Removal of pharmaceutical' estrogenic activity of sequencing batch reactor effluents assessed in the T47D-KBluc reporter gene assay

Teddy Kabeya Kasonga[a], Martie A.A. Coetzee[a], Catherina Van Zijl[b] and Maggy Ndombo Benteke Momba[a]

[a]Department of Environmental, Water and Earth Sciences, Faculty of Sciences, Tshwane University of Technology, P/B X 680, Pretoria, 0001, South Africa
[b]Department of Urology, University of Pretoria, Private Bag X323, Arcadia, 0007, Pretoria, South Africa

HIGHLIGHTS

- A fungal consortium was successful for the bioremediation of synthetic wastewater.
- Simultaneous degradation of carbamazepine, diclofenac and ibuprofen was observed.
- Estrogenic activity was detected with metabolites compounds than parents.
- Fungal consortium was found to remove the metabolites' estrogenic activity.

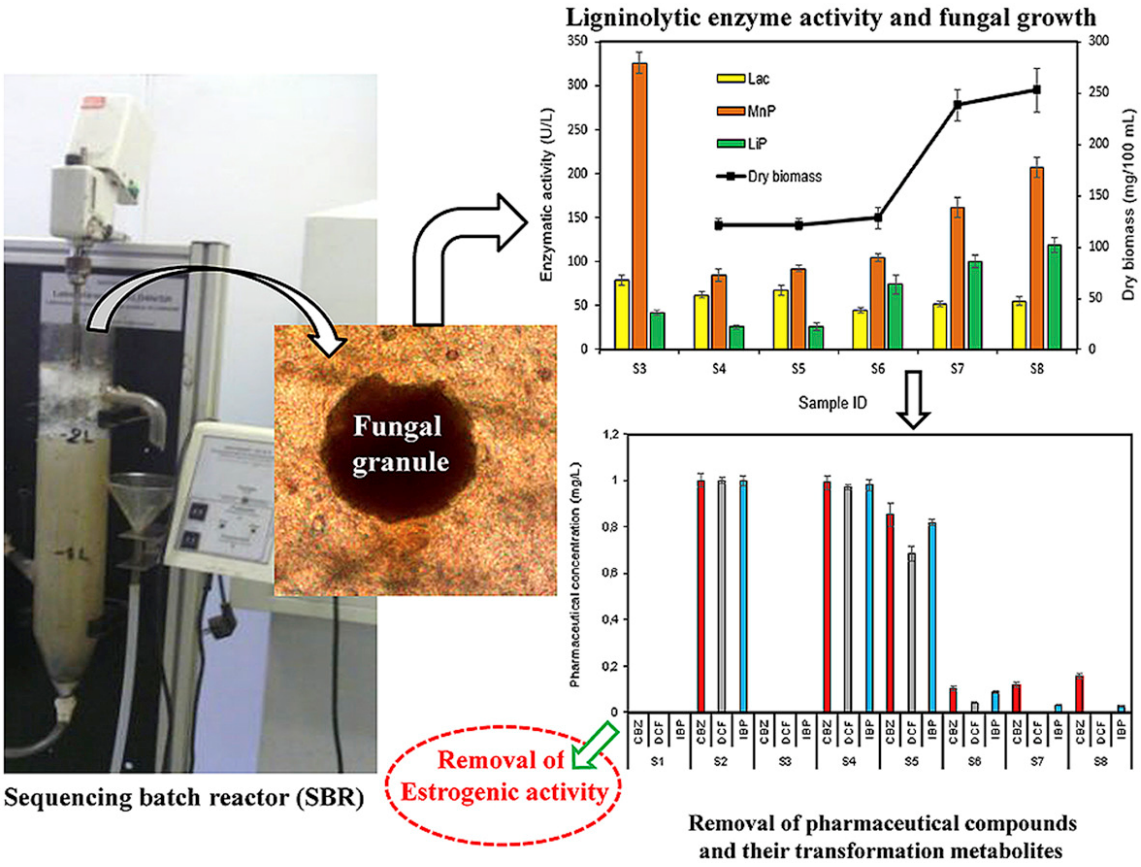
ABSTRACT

Various water treatment processes may be ineffective to remove pharmaceutical compounds (PhCs) and their by-products, leading to endocrine-disruptive activity that might be detrimental to wildlife and human health. This study investigated the degradation of carbamazepine (CBZ), diclofenac (DCF), ibuprofen (IBP), and their intermediates, as well as estrogenic activity that is not effectively removed by conventional methods. A consortium of isolated South African indigenous fungi *A. niger, M. circinelloides, T. polyzona, T. longibrachiatum* and *R. microsporus,* was used in a sequencing batch reactor (SBR) to remove PhCs, their intermediates and strongly reduce their estrogenic activity. The fungal ligninolytic enzymatic activity was determined for laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP) using a spectrophotometric method. The biodegradation of PhCs and their intermediates was monitored by SPE-UPLC/MS. The *in vitro* estrogenic activity was assessed in the T47D-KBluc

reporter gene assay. Lac, MnP and LiP production appeared to be biomass growth dependent. During a lag phase of growth, a constant biomass of about 122.04 mg/100mL was recorded with average enzymatic activity around 63.62 U/L for Lac, 151.91 U/L for MnP and 42.12 U/L for LiP. The exponential growth phase from day 7 to day 17, was characterised by a biomass increase of 124.46 units, and an increase in enzymatic activity of 9.91 units for Lac, 99.03 units for MnP and 44.24 units for LiP. These enzymes played an important synergistic role in PhCs degradation in the cytochrome P450 system. A decrease of 13.89 %, 29.7 % and 16.15 % in PhC concentrations was observed for CBZ, DCF and IBP, respectively, and their intermediates were identified within 4 h of incubation. The removal efficiency achieved after 24 h in the SBR was about 89.77%, 95.8% and 91.41% for CBZ, DCF and IBP, respectively. The estradiol equivalent (EEq) values of 1.71 ± 0.30 ng/L and 2.69 ± 0.17 ng/L were recorded at the start-up time and after 4 h, respectively. The presence of intermediates was found to induce estrogenic activity. The EEq values after 24 h incubation was found to be below the LoQ and below the LoD of the assay. None of the samples exhibited any anti-estrogenic activity. The fungal consortium inoculum was found to induce toxicity at a 0.4x concentration, as observed under a microscope. This study revealed that the use of the fungal consortium can remove the estrogenic activity of pharmaceutical metabolites, which appeared to be the most significant contributors to the endocrine-disrupting activity of the wastewater treatment plant effluents.

Key words: EDCs, effluents, T47D-KBluc reporter gene assay, estrogenic activity, Pharmaceutical compounds.

GRAPHICAL ABSTRACT



1 INTRODUCTION

Access to fresh water in South Africa is challenging, as the country receives average annual rainfall of 450 mm which is lower than the global annual rainfall of 860 mm (Benhin, 2015). Therefore, the implementation of micropollutant bioremediation management strategies for water bodies is necessary. Numerous pharmaceutical compounds (PhCs) are recalcitrant, are not effectively removed by conventional wastewater treatment plants (WWTPs) and are regarded as emerging environmental pollutants (Lloret et al., 2010). In addition, several emerging pollutants including PhCs found in the environment, have been revealed to be able to mimic or to inhibit the actions of the natural estrogen 17-β estradiol (E_2) and as such have been named environmental estrogens (Kiyama and Wada-Kiyama, 2015). These have been detected

in WWTP effluents, rivers, groundwater and drinking water (Lloret et al., 2010; Van Zijl et al., 2017).

