Appl Microbiol Biotechnol (2016) 100:2381–2399
DOI 10.1007/s00253-015-7113-0

ENVIRONMENTAL BIOTECHNOLOGY

Biochemical and physicochemical processes contributing to the removal of endocrine-disrupting chemicals and pharmaceuticals by the aquatic ascomycete *Phoma* sp. UHH 5-1-03

Ulrike Hofmann[1] · **Dietmar Schlosser**[1]

Received: 31 July 2015 / Revised: 7 October 2015 / Accepted: 16 October 2015 / Published online: 5 November 2015

Abstract The environmentally widespread micropollutants bisphenol A (BPA), carbamazepine (CBZ), 17α-ethinylestradiol (EE2), diclofenac (DF), sulfamethoxazole (SMX), technical nonylphenol (t-NP) and triclosan (TCS) were used to assess the potential of the laccase-producing freshwater ascomycete *Phoma* sp. strain UHH 5-1-03 for micropollutant removal and to provide quantitative insights into the mechanisms involved. Biotransformation rates observed with whole fungal cells followed the rank order EE2 ≫ BPA > TCS > t-NP > DF > SMX > CBZ. Biosorption onto fungal mycelia was prominent for BPA, EE2, TCS and t-NP and insignificant for CBZ, DF and SMX. Enzymatic removal rates investigated with cell-free, laccase-containing culture supernatants of *Phoma* sp. followed the rank order EE2 > BPA > DF > t-NP > TCS and were insignificant for SMX and CBZ. Mass spectrometry-assisted investigations addressing metabolite formation from unlabelled and $^{13}C_6$-labelled DF and SMX yielded DF metabolites indicating hydroxylation, cyclisation and decarboxylation reactions, as well as oxidative coupling typical for laccase reactions. For SMX, several products characterised by lower molecular masses than the parent compound were found, and indications for deamination and formamide formation were obtained. Summarising, the obtained results suggest that the extracellular laccase of *Phoma* sp. largely contributes to fungal biotransformation of EE2, BPA, DF, TCS and t-NP, together with cell-associated enzymes such as, e.g. cytochrome P450 monooxygenases suggested by the appearance of hydroxylated metabolites from DF. Laccase does not seem to play any role in the metabolisation of SMX and CBZ, where yet to be identified cell-associated enzymes have to be considered instead.

Keywords Aquatic fungi · Biosorption · Biotransformation · Laccase · Metabolite formation · Micropollutants

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-7113-0) contains supplementary material, which is available to authorized users.

✉ Dietmar Schlosser
dietmar.schlosser@ufz.de

[1] Department of Environmental Microbiology, Helmholtz Centre for Environmental Research - UFZ, Permoserstrasse 15, 04318 Leipzig, Germany

Introduction

Micropollutants such as pharmaceuticals and personal care products (PPCPs) as well as various industrials, sometimes concomitantly representing endocrine-disrupting chemicals (EDCs), are continually released into the aquatic environment through different anthropogenic sources (Grassi et al. 2013; Murray et al. 2010). These compounds are commonly present in waters at trace concentrations, ranging from a few nanograms to several micrograms per litre (Luo et al. 2014). Despite such minute concentrations, they are suspected to have a long-term detrimental impact on aquatic life and human health (Yang et al. 2013b). Wastewater treatment plant effluents represent major sources of surface water contamination with micropollutants, because of the only limited ability of conventional activated sludge processes to remediate the more persistent representatives of this group of environmental contaminants (Castiglioni et al. 2005, 2006; Jackson and Sutton 2008). Activated sludge processes have been reported to remove sufficiently hydrophobic and/or easily biodegradable micropollutants such as ibuprofen and bisphenol A quite well. However, an only poor removal of semi-polar and biologically persistent micropollutants such as carbamazepine and

diclofenac has been recorded (Suárez et al. 2008). Some metabolites of micropollutants are still bioactive or even more toxic than their respective parent compound, and they may have a high stability and mobility in the aquatic environment (Delgado et al. 2011; Kümmerer 2011; van Leeuwen et al. 2011). Therefore, addressing both metabolite formation and the potentially related endocrine-disrupting and ecotoxicological effects are important components of assessing the environmental and human health risks associated with the release of micropollutants.

Lignin-degrading white-rot basidiomycetes have gained much attention with respect to their potential applicability for the biological removal of micropollutants, due to the ability to efficiently degrade a wide range of organic compounds also including rather 'classical' mass pollutants like aminonitrotoluenes, azo dyes, chlorophenols, polycyclic aromatic hydrocarbons, and others with the help of extracellular lignin-modifying enzymes such as lignin peroxidases, manganese peroxidases, versatile peroxidases and laccases (Harms et al. 2011). The removal of micropollutants by lignin-modifying exoenzymes from ligninolytic fungi has also widely been described (Cabana et al. 2007; Kim and Nicell 2006; Spina et al. 2015; Yang et al. 2013a, b; Zhang and Geissen 2010). Exoenzyme catalysis further can be enhanced in the presence of low-molecular-weight redox mediators, which act as electron shuttles between enzymes and target compounds (Hata et al. 2010b; Murugesan et al. 2010). Nevertheless, intracellular fungal enzyme systems like cytochrome P450 monooxygenases also contribute to the biotransformation of micropollutants, as has been demonstrated for, e.g. pharmaceuticals like diclofenac and carbamazepine (Golan-Rozen et al. 2011; Hata et al. 2010a). In fact, it has been demonstrated that a wider range of micropollutants can be removed by whole-cell fungal treatment compared to enzymatic (i.e. cell-free) treatment, due to combined effects of extracellular, mycelium-bound and intracellular enzymes and of sorption of contaminants to fungal biomass (Nguyen et al. 2014). Notwithstanding the above, in-depth mechanistic investigations addressing the respective contribution of extracellular and cell-associated biocatalytic processes, as well as biosorption to fungal micropollutant removal, are scarce and limited to a few examples (Nguyen et al. 2014; Tran et al. 2010; Yang et al. 2013b). Moreover, most reports addressing the fungal degradation and biotransformation of micropollutants are dealing with ligninolytic (white-rot) fungi and EDCs, with a reduction of the endocrine-disrupting activity or ecotoxicity of such compounds sometimes also being observed (Cajthaml 2014; Spina et al. 2015). On the contrary, clearly fewer studies focus on the fungal metabolisation and removal of pharmaceutical compounds other than EDCs (Hata et al. 2010a; Kang et al. 2008). Last but not least, knowledge about the potential to attack micropollutants of fungal groups other than terrestrial white-rot basidiomycetes is still limited. In this context, fungi dwelling in and hence being adapted to aquatic habitats may offer new biotechnological perspectives in the removal of micropollutants from wastewater (Junghanns et al. 2005, 2008a; Martin et al. 2007).

The aim of the present study was to (i) assess the potential of a laccase-producing freshwater ascomycete (*Phoma* sp. strain UHH 5-1-03; Junghanns et al. 2008a, 2009) for micropollutant removal, using a representative range of micropollutants covering members of the PPCPs, industrials and EDCs, (ii) provide quantitative insights into the biocatalytic and physicochemical mechanisms involved and (iii) shed light on the metabolites formed from selected micropollutant representatives, thereby also enabling to compare micropollutant metabolism amongst different fungal groups. The PPCPs carbamazepine (CBZ, anti-epileptic), 17α-ethinylestradiol (EE2; contraceptive), diclofenac (DF, anti-inflammatory), sulfamethoxazole (SMX, antibiotic) and triclosan (TCS, antimicrobial) and the industrials bisphenol A (BPA; used in polycarbonate polymer and epoxy resin production) and technical nonylphenol (t-NP, surfactant metabolites) were chosen as target compounds representing prominent and environmentally widely distributed (ubiquitous) micropollutants, with BPA, EE2, t-NP and TCS at the same time exhibiting endocrine activity thus also being referred to as (suspected in case of TCS) EDCs (Cajthaml 2014; Kümmerer 2011; Murray et al. 2010; Witorsch 2014).

Materials and methods

Sources of micropollutants

All chemicals were of analytical grade (gradient grade in the case of chromatography solvents), if not otherwise stated. t-NP (purity 85 %) was purchased from Merck (Darmstadt, Germany), and BPA (purity 95 %) was provided by Fluka (Buchs, Switzerland; now belonging to Sigma-Aldrich, Saint Louis, MO, USA). TCS (purity>99 %) was purchased from LGC Promochem (Wesel, Germany). DF (purity>99 %) was provided by MP Biomedicals (Eschwege, Germany), and SMX (purity>98 %) was obtained from ABCR (Karlsruhe, Germany). EE2 (purity>98 %), CBZ (purity>99 %), and the [^{13}C]-labelled substances diclofenac-(acetophenyl ring-$^{13}C_6$) sodium salt hemi(nonahydrate) and sulfamethoxazole-(phenyl-$^{13}C_6$) were purchased from Sigma-Aldrich (Munich, Germany).

Fungal strain

The isolation, identification and maintenance of *Phoma* sp. strain UHH 5-1-03 (available as *Phoma* sp. DSM 22425 from the German Culture Collection of Microorganisms and Cell

Cultures, Braunschweig, Germany) have previously been described (Junghanns et al. 2008a).

Micropollutant removal experiments using whole cells of *Phoma* sp.

Fungal pre-cultures of *Phoma* sp. were established for subsequent batch tests with live and NaN_3-inactivated fungal cultures. For this, *Phoma* sp. was pre-cultivated in 100-ml Erlenmeyer flasks containing 30 ml of a 2 % (*w*/*v*) malt extract medium (pH 5.7), which was additionally supplemented with 50 μM $CuSO_4$ and 1 mM vanillic acid to stimulate laccase production (Junghanns et al. 2008b, 2009). Each flask was inoculated with 0.5 ml of a mycelial suspension prepared as previously described (Junghanns et al. 2008b). Thereafter, flasks were incubated on a rotary shaker (Infors HT, Infors, Bottmingen, Switzerland) at 14 °C and 120 rpm in the dark. After 8 days of incubation, half of the growing fungal cultures were inactivated with 0.5 g l^{-1} NaN_3. After a further day of incubation, fungal biomass was harvested by sterile filtration over Whatman no. 1 paper filters (GE Healthcare, Freiburg, Germany), washed three times with 30 ml of a synthetic mineral salts medium devoid of a source of carbon and energy (pH 6.8; Stanier et al. 1966) and transferred into new 100-ml Erlenmeyer flasks containing 30 ml of the aforementioned mineral salts medium. For subsequent micropollutant removal experiments, the fungus thus was applied in the form of resting cells. Micropollutants were added from 50 mM stock solutions in methanol containing 10 % (*w*/*v*) Tween 80 in addition in order to improve their aqueous solubility, to yield a final concentration of 250 μM (thus corresponding to 0.5 % (*v*/*v*) methanol and 0.05 % (*w*/*v*) Tween 80; modified from Jahangiri et al. 2014; Junghanns et al. 2005; Libardi Junior et al. 2012). Micropollutant-containing fungal cultures were incubated on a rotary shaker at 70 rpm and 28 °C in the dark. Triplicate experiments were always performed.

The initial (maximal) removal rates of micropollutants were determined from fitting of data of pollutant concentration versus time plots. Exponential regression assuming first-order removal kinetics was applied to active fungal cultures containing BPA, EE2, DF, TCS and t-NP and also to NaN_3-inactivated cultures supplemented with t-NP (controls) (Fig. 1), using the following formula:

$$c_t = c_0 \times e^{-k't} \quad (1)$$

where c_t is the micropollutant concentration at a given time point t (μM), c_0 refers to the initial micropollutant concentration at t=0 (μM), k' represents the apparent first-order decay constant (h^{-1}) and t is the time of incubation in presence of micropollutant (h). The corresponding coefficients of determination (COS) were always >0.96 (>0.93 for t-NP-containing inactive cultures). The initial rates of micropollutant removal were calculated by multiplying the respective c_0 and k' values.

In cases where micropollutants did not completely disappear and their concentrations tended to level off during the experiment as observed for NaN_3-inactivated fungal cultures containing BPA, EE2 and TCS (Fig. 1), Eq. (1) was modified as follows:

$$c_t = c_a + c_s \times e^{-k't} \quad (2)$$

where c_a is a bottom asymptode concentration of micropollutant approached at infinite time (μM), c_s corresponds to a removal rate-governing micropollutant concentration at t=0 (μM) (with the sum of c_a and c_s yielding c_0, i.e. the total initial micropollutant concentration at t=0), k' refers to the apparent first-order decay constant (h^{-1}) and t is the time of incubation in presence of micropollutant (h). The corresponding COD values were >0.82 for BPA, >0.87 for EE2 and >0.98 for TCS. The initial rates of micropollutant removal were calculated by multiplying the respective c_s and k' values.

