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# Removal of trace organic contaminants by nitrifying activated sludge and whole-cell and crude enzyme extract of Trametes versicolor

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**Abstract**

The resistance of certain anthropogenic trace organic contaminants (TrOCs) to conventional wastewater treatment and their potential adverse effects on human and ecological health raise significant concerns and have prompted research on their bioremediation by white-rot fungi. This study compared the removal efficiencies of four widespread TrOCs: carbamazepine (CBZ), sulfamethoxazole (SMX), bisphenol A (BPA) and diclofenac (DCF), by nitrifying activated sludge as well as whole-cell and extracellular enzyme (laccase) extract of the white-rot fungus Trametes versicolor. Fungal whole-cell culture removed only BPA and DCF but with high efficiencies (>90%) while the mixed nitrifying culture removed all compounds, although by levels of only 5-40%. Rapid initial sorption on fungal mycelium (44 ±13% for DCF) was observed; however, biodegradation governed the overall removal. Performance comparison between fungal whole-cell and extracellular extract revealed that, unlike BPA, a catalytic pathway independent of extracellular laccase was responsible for DCF removal. Addition of mediator (1-hydroxybenzotriazole) to extracellular extract improved the removal of SMX which bears an electron donor group, but not that of the resistant compound CBZ.

**Keywords**

trametes, extract, enzyme, crude, cell, whole, sludge, versicolor, activated, removal, nitrifying, contaminants, organic, trace, GeoQuest, CMMB

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**Abstract**
The resistance of certain anthropogenic trace organic contaminants (TrOCs) to conventional wastewater treatment and their potential adverse effects on human and ecological health raise significant concerns and have prompted research on their bioremediation by white-rot fungi. This study compared the removal efficiencies of four widespread TrOCs: carbamazepine (CBZ), sulfamethoxazole (SMX), bisphenol A (BPA) and diclofenac (DCF), by nitrifying activated sludge as well as whole-cell and extracellular enzyme (laccase) extract of the white-rot fungus *Trametes versicolor*. Fungal whole-cell culture removed only BPA and DCF but with high efficiencies (>90 %) while the mixed nitrifying culture removed all compounds, although by levels of only 5 - 40 %. Rapid initial sorption on fungal mycelium (44±13 % for DCF) was observed; however, biodegradation governed the overall removal. Performance comparison between fungal whole-cell and extracellular extract revealed that, unlike BPA, a catalytic pathway independent of extracellular laccase was responsible for DCF removal. Addition of mediator (1-hydroxybenzotriazole) to extracellular extract improved the removal of SMX which bears an electron donor group, but not that of the resistant compound CBZ.

**Keywords**
Trace organic contaminants; water reuse; nitrifying activated sludge; white-rot fungus; crude enzyme extract; mediator

## INTRODUCTION

There is increasing concern about the occurrence of trace organic contaminants (TrOCs) such as industrial chemicals, pesticides, pharmaceuticals and personal care products, and endocrine disrupting chemicals in wastewater-impacted freshwater systems worldwide (Cirja et al., 2008). These compounds are suspected of having a long-term detrimental impact on aquatic life and human health. Efficient removal of TrOCs is a prerequisite to water reuse which is an important factor in meeting the ever increasing demand for water from a growing population, changing lifestyle patterns due to urbanization, and diminishing and uncertain natural water resources due to erratic weather patterns (Melin et al., 2006). However, the limitations of conventional activated sludge-based treatment processes in removal of resistant TrOCs from wastewater have been widely reported. For example, the removal of diclofenac has been observed to be low and highly variable ranging from negligible to about 50% (Cirja et al., 2008, Tadkaew et al., 2011).

Due to the limitations of TrOC removal by the activated sludge processes, there have been a number of recent studies on the application of white-rot fungal bioremediation (Kim and Nicell, 2006, Marco-Urrea et al., 2009, Rodarte-Morales et al., 2010). White-rot fungi have been reported to efficiently degrade a wide range of xenobiotics such as azo dyes, polycyclic aromatic hydrocarbons, chlorophenols, nitrotoluenes and polychlorinated biphenyl via one or

more extracellular enzymes including lignin peroxidases (LiP), manganese-dependent peroxidases (MnP) and laccases. Recent work has also reported efficient removal of several TrOCs such as diclofenac and carbamazepine which normally exhibit strong resistance to bacterial degradation (Marco-Urrea et al., 2009, Rodarte-Morales et al., 2010, Tran et al., 2010). However, the removal efficiency has been observed to largely depend on fungal species and their specific enzyme systems (Marco-Urrea et al., 2009, Rodarte-Morales et al., 2010). An alternative which decouples the fungal growth and bioremediation stages is to utilise extracellular culture extract ('crude enzyme') or purified enzymes instead of whole-cell preparations (Kim and Nicell, 2006). Enzyme-catalysis has been shown to be enhanced by the addition of low molecular weight mediator compounds which act as an 'electron shuttle' between the oxidizing enzyme and target compounds (Kurniawati and Nicell, 2007). The degree of enhancement, however, has been observed to depend predominantly on the type of mediator and the TrOC structure (Baiocco et al., 2003, Tran et al., 2010).

