

2015

Trace organic contaminant removal by fungal membrane bioreactors and enzymatic membrane reactors

Luong Ngoc Nguyen
University of Wollongong

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Nguyen, Luong Ngoc, Trace organic contaminant removal by fungal membrane bioreactors and enzymatic membrane reactors, Doctor of Philosophy thesis, School of Civil, Mining and Environmental Engineering, University of Wollongong, 2015. <https://ro.uow.edu.au/theses/4540>

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School of Civil, Mining and Environmental Engineering

Faculty of Engineering and Information Sciences

University of Wollongong, Australia

**Trace organic contaminant removal by fungal membrane bioreactors and
enzymatic membrane reactors**

A thesis submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

from

UNIVERSITY OF WOLLONGONG

by

Luong Ngoc Nguyen

October, 2015

CERTIFICATION

I, Luong Ngoc Nguyen, hereby declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy to the School of Civil, Mining and Environmental Engineering, Faculty of Engineering and Information Sciences, University of Wollongong is wholly my own work unless otherwise acknowledged. The document has not been submitted for qualification at any other academic institution.

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THESIS RELATED PUBLICATIONS

Journal articles

1. **Luong N Nguyen**, Jason P. van de Merwe, Faisal I Hai, Frederic DL Leusch, Jinguo Kang, William E Price, Felicity Roddick, Saleh F. Magram and Long D Nghiem. (2015) Laccase - syringaldehyde (SA) - mediated degradation of trace organic contaminants (TrOCs) in an enzymatic membrane reactor (EMR): Removal efficiency and effluent toxicity. *Bioresource Technology*, (Accepted, October 2015).
2. **Luong N Nguyen**, Faisal I Hai, William E Price, Jinguo Kang, Frederic DL Leusch, Felicity Roddick, Jason P van de Merwe, Saleh F Magram, Long D Nghiem. (2015) Degradation of a broad spectrum of trace organic contaminants by an enzymatic membrane reactor: Complementary role of membrane retention and enzymatic degradation. *International Biodeterioration & Biodegradation*, 99, 115-122.
3. **Luong N Nguyen**, Faisal I Hai, William E Price, Frederic DL Leusch, Felicity Roddick, Hao H Ngo, Wenshan Guo, Saleh F Magram, Long D Nghiem. (2014) The effects of mediator and granular activated carbon addition on degradation of trace organic contaminants by an enzymatic membrane reactor. *Bioresource Technology*, 167, 169-177.
4. **Luong N Nguyen**, Faisal I Hai, William E Price, Frederic DL Leusch, Felicity Roddick, Ewan J McAdam, Saleh F Magram, Long D Nghiem. (2014) Continuous biotransformation of bisphenol A and diclofenac by laccase in an enzymatic membrane reactor. *International Biodeterioration & Biodegradation*. 95, 25-32.
5. **Luong N Nguyen**, Faisal I Hai, Jinguo Kang, Frederic DL Leusch, Felicity Roddick, Saleh F Magram, William E Price, Long D Nghiem. (2014) Enhancement of trace organic contaminant degradation by crude enzyme extract from *Trametes versicolor* culture: Effect of mediator type and concentration. *Journal of the Taiwan Institute of Chemical Engineers*. 45, 1855-1862.
6. **Luong N Nguyen**, Faisal I Hai, Shufan Yang, Jinguo Kang, Frederic DL Leusch, Felicity Roddick, William E Price, Long D Nghiem. (2014) Removal of pharmaceuticals, steroid hormones, phytoestrogens, UV-filters, industrial chemicals and pesticides by *Trametes versicolor*: Role of biosorption and biodegradation. *International Biodeterioration & Biodegradation*, 88, 169-175.

7. **Luong N Nguyen**, Faisal I Hai, Jinguo Kang, Saleh F Magram, William E Price, Long D Nghiem. (2014). Impact of 1-hydroxybenzotriazole dosing on trace organic contaminant degradation by laccase. *Journal of Water Sustainability*, 4, 41-48.
8. **Luong N Nguyen**, Faisal I Hai, Shufan Yang, Jinguo Kang, Frederic DL Leusch, Felicity Roddick, William E Price, Long D Nghiem. (2013) Removal of trace organic contaminants by an MBR comprising a mixed culture of bacteria and white-rot fungi, *Bioresource technology*, 148, 234-241.
9. Shufan Yang, Faisal I Hai, **Luong N Nguyen**, Long D Nghiem, Felicity Roddick, William E Price. (2013) Removal of bisphenol A and diclofenac by a novel fungal membrane bioreactor operated under non-sterile conditions. *International Biodeterioration & Biodegradation*, 85, 483-490.

Book chapters:

1. Faisal I. Hai, **Luong N. Nguyen**, Long D. Nghiem, Bao-Qiang Liao, Ismail Koyuncu, and William E. Price, 2015. Trace Organic Contaminants Removal by Combined Processes for Wastewater Reuse. In D. Fatta-Kassinos, D.D. Dionysiou and K. Kümmerer (Eds) *Emerging Challenges in Wastewater Reuse: Contaminants, Treatment, Effects*, Springer, Germany, pp. 519-568.
2. Faisal I. Hai, Abdulhakeem Alturki, **Luong N. Nguyen**, William E. Price and Long D. Nghiem, 2015. Removal of Trace Organic Contaminants by Integrated Membrane Processes for Water Reuse Application. In Hao H. Ngo, Wenshan Guo, Rao Y. Surampalli and Tian C. Zhang (Eds). *Membrane Reactors for Energy Applications*, ASCE, USA, Accepted, In press.

Conference presentation:

1. **Luong N. Nguyen**, Faisal I. Hai, Anthony Dosseto, William E. Price, and Long D. Nghiem. (2015) Continuous adsorption and biotransformation of micropollutants by granular activated carbon-bound laccase in a packed-bed immobilized enzyme reactor. 8th Challenges in Environmental Sciences and Engineering, Sydney, Australia (September 2015) (Received Best Oral Presentation Award).