Linear regression was applied to both active and NaN_3-inactivated fungal cultures supplemented with CBZ and SMX and also to NaN_3-inactivated cultures containing DF (Fig. 1) according to the following linear equation:

$$c_t = c_0 + r' \times t \quad (3)$$

where c_t is the micropollutant concentration at a given time point t (μM), c_0 refers to the initial micropollutant concentration at t=0 (μM), r' represents the apparent rate of micropollutant removal (μM h^{-1}) and t is the time of incubation in the presence of micropollutant (h). The corresponding COD values for variants displaying noticeable micropollutant removal (Table 1) were >0.8 (active fungal cultures supplemented with CBZ) and >0.95 (active cultures containing SMX). Data fitting was always performed using the software OriginPro 8.6.0 Sr2 (OriginLab Corp., Northampton, MA).

Micropollutant removal experiments using laccase-containing crude culture supernatants of *Phoma* sp.

Laccase-containing concentrated crude culture supernatants of *Phoma* sp. served as enzyme source in experiments addressing the enzymatic degradation of micropollutants. *Phoma* sp. was cultivated in 2-l Erlenmeyer flasks containing 500 ml 2 % (*w*/*v*) liquid malt extract medium (pH 5.7) additionally containing 50 μM $CuSO_4$ and 1 mM vanillic acid to stimulate laccase production (Junghanns et al. 2008b, 2009). Each flask was inoculated with 25 ml of a mycelial suspension prepared as previously described (Junghanns et al. 2008b). The flasks were then incubated on a rotary shaker at 120 rpm and 14 °C in the dark. The cultures usually reached their maximum laccase activity after 7 days of incubation, and then mycelia were separated from culture supernatants by filtration

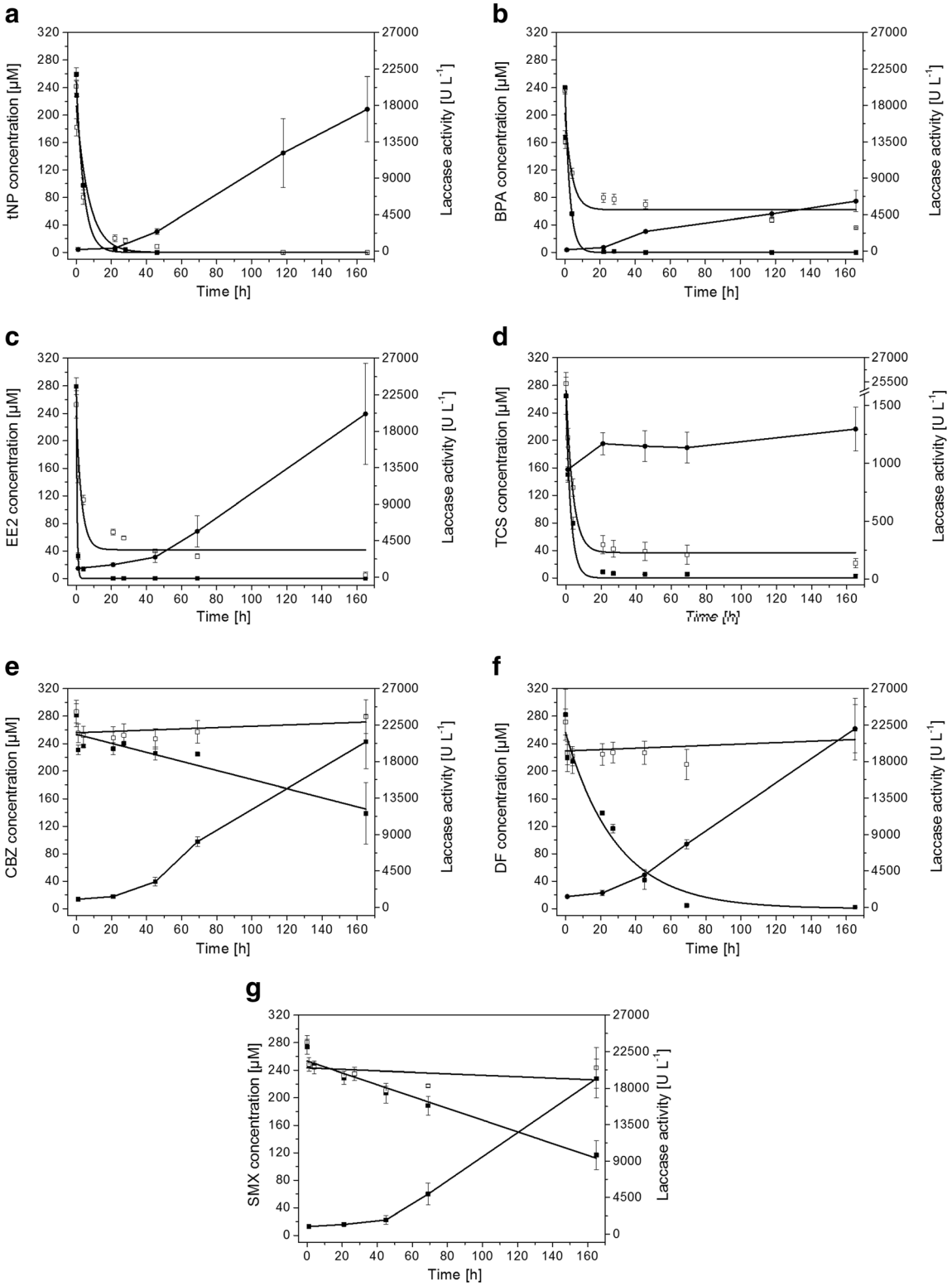


Fig. 1 Time courses of t-NP (**a**), BPA (**b**), EE2 (**c**), TCS (**d**), CBZ (**e**), DF (**f**), and SMX concentrations (**g**) in active (*black square*) and NaN_3-inactivated cultures of *Phoma* sp. (*white square*). The corresponding *solid lines* arise from data fitting of measured micropollutant concentrations (*black square* and *white square*) as described in the 'Materials and methods' section. Extracellular laccase activity is concomitantly shown for active fungal cultures (*black circle*). *Symbols* represent means±standard deviations from triplicate cultures

(Whatman no. 1 filter papers). Cell-free culture supernatants were concentrated by ultrafiltration using a 400-ml stirred cell (Model 8400, Merck Millipore, Billerica, MA, USA) equipped with an Omega polyethersulfone membrane

Table 1 Initial and specific (fungal dry biomass-based) initial removal rates of micropollutants observed with active and NaN_3-inactivated cultures of *Phoma* sp.

Micropollutant	Active fungal cultures		NaN_3-inactivated fungal cultures		Biotransformation
	Initial removal rate [μM h^{-1}]	Specific initial removal rate [μM h^{-1} g^{-1}][a]	Initial removal rate [μM h^{-1}]	Specific initial removal rate [μM h^{-1} g^{-1}][a]	Specific initial removal rate [μM h^{-1} g^{-1}][a]
t-NP	59.14±4.35	6.05±0.71	29.88±4.90	3.38±0.57	2.68±0.91
BPA	86.35±7.62	8.84±1.13	35.81±21.95	4.05±2.48	4.79±2.73
EE2	598.49±49.94	70.33±5.97	63.52±27.91	9.38±4.15	60.95±7.27
TCS	88.56±15.44	10.41±1.82	59.08±9.94	8.73±1.53	1.68±2.38
CBZ	0.66±0.12	0.08±0.01	0±0	0±0	0.08±0.01
DF	8.75±1.30	1.03±0.15	0±0	0±0	1.03±0.15
SMX	0.86±0.07	0.10±0.01	0.10±0.15	0.02±0.02	0.08±0.02

Specific removal rates attributed to fungal biotransformation reactions were calculated as the difference between the specific removal rates obtained from active (representing biotransformation + biosorption) and NaN_3-inactivated fungal cultures (representing biosorption only). Data represent means± standard errors from triplicate cultures (calculated according to Gaussian error propagation rules)

[a] For t-NP and BPA-supplemented cultures, the fungal dry biomass at the time point of pollutant addition was 9.77±0.90 and 8.85±0.33 g l^{-1} (means± standard deviations from triplicate cultures) for active and NaN_3-inactivated cultures, respectively. For all other micropollutants, a fungal dry biomass of 8.51±0.13 and 6.77±0.33 g l^{-1} was applied for active and NaN_3-inactivated cultures, respectively

(10 kDa cutoff, Pall GmbH Life Sciences, Dreieich, Germany).

For enzymatic degradation tests, micropollutants were added at a final pollutant concentration of 250 μM from 50 mM stock solutions (already described for whole-cell experiments before) to 5-ml reaction mixtures additionally comprising crude culture supernatant (corresponding to a laccase activity of 3 U ml^{-1}) and 100 mM citrate-phosphate buffer in 22-ml glass vials (a pH value of 5.0 was chosen to meet conditions previously found to be optimal for the oxidation of common phenolic laccase substrates by the laccase from *Phoma* sp.; Junghanns et al. 2009; Libardi Junior et al. 2012). Controls contained crude culture supernatant that was heat-inactivated by boiling in a water bath for 30 min. Additional samples were incubated to assess the effect of the addition of 500 μM syringaldehyde (SA) as redox mediator (added from a 50 mM stock solution dissolved in methanol). Furthermore, the impact of 500 μM 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), *p*-coumaric acid and SA on the degradation efficiency of 50 μM CBZ was investigated under otherwise identical conditions. In addition, the enzymatic degradability of CBZ (again applied at 50 μM) was assessed in the presence and absence of 500 μM HBT, using 3 U ml^{-1} commercial laccase from *Trametes versicolor* (Sigma-Aldrich) under conditions otherwise identical to those applied for experiments with *Phoma* sp. crude culture supernatants. Enzymatic incubations were carried out on a horizontal shaker at 120 rpm in the dark. An incubation temperature of 22 °C was chosen in order to ensure a sufficient maintenance of the activity of the isolated laccase from *Phoma* sp. over the experimental time period (Junghanns et al. 2009). Triplicate experiments were always performed. As for experiments employing whole fungal cells, the initial (maximal) removal rates of micropollutants were determined via fitting of data of pollutant concentration versus time plots, employing Eq. (1) for reaction mixtures containing active laccase together with BPA, EE2 and DF and Eq. (3) for all other types of enzymatic reaction mixtures. The corresponding COD values for enzymatically active test mixtures showing noticeable micropollutant removal (Table 2) were >0.99 for DF, >0.96 for BPA, >0.93 for EE2, >0.93 for t-NP and >0.86 for TCS.

Analysis of micropollutants by ultra performance liquid chromatography (UPLC)

Aqueous samples (0.5 ml) taken from cell-free supernatants of fungal cultures and enzymatic reaction mixtures at the time points indicated in the text were placed in 1.5-ml Eppendorf tubes, supplemented with 0.5 ml methanol, thoroughly mixed and stored at −20 °C until further use. Before analysis, samples were centrifuged at 14,000 rpm and 4 °C for 15 min (Eppendorf centrifuge 5804R, rotor type 16 F24-11, Eppendorf, Hamburg, Germany). Aliquots (3.3 μl) of the resulting supernatants were directly subjected to an Acquity™ UPLC system (Waters, Eschborn, Germany) comprising of a Binary Solvent Manager (BSM), a Sample Manager (SM), and a PDA eλ photo diode array detector, and equipped with an Acquity™ UPLC BEH C18 column (1.7 μm particle size; 2.1×50 mm; Waters) operated at a column temperature of 40 °C. The following solvents served as mobile phases: solvent A—10 % methanol (gradient grade, Th. Geyer, Renningen, Germany) in deionised water (Q-Gard 2, Millipore, Schwalbach, Germany) and solvent B—100 % methanol; both acidified to pH 3.0 with concentrated

Table 2 Initial removal rates [μM h^{-1}] of micropollutants determined for enzymatic experiments employing active and heat-inactivated laccase-containing culture supernatants of *Phoma* sp.

Micropollutant	Active fungal laccase	Heat-inactivated fungal laccase	Enzymatic removal
t-NP	2.15±0.28	0.34±0.16	1.81±0.28
BPA	67.94±17.29	0±0	67.94±17.29
EE2	76.67±32.18	0.26±0.10	76.41±32.18
TCS	0.58±0.09	0±0	0.58±0.09
CBZ	0±0	0±0	0±0
DF	5.91±1.04	0±0	5.91±1.04
SMX	0.24±0.06	0.02±0.04	0.22±0.07

The enzymatic removal rates were calculated as the difference between the removal rates obtained with active and heat-inactivated enzyme, respectively. Data represent means±standard errors from triplicate experiments (calculated according to Gaussian error propagation rules)

phosphoric acid. The following elution profile was applied: isocratic elution at 20 % B for 0.14 min, linear increase to 100 % B until 2.8 min, isocratic elution at 100 % B until 3.2 min, linear decrease to 20 % B until 3.25 min and isocratic elution at 20 % B until 3.5 min (0.5 ml min^{-1} flow rate). The detection wavelength was set to 278 nm. Calibration of the method was carried out using external standards.

