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5 **The white-rot fungus *Trametes versicolor* reduces the estrogenic activity of a mixture of emerging**
6 **contaminants in wastewater treatment plant effluent**
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Abstract

This study investigated the removal of common emerging contaminants (ECs) and related estrogenic activity from wastewater effluent by a strain of the white-rot fungus *Trametes versicolor* with previously unreported bioremediation potential. *T. versicolor* NRRL 66313 was grown in carbon-amended sterile secondary wastewater treatment plant (WWTP) effluent. Aerated batch reactors containing sterile, glucose-amended (5 g l^{-1}) wastewater were inoculated, incubated for eight days, and then spiked with either 5 mg l^{-1} of 17β -estradiol (E2), or a mixture of E2, atrazine (ATZ), bisphenol A (BPA), carbamazepine (CBZ), N,N-diethyl-3-methylbenzamide (DEET), estrone (E1), 17α -ethynylestradiol (EE2), oxybenzone (OBZ), and triclosan (TCS) to a final concentration of $350 \text{ }\mu\text{g l}^{-1}$ each. Abiotic and heat-killed fungus controls were also prepared. EC and metabolite concentrations were measured over time using HPLC and Gas Chromatography coupled with Time-Of-Flight Mass Spectrometry (GC-TOFMS). Estrogenic activity was measured on the same samples using the Yeast Estrogen Screen. In less than 5 hours, active fungi decreased the concentration of E2 from 5 mg l^{-1} to below detection, producing E1 as a metabolite and subsequently removing it from solution. Acting on the mixture of ECs, fungi removed BPA, E1, E2, and EE2 to a significant degree (62-100%) vs. controls (0-28%) in only 3.5 hours, reducing the estrogenic activity of the mixture by 77% (vs. 4-8% for controls). After 12 hours, the total reduction of estrogenic activity was greater than 98% (vs. 24-42% for controls). These results show that *T. versicolor* NRRL 66313 can reduce the estrogenic activity of mixtures of estrogens and BPA, which are typically the most significant contributors to the hormone disrupting activity of domestic WWTP effluent.

Keywords: Pharmaceuticals and Personal Care Products; Endocrine Disrupting Compounds; Estrogenic activity; domestic wastewater; *Trametes versicolor*; Yeast Estrogen Screen

1 Introduction

Emerging contaminants (ECs) often pass through domestic wastewater treatment plants (WWTPs) and are discharged into surface waters, where they can threaten aquatic ecosystems and human health by mimicking natural hormones (e.g. estrogen) and disrupting the endocrine systems of exposed individuals. The potential for an EC, or a mixture of ECs, to mimic estrogen can be quantified in terms of its estrogenic activity. Among the ECs detected in WWTP effluent, estrogen compounds (e.g. 17 β -estradiol, 17 α -ethynylestradiol, and estrone) have been found to contribute the majority of hormone disrupting activity to receiving waters (Desbrow et al. 1998; Aerni et al. 2004; Metcalfe et al. 2013). In addition, risk-based prioritization schemes which consider occurrence, exposure, and hazard effects have been used to narrow the field of potentially harmful ECs and focus research efforts (Murray et al., 2010; Kumar and Xagorarakis 2010). On the basis of these prior works, nine chemicals were chosen as model ECs for use in this study: atrazine (ATZ), bisphenol A (BPA), carbamazepine (CBZ), N,N-diethyl-3-methylbenzamide (DEET), estrone (E1), 17 β -estradiol (E2), 17 α -ethynylestradiol (EE2), oxybenzone (OBZ), and triclosan (TCS).

While various physical/chemical technologies (e.g. membrane separation, advanced oxidation, and carbon adsorption) show some promise for removing ECs during wastewater treatment, drawbacks include added costs associated with plant modification, energy/chemical consumption, and disposal of concentrated waste streams. In contrast, advanced biocatalysis results in the degradation, or detoxification, of ECs and the potential to enhance existing biological WWTP unit operations through bioaugmentation. White-rot fungi (WRF) have received a great deal of attention in recent years for their ability to transform a variety of recalcitrant ECs in bench-scale experiments via their very powerful and non-specific extracellular oxidative enzymes (Cabana et al. 2007; Yang et al. 2013). In some cases, extracellular fungal enzymes have been isolated and immobilized for use in specialized reactors (Gasser et al. 2014). However, using whole-cell cultures as biocatalysts allows for the potential to exploit intracellular fungal enzymes and potentially degrade a broader range of ECs (Yang et al. 2013; Nguyen et al. 2014). In addition, whole-cell cultures of fungi could be used to enhance microbial populations in existing wastewater treatment processes such as activated sludge, or as a tertiary treatment step to further treat the effluent from existing WWTP operations.

To date, nearly all of the bench-scale experiments involving EC degradation by whole-cell fungal cultures have been conducted in nutrient media or synthetic wastewater (Yang et al. 2013). The use of whole-cell cultures of WRF to treat *actual domestic* wastewater for EC removal has been reported in only a limited number of cases (Zhang and Geißen, 2012; Cruz-Morató et al., 2013). The WRF used in these studies were grown in nutrient media prior to being introduced into wastewater. In order to use WRF as a tertiary treatment step in existing domestic WWTPs, their ability to grow, produce, enzymes, and remove ECs in a wastewater matrix with minimal nutrient supplementation is of interest. With any process involving biological removal of ECs, it is also critical that the overall effect of fungal metabolism on the hormone disrupting potential of mixtures of ECs be investigated. Prior studies using whole-cell cultures of WRF to treat domestic wastewater and hospital wastewater, assess overall toxicity using the Microtox assay, but do not assess the hormone disrupting potential of the treated effluent (Cruz-Morató et al., 2014, 2013). In the present study, these gaps in the literature are addressed by comparing the bioremediation potential of whole-cell cultures of a WRF cultured in nutrient media and domestic wastewater and examining the fungal degradation of a mixture of ECs in actual domestic WWTP effluent in terms of the removal of individual chemicals *and* the overall estrogenic activity of the mixture.

