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**Biocatalytic degradation of pharmaceuticals, personal care products,
industrial chemicals, steroid hormones and pesticides in a membrane
distillation-enzymatic bioreactor**

**Muhammad B. Asif ^a, Faisal I. Hai ^{a,*}, Jinguo Kang ^{a,b}, Jason P. van de Merwe ^c, Frederic
D.L. Leusch ^c, William E. Price ^b, Long D. Nghiem ^a**

^a Strategic Water Infrastructure Lab, School of Civil, Mining and Environmental Engineering,
University of Wollongong, Wollongong, NSW 2522, Australia.

^b Strategic Water Infrastructure Lab, School of Chemistry, University of Wollongong,
Wollongong, NSW 2522, Australia.

^c Australian Rivers Institute and Griffith School of Environment, Griffith University, QLD
4222, Australia.

*** Corresponding Author:** faisal@uow.edu.au; Tel.: +61-2-42213054

Abstract:

Laccase-catalyzed degradation of a broad spectrum of trace organic contaminants (TrOCs) by a membrane distillation (MD)-enzymatic membrane bioreactor (EMBR) was investigated. The MD component effectively retained TrOCs (94-99%) in the EMBR, facilitating their continuous biocatalytic degradation. Notably, the extent of TrOC degradation was strongly influenced by their molecular properties. A significant degradation (above 90%) of TrOCs containing strong electron donating functional groups (*e.g.*, hydroxyl and amine groups) was achieved, while a moderate removal was observed for TrOCs containing electron withdrawing functional groups (*e.g.*, amide and halogen groups). Separate addition of two redox-mediators, namely syringaldehyde and violuric acid, further improved TrOC degradation by laccase. However, a mixture of both showed a reduced performance for a few pharmaceuticals such as primidone, carbamazepine and ibuprofen. Mediator addition increased the toxicity of the media in the enzymatic bioreactor, but the membrane permeate (*i.e.*, final effluent) was non-toxic, suggesting an added advantage of coupling MD with EMBR.

Keywords: biocatalysis; laccase; membrane distillation; redox-mediator; trace organic contaminants

1. Introduction

Membrane distillation (MD) is a low temperature distillation process in contrast to conventional distillation processes such as fractional or steam distillation. It essentially relies on the transport of water in the vapor phase from a feed solution through a microporous hydrophobic membrane to the permeate or distillate. Among different MD configurations, direct contact membrane distillation (DCMD) has been predominantly studied due to the ease of its operation (Alkhudhiri et al., 2012; Curcio & Drioli, 2005). In DCMD, the temperature of the feed solution is maintained at 15-20 °C higher than the permeate to create an adequate vapor pressure difference, which allows water to pass through a microporous membrane in vapor form *via* diffusion (Alkhudhiri et al., 2012; Duong et al., 2017). Since mass transfer occurs in gaseous phase, MD can theoretically achieve complete rejection of all non-volatile compounds (Martinetti et al., 2009; Wijekoon et al., 2014a).

Due to efficient separation efficiency, low fouling propensity and potentially low energy requirement (subject to the availability of low grade heat), stand-alone MD has been studied for applications such as protein recovery in dairy processing (Hausmann et al., 2013), treatment of industrial (Khaing et al., 2010) and municipal wastewater (Phattaranawik et al., 2008; Wijekoon et al., 2014b), as well as for the removal of trace organic contaminant (TrOCs), such as pharmaceuticals and personal care products, pesticides and industrial chemicals, from wastewater (Darowna et al., 2014; Wijekoon et al., 2014a). Recently, TrOC removal has also been investigated by coupling an activated sludge based bioreactor to MD that achieved excellent (95-99%) TrOC retention (Wijekoon et al., 2014b). Since effective retention of TrOCs by the MD theoretically decouples organic retention time from hydraulic retention time of a bioreactor, the degradation of TrOCs is expected to improve due to prolonged contact time between the recalcitrant compounds and the microorganisms (Luo et al., 2014a). However, it was found that the biodegradation of resistant TrOCs, such as those containing strong electron withdrawing functional groups (EWGs), by the activated sludge in the MD-coupled bioreactor did not improve, and eventually these TrOCs accumulated in the bioreactor (Hai et al., 2014; Wijekoon et al., 2014b). Hence, to realize the full potential of a

combined biological - MD process, it is necessary to find the means to improve biodegradation of TrOCs retained in the bioreactor by the MD membrane. In this context, it is noteworthy that the oxidoreductase enzyme laccase (EC 1.10.3.2) can degrade TrOCs that are less susceptible to degradation by the activated sludge process (Cruz-Morató et al., 2014; Yang et al., 2013).

