Green Solutions for Wastewater Reclamation: Harnessing Trichoderma Bioinoculants for Micropollutant Removal in Constructed Wetlands and Agricultural Soils

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INTRODUCTION

Utilizing wastewater irrigation in agricultural practices offers the dual benefit of reducing water consumption while enriching soils with nutrients. However, this practice also presents inherent risks due to the presence of micropollutants. Moreover, the formation of potentially hazardous transformation products within the soil and rhizosphere, which may subsequently be absorbed by plants, further exacerbates concerns [1].

Trichoderma is a genus of fungi found ubiquitously in soils. Many Trichoderma species can colonize root surfaces and establish mutualistic relationships with plants and in some cases act as endophytes, providing benefits such as enhanced growth, stress tolerance, and disease resistance [2]. Used in agriculture as biocontrol agents, their application to remediate micropollutant contamination in constructed wetlands or in agricultural soils irrigated with reclaimed wastewater has been scarcely investigated.

Here, we present the results of our studies using commercially available products containing spores of symbiotic fungi for enhancing the biodegradation of micropollutants. *Trichoderma asperellum* (strain T34) was tested for the removal of organic micropollutants and antibiotic resistance genes (ARGs) in a pilot-scale partially saturated vertical flow constructed wetland. In a second study, we explored the impact of the *fungus Trichoderma harzianum* on the distribution of carbamazepine and climbazole, and their primary transformation products (TPs) in soils and lettuce tissues under controlled conditions.

MATERIAL AND METHODS

Constructed Wetland

The present study was carried out with *Phragmites australis* based vertical subsurface constructed wetlands (VSSF-CW) units located outdoors at the facilities of the experimental platform for wastewater reuse in irrigation in Murviel-Lès-Montpellier (Southern France). The system was operated as tertiary treatment and was fed with real secondary treated domestic wastewater obtained by an aerated CW (3000 IE). The treatment system consisted of three lines of three CW units each and operated in parallel (planted, unplanted, and planted and inoculated with *T. asperellum*). Each unit (0.48 m^2 surface area) was designed to treat 96 L/d distributed in 8 batches along the day and was partially saturated with water (3). The average organic loading rate (OLR) and hydraulic loading rate (HLR) applied to each CW unit during the study were 10 g COD m-2/ d and 200 mm/d, respectively. Inoculation of *Trichoderma* was performed by adding 12 g of spores to each bed (100 g of spores / m3 of soil, i.e., the filtration layer) and repeated three times with a 7-day interval. The population density of *Trichoderma* was assessed by plate-counting on semi-selective media designed for *Trichoderma* [3].

Lettuce plants

Lettuce plantlets (*Lactuca sativa* var. *Batavia*) were grown in pots containing a synthetic soil mix (35% peat, 10% clay soil, 25% sand, and 30% perlite) under controlled conditions (16 h of light, 16-25°C and a relative air humidity of 60%). Three conditions were studied: plants irrigated with wastewater, plants irrigated with spiked wastewater, and plants inoculated with *T. harzianum* and irrigated with spiked wastewater. Wastewater was collected after secondary lagoon-based treatment at the municipal wastewater treatment plant of Murviel-les-Montpellier, France and autoclaved at 121°C for 20 min to reduce the impact of wastewater-borne microbes on pharmaceutical metabolism. Wastewater was spiked with carbamazepine and climbazole at a final concentration of 200 µg/L, allowing the identification of TPs in our experimental conditions. Inoculation with *T. harzanium* was done using the commercial product Canna AkTRIvator®, purchased from Canna International BV (Breda, the Netherlands) following the recommendations of the manufacturer.

