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Ability of filamentous fungi to degrade four emergent water priority pollutants

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ABSTRACT

This study was conducted to investigate biodegradation of four emergent water priority pollutants, di(2-ethylhexyl)phthalate (DEHP), fluoranthene, aminomethylphosphonic acid (AMPA), and estrone (EST), by filamentous fungi (*Fusarium oxysporum*, *Geotrichum galactomyces*, *Trichoderma harzianum*, and *Fusarium solani*). These pollutants are commonly found at high occurrence in French wastewater treatment plants. In acute toxicity tests, a weak sensitivity of fungal growth to the pollutants was observed with *F. oxysporum* showing the greatest growth inhibition (19.3%) in the presence of DEHP after four days of incubation. In addition, degradation experiments were conducted in mineral medium for each pollutant incubated with each filamentous fungus for 10 d. With the exception of EST, which was not degraded by any fungal isolate tested, the fungi degraded these emergent water priority pollutants, with *F. solani* and *T. harzianum* degrading 100% of DEHP and 69% of AMPA, respectively.

Keywords: Biodegradation; Emergent pollutant; Filamentous fungi; Wastewater treatment

1. Introduction

Wastewater treatment plants (WWTPs) receive raw water from domestic and industrial discharges containing various pollutants. A recent research program studying the elimination of 127 contaminants in 21 French WWTPs demonstrated the persistence of pollutants at concentrations ranging from 10 to 100 mg/kg in raw water, treated water, and sewage sludge with high occurrence [1]. The actual limit of pollutant removal at different treatment stages in a WWTP might lead to an important input source of pollutants to the environment via water release or sewage sludge applied as soil amendments.

This study was conducted to investigate the potential capacity of fungal degradation of four emergent water priority pollutants. Di(2-ethylhexyl)phthalate (DEHP) is probably the most used phthalate in polyvinylchloride (PVC) manufacturing and is also applied as an additive in paints, glues, and inks. Because of its toxicity, especially towards the reproduction system, DEHP is classified as a priority hazardous substance according to the 2013/39/EU directive of the European union [2]. The French research program studying the elimination of contaminants from WWTPs also revealed the persistence

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of DEHP at concentrations ranging from 10 to 100 mg/kg in sewage sludge owing to its high hydrophobicity (log K_{ow} 7.45) [1].

The second pollutant selected was fluoranthene (FLU). This compound is one of the US Environmental Protection Agency's 16 priority pollutant polycyclic aromatic hydrocarbons (PAHs) and is also classified as a priority substance by the European Union [2]. FLU, which has a four-membered rings in its molecular structure, was selected as a model of high-molecular-weight PAH with low water solubility (0.27 mg/l) and an elevated octanol/water partitioning coefficient (log K_{ow} 5.33). These parameters result in high affinity to organic matter and consequently slow intrinsic degradation and continuous accumulation and persistence in soils and sewage sludges. This pollutant was found at concentrations ranging from 0.1 to 1 mg/kg in 70% of 21 French WWTPs [1].

Aminomethylphosphonic acid (AMPA), the third pollutant studied, is the primary product of glyphosate (N-(phosphonomethyl)glycine) degradation by microbial activity in soils [3]. Glyphosate, which is also known as Roundup, is a foliar herbicide widely used throughout the world for weed control, as well as in new agricultural systems, notably those cultivating glyphosate-tolerant crops. This widespread use led to an universal input of this molecule and AMPA to the environment and consequently its presence in WWTPs [4]. Owing to its very high water solubility (10,000 mg/l) and its low octanol/water partitioning coefficient (log K_{ow} −3.5), AMPA was found in both water and sewage sludge of WWTPs at concentrations ranging from 0.1 to 1 mg/kg in 70% of 21 French WWTPs [1].