The investigation consisted of two phases: First, a preliminary experiment compared small batch cultures (150 ml) of the WRF *Trametes versicolor* grown in carbon-amended wastewater (with no additional nutrient supplementation) to those grown in a defined nutrient medium with a focus on quantifying biomass growth and enzyme production. The strain of *T. versicolor* used in this study (NRRL 66313) had not previously been examined with regard to its bioremediation potential. During this phase, E2 (10 mg l⁻¹) was added to assess whether the viability of *T. versicolor* would be negatively affected in the subsequent experimental phase, where high concentrations of E2 were used to evaluate metabolite production. In the second phase, carbon-amended wastewater cultures of *T. versicolor* were grown in larger (1 l) reactors allowing for more efficient collection of time-series EC and estrogenic activity removal data. Two groups of these larger reactors were prepared: the 'estrogen group' was spiked with an elevated concentration of E2 (5 mg l⁻¹) in order to ease the detection of metabolites, while the 'mixed group' was spiked with the aforementioned mixture of nine common ECs at a lower concentration (350 μ g l⁻¹ each).

2 Materials and Methods

2.1 Model emerging contaminants and stock solutions

Bisphenol A (BPA), carbamazepine (CBZ), N,N-diethyl-3-methylbenzamide (DEET), estrone (E1), 17 β -estradiol (E2), 17 α -ethynylestradiol (EE2), oxybenzone (OBZ), and triclosan (TCS) were purchased from SigmaAldrich (St. Louis, MO, USA) at a purity of 97%, or greater. Atrazine (ATZ) was purchased from Cayman Chemical (Ann Arbor, MI, USA) at a purity of 98%. All model ECs were dissolved in N,N-dimethylformamide (DMF; EMD Millipore, Billerica, MA, USA) prior to spiking into bioreactors.

2.2 Carbon-amended wastewater and nutrient media

Nutrient media used in batch reactors was prepared after Blázquez and Guieysse (2008), where the micronutrient medium was that of Tien and Kirk (1988). Wastewater (secondary effluent, pre-chlorination) was collected from the overflow weir of the secondary clarifier at The Pennsylvania State University WWTP (University Park, PA, USA). The wastewater was prepared for use in batch reactors by chemically adjusting 2 l aliquots as follows: alkalinity was removed by adding 1 M hydrochloric acid (EMD, USA) to a pH of 3.5, followed by the addition of 1 M sodium hydroxide (NaOH; J.T. Baker, Phillipsburg, NJ, USA) to a pH of 4.5 over a period of 3 hours, while vigorously stirring. Glucose (EMD, USA) was added to a final concentration of 5 g l⁻¹ in both nutrient media and wastewater.

2.3 Organism, maintenance, and cultivation conditions

T. versicolor (WC 861) was obtained from the Mushroom Spawn Laboratory at The Pennsylvania State University, and was deposited in the United States Department of Agriculture Agricultural Research Service (USDA ARS) Culture Collection as strain number NRRL 66313. A partial 18s rRNA gene sequence was obtained via DNA extraction from the pure culture using a PowerSoil[®] DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) followed by PCR amplification using the primer set nu-SSU-0817-5'/nu-SSU-1536-3' (Borneman and Hartin 2000) and subsequent Sanger sequencing. The sequence was deposited in GenBank at the National Center for Biotechnology Information (NCBI) under accession number KR869151 (<http://www.ncbi.nlm.nih.gov/GenBank/index.html>). Solid-phase, stock cultures were maintained on YMPG agar media (Tien and Kirk 1988). Colonized YMPG plates were stored at 4 °C and *T. versicolor* was transferred to fresh media every 1-2 months.

2.3.1 Preparation of fungal inoculum

Agar plugs were aseptically harvested from a *T. versicolor* populated plate and incubated in YMPG media for 7 days at 30 °C and 120 rpm (MaxQ 4000; Thermo Scientific, Waltham, MA, USA), yielding spherical pellets of *T. versicolor* mycelia with a wide size distribution. These pellets were rinsed of spent growth medium using sterile distilled deionized (DDI) water and blended (WSB33 Quik Stik; Waring, USA) with additional sterile DDI water in an approximate volumetric ratio of 1:3 (DDI:mycelia) for 3 min in order to prepare a homogenous suspension of mycelia. The homogenate was then added to fresh YMPG media in a ratio of 0.8 - 1.4 % (v/v) and incubated again under the above conditions. The second growth phase yielded pellets of uniform size which were rinsed with, and stored in, 0.85% NaCl at 4 °C for up to two months (Romero et al. 2006). As needed, the stored pellets were recovered from the storage solution, rinsed, and blended in equal parts (v/v) with additional sterile DDI water. The resulting inoculum was stirred continuously as aliquots were distributed to the appropriate reactors. Before and after distribution of the inoculum, three 5 ml aliquots were placed on dry, pre-weighed glass fiber qualitative filters (Grade 696; VWR, USA) and dried to a constant weight at 60 °C in order to determine the approximate dry mass of mycelia per ml of inoculum.

