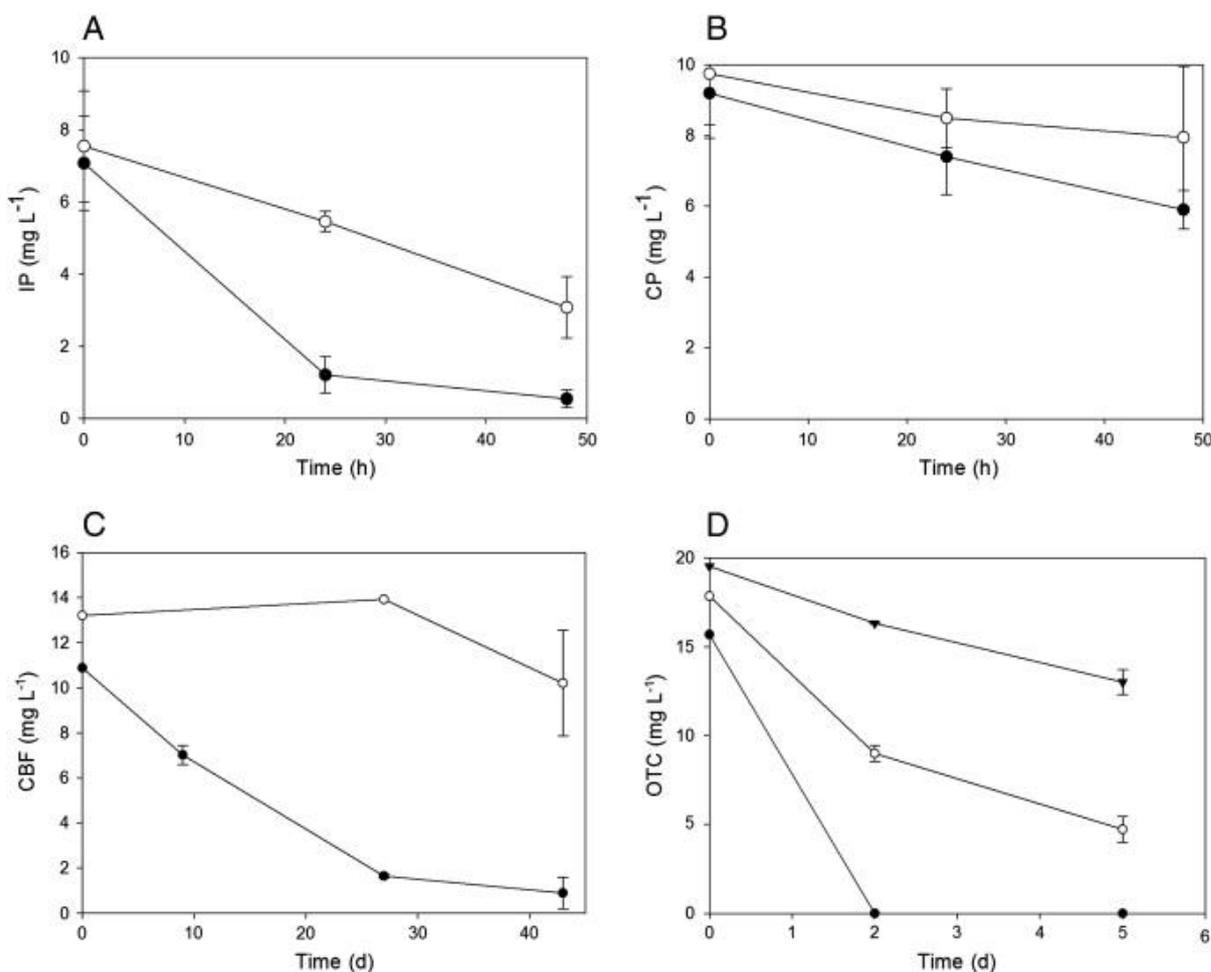


Fig. 4. Degradation profiles of IP (A), CP (B), CBF (C) and OTC (D) by *T. versicolor* in the presence of ABT as cytochrome P450 inhibitor. Symbols: inhibitor-free cultures (●), cultures supplemented with ABT (○), abiotic control (▼). Values plotted are the means \pm SD for triplicate cultures.

