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## Elucidating the performance of an integrated laccase- and persulfate-assisted process for degradation of trace organic contaminants (TrOCs)

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# Elucidating the performance of an integrated laccase- and persulfate-assisted process for degradation of trace organic contaminants (TrOCs)

## Abstract

2020 The Royal Society of Chemistry.. Laccase-catalysed degradation is a promising technology for the degradation of trace organic contaminants (TrOCs). However, depending on their physicochemical properties (e.g., chemical structures), non-phenolic TrOCs appear to be resistant to laccase-catalysed degradation, thus requiring an additional treatment process. In this study, the performance of an integrated laccase and persulfate (PS) mediated oxidation process was explored and elucidated for the first time. Compared to that achieved during treatment with laccase alone, the addition of PS at 1-10 mM concentration into batch enzymatic bioreactors showed concentration-dependent improvement in the degradation of the five TrOCs selected, namely diclofenac, sulfamethoxazole, carbamazepine, bisphenol A and oxybenzone. The results obtained from a series of batch tests also confirmed that the structural components (e.g., carbohydrate moieties in the enzyme) as well as the possibility of PS acting as a final acceptor of electrons from the type II and type III active sites of laccase may have led to PS activation. A continuous treatment system was developed by integrating a nanofiltration (NF) membrane with the laccase/PS process (NF-MBRlaccase/PS), which achieved TrOC-specific degradation. Importantly, the degradation of non-phenolic TrOCs (diclofenac, sulfamethoxazole and carbamazepine) further improved by 10 to 65% in the NF-MBRlaccase/PS system. This improvement could be attributed to: (i) the prolonged contact time between laccase/PS and TrOCs, which was possible due to their complete retention by the NF membrane; and (ii) retention of oxidative cross coupling agents or secondary radicals enhancing TrOC degradation. The NF membrane not only retained the moderately degraded carbamazepine and sulfamethoxazole effectively but also effectively removed toxic transformation products and residual estrogenicity of the final effluent as evaluated by cytotoxicity and estrogenicity assays, respectively. These observations indicate the significance of the high retention membrane in the NF-MBRlaccase/PS system for producing high quality permeate suitable for disposal and potentially for water reuse applications.

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**Elucidating the performance of an integrated laccase- and persulfate-assisted process  
for degradation of trace organic contaminants (TrOCs)**

**(Accepted Manuscript)**

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**Water impact:**

Trace organic contaminants (TrOCs) in wastewater-impacted waterbodies raises concern because of their adverse impacts on aquatic organisms and humans. In this study, the performance of an integrated biocatalytic (laccase) and persulfate (PS) oxidation process for TrOC degradation was explored and performance governing factors elucidated. The integrated treatment process achieved improved TrOC degradation as compared to that achieved by either laccase alone or PS alone. Laccase was particularly effective for ecotoxicity and estrogenicity removal. PS-addition significantly improved TrOC degradation but raised the ecotoxicity of the treated solution. We demonstrate that a nanofiltration membrane combined with the laccase/PS treatment process can achieve nontoxic and estrogenicity-free treated effluent.

**Highlights:**

- First study on integrated laccase and persulfate (PS) oxidation for TrOC degradation
- PS and laccase achieved concentration-dependent and TrOC-specific degradation
- Laccase was particularly effective for estrogenicity removal
- Laccase/PS-membrane bioreactor showed 95-100% TrOC removal
- Membrane permeate was nontoxic, and did not exhibit estrogenic activity

**Abstract:**

Laccase-catalysed degradation is a promising technology for the degradation of trace organic contaminants (TrOCs). However, depending on their physicochemical properties (*e.g.*, chemical structures), non-phenolic TrOCs appear to be resistant to laccase-catalysed degradation, thus requiring an additional treatment process. In this study, performance of an integrated laccase and persulfate (PS) mediated oxidation process was explored and elucidated for the first time. Compared to that achieved during treatment with laccase alone, addition of PS at 1-10 mM concentration in batch enzymatic bioreactors showed concentration-dependent improvement in the degradation of the five TrOCs selected, namely diclofenac, sulfamethoxazole, carbamazepine, bisphenol A and oxybenzone. The results obtained from a series of batch tests also confirmed that the structural components (*e.g.*, carbohydrate moieties in the enzyme) as well as the possibility of PS acting as final acceptor of electron from Type II and Type III active sites of laccase may have led to PS activation. A continuous treatment system was developed by integrating a nanofiltration (NF) membrane with laccase/PS process (NF-MBR<sub>laccase/PS</sub>), which achieved TrOC-specific degradation. Importantly, degradation of non-phenolic TrOCs (diclofenac, sulfamethoxazole and carbamazepine) further improved by 10 to 65% in NF-MBR<sub>laccase/PS</sub> system. This improvement could be attributed to: (i) the prolonged contact time between laccase/PS and TrOCs which was possible due to their complete retention by the NF membrane; and (ii) retention of oxidative cross coupling agents or secondary radicals enhancing TrOC degradation. The NF membrane not only retained the moderately degraded carbamazepine and sulfamethoxazole effectively but also effectively removed toxic transformation products and residual estrogenicity of the final effluent as evaluated by cytotoxicity and estrogenicity assays, respectively. These observations indicate the significance of the high retention membrane in NF-MBR<sub>laccase/PS</sub> system for producing high quality permeate suitable for disposal and potentially water reuse applications.

**Keywords:** Ecotoxicity and estrogenic activity; Laccase-catalysed degradation; Nanofiltration (NF) membrane; Persulfate activation; Sulphate radicals; Trace organic contaminant (TrOC)

## 1. Introduction

Occurrence of trace organic contaminants (TrOCs) such as ingredients of pharmaceuticals and personal care products and industrial chemicals in wastewater-impacted waterbodies raises concern because of their adverse impact on aquatic organisms and humans <sup>1, 2</sup>. Compared to conventional biological processes, laccase-catalysed degradation has been reported to be effective for a broad range of TrOCs <sup>3, 4</sup>. Laccases (EC 1.10.3.2) are copper containing oxidoreductase enzymes that can effectively catalyse the degradation of a wide range of aromatic pollutants by using molecular oxygen as a co-factor <sup>5-7</sup>. The active sites of laccase contain four copper ions: (i) one copper ion at the Type I active site; (ii) one copper ion at the Type II active site; and (iii) two copper ions at the Type III active site. The degradation of a substrate occurs at the Type I active site that acts as the primary electron acceptor. The electron accepted by the Type I active site is transferred to the Type II and Type III active sites, where molecular oxygen is reduced to water <sup>8-10</sup>.

Recent studies have demonstrated that the performance of laccase-catalysed treatment systems is governed by the physicochemical properties of target TrOCs such as chemical structure and hydrophobicity <sup>11, 12</sup>. In general, TrOCs containing a phenolic moiety or electron donating functional groups (EDGs) are effectively degraded (70-99%) by laccase, while degradation of TrOCs containing electron withdrawing functional groups has been reported to be unstable/poor <sup>3, 11</sup>.

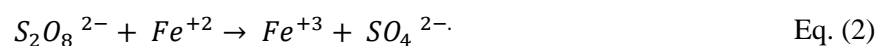
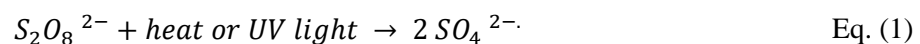
The application of laccase in continuous flow systems such as wastewater treatment plants remains a challenge. This is because laccase added into the process gets depleted as it continuously flows out with the treated effluent. This loss of laccase in a continuous treatment system could be prevented by integrating a membrane with the enzymatic bioreactor that can retain the enzyme. Lloret et al. <sup>13</sup> and Nguyen et al. <sup>14</sup> developed an enzymatic membrane bioreactor by coupling ultrafiltration (UF) membrane with the enzymatic bioreactor. Subsequent developments have seen integration of high retention (HR) membranes such as nanofiltration (NF) and membrane distillation (MD) with enzymatic membrane bioreactors <sup>9, 15</sup>. During the operation of HR-MBR, laccase and TrOCs are simultaneously retained by the high retention membrane separation process, thereby providing a prolonged contact time between laccase and TrOCs for enhanced degradation. In a recent study, Asif et al. <sup>9</sup> compared the performance of UF- and NF-MBR<sub>laccase</sub> for the treatment of a synthetic wastewater containing a mixture of TrOCs under identical operating conditions. Compared to UF-MBR<sub>laccase</sub>, the NF-MBR<sub>laccase</sub> achieved 15–30% better degradation of the selected TrOCs <sup>9</sup>. In addition to the effective retention (92-99%) of TrOCs by the NF membrane, degradation of TrOCs by laccase in the bioreactor ranged between 30 and 80% <sup>9</sup>. The partially degraded TrOCs accumulated in the bioreactor. Therefore, further efforts to improve the extent of TrOC degradation are required.

Degradation of TrOCs by laccase could be improved by introducing a redox-mediator. Redox mediators are low molecular weight phenolic compounds that can act as electron carriers between laccase and target TrOCs <sup>8</sup>. In a study by Ashe et al. <sup>16</sup>, performance of seven redox-

mediators for improving TrOC degradation was assessed. They observed that the effectiveness of redox-mediators depends on their type and concentration. For instance, 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) achieved the highest degradation for phenolic TrOCs, while 1-hydroxybenzotriazole (HBT) and violuric acid (VA) were the best redox-mediators for non-phenolic TrOCs<sup>16</sup>. In another study, the performance of syringaldehyde (SA) and HBT was compared in a continuous-flow UF-MBR<sub>laccase</sub>. The tested redox-mediators were found to achieve TrOC-specific improvement in their degradation<sup>12</sup>. However, the laccase—mediator systems suffer from limitations such as significant laccase inactivation and elevated toxicity of treated media<sup>8</sup>.