2.4 Experimental procedures

2.4.1 Effect of wastewater and estradiol on growth and enzyme production (‘preliminary growth experiment’)

A preliminary investigation was conducted to compare the growth of *T. versicolor* and production of extracellular enzymes in both carbon-amended wastewater and a defined nutrient medium. Erlenmeyer flask reactors (150 ml)

were assembled aseptically, sealed, and vented through sterile barriers to allow for axenic operation following fungal inoculation. Abiotic and heat-killed controls were prepared in duplicate, while treatments containing live fungus were prepared in triplicate. Humidified, filtered (0.2 μm) lab air was delivered to each reactor at a rate of 0.7 vvm through an 18G needle (BD, Franklin Lakes, NJ, USA). In order to promote attached growth of fungal mycelia, each reactor contained Tri-Packs[®] (1" diameter; Jaeger Products Inc., Houston, TX, USA) in a ratio of 1 piece per 15 mL of reactor solution, yielding a submerged surface area of approximately 0.16 m² l⁻¹.

Pre-assembled reactors containing either nutrient media or carbon amended wastewater were autoclaved (15 min, 121 °C, 14 psi). After cooling, fungal inoculum was added to bring the total volume to 135 ml and the dry mass of fungus to 0.02% (w/v). In a subset of reactors, additional sterile nutrient media or sterile carbon amended wastewater was substituted for the fungal inoculum; these reactors served as abiotic controls. All reactors were incubated at room temperature (25 $\pm$ 2°C) for 8 days, at which time a subset of the inoculated reactors was inactivated at a relatively low temperature (60°C, 24 hrs.) in order to serve as heat-killed controls (van der Kooij et al. 1982; Logan et al. 1994). Sodium azide (NaN₃, 10 mM final concentration; Alfa Aesar, Ward Hill, MA, USA) was added to heat-killed controls and abiotic controls to ensure the complete absence of extracellular enzyme activity (Marco-Urrea et al. 2010). All eight day old reactors were spiked with a stock solution of E2 in DMF (10 mg l⁻¹ final E2 concentration) to determine its effect on the growth and viability of fungal mycelia. In all reactors, the final concentration of DMF was 0.5% (v/v) (Cajthaml et al. 2009).

Flask reactors were sacrificially sampled at designated time points over a 30 day period in order to quantify dry biomass, enzyme activity, and total carbohydrates. E2 concentration in the reactors was tracked for a period of 72 hours after spiking. At each sampling event, air flow was shut off and reactor solution was withdrawn carefully to avoid disturbing attached fungal growth and filtered through a 0.3 μm glass fiber filter (GF75; Advantec MFS, Inc., Dublin, CA) into appropriate sample containers for the intended analyses. For reactors with living fungus, sample vials contained enough NaN₃ to provide a final concentration of 10 mM, prohibiting any further enzyme activity. Samples intended for enzyme activity analysis were not exposed to NaN₃ after sample collection. Samples for E2 analysis were analyzed immediately via high-performance liquid chromatography with photodiode array detection (HPLC-PDA). All other samples were stored at -20 °C for later analysis.

After sampling was complete, the remainder of the reactor solution was withdrawn and the volume recorded to account for liquid losses due to aeration. Tri-Packs[®] were carefully removed and placed onto a Buchner funnel apparatus fitted with a dried, pre-weighed, qualitative glass fiber filter (Grade 696; VWR, USA). The attached fungus was gently removed from each of the Tri-Packs[®] by spraying with a thin stream of DDI water. The filter paper and fungus were then dried to constant weight at 60 °C to determine the dry mass of fungus per unit surface area of attachment media.

2.4.2 Removal of 17 β -estradiol and associated estrogenic activity from wastewater ('estrogen group')

To more conveniently track estrogen metabolism, estrogenic activity, and enzyme activity for *T. versicolor* grown in carbon amended wastewater, a 1 l media bottle reactor was designed. In these axenic 1 l reactors, the total volume of reactor solution was scaled up by a factor of five (to 675 ml) and a sampling port was integrated into the design to allow for repeated aseptic sampling of a single reactor without significant volume losses. Treatments containing live mycelia were prepared in triplicate. Abiotic and heat-killed controls were prepared in duplicate, as described for the preliminary growth experiment. In addition, a single blank control was prepared which did not contain E2 or fungus. All previously stated set-up procedures and operational parameters hold for these larger reactors, except that carbon-amended wastewater was filtered into each sterile pre-assembled vessel using a sterilizing bottle-top filter (0.2 μm Rapid-Flow[™]; Nalge Nunc Int., Penfield, NY, USA), rather than being autoclaved. Filtered (0.2 μm) lab air was delivered to each reactor at a rate of 0.7 vvm through a submerged stainless steel sparger (10 μm pore size, Upchurch Scientific, West Berlin, NJ, USA). Near the end of the exponential growth phase (8 days), all reactors were spiked to a final concentration of 5 mg l⁻¹ with E2 stock solution (final DMF concentration 0.5% v/v). These reactors are referred to as the 'estrogen group' in subsequent sections of this paper.

