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Integration of an enzymatic bioreactor with membrane distillation for enhanced biodegradation of trace organic contaminants

Muhammad Bilal Asif
University of Wollongong, mba409@uowmail.edu.au

Luong Nguyen
Nanyang Technological University, University of Wollongong, luong@uow.edu.au

Faisal I. Hai
University of Wollongong, faisal@uow.edu.au

William E. Price
University of Wollongong, wprice@uow.edu.au

Long D. Nghiem
University of Wollongong, longn@uow.edu.au

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Abstract

A novel membrane distillation - enzymatic membrane bioreactor (MD-EMBR) system was developed for efficient degradation of trace organic contaminants (TrOCs). Degradation of five TrOCs, namely carbamazepine, oxybenzone, diclofenac, atrazine and sulfamethoxazole was examined using two commercially available laccases (from *Trametes versicolor* and *Aspergillus oryzae*). The MD system ensured complete retention (>99%) of both enzyme and TrOCs. Of particular interest was that the complete retention of the TrOCs resulted in high TrOC degradation by both laccases. Oxybenzone and diclofenac degradation in the MD-EMBR ranged between 80 and 99%. Compared to previously developed EMBRs, as much as 40% improvement in the removal of resistant non-phenolic TrOCs (e.g., carbamazepine) was observed. Laccase from A. *oryzae* demonstrated better TrOC degradation and enzymatic stability. With the addition of redox mediators, namely 1-hydroxybenzotriazole (HBT) or violuric acid (VA), TrOC degradation was improved by 10-20%. This is the first demonstration of a laccase-based high retention membrane bioreactor for enhanced biodegradation of TrOCs.

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Muhammad B. Asif [a], Luong N. Nguyen [a,b], Faisal I. Hai [a]*, William E. Price [c], Long D. Nghiem [a]

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

[b] School of Civil and Environmental Engineering, Nanyang Technological University, 50 Nanyang Avenue 639798, Singapore.

[c] Strategic Water Infrastructure Lab, School of Chemistry, University of Wollongong, Wollongong, NSW 2522, Australia.

Corresponding author: Faisal I. Hai (E-mail: faisal@uow.edu.au; Ph: +61 02 42213054)

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Highlights:

- Coupling membrane distillation to enzymatic reactor (MD-EMBR) improved TrOC removal
- Enzymatic degradation was the main mechanism of TrOC removal by MD-EMBR
- Degradation of resistant non-phenolic TrOCs was significantly improved
- Redox-mediator addition achieved rapid and improved TrOC removal (10-20%)
- Laccase$_{A.\ oryzae}$ showed better stability and TrOC removal than laccase$_{T.\ versicolor}$

Abstract:

A novel membrane distillation – enzymatic membrane bioreactor (MD-EMBR) system was developed for efficient degradation of trace organic contaminants (TrOCs). Degradation of five TrOCs, namely carbamazepine, oxybenzone, diclofenac, atrazine and sulfamethoxazole was examined using two commercially available laccases (from *Trametes versicolor* and *Aspergillus oryzae*). The MD system ensured complete retention (>99%) of both enzyme and TrOCs. Of particular interest was that the complete retention of the TrOCs resulted in high TrOC degradation by both laccases. Oxybenzone and diclofenac degradation in the MD-EMBR ranged between 80 and 99%. Compared to previously developed EMBRs, as much as 40% improvement in the removal of resistant non-phenolic TrOCs (*e.g.,* carbamazepine) was observed. Laccase from *A. oryzae* demonstrated better TrOC degradation and enzymatic stability. With the addition of redox mediators, namely 1-hydroxybenzotriazole (HBT) or violuric acid (VA), TrOC degradation was improved by 10-20%. This is the first demonstration of a laccase-based high retention membrane bioreactor for enhanced biodegradation of TrOCs.

Keywords: High retention membrane; Membrane distillation (MD); Enzymatic membrane bioreactor (EMBR); Laccase; Redox mediator; Trace organic contaminants (TrOCs)

1. Introduction:

Trace organic contaminants (TrOCs) include a wide array of natural or anthropogenic chemicals including pesticides, pharmaceuticals and personal care products. Recent studies have confirmed the potentially harmful effects of TrOCs on the growth and reproduction patterns of aquatic flora and fauna and also on human health due to prolonged ingestion (Flint et al., 2012; Gavrilescu et al., 2015). Conventional wastewater treatment processes cannot effectively remove certain groups of TrOCs, resulting in their widespread occurrence in freshwater sources (Deblonde et al., 2011; Luo et al., 2014). Therefore, the scientific community is in constant pursuit of an effective wastewater treatment process for TrOC removal.

Different physicochemical and biological wastewater treatment processes have been investigated over the years for TrOC removal (Gao et al., 2012; Hai et al., 2014; Navaratna et al., 2017; Silva et al., 2012). TrOC degradation by biocatalysts such as laccase, peroxidase and proteases is a promising eco-friendly technique (Demarche et al., 2012; Hai et al., 2013). Enzymatic transformation of TrOCs is governed by a number of factors such as pH, temperature, TrOC properties and characteristics of enzymes (Yang et al., 2013). Laccase is an oxidase enzyme that can degrade a broad spectrum of TrOCs over a wide range of pH by utilizing the dissolved oxygen in water (Chea et al., 2012; Nguyen et al., 2015). Particularly mention worthy is the ability of laccase to oxidize the phenolic TrOCs including aromatic/aliphatic amines, diphenols and methoxy-substituted monophenols (Yang et al., 2013). Molecular structure, namely distribution of the functional groups, *i.e*., electron withdrawing group (EWG) and electron donating groups (EDG), governs the extent of TrOC removal by laccase. The oxidation of TrOCs containing EWGs such as amide, halogen and nitro groups is slower as compared to those containing EDGs (Asif et al., 2017; Yang et al., 2013). TrOC oxidation can be enhanced by

introducing a redox mediator, which can act as an electron shuttle between the target compounds and enzyme. Depending on the type of mediator and TrOC structure, laccase-mediator systems can achieve significant improvement in the removal of target compounds (Ashe et al., 2016; Nguyen et al., 2014a).

Enzyme washout is a major constraint in the large scale application of an enzymatic bioreactor. To mitigate this problem, laccase can be immobilized onto or entrapped within different supports (Ba et al., 2013; Yang et al., 2013). Alternatively, enzymatic bioreactor can be coupled with a membrane having a suitable molecular cutoff. For example, Nguyen et al. (2015) and Lloret et al. (2012) achieved complete retention of laccase with ultrafiltration (UF) membranes. The use of enzymatic membrane bioreactor (EMBR) can avoid the mass transfer limitations associated with laccase immobilization onto support media. Although TrOCs are not expected to be retained by UF membranes, Nguyen et al. (2015) observed the formation of an enzyme gel layer on the surface of the membrane that effectively adsorbed non-phenolic hydrophobic TrOCs such as octocrylene, amitriptyline and benzophenone. This resulted in enhanced degradation of these compounds. However, enzyme gel layer could not adsorb hydrophilic non-phenolic TrOCs such as atrazine and carbamazepine, and their overall removal was less than 10% (Nguyen et al., 2015). Hence, it was postulated that the use of high retention membranes, which will retain both laccase and TrOCs, can facilitate the degradation of resistant TrOCs.

In recent years, high retention membranes, namely membrane distillation (Wijekoon et al., 2014b), nanofiltration (Phan et al., 2016) and forward osmosis (Alturki et al., 2012; Holloway et al., 2014; Luo et al., 2015), have been integrated with the conventional activated sludge bioreactors to achieve complete TrOC retention, resulting in their high aqueous phase removal.

However, these short term studies have revealed accumulation of membrane-retained recalcitrant compounds in the bioreactor, indicating the need for enhancement of biodegradation. Although laccase has been reported to achieve better biodegradation than conventional activated sludge, no study has explored a high retention membrane - enzymatic bioreactor.

In this study, a laccase-based high retention membrane bioreactor was investigated for the first time to achieve enhanced degradation of five hardly degradable TrOCs. For this, a membrane distillation system was integrated with an enzymatic bioreactor (MD-EMBR). A series of experiments were performed to elucidate the performance of two commercially available laccases – one from genetically modified *Aspergillus oryzae* (*A. oryzae*) and the other from *Trametes versicolor* (*T. versicolor*). Impacts of two N=OH type redox mediators, namely 1-hydrozybenzotriazole (HBT) and violuric acid (VA) on TrOC degradation as well as on enzyme stability were also studied.

