Bioresource Technology 102 (2011) 5602–5608

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Solid-phase treatment with the fungus *Trametes versicolor* substantially reduces pharmaceutical concentrations and toxicity from sewage sludge

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ARTICLE INFO

Article history:
Received 26 November 2010
Received in revised form 4 February 2011
Accepted 5 February 2011
Available online 4 March 2011

Keywords:
Sewage sludge
Trametes versicolor
Pharmaceuticals
Solid-phase
Biodegradation

ABSTRACT

For safe biosolid-land-applying, sludge should be contaminant-free. However, it may contain important amounts of micropollutants, not removed in the wastewater-treatment-processes. An alternative treatment with the fungus *Trametes versicolor* was applied in sterile solid-phase systems consisting of sludge and a lignocellulosic substrate. Fungal colonization and activity were demonstrated during the process, according to monitoring of ergosterol, laccase activity and the naproxen-degradation test (ND24). Fourteen out of 43 analyzed pharmaceuticals were found in the raw sludge. After treatment, phenazone, bezafibrate, fenofibrate, cimetidine, clarithromycin, sulfamethazine and atenolol were completely removed, while removals between 42% and 80% were obtained for the remaining pharmaceuticals. Toxicological analyses (*Daphnia magna*, *Vibrio fischeri* and seed germination) showed an important reduction in sludge toxicity after treatment. Results suggest that a solid-phase treatment with *T. versicolor* may reduce the ecotoxicological impact of micropollutants present in sewage sludge. This is the first report of a fungal-approach for elimination of emerging pollutants from biosolids.

1. Introduction

Sewage sludge is a by-product generated during the wastewater treatment process. Treated sewage sludge meeting specific regulations for microbial pathogens, nutrients and metal concentrations is known in the EU and the US by the term biosolids (Wu et al., 2009). Biosolids are used as soil amendment; however, scarce jurisdiction exists for the regulation of concentrations of organic pollutants. Regulation initiatives on biosolids in EU, US and Canada include the control of heavy metals and microbial pathogens, while the more recent concern in organic pollutants focuses on halogenated compounds, alkylbenzene sulphonates, phthalates, nonylphenols, polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH) and dioxins (European Union, 2000; Hébert, 2008). However, legislation regarding the limits of emerging organic micropollutants such as pharmaceuticals is not included. Therefore, municipal biosolids may contain important amounts of contaminants, previously sequestered by suspended solids in the wastewater (Kinney et al., 2006). If high enough concentrations are repeatedly applied on soil, these micropollutants might reduce or counteract the benefits of land applying biosolids, resulting in contamination of arable soils. Results of the present work expect to contribute, among others in the field, to provide the information required to include the control of emerging pollutant concentrations in the future legislation.

In Europe, around 4000 pharmaceutical active compounds employed both for human or veterinary purposes are susceptible to reach the environment (Mompelat et al., 2009). The annual consumption of the most common pharmaceuticals reaches hundreds tons in Europe; in particular, anti-inflammatory drugs like aspirin, paracetamol, ibuprofen and diclofenac were produced in Germany (by 2001) at amounts ranging from 836 to 86 t, meanwhile the antiepileptic carbamazepine was produced at 88 t (Fent et al., 2006). On the other hand, annual antibiotic consumption may reach thousands tons, more than 13 000 t in EU in 1999, especially due to their wide use in medicine, veterinary, farming and aquaculture (Kemper, 2008). Pharmaceuticals and their metabolites may

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doi:10.1016/j.biortech.2011.02.029

reach the environment via WWTP discharges, manufacturing and hospital effluents, concentrated animal feeding operations, direct disposal and land application of biosolids (Daughton and Ternes, 1999).

The scientific community widely agrees with the possibility that negative ecotoxicological effects may arise from the presence of pharmaceuticals in the environment (Santos et al., 2010). In particular, since antibiotics have the potential to affect microbial communities, the inhibition of natural occurring processes such as degradation of organic matter in water and sediments, anaerobic digestion or processes related to the N_2 cycle may occur (Kümmerer 2009); moreover, it is still unknown whether their presence in nature contributes to the spread of microbial antibiotic resistance (Kümmerer, 2009). Although chronic ecotoxicity data are scarce if compared to acute studies, accumulative effects have been shown to damage some ecosystems (Daughton and Ternes, 1999). Acute and chronic ecotoxicology of different groups of pharmaceuticals are reviewed by Santos et al. (2010).

A promising approach to reduce organic pollution is the application of natural-degrading microorganisms, since bioremediation techniques are increasing attention as more environmentally friendly alternatives than conventional physicochemical treatments used for cleaning up of contaminated sludge. White-rot fungi (WRF) are considered as an interesting group of microorganisms from the biodegradation point of view, due to their non-specific extracellular ligninolytic enzymatic system, which includes laccases and high redox potential peroxidases such as lignin peroxidase, manganese peroxidase and versatile peroxidase (Martínez et al., 2005). Additionally, some xenobiotics can be potentially metabolized by WRF by means of the intracellular cytochrome P450 complex, which acts in a similar way in mammals (Doddapaneni and Yadav, 2004). In this respect, efficient colonization and degradation of spiked pharmaceuticals in sludge has been recently demonstrated by the fungus *Trametes versicolor* (Rodríguez-Rodríguez et al., 2010a), thus opening a whole new spectrum of interesting and promissory environmental-friendly decontamination processes.