Considering the complex structures and low bioavailability of most of these pollutants, their partial biodegradation from conventional biological or physicochemical treatment processes remains a challenge. Incomplete removal from conventional wastewater treatment processes necessitates investigation of alternative processes. Chemical/physical treatment such as advanced oxidation processes including ozonisation or ultraviolet (UV) exposure, have demonstrated variable degradation yields (Lloret et al., 2010). Another treatment option seems to be bioremediation and in this field, fungal strains and their ligninolytic enzymes have been found a valuable potential alternative for the removal of these recalcitrant pollutants and the reduction of their estrogenicity from the environment (Mao et al., 2010).

One of the challenges encountered in the application of advanced and biological treatment processes is the formation of intermediate compounds which display even higher estrogenicity than the parent compounds (Noguera-Oviedo and Aga, 2016; Rivera-Jaimes et al., 2018). Several *in vitro* assay methods have been used to assess estrogenic activity in aquatic environmental samples including yeast-based reporter assay and mammalian-based reporter gene assays. These assays include the yeast estrogen screen (YES), luciferase-transfected human breast cancer cell line (MELN) reporter gene assay, estrogen receptor chemically activated luciferase gene expression (ER-CALUX), and the T47D-KBluc reporter gene bioassay (Könemann et al., 2018; Van Zijl et al., 2017; Wilson et al., 2004).

In the present study which intended to evaluate the estrogenic and antiestrogenic activities of the sequencing batch reactor (SBR) effluents, the T47D-KBluc reporter gene bioassay was used. (Wilson et al., 2004).

The underlying principle of the method is that compounds that bind to and activate the ER will result in the ER dependent production of the luciferase enzyme. Agonist compounds induce luciferase expression and are compared to the vehicle control (media plus ethanol) or to the positive control 17β-estradiol (E_2, Figure 1.A). The synthetic estrogen receptor antagonist ICI 182,780 (Figure 1.B) is used in the bioassay as the positive control for anti-estrogenic activity. In the bioassay using the T47D-KBluc cells, an estrogen is defined as a chemical that induces dose dependent luciferase activity that can be specifically inhibited by the anti-estrogen ICI 182,780 (Wilson et al., 2004).

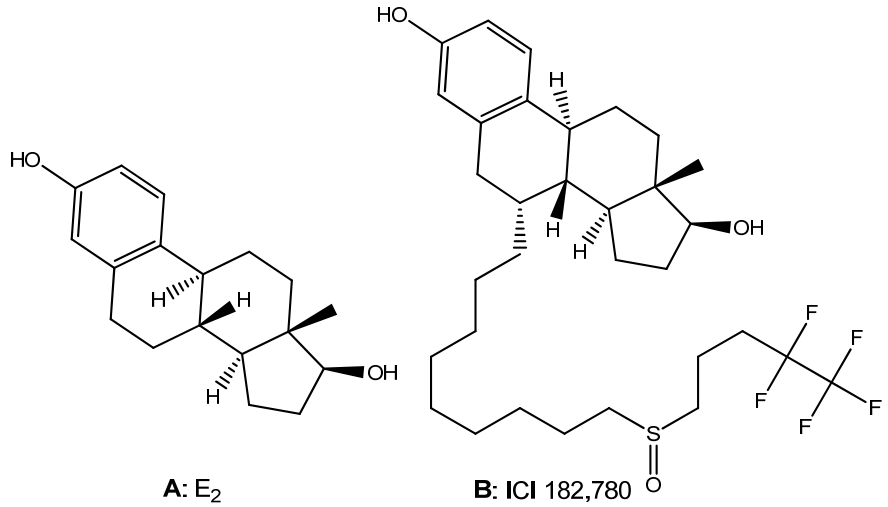


Figure 1: Chemical structures of the natural steroidal estrogen 17-ß estradiol, E_2 (A) and the steroidal antiestrogen, ICI 182,780 (B)

Based on their chemical structures, a number of estrogenic compounds are found to be organic, in particular phenolics or carbon ring structures of varying structural complexity (Darbre, 2006). A list of phenolic compounds as reported by Kiyama and Wada-Kiyama, (2015) is

provided by Kasonga et al. (2019). However, the majority of endocrine-disrupting compounds found in effluents, especially PhCs that show limited removal in wastewater treatment processes are polar compounds with complex aromatic chemical structures. These compounds have molecular masses normally ranging from 200 to 1000 Da. Therefore, a minor modification in the chemical structure might have an important impact on physicochemical properties that govern their environmental fate to some extent, e.g. their potential to interact with ERα and ERβ (Hui et al., 1998; Kiyama and Wada-Kiyama, 2015). When these compounds end up in drinking water or food, they may have adverse health effects in humans (Aneck-Hahn et al., 2009; Mahomed et al., 2008). The three selected PhCs in this study, namely carbamazepine (CBZ), diclofenac (DCF) and ibuprofen (IBP), also have aromatic chemical structures as shown in Figure 2. Their environmental concentrations range from a few ng/L to 100 µg/L (Agunbiade & Moodley, 2016; Ferrari et al., 2003). Higher drug concentrations can be found discharged in the aquatic environment from pharmaceutical factories ≥ 1 mg/L (Cardoso et al., 2014). Several studies have reported the estrogenic activity of PhCs, especially their intermediates rather than the parent compounds. Chronic exposure may lead to toxic effects (Efosa et al., 2017; Gröner et al., 2017; Jelic et al., 2012; Yu and Chu, 2009).

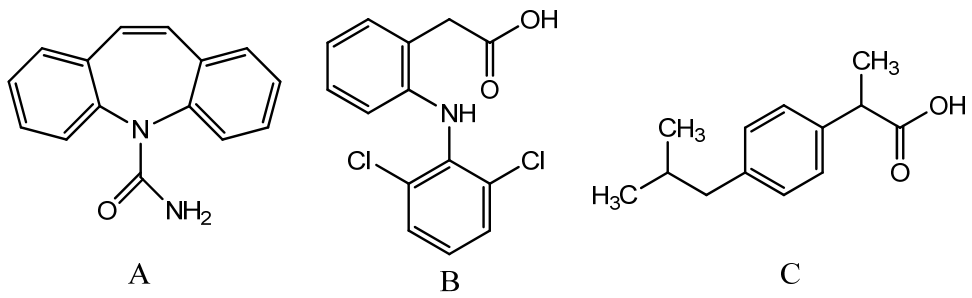


Figure 2: Selected pharmaceuticals carbamazepine (CBZ: A), diclofenac sodium (DCF: B) and ibuprofen (IBP: C)

Because of the poor removal of the selected PhCs CBZ, DCF and IBP from conventional WWTPs, the present study intended to use a consortium of five previously isolated and identified South African indigenous fungal strains in the SBR. This led to evaluate the fungal

ligninolytic enzyme activity, as well as the ability of the fungal consortium to degrade these recalcitrant compounds at a retention time of 24 h in the SBR. Moreover, the bioassay was conducted to evaluate the risk involved in the emission of the treated SBR effluents in reducing the production of intermediates and therefore, reduce their estrogenic activity.

2 MATERIALS AND METHODS

2.1 Sequencing batch reactor configuration and operational conditions

An SBR system was designed using a single glass cylindrical tank with a conical base and was filled with a working volume of 2 L of medium under non-sterile conditions out of a total volume of 3 L. The SBR had an inner diameter of 9 cm and a height of 47 cm, of which the conical base had a height of 11.5 cm (Figure 3). The SBR driven by a fungal consortium of five isolated South African indigenous fungi inoculated at 30% (v/v) of mycelium solution, was run at room temperature under continuous air-lift flow pressure of 8 mmHg, and at a stirrer shear force of 120 rpm. The level of air flow was displayed by a gas flow meter to monitor a continuous air supply. The SBR was run for a period of 17 days and working from day 3 at a retention time of 24 h and a settling time of 30 min to 45 min, for effluent collection and renewing media. Thereafter 200 mL solutions of 5-day-old fungal mycelium of each isolated fungus cultivated in the LN-m were combined in a 2 L flask to make 1 L of fungal consortium. Afterward, this was allowed to grow in the consortium for 3 days, without additional nutrients.