Overall, several studies, as noted above, have investigated the TrOC removal capacity of different fungal species. The efficiency of different components of activated sludge has also been compared. For example, Tran et al. (2009) reported better capacity of the nitrifiers over the heterotrophs. However, to date, only a few reports have specifically compared the capacity of fungus and activated sludge particularly in relation to TrOC (Hai et al., 2012) or other persistent compounds (Hai and Yamamoto, 2010). As an additional notable omission, the relative contribution of biosorption and biodegradation during fungal removal of TrOCs remains largely unexplored (Cravotto et al., 2008, Hai et al., 2012) although several studies have reported that biosorption plays a significant role in removal of TrOCs by activated sludge (Cirja et al., 2008, Tran et al., 2009, Yang et al., 2011).

With the aim of addressing the research gaps outlined above, a series of batch tests was conducted in this study to compare the removal of selected TrOCs by a nitrifying activated sludge culture, a whole-cell pure fungus culture and a fungal extracellular enzyme extract. In discussing the removal efficiency, the relative contribution of biosorption and biodegradation is addressed, as well as potential catalytic modes (extracellular enzyme dependent/independent) responsible for fungal degradation of trace organic contaminants. The effect of augmenting enzymatic transformation with a redox mediator was also studied.

## METHODS

### Microorganisms and culture and test media

Conventional bacteria-dominated activated sludge was obtained from the biological nutrient removal clarifier of the Wollongong wastewater treatment plant (Wollongong, NSW, Australia). In that plant, primary treated wastewater passes through biological nutrient removal tanks (anaerobic-anoxic-aerobic-clarifier). The white-rot fungus *Trametes versicolor* ATCC 7731 was procured from the ATCC (USA). Nitrifier enrichment media (Tran et al., 2009) and malt extract broth (1 g/L) were used as culture media for the nitrifying activated sludge and fungus, respectively. The initial pH of the activated sludge and fungus culture media were 9 and 4.5, respectively. A pure culture of fungus (0.022 g dry wt.) was aseptically inoculated into 50 mL malt extract broth and grown at 25 °C on a rotary shaker (Bioline incubator shaker 8500, Edwards Instrument Co, Australia) at 120 rpm. The resulting fungal biomass was harvested after seven days for *in vivo* testing, while the filtered extracellular extract (0.45 μm cellulose acetate filter, Advantec) was utilised as a crude enzyme solution for an *in vitro* test. Under the conditions tested, the fungus secreted laccase predominantly. The enzyme solution typically had a pH of 5 and showed an activity of 40 μM / min (measured using 2,6-dimethoxy phenol as

substrate). For batch tests with nitrifying activated sludge and whole-cell fungus, the test media comprised the respective culture media and TrOCs. For enzymatic tests, TrOCs were added to previously harvested culture extract of known laccase activity. During the initial screening tests, each compound was incubated separately with activated sludge culture, pure fungal whole-cell culture and crude enzyme solution. However, for the final set of experiments conducted in duplicate, SMX and CBZ were incubated together, as were BPA and DCF. Trials showed that under these conditions there was no influence on the degradation of the individual compounds or on their detection by high performance liquid chromatography (HPLC)-UV analysis.

**Batch test protocol**

All test media (excluding crude enzyme solution) and laboratory apparatus were sterilized by autoclaving before use to maintain aseptic conditions. Comparison of performance was planned to be conducted at an initial TrOC concentration of approximately 1,000 μg/L by adding a required volume of TrOCs stock solution into different test media. However, the actual initial concentrations of the TrOCs during the activated sludge, whole-cell fungus, and fungal extracellular extract tests as determined by HPLC analysis were 1,200±250, 1,330±350 and 1,450±360 μg/L, respectively.

In the activated sludge batch test, TrOCs were added in pairs (BPA and DCF, and CBZ and SMX) at an initial concentration of 1,200±250 μg/L into 100 mL nitrifier enrichment medium in 400 mL beakers covered with aluminium foil. The nitrifying capacity of the sludge was confirmed before inoculation (Tran et al., 2009). The sludge demonstrated a nitrifying activity of 10 mg $NH_4$-N/g MLSS.h. The initial mixed liquor suspended solids concentration was approximately 0.2 g/L. To assess the relative contribution of biodegradation and biosorption, tests with chemically inactivated sludge were also conducted. Sodium azide was added to the test medium (0.1 mM) to inactivate the bacteria so that the extent of biosorption could be ascertained (Yang et al., 2011). Control samples comprised trace organic contaminants in Milli-Q water. All tests were conducted in duplicate. All test beakers were incubated in a rotary shaker at 120 rpm and 25 °C , and samples were collected at 4, 8 and 22 h for TrOC analysis.

Batch tests with live and inactivated (with sodium azide) whole-cell fungal cultures were conducted using malt extract broth maintaining similar conditions as described above. However, to obtain a clearer understanding of the removal capacity of the fungus, in addition to an initial concentration of approximately 1,000 μg/L, tests were also conducted using other initial concentrations. For CBZ and SMX, these were 380±75 μg/L and 8,800±80 μg/L (CBZ) and 680±110 μg/L and 10,800±430 μg/L (SMX), respectively. For BPA and DCF, the additional initial concentration tested was 6,000±50 μg/L. Immediately after collection, the supernatant sample was mixed with methanol in 1:1 volumetric ratio to stop further biodegradation due to the enzyme in the supernatant.