This work aimed to demonstrate the biodegradation of pharmaceuticals in sterilized sewage sludge under solid-phase conditions with *T. versicolor*, as a first approach for the removal of emerging pollutants from biosolids.

2. Methods

2.1. Chemicals

Ergosterol (ergosta-5,7,22-trien-3β-ol, >95%) was obtained from Sigma–Aldrich Co. (St. Louis, MO). The pharmaceutical standards used for analysis were of high purity grade (>90%). Ibuprofen, Naproxen, Ketoprofen, Diclofenac and Gemfibrozil were supplied by Jescuder (Rubí, Spain). Acetaminophen, Indometacin, Mefenamic acid, Phenazone, Bezafibrate, Mevastatin, Fenofibrate, Pravastatin (as sodium salt), Carbamazepine, Famotidine, Ranitidine (as hydrochloride), Cimetidine (as hydrochloride), Erithromycin (as hydrate), Azithromycin (as dehydrate), Roxitromycin, Clarithromycin, Josamycin, Tylosin A, Sulfamethazine, Trimethoprim, Chloramphenicol, Atenolol, Sotalol, Metoprolol (as tartrate), Timolol, Pindolol, Nadolol, Salbutamol, Clenbuterol (as hydrochloride), Enalapril (as maleate), Glibenclamide, Furosemide, Hydrochlorothiazide and Metronidazole were purchased from Sigma–Aldrich (Steinheim, Germany). Standard Atorvastatin (as calcium salt) was provided by LGC Promochem (London, UK), while Diazepam, Lorazepam and Butalbital were from Cerilliant (Texas, USA).

The deuterated or ^{13}C-labeled compounds, used as internal standards, were Sulfathiazole-d_4, Famotidine-$^{13}C_3$, rac-Timolol-d_5 maleate, Clarithromycin-*N*-methyl-d_3, Atorvastatin-d_5 sodium salt, Azithromycin-d_3, Fenofibrate-d_6, Metronidazole hydroxyl-d_2, Phenacetine-^{13}C, Ketoprofen-$^{13}C,d_3$, Indomethazine-d_4, rac-Naproxen-d_3, Mefenamic acid-d_3, Gemfibrozil-d_6 and Bezafibrate-d_4 from Toronto Research Chemicals; Diazepam-d_5 and Phenobarbital-d_3 from Cerilliant (Texas, USA); Atenolol-d_7, Carbamazepine-d_{10}, Ibuprofen-d_3, Enalapril–d_5, Glyburide-d_3, Albuterol-d_3, Cimetidine-d_3, Antipyrine-d_3, Diclofenac-d_4, Hydrochlorothiazide-3, 3-d_2 from CDN Isotopes (Quebec, Canada); Sotalol hydrochloride-d_6 from Dr. Ehrenstorfer (Augsburg, Germany) and Erythromycin-$^{13}C,d_3$ (*N*-Methyl-$^{13}C,d_3$) from Isotec (Ohio, USA).

The individual standard solutions as well as isotopically labeled internal standard solutions were prepared according to Jelić et al. (2009).

2.2. Fungal strain

The strain *T. versicolor* ATCC 42530 was acquired from the American Type Culture Collection, and maintained by subculturing every 30 days on 2% malt extract agar slants (pH 4.5) at 23 °C. *T. versicolor* blended mycelial suspension was prepared according to Font Segura et al. (1993).

2.3. Sewage sludge and bulking material

Dry sewage sludge was obtained from the wastewater treatment plant of El Prat de Llobregat. The plant is located near Barcelona, Spain and it has a total treatment capacity of two million equivalent inhabitants. It is a typical biological activated sludge plant with sludge anaerobic digestion and thermal dehydration. Sludge employed in the experiments was obtained from the final stage of processing, i.e., after thermal dehydratation (~10% water content). The wheat-straw pellets (WSP, ATEA Praha s.r.o., Czech Republic) used as bulking material and substrate in solid-phase cultures were kindly provided by Č. Novotný.

2.4. Solid-phase treatment

Solid-phase systems with a total dry solid weight content of 6.5 g were performed in 24 × 150 mm tubes (Barloworld Scientific Ltd., Staffordshire, UK), containing sterile-sewage sludge and 38% (w/w, dry basis) *T. versicolor* inoculum. Inocula were prepared by adding blended mycelium suspension to sterile WSP, 0.65 mL per gram of dry WSP and pre-growing for 7 d at 25 °C. The WSP were hydrated in a 1:2 ratio (w/v) prior mycelium inoculation. Sterilization process consisted of autoclaving at 121 °C for 30 min in every case. The solid-phase systems were incubated for up to 42 d at 25 °C, periodically homogenized and often sprinkled with sterile distilled water to provide moisture. Triplicate cultures were sacrificed for time-course and final-point analytical determinations. Uninoculated cultures consisting of sterile-sludge amended with 38% WSP were used as controls, and are referred to as "untreated sludge", unless otherwise stated. Unamended sterile sludge (lacking WSP) is referred to as "raw sludge".

2.5. Analytical methods

2.5.1. Sample preparation and analysis of pharmaceuticals

The samples were prepared according to a previously developed multi-residue method for analysis of 43 pharmaceuticals in sludge (Jelić et al., 2009). The pharmaceuticals were isolated from solid samples by pressurized solvent extraction using Dionex ASE 200 (Dionex; Sunnyvale, CA), followed by a solid-phase extraction clean-up step onto a lipophilic–hydrophilic balanced Oasis HLB (60 mg, 3 mL) cartridge.