Figure 3: Sequencing batch reactor (SBR)

2.2 Experimental procedure

2.2.1 Preparation of media and solutions

The fungal mycelium solution

The isolated fungal strains were separately prepared in liquid LN-m at 30±1.5 °C for 5 days under static conditions in the dark as described by Tien and Kirk, (1988) and inoculated in the non-sterile SBR at a total consortium concentration of 30% (v/v). The composition of the LN-m solution is described in Table 1.

Table 1: Composition of the LN-m

Compound	Quantity	Compound	Quantity
D-(+)-glucose anhydrous (Sigma Aldrich Ltd, South Africa),	12000 mg/L	$CoCl_2.6H_2O$	7 mg/L
Trans aconitic acid 0.1 M (pH 4.3) (Sigma Aldrich Ltd, South Africa),	100 mL/L	$ZnSO_4.7H_2O$	7 mg/L
Ammonium tartrate dibasic (Sigma Aldrich Ltd, South Africa)	200 mg/L	$CuSO_4.5H_2O$	7 mg/L
$MgSO_4.7H_2O$ (Sigma Aldrich Ltd, South Africa)	521 mg/L	$Al_2(SO_4)_3.18H_2O$	0.7 mg/L
$CaCl_2$ (Sigma Aldrich Ltd, South Africa)	100 mg/L	H_3BO_3	0.7 mg/L
Nitrilotriacetic acid (MINEMA Chemicals, South Africa)	105 mg/L	$Na_2MoO_4.2H_2O$	0.7 mg/L
$MnSO_4.H_2O$ (Sigma Aldrich Ltd, South Africa)	35 mg/L	Thiamine chloride solution 1 g/L	1 mL/L
NaCl (Sigma Aldrich Ltd, South Africa)	70 mg/L	Veratryl alcohol (MINEMA Chemicals, South Africa)	100 mL/L
$FeSO_4.7H_2O$ (Sigma Aldrich Ltd, South Africa)	7 mg/L		

Medium composition in the sequencing batch reactor

The 2 L SBR was supplemented with 10 g/L of D-(+)-glucose anhydrous and 2 g/L of ammonium tartrate dibasic, as carbon and nitrogen sources, respectively. A volume of 2.5 mL/L of 1% Tween® 80 solution (polysorbate 80, MINEMA Chemicals, South Africa) was added as surfactant to protect fungi granules and enzymes from stirrer shear force (Liu and Wu, 2012; Tien and Kirk, 1988). A volume of 600 mL of fungal mycelium solution containing compounds as indicated in Table 1 was added. A 20 mL aliquot from 100 mg/L stock solution of the three PhCs was spiked in the SBR on day 3 so that the starting concentration was 1 mg/L. The typesetting of the SBR constitutes the synthetic wastewater composition. The synthetic wastewater chemical oxygen demand (COD) could be evaluated about 14511.2 mgO_2/L.

Solution of PhCs

The solution of CBZ, DCF and IBP combined was prepared by diluting 10 mL of the stock solution (100 mg/L) in a 1000 mL volumetric flask, to make a PhC solutions at a final concentration of 1 mg/L of each (S_2), using tap water.

2.2.2 Isolated fungal strains and targeted PhCs

The 5-day-old fungal mycelium solutions of the five isolated fungi were previously cultivated individually in a low nitrogen medium (LN-m) at 30 ± 1.5 °C as described by Tien and Kirk, (1988). After a start-up period of 3 days to allow the fungal consortium of previously isolated South African indigenous fungi, *A. niger* (Figure 4.A), *M. circinelloides* (Figure 4.B), *T. longibrachiatum* (Figure 4.C), *T. polyzona* (Figure 4.D) and *R. microsporus* (Figure 4.E) to accommodate the working conditions in the SBR, the mixture of PhCs was added. The selected PhCs were spiked in the SBR for the study: CBZ (Figure 2.A), DCF (Figure 2.B) and IBP (Figure 2.C). Fungal mycelial morphology (Figure 4.F) was observed during the lag phase of growth, and granule morphology (Figure 4.G) appeared from day 6.

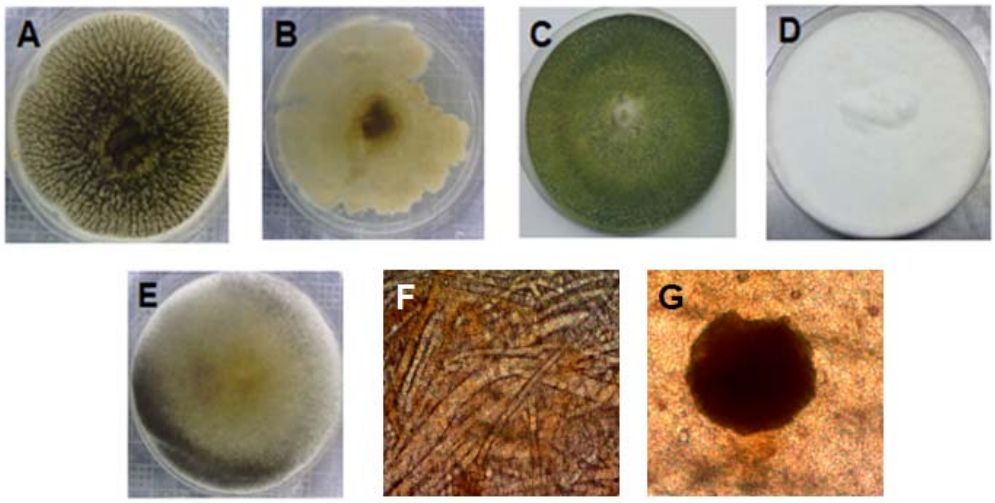


Figure 4: Isolated and identified South African indigenous fungal strains: *Aspergillus niger* (A), *Mucor circinelloides* (B), *Trichoderma longibrachiatum* (C), *Trametes polyzona* (D) and *Rhizopus microsporus* (E) cultured in solid medium (YMPG according to Tien & Kirk, 1988), mycelium morphology (F) and granule morphology (G) of the fungal consortium in the SBR (in Section 2.1).

2.2.3 Sample ID

Apart from tap water (S_1), a solution of the combined selected PhCs CBZ, DCF and IBP, at a concentration of 1 mg/L of each (S_2) and fungal consortium medium in LN-m (S_3), samples were collected from different day's effluents of the SBR performing with the consortium of isolated fungi. The effluent samples were collected after spiking selected PhCs (on the third day: S_4), after 4 h (S_5), on the 7th day (S_6), on the 12th day (S_7) and on the 17th day (S_8) as shown in Table 2.

Table 2: Sample descriptions

Sample ID	Description	Appearance after SPE
S_1	Tap water	Clear and colourless
S_2	CBZ+DCF+IBP solution at the concentration of 1 mg/L of each	Clear and colourless
S_3	Fungal consortium medium in LN-m	Brownish
S_4	SBR effluent of 3rd d (spiking day)	Brownish
S_5	SBR effluent of 4 h after spiking	Brownish
S_6	SBR effluent of 7th day	Slightly brownish
S_7	SBR effluent of 12th day	Yellowish
S_8	SBR effluent of 17th day	Yellowish

2.3 Enzymatic activity assay and fungal growth evaluation

The enzymatic activity assay of samples S_3 to S_8 was performed for the three targeted fungal ligninolytic enzymes. Lac (E.C. 1.10.3.2), MnP (E.C. 1.11.1.13) and LiP (E.C. 1.11.1.14) activities were assessed by oxidation of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma Aldrich, South Africa), MnP by using 2,6-dimethoxyphenol (2,6-DMP, Sigma Aldrich, South Africa) and LiP with veratryl alcohol (VA, Sigma Aldrich, South Africa), in the presence of H_2O_2, according to Tien and Kirk, (1988), Ryan et al., (2005) and Silva Lisboa et al., (2017). Therefore, a UV-Vis spectrophotometer HACH model DR 6000^{TM} (Colorado, USA) provided with 10 mm glass cells was used to monitor the oxidation of ABTS at 420 nm,

VA at 310 nm and 2,6-DMP at 470 nm. Fresh filtered SBR's effluent samples were used for the enzymatic activity assays.