An alternative and innovative approach to improving TrOC degradation could be to combine an advanced oxidation process (AOPs) with laccase-catalysed degradation process in a bioreactor. AOPs can be either hydroxyl radical-based such as ultraviolet (UV) photolysis<sup>17</sup>, or sulphate radical-based such as activated persulfate (PS) assisted-oxidation process<sup>18,19</sup>. In recent years, sulphate radical-based oxidation has received significant attention due to its effectiveness for a wide range of TrOCs<sup>19</sup>. To-date, a combined laccase/PS treatment system is yet to be studied.

Persulfate is stable at room temperature (*i.e.*, 20 °C) and requires an activator such as heat (as low as 30 °C), transition metals (*e.g.*, iron) and UV light to generate highly reactive  $SO_4^{\cdot -}$  radicals. Depending on the type of activator, PS produces one (*e.g.*, in presence of transition metals, *see* Equation 1) or two (*e.g.*, in presence of heat or UV light, *see* Equation 2)  $SO_4^{\cdot -}$  radicals<sup>19,20</sup>. It is worth mentioning here that phenolic compounds<sup>21</sup>, humic substances containing quinone functional groups<sup>22</sup>, graphene<sup>23</sup>, activated carbon<sup>19</sup>, ultrasonication<sup>19</sup> and sub-surface minerals<sup>24</sup> have also been reported to activate PS. Similarly, PS activation by oxidoreductase enzymes is possible but has not been assessed so far.



To date, most studies have focused on PS activation and the formation of reactive radicals. Only a few studies have studied TrOC degradation by PS<sup>19,25</sup>. It is important to assess the performance of laccase and PS for a mixture of TrOCs. Also, the extent of degradation for a single TrOC may change in a reaction media containing a mixture of TrOCs. Importantly, the toxicity of the treated effluent should be analysed for safe disposal and reuse.

The aim of this study was to elucidate the degradation of five TrOCs in a mixture by a laccase/persulfate (PS) oxidation-assisted nanofiltration membrane bioreactor (NF-MBR<sub>laccase/PS</sub>) for the first time. Initially, performance of laccase, PS and laccase/PS were assessed in batch experiments to understand the effect of initial PS concentration and effect of incubation time, as well as possible PS activation mechanisms. In addition, the TrOC removal achieved by both batch and continuous-flow bioreactors, and the effect of the physicochemical properties of TrOCs were considered to provide an in-depth understanding. The estrogenicity



and toxicity of the bioreactor media and membrane permeate were analysed and discussed. Finally, the hydraulic performance of the NF membrane in the NF-MBR<sub>laccase/PS</sub> was presented to confirm the stability of the developed process.

## 2. Materials and methods

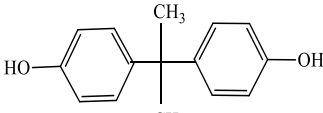
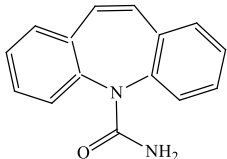
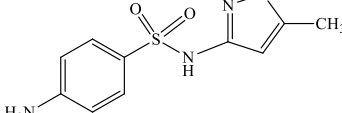
### 2.1. Trace organic contaminants, laccase and persulfate

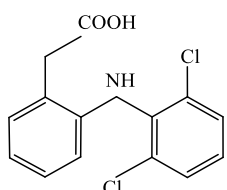
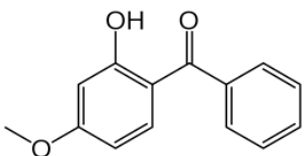
In this study, one industrial chemical (bisphenol A) and four pharmaceuticals and personal care products, namely diclofenac, sulfamethoxazole, carbamazepine and oxybenzone were selected based on their ubiquitous presence in wastewater and freshwater bodies <sup>1</sup>. For both batch and continuous experiments, a synthetic wastewater containing the selected TrOCs each at a concentration of 500 µg/L in ultrapure Milli-Q water was prepared. All the TrOCs were of analytical grade (purity >98%), and were procured from Sigma–Aldrich (Sydney, NSW, Australia). The main physicochemical properties of the selected TrOCs are summarised in Table 1.

Laccase from genetically modified *Aspergillus oryzae*, supplied by Novozymes Australia Pty. Ltd. (Sydney, NSW, Australia), was used in this study. The density and molecular weight of the laccase solution was 1.12 g/mL and 56 kDa, respectively. Laccase activity of the stock solution measured using 2,6-dimethoxy phenol (DMP) as substrate at 20 °C and pH=4.5 was 190,000 µM<sub>(DMP)</sub>/min. The oxidation-reduction potential (ORP) of laccase measured using an ORP meter (WP-80D dual pH-mV meter, Thermo Fisher Scientific, Australia) was 0.27 mV.

Reagent grade (purity ≥99%) potassium persulfate (PS) was purchased from Sigma-Aldrich (Sydney, NSW, Australia). A stock solution (50 mM) of PS was prepared in ultrapure Milli-Q water and stored at 4°C before use.

**Table 1.** Selected physicochemical properties of the TrOCs used in this study

TrOCs	Molecular structure	Molecular weight (g/mole)	Water solubility at 25 °C (mg/L)	Acid dissociation coefficient (pK <sub>a</sub> )	log D at pH=7
Bisphenol A		228.29	120	10.29	3.64
Carbamazepine		236.27	220	13.94	1.89
Sulfamethoxazole		253.28	410	5.6	-0.22

Diclofenac		296.15	30	4.18	1.77
Oxybenzone		228.24	100	7.56	3.99

## 2.2. Performance of laccase and persulfate in batch bioreactor

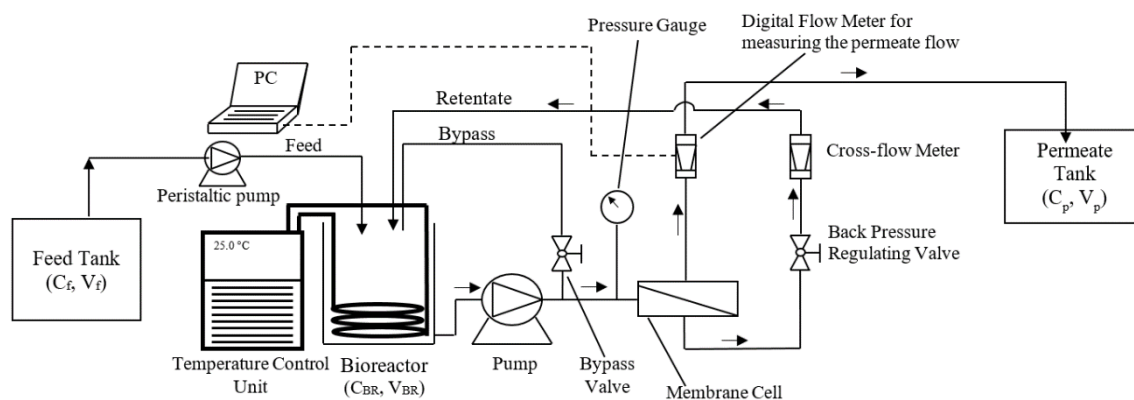
The performance of laccase/PS was initially assessed in a batch bioreactor at different PS concentrations (*i.e.*, 1, 2, 5 and 10 mM) for an incubation period of 24 h. All the experiments were conducted in 250 mL conical flasks. The concentrated laccase stock solution (70  $\mu$ L) was diluted to a final volume of 100 mL in conical flasks to yield an initial enzymatic activity of 90-95  $\mu$ M<sub>(DMP)/min</sub>. PS and the selected TrOCs were added to the flasks at an initial concentration of 1-10 mM and 500  $\mu$ g/L, respectively. The initial measured TrOC concentrations of bisphenol A, diclofenac, sulfamethoxazole, carbamazepine and oxybenzone were 510 $\pm$ 15, 485 $\pm$ 10, 525 $\pm$ 20, 510 $\pm$ 10 and 480 $\pm$ 5  $\mu$ g/L (n=12), respectively. The initial pH of the reaction media was 6.85 $\pm$ 0.1 and was not adjusted during the experiments. All the flasks were incubated in an orbital shaker incubator (Model 8500, Bioline Global Pty Ltd. Australia) at 80 rpm and 25°C. Batch bioreactor tests at each PS concentration were conducted in triplicates. Samples (2 mL each) were collected at 2, 4, 8 and 24 h for TrOC analysis. Samples for measuring the laccase activity, PS consumption, estrogenic activity and ecotoxicity were also collected at the end of each batch experiment. To verify the contribution of laccase and PS in TrOC degradation, ‘control’ batch tests were performed by studying the performance of laccase alone, PS alone, and PS plus heat inactivated laccase.