2. Materials and methods

2.1. Trace organic contaminants

Four pharmaceutical and personal care products, namely sulfamethoxazole, carbamazepine, diclofenac and oxybenzone, and one pesticide (atrazine) were selected for this study due to their widespread occurrence in environmental systems (Luo et al., 2014). Analytical grade (>98% purity) standards of these TrOCs were purchased from Sigma–Aldrich (Australia). The physicochemical properties including molecular weight, chemical structure, hydrophobicity (log D) and volatility (pK_H) of the tested TrOCs are given in Table 1. A stock solution (2 g/L) of these compounds was prepared and stored at -18 ^{o}C in the dark.

[Table 1]

2.2. Enzyme solutions and mediators

Commercially available laccase purified from *T. versicolor* (CAS No. 80498-15-3) purchased from Sigma–Aldrich (Australia) was used in this study. Laccase from genetically modified *A. oryzae* (Novozym 51030) was the second source of laccase used in this study, and it was supplied by Novozymes Pty. Ltd, Australia. These laccases have been investigated recently for the removal of a broad spectrum of TrOCs, showing promising results (Nguyen et al., 2015; Nguyen et al., 2014a; Nguyen et al., 2016b). The w/w composition of *A. oryzae* laccase solution is as follows: 66% water, 25% propylene glycol, 4% glucose, 3% laccase and 2% glycine. Moreover, its molecular weight, activity (measured by monitoring the oxidation of 2,6-dimethoxy phenol, DMP, as substrate at pH 4.5 and 20°C) and density is 56 kDa, 150,000 $\mu M_{(DMP)}$/min and 1.12 g/mL, respectively. Laccase from *T. versicolor* was received in powdered form. After dissolving 10 mg of *T. versicolor* laccase in 1 L Milli-Q water, *T. versicolor* laccase showed an activity of 8.5 $\mu M_{(DMP)}$/min at pH 4.5 and 20°C.

Two N=OH type mediators, namely HBT and VA were selected for this study. HBT and VA are particularly effective for improving the degradation of non-phenolic compounds. Both HBT and VA follow hydrogen atom transfer (HAT) mechanism, producing highly stable and reactive aminoxyl radicals (Asif et al., 2017). Mediators were also purchased from Sigma–Aldrich (Australia), and separate stock solutions (50 mM) for HBT and VA were kept at 4 ^{o}C before use.

2.3. MD-EMBR experimental setup

A laboratory scale MD-EMBR setup consisting of a glass enzymatic bioreactor (1.5 L) and an external direct contact membrane distillation (DCMD) module was used (Supplementary Data Figure S1). The enzymatic bioreactor was covered with aluminum foil to avoid TrOC photolysis.

An immersion heating unit (Julabo, Germany) was immersed in the water bath to maintain the temperature at 30±0.2 ^{o}C. Moreover, air diffuser connected with an air pump (ACO-002, Zhejiang Sensen Industry Co. Ltd., Zhejiang, China) was placed at the bottom of the bioreactor to maintain homogeneity, and to keep dissolved oxygen (DO) above 3 mg/L.

To minimize heat losses, acrylic glass material was used to prepare the DCMD module. The feed and distillate flow channels (Dimensions: 145 mm×95 mm×3 mm) were engraved on each acrylic block. Water from the enzymatic bioreactor and the distillate container was continuously passed from the DCMD module and then recirculated back to the enzymatic bioreactor and distillate container, respectively. A temperature sensor was placed at the inlet of the DCMD module to monitor the temperature of the feed. Distillate temperature was maintained at 10±0.1 ^{o}C using a chiller (SC100-A10, Thermo Scientific, USA). A stainless steel heat exchanging coil connected with the chiller was immersed in the distillate container placed on a precision balance (Mettler Toledo Inc, USA) to monitor permeate flux. The recirculation flow rate of both feed and the distillate was kept at 1 L/min (corresponding to the cross flow velocity of 9 cm/s) using two rotameters.

Hydrophobic microporous polytetrafloroethylene (PTFE) membranes (GE, Minnetonka, MN) were used during all experiments. Properties of PTFE membrane are given elsewhere (Nghiem and Cath, 2011; Wijekoon et al., 2014b). Briefly, nominal pore size, thickness, active layer thickness and porosity of the PTFE membrane was 0.22 µm, 175 µm, 70% and 5 µm, respectively.

2.4. Experimental protocol

Initially, the MD-EMBR system was operated without the addition of enzyme and mediators to determine the loss in TrOC concentrations due to adsorption and/or evaporation. Laccase from *T. versicolor* was tested alone and then both mediator, HBT and VA (at 1 mM concentration), were added separately to investigate the improvement in the degradation of TrOCs. Similarly, laccase from genetically modified *A. oryzae* was also tested with and without the addition of HBT and VA separately. At the start of each experiment with laccase, 1 mL and 0.1 g of *A. oryzae* and *T. versicolor*, respectively, were added to 1.5 L Milli-Q water separately for achieving an initial enzymatic activity of 95-100 $\mu M_{(DMP)}$/min. TrOCs were each added at a nominal concentration of 1 mg/L. However, the actual measured concentrations of sulfamethoxazole, diclofenac, carbamazepine, atrazine and oxybenzone were 948±90, 923±76, 873±137, 855±140, 771±210 µg/L (n=8), respectively. The difference in theoretical and measured concentrations of TrOCs may be attributed to the purity of each compound, since the actual purity of a compound may differ from that claimed by the manufacturer (Betz et al., 2011). Samples from enzymatic bioreactor and distillate were taken at every three hours over a period of 12 hours to monitor TrOC removal and enzymatic activity. The enzymatic degradation of the TrOCs was determined as $R(\%) = 100 \times (1 - \frac{m_t}{m_0})$, where m_o and m_t are initial mass (0 h) and mass at the time of sampling, respectively.

2.5. Analytical methods

2.5.1. Analysis of TrOCs

TrOC concentration in the enzymatic bioreactor and permeate was measured at different time intervals using HPLC (Shimadzu, Kyoto, Japan) at the detection wavelength of 280 nm. The HPLC system was equipped with a UV-Vis detector and C-18 column (300×4.6 mm) having a

pore size of 5 mm (Supelco Drug Discovery, Sigma Aldrich, Australia). Milli-Q water buffered with 25 mM KH_2PO_4 and HPLC grade acetonitrile were used as the mobile phase for TrOC quantification. Two eluents, namely eluent A (20% acetonitrile + 80% buffer, v/v) and eluent B (80% acetonitrile + 20% buffer, v/v), were passed through the C-18 column at a flow rate of 0.7 mL/min for 30 min in time dependent gradients as follows: [Time (min), A (%)]: [0, 85], [8, 40], [10, 0], [22, 0], [24, 85]. The limit of detection (LOD) for this method was approximately 10 µg/L. Since any residual enzymatic activity in samples may interfere with the accuracy of the results, samples were diluted (2 folds) with methanol to inactivate laccases (Nguyen et al., 2014a). Before TrOC analysis, known standards of each TrOC were analyzed to determine the time at which the peak of specific TrOC appears. After that, standards prepared from stock solution containing the mixture of selected TrOCs were analyzed to prepare the calibration curve (peak area *vs* concentration). Coefficient of determination (R^2) for all the calibration curves was greater than 0.99.

2.5.2. Laccase assay and ORP

Laccase activity was measured at an interval of three hours using a method previously described by Nguyen et al. (2014a). Oxidation of 2,6-dimethoxyl phenol (DMP) 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, Colorado, USA). Enzymatic activity ($\mu M_{(DMP)}$/min) was then calculated from a molar extinction coefficient of 49.6 mM/cm. Oxidation-reduction potential (ORP) of laccases with and without the addition of mediators were measured using an ORP meter (WP-80D dual pH-mV meter, Thermo Fisher Scientific, Australia).