The last selected pollutant was estrone (EST), which is a hormone well known for its estrogenic activity and endocrine-disrupting characteristics in municipal/industrial wastewaters and landfill leachates. Owing to its high octanol/water partitioning coefficient (log K_{ow} 3.13), EST is an hydrophobic compound found in treated water of WWTPs as well as in sewage sludge [1], indicating that conventional physicochemical or biological treatment can lead to only a partial degradation.

In this study, the potential degradation of these four pollutants (DEHP, FLU, AMPA, and EST) by four filamentous fungi isolated from either French WWTPs (*Fusarium oxysporum*, *Geotrichum galactomyces*, and *Trichoderma harzianum*) or a petroleum-contaminated soil (*Fusarium solani*) was investigated by acute toxicity tests and pollutant degradation ones conducted in mineral medium (MM).

2. Materials and methods

2.1. Chemicals and media

DEHP, FLU, AMPA, EST, and p-toluenesulfonyl chloride (TsCl) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), and chloroform (TCM) were obtained in the highest purity grade available from Fisher Scientific (Illkirch, France). Acetone (ACT) and dimethyl sulfoxide (DMSO) were supplied by Panreac Química SA (Barcelona, Spain). Distilled deionized water was used throughout this study. Malt yeast extract agar (MYEA) medium was used for routine fungal growth. Screening studies were conducted in MM composed of (g/l): KCl (0.25); $NaH_2PO_4{\cdot}2H_2O$ (3.235); $Na_2HPO_4{\cdot}2H_2O$ (5.205); $MgSO_4$ (0.244); and NH_4NO_3 (1.0). Trace element solution consisting of the following materials was also used (mg/l): $ZnSO_4{\cdot}7H_2O$ (1.0); $MnCl_2{\cdot}H_2O$ (0.1); $FeSO_4{\cdot}7H_2O$ (1.0); $CuSO_4{\cdot}5H_2O$ (0.5); $CaCl_2{\cdot}2H_2O$ (0.1); and MoO_3 (0.2). The culture medium was about pH 5.5.

2.2. Micro-organisms

Three fungal strains were obtained by direct isolation onto Sabouraud agar medium with chloramphenicol (BK027HA, Biokar Diagnostics, Beauvais, France) from WWTP sludges. These fungi were identified by molecular approaches conducted by BCCM™/MUCL (Louvain-la-Neuve, Belgium) as *F. oxysporum*, *G. galactomyces*, and *T. harzianum*. Pure cultures were deposited in the BIOVITIS culture collection (Clermont-Ferrand, France). *F. solani*, previously isolated from petroleum-contaminated soil, was obtained from the UCEIV mycology collection (Dunkerque, France) [5,6]. Stock cultures were transferred to MYEA plates at 25°C for use as inocula.

2.3. Preparation of each pollutant

Two sets of experiments were conducted in MM (25 ml) in 250 ml Erlenmeyer flasks containing glucose (10 g/l). An acute toxicity test was conducted after 96 h of incubation (AT_{96h}) by assessing fungal dry biomass in the presence of each pollutant. Additionally, pollutant degradation tests were conducted after 10 d of incubation. A total of 500 µl of a stock solution of DEHP (5 g/l) dissolved in ACT was transferred into individual 250 ml Erlenmeyer flasks. The solvent was then allowed to evaporate, after which 25 ml of MM were added to each flask to give a final DEHP dose of 2.5 mg/flask for both the AT_{96h} and pollutant

degradation tests. For FLU, an appropriate volume of a stock solution of FLU initially dissolved in ACT (2 g/l) was transferred into each 250 ml Erlenmeyer flasks. After solvent evaporation, 25 ml of MM were added per flask, giving a final FLU dose of 250 μg and 1 mg/flask for the AT_{96h} and pollutant degradation tests, respectively. About 25 ml of a stock solution of AMPA initially dissolved into MM were transferred into individual 250 ml Erlenmeyer flasks containing 25 ml of MM, giving a final AMPA dose of 2.5 μg and 250 μg/flask for the AT_{96h} and pollutant degradation tests, respectively. A total of 50 μl of a sterile EST stock solution (2 g/l) dissolved in DMSO was transferred into individual 250 ml Erlenmeyer flasks containing 25 ml of MM, giving a final EST dose of 10 ng and 10 mg/flask for the AT_{96h} and pollutant degradation tests, respectively. After sterilization (121˚C for 20 min), sterile glucose (at a final concentration 10 g/l) was added to each flask.