2. Bradley Ashe, **Luong N. Nguyen**, Faisal I. Hai, William E. Price, and Long D. Nghiem. (2015)
Impacts of redox-mediator type on enzymatic degradation of trace organic contaminants:
Degradation efficiency, enzyme stability and effluent toxicity. 8th Challenges in
Environmental Sciences and Engineering, Sydney, Australia (September 2015).
3. **Luong N Nguyen**, Faisal I Hai, William E Price, Frederic DL Leusch, Felicity Roddick, Ewan
J McAdam, Saleh F Magram, Long D Nghiem. (2013) Continuous biotransformation of
bisphenol A and diclofenac by laccase in an enzymatic membrane reactor. 6th Challenges
in Environmental Sciences and Engineering, Daegu, Korea (November 2013).

ABSTRACT

The occurrence of trace organic contaminants (TrOCs) in raw and secondary treated effluent is of significant human and ecological health concern, particularly with regard to the safety of the downstream production of water for human consumption. Efficient removal of TrOCs is a prerequisite to wastewater reclamation which is regarded as an important means of tackling the ever increasing demand for water. This thesis work investigated the removal of TrOCs by whole-cell white rot fungi (WRF) and their extracellular enzyme (*i.e.*, laccase) in membrane bioreactors (MBRs) and enzymatic membrane reactors (EMRs).

The first step of this research compared the TrOC removal performance of live (biosorption + biodegradation), intracellular enzyme-inhibited and chemically inactivated (biosorption only) whole-cell preparations and the fungal extracellular enzyme extract (predominantly laccase) from *Trametes versicolor* (ATCC 7731). Because phenolic substrates are amenable to degradation by laccase, all 14 phenolic TrOCs were readily biodegraded by the live whole-cell culture. On the other hand, only eight of the 16 non-phenolic TrOCs were readily biodegraded, while the removal of hydrophilic TrOCs ($\log D < 3$) was negligible. With the exception of diclofenac, no non-phenolic TrOCs were degraded by the extracellular enzyme extract. The whole-cell culture showed considerably higher degradation of at least seven compounds, indicating the importance of biosorption and subsequent degradation by intracellular and/or mycelium associated enzymes. TrOC removal performance of laccase was improved by the addition of redox-mediator (1-hydroxybenzotriazole (HBT) and syringaldehyde (SA)). The laccase-mediator system extended the spectrum of efficiently degraded TrOCs to 13 phenolic and three non-phenolic compounds, with moderate improvements in removal of a few other non-phenolic compounds. TrOC removal efficiency improved significantly as the HBT dose was increased from 0.1 to 0.5 mM, while SA achieved similar removal over dosage range of 0.1–1 mM. However, a particular concern was the toxicity of the treated media (1200–2200 times that of the control) for all SA dosages applied. Overall, HBT at a concentration of 0.5 mM achieved the best removal without raising concern regarding toxicity of the treated media. The results have been discussed in the light of the redox potential of the enzyme-mediator cocktail,

the balance between the stability and reactivity of the radicals generated, and their cytotoxic effects.

The batch tests confirmed significant removal of various TrOCs by whole-cell *T. versicolor* and its extracellular extract under sterile conditions. However, little is known about TrOC removal in continuous flow fungal reactors in a non-sterile environment. The degradation of 30 TrOCs by a white-rot fungus-augmented MBR was investigated. Overall, the fungus-augmented MBR showed better TrOC removal compared to a system containing conventional activated sludge. The major role of biodegradation in removal by the MBR was noted. Continuous mediator dosing to MBR may potentially enhance its performance, although not as effectively as for mediator-enhanced batch laccase systems. A toxicity bio-assay revealed no significant increase in the toxicity of the effluent during MBR treatment of the synthetic wastewater comprising TrOCs, confirming that no toxic by-products were produced.

One of the challenges facing the application of laccase in a continuous flow wastewater treatment process is the wash out of the enzyme along with the treated effluent. In this thesis work, an EMR equipped with an ultrafiltration (UF) membrane was developed to facilitate retention of laccase and its separation from treated effluent. A commercially available laccase purified from *Aspergillus oryzae* (Novozymes, Australia) was used. The UF membrane completely retained laccase but periodic enzyme addition was required due to its denaturation. Under the operation condition in this thesis, the laccase activity was maintained by addition of a laccase dose of 23 mg/L d every 12 h. The EMR was observed to facilitate degradation of a number of TrOCs originally showing resistance to enzymatic degradation. This was attributed to the formation of a dynamic layer of laccase over the membrane surface which retained TrOCs and facilitated their subsequent enzymatic degradation. A complementary role of enhanced degradation by the laccase-mediator system and retention by the membrane gel layer was revealed. Membrane retention particularly enhanced the degradation of the compounds which are less amenable to mediator-enhanced enzymatic degradation.

The performance of the EMR was further evaluated in terms of removal efficiency and toxicity of the treated effluent under different concentrations of the TrOCs and a redox-mediator syringaldehyde (SA). The results showed that SA can improved laccase-catalyzed degradation of

TrOCs in EMR but may increase effluent toxicity. A specific emphasis was placed on the investigation of the toxicity of the enzyme (laccase), SA, TrOCs and the treated effluent. Batch tests demonstrated significant individual and interactive toxicity of the laccase and SA preparations. TrOC removal efficiency by the EMR was observed to increase with influent TrOC concentration up to 50 µg/L, but decreased for some compounds when the influent TrOC concentration was further increased to 100 µg/L, indicating kinetic limitations. On the other hand, SA addition at a concentration of 10 µM significantly improved TrOC removal, but no removal improvement was observed at the elevated SA concentrations of 50 and 100 µM. The treated effluent showed significant toxicity at SA concentrations beyond 10 µM, providing further evidence that higher dosage of SA must be avoided.

Finally, the effect of granular activated carbon (GAC) addition on TrOC removal performance, effluent toxicity and membrane fouling was investigated. In this part of the study, four non-phenolic resistant TrOCs, namely carbamazepine, diclofenac, sulfamethoxazole and atrazine were used. A one-off dose of 3 g/L GAC was added into the EMR. The results showed that GAC addition could enhance the TrOC degradation by the laccase-SA system, reduce the mediator-induced effluent toxicity and mitigate membrane fouling by preventing excessive accumulation of a gelatinous layer of laccase over the membrane. Mass balance analysis reveals that the improvement was not due solely to adsorption on GAC but also enhanced biodegradation.

ACKNOWLEDGEMENT

My PhD journey at the University of Wollongong has been full of exciting challenges and opportunities.

I express my deepest gratitude to Dr Faisal I. Hai, who has been a dedicated supervisor. I am honoured to work under his supervision for the last five years – first in connection to my Masters degree and then the PhD. Without his continuous support and guidance, it would not be possible to complete my thesis.