In order to study TrOC degradation by the extracellular fungal enzyme, 5 mL crude enzyme extract was incubated with TrOCs at an initial concentration of 1,450±360 μg/L in 10 mL screw-capped test tubes in duplicate. Additional tubes were incubated to assess the effect of addition of 1 mM of the redox mediator, 1- hydroxybenzotriazole (HBT) (Tran et al., 2010). The tubes were incubated in a rotary shaker at 70 rpm and 25 °C, and samples were taken at 2, 4, 8 and 22 h. As the entire volume of test medium in each tube was used at each sampling, separate tubes (in duplicate) were incubated for the different sampling times. The enzymatic activity (laccase) of the culture supernatant was measured for the tests with both fungal whole-cell and extracellular enzyme extract.

**Analytical methods**

A Shimadzu HPLC system (Shimadzu, Kyoto, Japan), equipped with a Supelco Drug Discovery 300 x 4.6 mm C-18 column (5 μm pore size) and a UV–vis detector, was used to measure the concentrations of TrOCs. The detection wavelength was 280 nm and the column temperature was 20 °C. The sample injection volume was 50 μL. The mobile phase comprised acetonitrile and Milli-Q grade deionized water buffered with 25 mM $KH_2PO_4$ (pH = 4.8). Two eluents, A (80 % acetonitrile + 20 % buffer, v/v) and B (20 % acetonitrile + 80 % buffer, v/v) were delivered at 0.7 mL $min^{-1}$ through the column in time-dependent gradient proportions (Supplementary Data Table S2). The limit of quantification for the analytes under investigation using these conditions was approximately 10 μg/L. Laccase activity was measured by monitoring the change in absorbance at 468 nm due to the oxidation of 2,6-dimethoxy phenol at room temperature over 2 min using a spectrophotometer (PharmaSpec UV-1700, Shimadzu, Kyoto, Japan) (Hai et al., 2009). Laccase activity was calculated from the molar extinction coefficient ε = 49.6/ (mM. cm) and expressed in μM substrate/ min (Hai et al., 2009).

## RESULTS AND DISCUSSION

The activated sludge and fungus cultures demonstrated significantly different levels of initial biosorption and overall removal of the compounds investigated from the liquid phase (Table 1). Noticeable differences were also observed between the performance of whole-cell culture and the extracellular extract of the fungus.

**[TABLE 1]**

### TrOC removal by activated sludge

Based on the change in aqueous phase TrOC concentrations during incubation with activated sludge, chemically inactivated sludge and Milli-Q water (Figure 1), it can be concluded that within 22 h, activated sludge could remove 5 - 40 % of the target compounds, while biosorption accounted for 0 - 25 % of the removal. The activated sludge achieved the highest overall removal (40 %) as well as the highest biosorption (25 %) for BPA. This observation is consistent with the relatively higher hydrophobicity (log D=3.62 at pH=9) and greater degradability of BPA due to the presence of the strong electron donating –OH group in its structure (Tadkaew et al., 2011). However, compared with the high removal (≥ 95 %) of BPA in a previous study (Zhao et al., 2008), a significantly lower removal was achieved in this study. This discrepancy may be attributed to the fact that the current study was conducted at a significantly higher initial concentration (1490 μg/L compared with a few μg/L). Approximately 23 % overall removal of DCF (10 % biosorption) was achieved in this study. This removal efficiency is consistent with the range (30 - 75 %) reported for DCF in relation to a nitrifying culture by Tran et al. (2009) who used an initial concentration one-tenth of that used in this study. On the other hand, in line with its extremely resistant nature (Tran et al., 2009), a low and highly variable (13±7 %) removal of CBZ was achieved (Figure 1). Meanwhile, the activated sludge culture achieved an overall SMX removal of approximately 15 %, with negligible biosorption. Our results are in good agreement with those in the few batch studies available which investigated SMX removal (Hai et al., 2011, Yang et al., 2011).

**[FIGURE 1]**

### TrOC removal by whole-cell fungus

Similar overall removal of BPA and DCF from the liquid phase was achieved at initial concentrations of 1,420±40 μg/L (Supplementary Data Figure S3) and 6,000±50 μg/L (Figure 2). In order to demonstrate the excellent removal achieved for these two compounds, the

removal data corresponding to the higher initial concentration has been discussed here. Both BPA and DCF were partially removed (ca. 50 %) from the liquid phase by the live fungi within 10 h of incubation (Figure 2). Further gradual decrease in liquid phase concentration occurred and complete removal of DCF and BPA was achieved in less than 22 and 72 h, respectively. Our results regarding high removal of BPA and DCF by fungus are consistent with those reported in the literature (Lee et al., 2005, Marco-Urrea et al., 2010, Tran et al., 2010).

A significant reduction in the liquid phase concentration of both compounds (44±13 and 40±12 % for BPA and DCF, respectively) was also observed in the test media incubated with chemically inactivated fungi within 10 h, however, no further removal was observed thereafter (Figure 2 a). In this study, sodium azide completely stopped fungal growth (Supplementary Data Figure S4) and enzyme production (Figure 2 b), meaning that inactivated fungus removed the TrOCs solely by sorption. Notably, the extent of biosorption was slightly lower for the activated sludge (25 and ~10 %, for BPA and DCF, respectively) (Table 1). The initial pH of the activated sludge and fungus culture media were 9 and 4.5, respectively. DCF has a lower hydrophobicity at alkaline pH (log D of 4.33 and 0.83 at a pH of 4 and 9, respectively) as it is a charged compound. Therefore, the lower sorption of DCF onto activated sludge can be explained by the difference in pH of bacterial and fungal media. However, because the neutral compound (BPA) also demonstrated somewhat lower sorption onto bacterial biomass, it is likely that differences in both media pH and in adsorption capacity of the activated sludge and pure fungal mycelium were responsible for the different amount of biosorption. No other study comparing adsorption of TrOCs on bacterial and fungal biomass could be identified in the literature; however, the observed discrepancy may be due to the nature of the cell wall constituents and functional groups involved in binding (Kotrba et al., 2011). Nevertheless, the comparative performance of live and inactivated fungus confirmed the major role of biodegradation in the overall liquid phase removal of the compounds. This is an important additional outcome from our study.