Analyses of biotransformation products

Biotransformation products formed from DF and SMX by whole cells of *Phoma* sp. were analysed by liquid chromatography-mass spectrometry (LC-MS). For this, whole-cell biotransformation experiments were carried out as already described. Non-labelled DF and SMX were applied at 250 μM, respectively, and $^{13}C_6$-labelled DF and SMX were employed at 50 μM, each. NaN_3-inactivated cultures containing the respective pharmaceutical served as controls. Fungal cultures containing DF and SMX were harvested after 48 and 168 h of incubation, respectively. The analysis of biotransformation products was carried out using the crude culture supernatant only, and mycelia were removed by filtration over paper filters (Whatman no. 1) prior to metabolite extraction. Afterwards, the cell-free culture supernatants containing DF and transformation products were acidified to pH 2.8 with sulphuric acid, whilst the culture liquids containing SMX and possible metabolites were adjusted to pH 7.5, and then both were concentrated by solid phase extraction. DF and its metabolites were extracted using Oasis MCX cartridges (3 cc, 60 mg, Waters), preconditioned with 1×2 ml *n*-heptane, 1×2 ml acetone, 3×2 ml methanol and 4×2 ml Millipore water acidified to pH 2.8 with sulphuric acid. Samples containing SMX were extracted using Oasis HLB cartridges (6 cc, 200 mg, Waters). The cartridges were conditioned as described above but with Millipore water at pH 7.5 as final step. After the enrichment, the cartridges were dried under an argon stream for 1 h. Finally, 4×1 ml of acetone was used to elute the adsorbed biotransformation products of DF from the cartridges. The SMX metabolites were eluted from the cartridges using 4×2 ml methanol.

The extracts were evaporated under a gentle stream of nitrogen and redissolved in 1 ml methanol, and 10 μl aliquots were injected into the HPLC-MS system. The apparatus consisted of an 1100 series binary HPLC pump with an 1100 series autosampler (Agilent Technologies, Waldbronn, Germany) connected to an API 2000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Waltham, MA, USA) with electron spray ionisation (ESI). High-performance liquid chromatography (HPLC) separation was performed on a Supelco Ascentis Express C_{18} column (100 mm×3.0 mm, particle diameter 2.7 μm, Sigma-Aldrich) at a flow rate of 0.3 ml min^{-1}. As solvents, water acidified with 0.1 % acetic acid (A) and methanol acidified with 0.1 % acetic acid (B) were used to following gradient: 0 min, 80 % A; 5 min, 50 % A; 3 min, 0 % A (7 min isocratically); and 10 min, 80 % A (5 min isocratically). The ESI source was operated at 400 °C, and the spray capillary voltage was set to 5 kV (ESI pos). The quadrupole scanned from 100 to 700 u.

The identification of metabolites was further supported by analysing the samples with an UPLC-quadrupole time-of-flight (QTOF) mass spectrometer system (see supporting information).

Size exclusion chromatography (SEC) was employed in the degradation experiments with laccase-containing concentrated crude supernatants of fungal cultures described above (final pollutant concentration always 250 μM), to detect degradation products with higher molecular masses than that of the respective parent compound. Samples (1.5 ml) were acidified with 150 μl 1 M HCl and centrifuged at 20,817×*g* and 4 °C for 30 min. The pellets were redissolved in 150 μl 0.1 M NaOH and subjected to SEC. Separations were carried out on a HEMA Bio-40 SEC column (8×300 mm, PSS GmbH, Mainz, Germany), under conditions previously described (Singh et al. 2014). The absorbance was recorded over a wavelength range of 230–600 nm. The molecular masses of degradation products were estimated by comparing the

retention times of degradation products with that of polystyrene sulfonate sodium salt molecular mass standards (PSS GmbH).

Determination of laccase activity

Laccase activity was routinely determined following the oxidation of 2 mM ABTS in 0.1 M citrate-phosphate buffer (pH 4.0) at 420 nm [ε_{420}=36 mM^{-1} cm^{-1}] (Johannes and Majcherczyk 2000). Activities are expressed in international units (U), where 1 U is defined as the amount of laccase capable of oxidising 1 μmol ABTS per minute.

Fungal dry mass determination

Fungal dry masses of the active and NaN_3-inactivated fungal cultures were determined at the time point of micropollutant addition. Fungal biomasses were separated from culture supernatants by filtration through pre-weighed filter papers (Whatman no. 1), washed with 50 ml distilled water, dried at 80 °C for 24 h and the biomass-containing filter papers were weighed again.

Results

Micropollutant removal by fungal cultures

The removal of the seven target pollutants was followed by UPLC analysis of fungal culture supernatants. All compounds were removed by *Phoma* sp. cultures to varying degrees (Fig. 1). In active fungal cultures, concentrations of EE2, BPA, TCS and t-NP had decreased to about 14, 56, 80 and 97 μM already after 4 h of incubation, respectively, corresponding to relative removals (i.e. relative to the actually quantified initial concentration) of approximately 95, 77, 70 and 62 %. Complete removal of the aforementioned pollutants was observed within 24 h. At the same time, a significant reduction of their concentrations (by about 73, 66, 83 and 91 % for EE2, BPA, TCS and t-NP, respectively) was also recorded for sodium azide-inactivated fungal cultures (Fig. 1), indicating pollutant removal by sorption onto the fungal biomass. Whereas t-NP also completely disappeared rapidly in inactive cultures, the concentrations of the other pollutants rather tended to level off during the time course of the experiment under such conditions, suggesting that sorption equilibria were reached (Fig. 1). Extracellular laccase activities, which were concomitantly monitored with pollutant concentrations in fungal culture supernatants, continuously increased in active fungal cultures until the end of cultivation, with the exception of TCS-containing cultures (Fig. 1). Values of about 20,000, nearly 18,000 and close to 7,000 U l^{-1} were finally observed in EE2-, t-NP- and BPA-supplemented cultures, respectively. By contrast, laccase activities in active fungal cultures containing TCS did not substantially increase and remained at a comparatively low level ranging from about 1,000 to 1,250 U l^{-1}.

Compared to the tested EDCs mentioned before, removal of the pharmaceuticals DF, SMX and CBZ was clearly less efficient (Fig. 1). In active fungal cultures, DF had completely disappeared within 3 days, whereas the concentrations of SMX and CBZ had decreased to about 117 and 138 μM at the end of cultivation (corresponding to relative removals of approximately 57 and 51 %). Final SMX and CBZ concentrations in active fungal cultures were significantly lower (P<0.02) than in the corresponding controls according to Student's t tests, which were performed to address the comparatively high variability of compound concentrations (Fig. 1e, g). Only about 2, 4 and 13 % of the initially added CBZ, DF and SMX, respectively, had finally been removed in sodium azide-inactivated fungal cultures, suggesting that biosorption of these compounds could be essentially neglected. Similar to cultures amended with EE2 and t-NP, laccase activities rising up to more than 20,000 U L^{-1} at the end of cultivation were also recorded for DF-, SMX- and CBZ-containing active fungal cultures.

In order to calculate the initial (maximal) removal rates of target pollutants, exponential fits (linear fits for CBZ, DF and SMX controls and for active cultures containing CBZ and SMX) were applied to time courses of pollutant concentrations in fungal cultures (Fig. 1 and Table 1). The thus obtained rates were based on corresponding fungal dry weights, to yield specific initial removal rates enabling for direct comparison. Specific initial rates of biological removal (further on referred to as biotransformation rates) were calculated as the difference between specific initial removal rates observed with active fungal cells and those attributable to biosorption as obtained from sodium azide-inactivated cells (Table 1). The resulting specific initial biotransformation rates followed the rank order EE2 ≫ BPA > TCS > t-NP > DF > SMX > CBZ. Their biosorption counterparts followed the rank order EE2 ≥ TCS > BPA > t-NP and were insignificant for CBZ, DF and SMX.

The logarithm of the distribution coefficient (log D_{ow}) at pH 7, a form of the octanol-water partition coefficient (log K_{ow}) accounting for the dissociation or protonation of a target molecule at a given pH (de Ridder et al. 2010), was used as a measure for the hydrophobicity of the tested micropollutants at a pH value essentially being comparable to that applied for fungal degradation experiments within the present study (i.e. pH 6.8; compare 'Materials and methods/experimental' section). The corresponding log D_{ow} values were obtained from Margot et al. (2013). In order to link both biological and abiotic micropollutant removal to compound hydrophobicity, the specific (i.e. dry biomass-based) initial removal rates observed with active fungal cultures and the corresponding inactivated controls according to Table 1 were plotted against log D_{ow}

values of the micropollutants (Fig. 2a), with the difference between either pair of data representing compound removal attributable to biotransformation. Furthermore, the dry biomass-based micropollutant biosorption was calculated on the basis of data fitting of biologically inactive control cultures shown in Fig. 1 (i.e. as the difference between the fitting-derived initial and sorption equilibrium-suggesting asymptote concentrations for BPA, EE2 and TCS and as the difference between initial concentrations and those at the end of the respective experiment for t-NP, CBZ, DF and SMX) and plotted against log D_{ow} values of the micropollutants (Fig. 2b). As could be seen from Fig. 2a, b, biosorption becomes an important removal mechanism starting from log D_{ow} 3.3 (BPA) onwards and is maximal at log D_{ow} 4.8 (TCS), whereas it is negligible for compounds with a log $D_{ow} \leq 3.0$ (DF, CBZ and SMX).

Biotransformation metabolites produced from DF and SMX by fungal cultures

In the present study, we focused on the analysis of biotransformation products of DF and SMX resulting from micropollutant metabolisation by fungal cultures, thereby targeting micropollutant representatives with intermediate and rather slow biotransformation, respectively (Fig. 1 and Table 1). DF is contained in the watch list of the European Water Framework Directive (EU 2013), and the comparatively scarce information regarding its fungal metabolism is so far limited to terrestrial basidio- and ascomycetes (Badia-Fabregat et al. 2014; Hata et al. 2010a). With respect to fungal attack on SMX, only one oxidation product resulting from the action of an isolated extracellular peroxidase of a terrestrial white-rot basidiomycete has been reported as yet (Eibes et al. 2011). SMX metabolism is well documented in bacteria, but related information is so far missing for whole fungal cells (Larcher and Yargeau 2012). Fungal metabolisation of EE2, BPA, t-NP, TCS and CBZ has been reported before (Cajthaml 2014; Golan-Rozen et al. 2011; Hundt et al. 2000; Junghanns et al. 2005; Kang et al. 2008; Kresinova et al. 2012; Wang et al. 2014).

The biotransformation of DF was reflected mainly by the formation of its oxidation and oligomerisation products. The confirmation of their identity as DF metabolites was supported by additional experiments using $^{13}C_6$-labelled DF. Tentative structures with mass spectral characteristics of the products of DF and the $^{13}C_6$-labelled DF, which were not present in the corresponding control cultures, are listed in Tables S1 and S2 of the Supplementary Material. Representative trace ion chromatograms of products of $^{13}C_6$-labelled DF obtained by UPLC-QTOF-MS analysis are exemplified in Fig. S1 of the Supplementary Material. Due to the low concentrations of the biotransformation products, their isolation from the biological matrix in amounts sufficient for confirmation by nuclear magnetic resonance spectroscopy was not possible. Four monomeric biotransformation products of DF were identified and confirmed in the respective experiments employing $^{13}C_6$-labelled DF. One monohydroxylated DF isomer (DF1 in Table S1 of the Supplementary Material) was detected. Its product ion spectrum points to an OH substitution at the acetophenyl ring (*m/z* 166 and 172 for the unlabeled and the $^{13}C_6$-labelled compound, respectively) as described before (Hata et al. 2010a; Webster et al. 1998). For the low-abundant compound DF2, two possible isobaric structures depicted in Table S1 (see Supplementary Material) remain uncertain due to too poor mass spectral information. Product

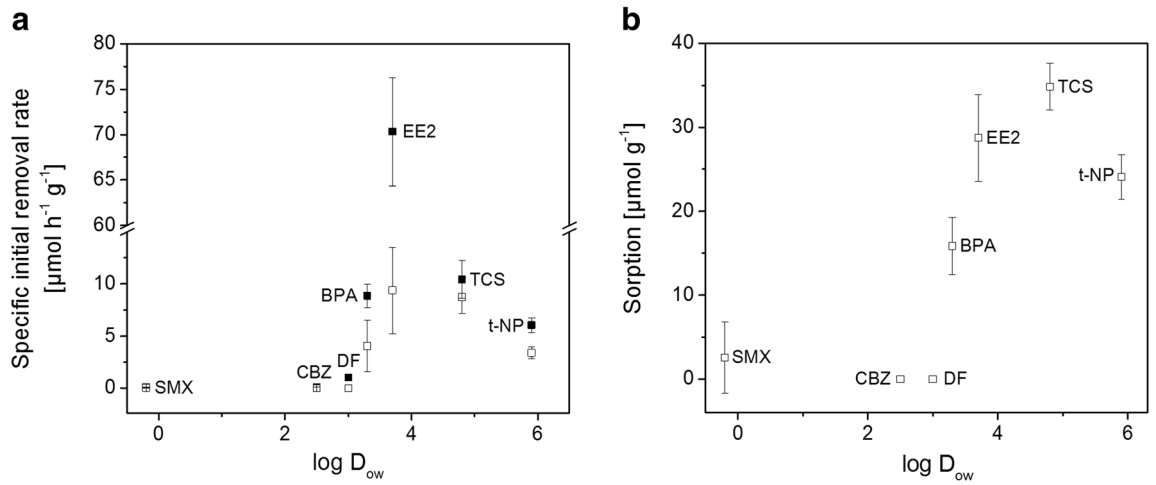


Fig. 2 Specific (fungal dry biomass-based) initial removal rates of micropollutants observed with active (*black square*) and NaN_3-inactivated cultures of *Phoma* sp. (*white square*) vs. corresponding log D_{ow} values of micropollutants (**a**). The fungal dry biomass-based micropollutant biosorption at the end of experiments, as calculated on the basis of data fitting of NaN_3-inactivated control cultures shown in Fig. 1, was also plotted against the corresponding log D_{ow} values of micropollutants (**b**). *Symbols* represent means±standard errors from triplicate cultures (calculated according to Gaussian error propagation rules)