Laccase can catalyze the degradation of a broad spectrum of pollutants including aromatic hydrocarbons, aliphatic amines and TrOCs by using dissolved oxygen as a co-substrate (Asif et al., 2017b; Hai et al., 2007; Yang et al., 2013). However, its larger scale application is restricted by the lack of a reactor system, which can prevent washout of enzymes along with treated effluent. In a recent study, Asif et al. (2017c) combined an enzymatic bioreactor with the MD (MD - Enzymatic membrane bioreactor or MD-EMBR), which retained both laccase and the tested TrOCs (carbamazepine, sulfamethoxazole, diclofenac, atrazine and oxybenzone). During a short term (12 h) batch operation of the MD-EMBR (Asif et al., 2017c), degradation of the investigated TrOCs by laccase was found to improve significantly compared to that achieved by an activated sludge-based MD bioreactor (Wijekoon et al., 2014b). Furthermore, TrOC degradation by the MD-EMBR was better than those achieved by previously developed ultrafiltration (UF)-EMBRs, which retained laccase but not TrOCs (Asif et al., 2017c; Nguyen et al., 2016). Apparently, the effective retention of the TrOCs by the MD membrane also improved their biodegradation. The initial observations were promising but it is necessary to assess the performance of MD-EMBR for a wide range of TrOCs during continuous operation.

This study aims to evaluate the performance of the MD-EMBR system for the degradation of a set of 30 TrOCs with diverse physicochemical properties following their effective retention by the MD membrane. Redox mediators, which are low molecular weight substrates of laccase, can enhance enzymatic degradation (Yang et al., 2013). Thus, additionally, the effect of dosing two redox-mediators *viz* violuric acid (VA) and syringaldehyde (SA), separately and as a mixture, on TrOC degradation and laccase stability was investigated. Redox mediators can improve degradation but may increase the toxicity of the treated effluent (Ashe et al., 2016; Nguyen et al., 2016), therefore, the toxicity of the bioreactor media and

MD permeate (*i.e.*, final effluent) to bacteria was monitored to clarify the applicability of this treatment process. Finally, during continuous operation, TrOC retention by MD can decrease over time due to ‘membrane wetting’ or loss of hydrophobicity (Alkhudhiri et al., 2012; Duong et al., 2017). Accordingly, the effect of laccase and redox-mediators on the MD performance was also investigated.

2. Materials and methods

2.1. TrOCs, laccase and mediators

A synthetic wastewater containing a mixture of 30 TrOCs in Milli-Q water was prepared for this study. These compounds were selected to represent different common classes of TrOCs, *viz* pharmaceutical and personal care products, industrial chemicals, steroid hormones and pesticides, which are commonly detected in different environmental systems (Luo et al., 2014b). A complete list along with their chemical structures appears in Supplementary Data. Relevant physicochemical properties of the selected TrOCs such as hydrophobicity ($\log D$) and volatility (pK_H) are given in Table 1. Analytical grade TrOCs were purchased from Sigma Aldrich (Australia). A stock solution (25 mg/L) containing the mixture of 30 TrOCs was prepared in methanol, and kept in dark at -18 °C prior to use.

[Table 1]

Laccase from genetically modified *Aspergillus oryzae* (Novozymes Australia Pty Ltd.) was used in this study. According to the supplier, the molecular weight of this laccase is 56 kDa. It has a purity of approximately 10% (w/w), density of 1.12 g/mL, and activity (measured using 2,6-dimethoxy phenol, DMP, as substrate) of 150,000 $\mu\text{M}_{(\text{DMP})}/\text{min}$. One of the main factors governing the laccase-catalyzed degradation of a substrate is the relative oxidation reduction potential (ORP) of that substrate and laccase (Yang et al., 2013). In this study, the ORP of the laccase, measured using an ORP meter (see section 2.4.2), was 0.3 V.