3. Results and discussion

3.1. TrOC retention by MD system

TrOC removal by an MD-EMBR system is governed by enzymatic transformation and retention by MD membrane. In this study, the MD membrane achieved complete retention (>99%) of the tested TrOCs *i.e.,* concentration of TrOCs in membrane permeate was below the detection limit of 10 µg/L during all experiments. Since mass transfer in MD occurs in vapor phase, volatility (pK_H) of target pollutants controls their transport from feed to distillate. The retention of TrOCs has been investigated recently in MD-only and MD coupled with conventional bioreactor (MDBR) systems (Wijekoon et al., 2014a; Wijekoon et al., 2014b), but not an enzymatic bioreactor. In these studies, retention of volatile TrOCs (pK_H <9) by MD system varied from 50-90%, while retention of most non-volatile TrOCs (pK_H >9) varied from 95-99%. Among the incompletely removed moderately-volatile TrOCs in previous studies was oxybenzone (Wijekoon et al., 2014a; Wijekoon et al., 2014b). Complete retention of all TrOCs including oxybenzone in the current study can be attributed to the lower operating temperature (*i.e.,* 30 ^{o}C vs. 40 ^{o}C) of the enzymatic bioreactor, which consequently lowered the vapor pressure.

The MD system was also operated without the addition of laccase to quantify the loss in the mass of tested TrOCs due to adsorption and/or evaporation. Sulfamethoxazole and diclofenac lost approximately 4.5 and 2.5% of its initial mass, respectively, at the end of control run, while the remaining compounds lost less than 1%. A negligible loss in the mass of TrOCs due to adsorption and/or evaporation during the control run suggests that membrane retention and enzymatic degradation were the main mechanisms of TrOC removal in MD-EMBR.

3.2. TrOC degradation by Laccase in MD-EMBR

Oxidation of TrOCs by laccase is principally controlled by two factors: (i) the nature of functional groups attached to the core part of the molecule *i.e.*, EDGs and EWGs; and (ii) relative redox potential of laccase and TrOCs. Laccase can efficiently degrade phenolic compounds. On the other hand, oxidation of non-phenolic compounds by laccase is possible but it may be restricted by kinetic limitations (Asif et al., 2017; Yang et al., 2013). In this study, significant enzymatic degradation of TrOCs was observed following their complete retention by the MD membrane in MD-EMBR (Figure 1).

[Figure 1]

The fate of each compound was analyzed by developing a mass balance considering the total input mass, mass in concentrate at the end of experiment, mass in permeate, adsorption and/or evaporation losses, and enzymatically degraded portion (Figure 2). It was observed that biodegradation was the main mechanism of TrOC removal in the enzymatic bioreactor. Laccase from *T. versicolor* and *A. oryzae* achieved 40-80 and 45-99% TrOC degradation, respectively (Figure 2). Laccase from *A. oryzae* demonstrated better overall performance possibly due to its higher ORP, as discussed further later.

[Figure 2]

This is the first demonstration of a laccase-based high retention membrane bioreactor *i.e.*, MD-EMBR. Thus, the results are compared with previous UF-EMBR studies to highlight the synergistic effect of integrating a high retention membrane with an enzymatic bioreactor. Given the high TrOC retention by the MD membrane, depending on the level of biodegradation, TrOC concentration in the bioreactor and the MD-permeate may be significantly different. By contrast, in an UF-EMBR, due to the limited TrOC retention by the cake-layer on UF membrane, the

TrOC concentration in the bioreactor and in permeate are usually close *i.e.,* permeate to supernatant ratio is usually within 0.8-1.0 as reported by Nguyen et al. (2015). Therefore, for simplicity we compare overall removal by UF-EMBR (biodegradation plus retention on membrane cake-layer) with biodegradation in MD-EMBR.

The highest degradation (80-99%) was achieved for a phenolic compound oxybenzone containing two EDGs, namely methoxy and hydroxyl groups. Previously, Nguyen et al. (2015) also observed high overall removal (>80%) of oxybenzone in an UF-EMBR. Conversely, in comparison to the very low overall removal (>10%) of carbamazepine (containing a strong EWG amide) in previous studies (Hata et al., 2010; Nguyen et al., 2015), its degradation was 43% with laccase from *T. versicolor* and 53% with laccase *A. oryzae* in the MD-EMBR. In general, diclofenac is well removed (>60%) by laccase in UF-EMBR due to the presence of EDGs aromatic amine and aniline, providing active sites for enzymatic attack (Lloret et al., 2012; Nguyen et al., 2015). In this study, degradation of diclofenac by laccases from *T. versicolor and A. oryzae* was observed to be 82 and 90%, respectively.

Despite the presence of methyl and amine EDGs, atrazine, a non-phenolic pesticide, is resistant to laccase based treatment systems due to the steric hindrance caused by chloride, a strong EWG (Ashe et al., 2016; Nguyen et al., 2014b). In this study, enhanced degradation of atrazine by laccases from *T. versicolor* (54%) and *A. oryzae* (67%) compared to its previously reported overall removal of less than 5% in UF-EMBR (Nguyen et al., 2015) highlights the importance of complete TrOC retention for efficient enzymatic degradation of recalcitrant TrOCs. Sulfamethoxazole, a significantly hydrophilic compound (log D = -0.22), contains amine (EDG) and sulfonamide (EWG) groups. Depending on the source of fungal laccase and bioreactor type, sulfamethoxazole degradation has been reported to vary significantly (20-80%). For instance,

Nguyen et al. (2014c) achieved less than 20% degradation of sulfamethoxazole by laccase from *A. oryzae* in an UF-EMBR. Conversely, Rodarte-Morales et al. (2011) reported around 80% degradation of sulfamethoxazole with laccases from *Bjerkandera sp.*, *Phanerochaete chrysosporium* and *Bjerkandera adusta* in a batch reactor. In this study, MD-EMBR achieved approximately 40 and 46% degradation of sulfamethoxazole with laccases from *T. versicolor* and *A. oryzae*, respectively.

Integration of a DCMD system with conventional thermophilic bioreactor (MDBR) was investigated by Wijekoon et al. (2014b). As expected, the performance of MDBR and MD-EMBR (this study) is comparable based on their permeate quality. However, it is indeed important to compare the extent of biodegradation achieved in the bioreactors. The comparison of MD-EMBR with MDBR (Wijekoon et al., 2014b) and UF-EMBR (Nguyen et al., 2015; Nguyen et al., 2016b) suggests better TrOC removal in the bioreactor of MD-EMBR. For instance, degradation of diclofenac in this study ranges from 80-90%, while MDBR achieved 25% degradation (Wijekoon et al., 2014b). Similarly, while the conventional activated sludge in MDBR achieved 10% removal of carbamazepine, its enzymatic degradation in MD-EMBR was in the range of 43-55%.

3.3. Effect of redox mediator addition

3.3.1. TrOC degradation

Oxidation of phenolic and non-phenolic compounds by laccase can be possible *via* mono-electronic oxidation subject to their ORP. Low removal of non-phenolic compounds by laccase is due to: (i) their higher ORP than laccase; and/or (ii) steric hindrance caused by EWGs such as chloride and amide functional groups (d'Acunzo et al., 2006). A number of different redox-

mediators such as 1-hydroxybenzotriazole (HBT), VA and SA have been studied to improve the ORP of laccase, consequently improving TrOC degradation (Ashe et al., 2016; Nguyen et al., 2015). In a laccase-mediator system, laccase reacts with redox mediators to produce reactive radicals that can improve the effectiveness of laccase based treatment systems. Moreover, effectiveness of this system depends on mediator type and concentration, chemical structure of the substrate and ORP of laccase (Kurniawati and Nicell, 2007). The efficacy of N=OH type mediators for non-phenolic TrOC degradation is evident from literature (Ashe et al., 2016; d'Acunzo et al., 2006). Therefore, two N=OH type mediators, namely HBT and VA, at 1 mM concentration were added separately to the enzymatic bioreactor at the start of the experiment. Both mediators follow hydrogen atom transfer (HAT) mechanism and produce highly reactive aminoxyl radical (Asif et al., 2017).

Regardless of the laccase source, the tested mediators achieved the highest degradation for oxybenzone and diclofenac (Figure 3), probably because these compounds were already well removed by laccase (Figure 2). Overall, an improvement of 5-10% in TrOC degradation was achieved in *T. versicolor*-HBT system, while *T. versicolor*-VA yielded 10-20% improvement. Separate addition of HBT and VA with laccase from *A. oryzae* improved the degradation of TrOCs by 12-15 and 15-20%, respectively. Importantly, mediator addition significantly improved carbamazepine and atrazine removal as compared to oxybenzone that was already well removed in the absence of any mediator (Figure 3).