In addition to HPLC-PDA determination of E2 concentration, liquid-liquid extraction followed by Gas Chromatography coupled with Time-Of-Flight Mass Spectrometry (GC-TOFMS) was used to allow for detection of low concentrations of E2 metabolites. Sampling procedures were identical to those of the smaller flask reactors, except for the following: 16 ml of reactor solution was sampled at designated time points over a 12 hour period

following E2 addition; samples for estrogen analysis via GC-TOFMS were subjected to liquid-liquid extraction within 24 hours of collection, and extracts were stored at 4 °C until analysis (up to 45 days); additional sample aliquots were stored at -20 °C for later determination of estrogenic activity.

2.4.3 Removal of a mixture of ECs and associated estrogenic activity from wastewater ('mixed group')

A second group of axenic 1 l reactors was prepared to investigate the overall effect of fungal metabolism on a suite of common ECs and associated estrogenic activity in a wastewater matrix. These reactors are referred to as the 'mixed group' in subsequent sections of this paper. Set-up and operation of these 1 l media bottle reactors was identical to that described for the estrogen group except that all eight day old reactors were spiked with a mixture of nine ECs (ATZ, TCS, DEET, CBZ, OBZ, BPA, E2, EE2, E1) to an individual final concentration of 350 µg l⁻¹ (final DMF concentration 0.5% v/v) and ECs and their metabolites were quantified using only GC-TOFMS (not HPLC-PDA). While typical EC concentrations in wastewater are in the range of ng/L, repeated sampling of the reactors required that small aqueous samples be taken, which limited the concentration factor that could be achieved by liquid-liquid extraction. A final spiked concentration of 350 µg/L was selected in order to allow for the quantification of up to 99% removal given these constraints. Sampling procedures were identical to those described for the estrogen group.

2.5 Analytical methods

2.5.1 Enzyme assays

The activities of lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) were measured spectrophotometrically using modified versions of the methods of Tien and Kirk (1984), Paszczyński, et. al. (1988), and Blánquez and Guieysse (2008), respectively. Each enzyme assay was performed in a 1.5 ml cuvette with a total reaction mixture volume of 1 ml. DDI water was used to make up the balance of the reaction mixture where necessary. For LiP, 100 µl of 0.2 M sodium tartrate buffer (pH 3.0), 50 µl of 20 mM veratryl alcohol, 100 µl of 4 mM H₂O₂, and up to 750 µl of sample were used, depending on the activity of the sample. For MnP, 100 µl of 0.5 M sodium tartrate buffer (pH 4.5), 50 µl of 20 mM 2,6-dimethoxyphenol, 50 µl of 20 mM MnSO₄, 100 µl of 4 mM H₂O₂, and up to 700 µl of sample were used. The reaction mixture for Lac was the same as that for MnP; however, the MnSO₄ and H₂O₂ were omitted and up to 850 µl of sample was used. Absorbance measurements at 310 nm (LiP) and 468 nm (MnP and Lac) were recorded for 2 min and converted to units per liter of enzyme activity, where one unit (U) is equivalent to the transformation of one µmol of substrate per min. The extinction coefficient for 2,6 dimethoxyphenol is 10,000 M⁻¹ cm⁻¹ and the extinction coefficient for veratryl alcohol is 9,256 M⁻¹ cm⁻¹. Corrected MnP activity was calculated as the MnP activity measured above minus the activity of Lac, as measured on a separate aliquot of the same sample.

2.5.2 Liquid-liquid extraction

Extractions were performed on samples to be run via GC-TOFMS. Deuterium labeled surrogate compounds (CBZ-d10 and E1-d4) were added to 5 ml of aqueous phase sample for final concentrations of 200 pg µl⁻¹ (assuming 100% recovery) prior to being extracted by a 3x5 ml addition of dichloromethane (DCM) under acidic conditions (pH ≈ 2). This allowed for calculation of extraction recovery based on compounds that are chemically equivalent to the targets. Each solvent addition was shaken for 2 min, and then the DCM was transferred to a clean vial, prior to the addition of the next 5 mL aliquot of solvent. The three extracts were combined and the volume reduced to ~1-2 mL using nitrogen blow-down. The extract was then dried using an in-line granular sodium sulfate drying cartridge with 0.45 µm filtration using disposable PTFE filters (Whatman Autovial; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The final extract volume was adjusted to 1.0 ml with additional nitrogen blow-down. The extraction method was qualified prior to this study with spike-recovery studies wherein target analytes were added at a final extract concentration of 10 ng µl⁻¹. Results from the spike study demonstrate acceptable recovery for the analytes of interest in this work (66-106%).

2.5.3 Quantification of emerging contaminants

Aqueous samples from the preliminary growth experiment (150 ml Erlenmeyer flask reactors) and the estrogen group (1 l reactors) were analyzed via an HPLC (UFLC; Shimadzu Scientific Equipment, Columbia, MD, USA)

fitted with an Ultra II Biphenyl reverse phase HPLC column (250 mm x 4.6 mm I.D. x 5 μ m; Restek Corp., Bellefonte, PA, USA) and a photodiode array detector (Shimadzu Scientific Equipment, Columbia, MD, USA). Samples and external calibration standards were injected at a volume of 100 μ l into an isocratic mobile phase consisting of 50% acetonitrile and 50% DDI water with a flow rate of 1 ml min⁻¹ through the column which was maintained at 40°C. The instrument detection limit for E2 was 0.1 mg l⁻¹.