Two analytical grade natural redox-mediators, namely violuric acid (VA) and syringaldehyde (SA) (Sigma Aldrich, Australia), were used. A separate stock solution (50 mM) of each mediator was prepared in ultrapure Milli-Q water, and stored at 4 °C in the dark. SA and VA produce highly reactive phenoxyl

and aminoxyl radicals, respectively. They can mediate TrOC degradation by following a hydrogen atom transfer pathway (Ashe et al., 2016; Asif et al., 2017b). [The physicochemical properties of redox-mediators are presented in Supplementary Data.](#)

2.2. The MD-EMBR System

A laboratory scale MD-EMBR system was used comprising a glass enzymatic bioreactor (1.5 L) and an external direct contact membrane distillation (DCMD) module. A schematic of the setup is available in Supplementary Data. The enzymatic bioreactor was covered with aluminum foil and was placed in a water bath maintained at 30 ± 0.2 °C using an immersion heating unit (Julabo, Germany). The enzymatic bioreactor was equipped with an air pump (ACO-002, Zhejiang Sensen Industry Co. Ltd., Zhejiang, China) to maintain the dissolved oxygen concentration at around 3 mg/L.

The external DCMD module contained an acrylic glass membrane cell, two circulation pumps (Micropump Inc., USA) and a glass permeate tank. Feed and permeate flow channels were engraved on each block of the membrane cell. The length, width and height of each flow channel were 145, 95 and 3 mm, respectively. The media from the glass enzymatic bioreactor and water from the permeate tank were passed through the membrane cell and then returned back to the enzymatic bioreactor and permeate tank, respectively. A chiller (SC100-A10, Thermo Scientific, USA) was used to regulate the temperature of the permeate tank at 10 ± 0.1 °C. The permeate tank was also placed on a precision balance (Mettler Toledo Inc, USA) to monitor permeate flux. The recirculation flow rate of both feed and the distillate was controlled at 1 L/min (corresponding to a cross flow velocity of 9 cm/s) using two rotameters.

A hydrophobic microporous flat-sheet polytetrafluoroethylene (PTFE) membrane (GE, Minnetonka, MN) was used in this study. The PTFE membrane had a nominal pore size of 0.2 µm, thickness of 175 µm, porosity of 70% and an active layer thickness of 5 µm (Nghiem & Cath, 2011).

2.3. Experimental protocol

After confirming the retention of laccase by the MD membrane, a series of experiments were conducted to investigate TrOC retention (by MD membrane) and enzymatic degradation with and without the

addition of mediators (*i.e.*, SA and VA). The initial laccase activity and TrOC concentration in the enzymatic bioreactor of MD-EMBR was 95-100 $\mu\text{M}_{(\text{DMP})}/\text{min}$ and 20 $\mu\text{g}/\text{L}$, respectively. It is noteworthy that laccase activity in the enzymatic bioreactors may gradually diminish due to various physicochemical and biological inhibitors such as shear stress caused by membrane filtration (Asif et al., 2017a). Hence, the laccase activity was maintained at 95-100 $\mu\text{M}_{(\text{DMP})}/\text{min}$ by injecting a small dose of laccase (275 and 400 μL per liter of reactor volume for laccase and laccase-mediator, respectively) every 12 h to sustain MD-EMBR operation.

The MD-EMBR was first operated for a period of 60 h (*i.e.*, $2\times$ HRT) in a continuous mode (*i.e.*, continuous withdrawal of treated effluent) without the addition of mediators. The enzymatic bioreactor was replenished with synthetic wastewater every time the water recovery reached 70% (*i.e.*, approximately around every 24 h). Feed, bioreactor supernatant and treated effluent (*i.e.*, MD-permeate) samples were collected after 30 and 60 h of MD-EMBR operation for TrOC quantification. The effect of individual mediators and SA-VA mixture on TrOC degradation was investigated in additional runs. A single dose of an individual redox-mediator (SA or VA at 0.5 mM) or their mixture (0.25 mM SA and 0.25 mM VA) was added to the enzymatic bioreactor at the beginning of a run. Again, two sets of feed, supernatant and permeate samples for TrOC quantification were collected.