2.4. Acute toxicity test (AT_{96h}) by fungal dry biomass assessment

Samples were inoculated by adding a spore suspension of each fungus, prepared by washing a two-week-old Petri dish culture of each isolate on MYEA with 4 ml of sterile deionized water [5]. The spore suspension was estimated using a Malassez haemocytometer, to give a final concentration of 10^4 spores per ml of MM. Three replicates were run for each fungal isolate in the presence or absence (control) of pollutant. Incubation was conducted at room temperature on a reciprocating shaker (Infors, 175 rpm). Acute toxicity was estimated by biomass produced over a total period of 96 h. Mycelium was filtered through pre-dried cellulose filters (55 mm diameter, 25 μm porosity) in a vacuum filtration apparatus and weighed after complete drying in an oven (3 d). The percentage growth inhibition in the presence of each pollutant was then calculated. Differences between treatments were identified by a two-sample *t*-test at 95% confidence.

2.5. Pollutant degradation test

The degradation experiment was performed for 10 d with pollutant prepared as detailed in Section 2.3. Inoculation was performed with a spore suspension to give a final concentration of 10^4 spores/ml of MM. At the end of the incubation period, the cultures were lyophilized for 4 d. For DEHP and FLU, total lyophilized cultures were scraped and then extracted for 16 h in a Soxhlet apparatus using DCM. The residual pollutants in each sample were concentrated in 25 ml of TCM/MeOH solution (50:50 v/v) for DEHP and in 25 ml of DCM/MeOH solution (75:25 v/v) for FLU. For AMPA, the lyophilized samples were tosylated before extraction and dosage [7]. The tosylated samples were again lyophilized for 4 d and then extracted at 60˚C in a series of DCM/MeOH mixtures of 25:75 v/v, 50:50, 75:25, and 100:0. The pollutant fraction was concentrated in 20 ml DCM/MeOH (50:50 v/v). For EST, cultures were extracted at 60˚C four times in DCM/MeOH (50:50 v/v) before being concentrated in 20 ml DCM/MeOH (50:50 v/v).

Pollutant concentrations were determined using an High-performance liquid chromatography (HPLC) Waters 2,690 system fitted with a Waters XTerra® 5 μm C18 column and a Waters 996 Photo Diode Array Detector. Detector separation was achieved at a solvent flow rate of 1 ml/min with an ACN/MeOH solution (95:5 v/v) for DEHP, an ACN/H_2O solution (90:10 v/v) for FLU, a 0.2 M phosphate buffer/ACN solution (85:15 v/v) for AMPA, and an ACN/H_2O solution (50:50 v/v) for EST. Concentrations were determined based on the UV absorbance at 257 nm for DEHP, 236 nm for FLU, 240 nm for AMPA, and 280 nm for EST. To evaluate the pollutants adsorbed by the fungal hyphae and abiotic degradation (extraction controls), Erlenmeyer flasks containing 25 ml MM without pollutant were inoculated with each isolate. At the end of the experiment, the obtained cultures (mycelia + filtrate) were suspended in MM containing the same dose of pollutant as in biotic treatments and stirred for 4 h on a reciprocating shaker at 4˚C. These treatments enabled determination of the adsorption processes on hyphae and abiotic degradation, which were used for further calculations of the degradation of each pollutant. All treatments were conducted in triplicate, and the percentage of pollutant degradation was calculated by the formula: $[(m_{EC} - m_T/m_{EC})] \times 100$, in which m_{EC} was the quantity of pollutant recovered in extraction controls (conducted to detect pollutants adsorbed by fungal hyphae) and m_T was the quantity of pollutant obtained in each treatment. Treatments were compared by two-sample *t*-tests at 95% confidence.