Analysis of micropollutants

Extraction of micropollutants from water and solid (leaves, roots and soil) samples was performed by SPE (Oasis HLB) and QUECHERS methods, respectively. Extracts were analyzed on an HPLC Accelera 600 pump coupled to a Q-Orbitrap HRMS mass spectrometer. Chromatographic separation was conducted using a Waters XBridge BEH C18 (2.1 × 150 mm and 2.5 μm particle size) analytical column equipped with a pre-column. The chromatography assays involved a 10 µL injection volume, a 0.30 mL/min flow rate, and a binary gradient of water and acetonitrile, both containing 0.1 % formic acid. The non-target screening was performed with a home-made tool [4]. All the selected compounds were also manually reviewed to validate their identification, with experimental mass spectra compared to reference spectra and comparison of isotopic profiles.

Quantification and analysis of antibiotic resistance genes in effluents

Environmental DNA was extracted using the DNeasy PowerWater Kit (Qiagen, Venlo, Netherlands). Gene detection and quantification were performed using the SmartChip™ Real-Time PCR system (TakaraBio, CA, USA) by Resistomap (Helsinki, Finland). 72 primer sets were used to assay the genes of interest. ARGs abundances and profiles were analyzed using (version 4.2.0). ARG abundances were normalized by 16s rRNA abundances.

RESULTS

Removal of micropollutants in bioaugmented CWs

Successful bioaugmentation was achieved in CWs, with Trichoderma exhibiting growth in competitive conditions using secondary treated domestic wastewater. A large number of chemicals was quantified by non-target LC-HRMS analysis in wastewater effluents (Fig.1).

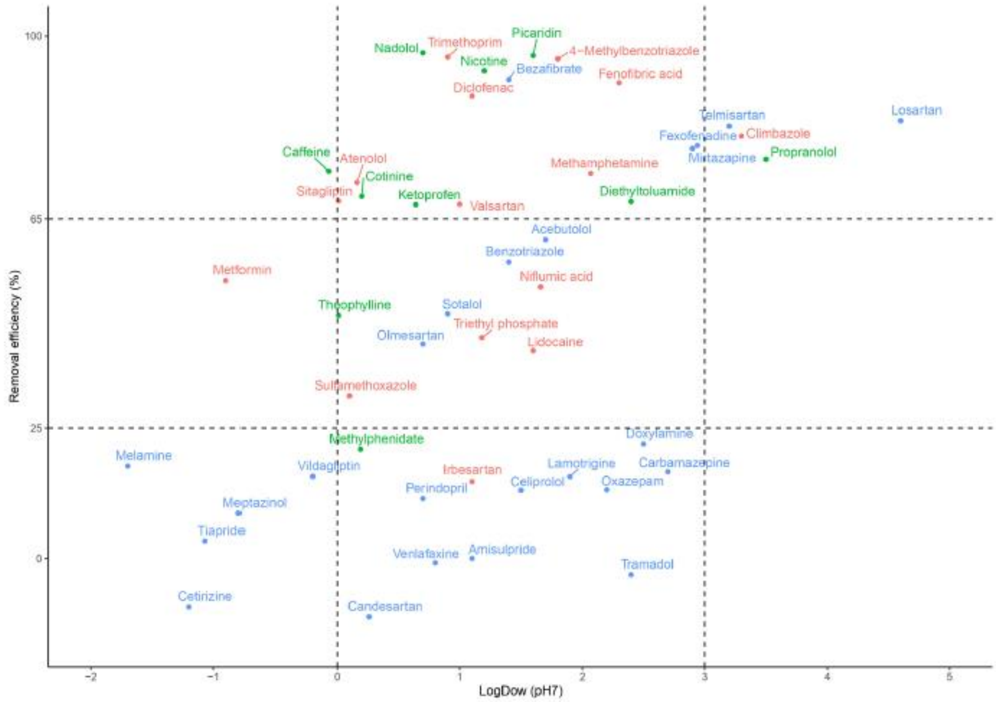

Figure 1: Removal efficiency (in %) of organic micropollutants as a function of their log Dow and their biodegradability in CW units with plants inoculated with Trichoderma. *Different colors are used to classify the significance of each chemical biodegradability: Green color for quickly biodegradable compounds (dissipation time (DT50) <2 d), blue color for moderately biodegradable compounds (2 < DT50 > 10 d) and red color for slowly biodegradable compound (DT50 > 10 d) following DT50 literature available data.*