2.4. Analytical methods

2.4.1. TrOC analysis

TrOCs were analyzed by solid phase extraction (SPE) and quantitative determination by a Shimadzu GC/MS (QP5000) system (Hai et al., 2011; Wijekoon et al., 2014b). TrOCs present in the feed, supernatant and permeate samples were extracted using 6 mL 200 mg Oasis HLB cartridges (Waters, Milford, MA, USA). The TrOC extraction procedure was as follows: (i) pre-conditioning of HLB cartridge with 5 mL dichloromethane and methanol solution (1:1 v/v), 5 mL methanol and 5 mL Milli-Q water; (ii) loading of acidified (pH 2-2.5) samples onto the cartridges at a flow rate of 1–4 mL/min; and (iii) drying of cartridges with nitrogen for 30 min. The TrOCs were subsequently eluted from the

cartridge using 5 mL of methanol followed by dichloromethane and methanol mixture (1:1 v/v) at a flow rate of 1–4 mL/min. The effluent was subsequently evaporated at 40 °C under a gentle stream of nitrogen. The residual after evaporation was re-dissolved in 200 µL methanol containing an internal standard (5 mg bisphenol A-d16) before its transfer into 1.5 mL vials. The mixture present in 1.5 mL vials was again evaporated under gentle stream of nitrogen. Finally, the extracts were derivatized by adding 100 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (1% trimethylchlorosilane) and pyridine (dried with KOH solid), then heated on a heating block (60–70 °C) for 30 min. The derivatives were cooled to room temperature and analyzed using the Shimadzu QP5000 GC–MS (Shimadzu, Kyoto, Japan). The limit of detection (LOD) for this method is compound specific and ranged from 1-20 ng/L as listed in Supplementary Data. Removal efficiency by enzymatic reactor (R_1) and MD-EMBR (R_2) was calculated as shown in equation (1) and (2), respectively:

$$R_1 = 100 \times \left(1 - \frac{C_{su}}{C_f}\right) \quad (1)$$

$$R_2 = 100 \times \left(1 - \frac{C_p}{C_f}\right) \quad (2)$$

Where, C_f , C_{su} and C_p are the concentration (ng/L) of a specific TrOC in the feed, supernatant and permeate, respectively. The enzymatic transformation/degradation was calculated as follows:

$$C_f \times V_f = (C_{su} \times V_{su}) + (C_p \times V_p) + \text{biodegradation/biotransformation} \quad (3)$$

Where, V_f , V_{su} and V_p represents the volume of feed, supernatant and permeate, respectively.

2.4.2. Laccase activity, ORP, toxicity and contact angle

Laccase activity and effluent toxicity were examined as described elsewhere (Nguyen et al., 2016).

Enzymatic activity was determined by recording the change in absorbance at 468 nm due to the oxidation of 2,6-dimethoxyl phenol (DMP) in the presence of 100 mM sodium citrate (pH 4.5). Laccase activity expressed as $\mu\text{M}_{(\text{DMP})}/\text{min}$ was then calculated using its molar extinction coefficient of 49.6/mM.cm. ORP was measured using an ORP meter (WP-80D dual pH-mV meter, Thermo Fisher Scientific, Australia).

Samples for toxicity analysis were collected from the enzymatic bioreactor and permeate tank at the end of each experiment. Toxicity, expressed as a relative toxicity unit (rTU), was analyzed by measuring the inhibition of luminescence in the naturally bioluminescent bacteria *Photobacterium leiognathi* using the BLT-Screen as previously described by van de Merwe and Leusch (2015).

The contact angle of the membrane surface was measured using a Rame-Hart Goniometer (Model 250, Rame-Hart, Netcong, New Jersey, USA) using the standard sessile drop method, with Milli-Q water being used as the reference liquid (Duong et al., 2016).