Fungal growth evaluation was carried out by determining the fungal consortium dry biomass mg per 100 mL of samples from different days (3, 6, 9, 12 and 15) as described by previous investigators (Yamamoto et al., 2017).

2.4 Chemical analysis using SPE-UPLC/MS method

Targeted PhC concentrations of SBR effluent samples were analysed using the SPE-UPLC/MS method previously developed and validated. Solid phase extraction (SPE) was performed using Supel^{TM}-Select Supelco-HLB 500 mg, 12 mL plastic cartridges (Sigma Aldrich, South Africa) for samples S_4 to S_8 as described in the literature, with slight modifications (Agunbiade and Moodley, 2016; Van Zijl et al., 2017). The SPE cartridge columns were pre-conditioned with 5 mL of methanol (HPLC grade, Sigma Aldrich, South Africa) and equilibrated using 5 mL of 10% methanol followed by 5 mL of double de-ionized water (ddH_2O) at pH 2.5. Filtered samples of 1 mL diluted at 10 mL with ddH_2O at pH 2.5 (adjusted with 32% HCl, Sigma Aldrich, South Africa) were loaded onto the column at a flow rate of ± 1 mL/min and air dried by vacuum. The PhCs were eluted with 6 mL of methanol in 10 mL tubes. The solvent was evaporated at room temperature under a gentle nitrogen stream using an evaporation unit (Thermo Scientific Reacti-Vap 1, Germany) to bring the sample volume to ±1 mL, which was transferred into a 1.5 mL amber vial. Thereafter, the solvent was evaporated to dryness and sample residues were reconstituted to a volume of 1 mL of water-methanol (1:1, v/v HPLC grade, Water Microsep Pty Ltd, South Africa) before injection into UPLC/MS.

The UPLC/MS system used was the Waters Aquity UPLC® system hyphenated to a quadrupole-time-of-flight (QToF: Waters Inc., Milford, Massachusetts, USA) using a Titan C_{18} 80 Å column (2.1 mm ID x 100 mm length, 1.9 µm particle size, Supelco, Sigma-Aldrich, South Africa). Data acquisition was performed using MassLynx™ (version 4.1) software (Waters Inc., Milford, Massachusetts, USA). Selected PhCs were identified under positive mode at a protonated or sodium adduct ion mass *m/z* of 237.10 for CBZ at 2.750 ± 0.1 min, of 296.02 for DCF at 4.90 ± 0.2 min and of 229.12 for IBP at 4.99 ± 0.3 min (Kasonga et al., 2019). A full-scan resolution of *m/z* between 50 and 1200 Da was recorded for PhC intermediates detection. A reverse phase step gradient elution scheme was used to complete the separation from 60% ddH_2O (0.1% formic acid) to 95% methanol (0.1% formic acid). The gradient started with an isocratic flow (hold 0.5 min) followed by a linear increase to 95% methanol (6.5 min); subsequently, the column was washed for 0.8 min followed by conditioning and re-establishment of initial conditions to allow for equilibration before the start of the next run for the complete elution scheme. The flow rate was set at 0.3 mL/min for the entire run giving a total run time of 9 min, in a column kept at a constant temperature of 40 °C. Injection volumes were set at 5 µL (Gracia-Lor, Sancho, & Hernández, 2010; Wang et al., 2011). The method was thereafter validated according to parameters such as linearity, limit of detection (LoD), limit of quantification (LoQ), accuracy and precision (Lee et al., 2008; Zhao et al., 2018).

2.5 Assessment of estrogenic and anti-estrogenic activities in the T47D-KBluc assay

To assess the estrogenic activity in the T47D-KBluc assay of the pre-filtered effluents (S_4 to S_8) from the SBR and of CBZ, DCF and IBP mixture aqueous solution (S_2), both estrogenic activity and anti-estrogenic activity were assessed as described below. Tap water (S_1) used for the preparation of non-sterile media and fungal consortium medium without the selected PhCs (S_3), was also tested.

2.5.1 Solid phase extraction procedure

SPE Oasis® HLB 60 µm (LP), 500cc glass cartridges (Water Microsep Pty Ltd, South Africa) were used for T47D-KBluc's samples. Prior to carrying out the T47D-KBluc bioassay of the pre-filtered samples (S_1 to S_8, at adjusted pH 3) from the SBR, the sorbent Oasis® HLB 60 µm (LP), 500cc glass cartridges (Water Microsep Pty Ltd, South Africa) were used to carry out the SPE method according to the slightly modified extraction protocol of the WRC toolbox of bio-assays for detection of estrogenic and anti-estrogenic activities in water samples (de Jager et al., 2011).

Briefly described: the SPE Oasis® HLB cartridges were pre-conditioned for 30 min using 5mL of methanol (HPLC grade, Sigma Aldrich, South Africa), followed by 5 mL of 10% solution ddH_2O-methanol and 5mL of ddH_2O (pH 3, adjusted using a solution of 32% HCl or 5M NaOH, Sigma Aldrich, South Africa), all at a flow rate of 1mL/min (drop by drop). Thereafter, 200 mL samples were loaded under vacuum at a pressure of 5mmHg. The cartridges were dried for 30 min using vacuum and elution was performed at a flow rate of 1mL/min (drop by drop) with 5 mL of methanol maintained for 30 min before elution and a vacuum was used for the collection of the eluate. The eluates were collected in 10 mL tubes and completely dried under a gentle stream of nitrogen, before being reconstituted with 1mL of ethanol (HPLC grade, Sigma Aldrich, South Africa). The reconstituted samples were transferred to 1.5 mL amber vials and stored at -20 °C.

2.5.2 T47D-KBluc reporter gene bioassay procedure

The estrogenic and anti-estrogenic activities of the different samples (Table 2) were evaluated using the estrogen dependent reporter gene bioassay in T47D-KBluc breast cancer cells, according to the method developed by the USEPA to screen environmental samples and

chemicals (Wilson et al., 2004). The experiment was performed at the University of Pretoria's Environmental Chemical Pollution and Health Research Unit laboratory as described by previous investigators (de Jager et al., 2011; Van Zijl et al., 2017; Wilson et al.2004). The T47D-KBluc cells were purchased from the American Type Culture Collection (ATCC, USA). Cells were maintained in RPMI medium (Sigma Aldrich, USA), supplemented with 10% foetal bovine serum, characterised (FBS, Hyclone Laboratories, USA) and antibiotic/antimycotic solution (Sigma Aldrich, USA).