**[FIGURE 2]**

In contrast to BPA and DCF, negligible removal of CBZ and SMX at initial concentrations of 1290±440 μg/L was observed in this study, although the laccase activity in the test culture reached 10 μM / min (measured using 2,6-dimethoxy phenol as substrate) within 72 h (Supplementary Data Figure S5). The resistance of CBZ and SMX to degradation was further confirmed by additional experiments employing initial concentrations of approximately 500 and 10,000 μg/L, and incubation for up to 10 days. The observed resistance of CBZ toward fungal degradation in this study was not entirely unexpected as disparate removal efficiencies of CBZ by different white-rot fungal species (Marco-Urrea et al., 2009, Rodarte-Morales et al., 2011), and often by different strains of the same fungal species (Marco-Urrea et al., 2009, Rodarte-Morales et al., 2010), have been previously reported. For example, complete removal of CBZ by *Bjerkandera sp.* (Rodarte-Morales et al., 2011) and *Phanerochaete chrysosporium* (Rodarte-Morales et al., 2010) was reported, while work by Marco-Urrea et al. (2009) showed negligible removal of CBZ by *P. chrysosporium* and *Irpex lacteus*. Tran et al. (2010) found 76 % removal of CBZ by a different strain of *T. versicolor* (ATCC 42530) than that used in this study. However, a different initial CBZ concentration (10 μg/L) and culture medium (basal liquid medium) were used in that study, and as noted above, significantly different levels of degradation of CBZ by different strains of the same fungal species (e.g. *P. chrysosporium* (Marco-Urrea et al., 2009, Rodarte-Morales et al., 2010)) is not uncommon. On the other hand, Rodriguez-Rodriguez et al. (2012) reported degradation of the sulphonamides sulfapyridine and sulfathiazole by *T. versicolor*; however, no investigations on the SMX removal capacity of *T. versicolor* could be found in the literature.

In this study, the fungal culture demonstrated significantly higher removal of BPA and DCF and no removal of CBZ and SMX, whereas the activated sludge culture achieved low removal of all four compounds. In a recent study by Hai et al.(2012) a mixed culture of bacteria and fungus achieved better removal of selected pesticides than the bacterial or fungal culture alone. The results from this study also indicate potential advantages of combining fungus and bacterial cultures; however, such an attempt was beyond the scope of this study.

**TrOC removal by crude enzyme extract**

BPA was almost completely (96 %) degraded by the crude enzyme extract within 4 h of incubation regardless of the presence of the mediator HBT (Figure 3 a). This is consistent with previous reports regarding a high degree of laccase-catalysed conversion of the phenolic structure of BPA (Kim and Nicell, 2006, Cabana et al., 2007). On the other hand, contrary to complete removal by whole-cell fungal culture, no removal of DCF was observed by the extracellular enzyme extract (Figure 3 a). Only moderate removal (16±11 %) was achieved in the presence of HBT. By assessing the effect of chemically inactivating intracellular cytochrome P450 (CYP) enzymes in a strain of *Phanerochaete sordida*, Hata et al. (2010) showed that intracellular enzymes, and not extracellular ones, were involved in the elimination and detoxification of DCF. Our results also indicate a negligible role of extracellular enzymes in DCF removal. In order to clarify the underlying reasons, further study on the role of mycelium-bound enzyme (Valaskova and Baldrian, 2006) and intracellular CYP is deemed necessary; however, that is beyond the scope of this study.

**[FIGURE 3]**

Similar to the observation for fungal whole-cell culture, no CBZ removal was observed during its incubation with extracellular enzyme extract with or without HBT (Figure 3 a). Tran et al. (2010) also reported that a crude enzyme preparation (laccase and MnP) of *Trametes versicolor* achieved only 5 % removal of CBZ, and the addition of HBT to commercial laccase resulted in only 19 % removal. In contrast to the case of CBZ, the mediator HBT improved the degradation of SMX from a negligible level to 41 % (Figure 3 a). HBT, as an N-OH derivative mediator, is converted to a radical cation by mono-electronic enzymatic oxidation (Baiocco et al., 2003). SMX contains an amine group, which is a strong electron-donating group, while CBZ contains a strong electron-attracting group amide. Knackmuss et al. (1996) reported that electron donating functional groups render molecules more prone to electrophilic attack by oxygenases of aerobic bacteria. Therefore, it is possible that the amine group of SMX is also more easily oxidised by the radical cation released from the laccase-HBT system. However, although the presence of HBT enhanced SMX degradation, complete removal was not achieved. This may be attributed to the faster inactivation of laccase in the presence of HBT (Figure 3 b). According to Kurniawati et al.(2007), mediators such as HBT can cause inactivation of laccase by free radical attack on the catalytic site of the enzyme.