DF3 (Table S1 in the Supplementary Material) is suggested to originate from DF1 by cyclization along with water loss. Although abiotic water elimination and cyclization of DF are known to occur under acidic conditions (Palomo et al. 1999) as was also applied during DF metabolite extraction in our study (see 'Materials and methods' section), we did not observe the cyclization product of the original DF. Thus, the formation of DF3 can be assumed to be initiated by the fungus. The molecular ion of compound DF4 (Table S1 in the Supplementary Material) suggests a dihydroxy-DF as previously reported for DF metabolism by white-rot basidiomycetes (Hata et al. 2010a). The positions of the hydroxyl groups in DF4 remain uncertain due to insufficient mass spectral information. The presence of products with higher molecular masses than that of DF (DF5-12 in Table S2 of the Supplementary Material) suggests the formation of oligomers from DF and/or its monomeric metabolites. For the non-labelled DF, several products with molecular masses ranging from 521 to 1,042 g/mol were detected (D5–D7 in Table S2, Supplementary Material). In experiments with $^{13}C_6$-labelled DF, various compounds with molecular masses between 410 and 670 g/mol were produced (D8-D12 in Table S2 of the Supplementary Material). All of these products contained either two or gour chlorine atoms per molecule, thus partially indicating dechlorination along with oligomerisation. For DF8 and DF9 (Table S2), the elemental composition deduced from the exact molecular mass also allows to suggest the formation of adducts of DF with hydrocarbon compounds, but structural confirmation was not possible. Furthermore, the large number of possible structures of the products with higher molecular mass complicates reliable structure determinations. The dissimilarities especially observed between the oligomeric product patterns of experiments with $^{13}C_6$-labelled and non-labelled DF seem to indicate randomly occurring oligomerisation processes involving DF and different monomeric DF metabolites. The fungal transformation of SMX predominantly generated products characterised by molecular masses at 162, 165, 169, 181 and 238 g/mol, clearly indicating a degradation of SMX (compiled in Tables S3 and S4 with exact masses). The formation of oligomeric products was not observed, despite the product SMX6 yielded a fairly high molecular ion mass ($M + H^+$) at m/z=623.3345 (Table S3 in the Supplementary Material). However, the exact ion mass of SMX6 does not fit with hypothetical products potentially formed by oligomerisation of SMX and/or a SMX metabolite. One possible elemental composition of SMX6 ($C_{22}H_{34}N_3O_{14}S$; Table S3 in the Supplementary Material) would be in line with the formation of a conjugate or an adduct of a SMX metabolite and, e.g. a carbohydrate. Alternative elemental compositions such as $C_{21}H_{47}N_{14}O_4S_2$ or $C_{36}H_{43}N_6O_4$ differ by 0.00012 and 0.00012 u from the experimentally determined exact ion mass (Table S3 in the Supplementary Material) and cannot be linked to a plausible structure proposal related to SMX. A rare fragmentation observed in high collision-induced fragmentation mode (MS^E) was not helpful for further identification, too.

The metabolite patterns observed in the experiments with non-labelled and $^{13}C_6$-labelled SMX were not compatible, most likely since the only quite slow biotransformation of SMX (Fig. 1 and Table 1) did not always result in metabolite quantities that were sufficient high for data interpretation. For only two products obtained from the non-labelled SMX, the respective $^{13}C_6$-labelled counterparts were detected in experiments employing $^{13}C_6$-labelled SMX (SMX8 and SMX9 in Table S4, Supplementary Material).

Deamination of SMX produced a product of low abundance (SMX1, Table S3 in the Supplementary Material), which was suggested as *N*-(5-methylisoxazol-3-yl)-benzenesulfonamide. Structures could not be proposed for the transformation metabolites SMX2–SMX4 (Table S3 in the Supplementary Material), due to their low signal abundance in the analysis. For compound SMX5, an elemental composition of $C_{12}H_{21}N_2O_6S$ ($M + H^+$) was determined, which fits to different possible SMX metabolite structures (Table S3 in the Supplementary Material). Structural features such as carboxylic and hydroxyl functionalities were deduced from the corresponding fragment ions observed in MS^E mode.

The UPLC-ToF MS analysis at negative electrospray ionisation indicated three more products of SMX transformation (SMX7–SMX9 in Table S4 of the Supplementary Material). The mass spectral data of SMX7 suggest a formamide derivative of SMX, with a molecular ion mass at m/z 280.0409. Products SMX8 and SMX9 are likely related to the 1,2-oxazol ring moiety of SMX and clearly represent SMX breakdown products, as confirmed by the detection of their $^{13}C_6$-labelled counterparts. Both metabolites share two identical ions (m/z 161.0242 and 91.0182, respectively) pointing to their structural similarity.

Micropollutant removal by laccase-containing crude culture supernatants

Cell-free, laccase-containing concentrated crude culture supernatants of *Phoma* sp. were used as enzyme sources in experiments addressing the susceptibility of the target pollutants to oxidation by laccase. BPA and EE2 were mostly degraded by concentrated crude culture supernatant containing active laccase within 4 h of incubation, corresponding to a removal of about 79 and 82 % of the BPA and EE2 initially recorded, respectively (Fig. 3). DF and t-NP were comparatively more slowly oxidised. After 22 h of reaction, the pollutant concentration had decreased to approximately 119 and 163 μM (corresponding to about 45 and 32 % compound removal) for DF and t-NP, respectively. Contrary to its complete disappearance in whole fungal cultures, TCS was only moderately removed (by about 14 % within 52 h) by the laccase-containing culture

supernatant (Fig. 3). A very slight decrease in the SMX concentration by about 9 % within 52 h was visible in reaction mixtures containing active laccase (Fig. 3), albeit the final SMX concentration formally was not significantly lower than its initial one according to Student's *t* test. CBZ resisted attack by laccase. Pollutant removal in the respective controls containing heat-inactivated enzyme was always marginal. Similar to the whole-cell approaches described before, linear fits (exponential fits for BPA, EE2 and DF degradation by active laccase) were applied to time courses of pollutant concentrations in enzymatic degradation mixtures (Fig. 3 and Table 2). Initial (maximal) rates of enzymatic removal were calculated as the difference between the initial removal rates observed with reaction mixtures containing active laccase and those obtained from reaction mixtures containing heat-inactivated enzyme (Table 2). The hence resulting initial enzymatic removal rates followed the rank order EE2 > BPA > DF > t-NP > TCS, with only very little and insignificant removal of SMX and no removal at all being observed for CBZ.

The use of low-molecular weight redox mediators can enhance the laccase-catalysed transformation of recalcitrant pollutants (Murugesan et al. 2010). The effects of the natural redox mediator SA (Camarero et al. 2007), which was applied at 500 μM, on the removal of the tested micropollutants are depicted in Fig. 4. DF and t-NP completely disappeared, and about 87 and 90 % of the initially applied SMX and TCS, respectively, had been removed in the presence of SA; indicating a remarkable enhancement of the oxidation of these pollutants by SA compared its absence. No enhancement by SA of the removal of BPA and EE2, which were already efficiently degraded by laccase without SA (Fig. 3b, c), could be recorded after 22 h (Fig. 4). In contrast to all aforementioned target pollutants, a removal of CBZ by the laccase-containing crude culture supernatant could not be observed even in the presence of SA.

Laccase activities were monitored during the target pollutant transformation experiments employing laccase-containing concentrated crude culture supernatants described before. Depending on the respective micropollutant and the presence (or absence) of SA, observed decreases in the initial laccase activities by only about 10 to 28 % after 2 days of incubation indicate a maintenance of substantial laccase activity during all of these experiments.

The synthetic redox mediators ABTS and HBT and the natural redox mediators *p*-coumaric acid and SA were additionally tested for their potential to enable the degradation of CBZ by laccase at a redox mediator/CBZ concentration ratio of 10:1, due to the unsuccessful application of SA at a mediator/pollutant concentration ratio of 2:1 as described before, and since HBT was previously described to mediate the laccase-catalysed oxidation of CBZ at a mediator/CBZ concentration ratio of 10:1 (Hata et al. 2010b). However, CBZ persisted regardless the presence or absence of also these

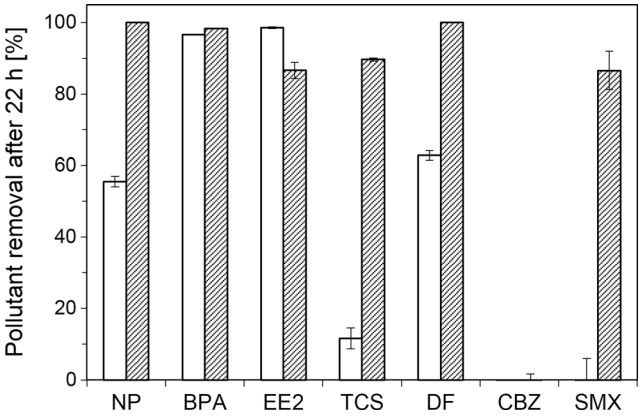
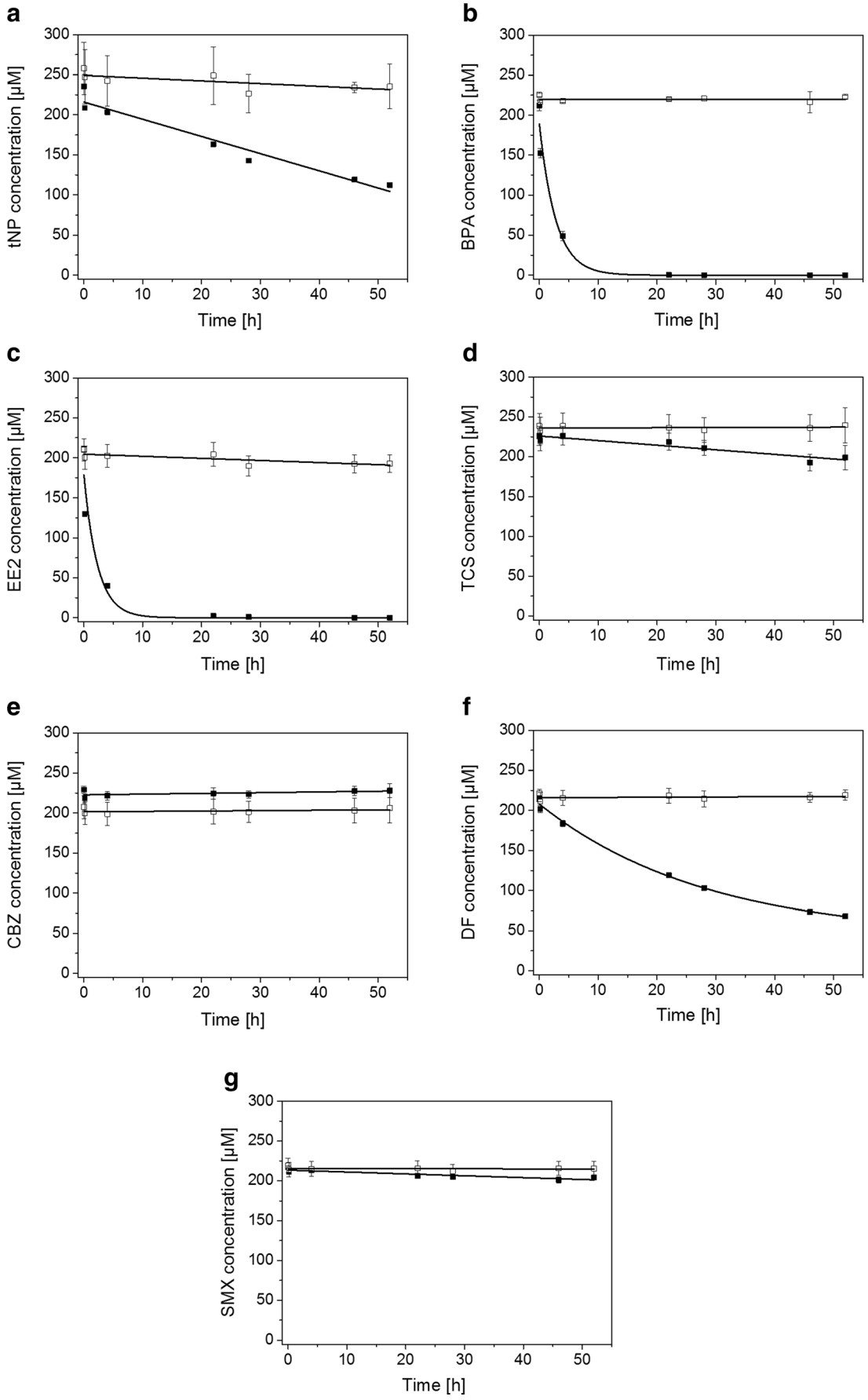


Fig. 4 Effect of 250 μM SA (*hatched bars*) and its absence (*empty bars*) on the removal of micropollutants after a 22-h treatment using laccase-containing culture supernatant of *Phoma* sp. *Bars* represent means± standard deviations from triplicate experiments

redox mediators in our experiments (data not shown). Furthermore, an attempt was made to reproduce previously published results describing the degradation of CBZ by laccase from *T. versicolor* in combination with HBT (Hata et al. 2010b) under conditions similar to those of the aforementioned study (please refer to the 'Materials and methods' section for details). However, CBZ degradation could not be observed (data not shown).