I thank my co-supervisor Professor William E. Price for his insightful guidance and encouragement throughout my PhD studies.

I am grateful to Professor Long D. Nghiem for his kind support and constructive comments during the research meetings.

The University of Wollongong is greatly appreciated for the provision of a PhD scholarship. My research project was funded by the UOW GeoQuEST Research Centre.

Dr Jinguo Kang is thanked for his assistance in GC-MS analysis.

It has been a great pleasure to work in a dynamic research group with full of motivated and helpful colleagues. They are the source of my motivation, and I appreciate their help during my PhD studies. The long list includes: Abdulhakeem Ali Alturki, Ming Xie, Takahiro Fujioka, Kaushalya Wijekoon, Wenhai Luo, and Galilee Semblante. The technical staffs of the Faculty of Engineering and Information Sciences, particularly Frank Crabtree, are also thanked for their valuable help.

I would like to thank my parents and brothers for their continuous inspiration. I wish them happiness and health every day.

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AOPs	Advanced oxidation processes
CAS	Conventional activated sludge
COD	Chemical oxygen demand
DCM	Dichloromethane
DMP	2,6-dimethoxyphenol
DO	Dissolved oxygen
GAC	Granular activated carbon
GC-MS	Gas chromatography- mass spectrometry
EDCs	Endocrine disrupting chemicals
EDG	Electron donating group
EMR	Enzymatic membrane reactor
EWG	Electron withdrawing group
HBT	1-hydroxylbenzotriazole
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
LC-MS	Liquid chromatography- mass spectrometry
LME	Lignin modifying enzyme
LiP	Lignin peroxidase
MBE	Malt extract broth
MBR	Membrane bioreactor
MnP	Manganese peroxidase
MLNVSS	Mixed liquor non-volatile suspended solids
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
MWCO	Molecular weight cut off
NF	Nanofiltration
NPOC	Non purgeable organic carbon
PAC	Powdered activated carbon

PhACs	Pharmaceutically active compounds
RO	Reverse osmosis
SA	Syringaldehyde
SPE	Solid phase extraction
TEMPO	2,2,6,6-tetramethylpiperidin-1-yloxy
TMP	Transmembrane pressure
TN	Total nitrogen
TOC	Total organic compounds
TrOCs	Trace organic contaminants
WRF	White-rot fungi
WWTPs	Wastewater treatment plants

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

The term trace organic contaminants (TrOCs) refer to several groups of pollutants present in water and wastewater at very low concentration (*i.e.*, several micrograms per litre or lower). Classes of TrOCs include endocrine disrupting compounds (EDCs), pharmaceutical and personal care products (PPCPs), disinfection by-products (DBPs), UV filters, steroid hormones, phytoestrogens and industrial chemicals. These have been detected in sewage and sewage-impacted surface waters all over the world, including Australia (Schwarzenbach et al., 2006; Ort et al., 2010; Saba et al., 2012).

The occurrence of these TrOCs in secondary treated effluent is of significant human and ecological health concern because TrOCs have been found to cause adverse physiological changes in aquatic organisms and can potentially affect human health on prolonged exposure. Thus, efficient removal of TrOCs is a prerequisite to wastewater reclamation which is regarded as an important means of tackling the ever increasing demand for water from a growing population, changing lifestyle patterns due to urbanization, and diminishing and uncertain natural water resources due to erratic weather patterns (Radcliffe 2006; Fane 2007). It has been a matter of concern that conventional wastewater treatment plants do not remove certain groups of TrOCs effectively. The TrOC removal capacity of conventional wastewater treatment depends significantly on the biological treatment stage where trace organics are removed by adsorption on suspended solids possibly followed by biodegradation. Removal of some hydrophobic compounds has been reported to be mainly due to adsorption. Wick et al. (2009) confirmed that no significant removal of certain hydrophilic and resistant pharmaceuticals (*i.e.*, carbamazepine and diclofenac) was observed in the conventional wastewater treatment plant. Incomplete removal of pharmaceuticals such as naproxen, ketoprofen and diclofenac during conventional wastewater treatment has also been reported by Kimura et al. (2007). Over the last decade, many studies have investigated TrOC removal from water and wastewater using physicochemical, biological, or advanced oxidation processes, or a combination of different processes. These

studies have led to encouraging outcomes; however, a universal end-of-pipe treatment process is yet to be formulated. The available technologies have their own pros and cons. For example, membrane filtration processes such as nanofiltration or reverse-osmosis have the potential to retain TrOCs via size exclusion and/or charge repulsion; however, hydrophobic TrOCs can accumulate on membrane and subsequently diffuse through it (Alturki et al., 2010). Similarly, physicochemical treatment processes such as adsorption only transfer TrOCs from one form to another and produce large volumes of secondary waste.

Biodegradation is an attractive technology that utilizes microorganisms to clean up the environment (Watanabe 2001). A potential alternative to the application of conventional activated sludge process is the use of white-rot fungi (WRF) cultures and their lignin modifying enzymes (LME). Fungi display many features and components that could be used to remediate polluted soil and water. WRF have the potential to be useful biocatalysts due to their broad substrate coverage through a battery of extracellular (*i.e.*, laccases, manganese peroxidases (MnP) and lignin peroxidases (LiP)) and intracellular enzymes (*i.e.*, cytochrome P450) (Cabana et al., 2007; Marco-Urrea et al., 2009; Harms et al., 2011) (**Figure 1.1**). Initially extracellular or intracellular enzyme systems attack the target pollutant. Metabolites generated during extracellular pollutant oxidation may be further subject to intracellular catabolism. Metabolites arising from intracellular attack may be excreted and can then either undergo further extracellular enzymatic reactions or may undergo further intracellular catabolism. This may result in mineralization or, again, in metabolite excretion at various oxidation stages if subsequent oxidation is impeded (Harms et al., 2011). WRF can degrade a range of compounds that are inefficiently degraded by bacteria. For example, although low molecular mass polycyclic aromatic hydrocarbons are readily degraded by bacteria, however, as molecular mass increases, their bioavailability strongly decrease which make them resistant to bacterial degradation. Fungi could intracellularly hydroxylate polycyclic aromatic hydrocarbons and further convert them to water soluble products (Juhasz et al., 2000). WRF and their LMEs have been reported to degrade pollutants that are not amenable to bacterial degradation such as azo dyes, polycyclic aromatic hydrocarbons, chlorophenols, nitrotoluenes and polychlorinated biphenyls (Hai et al., 2007; Hai et al., 2012). The ability to degrade a wide variety of xenobiotics makes these microorganisms potentially useful in application for TrOC removal.