## CONCLUSIONS

This study compared the removal efficiency of four common trace organic contaminants from wastewater by a nitrifying activated sludge culture and whole-cell and extracellular enzyme extract of the white-rot fungus *Trametes versicolor* ATCC 7731. The whole-cell fungus culture achieved complete removal of bisphenol A and diclofenac, but no removal of carbamazepine and sulfamethoxazole. Although initial biosorption was evident, biodegradation governed the overall removal of bisphenol A and diclofenac. The fungal culture extract only removed bisphenol A, indicating possible roles of intracellular and/or mycelium-associated enzymes in fungal degradation of diclofenac. Of special interest was the capacity of the 1-hydroxybenzotriazole (mediator)- enhanced laccase catalysis to improve the removal of

sulfamethoxazole, but not carbamazepine, presumably due to the existence of a strong electron donating group (amine) in the former. In comparison with the fungus, biosorption played a more significant role in the removal of bisphenol A and diclofenac from the liquid phase by the activated sludge culture. In contrast to the fungal whole-cell culture, the activated sludge culture was able to achieve a low removal of carbamazepine and sulfamethoxazole. These results provide an important foundation for future studies exploring a combination of activated sludge and fungus culture for enhanced removal of the investigated groups of compounds.

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**LIST OF TABLES**

**Table 1** Removal efficiencies achieved by the options studied

| Compound | Removal from liquid phase[a] (%) | | | | | |
|---|---|---|---|---|---|---|
| | Activated sludge | | Fungus (whole-cell) | | Fungus (extracellular extract) | |
| | Inactivated[b] | Active | Inactivated[b] | Active | Without mediator[c] | With mediator[c] |
| Bisphenol A | ≈25 | 38 - 44 | 31 - 57 | 85 - 95 | 94 - 98 | ≈93 |
| Diclofenac | ≈9 | ≈23 | 28 - 52 | > 99 | 0 | 5 - 27 |
| Carbamazepine | ≈0.1 | 6 - 20 | 0 | 0 | 0 | 0 |
| Sulfamethoxazole | 0 | 13 - 17 | 0 | 0 | 0 | ≈41 |

[a] After an incubation period of 22 h at initial concentrations of 1200±250 (activated sludge), 1330±350 (whole-cell fungus) and 1450±360 (fungus extracellular extract) μg/L, respectively. Removal percentage values indicate the range observed between two replicates.

[b] Inactivated with 0.1 mM sodium azide to assess biosorption.

[c] 1 mM 1-hydroxybenzotriazole (HBT).

## LIST OF FIGURES

**Figure 1** Removal of trace organic contaminants from liquid phase during incubation of nitrifying sludge and chemically inactivated sludge in nitrifier enrichment media under continuous shaking at 120 rpm (25 °C). The initial pH and fungus concentration were 9 and 0.15 g/L, respectively. The measured initial concentration of bisphenol A, diclofenac, carbamazepine and sulfamethoxazole were 1500 μg/L, 1300 μg/L, 930 μg/L and 1100 μg/L, respectively. Control samples comprised trace organic contaminants in Milli-Q water. Sludge was inactivated by adding 0.1 mM sodium azide to the test solution. Each data point represents average of two replicates.

**Figure 2** (a) Removal of bisphenol A and diclofenac from liquid phase, and (b) the laccase activity of culture supernatant, during incubation of active and chemically inactivated fungus, in malt extract broth under continuous shaking at 120 rpm (25 °C). The initial pH and fungus concentration were 4.5 and 0.2 g/LL, respectively. The initial concentration of both bisphenol A and diclofenac was 6000 μg/L. Fungus was inactivated by adding 0.1 mM sodium azide to the test medium. Each data point represents average of two replicates.

**Figure 3** (a) Removal of trace organic contaminants from liquid phase, and (b) the change in enzymatic activity, during incubation with crude enzyme extract with and without a mediator (1-hydroxybenzotriazole) under continuous shaking at 70 rpm (25 °C). The measured initial concentration of bisphenol A, diclofenac, carbamazepine and sulfamethoxazole were 1700 μg/L, 1800 μg/L, 1200 μg/L and 1100 μg/L, respectively. The mediator was added to the test medium at a concentration of 1 mM. Each data point represents average of two replicates.