Ecotoxicological evaluation was performed as most of the pyrethroids produce metabolites of higher toxicity than the parent compounds ([Laffin et al., 2010](#)). To assess the risk involved with the disposal of a potentially fungal-treated effluent containing CP and IP into the environment, a standard acute toxicity bioassay was performed with the bacterium *V. fischeri*. The initial pyrethroid concentration in the medium showed statistically the same 4 TU (with a 95% of confidence) observed by the end of the process, probably due to the accumulation of similarly toxic transformation products.

3.2. Degradation of CBF

The degradation profile of CBF is shown in [Fig. 1C](#). Abiotic losses and adsorption were negligible, according to control profiles. Although complete removal of the carbamate was not achieved, more than 96% was eliminated by the day 44, with a half-life of around 22 d, and an experimental degradation rate of $3.42 \text{ ng h}^{-1} \text{ mg}^{-1} \text{ DCW}$ (dry cell weight). Removal of CBF is markedly slower than that of several organic pollutants tested with *T. versicolor* under similar conditions, including anti-inflammatories and antibiotics ([Marco-Urrea et al., 2009](#) and [Prieto et al., 2011](#)), and other agrochemicals

from the present work; this observation can be ascribed mostly to the differences in the chemical structure of this more toxic pesticide compared to those organic contaminants. However the estimated half-life in this case was shorter than most values reported for dissipation of CBF in previously unexposed soils ([Karpouzas et al., 2001](#)).

Laccase activity was detected throughout the process and levels reached a maximum of 111 U L^{-1} by time 32 d. However, assays conducted with purified laccase or laccase/mediators failed to reduce CBF concentration, thus indicating that this enzymatic complex is not involved in the transformation of the carbamate. Therefore, participation of cytochrome P450 was also evaluated. When the cytochrome P450-inhibitor ABT was added to the fungal cultures, a marked suppression of the removal was observed (0% elimination at time 27 d and 22% after 44 d, respect $> 87\%$ in the free-inhibitor cultures, [Fig. 4C](#)). This finding strongly suggests that cytochrome P450 is involved in the transformation of CBF by *T. versicolor*.

The detection of one transformation product in the reaction cultures permitted to confirm that the removal of CBF was due to a degradation process. The profile of 3-hydroxycarbofuran production is shown in [Fig. 5](#). This metabolite was detected for the first time after 9 d, time after which its concentration continuously increased to reach a maximum concentration of 4.01 mg L^{-1} at time 38 d. Only the final sampling point registered a slight decrease in concentration, when CBF was almost completely removed. 3-hydroxycarbofuran is regarded (alongside 3-ketocarbofuran) as one of the main CBF metabolites detected in diverse matrices, and shows more polarity but equally toxic effects on target and non-target organisms ([Gupta, 1994](#)). 3-ketocarbofuran was also analyzed; however it was not detected in the fungal cultures. Fungal degradation of CBF has been reported with a *Gliocladium* sp strain, which used the pesticide as a sole carbon and energy source ([Slaoui et al., 2007](#)), and with *Mucor ramannianus*, which in addition, produces the metabolite 3-hydroxycarbofuran-phenol from carbofuran-phenol ([Seo et al., 2007](#)), analogous to the 3-hydroxycarbofuran detected in this work with *T. versicolor*.

Evaluation of the toxicity of the treatment revealed a reduction from 42 TU at initial conditions to a residual 2 TU by time 22 d (half of the treatment) that was maintained at the end of the assay (3 TU), despite the accumulation of 3-hydroxycarbofuran.

3.3. Degradation of OTC

The removal of the antibiotic OTC is shown in [Fig. 1D](#). Results obtained from abiotic and heat-killed controls indicated that adsorption phenomena are negligible; similarly, these data also reveal high instability of the compound due to abiotic losses.