[Figure 3]

In line with the results of this study, degradation of oxybenzone and diclofenac in the range of 80-99% has been reported following the addition of HBT and VA at 1 mM concentration in batch reactors (Ashe et al., 2016; Lloret et al., 2010). Similarly, Nguyen et al. (2015) achieved

80-85% degradation of oxybenzone following the continuous addition of HBT at a low concentration of 0.01 mM in an UF-EMBR. It also suggests that high concentration (*e.g.,* 1 mM in this study) of mediators may not be required to improve the degradation of those TrOCs that are well degraded by laccase.

Improvement in the degradation of non-phenolic compounds has been observed to depend on the type and concentration of redox-mediators (Yang et al., 2013). Indeed, improvement in the degradation of non-phenolic TrOCs including carbamazepine, sulfamethoxazole and atrazine was in the range of 10-15 and 15-20% due to the addition of HBT and VA, respectively, (Figure 3). Based on the overall performance of both laccase sources with VA or HBT (Figure 3), the laccase from *A. oryzae* again outperformed that from *T. versicolor*.

Increase in the ORP of the reaction media has been suggested as one of the reasons for enhanced TrOC removal in laccase-mediator system (Asif et al., 2017). In this study, ORP of *A. oryzae* laccase was higher than *T. versicolor* laccase (Figure 4). Moreover, significant increase in ORP was also observed following the addition of mediators, and its highest value was obtained for VA regardless of the laccase source. Even though ORP of laccase-VA was higher than laccase-HBT, the results (Figure 3) suggest slightly better degradation of sulfamethoxazole and oxybenzone by laccase-HBT. Therefore, ORP is not the sole factor responsible for enhanced TrOC removal.

Figure [4]

3.3.2. Impact on enzymatic activity

Gradual enzyme denaturation can occur during the operation of an EMBR due to different physical, biological and chemical inhibitors (Purich, 2010). Moreover, rapid denaturation of enzymes has also been observed following mediator dosing (Ashe et al., 2016). In this study,

despite the absence of any chemical inhibitor, a continuous drop in enzymatic activity was observed due to hydrodynamic stress during all experiments (Supplementary Data Figure S2). Enzyme inactivation was significantly increased with the addition of HBT and VA (Figure 4). Purich (2010) suggested that the substrate and charged metabolites can inactivate enzyme in a number of ways such as: (i) substrate can block the active sites of the enzymes due to the electrostatic interactions between enzyme and charged metabolites; and (ii) metabolites can react with enzyme to convert it into nonproductive complexes.

The extent of inactivation in presence of mediators was different for laccase from *T. versicolor* and *A. oryzae*. A direct relation between ORP and enzyme inactivation was observed (Figure 4). For example, the highest inactivation was induced by VA having the highest ORP (>0.6 V). High ORP of laccase-mediator system indicates that radicals generated due to the oxidation of mediator by laccase can rapidly degrade TrOCs but at the same time they can inactivate laccase quicker. Therefore, for the development of a long term laccase-mediator based treatment process, mediator type, concentration and the characteristics of target compounds need to be taken into account.

3.3.3. Impact on contact time

Besides the assessment of the final degradation efficiency at the end of each experiment (*i.e.,* 12 h), TrOC concentration in the enzymatic bioreactor was measured at an interval of three hours. In absence of mediators, regardless of the laccase source, the TrOC concentrations showed a gradual drop over the entire operation period (Figure 5). On the other hand, in the presence of mediators, the maximum degradation of most TrOCs was achieved within six hours (Figure 5). Only oxybenzone degradation was completed within three hours irrespective of mediator addition. Thus, not only that 10-20% improvement in TrOC degradation was achieved (Figure 3)

but that was achieved rapidly (Figure 5) following the addition of mediators. The cease of TrOC oxidation after six hours was apparently due to the inactivation of the laccase as noted in the previous section. Reactive radicals produced due to the oxidation of mediators by laccase can react with the aromatic amino residues available on the outer surface of the enzyme, resulting in the inactivation of enzyme (Khlifi et al., 2010). Improved TrOC degradation at the expense of high laccase inactivation has been reported previously in batch tests involving laccase-mediator systems (Hata et al., 2010; Lloret et al., 2013). The current study extends such observation in case of an MD-EMBR for the first time (Figure 3 and Figure 4).

[Figure 5]

3.4. Hydraulic performance of membrane

[Figure 6]

Temperature difference between the feed and the distillate side has major influence over the permeate flux in MD process. Ideally, the temperature at the feed and distillate side is kept at >50 and 20-25ºC, respectively to produce adequate permeate flux (approximately 10 L/m^2 h) (Alkhudhiri et al., 2012). However, thermal stability of laccase at elevated temperature should be taken in to account before selecting the working temperature of enzymatic bioreactor in MD-EMBR. A few studies have covered the aspect of thermal stability of laccase under different experimental conditions. For instance, Nguyen et al. (2016a) observed stable laccase activity up to 40 °C when the enzyme solution was not spiked with TrOCs (*i.e.,* 'non-reacting' laccase solution). Conversely, in presence of TrOCs, Nair et al. (2013) and Kim and Nicell (2006) observed rapid drop in laccase activity beyond 30 °C. Therefore, in the current study, the temperature of the enzymatic bioreactor and permeate was kept at 30 and 10ºC, respectively.

The stability of the permeate flux was continually monitored during all experiments (Supplementary Data Figure S3). Permeate flux was stable during all experiments, and no significant decline was observed. Average permeate flux of 4.61±0.24, 3.78±0.35 and 3.74±0.46 L/m^2 h was obtained for MD only (control), MD-EMBR with *T. versicolor* and/or mediators and MD-EMBR with *A. oryzae* and/or mediators, respectively (Figure 6). Permeate flux depends more on the temperature of the feed side due to the exponential effect of increase in temperature on flux (Alkhudhiri et al., 2012). Thus, relatively low permeate flux in this study was expected.

4. Conclusion

This study investigated the removal of trace organic contaminants (TrOCs) by an integrated membrane distillation enzymatic bioreactor (MD-EMBR). Experiments were performed using laccase from two different sources, namely *Trametes versicolor* and genetically modified *Aspergillus oryzae*. Permeate flux of MD-EMBR was stable during all experiments. A mass balance revealed that enzymatic degradation was the major contributor in the overall removal of TrOCs. *A. oryzae* laccase demonstrated better TrOC removal and stable enzymatic activity at the end of experiments. Performance of MD-EMBR system was further improved with the addition of one natural (violuric acid, VA) and one synthetic (1-hydrozybenzotriazole, HBT) mediator at 1 mM concentration. Although HBT and VA both affected laccase stability, they increased the reaction rate, which resulted in rapid degradation of the selected compounds.

Acknowledgement

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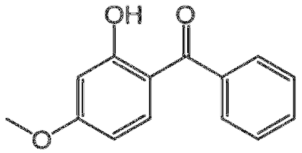
List of Tables

Table 1: Physicochemical properties of selected TrOC.

Compound	Molecular structure	Molecular weight (g/mol)	Log D at pH 7	Vapor pressure (mmHg)	Water solubility at 25 ^{o}C (mg/L)	H (atm m^3/mol)	pK_H at pH 7
Sulfamethoxazole		253.28	-0.22	1.87×10^{-09}	410	1.52×10^{-12}	11.81
Carbamazepine		236.27	1.89	5.78×10^{-07}	220	8.17×10^{-10}	9.08
Diclofenac		296.15	1.77	1.59×10^{-07}	30	2.06×10^{-09}	8.68
Oxybenzone		228.24	3.99	5.26×10^{-06}	100	1.58×10^{-08}	7.80
Atrazine		215.68	2.64	1.27×10^{-05}	69	5.22×10^{-08}	7.28

Note: Henry's law constant (H) at 25^{o}C (atm m^3/mol) = Vapor pressure × molecular weight/water solubility. The pK_H value is defined as $pK_H = -\log_{10}H$. Chemical structure, molecular weight (MW), log D, vapor pressure and water solubility values were taken from SciFinder Scholar.