All sample extracts were analyzed using a Pegasus 4D GC $\times$ GC-TOFMS instrument (LECO Corp., St. Joseph, MI) operated in one-dimensional (GC-TOFMS) mode. The gas chromatograph was a 7890A GC system with a 7683B Series injector (Agilent Technologies, DE, USA). The column ensemble consisted of an Rxi[®]-5Sil MS 60 m $\times$ 0.25 mm I.D. $\times$ 0.25 μ m df (Restek Corp., Bellefonte, PA, USA) coupled to a Rtx[®]-200 1.3 m $\times$ 0.25 mm ID $\times$ 0.25 μ m df (Restek Corp., Bellefonte, PA, USA), which was located inside the second dimension oven. The columns were connected using a capillary flow technology (CFT) union (Agilent Technologies, DE, USA) and UltiMetal Plus flexible metal ferrules (FMF) (Agilent Technologies, DE, USA). Helium was used as the carrier gas at a constant flow rate of 1 ml min⁻¹. The injection volume was 1 μ l of the final extract in DCM.

The final extracts were injected using a standard split/splitless injector held at 250 °C in splitless mode with a 1.5 min hold time, and equipped with a single taper gooseneck liner with wool (Restek Corp., Bellefonte, PA, USA). The primary oven was programmed at 40 °C for 2 min, 15 °C min⁻¹ to 325 °C with a final hold time of 10 min. The secondary oven offset was 5 °C, positive, and followed the same oven program ramp as in the primary oven. The modulator offset was 15 °C, positive to the oven temperature program. The MS transfer line temperature was 240 °C, constant. Ionization was with electron ionization (EI) energy of 70 eV and an ion source temperature of 250 °C. The collected mass range was 50-550 amu with an acquisition rate of 20 scans s⁻¹ after a solvent delay of 700 s. The detector voltage was set as the voltage needed to yield a S/N of approximately 1000 for the 284 ion of hexachlorobenzene injected on column at 200 pg prior to the analysis of any samples. Data was acquired using a mass defect of +20 mu per 100 u.

All data were processed using ChromaTOF software (LECO Corp., St. Joseph, MI, USA), version 4.51. Signal to noise minimum was set to 100, and the full chromatogram was processed over the complete mass range following the solvent delay. Positive identification of all compounds was based on the analysis of reference materials prior to the analysis of sample extracts. Mass spectral identification was also used for confirmation, and was based on both the spectra of the reference materials and also on the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) mass spectral library, version 2011.

Quantification was based on internal standards, using average relative response factors predetermined from the calibration curves for each of the target compounds. The internal standards used were deuterium-labeled polycyclic aromatic hydrocarbon compounds (cat # 31206, Restek Corp., Bellefonte, PA, USA), added to all sample extracts at a final concentration of 200 pg μ l⁻¹. Instrument detection limits for the target compounds ranged from 0.6-30.5 μ g l⁻¹ for the various compounds as determined in prior experiments.

2.5.4 Yeast Estrogen Screen (YES) assay

Prior to measuring the estrogenic activity of aqueous samples, NaAz was deactivated by reaction with nitrous acid in order to prevent inhibition of biological response during the YES assay. Sodium nitrite was added to each sample in excess (at least 1.5 g NaNO₂ for every g NaAz), and the pH was adjusted to ~3.0 using 1.8 M sulfuric acid. After 45 min, 1.7 M NaOH was added to neutralize pH. The estrogenic activity was then measured using a modified version of the YES assay. The assay was carried out in 96 well plates (1 ml well volume; VWR, West Chester, PA, USA). On each plate, an E2 dose-response standard and up to three aqueous samples were serially diluted in duplicate along rows. The expected estrogenic activity of the samples was considered when determining the concentration of the dose-response standard (0.5 to 10 mg l⁻¹) and the dilution factor (3 to 5) used for each plate. Plates containing 100 μ l per well of aqueous (0.5% DMF) sample dilutions, standard dilutions, and blanks, were incubated with genetically modified yeast following the methods of Chen et al. (2006) to produce a colorimetric response proportional to the estrogenic activity in each well. Measured optical densities yielded dose-response regression curves for samples and standards, which were further analyzed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) in order to determine the estrogenic potency of samples in terms of 17 β -estradiol equivalents (EEQ).

2.5.6 Carbohydrate quantification

Total carbohydrate was quantified spectrophotometrically using the phenol-sulfuric acid method (DuBois et al. 1956).

3 Results

3.1 Preliminary growth experiment

During the eight day growth phase, fungal dry mass increased from 1.2 g m⁻² in all freshly inoculated reactors, to approximately 3.2 g m⁻² in the reactors containing nutrient media and 3.8 g m⁻² in the reactors containing wastewater (Figure 1). *T. versicolor* continued to grow until at least day 22, reaching a maximum dry mass concentration of 5.14 g m⁻². In contrast, fungus grown in nutrient media reached its peak biomass concentration after approximately 12 days (3.47 g m⁻²) and then began to decline. The measured biomass decay in nutrient media cultures was corroborated by visual evidence, including browning of mycelia and apparent detachment from Tri-Packs®. Wastewater cultures maintained a healthy appearance for the duration of the experiment.

In nutrient media cultures, total carbohydrate was consumed at a constant rate until it was nearly depleted (< 5% remaining) after approximately 12 days. In wastewater cultures the amount of residual carbohydrate remained above 5% for at least 22 days. The biomass yield normalized to carbohydrate consumption was also different in the two cultures. After 8 days, fungus growing in nutrient media had added 11.6 mg of biomass (dry weight basis) per gram of carbohydrate consumed, while fungus growing in wastewater had added 15.5 mg g⁻¹. After 22 days, the biomass yield in wastewater cultures remained high (13.9 mg g⁻¹).