Nonetheless, comparison with reaction cultures showed a marked removal effect mediated by *T. versicolor*. That is, over 99% removal was achieved in the presence of viable biomass in 2 d, respect 25% from abiotic losses in the same period. Dissipation rate from abiotic controls was estimated in $1.87 \text{ mg L}^{-1} \text{ d}^{-1}$, far below the 9.96 mg L^{-1}

d^{-1} achieved with the fungus (equivalent to $65 \text{ ng h}^{-1} \text{ mg}^{-1} \text{ DCW}$). This value is slightly lower compared to analogous assays performed with *T. versicolor* and other antibiotics such as sulfamethazine, sulfapyridine and sulfathiazole ([García-Galán et al., 2011](#), [Rodríguez-Rodríguez et al., 2012a](#) and [Rodríguez-Rodríguez et al., 2012b](#)). Results from this work showed a faster removal by *T. versicolor* than the WRF *Pleurotus ostreatus*, which required around two weeks for complete elimination of OTC ([Migliore et al., 2012](#)).

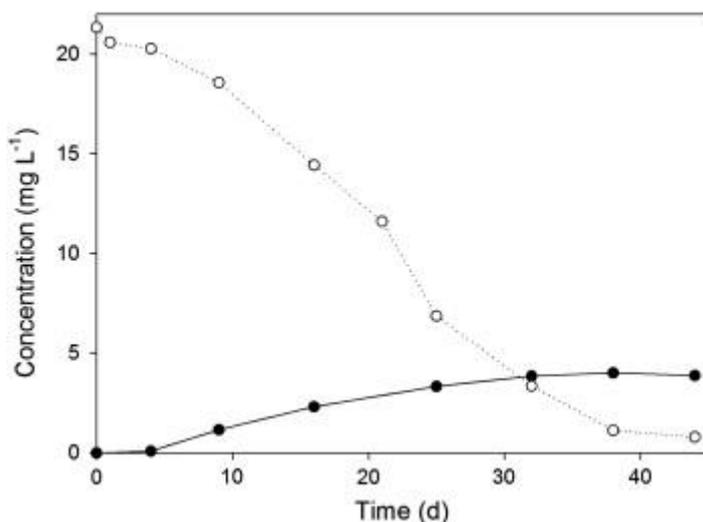


Fig. 5. Time-course evolution of 3-hydroxycarbofuran (●), major transformation product in the degradation of CBF (○) by *T. versicolor* pellets.

As for the case of CBF, laccase activity was detected during the whole period of the assay (maximum 156.7 UA L^{-1} at day 3). Similarly, enzymatic experiments with purified laccase and laccase/mediator systems demonstrated that this enzyme is not involved in the early transformation of OTC, as the antibiotic concentration was not reduced in any case. Crude extracts of manganese peroxidase and lignin peroxidase from the WRF *Phanerochaete chrysosporium* successfully removed OTC ($> 84\%$) at levels of 50 mg L^{-1} in 4 h (84%) and 5 min ($> 95\%$) respectively ([Wen et al., 2009](#) and [Wen et al., 2010](#)); however activity of both enzymes is not significant in *T. versicolor* at work conditions. On the contrary, when ABT was employed as an inhibitor of cytochrome P450, assays with whole cells resulted in the drop of the removal rate of OTC ([Fig. 4D](#)). This finding suggests that the intracellular enzymatic complex is somehow involved in the transformation of OTC by *T. versicolor*.

Acute toxicity assays with *V. fischeri* showed an abrupt decrease in the toxicity of the medium, from 11 TU at initial time to 3 TU after 3 d, which supports the environmental safety of the process.