3. Results and discussion

3.1. Pollutant toxicity to fungal growth

Table 1 shows the growth inhibition of isolates obtained in the presence of each pollutant after 96 h of incubation (AT_{96h}). The acute toxicity test was deliberately conducted at pollutant concentrations higher than the average levels commonly found in WWTPs in order to select isolates able to resist high

Table 1
Fungal growth inhibition (%) in the presence of each pollutant: DEHP (2.5 mg/flask), FLU (250 μg/flask), AMPA (2.5 μg/flask), and EST (10 ng/flask) after 4 d of incubation in MM

Fungus	Code	DEHP	FLU	AMPA	EST
F. oxysporum	1	19.3[a] (S)	0 (NS)	17 (S)	8 (NS)
F. solani	2	8.6 (NS)	0 (NS)	8 (S)	0 (NS)
G. galactomyces	3	0 (NS)	0 (NS)	0 (NS)	0 (NS)
T. harzianum	4	0 (NS)	0 (NS)	0 (NS)	0 (NS)

Note: S, significantly different; NS, not significantly different, as determined by a two-sample *t*-test at 95% confidence.
[a]The percentage of growth inhibition in the presence of each pollutant was calculated according to 2. Materials and Methods section.

pollution concentrations, which may occur in WWTPs during malfunctions or pollution peaks. The most sensitive isolates to DEHP were *F. oxysporum* and *F. solani*, which showed 19.3 and 8.6% growth inhibition relative to the control, respectively. *F. oxysporum* and *F. solani* showed weak sensitivity to AMPA, as indicated by 17 and 8% inhibition relative to the control, respectively, whereas *G. galactomyces* and *T. harzianum* were not affected by this pollutant. A null or weak decrease in biomass yield was observed in response to FLU and EST, indicating that the toxic effects of these pollutants under the experimental conditions were limited. The tested pollutants were not toxic to the chosen filamentous fungi even at high pollutant concentrations. This capacity to resist to pollutant is a prerequisite before conducting degradation studies.

3.2. DEHP degradation

The filamentous fungi were then evaluated for their capacity to degrade each pollutant at a given concentration in liquid MM. Additionally, this test employed a short incubation period (10 d) to evaluate the isolate during its active exponential growth phase. This short duration could also be compatible with the rather short residence time of wastewater and sludges in WWTPs.

All four isolates effectively degraded DEHP (Fig. 1), with *F. oxysporum* (1) and *F. solani* (2) showing the greatest degradation rates of 70 and 100%, respectively. In addition to bacteria, few filamentous fungi have been reported to degrade phthalates in other research fields. For example, *F. oxysporum* f. sp. *pisi*, a phytopathogenic fungus, produced extracellular cutinase, an hydrolytic enzyme that degrades cutin, which is a polyester consisting of hydroxy and epoxy fatty acids, usually with *n*-C16 and *n*-C18 of higher plants. This fungal cutinase showed high potential for degradation of DEHP and various phthalates [8,9]. Three novel phthalates utilizing fungi, *Aspergillus parasiticus*, *Fusarium subglutinans*, and *Penicillium funiculosum* were reported and their potential use was proposed for production of phthalate-free PVC [10]. In addition to results obtained in other fields (such as phytopathology or environment), this lab-scale experiment underlined the potential and innovative utilization of *Fusarium sp.* for biodegrading DEHP, a persistent priority pollutant that is present at high concentrations in wastewater sludge. This promising result is especially interesting as *Fusarium* are commonly reported in sewage sludge among other filamentous fungi and are thus well adapted to such real and complex environments [11].