To date, there have been a number of small-scale and batch studies on the application of WRF and their LMEs for TrOC degradation via whole cell fungi culture or extracellular enzyme preparations under different operating conditions (*e.g.*, pH and culture media). Evidence from literature indicates that WRF and their LMEs can degrade various TrOCs, and their removal efficiency is governed by the physicochemical properties of TrOCs, fungus strains and enzyme types (Marco-Urrea et al., 2009; Tran et al., 2010). Laccase is an important oxidoreductase enzyme available predominantly from fungi, but also from bacteria. The substrate range of laccase can be expanded in the presence of small molecular weight redox-mediators that act as an ‘electron shuttle’ between the enzyme and the target compounds. The degree of enhancement depends predominantly on the type of mediator and the TrOC structure.

Despite the potential, only a few studies have investigated TrOC removal by WRF in continuous flow reactors (Blázquez et al., 2008; Jelic et al., 2012). Furthermore, there are only two available studies on the removal of TrOC under non-sterile conditions as would likely be experienced in practice (Zhang et al., 2012; Yang et al., 2013). Adsorption on fungal mycelium and biodegradation by the fungal enzymes (extracellular and/or intracellular enzymes) are the two main mechanisms for TrOC removal in a continuous flow fungal reactor. However, there are two important inherent limitations of continuous flow fungal reactors i) washout of extracellular enzyme (which is necessary for the degradation) with treated effluent and ii) inhibition due to bacterial presence (deactivating enzyme and inhibiting fungal growth). Therefore, it is clear that more systematic studies on the enhancement of fungal degradation of TrOCs in continuous flow reactors are necessary.

Pollutant degradation via harvested enzymes, in contrast to degradation by live whole-cells, can help separating the ‘microbial growth’ and ‘microbial degradation’ steps. Thus enzymatic degradation of wastewater-borne resistant pollutants has gained much attention in recent years. Compared to conventional chemical oxidation, degradation by enzyme such as laccase can be achieved under milder conditions, while realizing higher rates and reaction specificity. However, enzyme-washout with treated effluent is a critical problem encountered during their application in continuous systems such as wastewater treatment plants. The application of a membrane with a smaller pore size relative to the enzyme molecule inside the enzymatic reactor forms an enzymatic membrane reactor (EMR). This approach offers several advantages over other

alternatives: (i) EMR retains enzymes more effectively than conventional packed bed reactors; (ii) operation with free enzyme avoids limitation of mass transfer associated with immobilization on carriers; and (iii) enzyme can be easily replenished during long term operation (Modin et al., 2014). Notably, most of the available studies on enzymatic degradation of resistant compounds in general and TrOCs in particular have been conducted in small scale and batch mode. Investigation of TrOC removal by EMR thus remains largely an unexplored topic.

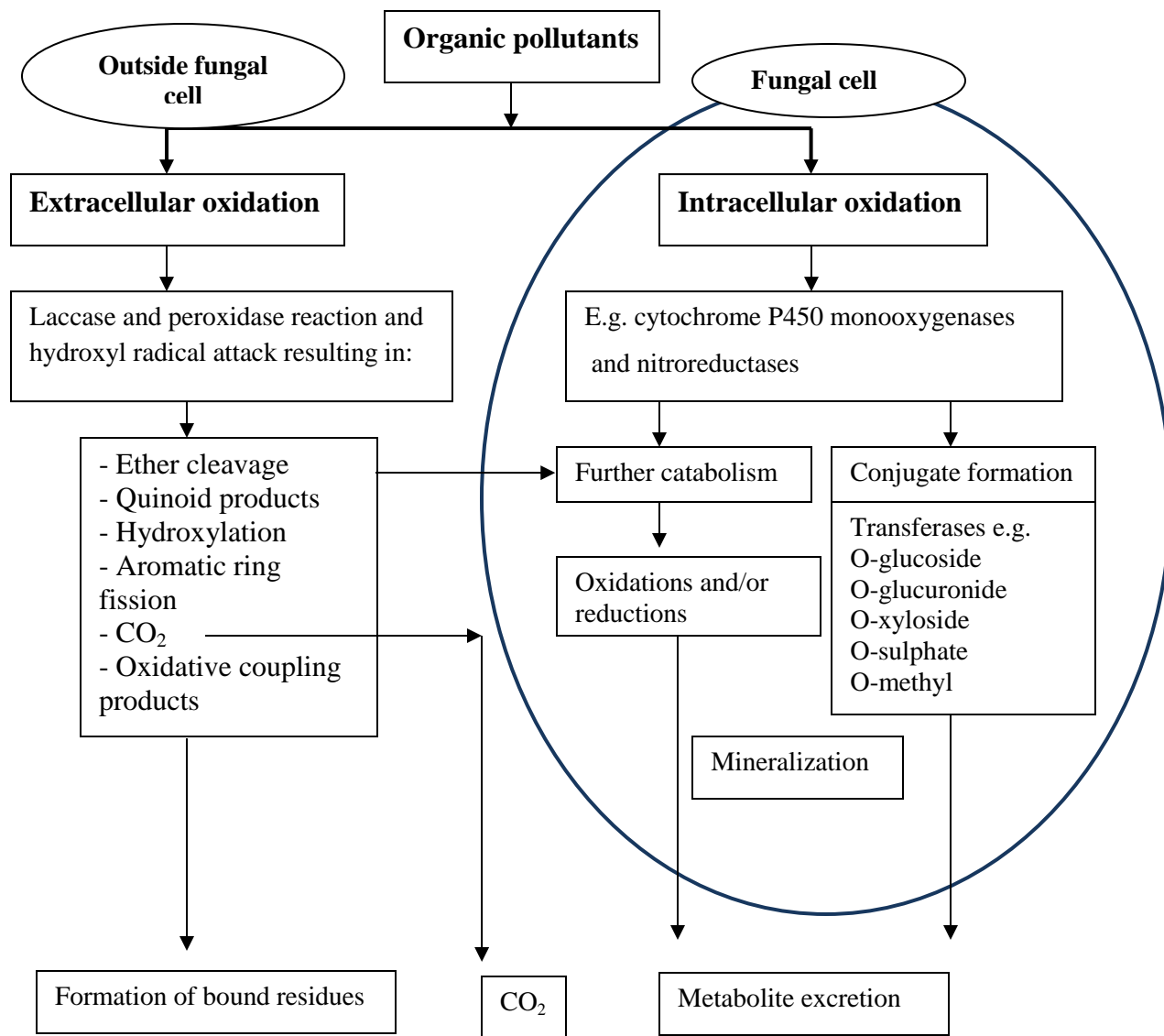


Figure 1.1: Principal methods used by fungi to degrade organic chemicals (adapted from Harms et al., 2011).