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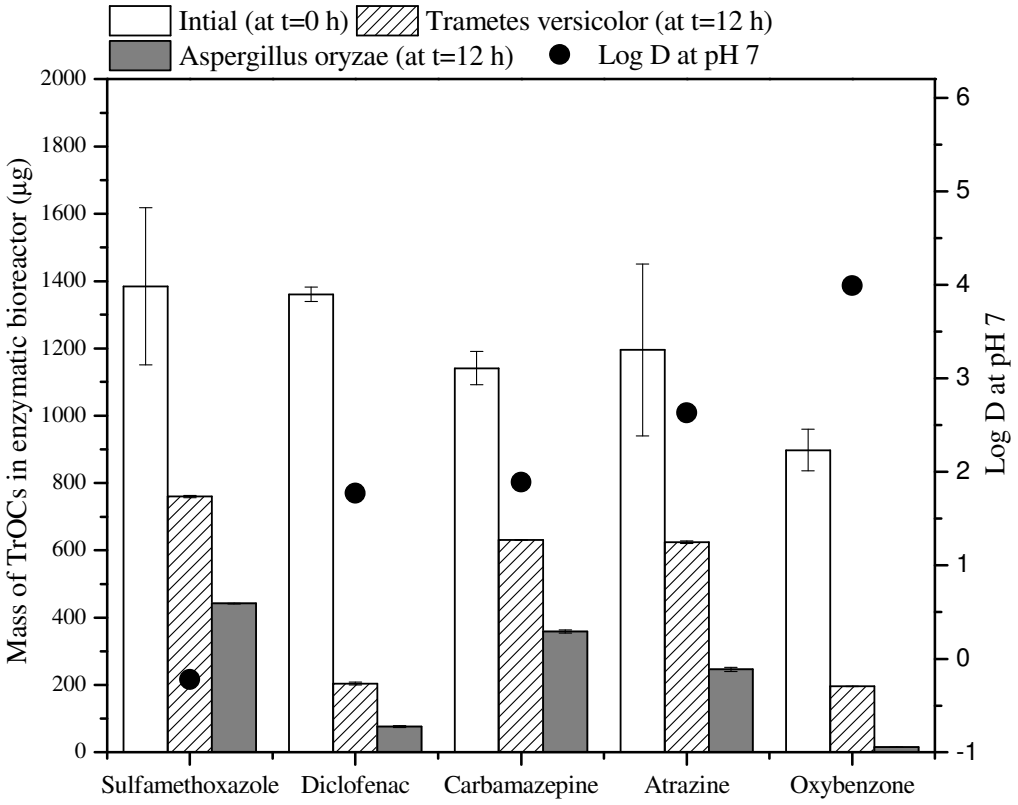
Intial (at t=0 h)
Trametes versicolor (at t=12 h)
Aspergillus oryzae (at t=12 h)
Log D at pH 7
Mass of TrOCs in enzymatic bioreactor (µg)
Log D at pH 7
0
200
400
600
800
1000
1200
1400
1600
1800
2000
-1
0
1
2
3
4
5
6
Sulfamethoxazole
Diclofenac
Carbamazepine
Atrazine
Oxybenzone

Figure 1

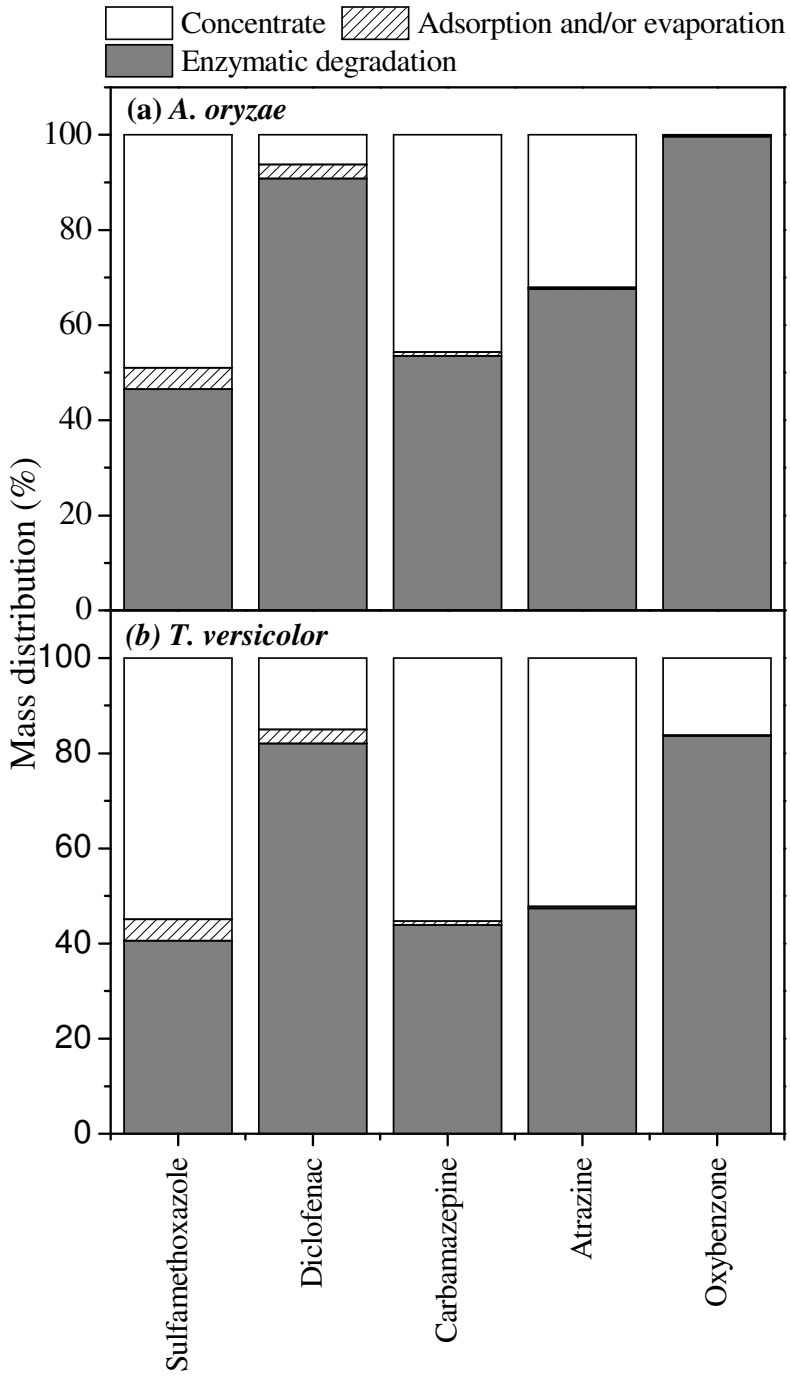
Concentrate
Adsorption and/or evaporation
Enzymatic degradation
(a) A. oryzae
(b) T. versicolor
Mass distribution (%)
0
20
40
60
80
100
Sulfamethoxazole
Diclofenac
Carbamazepine
Atrazine
Oxybenzone