3. Results and discussion

3.1. Overall TrOC removal by MD-EMBR

Retention by the MD membrane and degradation in the enzymatic bioreactor are two major mechanisms for TrOC removal in the MD-EMBR system. TrOC retention/removal by the MD membrane is governed by the vapor pressure (indicated by Henry's constant, H or, $pK_H = -\log H$; Table 1) and the water partition coefficient ($\log D$; Table 1) of the target TrOC. In a stand-alone MD system, a low (<2.5) ' $pK_H / \log D$ ' ratio suggests poor removal of the target compound (Wijekoon et al., 2014a). By contrast, the MD membrane coupled to an activated sludge bioreactor may achieve high removal of the target compounds irrespective of their $pK_H / \log D$ ratio. This is because a compound with a low $pK_H / \log D$ ratio tends to be adsorbed on the bioreactor particles (Wijekoon et al., 2014b). Although the enzymatic bioreactor was free of any suspended particles that can potentially adsorb TrOCs, MD-EMBR still achieved 94 to over 99% removal for the 30 TrOCs tested (Figure 1). It is noteworthy that compared to their partial removal (54-70%) in a stand-alone MD system (Wijekoon et al., 2014a), the permeate from the MD-EMBR in the current study achieved over 99% removal of some TrOCs including 4-tert-octylphenol ($pK_H / \log D = 0.98$), octocrylene ($pK_H / \log D = 1.21$), 4-tert-butylphenol ($pK_H / \log D = 1.51$), benzophenone ($pK_H / \log D = 1.83$) and oxybenzone ($pK_H / \log D = 2.1$). This significant improvement can be attributed to the efficient degradation of these TrOCs by laccase in MD-EMBR as discussed in the following sections.

[Figure 1]

3.2. TrOC degradation in MD-EMBR

Laccase degrades a substrate *via* a radical-catalyzed mechanism. In this process, transfer of one electron from a substrate to laccase occurs, and molecular oxygen is reduced to water. Laccase can efficiently degrade phenolic pollutants *i.e.*, substrates containing a hydroxyl (–OH) group attached to a benzene ring. On the other hand, non-phenolic pollutants are less amenable to laccase-catalyzed degradation (Asif et al., 2017b; Yang et al., 2013). Therefore, degradation of phenolic and non-phenolic TrOCs are discussed separately.

3.2.1. Degradation of phenolic TrOCs

Of the 13 phenolic TrOCs tested (Figure 1), laccase achieved significant degradation (95-99%) of 10 compounds including five steroid hormones, three industrial chemicals (bisphenol A, 4-tert-butylphenol and 4-tert-octylphenol) and two ingredients of personal care products (triclosan and oxybenzone). The observation of efficient enzymatic degradation of these TrOCs in MD-EMBR is consistent with the literature regarding previously developed enzymatic bioreactors. For example, Lloret et al. (2012) achieved 95-99% removal of two steroid hormones (estrone and 17 β -estradiol) in a batch enzymatic bioreactor. Similarly, efficient degradation (>90%) of oxybenzone, bisphenol A, triclosan and 4-tert-butylphenol has been achieved by batch or continuous flow enzymatic bioreactors (Margot et al., 2013; Nguyen et al., 2016). [Notably, compared to that achieved by UF-EMBRs \(Nguyen et al., 2015\), in this study, degradation of estrone, 17 \$\beta\$ -estradiol and estriol was greater than 99%, which indicates that effective retention of these TrOCs by the MD membrane facilitated their degradation by laccase in MD-EMBR.](#)

Although phenolic TrOCs are especially amenable to laccase-catalyzed degradation, moderate degradation of a few phenolic compounds has been previously attributed to the presence of EWG(s) in their molecular structure (d'Acunzo et al., 2006). Due to the steric hindrance caused by the concomitant presence of an EWG, phenolic TrOCs cannot access the active sites of laccase for efficient degradation

(d'Acunzo et al., 2006; Nguyen et al., 2016). In line with this, a moderate degradation (44-65%) was observed for three phenolic TrOCs, namely salicylic acid, pentachlorophenol and enterolactone in this study, which contain an EWG (*i.e.*, carbonyl or halogen) in their molecule (Figure 1).

3.2.2. Degradation of non-phenolic TrOCs

Laccase can oxidize non-phenolic TrOCs, but the extent of the degradation may not be significant (Yang et al., 2013). In previous studies, two distinct trends were observed for the degradation of non-phenolic TrOCs by laccase: (i) poor removal (*e.g.*, less than 5%) of those that only contain strong EWGs such as halogen ($-X$), amide ($-CONR_2$) and carbonyl ($-C=O$); and (ii) moderate to high removal of those that contain both EWGs and electron donating functional groups (EDGs) such as amine ($-NH_2$) or alkoxy ($-OR$) (Asif et al., 2017a; Ji et al., 2016; Yang et al., 2013). In this study, benzophenone, octocrylene and amitriptyline were significantly degraded (>95%) by laccase. On the other hand, a moderate degradation (45-75%) was observed for the remaining non-phenolic TrOCs (Figure 1).