1.2. RESEARCH OBJECTIVES

The overall goal of this research is to systematically investigate TrOC removal by a fungal-augmented membrane bioreactor and an enzymatic membrane reactor (EMR). The specific objectives are to:

1. Evaluate TrOC removal efficiency of whole-cell WRF and their enzyme systems in batch conditions.
2. Assess TrOC removal performance of a fungal membrane bioreactor.
3. Investigate the role of enzymatic degradation and membrane retention in TrOC removal by EMR.
4. Investigate the influence of redox-mediators on TrOC removal by EMR.
5. Assess the impact of granular activated carbon addition on TrOC removal by EMR.

1.3. THESIS OUTLINE

This thesis contains eight chapters as schematically presented in **Figure 1.2**. Chapter 1 introduces the background, and research objectives of this study. Chapter 2 provides a comprehensive literature review of the current technologies for TrOC removal, and current knowledge regarding application of WRF and their LMEs for TrOC removal. The results are discussed under two broad sections. The first section, including Chapter 3 and 4, presents the removal of TrOCs by whole-cell WRF and their LMEs under batch test conditions (Chapter 3) and by a whole-cell fungal membrane bioreactor (Chapter 4). The fungal biomass and crude extracellular enzyme solution from the culture of WRF *Trametes versicolor* was used in Chapter 3 and 4. The second section includes the rest of the chapters, discusses results from a series of investigations on TrOC removal by EMRs. In chapter 5, the performance of an EMR utilizing a commercially available laccase from *Aspergillus oryzae* for the removal of 30 chemically diverse TrOCs was presented. Chapter 6 focuses on the impact of redox-mediator concentration on TrOC removal by EMR. Particularly, the toxicity of treated effluents is systematically investigated in this chapter. Chapter 7 assesses the impact of simultaneous dosing of mediator and an adsorbent (*i.e.*, granular activated carbon) on TrOC removal by EMR. Finally, chapter 8 summarizes the overall outcomes of work and the recommendations for future research.

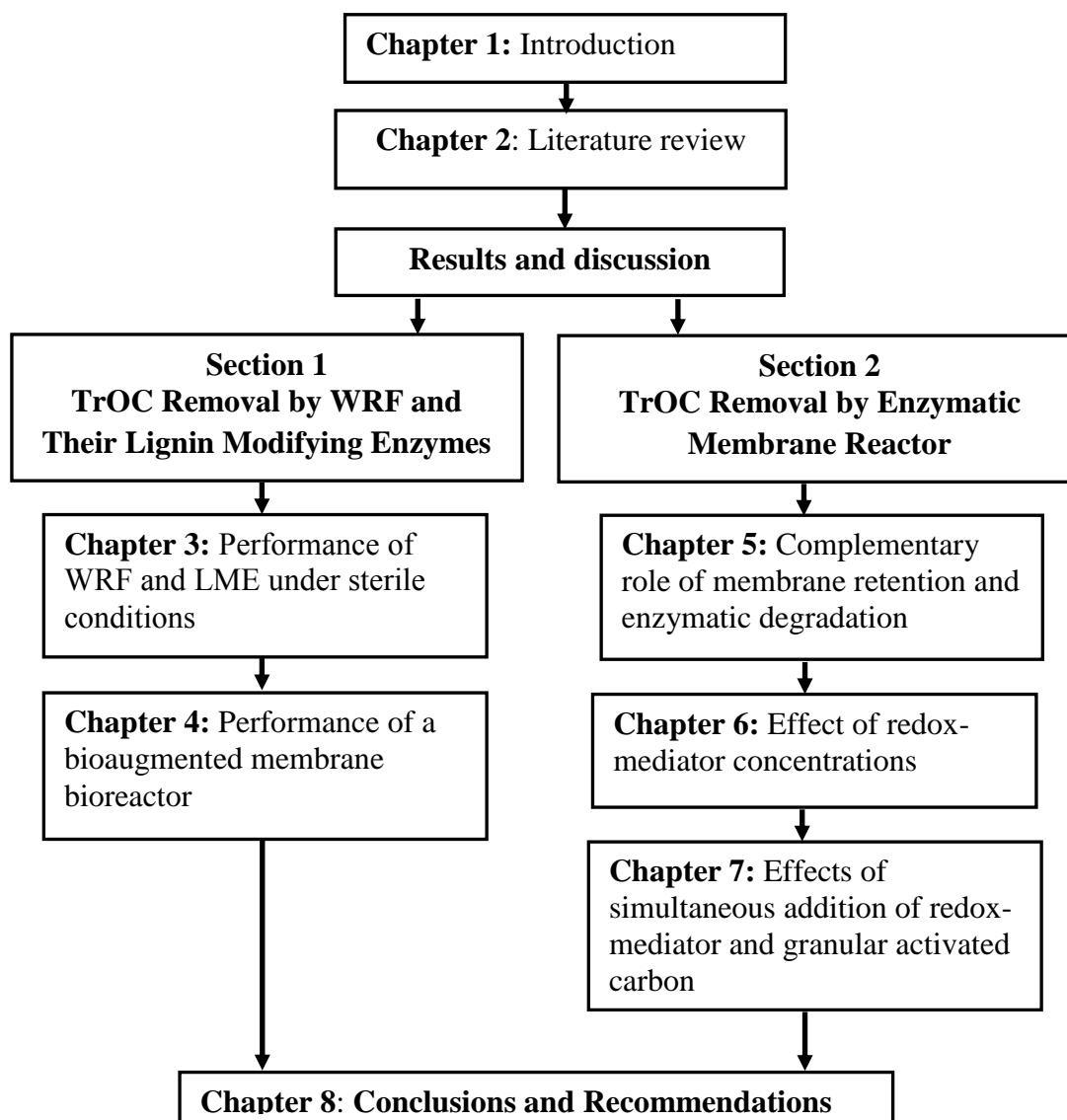


Figure 1.2: Schematic description of the thesis outline.