## 2.3. Continuous nanofiltration-membrane bioreactor setup and experimental protocol

### 2.3.1. Description of experimental setup

For elucidating the performance of laccase/PS in continuous-flow mode, a lab-scale cross-flow nanofiltration (NF) setup coupled to a bioreactor (3 L working volume) was used as shown in Figure 1. The details of the cross-flow nanofiltration setup used in study has been reported previously<sup>26</sup>. Briefly, the main components of this system include a stainless-steel bioreactor, a Hydra-cell pump (Wanner Engineering Inc., Minneapolis, MN, USA) and a stainless-steel flat-sheet membrane holder as well as backpressure and by-pass valves (Swagelok, Solon, OH, USA). The stainless-steel membrane cell had a channel height of 2 mm and a flat-sheet NF membrane with an active surface area of 40 cm<sup>2</sup>. All the experiments were conducted at a hydraulic pressure of 8 bar, cross-flow velocity of 40 cm/s, and temperature of 25 °C. The permeate line was attached to a digital flow meter (GJC Instruments Ltd, UK) for measuring the permeate flow rate.

A commercially available flat-sheet NF90 membrane (Dow/Filmtec, USA) was used in this study. It was a thin-film composite membrane with polyamide based active layer, and its molecular weight cut-off (MWCO) was 200 Da.



**Figure 1.** A schematic representation of the lab-scale cross-flow nanofiltration (NF) setup coupled to a bioreactor (NF-MBR). The direction of flow is shown with arrows. The concentration ( $\mu\text{g/L}$ ) of a specific micropollutant in the feed, bioreactor and permeate is represented with  $C_f$ ,  $C_{BR}$  and  $C_p$ , respectively.  $V_f$ ,  $V_{BR}$  and  $V_p$  represent the volume of feed, bioreactor and permeate, respectively.

### 2.3.2. *NF-MBR<sub>laccase/PS</sub> experimental protocol*

The *NF-MBR<sub>laccase/PS</sub>* experiment was started by compacting the membrane at an initial hydraulic pressure of 10 bar for at least 1 h or until the stabilization of the permeate flow rate. The synthetic wastewater (3 L volume) containing the mixture of the TrOCs each at a concentration of 500  $\mu\text{g/L}$  was added to the bioreactor. Laccase and PS from their respective stock solutions were directly added to the bioreactor to maintain an initial laccase activity of 90-95  $\mu\text{M}_{(\text{DMP})}/\text{min}$ , and PS concentration of 5 mM. The *NF-MBR<sub>laccase/PS</sub>* system was then operated at a hydraulic pressure of 8 bar and cross-flow velocity of 40 cm/s. This resulted in an initial permeate flux of 6.8  $\text{L}/\text{m}^2 \text{ h bar}$ . The synthetic wastewater containing TrOC mixture was continuously fed to the bioreactor *via* a peristaltic pump (Masterflex, USA) for a period of 64 h (*i.e.*, approximately  $4 \times$  hydraulic retention time, HRT). Triplicate samples (2 mL each) from the bioreactor and membrane permeate were collected at 0, 6, 12, 24, 36, 48, 64 h for TrOCs analysis. In addition, samples were obtained regularly every 12 h for measuring the laccase activity and PS consumption in the bioreactor and membrane permeate. At the end of experiment, samples from feed, bioreactor and membrane permeate were collected for the evaluation of estrogenic activity and ecotoxicity as explained in Section 2.4. Hydraulic performance of the NF membrane was studied by monitoring the permeate flux. At the conclusion of the experiment, the membrane was cleaned with Milli-Q water for 1 h to check flux recovery.

Laccase activity has been observed to drop during continuous operation due to different physicochemical and biological inhibitors as explained previously<sup>9, 27</sup>. A protocol was developed to replenish the laccase activity by adding approximately 150  $\mu\text{L}$  per litre of

bioreactor volume every 24 h. Importantly, PS at a concentration of 5 mM was added only once at the start of operation of the NF-MBR<sub>laccase/PS</sub>.

## 2.4. Analytical methods

### 2.4.1. TrOC analysis

TrOC in samples collected from batch and continuous-flow bioreactors was quantified by High-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) using a method reported previously<sup>9</sup>. The detection limit of this method was 10 µg/L. Removal efficiency by laccase/PS ( $R_{\text{degradation}}$ ) and the membrane ( $R_{\text{degradation+membrane retention}}$ ) was measured using equation (3) and (4), respectively:

$$R_{\text{biodegradation}} = 100 \times (1 - C_{\text{BR}}/C_{\text{f}}) \quad (3)$$

$$R_{\text{(degradation+membrane retention)}} = 100 \times (1 - C_{\text{p}}/C_{\text{f}}) \quad (4)$$

where,  $C_{\text{f}}$ ,  $C_{\text{BR}}$  and  $C_{\text{p}}$  are the concentration (µg/L) of a specific TrOC in the feed, bioreactor and membrane permeate, respectively. The mass of TrOCs degraded by laccase/PS during continuous-flow NF-BR operation was calculated as follows:

$$C_{\text{f}} \times V_{\text{f}} = (C_{\text{BR}} \times V_{\text{BR}}) + (C_{\text{p}} \times V_{\text{p}}) + \text{biodegradation/biotransformation} \quad (5)$$

where,  $V_{\text{f}}$ ,  $V_{\text{BR}}$  and  $V_{\text{p}}$  represents the volume of feed, bioreactor and permeate, respectively.

### 2.4.2. Enzymatic activity assay and PS concentration measurement

Laccase activity was measured as described elsewhere<sup>28</sup>. Briefly, oxidation of 2,6-dimethoxyl phenol (DMP) by laccase was monitored for two minutes in 100 mM sodium citrate buffer (pH 4.5). The change in the color due to the oxidation of the substrate (DMP) was measured at 468 nm using a UV-Vis spectrometer (DR3900, HACH, USA). Laccase activity (µM<sub>(DMP)</sub>/min) was then calculated from a molar extinction coefficient of 49.6/mM cm.

PS concentration may change following its addition to bioreactors. The change in PS concentration was monitored during each experiment by using a previously developed spectrophotometric method<sup>18,29</sup>.

### 2.4.3. Estrogenicity and ecotoxicity

Samples (110 mL each) collected from the batch and the continuous-flow bioreactors as well as from the membrane permeate were extracted using Oasis HLB cartridges and eluted in 5 mL methanol. This resulted in a relative concentration factor of 22 for each sample. Estrogenic activity was analysed by ER $\alpha$ -GeneBLazer assay (Life Technologies, USA) as described previously<sup>30,31</sup>. This is an estrogen receptor-mediated reporter gene assay that measures the presence of either estrogens or estrogen mimicking compounds. This assay was carried out in 384-well plate and run in both antagonist and agonist modes. A Fluostar plate reader (BMG Labtech, Germany) was used for measuring the fluorescence at wavelengths of 460 and 520 nm after excitation at 410 nm. The data from the plate reader was presented as the ratio of fluorescence obtained at 460 nm to that obtained at 520 nm. The results were compared with

the concentration-effect curve of reference standards and expressed as 17 $\beta$ -estradiol (agonist) and 4-hydroxytamoxifen (antagonist) equivalent concentration. The limits of detection for agonistic activity were 0.35 and 1.4 ng/L for 17 $\beta$ -estradiol (E2-EQ) in batch and continuous flow-experiments, respectively. The detection limit for anti-estrogenicity was 20  $\mu$ g/L for 4-hydroxytamoxifen (4-OHTMX-EQ) in all experiments.

Ecotoxicity of the samples (2 mL each) collected from the bioreactor and membrane permeate was analysed without solid phase extraction. The inhibition of luminescence in the naturally bioluminescent bacteria (*Photobacterium leiognathi*) was monitored using the BLT-Screen as described elsewhere<sup>32</sup>. The ecotoxicity is expressed as a relative toxicity unit (rTU, the reciprocal of the EC<sub>20</sub> value), and had a detection limit of 1 rTU.