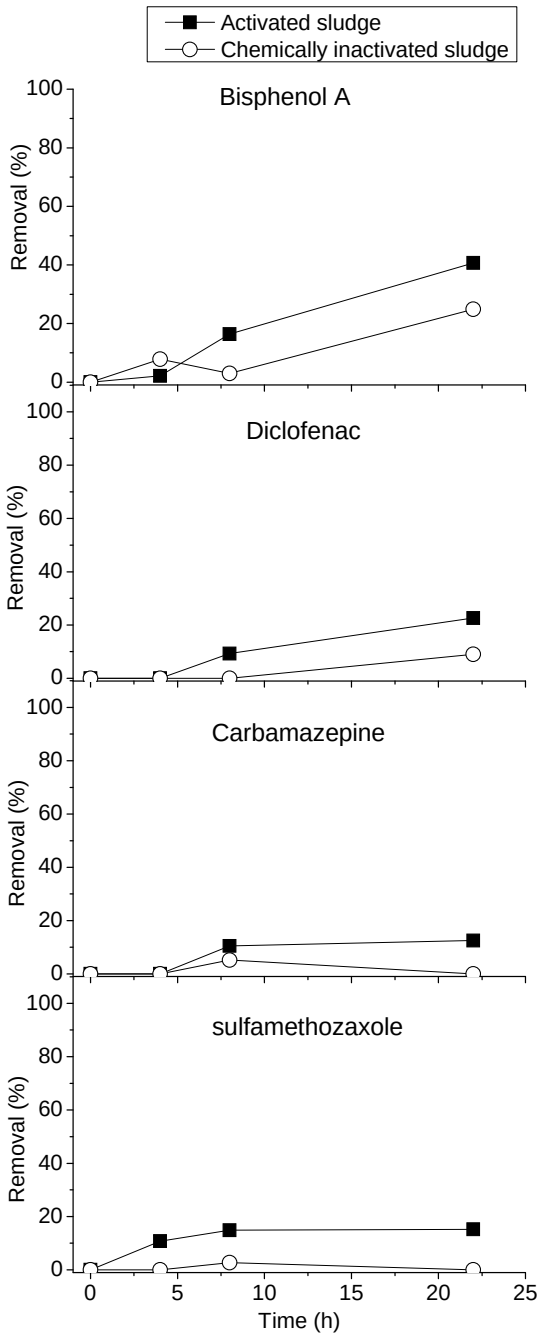
Activated sludge
Chemically inactivated sludge
Bisphenol A
Diclofenac
Carbamazepine
sulfamethozaxole
Removal (%)
Time (h)
0
5
10
15
20
25
0
20
40
60
80
100

**Figure 1**

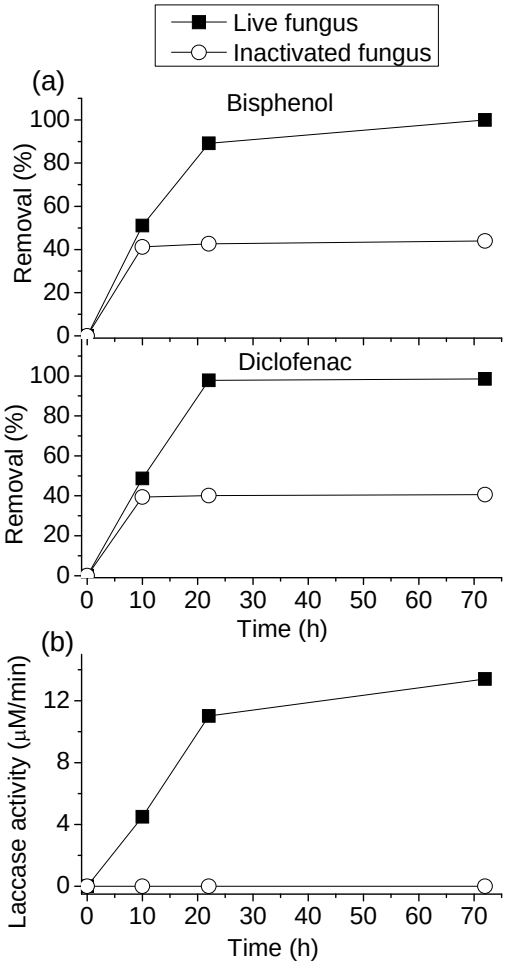
Live fungus
Inactivated fungus
(a)
Bisphenol
Removal (%)
Diclofenac
Removal (%)
Time (h)
(b)
Laccase activity (μM/min)
Time (h)

**Figure 2**

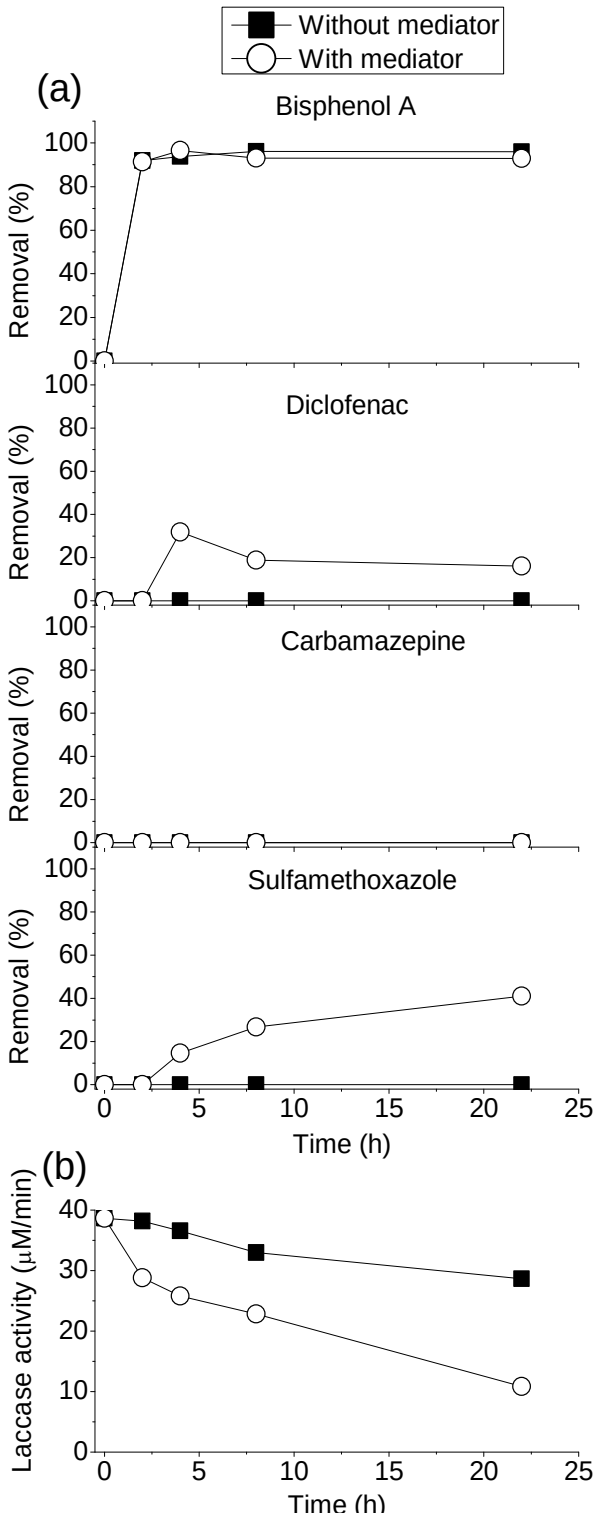
Without mediator
With mediator
(a)
Bisphenol A
Diclofenac
Carbamazepine
Sulfamethoxazole
Removal (%)
Time (h)
(b)
Laccase activity (μM/min)
Time (h)