Removal efficiency (RE) showed a clear correlation with compound biodegradability and sorption capacity, with easily biodegradable compounds showing higher elimination rates. Compounds with optimal plant uptake (LogDow between 1 and 3) demonstrated significant removal efficiency. Notably, benzotriazole and diclofenac showed the most beneficial effects. Polar compounds like melamine proved most challenging to eliminate. However, high variability in removal efficiency over the experiment's duration limited treatment performance assessment. Transformation products (TPs), including N-oxide TPs and 14-hydroxyclarithromycin, were identified, with some undergoing partial elimination. Bioaugmented treatments led to a shift in ARG composition, though average removal rates remained statistically unchanged.

Micropollutants in agricultural soils irrigated with wastewater

Even if signs of toxicity were not visually observed, exposure to carbamazepine and climbazole (200 µg/L) resulted in a loss of biomass in lettuce plants irrigated with wastewater. While inoculation with *T. harzianum* did not notably influence the uptake of parent compounds, it did elevate TP concentrations in soil while reducing them in plant leaves after three weeks (Fig. 2). Fungal inoculation also led to improved biomass and altered levels of certain phytohormones implicated in defense mechanisms and microbiome recruitment in roots and soils such as salicylic acid.

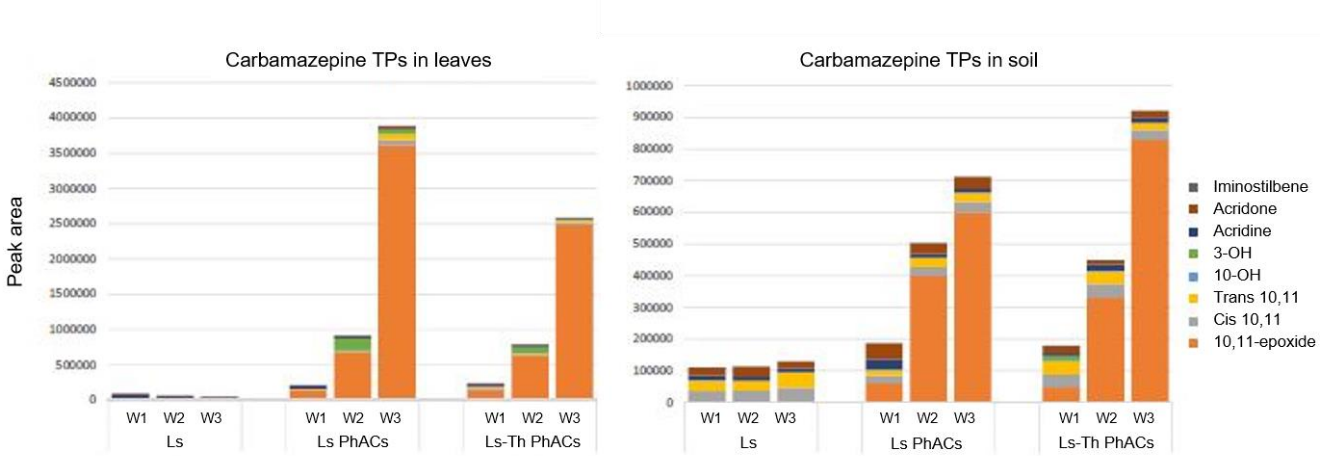

Figure 2: Distribution of CBZ transformation products in lettuce leaves and soils of plants irrigated with wastewater (Ls), plants irrigated with wasteqapetr spiked with carbamazepine and climbazole (200 µg/L) and plants irrigated with spiked wastewater and inoculated with T. harzianum. Results are expressed as peak areas (n=3)..

CONCLUSION

Our findings offer valuable insights applicable to sustainable NBS-based wastewater treatment and crop cultivation, particularly under irrigation using reclaimed wastewater, leveraging accessible biological products for enhanced management practices.

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