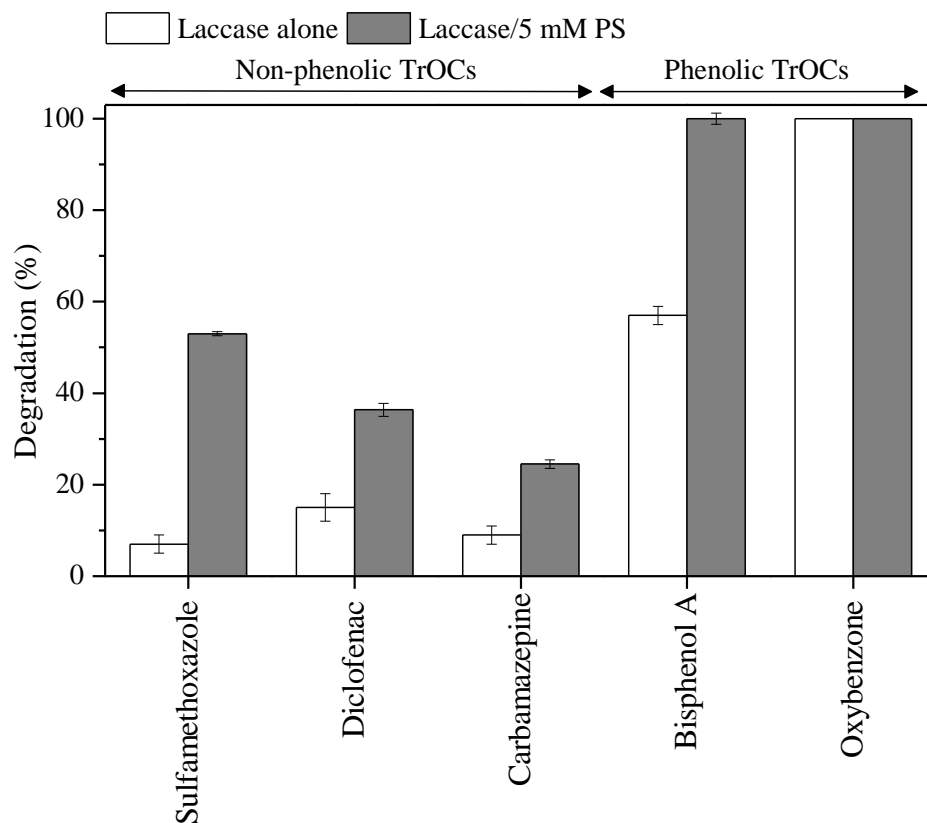
### 3. Results and discussion

#### 3.1. TrOC removal in batch experiments

##### 3.1.1. Preliminary performance of integrated laccase/PS system

Laccase is particularly suitable for the degradation of phenolic compounds but can also catalyse the degradation of non-phenolic compounds. The extent of degradation of non-phenolics is dependent on the relative ORP of laccase and the target TrOC<sup>33, 34</sup>. In this study, the non-phenolic TrOCs were only fractionally degraded (less than 15%) by laccase (Figure 2). At the end of the incubation period of 24 h, laccase achieved 7, 9 and 15% degradation of sulfamethoxazole, carbamazepine and diclofenac, respectively. The recalcitrance of the non-phenolic TrOCs may be attributed to their chemical structure. All the tested non-phenolic TrOCs contain strong electron withdrawing functional groups (EWGs). For example, carbamazepine and sulfamethoxazole contain amide ( $-\text{NH}_2$ ) functional group, and diclofenac contains both halogen ( $-\text{X}$ ) and carboxylic ( $-\text{CO}_2$ ) functional groups in its molecule (*see* Table 1). These EWGs make TrOCs resistant to laccase because they can release electrons to stabilise the electron deficiency caused by the degradation process<sup>7, 35</sup>. Indeed, poor or unstable removal of non-phenolics during laccase-catalysed degradation is also evident from available literature with reported removal often ranging between 10 and 25%<sup>3, 7, 36</sup>.

Although phenols have been recognised as a typical substrate of laccase<sup>11, 35</sup>, not all phenolic TrOCs are effectively degraded. In the current study, out of five tested TrOCs, two compounds (oxybenzone and bisphenol A) contain phenolic moiety in their chemical structures. Laccase achieved 57% and complete degradation for bisphenol A and oxybenzone, respectively (Figure 2). Almost complete degradation of bisphenol A by laccase in batch enzymatic bioreactors treating the mixture of bisphenol A and diclofenac (non-phenolic) was reported previously<sup>14</sup>. However, in this study, moderate degradation of bisphenol A could be due to the competition between the two phenolic TrOCs - oxybenzone and bisphenol A - for transferring an electron to active sites of laccase for degradation. This competition for transferring an electron to active sites of laccase has been observed previously for other substrates and/or pollutants such as 2,5-xylidine and ABTS<sup>37, 38</sup>.

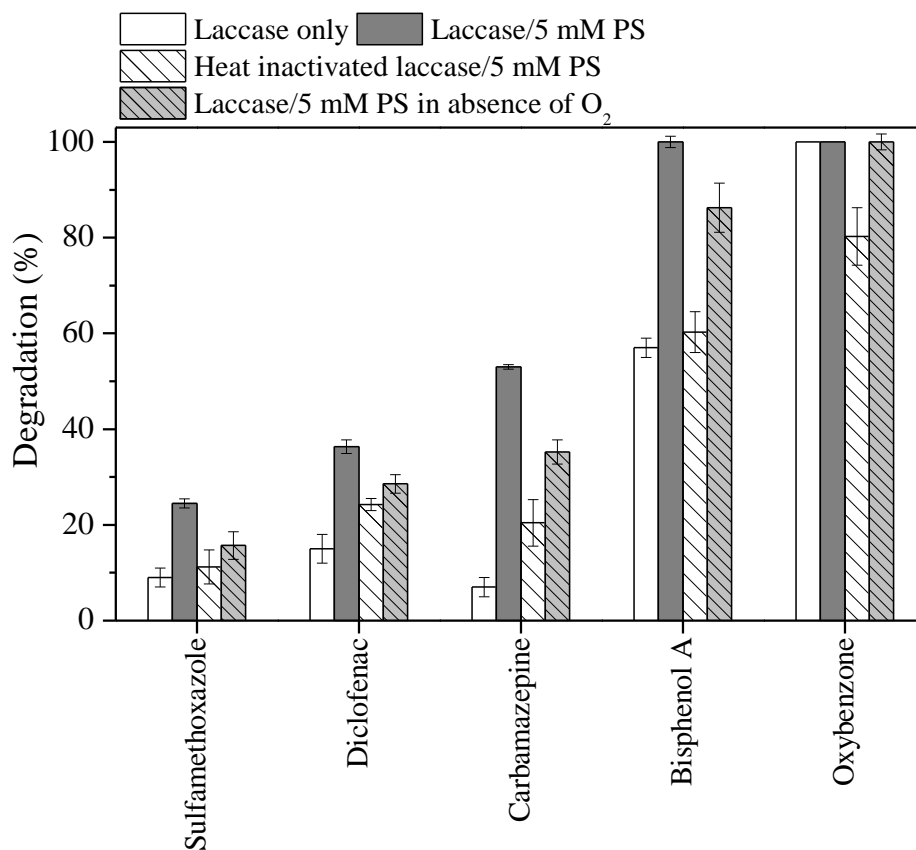


**Figure 2.** Degradation of the selected TrOCs by laccase and laccase/PS in batch tests over an incubation period of 24 h. PS (potassium persulfate) was added at a concentration of 5 mM, while the initial laccase activity was 90-95  $\mu\text{M}_{(\text{DMP})}/\text{min}$ . Results presented as average  $\pm$  standard-deviation ( $n=3$ ). Note: TrOC degradation performance of PS alone at a concentration of 5 mM was assessed, and no TrOC removal was observed.

Simultaneous dosing of laccase and PS significantly improved (15-46%) the extent of TrOC degradation, indicating the complementarity of combining laccase and PS oxidation processes. Compared to less than 20% degradation of non-phenolics by laccase-only, laccase/PS achieved 24, 36 and 53% degradation of carbamazepine, diclofenac, and sulfamethoxazole, respectively (Figure 2). PS addition achieved 43% improvement in the degradation of the phenolic TrOC bisphenol A that was moderately degraded (57%) by laccase. Notably, following treatment with and without PS addition, oxybenzone concentration was below the limit of detection (*i.e.*, 10  $\mu\text{g/L}$ ). This is the first report on the performance of an integrated laccase/PS oxidation process for TrOC degradation.

It is important to note that additional agents such as transition metals, heat or UV light are required to activate PS for the generation of  $\text{SO}_4^{\cdot-}$  and/or  $\text{OH}^{\cdot-}$  radicals<sup>19</sup>. Despite the absence of any known activators in the reaction media, the enhanced TrOC degradation by laccase/PS process in this study indicated that PS was activated in presence of laccase. For understanding the possible routes of PS activation, the mechanisms of laccase-catalysed degradation process need to be revisited. Oxidation of a substrate by laccase occurs following the transfer of an electron from the substrate to the Type I active site of laccase. This is followed by the transfer of electrons to Type II and III active sites, where reduction of the cofactor oxygen to water

molecules occurs. During the reduction of O<sub>2</sub>, formation of peroxide intermediates has been observed<sup>10</sup>. The production of peroxide intermediates can activate PS to produce reactive radicals<sup>39</sup>. To produce SO<sub>4</sub><sup>-•</sup> radicals, PS needs an electron from any source<sup>10</sup>. Hence, there is a possibility that both oxygen and PS may have acted as the cofactor and may accept electron from Type II and III active sites of laccase for completing the catalytic cycle. In the current study, the possibility of PS acting as a final electron acceptor was investigated by removing the dissolved oxygen from reaction media *via* autoclaving (Figure 3). Dissolved oxygen in the reaction media measured using a DO meter (YSI, USA) was less than 0.01 mg/L. In absence of oxygen, TrOC degradation occurred in laccase/PS system but the extent of degradation of all the tested TrOCs (except for oxybenzone) reduced by 5 to 20% as compared to that achieved by laccase/PS system in presence of oxygen. Although TrOC degradation was affected in absence of oxygen, PS activation still occurred as evident from the better TrOC degradation by laccase/PS system without oxygen as compared to laccase alone (Figure 3). An additional batch test was performed by adding heat-inactivated laccase and PS in the bioreactor for a period of 24 h. Like the performance of the laccase/PS system, degradation of TrOCs was reduced significantly (5-40%) in the heat-inactivated laccase /PS system (Figure 3). These results indicate that PS activation is possibly caused by the structural components (*e.g.*, polypeptide chain and carbohydrate moieties) of laccase<sup>40</sup>. Notably, TrOC degradation was affected more in the heat-inactivated laccase/PS system as compared to that achieved by laccase/PS system in absence of oxygen, thus indicating the significance of active laccase in an integrated laccase/PS treatment system. Based on the above observations, PS activation may have been caused by both the structural components of laccase as well as the possibility of PS acting as a final acceptor of electrons transferred by Type II and III active sites of laccase. This is the first study to report PS activation in presence of laccase.