Products formed from target pollutants by laccase reactions

SEC was employed in experiments addressing micropollutant degradation by laccase-containing crude culture supernatants, in order to detect products with higher molecular masses than that of the respective parent compound. Samples from corresponding degradation experiments were subjected to SEC after 2 days of incubation. Related SEC chromatograms and UV/Vis spectra of detected peaks representing products of enzymatic micropollutant oxidation are exemplified for BPA and DF in Fig. 5. SEC analysis of DF degradation led to the detection of two distinct peaks, which were absent in heat-inactivated controls (Fig. 5 and Table 3). During degradation of the other micropollutants, at least one new distinct peak (two peaks for t-NP) was always detected (Fig. 5 and Table 3), respectively, which was not observed in the respective control experiments employing heat-inactivated enzyme. Strong absorbance maxima of the corresponding UV/Vis spectra indicated the presence of aromatic structures also present in the respective parent micropollutants (exemplified for products yielded from BPA and DF oxidation in Fig. 5, where absorbance was maximal at 257 nm for the product obtained from BPA oxidation, and maxima at 248 and 269 nm were observed for the two DF products). The molecular masses corresponding to the respective absorbance maxima of those peaks in SEC chromatograms indicating newly formed


a
tNP concentration [µM]
Time [h]
b
BPA concentration [µM]
Time [h]
c
EE2 concentration [µM]
Time [h]
d
TCS concentration [µM]
Time [h]
e
CBZ concentration [µM]
Time [h]
f
DF concentration [µM]
Time [h]
g
SMX concentration [µM]
Time [h]

◀ **Fig. 3** Time courses of t-NP (**a**), BPA (**b**), EE2 (**c**), TCS (**d**), CBZ (**e**), DF (**f**), and SMX concentrations (**g**) in enzymatic experiments employing active (*black square*) and heat-inactivated laccase-containing culture supernatants of *Phoma* sp. (*white square*). The corresponding *solid lines* arise from data fitting of measured micropollutant concentrations (*black square*, *white square*) as described in the 'Materials and methods' section. *Symbols* represent means±standard deviations from triplicate experiments

products were roughly estimated by comparison with retention times of molecular mass markers. The obtained values indicate that dimers were produced during EE2 degradation, dimers and tetramers were formed from DF and polymers with sizes ranging from about 39 to more than 80 kDa resulted from t-NP, BPA and TCS oxidation (Table 3).

Discussion

The present study demonstrates that the aquatic ascomycete *Phoma* sp. UHH 5-1-03 is able to biotransform all of the tested micropollutants to individual extents (Fig. 1 and Table 1), thereby extending the range of xenobiotics reported to be biocatalytically attacked by this fungus beyond synthetic dyes and the EDCs BPA, EE2 and t-NP (Junghanns et al. 2008a, 2012; Libardi Junior et al. 2012). In addition to biotransformation, sorption onto fungal mycelia played a prominent role in the removal of BPA, EE2, TCS and t-NP. Previous studies addressing bacteria-dominated activated sludge as well as fungal processes demonstrated that the hydrophobicity of environmental pollutants plays a significant role with respect to their biosorption (Chen et al. 2010; Suárez et al. 2008). All EDCs tested within the present study are hydrophobic compounds possessing a pH-dependent effective octanol-water partition coefficient (log D_{ow}) of higher than 3 at a pH of 6.8 (Hai et al. 2013; Margot et al. 2013), which was applied in the whole-cell experiments of our study. The observation that biosorption of the most hydrophobic micropollutant applied (t-NP, log D_{ow}= 5.9; Margot et al. 2013) was less pronounced than that of the less hydrophobic TCS (log D_{ow}=4.8; Fig. 2) may reflect the individual cell surface properties of *Phoma* sp. It is well-known that fungi differ in their cell surface hydrophobicity and can alter their cell surface properties in response to varying growth conditions, which also applies to the presence of hydrophobic hydrocarbon pollutants (Chau et al. 2009; Hanano et al. 2015; Linder et al. 2005). Previously, biosorption of polycyclic aromatic hydrocarbons (in terms of the corresponding carbon-normalised partition coefficients) was found to be linearly correlated with compound hydrophobicity (in terms of octanol-water partition coefficients) for a consortium of white-rot

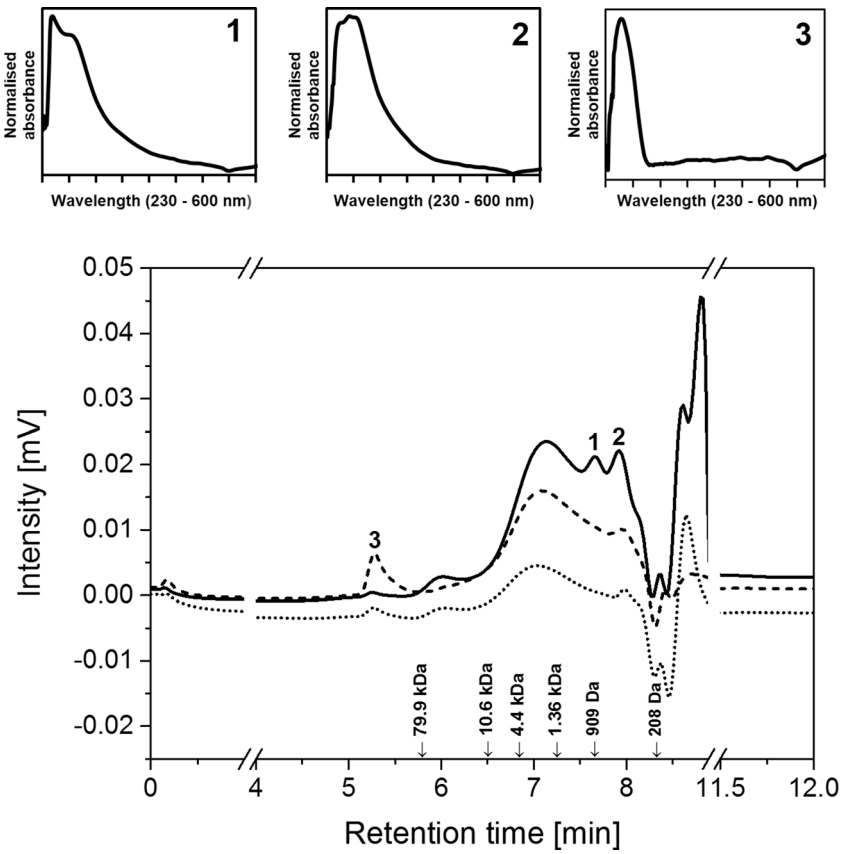


Fig. 5 Representative SEC chromatogram of samples from DF (*solid line*) and BPA degradation (*dashed line*) by active laccase-containing culture supernatant of *Phoma* sp. The *dotted line* represents the control containing heat-inactivated enzyme. Intensity signals (*y*-axis) were obtained from the integration of the respective absorbances over a wavelength range of 230–600 nm. The *insets* show the normalised UV/Vis spectra of labelled peaks indicating oxidative coupling products of DF (*1*, *2*) and BPA (*3*). The retention times of molecular mass markers are indicated by *arrows*

basidiomycetes (Chen et al. 2010). In addition to individual fungal characteristics, a significant biosorption of DF previously reported for the white-rot basidiomycete *T. versicolor* (Yang et al. 2013b), which is different from negligible DF biosorption found in our study (Figs. 1 and 2 and Table 1), most likely reflects the importance of the pH value for biosorption processes: DF is a charged compound and has a lower hydrophobicity at a nearly neutral pH applied in our study compared to a more acidic pH of 4.5 employed by Yang et al. (2013b) (log D_{ow} of 4.51 and 3.0 at pH 4.0 and 7.0, respectively; Margot et al. 2013), obviously preventing remarkable sorption onto fungal mycelia in our study. A prominent biosorption of BPA and negligible biosorption of CBZ and SMX observed in our study (Figs. 1 and 2 and Table 1) are well in line with previously published results (Yang et al. 2013b).

Biotransformation is another important process contributing to the removal of the investigated micropollutants by whole cells of *Phoma* sp., as could be deduced from differences of the respective removal rates obtained with active and NaN_3-inactivated fungal cells (Fig. 1 and Table 1) and from detected biotransformation metabolites of selected micropollutants detailed in Tables S1–S4 of the Supplementary Material. Fungal degradation of EDCs as well as the formation of corresponding biotransformation metabolites is widely described in the literature. In the following, we will provide and discuss pertinent examples with particular relevance for our own results. Biodegradation of the synthetic hormone EE2, which was found to be the most efficiently biotransformed compound amongst the micropollutants tested within the present study (Figs. 1, 2 and 3 and Tables 1 and 2), was addressed with respect to particular enzymes and cell fractions of the white-rot fungus *Pleurotus ostreatus* (Kresinova et al. 2012). Several catabolic mechanisms were found to support an efficient EE2 degradation, which include intracellular degradation via microsomal enzymes, mycelium-associated laccase-like activity, extracellular attack by manganese peroxidase and extracellular laccase activity. The detected intermediates indicate efficient EE2 transformation by extracellular lignin-modifying enzymes as well as by the general eukaryotic machinery of cytochrome P450 monooxygenases. The described catabolic intermediates include methoxylated estron (E1) and dioxo-17β-estradiol (E2), which were probably formed from laccase catalysis. Dehydrogenated and hydroxylated EE2 was also detected.

The comparatively high biocatalytic removal rates of BPA observed in our study (Figs. 1, 2 and 3 and Tables 1 and 2) corroborate previously published data regarding fungal degradation of the compound (Lee et al. 2005; Wang et al. 2013, 2014; Yang et al. 2013b). A metabolic pathway for BPA degradation by the white-rot fungus *Phanerochaete sordida* YK-624 under non-ligninolytic conditions has been reported by Wang et al. (2013, 2014). According to the authors, BPA is at first monooxygenated at its *ortho*-position by intracellular cytochrome P450 and then further metabolised by intracellular *O*-methyltransferases, generating two additional methylated metabolites. It was also proven that all three BPA metabolites formed have a reduced estrogenic activity compared to BPA.

The biotransformation of t-NP by aquatic fungi has already been demonstrated in a previous study, where the aquatic ascomycetes *Myrioconium* sp. UHH 1-6-18-4 and *Clavariopsis aquatica* were applied as test organisms (Junghanns et al. 2005). The analysis of degradation products suggested intracellular hydroxylation of the nonyl moieties of individual t-NP isomers. Further detected metabolites also indicated shortening of the branched nonyl chains, and 4-hydroxybenzoic acid was identified as a t-NP breakdown product in *Myrioconium* sp.

The ability of the white-rot fungi *T. versicolor* and *Pycnoporus cinnabarinus* to metabolise TCS was investigated by Hundt et al. (2000). *T. versicolor* produced three metabolites, 2-*O*-(2,4,4′-trichlorodiphenyl ether)-β-D-xylopyranoside, 2-*O*-(2,4,4′-trichlorodiphenyl ether)-β-D-glucopyranoside and 2,4-dichlorophenol. Our own results obtained with *Phoma* sp. also indicate the formation of dichlorophenol from TCS (Hofmann and Schlosser, unpublished data). *P. cinnabarinus* converted triclosan to 2,4,4′-trichloro-2′-methoxydiphenyl ether and the glucoside conjugate known

Table 3 Products of enzymatic degradation of micropollutants with higher molecular masses than the respective parent compound as obtained from SEC analysis

Parent compound		Peak maximum of degradation product	
Compound name	Molecular mass [kDa]	Retention time [min]	Molecular mass [kDa]
Technical nonylphenol	0.220	5.79	59.10
		5.97	39.28
Bisphenol A	0.228	5.28	>80.84
17α-ethinylestradiol	0.296	7.81	0.60
Triclosan	0.289	5.28	>80.84
Diclofenac	0.296	7.65	0.87
		7.92	0.47

The molecular masses corresponding to maxima of peaks representing enzymatic degradation products were estimated by comparison with retention times of molecular mass markers

from *T. versicolor*. The conjugates showed a distinctly lower cytotoxic and microbicidal activity than triclosan did. TCS is an antimicrobial agent being reported to be effective against many types of fungi (Dann and Hontela 2011). Thus, its resistance to biotransformation by whole cells of *Phoma* sp. (Figs. 1 and 2 and Table 1) may at least partly have been caused by its toxic effects. This possibility is further supported by clearly lower laccase activities in the presence of TCS, as compared to those in the presence of other micropollutants (Fig. 1).

