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Removal of carbamates and detoxification potential in a biomixture: Fungal bioaugmentation versus traditional use

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ABSTRACT

The use of fungal bioaugmentation represents a promising way to improve the performance of biomixtures for the elimination of pesticides. The ligninolyitc fungus *Trametes versicolor* was employed for the removal of three carbamates (aldicarb, ALD; methomyl, MTM; and methiocarb, MTC) in defined liquid medium; in this matrix ALD and MTM showed similar half-lives (14 d), nonetheless MTC exhibited a faster removal, with a half-life of 6.5 d. Then the fungus was employed in the bioaugmentation of an optimized biomixture to remove the aforementioned carbamates plus carbofuran (CFN). Bioaugmented and non-bioaugmented systems removed over 99% ALD and MTM after 8 d of treatment, nonetheless a slight initial delay in the removal was observed in the bioaugmented biomixtures (removal after 3 d: ALD 87%/97%; MTM 86%/99%, in bioaugmented/non-bioaugmented systems). The elimination of the other carbamates was slower, but independent of the presence of the fungus: > 98% for MTM after 35 d and > 99.5% for CFN after 22 d. Though the bioaugmentation did not improve the removal capacity of the biomixture, it favored a lower production of transformation products at the first stages of the treatment, and in both cases, a marked decrease in the toxicity of the matrix was swiftly achieved along the process (from 435 to 448 TU to values < 1TU in 16 d).

1. Introduction

Biomixtures are used in biopurification systems for the treatment of pesticide-containing wastewater produced during agricultural labors. The point source release of such wastewaters due to their inadequate disposal constitutes one of the main causes of contamination of environmental compartments (Wenneker et al., 2010).

Biomixtures typically contain a lignocellulosic substrate, a humic-rich component and soil pre-exposed to the target pesticides, mixed at a volumetric proportion of 50:25:25 (Castillo et al., 2008). Most of the degrading microbiota in the biomixture is provided by the soil; nonetheless, the presence of the lignocellulosic substrate promotes the colonization and activity of ligninolytic fungi, which are known for their capacity to degrade diverse organic pollutants (Asgher et al., 2008; Gao et al., 2010). Given that the lignocellulosic substrate accounts for half of the volumetric content in the biomixture, the bioaugmentation of these matrices using ligninolyitc fungi represents a promising approach to enhance their performance (Rodríguez-Rodríguez et al., 2013). The degrading capacity of ligninolytic fungi is linked to the production of extracellular lignin-modifying enzymes and intracellular enzymatic complexes such as the cytochrome P450 (Asgher et al., 2008; Yang et al., 2013), and their versatility includes the transformation of pesticides (Mir-Tutusaus et al., 2014), polychlorinated biphenyls (Ruiz-Aguilar et al., 2002), pharmaceuticals, brominated flame retardants, and UV filters (Borràs et al., 2011; Cruz-Morató et al., 2013; Rodríguez-Rodríguez et al., 2014), among others.

The carbamates constitute a group of pesticides with the structure of N-substituted carbamic acid esters (Soriano et al., 2001), which act as inhibitors of the acetylcholinesterase (Karami-Mohajeri and Abdollahi, 2010). They are extensively used in pest control due to their broad spectrum of biological activities as insecticides, fungicides or herbicides. The carbamates exhibit high acute toxicity (Gupta, 1994; Rosman et al., 2009), and even if they are properly applied, their residues can be found in soil, run-offs from soil, food, crops and water. Among carbamates, carbofuran (CFN) and aldicarb (ALD) are employed as insecticides and nematicides; methiocarb (MTC) is a molluscicide and insecticide, and methomyl (MTM) is mostly used as insecticide (Lewis et al., 2016).

The aim of this work was to evaluate the ability of a bioaugmented biomixture to remove several carbamates. The ligninolytic fungus *Trametes versicolor* was employed as the bioaugmentation agent; the ability of this organism to individually remove the carbamates ALD,

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MTC and MTM was first assayed in liquid medium. The biomixture used in the removal of the mixture of carbamates contained rice husk, compost and soil, previously exposed to CFN (at a volumetric composition of 30:43:27), and had been previously optimized for the degradation of CFN (Ruiz-Hidalgo et al., 2016). As accelerated degradation and enhanced-cross degradation of carbamates has been reported (Arbeli and Fuentes, 2007; Chin-Pampillo et al., 2015a; Osborn et al., 2010), a similar behavior was expected to occur in the biomixture in the cases of ALD, MTC and MTM. Finally, in order to determine the potential environmental application of the optimized biomixture, an ecotoxicological test was performed to monitor the changes in the toxicity of the matrix during the treatment of the pesticides.

2. Materials and methods

2.1. Chemicals and fungal strain

Analytical standards CBF (2,2-dimethyl-2,3-dihydro-1-benzofuran-7-ylmethylcarbamate, > 99% purity), 3-hydroxycarbofuran (99.5%), 3-ketocarbofuran (99.5%), aldicarb (99%), methomyl (99.5%) and methiocarb (99%) were obtained from Chem Service (West Chester, PA). Carbendazim-d_4 (surrogate standard, 99.0%) and linuron-d_6 (internal standard, 98.5%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Commercial CFN formulation (Furadan® 48SC, 48% w/v) was acquired from a local store. Solvents with quality for organic residue analysis were purchased from J.T. Baker (Deventer, The Netherlands).