3.3. FLU degradation

F. oxysporum (1) and *F. solani* (2) degraded 42 and 12% of the FLU initially present, respectively (Fig. 1), while *G. galactomyces* (3) and *T. harzianum* (4) did not significantly degrade this pollutant. Inset in Fig. 1(B) shows an example of HPLC elution profiles of FLU in treatment with *F. oxysporum*. Knowledge regarding FLU degradation by fungal isolates in WWTPs is rather limited. However, the degradation of various PAHs, including FLU, was previously investigated by fungi in noncontaminated soils or contaminated ones [12,13]. Degradation of FLU as a sole carbon and energy source used by several pure bacterial strains from activated sludge has also been reported [14]. Accordingly, the use of different microbial consortia combining successively or simultaneously bacteria and fungi could have interesting benefits for degrading persistent pollutants, such as FLU in sewage sludges, and decreasing thus their toxicity prior their land application for environmental safety.

3.4. AMPA degradation

T. harzianum (4) showed the greatest AMPA degradation of 69% after only 10 d of incubation (Fig. 1). The three other isolates did not significantly degrade this pollutant. These results are consistent

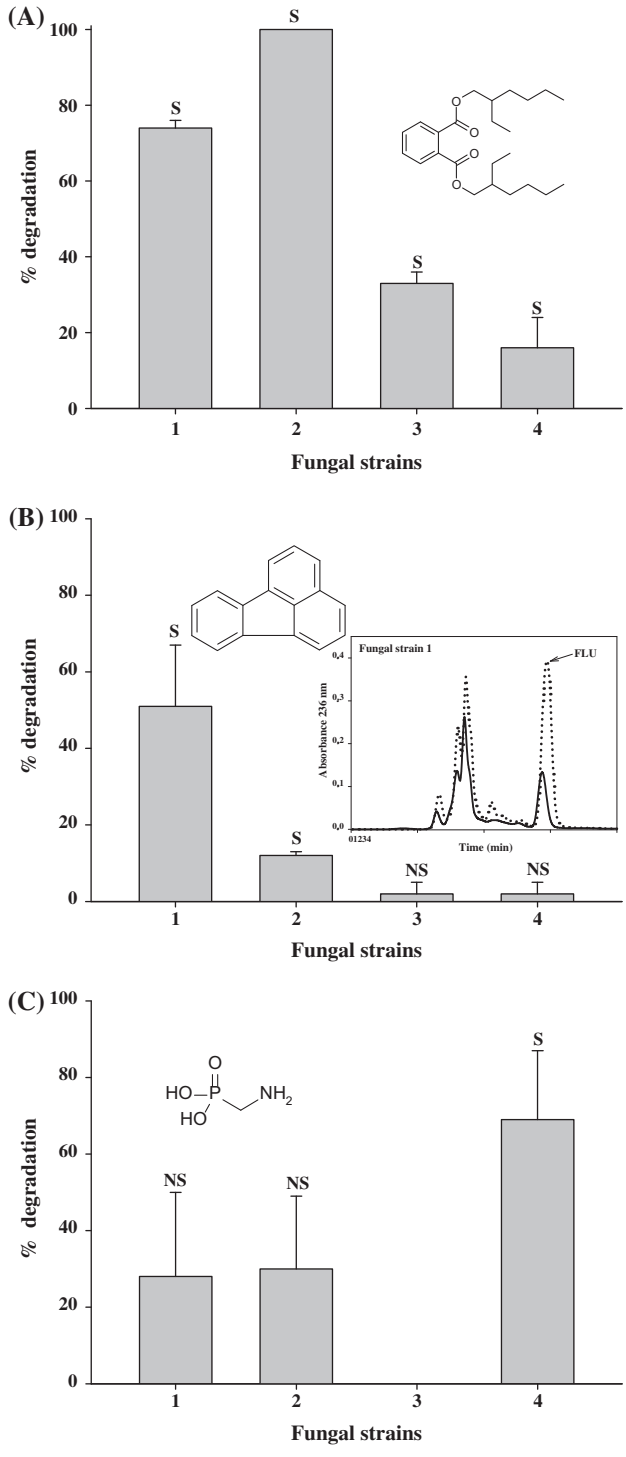

Fig. 1. Degradation of pollutant (%) in the presence of isolates after 10 d of incubation (Mean ± SE for triplicates). (A) DEHP (2.5 mg/flask); (B) FLU (1 mg/flask); and (C) AMPA (250 µg/flask). 1. *F. oxysporum*, 2. *F. solani*, 3. *G. galactomyces*, and 4. *T. harzianum*.
Note: Inset in Fig. 1(B) showed an example of HPLC elution profiles of FLU. Dotted line (···): FLU presents in abiotic control and plain line (—): FLU remained in treatment with *F. oxysporum*.

with previous studies of glyphosate in agricultural, horticultural, and timber production systems. Specifically, glyphosate has been found to exert diverse effects on the biology, ecology, and structure of soil fungal communities, especially in the rhizosphere of treated crops. It has frequently been reported that repeated and/or long-term exposure of soil microorganisms to glyphosate led to a fungal community dominated by *Fusarium* spp. [15]. The primary degradation pathway is the cleavage of glyphosate by glyphosate oxidoreductase to glyoxylate and AMPA, with the latter being subsequently degraded to methylamine and inorganic phosphate by C–P lyase enzymes. A second degradation pathway is the cleavage of inorganic phosphate from glyphosate by C–P lyase, resulting in production of the sarcosine metabolite. This metabolic pathway has been described for *T. harzianum* [16], clearly indicating that this species could degrade glyphosate or its degradation products. But even if the degradation of glyphosate in soil has been extensively documented for agricultural purposes, its degradation in biological wastewater treatment has not been thoroughly considered. The present results indicate that *T. harzianum* has been identified to possess AMPA-degrading capability on a laboratory scale. Therefore, this preliminary laboratory work needs to be followed by series of pilot scale for exploring potential fungal wastewater treatments applications.