1.4. REFERENCES

Alturki, A. A., N. Tadkaew, J. A. McDonald, S. J. Khan, W. E. Price and L. D. Nghiem (2010). Combining MBR and NF/RO membrane filtration for the removal of trace organics in indirect potable water reuse applications. *J. Membr. Sci.*, 365, 206-215.

- Blázquez, P. and B. Guieysse (2008). Continuous biodegradation of 17 β -estradiol and 17 α -ethynylestradiol by *Trametes versicolor*. J. Hazard. Mater., 150, 459-462.
- Cabana, H., J. P. Jones and S. N. Agathos (2007). Elimination of endocrine disrupting chemicals using white-rot fungi and their lignin modifying enzymes: A Review. Eng. Life Sci., 7, 429-456.
- Fane, A. G. (2007). Sustainability and membrane processing of wastewater for reuse. Desalination. 202, 53.
- Hai, F. I., K. Yamamoto and K. Fukushima (2007). Hybrid treatment systems for dye wastewater. Critical Rev Environ Sci Technol. 37, 315-377.
- Hai, F. I., K. Yamamoto, F. Nakajima and K. Fukushima (2012). Application of a GAC-coated hollow fiber module to couple enzymatic degradation of dye on membrane to whole cell biodegradation within a membrane bioreactor. J. Membr. Sci., 389, 67-75.
- Harms, H., D. Schlosser and L. Y. Wick (2011). Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat. Rev. Microbiol., 9, 177-192.
- Jelic, A., C. Cruz-Morató, E. Marco-Urrea, M. Sarrà, S. Perez, T. Vicent, M. Petrović and D. Barcelo (2012). Degradation of carbamazepine by *Trametes versicolor* in an air pulsed fluidized bed bioreactor and identification of intermediates. Water Res. 46, 955-964.
- Juhasz, A. L. and R. Naidu (2000). Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. Int. Biodeterior. Biodegrad. 45, 57-88.
- Kimura, K., H. Hara and Y. Watanabe (2007). Elimination of selected acidic pharmaceuticals from municipal wastewater by an activated sludge system and membrane bioreactors. Environ Sci Technol. 41, 3708-3714.
- Marco-Urrea, E., M. Perez-Trujillo, T. Vicent and G. Caminal (2009). Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. Chemosphere. 74, 765-772.
- Modin, O., F. I. Hai, L. D. Nghiem and A. Basile (2014). Gas-diffusion, extractive, and biocatalytic membrane biological reactors. Membrane Biological reactors. F. I. Hai, K. Yamamoto and C.-H. Lee. UK (ISBN:971780400655), IWA publishing: 299-333.
- Ort, C., M. G. Lawrence, J. Reungoat, G. Eaglesham, S. Carter and J. Keller (2010). Determining the fraction of pharmaceutical residues in wastewater originating from a hospital. Water Res. 44, 605-615.
- Radcliffe, J. C. (2006). Future directions for water recycling in Australia. Desalination. 187, 77.
- Saba, B., I. Hashmi, M. A. Awan, H. Nasir and S. J. Khan (2012). Distribution, toxicity level, and concentration of polycyclic aromatic hydrocarbons (PAHs) in surface soil and groundwater of Rawalpindi, Pakistan. Desalination Water Treat., 49, 240-247.
- Schwarzenbach, R. P., B. I. Escher, K. Fenner, T. B. Hofstetter, C. A. Johnson, U. von Gunten and B. Wehrli (2006). The Challenge of Micropollutants in Aquatic Systems. Science. 313, 1072-1077.
- Tran, N. H., T. Urase and O. Kusakabe (2010). Biodegradation characteristics of pharmaceutical substances by whole fungal culture *Trametes versicolor* and its laccase. J Water Environ Technol. 8, 125-140.
- Watanabe, K. (2001). Microorganisms relevant to bioremediation. Curr. Opin. Biotechnol., 12, 237-241.

- Wick, A., G. Fink, A. Joss, H. Siegrist and T. A. Ternes (2009). Fate of beta blockers and psycho-active drugs in conventional wastewater treatment. *Water Research*. 43, 1060-1074.
- Yang, S., F. I. Hai, L. D. Nghiem, L. N. Nguyen, F. Roddick and W. E. Price (2013). Removal of bisphenol A and diclofenac by a novel fungal membrane bioreactor operated under non-sterile conditions. *Int. Biodeterior. Biodegradation*. 85, 483-490.
- Zhang, Y. and S.-U. Geißen (2012). Elimination of carbamazepine in a non-sterile fungal bioreactor. *Bioresource Technol.* 112, 221-227.

CHAPTER 2: LITERATURE REVIEW

2.1. INTRODUCTION

The occurrence of trace organic contaminants (TrOCs) such as pesticides, pharmaceutically active compounds (PhACs), natural and synthetic hormones and various industrial compounds in the aquatic environment is of great concern due to their potential adverse effects on human and ecological health (Schwarzenbach et al., 2006). This chapter provides an overview of the current scientific research on the occurrences, fate and toxicity of TrOCs WWTPs and in natural systems. A comprehensive literature review on diverse treatment technologies that have been investigated for TrOC removal will be provided. In particular, the applications of WRF and their LMEs for TrOC removal are comprehensively reviewed.

2.2 TRACE ORGANIC CONTAMINANTS

2.2.1 Occurrence and fate of TrOCs

TrOCs are classified according to their intended use or function, *e.g.*, PhACs, pesticides, surfactants and industrial chemicals, steroid hormones, phytoestrogens and UV filters.

PhACs have been detected in water supplies and WWTP effluents around the world due to their widespread production and use (Kolpin et al., 2002; Loos et al., 2009; Tran et al., 2014). For example, Kolpin et al. (2002) investigated 139 water streams in US and observed 82 compounds with concentration ranging up to 2 µg/L. There are various pharmaceuticals in different therapeutic groups which have diverse physicochemical properties (*e.g.*, analgesics and anti-inflammatories, cholesterol-lowering agents, anti-epileptics, and others). Because they are developed with the intention of performing a biological effect, PhACs have many of the necessary properties to bio-accumulate and provoke effects in aquatic or terrestrial ecosystems (Halling-Sørensen et al., 1998). Pharmaceuticals in urine or faeces could exist in their original chemical structure or in the form of metabolites and conjugates (Halling-Sørensen et al., 1998).