The profiles of lignin modifying enzymes produced by *T. versicolor* were different for nutrient media and wastewater cultures (Table 1). The maximum observed enzyme activity was for Lac in the nutrient media culture (25.7 U l⁻¹). LiP activity was observed in wastewater cultures at a single time point, but not in any defined nutrient media cultures. MnP activities were similar in both nutrient media and wastewater cultures (~ 1 U l⁻¹). Live fungus grown in wastewater and nutrient media removed a majority of the E2 after 72 hours (>99% removal vs. 93.1% removal, respectively), while E2 removal occurred to a lesser extent in controls (maximum removal of approximately 65%, Table 1).

3.2 Estrogen group

After only 5 hours of exposure, active cultures of *T. versicolor* were able to remove 5 mg l⁻¹ of E2 to below detection, while 81.3% and 74.8% of E2 remained in abiotic and heat-killed controls, respectively (Figure 2). Twelve hours after the addition of E2, concentrations remained relatively high in both abiotic and heat-killed controls (78% and 58% remaining, respectively). GC-TOFMS analysis confirmed the removal of E2 and revealed that as E2 was removed from solution, E1 was formed (Figure 3). E1 was not added to any of the estrogen group reactors and was detected only in reactors with living fungus. E1 produced via degradation of E2 was also removed (> 99%) within five hours. A potential metabolite of E1, exhibiting mass spectrometry consistent with estradiol, 1,3,5(10),9(11)-tetraene-3,17β-diol was observed, with a reverse match probability as high as 860. The exact location of the double-bond on this compound is unknown, and would require further detailed investigation for confirmation. The removal of estrogenic activity closely tracked the removal of these estrogen compounds. After five hours, when E2 was below the level of detection for HPLC-PDA analysis, the removal of estrogenic activity was 96% in active cultures, but only 23% and 36% for heat-killed and abiotic controls, respectively. Twelve hours after the addition of E2, greater than 99% of the estrogenic activity had been removed in active cultures, and removal in controls remained low (43% and 20% for heat-killed and abiotic controls, respectively). The production of extracellular enzymes was modest here, as in the preliminary experiment, with less than 1 U l⁻¹ of MnP and Lac detected in all reactors at every time point and less than 1 U l⁻¹ of LiP detected in only one reactor at a single time point (Table 2).

3.3 Mixed group

Active cultures of *T. versicolor* were able to decrease the concentration of four of the nine contaminants in the EC mixture within 3.5 hours. The ability of *T. versicolor* to transform these ECs varied by compound, exhibiting the

following trend: E2 > E1 > EE2 > BPA (Figure 4; Table 2). None of the other ECs were removed to a significant degree when compared to controls. At 3.5 hours, the removal of BPA, EE2, E1, and E2 by living fungus corresponded with a 77% reduction of estrogenic activity, while only 8% of activity had been removed in controls. After 12 hours, the total removal of estrogenic activity in reactors with living fungus was >98% and removals in heat-killed and abiotic controls were only 42% and 24%, respectively. Extracellular enzyme activities were again low (less than 1 U l⁻¹ in all reactors) (Table 2).

4 Discussion

4.1 Preliminary experiment

The wastewater matrix itself did not appear to inhibit the growth of *T. versicolor*, indicating that it is feasible for WRF to grow axenically in secondary WWTP effluent containing supplemental carbon, but with no additional trace nutrients. In fact, the increased yield of dry biomass per gram of carbohydrate consumed suggests that the fungus grew more efficiently in wastewater, converting more of the carbohydrate to cell biomass. This could be due to the presence of more diverse trace nutrients or additional growth factors in the wastewater as compared to the nutrient media. The addition of E2 did not appear to inhibit the growth of fungus in either medium. The onset of cell decay in nutrient media was clearly correlated with the depletion of carbohydrates in those cultures.

Lac was the dominant extracellular enzyme produced by fungus grown in nutrient media, though the measured activity was low when compared to published studies investigating *T. versicolor* grown in identical nutrient media. For example, a series of investigations by Blázquez and others yielded Lac activities ranging from 37-2028 U l⁻¹ under batch and continuous-flow culture conditions (Blázquez and Guieysse, 2008; Blázquez et al., 2004, 2006). In the present study, Lac activity in nutrient media cultures of *T. versicolor* approached the lower limit of these reported values (maximum 25.7 U l⁻¹). Due to similarities in the culture conditions, it is likely that the lower levels of Lac activity in the present study are due to differences between the fungal strain used in this study (NRRL 66313) and that used in previously cited studies by Blázquez and others (ATCC# 42530).

In comparison to nutrient media cultures, wastewater cultures exhibited suppressed Lac activity. Additionally, LiP activity was detected for fungus grown in wastewater, but not nutrient media, suggesting that the wastewater matrix may have induced the production of LiP, potentially due to the presence of inducing substrates, or differences in the nutrient content. A number of factors, including the concentration and source of bioavailable nitrogen and carbon, the presence of metal ions, and the presence of xenobiotics are known to affect the regulation of extracellular enzymes in WRF (Janusz et al. 2013). However, there has been little focus in the literature on specific mechanisms regulating the production of LiP by *T. versicolor*. Further investigation is required to determine the cause of Lac suppression and whether the domestic wastewater matrix is capable of inducing LiP production by *T. versicolor*.