Of particular interest is the enhanced degradation of pharmaceuticals and pesticides (containing strong EWGs) that were previously reported to be poorly (<10%) degraded by laccase in both batch and continuous flow enzymatic bioreactors (Margot et al., 2013; Nguyen et al., 2016). These TrOCs include ketoprofen (EWG carboxylic; 52% removal), clofibric acid (EWG halogen; 55% removal), carbamazepine (EWG amide; 62% removal), metronidazole (EWG nitro; 67% removal), atrazine (EWG halogen; 59% removal), fenoprop (EWG halogen, 48% removal) and *N, N*-Diethyl-*meta*-toluamide (DEET; EWG amide; 69% removal) (Figure 1). In a previous study, significantly improved degradation of recalcitrant TrOCs such as carbamazepine, atrazine and diclofenac was attributed to simultaneous adsorption of laccase and TrOCs on granular activated carbon which allowed prolonged close contact between laccase and TrOCs (Nguyen et al., 2014b). Although our approach was different, the enhanced degradation of recalcitrant TrOCs in MD-EMBR can be ascribed to the increased contact time between laccase and TrOCs following their complete retention (95-99% removal) by the MD membrane.

It is also noteworthy that TrOCs containing EDGs such as hydroxyl and amine (*e.g.*, steroid hormones, bisphenol A and triclosan) can act as bi-functional substrates or redox-mediators (d'Acunzo et al., 2006; Hachi et al., 2017). Fragments of phenoxyl radicals or oxidative coupling agents (*e.g.*, dimers) produced due to the oxidation of bi-functional substrates can facilitate the degradation of recalcitrant TrOCs *via* enzymatic and/or non-enzymatic reactions (*e.g.*, polymerization or agglomeration). Indeed, Margot et al. (2013) reported significantly higher diclofenac removal in presence of the phenolic TrOC bisphenol A compared to that observed for diclofenac as a single compound. Similarly, Nair et al. (2013) observed above 90% removal of diclofenac in a mixture containing bisphenol A, 17 α -ethinylestradiol and diclofenac as compared to its 70% removal in absence of the phenolic TrOCs. In another study, Hachi et al. (2017) demonstrated that an oxidative coupling agent (*i.e.*, dimer) produced due to the degradation of acetaminophen containing an EDG (*i.e.*, amine) formed oligomers with carbamazepine. These oligomers were more susceptible to laccase catalyzed oxidation than the parent compound, which led to enhanced carbamazepine degradation (Hachi et al., 2017). Furthermore, in nature, laccase oxidizes the aromatic rings of lignin and produce phenoxyl radical, which are responsible for the degradation of non-phenolic components of lignin (Castro et al., 2003; d'Acunzo et al., 2006). Thus, there is a strong body of evidence of TrOCs containing EDGs working as redox mediators for enhancing degradation of non-phenolics, albeit from batch tests only. The synthetic wastewater used in this study contained a mixture of TrOCs containing EDGs and EWGs, as would be expected in practical wastewater conditions. These TrOCs were well retained by the MD component during the continuous operation of the MD-EMBR (see Section 3.1). Therefore, it is possible that radicals or oxidative coupling agents formed due to the oxidation of TrOCs containing hydroxyl and amine functional groups also contributed in achieving enhanced degradation (as compared to that achieved by previously developed enzymatic bioreactors) of resistant TrOCs containing EWGs by the MD-EMBR.

3.3. MD-EMBR performance with mediator addition

As noted in Section 3.2, efficient degradation (95-99%) by MD-EMBR was observed for 13 out of the 30 TrOCs investigated here, while the remaining TrOCs were moderately removed (44-75%). While these removal rates compare favorably with that in previous reports, two naturally occurring redox-mediators, namely SA and VA, were added to the EMBR separately and as a mixture in an attempt to further improve removal of the recalcitrant TrOCs.