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 Segura et al., 1993) using Sabouraud broth as the culture medium. Pellets of *T. versicolor* were produced by inoculating 1 mL of this mycelial suspension in an erlenmeyer flask (1 L) containing 250 mL of Sabouraud broth and continuous shaking (130 rpm), at 25 °C for 7 d.

2.2. Experimental procedures

2.2.1. Degradation experiments in liquid medium

Degradation experiments for ALD, MTC and MTM were performed in flasks containing 10 g of wet mycelial pellets per 100 mL (equivalent to 0.03 g DW) of a chemically defined medium (composition per liter: 8 g glucose, 1.0 g NH_4Cl, 4 g casein, 0.2 g $CaCl_2$, 0.2 g $MgSO_4$, final pH of 4.5). Glucose (8 g L^{-1}) was added every 3–4 days. Each unitary experiment was conducted in triplicate and included uninoculated controls to evaluate abiotic losses and heat-killed controls (autoclaved cultures cultivated under identical conditions to those of experimental cultures) to evaluate adsorption; all the controls contained 10 mM sodium azide. ALD and MTM were individually added into the flasks at a final concentration of 15 mg L^{-1} and MTC at 3 mg L^{-1}, and incubated under continuous shaking conditions (130 rpm) at 25 °C in the dark, to obviate the possible influence of light in the stability of the carbamates. Samples were withdrawn from triplicate cultures at each sampling point and analyzed as described in Section 2.3.2. Abiotic degradation of carbamates 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 carbamate concentrations between uninoculated and heat-killed controls; minimum biodegradation was defined as the difference between the final concentration of pesticides of the experimental flasks and that of the heat-killed control; total elimination (removal) is the sum of abiotic degradation, adsorption and minimum biodegradation.

2.2.2. Removal of carbamates in a rice husk-based biomixture

A biomixture containing rice husk, compost and soil pre-exposed to CFN at a volumetric composition of 30:43:27 was employed for the removal of the carbamates. The composition of this biomixture was previously optimized in order to maximize the removal of CFN and toxicity reduction, and to minimize the accumulation of 3-hydroxycarbofuran and 3-ketocarbofuran, both transformation products from CFN (Ruiz-Hidalgo et al., 2016). Removal assays were performed in trays (17×11×9.5 cm) containing ~500 mL of the biomixture. Flasks containing the proper amount of humidified rice husk (ratio 1:2, dry material/water, w/v) were sterilized at 121 °C for 15 min prior to the inoculation of blended *T. versicolor* mycelial suspension (0.35 mL g^{-1} of dry rice husk). After fungal colonization for 10 d at 25 °C, biomixtures were prepared by adding pre-exposed soil and compost at the above mentioned volumetric ratio. Six trays containing the bioaugmented biomixture were prepared; three trays were spiked with a mixture of carbamates, to give final concentrations of 12 mg kg^{-1} (CFN), 10 mg kg^{-1} (ALD), 0.4 mg kg^{-1} (MTC), 20 mg kg^{-1} (MTM); the other three trays were spiked with CFN solely. Three additional trays not inoculated with the fungus, and spiked with the mixture of carbamates, were employed as non-bioaugmented systems. All the trays were incubated in static conditions at 25 °C until the end of the assay; water was periodically added in order to keep constant water content in the matrix. Samples were periodically withdrawn over a 36 d-period to determine the concentration of carbamates, the transformation products of CFN and to perform ecotoxicological assays.

2.3. Analytical procedures

2.3.1. Sample processing

Samples from liquid medium assays 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 LC-MS/MS. Extraction from solid phase (biomixture) samples was carried out using a mixture of water and acidified acetonitrile (formic acid 1% v/v) as described in Ruiz-Hidalgo et al. (2014). Carbendazim-d_4 and linuron-d_6 were added as surrogate and internal standard, respectively, to samples obtained from degradation experiments.

2.3.2. Analysis of carbamates and transformation products of CFN

Analysis of carbamates was performed directly from centrifuged samples (liquid phase assays) or extracts (biomixtures) by LC-MS/MS using ultra high performance liquid chromatography (UPLC-1290 Infinity LC, Agilent Technologies, CA) coupled to a triple quadrupole mass spectrometer (model 6460). Chromatographic separation was done at 40 °C by injecting 6 µL samples in a Poroshell 120 EC-C18 column (100 mm×2.1 mm i.d., particle size 2.7 µm), and using acidified water (formic acid 0.1% v/v, A) and acidified methanol (formic acid 0.1% v/v, B) as mobile phases. The mobile phase flow was 0.3 mL min^{-1} at the following conditions: 30% B for 3 min, followed by a 15 min linear gradient to 100% B, 4 min at 100% B and 0.1 min gradient back to 30% B, followed by 5 min at initial conditions. Selected transitions, LOD and LOQ for the analytes are shown in Table 1. The mass spectrometer employed a jet stream (electrospray) ionization source operating at the following conditions: gas temperature 300 °C; gas flow 7 L min^{-1}; nebulizer 45 psi; sheath gas temperature 250 °C; sheath gas flow 11 L min^{-1}; capillary voltage 3500 V (for positive and negative); nozzle voltage 500 V (for positive and negative); heater MS1 and MS2 100 °C.

2.3.3. Laccase activity determination

Laccase activity was measured using a modified version of the method for manganese peroxidase determination (Wariishi et al., 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