Some reports portray the ability of *T. versicolor* to degrade pesticides other than those here studied. Previous works describe the increased removal of simazine, trifluralin and dieldrin when *T. versicolor* was added in wood chips to soil ([Fragoero and Magan, 2008](#)); in the meantime, [Karas et al. \(2011\)](#) demonstrated the degradation of imazalil,

thiophanate methyl, ortho-phenylphenol, diphenylamine and chlorpyrifos by the fungus in liquid media. Similarly, another species of the genus, *Trametes hirsuta*, has shown the ability to degrade endosulfan and endosulfan sulfate ([Kamei et al., 2011](#)) and γ -hexachlorocyclohexane (lindane) ([Singh and Kuhad, 1999](#)), both in liquid media. Likewise, transformation of pesticides by laccases from *T. versicolor* and other WRF has been reported for the phenylurea *N,N'*-(dimethyl)-*N*-(2-hydroxyphenyl)urea with immobilized enzyme ([Jolivalt et al., 2000](#)) and bromoxynil, niclosamide, bromofenoxim and dichlorophen with a laccase-syringaldehyde system ([Torres-Duarte et al., 2009](#)). The versatility shown by *T. versicolor* strongly supports the potential of the fungus to remove a wide range of agrochemicals.

3.4. Simultaneous degradation of agrochemicals

Since CBF and OTC are similarly applied on soils, the degradation of a mixture of OTC and CBF was described using fungal biomass ([Fig. 6A](#)). Although a slight lag-phase was observed in CBF removal, the graphically estimated half-life for this compound was the same (22 d) as for cultures without OTC, and only 6% remained by the end of the treatment. The degradation rate was $3.47 \text{ ng h}^{-1} \text{ mg}^{-1} \text{ DCW}$, practically the same observed without OTC. On the other hand, removal of OTC in the mixture was considerably delayed respect cultures without CBF (from 65 to $19.8 \text{ ng h}^{-1} \text{ mg}^{-1} \text{ DCW}$); nonetheless, OTC removal was still faster than in the abiotic controls. Assays in the culture broth showed a gradual reduction in toxicity compared to the initial toxicity (20 TU); it decreased to 9 TU by day 21 and only to 1 TU at the end of the experiment (44 d).

A second experiment with CBF/OTC mixture was performed. This assay included successive additions of OTC during the second half of the treatment ([Fig. 6 B](#)). The amount of added OTC was systematically removed, including one spike of 40 mg L^{-1} . As in the previous experiment, no apparent effect on the degradation of CBF was observed, and half-life was estimated in 21 d and degradation rate in $3.18 \text{ ng h}^{-1} \text{ mg}^{-1} \text{ DCW}$. Although the last parameter seems slightly lower than in the other experiments, it was constant throughout the assay, and did not change after the successive OTC additions.

The simultaneous removal of CBF, OTC, CP and IP was similarly evaluated for a 21 d period ([Fig. 7](#)). No inhibition on fungal metabolism was observed, as indicated by glucose consumption profile and a peak in laccase activity (37 U L^{-1}) similar to that obtained for this period in experiments solely with CBF or CBF/OTC. Moreover, the estimated half-life for CBF was 15 d, shorter than other values obtained in this work. The profile of CP removal followed the same trend observed in the CP/IP mixture. In the meantime, delayed elimination was only detected for IP and OTC; however the former was completely removed by day 7. Toxicological evaluation of the mixture showed a decrease in toxicity, from 10 TU at time zero, to 4 TU after 21 d of fungal treatment.