Oxidation of VA and SA by laccase produces highly reactive aminoxyl and phenoxyl radicals, respectively, that have higher ORP than laccase. Moreover, these radicals act as an electron shuttle between the substrate and laccase, thereby improving the degradation of the substrate *i.e.*, target pollutants (Ashe et al., 2016). In a study by Weng et al. (2012), addition of SA increased the ORP of the enzyme solution, consequently improving the degradation of sulphonamide antibiotics. Similarly, an increase in ORP was accompanied by an improved degradation of atrazine, pentachlorophenol, naproxen and oxybenzone following the addition of VA at a concentration of 0.5-1 mM in a batch enzymatic bioreactor (Ashe et al., 2016). In the current study, the ORP of EMBR-media increased from 0.3 to 0.39 and 0.45 V following the addition of SA and VA, respectively. This was accompanied by significant improvement in TrOC removal: an increase of 5-54% depending on the molecular structure of TrOCs and redox-mediator type as discussed below (Figure 2).

[Figure 2]

The performance of different mediators for enhanced TrOC degradation has been reported in only a few batch or UF-EMBR studies (Ashe et al., 2016; Lloret et al., 2013; Nguyen et al., 2016). In previous studies, a continuous supply of redox-mediator was required to sustain UF-EMBR operation, because UF membranes cannot retain redox-mediators (Nguyen et al., 2014a; Nguyen et al., 2016). A uniqueness of this study is that it demonstrates the effect of the single dose of mediators on TrOC degradation following the complete retention of laccase, TrOCs and mediators by the MD membrane. SA and VA demonstrated substrate specific improvements in the degradation of TrOC that were moderately degraded by laccase-only (Figure 1). Of the 17 moderately degraded TrOCs (Figure 1), the laccase-VA system achieved better

degradation for six compounds namely, ketoprofen, gemfibrozil, naproxen, primidone, carbamazepine and pentachlorophenol. By contrast, the laccase-SA system performed best for four compounds; fenoprop, clofibric acid, propoxur and atrazine (Figure 2). Similar degradation efficiency was achieved by both SA and VA for the remaining TrOCs. A comparison of TrOC fate in laccase and laccase-mediator based MD-EMBR revealed that the molecular structures of TrOCs significantly influence the effectiveness of laccase-mediator systems (Figure 3).

[Figure 3]

3.4. Effect of mediator mixture on TrOC degradation

Since in this study, SA and VA showed different patterns of TrOC-specific degradation-improvement (Figure 2), it was envisaged that a mediator-mixture would have further beneficial effects. Degradation of the phenolic TrOCs, which were already well removed by laccase-only, remained unaffected when a SA-VA mixture was used. The whole set of data is provided in Supplementary Data. However, compared to either SA-laccase or VA-laccase, the SA-VA-laccase system did not improve the degradation of any TrOCs (Figure 4). Furthermore, in comparison to TrOC degradation by laccase-only, the SA-VA-laccase system achieved somewhat reduced degradation of six pharmaceuticals, namely ketoprofen, naproxen, clofibric acid, primidone, carbamazepine and ibuprofen (Figure 4).

The performance of mediator mixtures has rarely been studied for the removal of TrOCs. Previously, Jeon et al. (2008) observed in batch tests that vanillin and acetovanillone mixture did not improve the degradation of pentachlorophenol, while enhanced pentachlorophenol degradation was found by adding a mixture of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and vanillin or acetovanillone (Jeon et al., 2008). It is possible that, in mixtures, some mediators can chemically interact with each other instead of acting as an electron shuttle for laccase. Moreover, simultaneous addition of some mediators can reversibly inhibit laccase, thereby inhibiting electron transfer between laccase and TrOCs (Dizhbite et al., 2004; Jeon et al., 2008). Indeed, laccase inactivation was significantly increased following the

addition of SA-VA mixture (*see* section 3.5). The current work demonstrates for the first time through continuous operation of the MD-EMBR that although VA and SA outcompete many other mediators tested to date (Ashe et al., 2016), using them together may be counterproductive. Further studies to screen redox-mediators and their mixtures are recommended, but that is beyond the scope of the current study.

[Figure 4]

3.5. Laccase stability in MD-EMBR

Laccase activity in enzymatic bioreactors may be affected by various physicochemical and biological factors (Asif et al., 2017a; Purich, 2010). Transformation byproducts or charged metabolites formed following the degradation of TrOCs can block the active sites of the laccase. Moreover, hydraulic stress during MD-EMBR operation can also cause laccase inactivation (Lloret et al., 2013). Although some laccase inhibition was observed during continuous operation of the MD-EMBR (Figure 5), a stable operation could be sustained by reinjecting as little as 275-400 μ L laccase solution per liter of reactor (working) volume every 12 h.