Previous reports dealing with the fungal degradation of the anti-inflammatory DF (Marco-Urrea et al. 2010; Tran et al. 2010; Yang et al. 2013b) are in accordance with the considerable biocatalytic removal observed for the compound within the present study (Figs. 1, 2 and 3 and Tables 1 and 2). In the Water Framework Directive of the European Union (2013/39/EU), DF is included in the watch list of substances established alongside the list of priority substances (EU 2013), and the inclusion of further pharmaceuticals in the future could be expected due to an increasing environmental burden caused by such compounds. As already stipulated above, there exists considerable knowledge about fungal degradation mechanisms of EDCs, and sometimes an accompanying reduction of the endocrine-disrupting activity or ecotoxicity has been described (Cajthaml 2014; Spina et al. 2015). The ability of fungi to degrade pharmaceuticals is not as well investigated yet. We therefore decided to study the biotransformation of selected pharmaceuticals by the freshwater-derived ascomycete *Phoma* sp. in more detail and attempted to detect and possibly to identify major biotransformation metabolites, also in order to establish the corresponding metabolic routes. For DF, HPLC-MS and UPLC-QTOF analysis enabled to suggest the biotransformation products listed in Tables S1 and S2 of the Supplementary Material. A corresponding (partial) catabolic pathway is depicted in Fig. 6. A hydroxylation at position 5 (metabolite DF1 in Fig. 6 and Table S1 in the Supplementary Material) seems to be most probable upon comparison with literature data (Hata et al. 2010a; Webster et al. 1998). Hydroxylated DF metabolites have been previously detected in white-rot fungi and bacteria (Hata et al. 2010a; Marco-Urrea et al. 2010; Webster et al. 1998). The biotransformation of DF by *P. sordida* yielded 4′,5-dihydroxy diclofenac and 4′-hydroxy- and 5-hydroxy-DF as confirmed by NMR spectroscopy (Hata et al. 2010a). The same hydroxy isomers were identified in experiments with *Trametes multicolor* (Marco-Urrea et al. 2010). In the ascomycete *Epicoccum nigrum*, 4′-hydroxy-DF and traces of 3′-hydroxy- and 5-hydroxy-DF were found as the major products of DF metabolism (Webster et al. 1998). Additionally, oligomer products (DF5-DF12, Table S2 in the Supplementary Material) from DF and/or its metabolites have been found, which is in favour of oxidative coupling reactions catalysed by the laccase of *Phoma* sp. (see below). Fairly high intensities of coupling products (DF8, 10, 11 and 12 in Fig. S1 of the Supplementary Material) compared to those of some monomeric metabolites likely produced via DF hydroxylation (DF2 and 3 in Fig. S1 of the Supplementary Material and in Fig. 6) suggest that oxidative coupling reactions contribute to DF removal by *Phoma* sp. to a considerable extent. However, this interpretation should be considered with caution since the unavailability of metabolite reference standards did impede the quantification of biotransformation products and also since a possible promotion of coupling reactions by the comparatively high initial DF concentration of 250 μM may have biased the related results.

The two particularly recalcitrant compounds SMX and CBZ were considerably slower removed than the EDCs and DF by active whole cells of *Phoma* sp. (Figs. 1 and 2 and Table 1). Concerning the removal of SMX, disparate efficiencies by different white-rot fungal species have been previously reported. For example, complete disappearance of SMX by *P. chrysosporium* (Rodarte-Morales et al. 2011) was observed, whilst Yang et al. (2013b) found only negligible removal of SMX by *T. versicolor*. Within the present study, HPLC-MS and UPLC-QTOF analysis enabled to detect several new SMX biotransformation products, which are characterised by lower molecular masses than the parent compound (Tables S3 and S4 in the Supplementary Material). The low-abundant compound SMX1 (Table S3 in the Supplementary Material), which is suggested as *N*-(5-methylisoxazol-3-yl)-benzenesulfonamide (desamino-SMX) and may arise from enzymatic reductive deamination of SMX, has already been reported as transformation product of SMX under denitrifying conditions (Nodler et al. 2012). The reductive deamination of further aromatic amines has been described for mammals (Woolley and Sigel 1982) and anaerobic bacteria (Travkin et al. 2002). Also, reductive deaminases acting on 3,4-dihydroxyphenylalanine are known from anaerobic bacteria (Ranjith et al. 2007). The formation of a formamide moiety from N-containing functional groups, as suggested for compound SMX7 (Table S4 in the Supplementary Material), resembles the detoxifying formation of formamide from cyanide by the enzyme formamide hydro-lyase (cyanide hydratase) in fungi such as, e.g. *Stemphylium loti* and *Gloeocercospora sorghi* (Nazly et al. 1983; Thatcher and Weaver 1976). The missing detection of oligomeric coupling products of SMX is in line with the inability of the *Phoma* sp. laccase to directly oxidise the compound (see below).

Only little CBZ degradation (Figs. 1 and 2 and Table 1) is in good accordance with results of a study from Marco-Urrea et al. (2009), where 57 and 46 % of CBZ were finally removed by the white-rot fungi *T. versicolor* and *Ganoderma lucidum*, respectively. In the same study, the involvement of cytochrome P450 systems in CBZ oxidation was deduced from indicative effects of cytochrome P450 inhibitors. Complete removal of CBZ from a mixture of eight different PPCPs

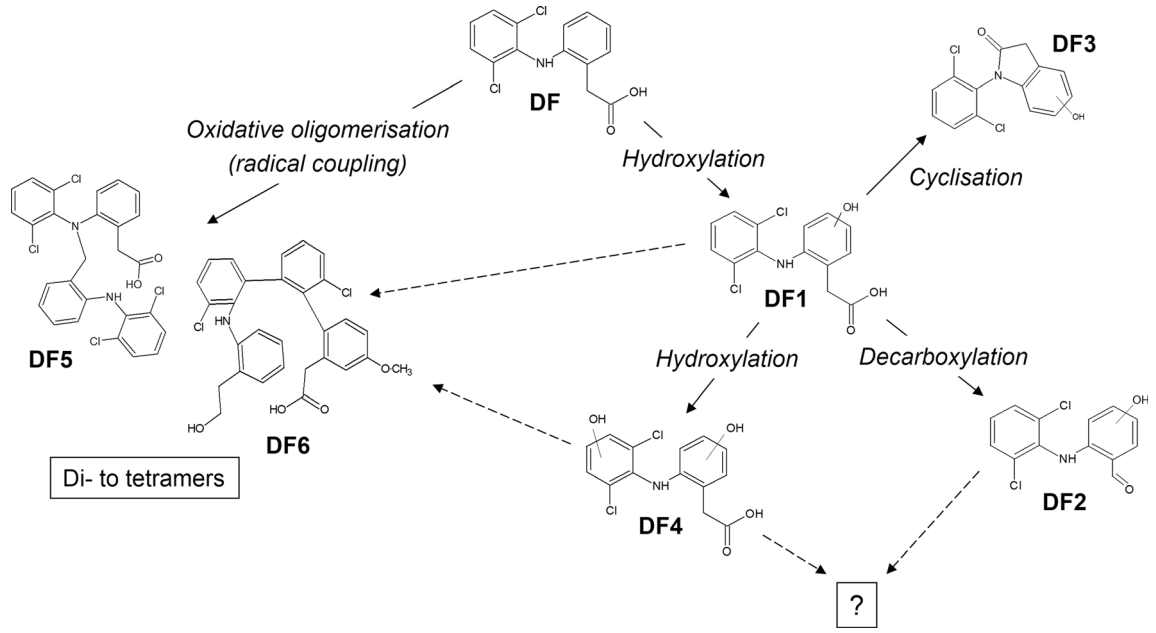


Fig. 6 Proposed pathway of DF metabolisation by *Phoma* sp. Labelling of chemical structures refers to that applied in Tables S1 and S2 of the Supplementary Material. Hydroxylation reactions are in favour of the action of intracellular cytochrome P450 monooxygenase systems, whereas the enzymes responsible for cyclisation and decarboxylation reactions remain to be defined. Oxidative coupling products most likely arise from extracellular laccase reactions

was described for *Bjerkandera* sp. and *P. chrysosporium* (Rodarte-Morales et al. 2010).

Besides cell-bound enzymes, fungi can employ extracellular oxidative enzymes to attack environmental pollutants (Harms et al. 2011). We therefore investigated cell-free laccase-containing culture supernatants of *Phoma* sp. with respect to micropollutant degradation and product formation (Figs. 3, 4 and 5 and Tables 2 and 3). The basic degradability of all tested EDCs by the laccase of *Phoma* sp. (Fig. 3 and Table 2) is consistent with previous reports regarding a high activity of laccases towards the phenolic compounds EE2, BPA and (albeit less pronounced) also t-NP (Cabana et al. 2007; Kresinova et al. 2012). The use of SA significantly increased the removal of TCS compared to the treatment without mediator (Fig. 4), as has already been shown in a former study of Murugesan et al. (2010) using laccase of *G. lucidum*. Important features of a potential redox mediator are the reversibility and stability of radicals during catalytic reactions. The mediator SA possesses a C-OH group, and hydrogen atom transfer is the assumed mechanism of radical formation (Murugesan et al. 2010). The formed phenoxy radicals (C-O·) have long half-lives and can undergo reversible reactions (Fernandez-Sanchez et al. 2002). The stability and reversibility of SA radicals are attributed to the presence of two methoxy groups in *ortho* positions to the phenolic group (Camarero et al. 2007). A comparatively high activity of the *Phoma* laccase in DF oxidation even in the absence of a redox mediator (Figs. 3, 4 and 5 and Tables 2 and 3) corroborates previous studies (Ammann et al. 2014; Ardao et al. 2015; Touahar et al. 2014). Also, a nearly negligible SMX removal by laccase without redox mediator (Fig. 3 and Table 2) and the greatly stimulated SMX oxidation observed in the presence of SA (Fig. 4) is consistent with a previous study, where Yang et al. (2013b) reported that the mediator HBT increased the laccase-catalysed degradation of SMX from a negligible level to 41 %. This may be due to the presence of a strong electron-donating aromatic amine group in the SMX structure, which possibly lowers its redox potential and makes it accessible to laccase oxidation when a redox mediator is added (Thatcher and Weaver 1976). Contrary to previously published data (Hata et al. 2010b), we were not able to verify a laccase-catalysed degradation of CBZ, regardless of the presence or absence of any of the redox mediators ABTS, HBT, *p*-coumaric acid and SA tested within our study. Tran et al. (2010) reported that crude enzyme extract, containing laccase and manganese peroxidase of *T. versicolor*, yielded only 5 % degradation of CBZ, and the addition of HBT to commercial laccase resulted in 19 % removal. The low removal of CBZ can be attributed to the presence of an amide group being electron-withdrawing in its structure (Yang et al. 2013a). Without doubt, redox mediators represent valuable experimental tools largely contributing to the mechanistic understanding of laccase reactions. However, concerns related to high costs and toxicity issues especially of some efficient synthetic redox mediators have been raised in the context of their practical applications (Camarero et al. 2007). The use of natural redox mediators, which may represent lignin-related plant phenols or could also be produced by fungi, has been

suggested to overcome the aforementioned problems (Camarero et al. 2007; Spina et al. 2015). Such naturally occurring redox mediators could also be of relevance for fungi dwelling on plant detritus in aquatic ecosystems (see also below).

Laccase-catalysed degradation of t-NP, BPA, EE2, TCS and DF led to the formation of products with higher molecular masses than that of the respective parent compound (Fig. 5 and Table 3), presumably arising from the primary formation of phenoxy radicals of these compounds catalysed by laccase and following radical coupling (Tsutsumi et al. 2001). The formation of polymerisation products from EDCs upon attack by lignin-modifying enzymes is well-known and has been reported to be accompanied by the loss of their estrogenic activities (Cabana et al. 2007; Junghanns et al. 2005; Suzuki et al. 2003; Tsutsumi et al. 2001; Uchida et al. 2001; Wang et al. 2012).