**Figure 3.** Monitoring degradation of the selected TrOCs by laccase and laccase/PS under different conditions for understanding the PS activation pathways. PS (potassium persulfate) was added at 5 mM concentration, while the initial laccase activity was 90-95  $\mu\text{M}_{(\text{DMP})}/\text{min}$ . Results presented as average  $\pm$  standard-deviation ( $n=3$ ). Note: TrOC degradation performance of PS alone at a concentration of 5 mM was assessed, and no TrOC removal was observed.

### 3.1.2. Effect of PS concentration

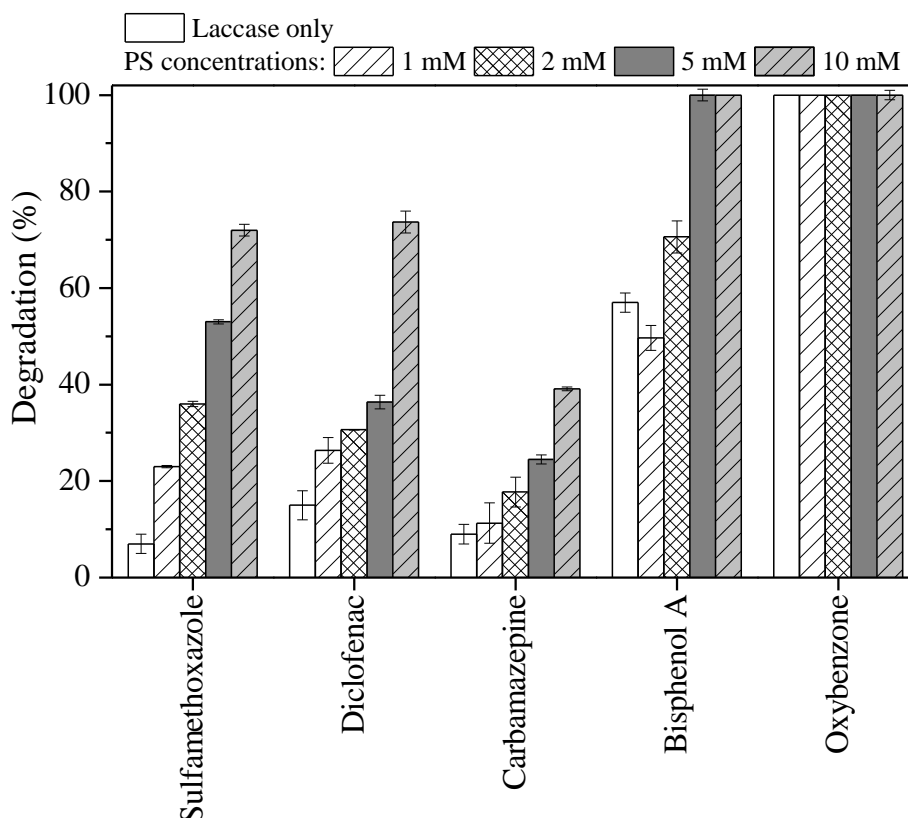
Improvement in TrOC degradation after combining PS and laccase processes has been shown in Section 3.1.1. This section illustrates the impact of PS concentration on the combined process. The performance of laccase/PS system was analysed at a range of PS concentrations (1-10 mM). Oxybenzone was completely degraded with and without PS addition. Degradation of the other TrOCs improved with increasing PS dose although the extent of improvement was compound-specific. For instance, degradation of sulfamethoxazole was 23, 36, 53 and 72% at an initial PS concentration of 1, 2, 5 and 10 mM (Figure 4). Similarly, carbamazepine degradation increased from 11% (at 1 mM PS) to 40% (at 10 mM PS).

Since the performance of the laccase/PS system was studied for the first time here, it was not possible to compare the results of this study directly with the literature. However, the trend in the improvement of TrOCs with increasing PS concentration seems to be consistent with available literature which tested PS only. According to the available literature, TrOC degradation generally improves with the increase in PS concentration. For instance, Ji et al.<sup>26</sup> reported an improvement of approximately 80% in the degradation of the pesticide atrazine by



heat-activated PS after increasing PS concentration from 0.1 to 2 mM. In another study, degradation of sulfamethoxazole increased from merely 10 to 70% following the increase in the concentration of bicarbonate-activated PS from 1 to 10 mM<sup>41</sup>.

The PS concentration in the laccase/PS system for achieving the highest degradation was different for the tested TrOCs. It was 1 mM for oxybenzone and 5 mM for bisphenol A, while 10 mM was the most effective PS concentration for diclofenac, carbamazepine and sulfamethoxazole (Figure 4).

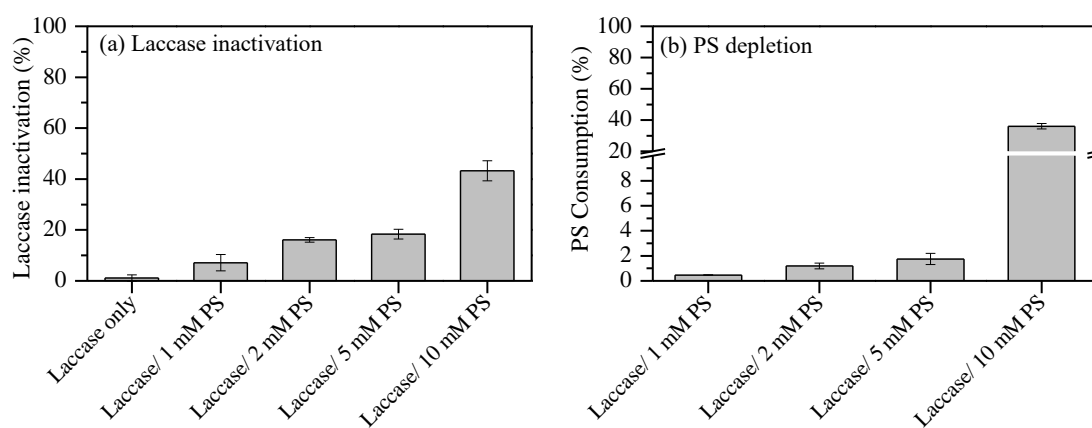


**Figure 4.** Effect of PS concentrations on TrOC degradation in batch laccase/PS system. PS concentration ranged from 1-10 mM, while the initial laccase activity was 90-95  $\mu\text{M}_{(\text{DMP})}/\text{min}$ . Data presented as average  $\pm$  standard-deviation ( $n=3$ ). Note: TrOC degradation performance of PS alone at a concentration of 1, 2, 5 and 10 mM was assessed, and no TrOC removal was observed.

Laccase inactivation during TrOC degradation can be a concern, requiring intermittent replenishment of laccase. Different inorganic (*e.g.*, salts and heavy metals) and organic (*e.g.*, organic acids and humic substances) co-occurring chemicals in wastewater can cause laccase inactivation<sup>7, 16</sup>. In absence of any known inhibitor, laccase activity was stable ( $\sim$  only 2% drop) during batch test with laccase-only. However, when PS was added to the enzymatic bioreactor, an increase in laccase inactivation was observed (Figure 5a). At the end of the operation of the batch laccase/PS system, laccase inactivation was 7% at 1 mM PS concentration, which increased to 16, 18, and 43% following addition of PS at 2, 5 and 10 mM, respectively (Figure 5a). Laccase inactivation could be due to the radicals produced by PS that can interact with the active sites of laccase, thereby affecting its activity. In previous studies,

redox-mediators were added to the enzymatic bioreactor for improving the degradation of TrOCs. Despite the TrOC-specific improvement in degradation, the radicals produced by redox-mediators have been reported to cause rapid laccase inactivation<sup>5, 8, 42</sup>. For instance, laccase was reported to lose 70-80% of its initial activity following the addition of violuric acid and 1-hydroxybenzotriazole separately at 1 mM concentration. Notably, laccase inactivation caused by PS radicals is significantly lower than that reported in presence of redox-mediators.

In addition to laccase inactivation, depletion of PS may occur due to the scavenging reactions in which radicals react with other radicals or non-target compounds. These scavenging reactions deplete PS by converting sulphate radicals into sulphate ions<sup>43, 44</sup>. In this study, in laccase/PS system, PS depletion over 24 h was insignificant (less than 2%) at a PS concentration of 1-5 mM but increased considerably (36%) at 10 mM (Figure 5b). Thus, it is important to consider the concentration of PS for developing a stable and efficient integrated laccase/PS treatment system.