3.5. EST degradation

The null or weak decrease in biomass yield for EST indicated that the toxic effects resulting from exposure to this pollutant under the experimental conditions were quite limited. Nevertheless, none of the fungal strains tested were able to degrade EST in the present study (data not shown). In the literature, biodegradation of estrogens has been attributed to ligninolytic enzymes produced by white-rot fungi. For example, manganese peroxidase, horseradish peroxidase, and laccase [17,18] produced by *Phanerochaete chrysosporium*, *Phanerochaete sordida*, *Trametes* sp., and *Pycnoporus* have been shown to degrade EST. The degradation of such compounds is challenging owing to their complex structures and their low bioavailability.

4. Conclusion

This study conducted in MM clearly demonstrated the potential for three among four filamentous fungi tested to resist and to degrade emergent water priority pollutants that are commonly found at high

occurrence in raw water, treated water, or sewage sludge in French WWTPs. This investigation could be considered as a first but necessary step underlying the potential of *F. oxysporum*, *F. solani*, and *T. harzianum* commonly reported in WWTPs among other filamentous fungi. Subsequent degradation of these recalcitrant organic pollutants could be carried out syntrophically by applying these fungal isolates alone or in combination with bacteria or other fungi. Moreover, fungi could be potential candidates because they could be highly resistant to toxic pollutants, even at high concentrations, owing to their hyphal growth, the presence of a cell wall, and pollutant degradation versatility through nonspecific oxidation reactions [19]. The effect of fungi can also increase the degradability and the settleability of wastewater sludge permitting thus important volume reductions, a requisite in sludge management strategy, while also offering an opportunity for by-products recovery during wastewater treatment, both important advantages considering the principle of sustainable development [11]. Therefore, these studies conducted on a laboratory scale need to be followed by additional research on pollutants degradation in more complex environments (sludge and/or water from WWTP) to enable industrial process applications of filamentous fungi [20].

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