Figure 2

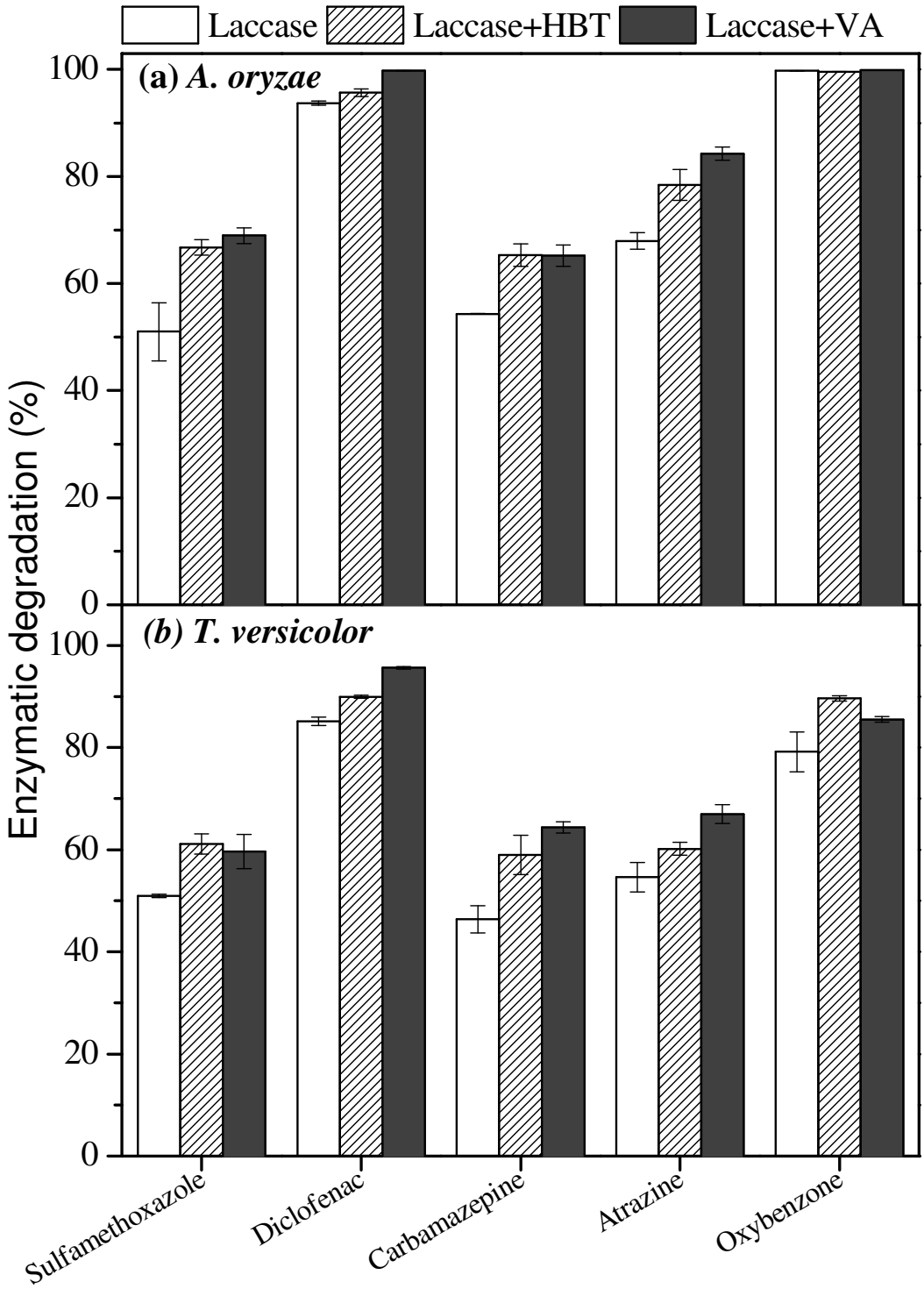
Laccase
Laccase+HBT
Laccase+VA
(a) A. oryzae
(b) T. versicolor
Enzymatic degradation (%)
0
20
40
60
80
100
Sulfamethoxazole
Diclofenac
Carbamazepine
Atrazine
Oxybenzone

Figure 3

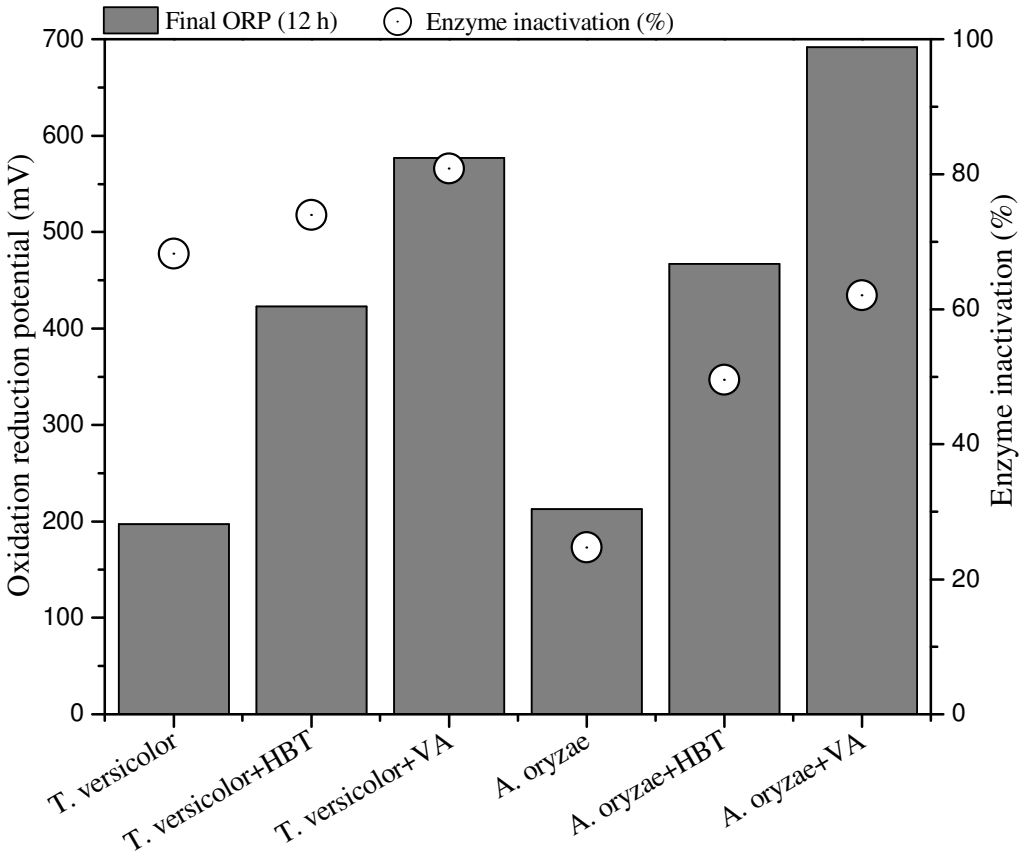
Final ORP (12 h)
Enzyme inactivation (%)
Oxidation reduction potential (mV)
Enzyme inactivation (%)
0
100
200
300
400
500
600
700
0
20
40
60
80
100
T. versicolor
T. versicolor+HBT
T. versicolor+VA
A. oryzae
A. oryzae+HBT
A. oryzae+VA

Figure 4

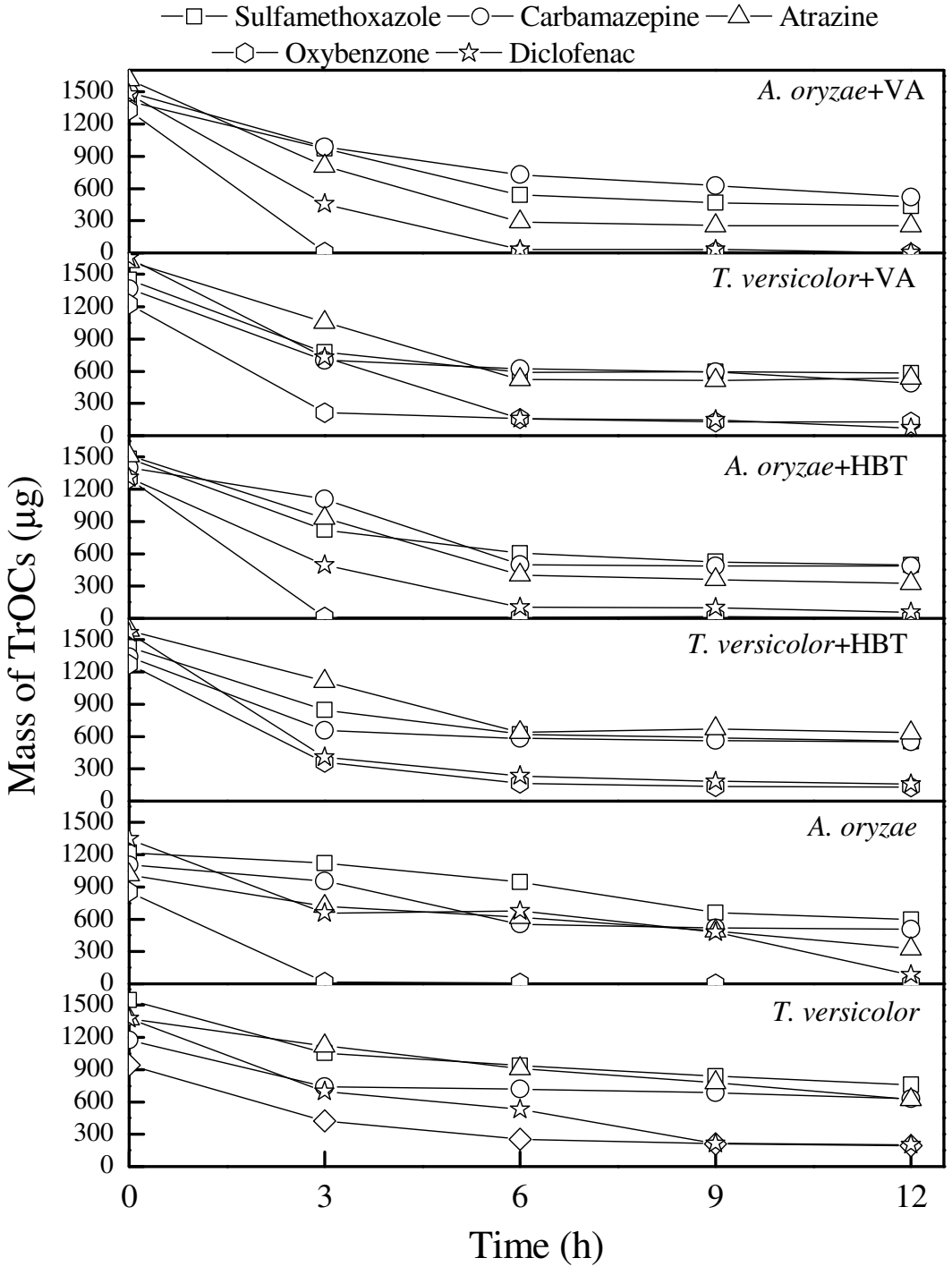
—□— Sulfamethoxazole —○— Carbamazepine —△— Atrazine
—⬡— Oxybenzone —☆— Diclofenac
A. oryzae+VA
T. versicolor+VA
A. oryzae+HBT
T. versicolor+HBT
A. oryzae
T. versicolor
Mass of TrOCs (μg)
Time (h)
0
300
600
900
1200
1500
3
6
9
12