4.2 Estrogen group

The removal of E2 by living fungus was rapid and corresponded with a reduction of the overall estrogenic activity, indicating that fungal metabolism directly resulted in a decrease in the estrogenic potency of the parent compounds. The partial removal (43%) of E2 in heat-killed controls may be explained in part by passive uptake into dead mycelia. It is also possible that some residual intracellular enzyme activity existed within the heat-killed mycelia; however, extracellular enzyme activity was confirmed to be eliminated by the addition of NaAz to the heat-killed reactors prior to the addition of E2. Blázquez and Guieysse (2008) reported similar results for E2 removal by active cultures grown in nutrient media (i.e. > 97% removal within 24 h) and heat-killed fungal biomass (approximately 40% removal).

E1 and its potential metabolite, estra-1,3,5(10),9(11)-tetraene-3,17β-diol, were not added to any reactor in the estrogen group and were found only in reactors containing living fungus, strongly suggesting that these compounds are byproducts of fungal metabolism. The transformation of E2 into E1, as well as dimers and trimers of E2, by commercially available Lac has been previously described in the literature (Nicotra et al. 2004; Lloret et al. 2013). However, rapid E2 removal in the presence of only modest Lac activity (< 1 U l⁻¹) in the estrogen group suggests the possible involvement of other removal mechanisms. For instance, some studies have found that CYP450s (a family of intracellular enzymes) from other species of WRF can contribute to the transformation of estrogens, such as EE2, in whole-cell cultures (Křesinová et al. 2012). Additional experiments using intracellular enzyme-inactivated

cultures would aid in determining the role, if any, of intracellular enzymes in the conversion of E2 by whole-cell cultures of *T. versicolor* NRRL 66313 grown in wastewater.

4.3 Mixed group

The removal of E1, E2, EE2, and BPA corresponded with the removal of estrogenic activity, suggesting that these four chemicals contribute significantly to the total estrogenic activity of the mixture, as expected, and that fungal metabolites had less estrogenic activity than the parent compounds. The high removals of E1, E2, EE2, and BPA observed here are consistent with existing literature, which shows that emerging contaminants with strong electron donating groups (such as the -OH present in these compounds) are more susceptible to electrophilic attack, and are therefore more efficiently removed by WRF (Yang et al. 2013). Recently, the whole-cell biocatalytic potential of *T. versicolor* to degrade a mixture of ECs (50 µg l⁻¹, each) in nutrient media over a 24 h incubation period was investigated (Nguyen et al. 2014). It was found that CBZ and ATZ were poorly removed, while BPA, EE2, E2, and E1 were effectively removed (more than 60%), as observed in the present study. Others have also observed the efficient removal of high concentrations of BPA (50 mg l⁻¹) as well as EE2 and E2 (10 mg l⁻¹) by whole-cell cultures of *T. versicolor* grown in nutrient media (Shin et al. 2007; Blázquez and Guieysse 2008).

In contrast to the present study and the results of Nguyen et al. (2014), CBZ has been found by other researchers to be removed by whole-cell cultures of *T. versicolor* grown in nutrient media (Marco-Urrea et al. 2009; Tran et al. 2010; Jelić et al. 2012). Also in contrast to the present study, Nguyen et al. (2014) found that TCS and OBZ were effectively removed by whole-cell cultures of *T. versicolor*, and others have published similar findings (Cajthaml et al. 2009; Gago-Ferrero et al. 2012; Badia-Fabregat et al. 2014). Though few studies have investigated fungal degradation of DEET, Seo et al. (2005) found that the soil fungi *Cunninghamella elegans* and *Mucor ramannianus* were able to transform DEET, while Lac from *T. versicolor* has been shown to be ineffective (Tran et al. 2013). Likewise, DEET was not significantly transformed in the present study.

Cajthaml et al. (2009) investigated the removal of TCS (2 mg l⁻¹), EE2 (10 mg l⁻¹), and BPA (10 mg l⁻¹) spiked into separate cultures of *T. versicolor* grown in nutrient media and found relative removal rates after 3 days of batch incubation (TCS > BPA > EE2) that were different than were found in the present study after 3.5 hours of batch incubation with a mixture of contaminants (EE2 > BPA > TCS). The discrepancies between findings in the literature and in the present study may be due to differences in incubation period, contaminant concentration, enzymatic cofactors, or inhibitory compounds present in the wastewater matrix, or differences in the nutrient content of the wastewater matrix as compared to the culture media used in other studies.

The removal of individual ECs in abiotic controls varied, roughly corresponding to the octanol-water partitioning coefficients (K_{ow}) of the respective compounds (which ranged from 2.5 to 4.8). For example, the highest removal in abiotic controls occurred with TCS (K_{ow} = 4.8) and OBZ (K_{ow} = 3.8), which are among the highest K_{ow} values of the nine contaminants examined here. However, E2 and EE2, which also have high K_{ow} values (4.0 and 3.7, respectively) were not removed in abiotic controls. In most cases, the 3.5 hour mean removals in heat-killed controls were not significantly different than those in abiotic controls, indicating that sorption to heat-killed fungal mycelia, passive uptake into mycelia, and degradation in the presence of non-living fungal biomass played a minor role in removal of these compounds. In the case of TCS, it appears that the presence of living fungus may have reduced the degree of removal by sorption as compared to the abiotic and heat-killed controls. One possible explanation is that compounds produced by the living fungus contributed to matrix effects during EC quantification via GC-TOFMS. Further investigation is required in order to better understand this result.