Prior to the bioassay, the T47D-KBluc cells were grown for 7 days in RPMI medium supplemented with 10% charcoal/dextran treated FBS (c/d FBS, Hyclone Laboratories, USA), without antibiotic to withdraw the cells from steroids. Cells were seeded at $5x10^4$ cells per well (100µL per well) in 96-well luminometer plates with clear bottoms (Corning Costar, Scientific group) using dosing RPMI medium supplemented with 5% c/d FBS and allowed to attach overnight. A dilution series of the controls E_2 and ICI 182,780, and of the samples were prepared in ethanol (EtOH). An aliquot of 2µL of the concentrations in EtOH was added to 1000 µL dosing medium. The solvent (EtOH) did not exceed 0.2% in the final dosing solutions. The medium in 96-well luminometer plates was replaced with 100 µL of the dosing medium. In addition to the samples, each plate contained an E_2 positive control standard curve (ranging from 0.1 nM to 0.3 pM), an antagonist control (10nM ICI 182,780 + 0.1 nM E_2), a solvent control [0.2% EtOH=vehicle control (V contr)] and a background control (10 nM ICI 182,780). An ICI 182,780 standard curve (ranging from 10 nM to 10 pM), co-incubated with 0.1 nM E_2 was used to determine the anti-estrogenic activity of antagonists. Each sample was tested alone, as well as in the presence of E_2 (0.1 nM) and ICI 182,780 (10 nM) respectively. The exposed plates were returned to the incubator (37 °C, 5% CO_2) for 24 h, after which, they were assessed under a microscope for any signs of cytotoxicity (e.g. condensed cell contents or "weathered"

cells). The dosing solutions were removed by gentle shaking of the plates over a waste tray (from this point forward aseptic conditions were not necessary). The cells were washed with 200 µL phosphate buffer saline (PBS, Gibco Life Technologies corporation, UK), at room temperature. The PBS was discarded and 25 µL lysis buffer (Promega, USA) was added to each well. The plates were placed in a -80 °C freezer overnight, as the lysis buffer was activated by one freeze/thaw cycle. The plates were thawed on a Thermostar plate warmer/shaker (BMG Labtech, Germany) and all reagents were allowed to reach room temperature before determining the luciferase activity using a LUMIstar OPTIMA luminometer (BMG Labtech, Germany) with two dispensers. The luminometer was set to inject into each well, 25 µL of reaction buffer, followed 5 sec later by 25 µL of 1 mM D-luciferin. The luciferase activity was quantified as relative light unit (RLU). The bioassay media preparation details are available in the section on supplementary materials (Kasonga et al., 2019).

2.5.3 Calculation of results

The estrogenic activity of the assay was reported for samples inducing dose dependent luciferase activity, which could be specifically inhibited by the anti-estrogen ICI 182,780 (Wilson et al., 2004). The RLU readings were transformed to fold induction relative to the solvent control (RLU/average RLU reading of solvent control) and expressed as a percentage of the maximum (0.1 nM E_2) response. EEq values were determined for samples using Graphpad Prism software (version 4). The X-values (concentrations 0.1 nM to 0.3 pM) were log transformed and the E_2 curve was fitted (sigmoidal function, variable slope). From the E_2 standard curve, the sample concentrations were calculated as unpaired Y-values and were corrected for the corresponding dilution factors to determine their EEq values. The EEq values were reported as the average ± SD (standard deviation) of triplicate plates (n = 3). The EC_{10} (10% effective concentration) of the assay was considered as the LoQ for agonist activity, and

the sample concentration for which the GraphPad Prism software cannot interpolate the value from the E_2 curve were considered below the LoD. The EC_{50} was calculated from the E_2 curve as the concentration at which 50% maximal luciferase activity was induced. The anti-estrogenic activity of the assay was quantified from the fitted ICI 182,780 curve (co-incubated with 0.1 nM E_2). The 50% inhibitory concentration (IC_{50}) was determined for the ICI 182,780 control curve for each plate. The LoQ for the anti-estrogenic activity was set as the concentration causing 20% inhibition (IC_{20}). Fitted figures of the standard curves of E_2 and ICI 182,780 are provided in Data in Brief (Kasonga et al., 2019).

3 RESULTS

Despite the agitations due to the continuous air supply and the stirrer shear force, the SBR operating in continuous mode appeared to be suitable for the fungal consortium of isolated fungi. Even though the renewal of the medium (inflow synthetic wastewater) was increasing the pH to around 3.9 to 4.6, the pH in the SBR was found to be decreasing below 3 in the samples after 24 h, indicating that the fungal consortium was active. In addition to that, the increase recorded in the fungal dry biomass described below also demonstrated the growth of the fungal consortium as well as its activity in the SBR.

3.1 Enzymatic activity and fungal consortium growth

Figure 5 displayed the enzymatic activities evaluated for samples S_3 to S_8. The results revealed that, MnP was the highest produced ligninolytic enzyme, followed by Lac for samples S_3, S_4 and S_5. More LiP than Lac appeared to be produced from day 6 in the SBR during the fungal exponential growth and granules development (samples S_6, S_7 and S_8). The 3-day-old fungal consortium in LN-m (S_3) exhibited higher Lac and MnP activities ($p < 0.05$), 88.99 ± 5.12 U/L and 325.53 ± 12.11 U/L, respectively than in the SBR initiated with 30% of fungal inoculum

solution. MnP was significantly higher in LN-m than in SBR ($p < 0.05$). However, LiP production (42.08 ± 3.14 U/L) was found to be lower compared to samples S_7 and S_8, 100.24 ± 7.41 U/L and 118.56 ± 8.27 U/L, respectively. The SBR seemed to be suitable for peroxidase enzymes production namely MnP and LiP. Hence, the MnP and LiP activities achieved in the SBR were found to increase from the PhC spiking day (day 3, S_4) 84.55 ± 7.54 U/L and 25.99 ± 2.15 U/L to the final day (day 17, S_8) 207.14 ± 11.11 U/L and 118.56 ± 8.27 U/L, respectively.

Although fungal consortium dry biomass was not evaluated in 3-day-old consortium grown in LN-m (S_3), fungal biomass was found to be constant during the lag phase of growth (day 0 to day 6), after which it increased on the exponential growth phase (day 6 to day 17) as shown in Figure 5. The influent containing fresh nutrients appeared to favour biomass growth in the working conditions of a retention time of 24 h. Therefore, on the starting day, a fungal dry biomass of 122.04 ± 6.01 mg/100 mL was determined (S_4 and S_5), and after 9 days (on day 12) 238.31 ± 15.13 mg/100 mL was recorded. The fungal consortium biomass generated after 12 and 17 days was found to be significantly higher in samples S_7 (238.31 ± 15.13 mg/100 mL) and S_8 (253.01 ± 21.11 mg/100 mL) ($p < 0.05$), compared to S_4, S_5 and S_6. This was an indication of the activity of the fungal consortium in the SBR during its exponential growth phase. However, after day 12, the fungal consortium seemed to begin the stationary phase of its growth. Lac, MnP and LiP production appeared to be biomass growth dependent.