Summarising, the results of the present study suggest that the extracellular laccase of *Phoma* sp. largely contributes to the fungal biotransformation of the EDCs EE2, BPA, TCS and t-NP, as well as of the anti-inflammatory DF. In addition, cell-associated enzymes such as cytochrome P450 monooxygenases indicated by the appearance of hydroxylated metabolites from DF, and other cell-bound enzymes not identified so far are likely involved in micropollutant catabolism. Our results also suggest that laccase does not play any role in the metabolisation of SMX and CBZ by whole cells of *Phoma* sp. and that yet to be identified cell-associated enzymes have to be considered instead. Apparently, the potential need for micropollutant concentrations sufficiently high enough to induce the expression of degradative fungal enzymes seems to be in contrast with the only minute environmental concentrations of such compounds. However, fungi dwelling in aquatic habitats usually colonise plant-derived detritus associated with considerable amounts of various lignin-related and other aromatic plant compounds, which serve as inducers for non-specific lignin-modifying enzymes like laccases (Krauss et al. 2011; Martin et al. 2007). Moreover, intracellular fungal enzymes acting on xenobiotics such as cytochrome P450 monooxygenases have also been implicated in the oxidation of plant-derived compounds (Syed et al. 2014) and hence may be induced by such naturally occurring substances. All in all, such effects could be expected to be in favour of the sufficient expression of micropollutant-degrading enzymes under conditions related to an aquatic lifestyle of fungi.

The biosorption of pollutants could facilitate their uptake by degrader organisms through shortening the distance between organism and target pollutant (Johnsen et al. 2005; Semple et al. 2007), thus promoting intracellular pollutant catabolism. Another possible consequence of the sorptive binding of environmental pollutants to fungal cell surfaces is related to extracellular polymeric substances (EPS), which are well-known to be frequently produced by fungi and often surround fungal hyphae (Gil-ad et al. 2001; Nicole et al. 1993; Synytsya and Novák 2013). For both asco- and basidiomycetes, such extracellular EPS sheaths have been found to harbour lignin-modifying exoenzymes like laccases and peroxidases (Blanchette et al. 1989; Daniel et al. 1989; Gil-ad et al. 2001; Nicole et al. 1993), which are well-known to attack a broad variety of environmental pollutants (Harms et al. 2011). Sorption of hydrophobic environmental pollutants to EPS indeed has been reported (Johnsen et al. 2005), which potentially could facilitate their attack by fungal exoenzymes likewise embedded in EPS sheaths. Our observation that for whole fungal cells the highest biotransformation rates were observed for the EDCs concomitantly showing the strongest biosorption (Fig. 2 and Table 1) would be in support of catabolic steps facilitated by biosorption and corroborates a previous study indicating the importance of biosorption for subsequent degradation of micropollutants by intracellular and/or mycelium-associated enzymes of *T. versicolor* (Nguyen et al. 2014). Such possible effects may help to increase biocatalytic removal rates, which are usually low due to the extremely low environmental concentrations of micropollutants, for those compounds undergoing biosorptive enrichment prior to biochemical attack. Concerning the potential application of fungi for micropollutant removal, e.g. from wastewaters using bioreactors (Yang et al. 2013a), a combination of biosorptive and biocatalytic removal steps hereby offered could provide advantages over the use of rather conventional adsorbents such as activated carbon and natural (clay) minerals especially with respect to costs, selectivity for contaminants and possible needs for regeneration and separation steps (Grassi et al. 2013).

The major aim of the present study was to explore the ability of the aquatic ascomycete *Phoma* sp. UHH 5-1-03 to remove representative micropollutants and to provide qualitative and quantitative insights into the mechanisms involved. Nevertheless, considering potential fungal applications for wastewater treatment, complex and potentially unfavourable conditions of real wastewater matrices (with respect to, e.g. pH, temperature, salinity, the presence of inhibitors, complex pollutant mixtures or suspended solids potentially causing mass transfer limitations) and problems especially related to the use of fungi in continuously operated bioreactors under non-sterile conditions (e.g. competition with bacteria for substrates, the necessity of co-substrates for cometabolic fungal biotransformations, damage or loss of fungal mycelia and/or loss of fungal enzyme activity over time) illustrate a challenging future scope of research (Yang et al. 2013a). In this context, we found whole cells of *Phoma* sp. to be also active in micropollutant removal when applied to real municipal wastewater, which contained micropollutants in the nanogram per litre range (Fenu et al., unpublished data). Other studies are encouraging with respect to the use of isolated fungal laccases for the removal of environmentally

relevant concentrations of micropollutants from real wastewater (Spina et al. 2015).

Acknowledgments We are thankful to Steffi Schrader, Monika Möder, Bettina Seiwert and Coretta Bauer (Department of Analytical Chemistry, UFZ, Leipzig) for performing mass spectral analyses and for corresponding data processing and interpretation. Funding from the EU project MINOTAURUS, EC Grant Agreement No. 265946, is gratefully acknowledged. This work was further supported by the Helmholtz Association and contributes to the integrated project 'Controlling Chemicals' Fate' in the Chemicals In The Environment (CITE) research programme conducted at the Helmholtz Centre for Environmental Research - UFZ.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

References

Ammann E, Gasser C, Hommes G, Corvini PX (2014) Immobilization of defined laccase combinations for enhanced oxidation of phenolic contaminants. Appl Microbiol Biotechnol 98(3):1397–1406. doi:10.1007/s00253-013-5055-y

Ardao I, Magnin D, Agathos SN (2015) Bioinspired production of magnetic laccase-biotitania particles for the removal of endocrine disrupting chemicals. Biotech Bioeng 112(10):1986–1996. doi:10.1002/bit.25612

Badia-Fabregat M, Rosell M, Caminal G, Vicent T, Marco-Urrea E (2014) Use of stable isotope probing to assess the fate of emerging contaminants degraded by white-rot fungus. Chemosphere 103:336–342. doi:10.1016/j.chemosphere.2013.12.029

Blanchette RA, Abad AR, Farrell RL, Leathers TD (1989) Detection of lignin peroxidase and xylanase by immunocytochemical labeling in wood decayed by basidiomycetes. Appl Environ Microb 55(6):1457–1465

Cabana H, Jiwan J-LH, Rozenberg R, Elisashvili V, Penninckx M, Agathos SN, Jones JP (2007) Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using enzyme preparation from the white rot fungus *Coriolopsis polyzona*. Chemosphere 67(4):770–778. doi:10.1016/j.chemosphere.2006.10.037

Cajthaml T (2014) Biodegradation of endocrine-disrupting compounds by ligninolytic fungi: mechanisms involved in the degradation. Environ Microbiol, article first published online: 18 April 2014 doi:10.1111/1462-2920.12460

Camarero S, Ibarra D, Martínez ÁT, Romero J, Gutiérrez A, del Río JC (2007) Paper pulp delignification using laccase and natural mediators. Enzyme Microb Technol 40(5):1264–1271. doi:10.1016/j.enzmictec.2006.09.016

Castiglioni S, Bagnati R, Calamari D, Fanelli R, Zuccato E (2005) A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. J Chromatogr A 1092(2):206–215. doi:10.1016/j.chroma.2005.07.012

Castiglioni S, Bagnati R, Fanelli R, Pomati F, Calamari D, Zuccato E (2006) Removal of pharmaceuticals in sewage treatment plants in Italy. Environ Sci Technol 40(1):357–363

Chau HW, Si BC, Goh YK, Vujanovic V (2009) A novel method for identifying hydrophobicity on fungal surfaces. Mycol Res 113(10):1046–1052. doi:10.1016/j.mycres.2009.06.007

Chen B, Wang Y, Hu D (2010) Biosorption and biodegradation of polycyclic aromatic hydrocarbons in aqueous solutions by a consortium of white-rot fungi. J Hazard Mater 179(1–3):845–851

Daniel G, Nilsson T, Pettersson B (1989) Intra- and extracellular localization of lignin peroxidase during the degradation of solid wood and wood fragments by *Phanerochaete chrysosporium* by using transmission electron microscopy and immuno-gold labeling. Appl Environ Microbiol 55(4):871–881

Dann AB, Hontela A (2011) Triclosan: environmental exposure, toxicity and mechanisms of action. J Appl Toxicol 31(4):285–311

de Ridder DJ, Villacorte L, Verliefde AR, Verberk JQ, Heijman SG, Amy GL, van Dijk JC (2010) Modeling equilibrium adsorption of organic micropollutants onto activated carbon. Water Res 44(10):3077–3086. doi:10.1016/j.watres.2010.02.034

Delgado LF, Faucet-Marquis V, Pfohl-Leszkowicz A, Dorandeu C, Marion B, Schetrite S, Albasi C (2011) Cytotoxicity micropollutant removal in a crossflow membrane bioreactor. Bioresource Technol 102(6):4395–4401. doi:10.1016/j.biortech.2010.12.107

Eibes G, Debernardi G, Feijoo G, Moreira MT, Lema JM (2011) Oxidation of pharmaceutically active compounds by a ligninolytic fungal peroxidase. Biodegradation 22(3):539–550

EU (2013) Directive 2013/39/EU of the European parliament and of the council of 12 August 2013 amending directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Brussels, Belgium

Fernandez-Sanchez C, Tzanov T, Gübitz GM, Cavaco-Paulo A (2002) Voltammetric monitoring of laccase-catalysed mediated reactions. Bioelectrochemistry 58(2):149–156

Gil-ad NL, Bar-Nun N, Mayer AM (2001) The possible function of the glucan sheath of *Botrytis cinerea*: effects on the distribution of enzyme activities. FEMS Microbiol Lett 199(1):109–113. doi:10.1016/S0378-1097(01)00165-3

Golan-Rozen N, Chefetz B, Ben-Ari J, Geva J, Hadar Y (2011) Transformation of the recalcitrant pharmaceutical compound carbamazepine by *Pleurotus ostreatus*: role of cytochrome P450 monooxygenase and manganese peroxidase. Environ Sci Technol 45(16):6800–6805. doi:10.1021/es200298t

Grassi M, Rizzo L, Farina A (2013) Endocrine disruptors compounds, pharmaceuticals and personal care products in urban wastewater: implications for agricultural reuse and their removal by adsorption process. Environ Sci Pollut R 20(6):3616–3628. doi:10.1007/s11356-013-1636-7

Hai FI, Nghiem LD, Khan SJ, Price WE, Yamamoto K (2013) Wastewater reuse: removal of emerging trace organic contaminants. In: Hai FI, Yamamoto K, Lee C-H (eds) Membrane biological reactors. IWA Publishing, London, UK, pp 163–203

Hanano A, Shaban M, Almousally I, Al-Ktaifani M (2015) *Saccharomyces cerevisiae* SHSY detoxifies petroleum n-alkanes by an induced CYP52A58 and an enhanced order in cell surface hydrophobicity. Chemosphere 135:418–426. doi:10.1016/j.chemosphere.2014.11.011

Harms H, Schlosser D, Wick LY (2011) Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat Rev Micro 9(3):177–192

Hata T, Kawai S, Okamura H, Nishida T (2010a) Removal of diclofenac and mefenamic acid by the white rot fungus *Phanerochaete sordida* YK-624 and identification of their metabolites after fungal transformation. Biodegradation 21(5):681–689. doi:10.1007/s10532-010-9334-3

Hata T, Shintate H, Kawai S, Okamura H, Nishida T (2010b) Elimination of carbamazepine by repeated treatment with laccase in the presence of 1-hydroxybenzotriazole. J Hazard Mater 181(1–3):1175–1178. doi:10.1016/j.jhazmat.2010.05.103

Hundt K, Martin D, Hammer E, Jonas U, Kindermann MK, Schauer F (2000) Transformation of triclosan by *Trametes versicolor* and *Pycnoporus cinnabarinus*. Appl Environ Microbiol 66(9):4157–4160. doi:10.1128/aem.66.9.4157-4160.2000

Jackson J, Sutton R (2008) Sources of endocrine-disrupting chemicals in urban wastewater, Oakland, CA. Sci Total Environ 405(1–3):153–160. doi:10.1016/j.scitotenv.2008.06.033

Jahangiri E, Reichelt S, Thomas I, Hausmann K, Schlosser D, Schulze A (2014) Electron beam-induced immobilization of laccase on porous supports for waste water treatment applications. Molecules 19(8): 11860–11882

Johannes C, Majcherczyk A (2000) Laccase activity tests and laccase inhibitors. J Biotechnol 78(2):193–199. doi:10.1016/S0168-1656(00)00208-X

Johnsen AR, Wick LY, Harms H (2005) Principles of microbial PAH-degradation in soil. Environ Pollut 133(1):71–84. doi:10.1016/j.envpol.2004.04.015

Junghanns C, Krauss G, Schlosser D (2008a) Potential of aquatic fungi derived from diverse freshwater environments to decolourise synthetic azo and anthraquinone dyes. Bioresource Technol 99(5): 1225–1235. doi:10.1016/j.biortech.2007.02.015

Junghanns C, Moeder M, Krauss G, Martin C, Schlosser D (2005) Degradation of the xenoestrogen nonylphenol by aquatic fungi and their laccases. Microbiology-SGM 151:45–57. doi:10.1099/mic.0.27431-0