The production of extracellular enzymes in the mixed group was once again modest (< 1 U l⁻¹) when compared to the typical values presented above. Again, it seems that the levels of enzyme activity observed throughout the present study are not likely to be sufficient to catalyze the rapid removal of ECs from solution and additional mechanisms, including intracellular enzymes, should be further examined.

4.4 Conclusions

A previously untested strain of *T. versicolor* (NRRL 66313) grew comparably well and produced modest amounts of extracellular enzymes in both nutrient media and carbon-amended secondary WWTP effluent. When grown in carbon-amended secondary WWTP effluent spiked with a complex mixture of common ECs, the overall estrogenic

activity of the mixture was rapidly reduced, corresponding with the removal of estrogen compounds and BPA. While the fungus could not remove every contaminant in the mixture, it preferentially removed those that are typically significant contributors to the overall estrogenic activity of domestic wastewaters. This study demonstrates the bioremediation potential of *T. versicolor* NRRL 66313 in a wastewater effluent matrix without the need for trace nutrient supplementation.

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Table 1 Summary of the maximum observed attached growth, enzyme activity, and 17 β -estradiol removal from the preliminary growth experiment with *T. versicolor* in 150 ml batch reactors containing Tri-Packs[®]. Attached growth and enzyme activity were measured over a period of 30 days following inoculation. The time, in days, at which maximum values were observed are noted in parentheses. 17 β -estradiol was added 8 days after inoculation and its aqueous concentration was monitored for a period of 72 hours.

Endpoint	Wastewater	Wastewater Controls ^a	Nutrient Media	Nutrient Media Controls ^a
Attached Growth (g _{dry mass} m ⁻²)	5.14 (22.9 d)	0	3.47 (12.7 d)	0
Enzymes (U l ⁻¹)				
Laccase	1.24 (5.0 d)	0	25.7 (29.9 d)	0
Lignin peroxidase	2.65 (7.9 d)	0	0	0
Manganese peroxidase	0.80 (19.8 d)	0	1.66 (12.7 d)	0
17 β -estradiol (% removal after 72 h)	> 99.0	64.1	93.1	63.1

a - Values are maximum values for all controls (abiotic and heat-killed) within a specified group

Table 2 Summary of attached growth, enzyme activity, emerging contaminants removal, and estrogenic activity removal from ‘estrogen group’ and ‘mixed group’ experiments with *T. versicolor* grown in 1 L batch reactors containing carbon-amended wastewater and Tri-packs®. Enzyme activities and contaminant removal were measured during a 12 hour contaminant removal phase which began following an 8 day growth phase. Attached growth was measured at the conclusion of the experiment.

Endpoint	Estrogen Group	Estrogen Group Controls ^a	Mixed Group	Mixed Group Controls ^a
Attached Growth (g _{dry mass} m ⁻²)	3.8	0	4.4	0
Enzymes (U l ⁻¹)				
Laccase	0.23 - 0.51	0	0.45 - 0.52	0
Lignin peroxidase	0 - 0.18	0	0	0
Manganese peroxidase	0.02 - 0.23	0	0 - 0.10	0
Contaminants (% Removal, 3.5 hrs)				
17β-estradiol	>99 ^b	25.2 ^b	>99	9.5
Estrone	--	--	83.5	19.8
17α-ethynylestradiol	--	--	71.3	28.4
Bisphenol-A	--	--	61.9	14.2
Estrogenic Activity (% Removal, 12 hrs)	>99	43	>98	42

a - Values are maximum values for all controls (abiotic and heat-killed) within a specified group

b - > 99% removal (25.2% in heat killed controls) occurred after 5 h in the estrogen group

Figure 1. Comparison of carbohydrate consumption (a) and growth (b) of triplicate batch cultures of the white rot fungus *Trametes versicolor* grown in carbon-amended sterile secondary wastewater treatment plant effluent amended with 5 g L⁻¹ glucose but no other nutrients (WW), and defined nutrient media with an equivalent amount of glucose as well as added trace minerals (NM).

Figure 2. Removal of 17 β -estradiol (E2) from triplicate 1 L media bottle reactors containing living fungus ('Live') and duplicate reactors containing heat-killed fungus ('Dead') or no fungus ('Abiotic'). Reactors were spiked with E2 to yield a theoretical starting concentration of 5 mg l⁻¹. Error bars represent one standard deviation from the mean.

Figure 3. Formation of estrone (E1) as 17 β -estradiol (E2) is removed from 1 L reactors containing living fungus. Concentrations are given as the concentration in the final extract following liquid-liquid extraction. Error bars represent one standard deviation of triplicate samples.

Figure 4. Percentage remaining, after 3.5 hours, of each of nine ECs and their overall combined estrogenic activity (EEQ). ECs were spiked as a mixture (350 μ g L⁻¹ each) into triplicate reactors containing living fungus ('Live'), as well as into duplicate abiotic and heat-killed ('Dead') controls. Error bars represent one standard deviation. Abbreviations are defined as follows: triclosan (TCS), N,N-diethyl-3-methylbenzamide (DEET), atrazine (ATZ), carbamazepine (CBZ), oxybenzone (OBZ), bisphenol A (BPA), 17 α -ethynylestradiol (EE2), estrone (E1), and 17 β -estradiol (E2).

Figure 1a

Figure 1b

Figure 2

Figure 3

Figure 4