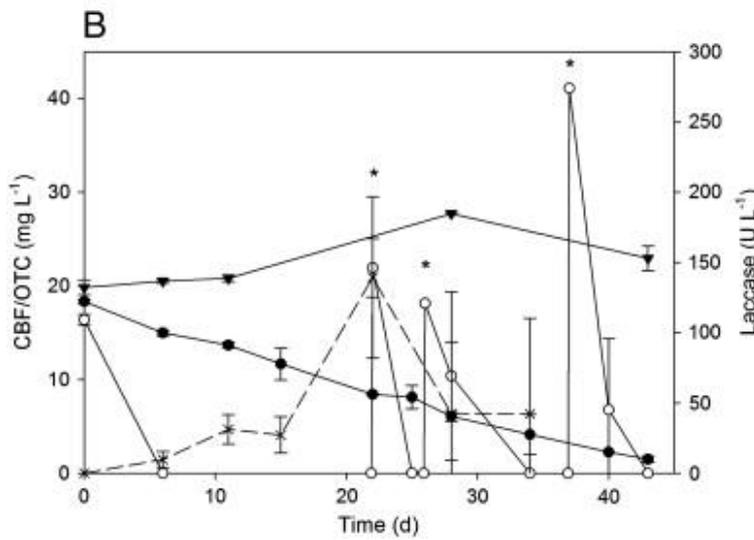
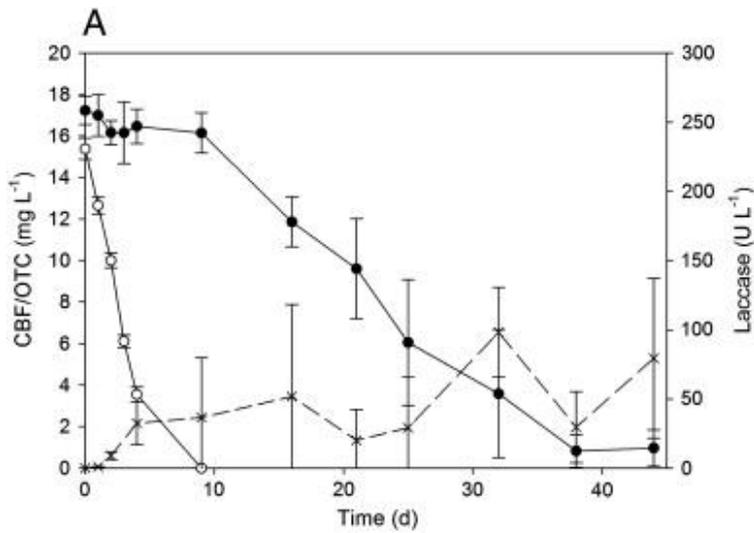


Fig. 6. Degradation profile of CBF/OTC mixtures: without successive additions of OTC (A); with successive additions of OTC (B). Symbols: CBF (●), OTC (○), CBF-abiotic control (▼), laccase activity (×). Time-points of OTC addition are marked with (*). Values plotted are the means \pm SD for triplicate cultures.

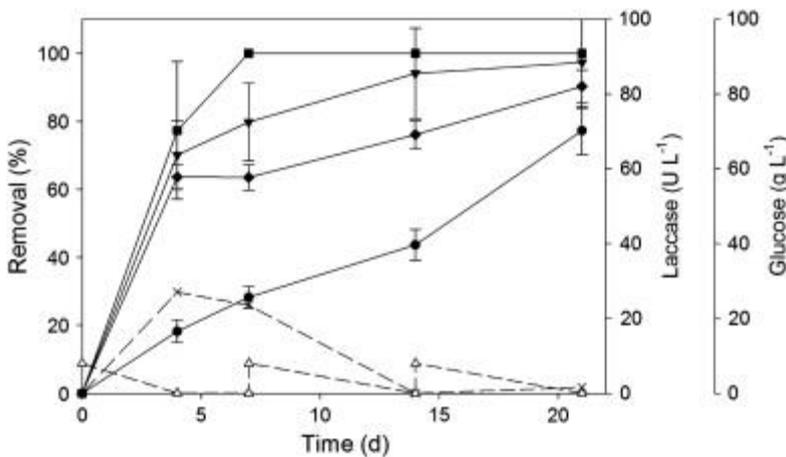


Fig. 7. Simultaneous degradation of IP (■), CP (◆), CBF (●) and OTC (▼) by *T. versicolor*. Glucose (Δ) and laccase activity (\times) are also shown. Values plotted are the means \pm SD for triplicate cultures.

4. Conclusions

Removal of diverse agrochemicals was achieved with *T. versicolor*. Degradation involved in the process was additionally confirmed in the cases of CBF and CP through the detection of transformation products. Simultaneous removal of the agrochemical mixtures supports not only the wide spectrum of oxidation capacity of the fungus, but also remarks its potential use to treat wastewaters of agro-industrial origin. Suitability of the process was evidenced by a reduction in the toxicity of the matrix after the fungal treatment. Slurry reactors and solid-phase biopile-like systems inoculated with *T. versicolor* have been successful in the removal of diverse organic emerging pollutants; similar configurations should be evaluated to assay their performance on the degradation of agrochemicals in the field.

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