[Figure 5]

It may be noted that the MD membrane can theoretically retain all non-volatile compounds including redox-mediator derived highly active radicals along with laccase and TrOCs. The radicals enhance laccase-catalyzed TrOC degradation (Hachi et al., 2017), but can also instigate laccase inactivation. It has been suggested that the highly reactive radicals produced due to the oxidation of mediators can react with laccase, consequently converting them into non-productive complexes (Khlifi et al., 2010; Lloret et al., 2013; Purich, 2010). Indeed, the extent of laccase inactivation increased during MD-EMBR operation after mediator addition. This data is available in Supplementary Data section. The average laccase inactivation was $53 \pm 11\%$ (no. of laccase injections, $n=4$) during MD-EMBR operation in absence of mediators, while the loss in laccase activity was 57 ± 11 , 62 ± 16 and $80 \pm 12\%$ ($n=4$) after the addition of a single dose of VA, SA and SA-VA mixture, respectively (Figure 5). To date higher laccase inactivation in

presence of mediators has mostly been reported in small scale batch enzymatic bioreactors. For instance, Nguyen et al. (2014a) reported rapid laccase inactivation following the addition of SA at the tested concentrations of 0.1-1 mM in a batch enzymatic bioreactor. In another study, a complete loss of enzymatic activity was observed in a batch enzymatic bioreactor by adding VA at a concentration of 0.5 mM (Ashe et al., 2016). In the current study, although laccase activity was significantly affected in the presence of redox-mediators, it was compensated for by the improvement in TrOC degradation (Figure 4).

3.6. Permeate toxicity and flux

Laccase-catalyzed degradation of TrOCs, particularly in the presence of mediators, produces reactive radicals and transformation products that may increase the toxicity of the treated effluent (Marco-Urrea et al., 2009; Nguyen et al., 2016). In this study, the overall bacterial toxicity of the media in the enzymatic bioreactor and MD-permeate (*i.e.*, final effluent) was measured at the end of each EMBR run (Table 2). The media in the enzymatic bioreactor showed an overall toxicity of 5 ± 1 , 14 ± 2 , 65 ± 3 and 130 ± 12 rTU ($n=2$) in presence of laccase, laccase-VA, laccase-SA and laccase-SA-VA, respectively. The observed increase in toxicity due to addition of VA and SA are consistent with previous studies (Ashe et al., 2016; Nguyen et al., 2016), however, this is the first study to report the toxicity in relation to mediator mixtures. A significantly increased toxicity following the addition of SA-VA mixture was observed. Despite the increase of toxicity in the enzymatic bioreactor, MD-EMBR permeate toxicity was below the limit of detection (*i.e.*, rTU <1), evidencing that in addition to laccase and TrOCs, the MD system retained reactive radicals and transformation products, which cause bacterial toxicity. This is an added advantage of integrating a high retention membrane with an enzymatic bioreactor.

[Table 2]

[Figure 6]

In this study, a stable MD-permeate flux of approximately 4 L/m².h was achieved in all experimental conditions irrespective of redox-mediator addition, which also indicates that membrane fouling did not

occur. The flux achieved here is consistent with the applied temperature differential for MD operation. Permeate flux achieved in each MD-EMBR run is given in Supplementary Data. During prolonged continuous operation, the performance of the MD process can be affected by the loss of hydrophobicity of the MD membrane (Alkhudhiri et al., 2012; Duong et al., 2017). Therefore, the integrity of the MD membrane was assessed by measuring the contact angle of the membrane after each experiment. The contact angle *i.e.*, the hydrophobicity was found to be not significantly affected (Figure 6), confirming the suitability of combining the MD membrane with the EMBR.

4. Conclusions

Efficient TrOC rejection by the MD membrane appeared to be beneficial for enhanced degradation of recalcitrant TrOCs. Addition of single redox-mediators, syringaldehyde (SA) and violuric acid (VA), significantly improved the degradation of those TrOCs that were moderately degraded by laccase. Despite the compound-specific improvement observed following the separate addition of SA and VA, a mixture of SA-VA was not effective for TrOC degradation. A mediator-specific increase in the toxicity of bioreactor media was observed, but no toxicity was detected in the membrane permeate. MD-EMBR operation was stable during all experiments, and membrane wetting or fouling was not observed.

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