Pesticides are substances or mixture of substances intended for preventing, destroying, repelling or mitigating any pest. Pesticides are categorised into four groups: pesticides, fungicides,

herbicides, and insecticides. The discovery of health and environmental hazards associated with pesticide use has brought about more stringent regulations and changes in pesticide formulation (Fu et al., 1999; Wilson et al., 2001). Hence, current pesticides are less persistent and harmful than before. However, harmful pesticides are still abundantly present in polluted sites due to widespread and indiscriminate use in the past (Fu et al., 1999; Wilson et al., 2001).

Surfactants are found in detergents, wetting agents, emulsifiers, foaming agents, and other formulations that are widely used in industrial processes. Hence, surfactants such as nonylphenol polyethoxylates and linear alkylbenzene sulfonates have been detected in certain effluents and water bodies in relatively high concentrations (*e.g.*, µg/L to mg/L) (Scott-Fordsmand et al., 2004; Song et al., 2010). Two other industrial chemicals that have significant impact on the environment are plasticizers and flame retardants. Plasticizers such as bisphenol A tend to exhibit low biodegradability, and may have high toxicity for aquatic and terrestrial organisms (Scott-Fordsmand et al., 2004). Flame retardants such as polybrominated diphenyl ethers have been found in relatively low concentrations in wastewater (*e.g.*, ng/L), but have high stability in the environment (Song et al., 2010).

Both natural and synthetic hormones have been detected in the aquatic environment. Although the full extent of the impact of natural and synthetic hormones on human health is still a subject of intense scientific debate (Rodgers-Gray et al., 2000; Cunningham et al., 2009), some of these compounds (*e.g.*, 17β-estradiol and 17α-ethinylestradiol) have been identified as the cause of adverse developmental and reproduction issues in fish exposed to municipal wastewater effluent (Jaser et al., 2003; Angus et al., 2005).

Phytoestrogens are widely found in plants such as soybeans, cabbages, and fruits. Thus, human diet is sometime rich in phytoestrogens. A variety of phytoestrogens and their metabolites have been identified in human body fluids. The compounds have also been often detected in urine or faeces, which can be one of the main sources of their occurrence in municipal wastewaters (Kang et al., 2009; Liu et al., 2010). Hence, phytoestrogens have been detected in certain effluents and water bodies. Kang et al. (2009) found high level of phytoestrogens such as enterolactone (581-2111 ng/L), daidzein (341-1688 ng/L) and enterodiol (60-834 ng/L) in raw sewage.

UV filters include both inorganic and organic sunscreen agents. The discharge of UV filters into the environment is likely to increase due to recommendations of health authorities on the prevention of skin cancer. UV filters such as benzophenone and oxybenzone have been detected in the environment in the range of ng/L to µg/L (Poiger et al., 2004; Gómez et al., 2009). Although only a limited number of studies on the environmental impact of UV filters is found, there is evidence that they adversely affect the reproduction of aquatic organisms (Kaiser et al., 2012).

The aforementioned TrOCs are introduced to the aquatic environment through various routes. Pharmaceuticals may come from discharges of households, animal husbandries, aquacultures, and manufacturing companies (Mompelat et al., 2009). Pesticides that were applied on soil during agricultural activities may leach into groundwater or nearby bodies of water (Wilson et al., 2001). Surfactants and industrial chemicals are found in industrial effluents due to typical production processes (Hall et al., 1989; Song et al., 2010). Natural hormones and phytoestrogens are excreted by humans, livestock, and plants (Kang et al., 2009; Combalbert et al., 2010). In addition, humans may excrete synthetic hormones (*e.g.*, 17 α -ethinylestradiol) that have been taken up for medicinal purposes (Johnson et al., 2004). UV filters may be directly discharged to surface water (released from the skin during swimming and bathing), or enter sewage (Poiger et al., 2004). TrOCs have been detected in different water bodies such as surface water, groundwater, treated effluent from WWTPs, and drinking water in a number of countries/regions including US, Austria, China, UK, Germany, France, Spain, Korea and Viet Nam. The concentration of the TrOCs in the environment shows significant spatial and temporal variations due to a number of factors such as production, specific sales, metabolism, and elimination efficiency of the wastewater treatment process (Luo et al., 2014). In general, the concentrations vary widely from the range of ng/L to few µg/L. TrOCs that are discharged into sewage enter wastewater treatment plants (WWTPs), wherein they may undergo biodegradation. However, conventional WWTPs are not designed to treat TrOCs and therefore, a significant amount may persist in waste activated sludge and effluent. The discharge or re-use sludge and effluents may cause the dispersion of TrOCs into the environment (Mompelat et al., 2009; Song et al., 2010).

2.2.2 TrOC toxicity

The potential effects of TrOCs have been well documented in various studies in the last decade (Schwaiger et al., 2004; Carlsson et al., 2006; Bolong et al., 2009). These compounds can disrupt the endocrine system by mimicking, blocking or also hampering functions of hormones, thereby affecting health of human and animal species (Bolong et al., 2009). Schwaiger et al. (2004) studied the possible effects in rainbow trout after prolonged exposure to diclofenac. They reported histopathological changes of kidney and liver when fish were exposed to 5 µg/L of diclofenac for 28 days. EDCs cause a wide range of adverse effects on aquatic organisms *e.g.*, feminisation of male fishes (Jobling et al., 1998), demasculinisation of alligators (Guillette et al., 2000), growth inhibition, immobilisation, mutagenicity, increased mortality and changes in population density (Alzieu 2000). For example, bisphenol A has been proven to have estrogenic effects in rats (Ishido et al., 2011). Bisphenol A has been shown to mimic estradiol in causing direct damage to the DNA of cultured human breast cancer cells (Iso et al., 2006). Some steroid hormones such as estrone, 17β- estradiol and 17α- ethinylestradiol have a high specific biological estrogenic activity even at extremely low concentrations (Bolong et al., 2009) and may cause feminisation in male fish. In the environment, TrOCs are present as a mixture of various parent compounds and their transformation products. Mixture of TrOCs may impose a more complicated effect when compared to that of a single compound (Fent et al., 2006; Backhaus et al., 2008; Klaus 2009). For example, eco-toxicity tests with antibiotics showed that combined toxicity of two antibiotics can lead to either synergistic, antagonistic or additive effects (Christensen et al., 2006). In general, knowledge about the toxicity of compound mixtures is limited. This is a new field of eco-toxicity and much remains to be studied.