Figure 5

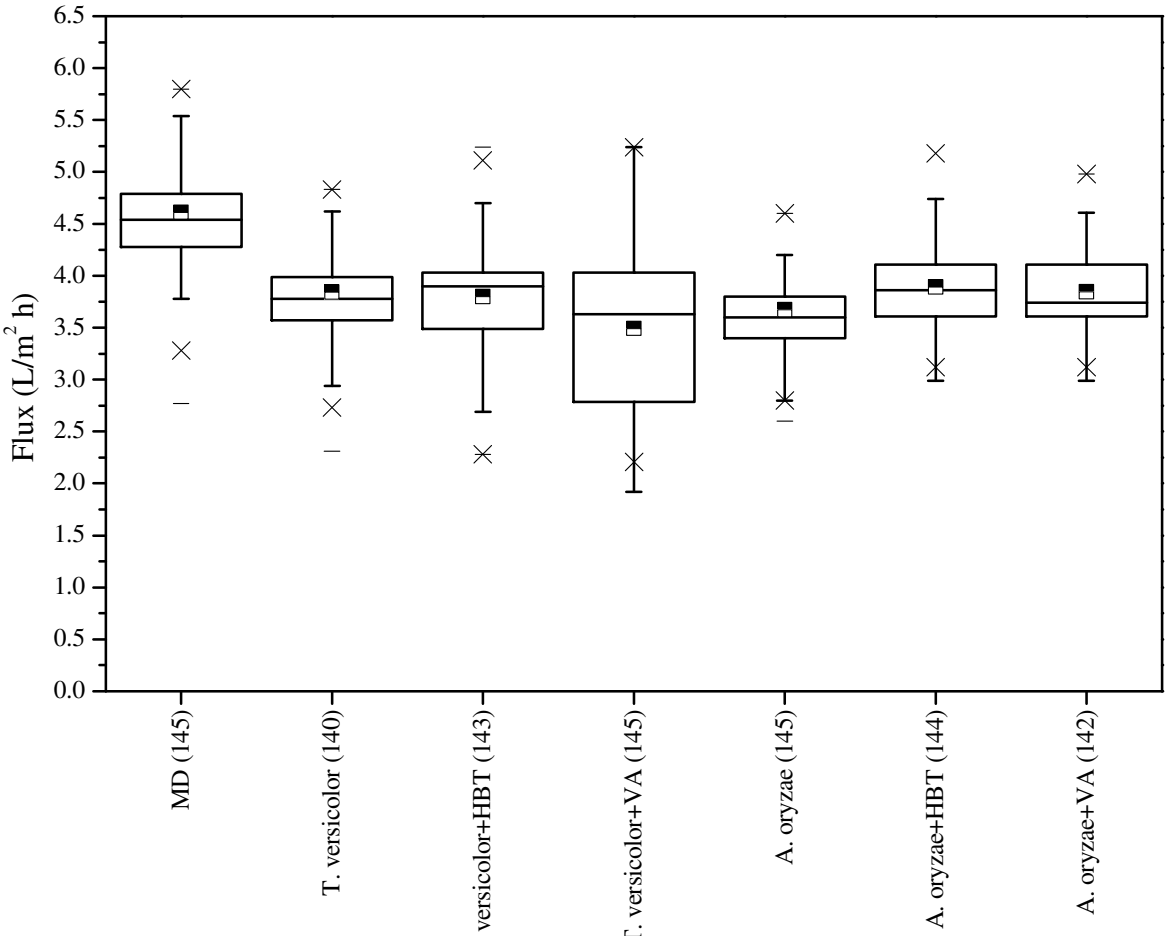
Flux (L/m^2 h)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MD (145)
T. versicolor (140)
versicolor+HBT (143)
T. versicolor+VA (145)
A. oryzae (145)
A. oryzae+HBT (144)
A. oryzae+VA (142)

Figure 6

Integration of an enzymatic bioreactor with membrane distillation for enhanced biodegradation of trace organic contaminants

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Muhammad B. Asif [a], Luong N. Nguyen [a,b], Faisal I. Hai [a]*, William E. Price [c], Long D. Nghiem [a]

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

[b] School of Civil and Environmental Engineering, Nanyang Technological University, 50 Nanyang Avenue 639798, Singapore.

[c] Strategic Water Infrastructure Lab, School of Chemistry, University of Wollongong, Wollongong, NSW 2522, Australia.

Corresponding author: Faisal I. Hai (E-mail: faisal@uow.edu.au; Ph: +61 02 42213054)

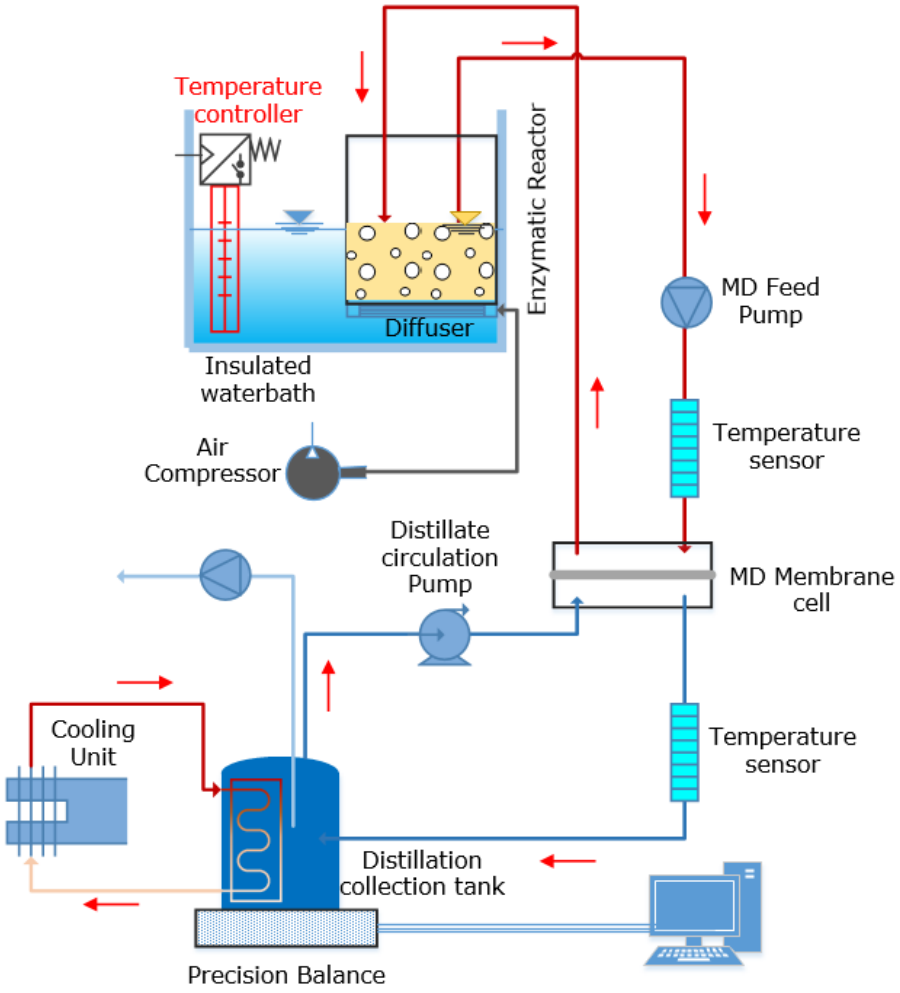

Figure S1. Schematics of membrane distillation-enzymatic membrane bioreactor (MD-EMBR)