**Figure 3**

# SUPPLEMENTARY DATA

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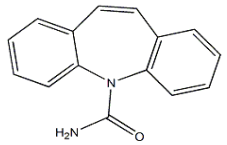
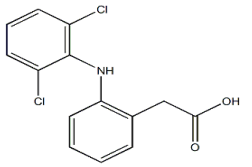
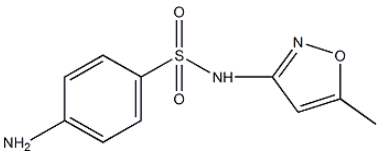
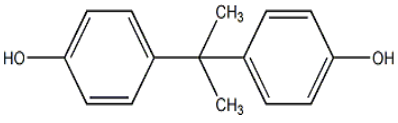
**Table S1:** Physicochemical properties of the selected trace organic contaminants.

| Category | Compound | **CAS number** | **Molecular weight (g/mol)** | **Log $K_{OW}$** [a] | **Log D (pH= 4)** [a] | Log D (pH= 9) [a] | **Dissociation constant** ( pKa) [a] | Water solubility (mg/L) [b] | Vapor pressure (mm Hg), at 25°C [a] | Boiling point (°C) [a] | Chemical structure |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pharmaceuticals | Carbamazepine ($C_{15}H_{12}N_2O$) | 298-46-4 | 236.27 | 1.89 ± 0.59 | 1.89 | 1.89 | 13.94 ± 0.20<br>-0.49 ± 0.20 | 17.7 | 5.78E-7 | 411 ± 48 |  |
| | Diclofenac ($C_{14}H_{11}Cl_2NO_2$) | 15307-86-5 | 296.15 | 4.55 ± 0.57 | 4.33 | 0.83 | 4.18 ± 0.10<br>-2.26 ± 0.50 | 2.37 | 1.59E-7 | 412 ± 45 |  |
| | Sulfamethoxazole ($C_{10}H_{11}N_3O_3S$) | 723-46-6 | 253.28 | 0.659 ± 0.409 | 0.65 | -1.28 | 5.81 ±0.50<br>1.39 ± 0.10 | 610 | 1.87E-9 | 482.1± 55.0 |  |
| Endocrine disrupting chemical | Bisphenol A ($C_{15}H_{16}O_2$) | 80-05-7 | 228.29 | 3.64 ± 0.23 | 3.64 | 3.62 | 10.29 ± 0.10 | 120 | 5.34E-7 | 401 ± 25 |  |

[a] Source: SciFinder database https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf
[b] Source: http://chem.sis.nlm.nih.gov/chemidplus/

**Table S2**: HPLC analysis methods

A Shimadzu HPLC system (Japan) equipped with a Supelco Drug Discovery C-18 column (with diameter, length and pore size of 4.6 mm, 300 mm and 5 μm, respectively) was used. The detection wavelength was 280 nm and column temperature was 20 °C. A sample injection volume of 50 μL was used.The mobile phase composed of acetonitrile and Milli-Q grade deionized water buffered with 25 mM $KH_2PO_4$. Two eluents, eluent A (80% acetonitrile + 20% buffer, v/v) and eluent B (20% acetonitrile + 80% buffer, v/v) were delivered at 0.7 mL/min through the column in time-dependent gradient proportions mentioned below:

| For carbamazepine and sulfamethoxazole | | | | | | |
|---|---|---|---|---|---|---|
| Time (min) | 0 | 5 | 8 | 10 | 11 | 20 |
| Eluent B, % | 100 | 100 | 40 | 40 | 100 | stop |
| **For bisphenol A and diclofenac** | | | | | | |
| Time (min) | 0 | 12 | 20 | 25 | 25.01 | 30.01 |
| Eluent B, % | 80 | 80 | 0 | 0 | 80 | stop |