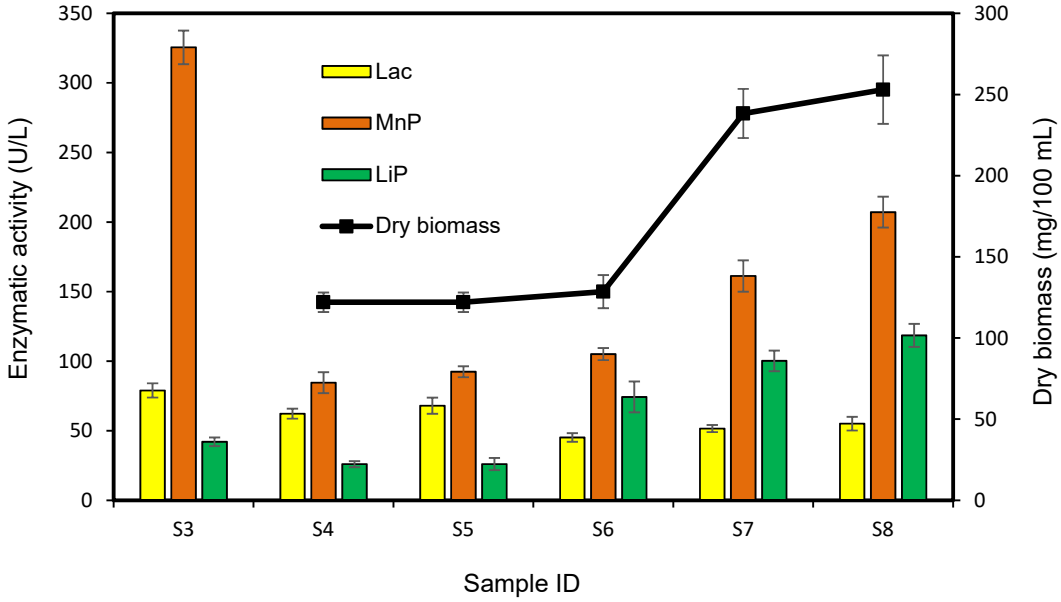


Figure 5: Evaluation of fungal enzymatic activity and dry biomass from collected samples

3.2 Analysis of selected PhCs

The SBR operating in continuous mode (retention time 24 h) was used with the aim of removing the PhCs, namely CBZ, DCF and IBP from the synthetic wastewater and also to limit the presence of their intermediate products, which could display estrogenic activities.

In respect of the matrix effect of 0.15%, less than 20% was recorded using the SPE-UPLC/MS method. The method developed was validated in terms of parameters including linearity, LoD, LoQ, accuracy and precision. Therefore, the correlation coefficient by linear curves of the PhCs was found to be greater than 0.95 (0.98 for CBZ, 0.99 for DCF and 0.96 for IBP, Kasonga et al., 2019). The mean recoveries and RCD calculated were found acceptable for the method validation at 106.20% and 8.45% for CBZ, 104.62% and 7.33% for DCF, and 102.89% and 8.5% for IBP. In addition, the LoD and LoQ of the SPE-UPLC/MS method were determined for the three selected compounds and values of $97x10^{-6}$ mg/L and $32.4x10^{-5}$ mg/L for CBZ,

$24x10^{-5}$ mg/L and $81x10^{-5}$ mg/L for DCF, and $44.7x10^{-4}$ mg/L and $17.16x10^{-3}$ mg/L for IBP were recorded (Kasonga et al., 2019).

Figure 6 shows the residual concentrations of the parent PhCs for the samples collected from SBR. Within 4 h of incubation (S_5), a decrease of 13.89%, 29.7% and 16.15% in PhC concentrations was already observed for CBZ, DCF and IBP, respectively, when compared to the inflow (S_4). The removal efficiency achieved within a period of 24 h (after 7 days, S_6) was 89.77%, 95.8% and 91.41% for CBZ, DCF and IBP. Significantly higher for S_6 ($p < 0.05$) compared to S_5. After 12 and 17 days from samples S_7 and S_8, the SBR accomplished a PhC removal efficiency of respectively 88.09% and 84.32% for CBZ, 99.77% and 99.84% for DCF and 97.03% and 97.48% for IBP. Although the SBR was run in continuous mode at a retention time of 24 h, the PhC removal appeared to increase over time, especially for DCF and IBP. This could be due to the acclimatisation of fungi to the medium, but also to the fungal consortium granulation, when comparing the residual concentrations from S_6 to S_8.

The active fungal consortium of the five isolated South African indigenous strains already biodegraded the spiked compounds within 4 h and produced the majority of previously identified PhC intermediate products detected in sample S_5, while the peaks of their mass *m/z* were found to be very low in sample S_6. Therefore, molecular ions of the transformation products and their sodium adduct showed accurate mass *m/z,* suggesting the intermediates identified as major transformation products of the selected PhCs in sample S_5, in Table 3. However, only very weak peak intensities of these transformation compounds were detectable in samples S_6 and S_8. See example of transformation fragment ion peaks in the section on supplementary materials (Kasonga et al., 2019).

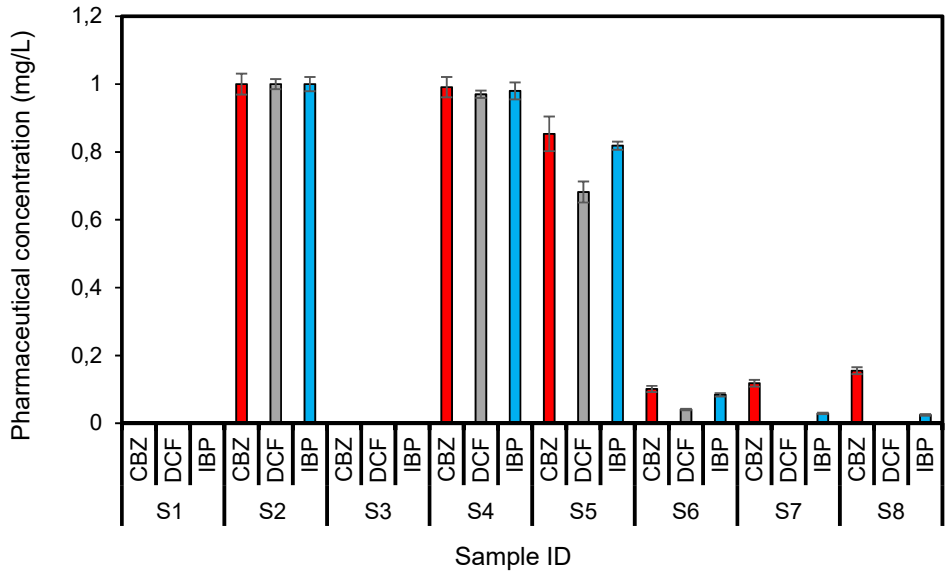


Figure 6: Residual concentrations (mg/L) of the selected pharmaceutical compounds in the collected samples from SBR. Not applicable for samples S_1 and S_3. S_2: prepared concentration of 1 mg/L.

Table 3: Accurate mass *m/z* of the suggested ionized structures of transformation intermediate compounds from CBZ, DCF and IBP determined by UPLC-(+)-ESI-QToF-MS

Parent	Suggested intermediate	*m/z*
CBZ	acridine	180.08
	iminoquinone	208.07
	9-acridine carboxaldehyde	208.07
	acridone	196.07
	10,11-dihydro-10,11-dihydroxy CBZ	293.09
	10,11-dihydro-10-hydroxy CBZ	255.11
	CBZ-2,3-quinone	267.07
	2,3-dihydroxy-CBZ	269.09
	2,3-dihydro-2,3-dihydroxy CBZ	271.10
	2-hydroxy imino stilbene	232.07
	9-hydroxymethyl-10-carbamoyl acridan	243.11
DCF	3'-hydroxy DCF	334.00
	4'-hydroxy DCF	
	5-hydroxy DCF	
	4',5-dihydroxy DCF	349.99
IBP	1-hydroxyl IBP	223.13
	2-hydroxy IBP	
	Carboxy IBP	237.11
	1,2-dihydroxy IBP	239.12

3.3 Bioassay for estrogenic and anti-estrogenic activities

The sample estrogenic and ant-estrogenic activities were interpolated from the E_2 and ICI 182,780 standard curves, respectively (Kasonga et al., 2019). EEq values were determined by

reading the samples from the E_2 curve on the same plate, in order to account for any inter-plate variation. An average EC_{50} value with standard deviation of 0.91 ± 0.07 ng/L was obtained. The EC_{10} of the assay for agonist activity (Kasonga et al., 2019) was calculated to be 0.31 ± 0.06 ng/L. Furthermore, for the antagonist activity, an IC_{50} from ICI 182,780 curve (Kasonga et al., 2019) for each plate was monitored and an average IC_{50} value of 1407 ± 215 ng/L was determined. The average IC_{20} value was 963 ± 408 ng/L.