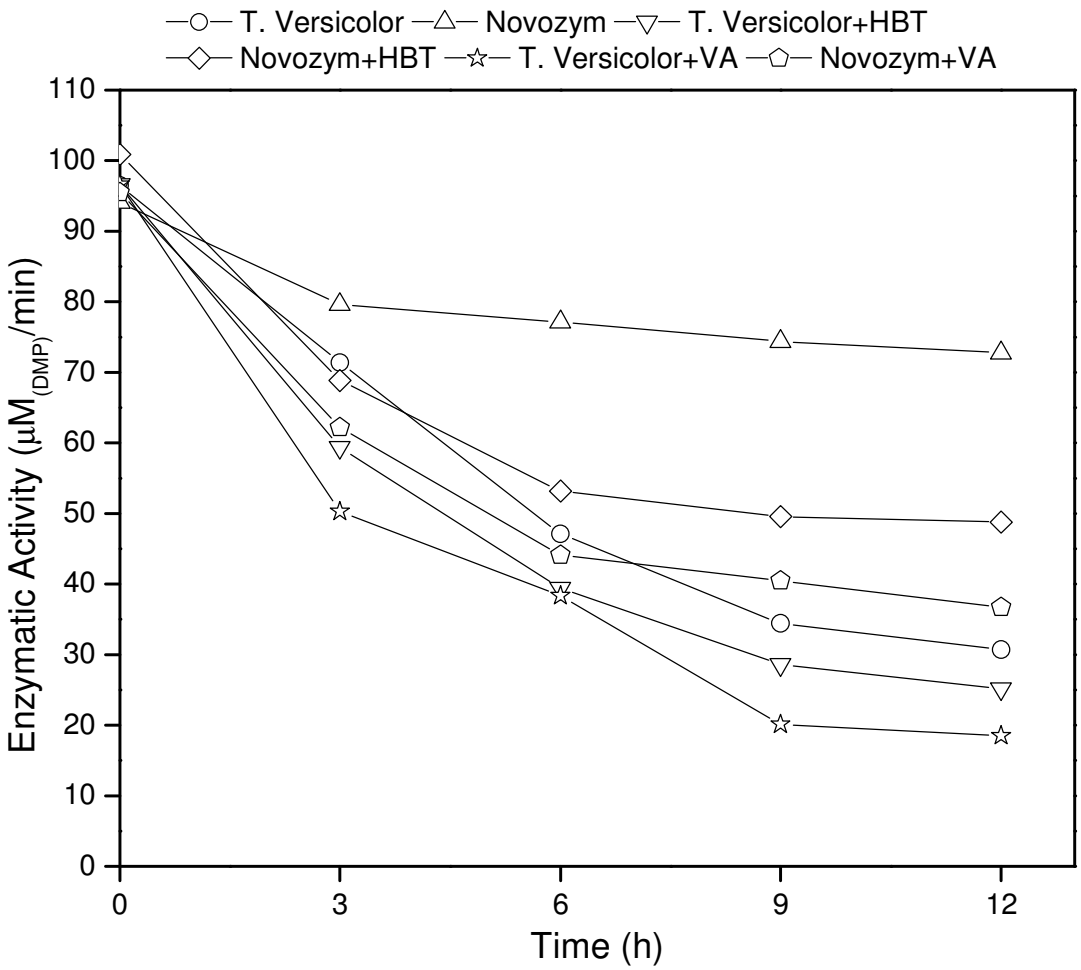

Figure S2. Enzymatic Activity profiles with and without the addition of redox mediator(s) in enzymatic membrane distillation system. Two N–OH type redox mediators namely, 1-hydrozybenzotriazole (HBT) and violuric acid (VA), were added separately at 1mM concentration.

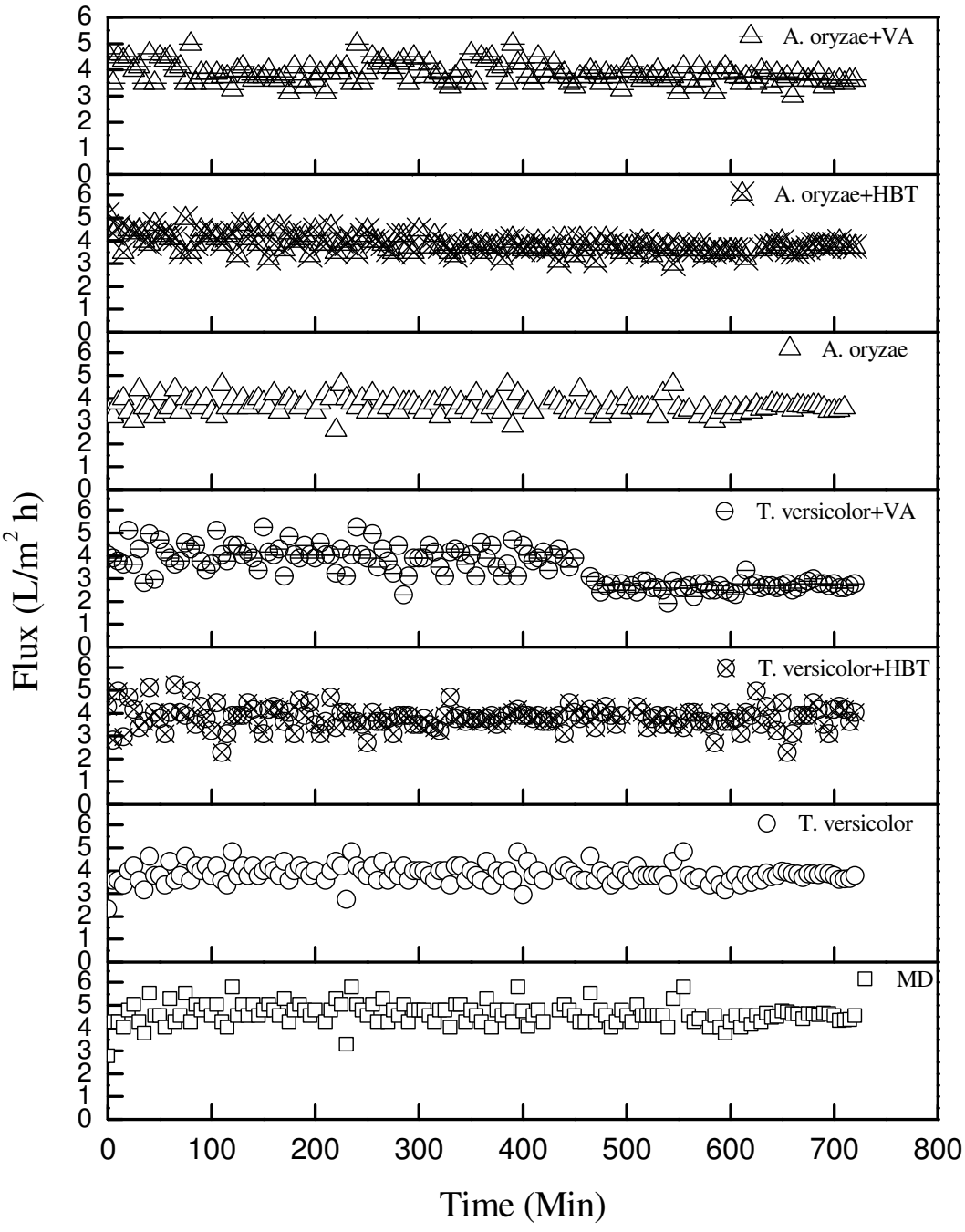

Figure S3. Variations in permeate flux during the operation of MD and MD-EMR systems. Feed and distillate temperature were controlled at 30 and 10 °C, respectively. The cross-flow rate of both feed and distillate side was set at 1 L/min (corresponding to a cross-flow velocity of 9 cm/s). Concentration of both HBT and VA was 1 mM.