Junghanns C, Neumann JF, Schlosser D (2012) Application of the aquatic fungus *Phoma* sp. (DSM22425) in bioreactors for the treatment of textile dye model effluents. J Chem Technol Biotechnol 87:1276–1283

Junghanns C, Parra R, Keshavarz T, Schlosser D (2008b) Towards higher laccase activities produced by aquatic ascomycetous fungi through combination of elicitors and an alternative substrate. Eng Life Sci 8(3):277–285. doi:10.1002/elsc.200800042

Junghanns C, Pecyna MJ, Böhm D, Jehmlich N, Martin C, von Bergen M, Schauer F, Hofrichter M, Schlosser D (2009) Biochemical and molecular genetic characterisation of a novel laccase produced by the aquatic ascomycete *Phoma* sp. UHH 5-1-03. Appl Microbiol Biotechnol 84(6):1095–1105. doi:10.1007/s00253-009-2028-2

Kang S-I, Kang S-Y, Hur H-G (2008) Identification of fungal metabolites of anticonvulsant drug carbamazepine. Appl Microbiol Biotechnol 79(4):663–669. doi:10.1007/s00253-008-1459-5

Kim YJ, Nicell JA (2006) Impact of reaction conditions on the laccase-catalyzed conversion of bisphenol A. Bioresource Technol 97(12): 1431–1442

Krauss G-J, Solé M, Krauss G, Schlosser D, Wesenberg D, Bärlocher F (2011) Fungi in freshwaters: ecology, physiology and biochemical potential. FEMS Microbiol Rev 35(4):620–651. doi:10.1111/j.1574-6976.2011.00266.x

Kresinova Z, Moeder M, Ezechias M, Svobodova K, Cajthaml T (2012) Mechanistic study of 17α-ethinylestradiol biodegradation by *Pleurotus ostreatus*: tracking of extracelullar and intracelullar degradation mechanisms. Environ Sci Technol 46(24):13377–13385. doi:10.1021/es3029507

Kümmerer K (2011) Emerging contaminants. Treatise on water science. Elsevier, Oxford, pp 69–87

Larcher S, Yargeau V (2012) Biodegradation of sulfamethoxazole: current knowledge and perspectives. Appl Microbiol Biotechnol 96(2): 309–318

Lee SM, Koo BW, Choi JW, Choi DH, An BS, Jeung EB, Choi IG (2005) Degradation of bisphenol A by white rot fungi, *Stereum hirsutum* and *Heterobasidium insulare*, and reduction of its estrogenic activity. Biol Pharm Bull 28(2):201–207

Libardi Junior N, Miranda Gern RM, Furlan SA, Schlosser D (2012) Laccase production by the aquatic ascomycete *Phoma* sp UHH 5-1-03 and the white rot basidiomycete *Pleurotus ostreatus* DSM 1833 during submerged cultivation on banana peels and enzyme applicability for the removal of endocrine-disrupting chemicals. Appl Biochem Biotech 167(5):1144–1156. doi:10.1007/s12010-012-9601-3

Linder MB, Szilvay GR, Nakari-Setälä T, Penttilä ME (2005) Hydrophobins: the protein-amphiphiles of filamentous fungi. FEMS Microbiol Rev 29(5):877–896. doi:10.1016/j.femsre.2005.01.004

Luo Y, Guo W, Ngo HH, Nghiem LD, Hai FI, Zhang J, Liang S, Wang XC (2014) A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci Total Environ 474:619–641

Marco-Urrea E, Pérez-Trujillo M, Cruz-Morató C, Caminal G, Vicent T (2010) Degradation of the drug sodium diclofenac by *Trametes versicolor* pellets and identification of some intermediates by NMR. J Hazard Mater 176(1–3):836–842. doi:10.1016/j.jhazmat.2009.11.112

Marco-Urrea E, Perez-Trujillo M, Vicent T, Caminal G (2009) Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. Chemosphere 74(6):765–772. doi:10.1016/j.chemosphere.2008.10.040

Margot J, Kienle C, Magnet A, Weil M, Rossi L, de Alencastro LF, Abegglen C, Thonney D, Chevre N, Scharer M, Barry DA (2013) Treatment of micropollutants in municipal wastewater: ozone or powdered activated carbon? Sci Total Environ 462:480–498

Martin C, Moeder M, Daniel X, Krauss G, Schlosser D (2007) Biotransformation of the polycyclic musks HHCB and AHTN and metabolite formation by fungi occurring in freshwater environments. Environ Sci Technol 41(15):5395–5402

Murray KE, Thomas SM, Bodour AA (2010) Prioritizing research for trace pollutants and emerging contaminants in the freshwater environment. Environ Pollut 158(12):3462–3471. doi:10.1016/j.envpol.2010.08.009

Murugesan K, Chang Y-Y, Kim Y-M, Jong-Rok J, Kim E-J, Chang Y-S (2010) Enhanced transformation of triclosan by laccase in the presence of redox mediators. Water Res 44(1):298–308. doi:10.1016/j.watres.2009.09.058

Nazly N, Knowles CJ, Beardsmore AJ, Naylor WT, Corcoran EG (1983) Detoxification of cyanide by immobilised fungi. J Chem Tech Biot B 33(2):119–126. doi:10.1002/jctb.280330207

Nguyen LN, Hai FI, Yang S, Kang J, Leusch FDL, Roddick F, Price WE, Nghiem LD (2014) Removal of pharmaceuticals, steroid hormones, phytoestrogens, UV-filters, industrial chemicals and pesticides by *Trametes versicolor*: role of biosorption and biodegradation. Int Biodeter Biodegr 88:169–175. doi:10.1016/j.ibiod.2013.12.017

Nicole M, Chamberland H, Rioux D, Lecours N, Rio B, Geiger JP, Ouellette GB (1993) A cytochemical study of extracellular sheaths associated with *Rigidoporus lignosus* during wood decay. Appl Environ Microbiol 59(8):2578–2588

Nodler K, Licha T, Barbieri M, Perez S (2012) Evidence for the microbially mediated abiotic formation of reversible and non-reversible sulfamethoxazole transformation products during denitrification. Water Res 46(7):2131–2139. doi:10.1016/j.watres.2012.01.028

Palomo ME, Ballesteros MP, Frutos P (1999) Analysis of diclofenac sodium and derivatives. J Pharmaceut Biomed 21(1):83–94. doi:10.1016/S0731-7085(99)00089-8

Ranjith NK, Sasikala C, Ramana CV (2007) Catabolism of L-phenylalanine and L-tyrosine by *Rhodobacter sphaeroides* OU5 occurs through 3,4-dihydroxyphenylalanine. Res Microbiol 158(6):506–511. doi:10.1016/j.resmic.2007.04.008

Rodarte-Morales AI, Feijoo G, Moreira MT, Lema JM (2011) Degradation of selected pharmaceutical and personal care products (PPCPs) by white-rot fungi. World J Microb Biot 27(8):1839–1846 doi:10.1007/s11274-010-0642-x

Rodarte-Morales AI, Moreira MT, Feijoo G, Lema JM (2010) Evaluation of two fungal strains for the degradation of pharmaceutical and personal care products. Chem Eng Trans 20:31–36

Semple KT, Doick KJ, Wick LY, Harms H (2007) Microbial interactions with organic contaminants in soil: definitions, processes and measurement. Environ Pollut 150(1):166–176. doi:10.1016/j.envpol.2007.07.023

Singh S, Harms H, Schlosser D (2014) Screening of ecologically diverse fungi for their potential to pretreat lignocellulosic bioenergy feedstock. Appl Microbiol Biotechnol 98(7):3355–3370. doi:10.1007/s00253-014-5563-4

Spina F, Cordero C, Schilirò T, Sgorbini B, Pignata C, Gilli G, Bicchi C, Varese GC (2015) Removal of micropollutants by fungal laccases in model solution and municipal wastewater: evaluation of estrogenic activity and ecotoxicity. J Clean Prod 100:185–194. doi:10.1016/j.jclepro.2015.03.047

Stanier RY, Palleroni NJ, Doudoroff M (1966) The aerobic pseudomonads: a taxonomic study. J Gen Microbiol 43(2):159–271

Suárez S, Carballa M, Omil F, Lema J (2008) How are pharmaceutical and personal care products (PPCPs) removed from urban wastewaters? Rev Environ Sci Biotechnol 7(2):125–138. doi:10.1007/s11157-008-9130-2

Suzuki K, Hirai H, Murata H, Nishida T (2003) Removal of estrogenic activities of 17beta-estradiol and ethinylestradiol by ligninolytic enzymes from white rot fungi. Water Res 37(8):1972–1975

Syed K, Shale K, Pagadala NS, Tuszynski J (2014) Systematic identification and evolutionary analysis of catalytically versatile cytochrome P450 monooxygenase families enriched in model basidiomycete fungi. PLoS One 9(1):e86683. doi:10.1371/journal.pone.0086683

Synytsya A, Novák M (2013) Structural diversity of fungal glucans. Carbohyd Polym 92(1):792–809. doi:10.1016/j.carbpol.2012.09.077

Thatcher RC, Weaver TL (1976) Carbon-nitrogen cycling through microbial formamide metabolism. Science 192(4245):1234–1235. doi:10.1126/science.192.4245.1234

Touahar IE, Haroune L, Ba S, Bellenger J-P, Cabana H (2014) Characterization of combined cross-linked enzyme aggregates from laccase, versatile peroxidase and glucose oxidase, and their utilization for the elimination of pharmaceuticals. Sci Total Environ 481:90–99. doi:10.1016/j.scitotenv.2014.01.132

Tran NH, Urase T, Kusakabe O (2010) Biodegradation characteristics of pharmaceutical substances by whole fungal culture *Trametes versicolor* and its laccase. J Water Environ Technol 8(2):125–140. doi:10.2965/jwet.2010.125

Travkin V, Baskunov BP, Golovlev EL, Boersma MG, Boeren S, Vervoort J, van Berkel WJH, Rietjens IMCM, Golovleva LA (2002) Reductive deamination as a new step in the anaerobic microbial degradation of halogenated anilines. FEMS Microbiol Lett 209(2):307–312. doi:10.1111/j.1574-6968.2002.tb11149.x

Tsutsumi Y, Haneda T, Nishida T (2001) Removal of estrogenic activities of bisphenol A and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes. Chemosphere 42(3):271–276

Uchida H, Fukuda T, Miyamoto H, Kawabata T, Suzuki M, Uwajima T (2001) Polymerization of bisphenol A by purified laccase from *Trametes villosa*. Biochem Biophys Res Commun 287(2):355–358

van Leeuwen JS, Vredenburg G, Dragovic S, Tjong TFJ, Vos JC, Vermeulen NPE (2011) Metabolism related toxicity of diclofenac in yeast as model system. Toxicol Lett 200(3):162–168. doi:10.1016/j.toxlet.2010.11.010

Wang J, Majima N, Hirai H, Kawagishi H (2012) Effective removal of endocrine-disrupting compounds by lignin peroxidase from the white-rot fungus *Phanerochaete sordida* YK-624. Curr Microbiol 64(3):300–303

Wang J, Yamada Y, Notake A, Todoroki Y, Tokumoto T, Dong J, Thomas P, Hirai H, Kawagishi H (2014) Metabolism of bisphenol A by hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 under non-ligninolytic condition. Chemosphere 109:128–133. doi:10.1016/j.chemosphere.2014.01.029

Wang J, Yamamoto R, Yamamoto Y, Tokumoto T, Dong J, Thomas P, Hirai H, Kawagishi H (2013) Hydroxylation of bisphenol A by hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 under non-ligninolytic condition. Chemosphere 93(7):1419–1423. doi:10.1016/j.chemosphere.2013.07.026

Webster R, Pacey M, Winchester T, Johnson P, Jezequel S (1998) Microbial oxidative metabolism of diclofenac: production of 4'-hydroxydiclofenac using *Epiccocum nigrum* IMI354292. Appl Microbiol Biotechnol 49(4):371–376

Witorsch RJ (2014) Critical analysis of endocrine disruptive activity of triclosan and its relevance to human exposure through the use of personal care products. Crit Rev Toxicol 44(6):535–555. doi:10.3109/10408444.2014.910754

Woolley JL Jr, Sigel CW (1982) The role of dietary nitrate and nitrite in the reductive deamination of sulfadiazine by the rat, guinea pig, and neonatal calf. Life Sci 30(25):2229–2234

Yang S, Hai FI, Nghiem LD, Price WE, Roddick F, Moreira MT, Magram SF (2013a) Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: a critical review. Bioresource Technol 141:97–108. doi:10.1016/j.biortech.2013.01.173

Yang S, Hai FI, Nghiem LD, Roddick F, Price WE (2013b) Removal of trace organic contaminants by nitrifying activated sludge and whole-cell and crude enzyme extract of *Trametes versicolor*. Water Sci Technol 67(6):1216–1223. doi:10.2166/wst.2013.684

Zhang Y, Geissen SU (2010) In vitro degradation of carbamazepine and diclofenac by crude lignin peroxidase. J Hazard Mater 176(1–3):1089–1092