The estrogenic activities assayed in different samples S_1 to S_8 using the T47D-KBluc bioassay are displayed in Table 4. The results demonstrated that none of the samples, from tap water used as solvent in non-sterile experimental working conditions, the parent PhCs CBZ, DCF and IBP, or the SBR effluents, exhibited anti-estrogenic activity. None of the samples co-incubated with E_2 demonstrated any antagonist activity by binding to the E_2 receptor and blocking the receptor, which could result in decreasing the E_2 response in inducing luciferase activity. The highest EEq value was 20.07 ± 10.29 ng/L in the 3-day-old fungal consortium medium, which was grown in LN-m (S_3). This sample exhibited toxicity to cells observed under a microscope at the highest concentration of 0.4x. An EEq value of 1.71 ± 0.30 ng/L was recorded on the starting day (S_4) and an EEq of 2.69 ± 0.17 ng/L in the sample collected after 4 h (S_5). Estrogenic activity was detected in samples S_3 and S_5 from much lower concentrations (0.012x and 0.12x concentrated samples respectively), compared to S_4, where estrogenic activity was only detected in the highest concentration tested (0.4x concentrated sample). Despite the high PhC concentration of 1 mg/L of each in the mixture solution used for the bioassay, the EEq values were found to be below the LoD. In addition, none of the SBR effluent collected (samples S_6, S_7 and S_8) was recorded above the LoQ in the T47D-KBluc bioassay for estrogenic activity. The fungal consortium appeared to remove the estrogenic activity noticed on the spiking day

and after 4 h, indicated in samples S_4 and S_5. After 4 h, estrogenic activity was found to increase in the SBR for the same medium from 1.71 ± 0.29 ng/L to 2.69 ± 0.17 ng/L.

Table 4: Estrogenic and anti-estrogenic activity assays

Sample	EEq (ng/L)	Comments
S_1	< LoQ	No anti-estrogenic activity detected
S_2	< LoD	No anti-estrogenic activity detected
S_3	20.07 ± 10.29	Toxicity observed under microscope at 0.4x concentration, and no anti-estrogenic activity detected
S_4	1.71 ± 0.29	No anti-estrogenic activity detected
S_5	2.69 ± 0.17	No anti-estrogenic activity detected
S_6	< LoQ	No anti-estrogenic activity detected
S_7	< LoD	No anti-estrogenic activity detected
S_8	< LoQ	No anti-estrogenic activity detected

< LoQ: below level of quantification of the assay
< LoD: below detection limit of the assay
Values in this table were adjusted for the 1000x concentrations from the SPE samples.

4 DISCUSSION

The goal of the current study was to evaluate the efficiency of the SBR driven by a fungal consortium of five isolated South African indigenous species in the removal of the estrogenic activity of PhC transformation metabolites using the T47D-KBluc gene reporter bioassay.

4.1 Enzymatic activity and fungal consortium growth

Based on the results, the consortium of isolated fungi in the SBR was found, in the experimental conditions, to produce a considerable amount of ligninolytic enzymes, namely Lac, LiP and MnP (Figure 5). Peroxidase enzymes were found to increase in SBR, while the pH of the medium tended to decrease. The increase in the peroxidase enzymes production, specifically MnP and LiP observed in the SBR, could be due to the pH of the medium that was found to be below 4, which is unfavourable for Lac enzyme production in working experimental conditions. The decrease in the pH of the fungal submerged culture has been attributed to fungal metabolites

such as citric acid production (Papagianni, 2004). This was indicative of fungal consortium activity in the SBR, which was confirmed by the increase in its dry biomass, especially during the exponential growth phase (Figure 5), as a result of total mutual intermingling of hyphae. These fungal ligninolytic enzymes, which are known for their ability to biodegrade organic pollutants (Tien and Kirk, 1988; Zhang and Geißen, 2010a) could contribute to the removal of the three selected PhCs recorded in the SBR, specifically CBZ, DCF and IBP, and therefore reduce or eliminate the estrogenic activity of their respective by-products, as noticed. Several studies have reported the high redox potency of ligninolytic enzymes from fungi which enable them to oxidise various organic pollutants (Pinar et al., 2017). For instance, a study conducted on the biodegradation of CBZ from wastewater using basidiomycetes white-rot fungi *Trametes versicolor* attributed that and the production of intermediates in particular to the ligninolytic enzymes (Jelic et al., 2012). The cytochrome P450 system was also reported to play a synergistic role with ligninolytic enzymes in the biodegradation of PhCs (Jelic et al., 2012; Marco-Urrea et al., 2009).

4.2 Removal of PhCs and intermediates

Fungal activity in the SBR has led to the biodegradation of the selected PhCs CBZ, DCF and IBP (Figure 6) and the production of corresponding intermediates that are supposed to be due to the catalysis of secreted ligninolytic enzymes and other metabolites by the fungal consortium. However, although the SBR was run at a retention time of 24 h, the ligninolytic enzymes production was evolving increasingly over time, while the residual concentrations of the spiked PhCs were decreasing. Hence, the lower residual concentration of the parent compounds achieved can lead to less production of intermediate compounds in the media as reported by Jelic et al., (2012), and therefore the suppression of estrogenic activity. However, while 73.97% of CBZ, 93.20% of DCF and 80.51% of IBP removals were reached in the first batch after 24

h, almost 89.88% of CBZ, 95.93% of DCF and 95.77% of IBP removals were achieved in the fourth batch (sample S_6). Only 54% of CBZ removal was reached by Jelic et al., (2012) using *Trametes versicolor* in SBR operating at a retention time of 3 days, and about 58% after 7 days of incubation reported by Marco-Urrea et al., (2009) using the same strain. Almost 100% of DCF was removed within 48 h by *A. niger* and *T. trogii* as reported by Aracagök et al., (2018). The findings of this study seemed closer to that reported by Collado et al. (2012), who reached IBP removal of 90% within 3 days. The current study could suggest repeated treatment of the first batches, which was effective in the elimination of residual concentrations of the selected PhCs and thus the reduction of intermediate compounds production (Hata et al., 2010). The decrease in the residual concentrations of the selected PhCs and the production of transformation intermediate products observed in the current study can serve as proof of the involvement of fungal enzymes as demonstrated by previous investigators using white-rot fungi (Golan-Rozen et al., 2015). The following intermediates (Table 3) were detected as CBZ, DCF and IBP corresponding transformation compounds, some especially in the 4 h effluent: acridine, iminoquinone, 9-acridine carboxaldehyde, acridone, 10,11-dihydro-10,11-dihydroxy CBZ, 10,11-dihydro-10-hydroxy CBZ, CBZ-2,3-quinone, 2,3-dihydroxy-CBZ, 2,3-dihydro-2,3-dihydroxy CBZ, 2-hydroxy imino stilbene (Kasonga et al., 2019), 9-hydroxymethyl-10-carbamoyl acridan, 3'-hydroxy DCF, 4'-hydroxy DCF, 5-hydroxy DCF (Kasonga et al., 2019), 1-hydroxyl IBP, 2-hydroxyl IBP (Kasonga et al., 2019), carboxyl IBP and 1;2-dihydroxyl IBP (Table 3). Even though a specific method such as the full ^{1}H and ^{13}C NMR assignment was not conducted to confirm the PhC metabolite structures definitely, the accurate mass *m/z* collected using the UPLC/MS method used was in accordance with previously identified metabolites generated by the same compounds (Jelic et al., 2012; Marco-Urrea et al., 2009; Sauvêtre et al, 2018).