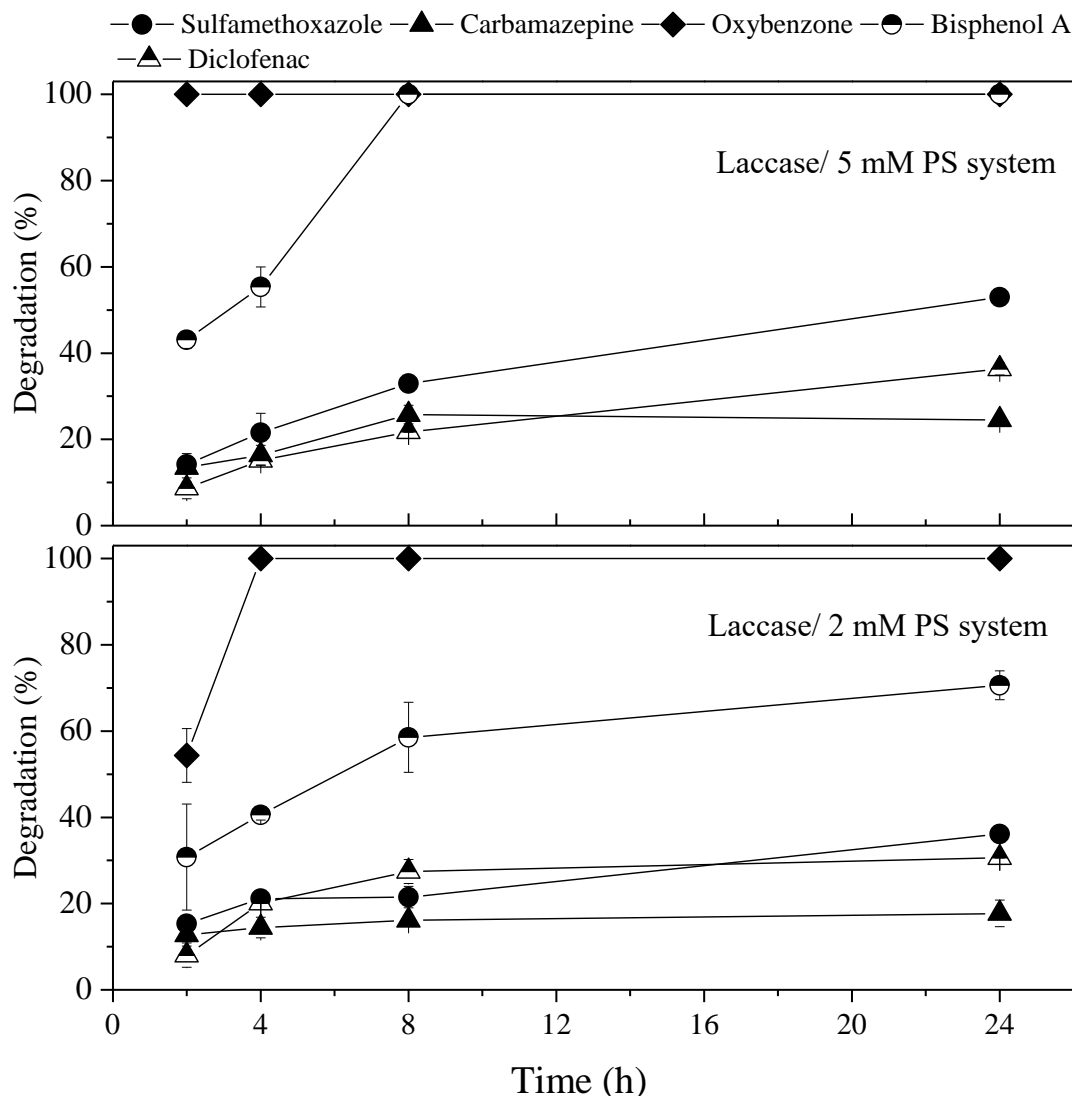


**Figure 5.** Laccase inactivation (a) and depletion of PS (b) at the end of batch tests (24 h) with and without the addition of PS at different concentrations. Error bars represent the standard deviation between duplicate samples.

### 3.1.3. Effect of incubation time

TrOC degradation performance of the integrated laccase/PS system was assessed at different incubation periods (*i.e.*, 2, 4, 8 and 24 h). To facilitate the discussion, performance of the integrated systems at 2 and 5 mM PS dose is presented in Figure 6. Laccase/PS system achieved rapid degradation of both phenolic TrOCs. Complete elimination of oxybenzone was observed after an incubation time of 2 h (at 5 mM PS concentration) and 4 h (at 2 mM PS concentration). On the other hand, 5 mM PS achieved 100% degradation of bisphenol A after 8 h (Figure 6). Regardless of PS concentration, the maximum degradation for non-phenolics was observed within first 8 h of incubation period, and the degradation slowed down considerably from 8 to 24 h. Similar data on laccase/PS combination is unavailable in the literature, but these results are consistent with the available literature related to the performance of PS alone for the degradation of individual TrOCs. For instance, when heat-activated PS at 0.5 mM was investigated for the degradation of an antibiotic (penicillin G), its maximum degradation occurred within 1 h<sup>45</sup>. Similarly, degradation of sulfamethoxazole by PS did not increase

significantly after 6 h in a PS/bicarbonate system<sup>41</sup>. The observations related to the incubation time are vital for designing a wastewater treatment system. This is because incubation time is an important parameter to estimate the size of the reactor required for effective treatment, and its overestimation may considerably increase the cost of the treatment system.



**Figure 6.** Effect of incubation time on TrOC degradation in batch laccase/PS system assessed separately at 2- and 5-mM PS concentration. Initial laccase activity was 90-95  $\mu\text{M}_{(\text{DMP})}/\text{min}$ . Data presented as average  $\pm$  standard-deviation (n=3).

### 3.1.4. Ecotoxicity and estrogenicity evaluation

TrOC degradation can result in transformation products that could cause more toxicity than the parent compound. To predict the risk associated with the disposal of treated effluent, bioassays have been developed to quantify the overall toxicity of the effluent<sup>16, 46</sup>. In the current study, the toxicity of the treated effluent was evaluated by measuring the inhibition of luminescence in the naturally bioluminescent bacteria (*Photobacterium leiognathi*) using the BLT-Screen<sup>32</sup>. Consistent with previous studies<sup>12, 46</sup>, following treatment with laccase alone, toxicity did not increase significantly, and ranged between < 1 and 2.3 rTU (Table 2). On the other hand, the

toxicity of the reaction media increased from 7.6 to 28.8 rTU in batch laccase/PS systems with the increase in PS concentration (Table 2). This may be attributed to the reactive radicals generated by PS and/or the generation of toxic transformation products. Reduction in toxicity following treatment of a single TrOC by PS has been reported previously<sup>47, 48</sup>. However, Kortenkamp et al.<sup>49</sup> observed that the toxic effects of the solution containing a mixture of compounds are often higher than the toxic effects of individual compounds. Overall, our data demonstrates that laccase/PS combination improves degradation of parent TrOCs (Figure 4), but even our lowest dose of PS (1 mM) produced a substantial increase in toxicity of the treated solution (Table 2). While toxicity in the BLT-Screen may not necessarily translate into a substantial detrimental impact on the receiving environment, it does warrant an additional barrier to mitigate the potential toxicity. The addition of NF dramatically reduced the toxicity of the final effluent (see Section 3.2.2).

**Table 2.** Estrogenic activity and ecotoxicity of samples collected at the end of different treatment options. Number of samples for each treatment is 2.

Reaction media of different treatment options	Toxicity (rTU)	E2-EQ (ng/L)	4-OHTMX-EQ (µg/L)
TrOCs only	<1	9.7 – 13.9	<20
TrOCs – Laccase	<1 – 2.3	< 1.4	<20
Laccase – TrOCs – Persulfate (1 mM)	7.6 – 11.3	2.3 – 2.6	<20
Laccase – TrOCs – Persulfate (2 mM)	3.9 – 5.6	1.8 – 2.6	<20
Laccase – TrOCs – Persulfate (5 mM)	10.3 – 11.2	3.3 – 3.9	<20
Laccase – TrOCs – Persulfate (10 mM)	24.8 – 28.8	2.0 – 2.2	<20

**Note:** The limit of detection of the toxicity assay was 1 rTU

The limit of detection for E2-EQ and 4-OHTMX-EQ was 1.4 ng/L and 20 (µg/L), respectively.

E2-EQ, 17β-estradiol equivalent concentration; 4-OHTMX-EQ, 4-hydroxytamoxifen equivalent concentration.

Prolonged exposure to TrOCs such as bisphenol A has been reported to induce endocrine disrupting effects in aquatic life and human<sup>46, 50</sup>. In this study, the estrogenic activity before and after laccase/PS treatment was evaluated using the ERα-GeneBLAzer assay, and the results are expressed as 17β-estradiol equivalent (E2-EQ) and 4-hydroxytamoxifen equivalent (4-OHTMX-EQ). E2-EQ concentrations of the influent ranged between 9.7 and 13.9 ng/L (n=2), but reduced after the treatments with laccase alone and laccase/PS system (Table 2). Importantly, laccase-catalysed degradation achieved complete elimination of estrogenic activity (*i.e.*, below the detection limit of 1.4 ng/L). Regardless of the treatment option, the 4-OHTMX-EQ (*i.e.*, antiestrogenic activity) of the influent and the treated effluent was below the limit of detection (*i.e.*, less than 20 µg/L) as shown in Table 2.

The reduction in estrogenic activity in laccase/PS system (Table 2) demonstrates that degradation of the parent estrogenic compounds (Figure 3) did not produce a mixture of transformation products with an overall increased total estrogenic activity. This is supportive of our previous study, which showed that while individual transformation products could be more potent endocrine disruptors than the parent compounds, it was likely that only a portion of the parent compound would produce the more potent transformation product and that the

estrogenic activity of the mixture of all transformation products would most likely decrease<sup>51</sup>. A few available studies have reported reduction in estrogenicity caused by TrOCs following separate treatment by laccase<sup>46, 52</sup> or PS<sup>53, 54</sup>. However, this is the first study to cast light on toxicity and estrogenicity of TrOCs treated by laccase/PS. Addition of the NF membrane further reduced the effluent estrogenicity to below detection limit (See Section 3.2.2).