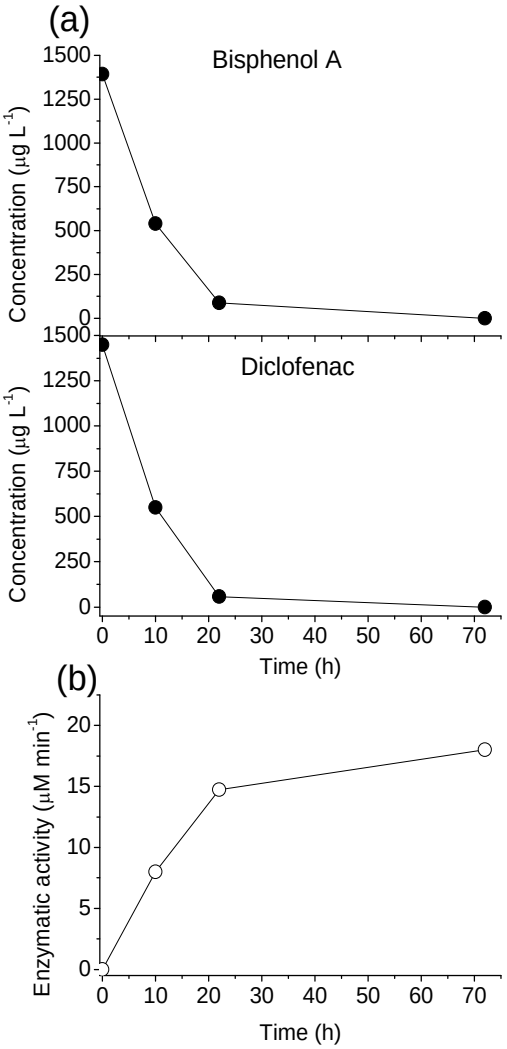



**Figure S3** (a) Removal of bisphenol A and diclofenac from liquid phase, and (b) the laccase activity of culture supernatant, during incubation of active and chemically inactivated fungus, in malt extract broth under continuous shaking at 120 rpm (25 °C). The initial pH, fungus concentration and concentration of the trace organic contaminants were 4.5, 0.2 g $L^{-1}$ and 1420±40 μg $L^{-1}$, respectively. Each data point represents the average of two replicates. Fungus was inactivated by adding 0.1 mM sodium azide to the test medium.

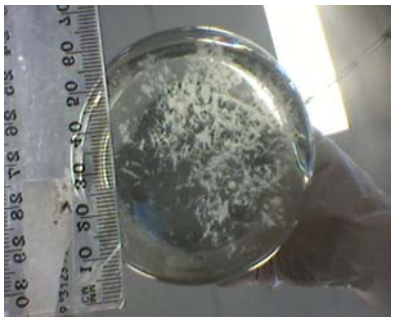
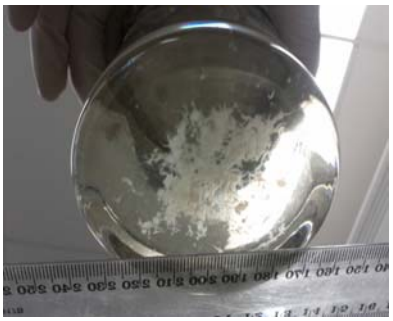
| | Active | Chemically inactivated |
|---|---|---|
| Day 0 |  |  |
| Day1 |  |  |
| Day3 |  |  |

**Figure S4**: Effect of chemical inactivation on fungus growth. Fungus culture was inactivated by adding sodium azide (0.1 mM).

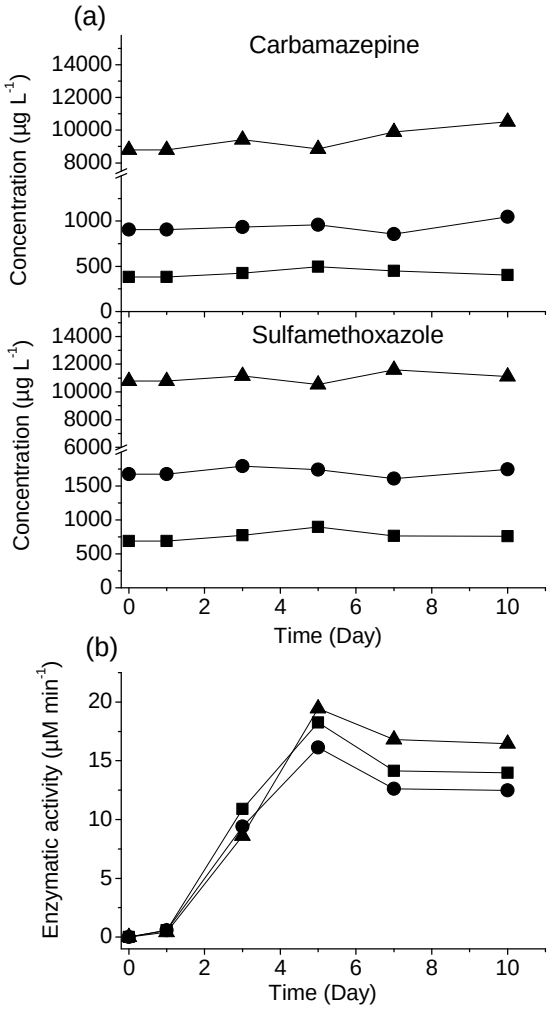



**Figure S5** (a) Removal of carbamazepine and sulfamethoxazole from liquid phase, and (b) the laccase activity of culture supernatant, during incubation of fungus in malt extract broth under continuous shaking at 120 rpm (25 °C). The initial pH and fungus concentration were 4.5 and 0.2 g $L^{-1}$, respectively. Three different initial trace organic contaminant concentrations (approximately 500 μg $L^{-1}$, 1,000 μg $L^{-1}$ and 10,000 μg $L^{-1}$) were tested. Each data point represents the