4.3 Bioassay for estrogenic and anti-estrogenic activities

None of the samples tested exhibited any antiestrogenic activity. Although EEq values ranging from 0.002 to 0.114 ng/L were detected from some drinking water distribution points in Pretoria (Van Zijl et al., 2017), the tap water used in the non-sterile experiment did not exhibit anti-estrogenic activities (Table 4). Its EEq calculated from the assays was found to be below the LoQ. The parent PhCs (CBZ, DCF and IBP) in the mixture did not display any anti-estrogenic activity at a concentration of 1 mg/L. The tap water was found unable to induce luciferase activities at the level of EC_{10} and was therefore considered below the LoQ, whereas the EEq tested for PhC parent compounds was below the LoD. Consequently, no estrogenic activities were detected for the tap water and for the selected PhCs in the mixture at a concentration of 1 mg/L of each in the SBR influent. However, the sample from 3-day-old fungal consortium grown in co-culture in LN-m demonstrated high estrogenic activity as measured by the T47D-KBluc assays, reaching EEq values of 20.07 ± 10.29 ng/L (Table 4). These values are higher than the trigger value of 0.7 ng/L for estrogenic activity in drinking water (Genthe et al., 2010). Toxicity (cell death) was also observed in the sample under a microscope at the highest concentration tested (0.4x concentrated sample). Therefore, the estrogenic activity observed in the SBR on day 3 could be induced by the fungal mycelium solution added to the batch at a concentration of 30 % (v/v). Hence the supplementation of the PhCs in the SBR could not have contributed to the estrogenic activity recorded, as the solution of parent compounds (CBZ, DCF and IBP) did not display any activity in the assay. The higher estrogenic activity of the fungal mycelium solution could not be induced by the ligninolytic enzymes either, but by the LN-m, which consisted of almost 17 compounds supplemented for the fungal mycelial growth from spores, as well as for enzymatic production (Kirk et al., 1978; Tien and Kirk, 1988). Although they were supplemented in LN-m as salts, metalloestrogens such as the metal ions aluminium, cobalt and copper (Kasonga et al., 2019) have been reported to display estrogenic activity

(Darbre, 2006). Alternatively, the increase in enzyme production, especially MnP and LiP from effluent after 4 h (S_5) MnP: 92.44 ± 3.95 U/L and LiP: 26.07 ± 4.38 U/L to MnP: 207.14 ± 11.11 U/L and LiP: 118.56 ± 8.27 U/L at 17 days (S_8), could have contributed to the increasing estrogenic activity in the assay. However, the increase observed in the estrogenic activity of the SBR effluent within 4 h (from 1.707 ± 0.298 ng/L to 2.687 ± 0.174 ng/L) occurs with the production of PhC transformation compounds by the fungal consortium. This could therefore be due to their presence in the samples. The suggested structures of all the detected transformation intermediate compounds identified after 4 h listed above (Table 3) have phenolic complex carbon ring structures, and thus have the ability to induce estrogenic activity (Kiyama and Wada-Kiyama, 2015) especially in a mixture. In addition to that, the absence of the estrogenic activity observed in the effluents collected on days 7, 12 and 17, and the reduction in intermediate peak intensities demonstrated the strong activity of the fungal consortium in the biodegradation of PhCs and elimination of the estrogenic activity with less production of transformation by-products. The EEq values of the 24 h samples were recorded below the LoQ for days 7 and 17, and below the detection limit for day 12. The isolated South African indigenous fungal strains were therefore capable of reducing the estrogenic activity of the PhC metabolites within 24 h. This could also be justified by the reduction in the intermediate compound peak intensities observed after 24 h, when compared to the result produced within 4 h. Therefore, the elimination of the estrogenicity in the medium could be attributed to the reactions mediated by fungal ligninolytic enzymes as reported earlier (Lloret et al., 2010; Mao et al., 2010).

These findings confirm the results from a previous study (Yu and Chu, 2009) in which the potential estrogenic active compounds detected in wastewater treatment influents, such as PhCs did not show any estrogenic activity, while the effluents exhibited higher estrogenic activities

attributed to the presence of transformation metabolites. These authors observed the estrogenic activity using YES assays in the wastewater treatment effluent containing IBP in combination with other compounds such as triclosan and bisphenol A (Yu and Chu, 2009). The authors confirmed that the estrogenic activity measured in the effluents near the outflow were much more than those estimated from parent compounds. This accordingly suggested, the presence of other unknown estrogenic metabolite compounds, as well as the additive effects of mixtures of estrogenic compounds at low concentration in the effluents, when considering that, despite the presence of the mentioned compounds in influents, no estrogenic activities were detected (Yu and Chu, 2009). This confirms the fact that numerous unknown PhC by-products could be more estrogenic and even toxic than the parent compounds (Noguera-Oviedo and Aga, 2016; Rivera-Jaimes et al., 2018).

Although the three selected PhCs have phenolic chemical structures as potential estrogenic active compounds with functional groups comparable with those of E_2 and ICI 182,780, their mixture solution did not induce any estrogenic and anti-estrogenic activities to corroborate preceding reports. Indeed, after a gene expression analysis conducted by RT-qPCR, the estrogenic activity and toxicity effects of the selected PhCs in the aquatic environment were reported in chronic exposure, which may accumulate over time and lead to adverse effects, such as changes in population structure and growth, as well as extinction (Gröner et al., 2017; Kruglova et al., 2014; Oliveira et al., 2018), but especially toxicity in the presence of undesirable metabolite transformation compounds determined by the *Vibrio ficheri luminescence reduction test* (Jelic et al., 2012; Yu and Chu, 2009).

The present study therefore confirms findings demonstrating that DCF does not exhibit any estrogenicity or toxicity, except under long exposure as well as in the presence of its

intermediate transformation compounds. However, Efosa et al., (2017) reported that DCF can act as EDC by displaying slight estrogenic modes of action observed, as found in their experiment. The authors demonstrated that, DCF altered the calling behaviour of the exposed adult male *Xenopus laevis* (aquatic frog of the family Pipidae) by potentially reducing mating and reproductive success. Toxicity associated with uptake and depuration of CBZ was also reported under chronic exposure in the clam *Scrobicularia plana* (Almeida et al., 2017).

5 CONCLUSIONS

The present study revealed that the mixture of CBZ, DCF and IBP did not exhibit any estrogenic or antiestrogenic activities. The ligninolytic enzymes Lac, MnP and LiP produced by the fungal consortium appeared to contribute to the removal of PhCs by producing known corresponding intermediate transformation compounds. No quantifiable levels of E_2 EEq were detected in the SBR effluent samples. Therefore, the SBR designed in the present study, and setup with the five isolated South African indigenous fungal strains was found able to reduce or eliminate the PhCs estrogenic activities efficiency by converting the parents into less estrogenic intermediate compounds. Hence, the use of the fungal consortium in an SBR might be a good strategy for the bioremediation of PhC contaminated wastewater. Nevertheless, further experiments have to be planned at pilot scale to confirm the findings of the study.

Availability of data and materials

The data generated in this paper, tables and figures are found inside the paper. Supplementary data related to this article can be found in Data in Brief article.

Authors' contributions

Teddy Kabeya Kasonga, Martie A. A. Coetzee and Maggy Ndombo Benteke Momba conceived and designed the experiments; Teddy Kabeya Kasonga performed the experiments and wrote the paper; Catherina Van Zijl conducted the T47D-KBluc bioassay and reviewed the paper, Martie A. A. Coetzee reviewed the paper; Martie A. A. Coetzee and Maggy Ndombo Benteke Momba contributed reagents/materials/analysis tools.

Competing interests

The authors declare that there are no competing interests.

Ethics approval

The human cells used in the present study were not harvested by ourselves. An established and modified cell line from the ATCC® CRL-2865™ was used.

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