### **3.2. Continuous TrOC removal by NF-MBR<sub>laccase/PS</sub> treatment system**

The NF-MBR<sub>laccase/PS</sub> was operated continuously for a period of 64 h (*i.e.*, 4 ×HRT) at a TrOC loading rate of 0.72 mg/L.d, and laccase activity of 90-95  $\mu\text{M}_{(\text{DMP})}/\text{min}$ . A PS concentration of 5 mM was selected based on the performance of batch laccase/PS system. PS was added only once at the start of the experiment. Coupling a membrane with an enzymatic bioreactor effectively retains laccase, thereby preventing laccase washout with treated effluent<sup>13, 14</sup>. To confirm this, permeate samples were collected after regular intervals for monitoring the residual laccase activity as well as PS concentration. In this study, the NF membrane effectively retained laccase. PS was also not detected in membrane permeate (data not shown); however, PS concentration may deplete over time due to the interaction of secondary radicals with sulphate radicals as explained in Section 3.1.2. Samples were collected every 12 h for monitoring PS concentration in the bioreactor. A gradual reduction in PS concentration was observed during the operation of the NF-MBR<sub>laccase/PS</sub> system. PS was not completely depleted by the end of continuous experiment (*i.e.*, after 64 h), and total reduction in PS concentration was approximately 55%. Degradation and overall removal of TrOCs in NF-MBR<sub>laccase/PS</sub> is discussed separately below for elucidating the performance of the developed treatment system.

#### **3.2.1. TrOC degradation**

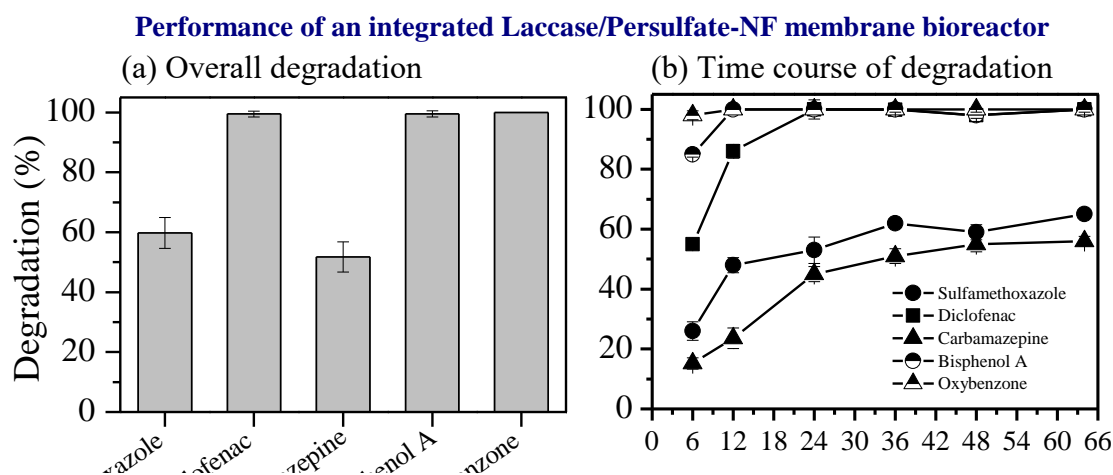
In the continuous-flow NF-MBR<sub>laccase/PS</sub> treatment system, mechanisms of TrOC removal included degradation by laccase/PS and membrane retention. The impact of membrane retention is discussed in section 3.2.2. TrOC degradation by laccase/PS during continuous treatment ranged between 56 and 100% (Figure 7a) with over 99% degradation achieved for one non-phenolic (diclofenac) and two phenolic TrOCs (bisphenol A and oxybenzone). The performance of the NF-MBR<sub>laccase/PS</sub> system was monitored continuously over the course of its operation for assessing the stability of the developed process. The time course of TrOC degradation during continuous operation, as presented in Figure 7b, indicates that the degradation of both phenolic TrOCs (bisphenol A and oxybenzone) stabilised within 12 h, while stable diclofenac degradation was achieved after 18 h. Degradation of sulfamethoxazole and carbamazepine increased up until 36 h, and remained almost constant from that point onward. Thus, it may be concluded that the developed system achieved stable TrOC degradation.

Although a direct comparison of data obtained from batch and continuous experiments may not be appropriate, it is worth noting that better degradation of TrOCs was observed in the NF-MBR<sub>laccase/PS</sub> system as compared to that achieved in the batch laccase/PS system (Figure 2). In the current study, degradation of carbamazepine, sulfamethoxazole, and diclofenac by the NF-MBR<sub>laccase/PS</sub> system was 52, 60 and 100%, respectively (Figure 7b), while their

degradation in batch tests under the same PS concentration (5 mM) was less than 55% (Figure 2).

The absence of any other NF-MBR<sub>laccase/PS</sub> studies restricts comparison of our data with that in the literature. However, a significant difference in TrOC degradation between batch enzymatic bioreactors and continuous-flow UF-MBR<sub>laccase</sub> was reported by Nguyen et al.<sup>12</sup>. They reported that the degradation of a few TrOCs such as oxybenzone and diclofenac improved by 10 to 60% in a continuous-flow UF-MBR<sub>laccase</sub> as compared to a batch enzymatic bioreactor<sup>12</sup>. Nguyen et al.<sup>12</sup> attributed this degradation-improvement to retention of TrOCs on enzyme-layer on the UF membrane-surface, which facilitated their subsequent enzymatic degradation. Furthermore, Asif et al.<sup>9</sup>, compared performance of UF-MBR<sub>laccase</sub> and NF-MBR<sub>laccase</sub> and observed that compared to UF-MBR<sub>laccase</sub>, degradation of the selected TrOCs, namely carbamazepine, sulfamethoxazole, diclofenac, atrazine and oxybenzone improved by 15 to 30% in NF-MBR<sub>laccase</sub>. Asif et al.<sup>9</sup> attributed this further improvement in TrOC degradation by NF-MBR<sub>laccase</sub> to the prolonged contact time between laccase and TrOCs following their complete retention by the NF membrane.

It is worth noting that the presence of TrOCs containing phenolic moiety can also facilitate the degradation of non-phenolic pollutants by acting as a redox-mediator. In natural environmental settings, during degradation of phenolic component of lignin by laccase, secondary radicals (*e.g.*, phenoxy radical) or cross-coupling agents are formed that can degrade the non-phenolic components of lignin<sup>35</sup>. In a recent study, the transformation by-products of a phenolic pharmaceutical (acetaminophen) was reported to directly oxidize a non-phenolic pharmaceutical carbamazepine<sup>55</sup>. In this study, it is possible that the phenolic TrOCs (oxybenzone and bisphenol A) that were effectively eliminated (~100%) may have contributed to the degradation of non-phenolic TrOCs. This phenomenon may not be apparent in batch experiments because the oxidative cross coupling agents or secondary radicals were not abundant enough as compared to that in the continuous-flow NF-MBR<sub>laccase/PS</sub> system. Since the NF membrane can achieve effective retention of laccase, PS and TrOCs, the transformation products will also stay in the bioreactor of the NF-MBR<sub>laccase/PS</sub> system to contribute in TrOC degradation *via* catalytic and non-catalytic pathways.

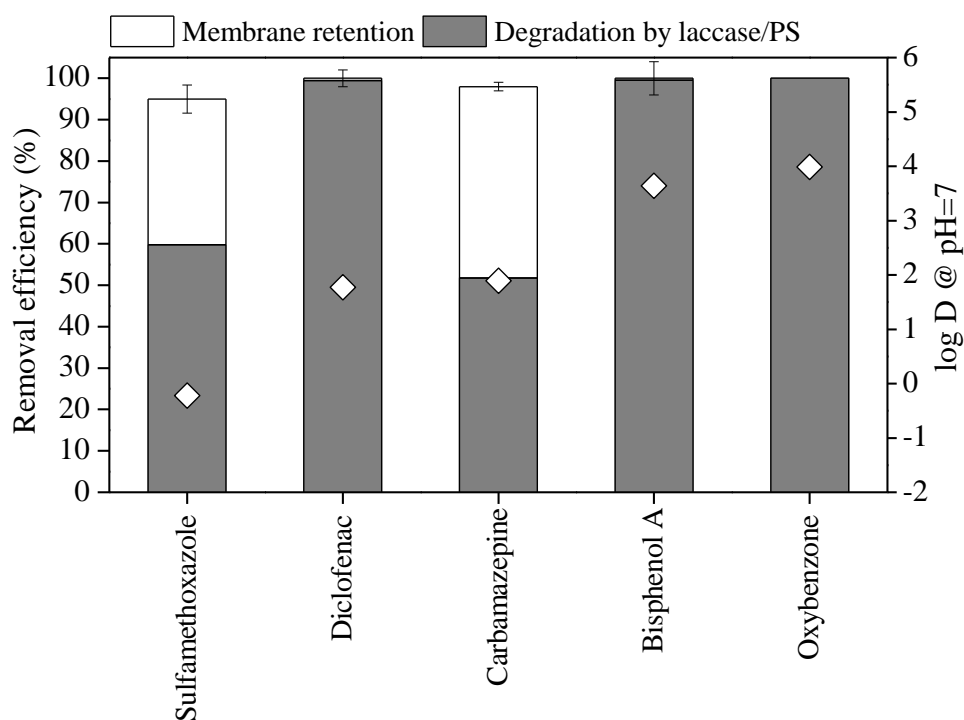


**Figure 7.** Performance of the NF-MBR<sub>laccase/PS</sub> treatment system for the degradation of the selected TrOCs. PS (potassium persulfate) was added at a concentration of 5 mM, while the initial laccase activity was 90-95  $\mu\text{M(DMP)/min}$ . The NF-MBR<sub>laccase/PS</sub> treatment system was operated at a TrOC loading rate of 0.72 mg/L.d and an HRT of 16 h. (a) Overall TrOC degradation: data is presented as average  $\pm$  standard-deviation calculated based on the triplicate samples that were collected at 24, 36, 48 and 64 h; (b) time course of TrOC degradation: error bars denote average  $\pm$  standard-deviation of triplicate samples .

### 3.2.2. Overall TrOC removal

The benefits of integrating the laccase/PS system with a high retention NF membrane (*i.e.*, NF-MBR<sub>laccase/PS</sub> system) can be realised by assessing the overall TrOC removal (*i.e.*, degradation + membrane retention). Despite the appreciable TrOC degradation, sulfamethoxazole and carbamazepine were resistant to laccase/PS system and were only moderately degraded (52-60%). The NF membrane effectively retained the TrOCs not completely degraded by laccase/PS, producing an effluent with over 95% overall TrOCs removal (Figure 8).

NF membrane can effectively retain a wide range of TrOCs *via* a combination of removal mechanisms. TrOC with molecular weight above 200 g/mol has been reported to be effectively retained (above 90%) by size exclusion. On the other hand, removal mechanisms for hydrophobic ( $\log D > 3$ ) and charged (*e.g.*, diclofenac) TrOCs include adsorption on membrane surface and charge repulsion, respectively<sup>56, 57</sup>. Since molecular weight of all the selected TrOCs in this study was above 200 g/mol (*see* Table 1), size exclusion appears to be the dominant mechanism of TrOC retention by the NF membrane.



**Figure 8.** Overall removal (degradation + membrane retention) of TrOCs by the NF-MBR<sub>laccase/PS</sub> system. Error bars represent average  $\pm$  standard-deviation ( $n=12$ ).  $\log D$  at  $\text{pH}=7$  represents the hydrophobicity of each TrOC. Experimental conditions are the same as in Figure 7.

As discussed in Section 3.1.4, toxicity of the treated effluent (as indicated by BLT-Screen assay) increased during the batch laccase/PS treatment depending on PS concentration. In a previous study on high retention membrane distillation MBR (MD-MBR<sub>laccase</sub>), TrOCs and their transformation products were retained, thus producing a non-toxic permeate<sup>27</sup>. The current study was conducted with NF membrane, and both laccase and PS were dosed into the bioreactor. To confirm that the membrane-permeate from NF-MBR<sub>laccase/PS</sub> was non-toxic, samples were collected at the end of continuous operation, and their toxicity was analysed. Although the toxicity of the bioreactor media was  $96.2 \pm 6.7$  rTU ( $n=3$ ), NF permeate samples were non-toxic (*i.e.*, less than 1 rTU). This confirmed that the high retention NF membrane not only retained the parent TrOCs but also the transformation products and reactive radicals generated by PS that may exhibit toxicity.

Estrogenic activity is another important parameter to evaluate the safety of treated effluent for disposal and reuse<sup>30, 58</sup>. The samples collected from NF-MBR<sub>laccase/PS</sub> bioreactor at the end of experiment showed an estrogenicity of  $0.6 \pm 0.2$  ng/L E2-EQ, while estrogenic activity in NF permeate was below the detection limit of 0.35 ng/L E2-EQ. The antiestrogenicity (expressed as 4-OHTMX-EQ) in both the NF-MBR<sub>laccase/PS</sub> bioreactor and NF permeate was below the detection limit of 20  $\mu\text{g/L}$ . This again confirms that both the parent TrOCs and any estrogenic transformation products were effectively retained by the NF membrane. Combining the laccase/PS degradation process with NF membrane helps develop a single-step process with

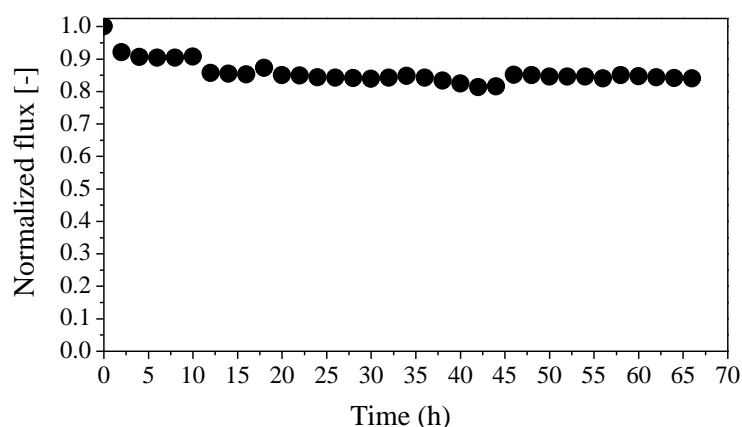


increased TrOC degradation and, therefore, less production of concentrated waste that is typical of standalone application of a high retention membrane process.

We have demonstrated the performance of an integrated biocatalytic (laccase) and persulfate (PS) oxidation process for TrOC degradation. In this context, impact of persulfate concentration and incubation time on TrOC degradation, effluent toxicity and estrogenic activity was elucidated. In addition, mechanisms of PS activation by the enzyme were elucidated and discussed. To facilitate this observation, a mixture of TrOCs in ultrapure water, in absence of any other organics, was used in this study. It is noted that real wastewater is a complex mixture of bulk organics including the TrOCs. Different dissolved organic and inorganic impurities co-occurring in wastewater <sup>7</sup> may affect the performance of the integrated biocatalytic (laccase) and persulfate (PS) oxidation process. We recommend future studies on this aspect. However, that is beyond the scope of the current study

### 3.3. Hydraulic performance of the NF membrane in NF-MBR<sub>laccase/PS</sub>

Permeate flux was monitored for analysing the hydraulic performance of the NF membrane. By the end of the experiment, permeate flux of the NF membrane reduced by almost 20% (Figure 9). The permeate flux reduced by 10% within the first 2 h of operation, possibly due to the adsorption of the reaction media consisting of TrOCs, laccase and PS. A similar trend was observed previously when an enzymatic bioreactor was integrated with NF membrane <sup>9,59</sup>. The reduction in permeate flux can also be attributed to concentration polarization that is caused by the accumulation of the bioreactor media on or near the membrane surface <sup>59,60</sup>. At the end of operation, cleaning the NF membrane with milli-Q water was effective to recover the flux by almost 95%. This indicates that the reduction in flux caused due to the adsorption of reaction media and/or concentration polarization was significantly reversible without chemical cleaning.



**Figure 9.** Hydraulic performance of the NF membrane expressed as normalised flux during the operation of the NF-MBR<sub>laccase/PS</sub> treatment system.

## 4. Conclusion

Performance of an integrated laccase and persulfate (PS) oxidation process in batch and continuous modes was systematically investigated for the degradation of five trace organic

contaminants (TrOCs), namely diclofenac, sulfamethoxazole, carbamazepine, bisphenol A and oxybenzone. Degradation of the selected TrOCs by laccase alone in batch experiments ranged between 7 (for sulfamethoxazole) and 100% (for oxybenzone) and was governed by the TrOCs' chemical structure. Addition of PS at different concentrations (1-10 mM) in the batch enzymatic bioreactor achieved TrOC-specific improvement in degradation, exhibiting the benefit of combining laccase and PS oxidation processes. Among the tested PS concentrations (1-10 mM), the best performance was achieved at 5 mM that achieved 100% degradation for two phenolics (bisphenol A and oxybenzone) and 25-53% degradation for three non-phenolic (diclofenac, sulfamethoxazole, and carbamazepine) TrOCs without significantly causing laccase inactivation and PS depletion. However, addition of PS significantly increased the toxicity of the reaction media (from <1 to 7.6-28.8 rTU). Laccase treatment, irrespective of PS addition, reduced the estrogenicity. Based on the batch tests, a treatment system by coupling a nanofiltration (NF) membrane with laccase/PS (5 mM) system *i.e.*, NF-MBR<sub>laccase/PS</sub> was also developed. The continuous NF-MBR<sub>laccase/PS</sub> treatment system achieved effective TrOC removal (95-100%). Around 10-65% better degradation of non-phenolic TrOCs was also achieved by the NF-MBR<sub>laccase/PS</sub> as compared to that obtained in the batch laccase/PS system. In addition, the NF membrane dramatically reduced the toxicity and estrogenicity of the final effluent to below detection limit of both assays (<1 rTU and <0.35 ng/L E2-EQ, respectively). Hydraulic performance of the NF membrane was monitored: cleaning the membrane only with Milli-Q water helped recover permeate flux by 95% without any chemical cleaning.

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