Table 2.1: Information on the adverse effects of TrOCs from recent studies (adapted from Pal et al., 2010).

Compounds	Concentration (µg/ L)	Type of risk	Reference
Diclofenac	0.5 -50	Affect tissues of gills and kidney of freshwater fish brown trout	(Hoeger et al., 2005)
Ibuprofen, diclofenac, 17β -estradiol and 17β -estradiol -17acetate	0.01	Risk to aquatic environment with chronic toxic effect (such as inhibited polyp regeneration and reduced reproduction in hydra)	(Carlsson et al., 2006)
17α-ethinylestradiol (EE2)	5- 50	Modulation of brain and inter-renal steroid genic acute in juvenile salmon	(Lyssimachou et al., 2007)
Carbamazepine	20*	Cause ataxia, movement disorders, anticholinergic toxidrome	(Soderstrom. et al., 2006)
Triclosan	0.3 -30	Disrupts gene expression in <i>Rana catesbeiana</i>	(Veldhoen et al., 2006)
Estriol	0.1 -30	Disruption of vitellogenesis (shrimp)	(Holbech et al., 2006)
Nonylphenol	100	Multi-generational reproductive process disruptions on <i>Daphnia magna</i>	(Brennan et al., 2006)

* mg/kg body weight

2.3 TrOC REMOVAL PROCESSES

2.3.1 Physicochemical treatment processes

2.3.1.1 Coagulation and flocculation

In water and wastewater treatment, coagulation and flocculation is typically applied prior to sedimentation and filtration (Matilainen et al., 2010). Coagulation process involves the addition of chemicals which can neutralise particle charge thus promoting formation of larger particles which can settle or get entrapped into filter material. Coagulation and flocculation have been employed in water treatment to decrease turbidity and colour and to remove pathogens. However, coagulation has been found inefficient in terms of TrOC removal. For example, Choi et al. (2006) reported that the removal of 14 EDCs was in the range of 0 to 7% by coagulation using five different coagulants. Negligible removal of estrone and estradiol has been reported in the

literature (Bundy et al., 2007; Le-Minh et al., 2010). Information regarding the removal efficiency of selected EDCs and PPCPs during coagulation is summarised in **Table 2.2**.

Table 2.2: Removal of selected TrOCs during coagulation/flocculation

Compounds	Primary coagulation/pH/Dose (mg/L)	Influent (ng/L)	Removal (%)	References
Pharmaceutically active compounds				
Acetaminophen	Alum, pH 6.8, 78	-	< 20	(Westerhoff et al., 2005)
Carbamazepine	Ferric, pH 8	1000	13	(Ternes et al., 2002a)
	Ferric, pH 8, 10.4-12.6	80-180	0-6	(Ternes et al., 2002a)
	Alum, pH 6.8, 78	-	< 20	(Westerhoff et al., 2005)
	Ferric, pH 6.8, 13.1	30	35	(Westerhoff et al., 2005)
Diclofenac	Ferric, pH 8	1000	6	(Ternes et al., 2002a)
	Ferric, pH 8, 10.4-12.6	65-140	8-25	(Ternes et al., 2002a)
	Alum, pH 6.8, 78	-	< 20	(Westerhoff et al., 2005)
Ibuprofen	Ferric, acidic pH	8.5-17.5	4	(Vieno et al., 2006)
	Alum, pH 6.8, 78	-	< 20	(Westerhoff et al., 2005)
Ketoprofen	Ferric, acidic pH	7-11.5	13	(Vieno et al., 2006)
Naproxen	Ferric, acidic pH	7-7.5	20	(Vieno et al., 2006)
	Alum, pH 6.8, 78	-	< 20	(Westerhoff et al., 2005)
Sulfamethazine	Alum, 20-107	50000	0	(Adams et al., 2002)
	Ferric, 25-169	50000	0	(Adams et al., 2002)
Trimethoprim	Alum, 20-107	50000	0	(Adams et al., 2002)
	Ferric, 25-169	50000	0	(Adams et al., 2002)
Primidone	Ferric, pH 8	1000	0	(Ternes et al., 2002a)

Personal Care Products				
DEET	Alum, pH 6.8, 78	78	0	(Westerhoff et al., 2005)
	Ferric, pH 6.8, 13.1	78	0	(Westerhoff et al., 2005)
Triclosan	Alum, pH 6.8, 78	78	0	(Westerhoff et al., 2005)
Steroids hormones				
17 α -Ethinylestradiol	Alum, pH 6.8, 78	95	0	(Westerhoff et al., 2005)
	Ferric, pH 6.8, 13.1	95	0	(Westerhoff et al., 2005)
	Alum, pH 5.4, 57-126	50000	0	(Ballard et al., 2005)
17 β -Estradiol	Alum, pH 6.8, 78	95	2	(Westerhoff et al., 2005)
	Ferric, pH 6.8, 13.1	95	0	(Westerhoff et al., 2005)

2.3.1.2 Granular and powdered activated carbon

Activated carbon can be used to remove TrOCs completely or partially from water and wastewater (Ternes et al., 2002b; Snyder et al., 2007). Activated carbon can be used in the following two types: powdered activated carbon (PAC) and granular activated carbon (GAC). PAC is usually added in contact basins while GAC is packed in a column. The performance of activated carbon adsorption depends on the properties of the activated carbon sorbent (*i.e.*, surface area, pore size distribution, surface charge, oxygen content) and on the properties of the solute (*i.e.*, shape, size, charge, and hydrophobicity). The influent concentrations, organic concentration and contact time can also affect the performance of activated carbon adsorption. Adsorption onto PAC or GAC can also be used to efficiently remove TrOCs from water. However, limited adsorption of ionic compounds, particularly those containing electron-withdrawing functional groups, has been reported (Ternes et al., 2002b). **Table 2.3** summarises the removal efficiency of selected TrOCs via adsorption on activated carbon.

Table 2.3: Removal of selected TrOCs through activated carbon adsorption

Compounds	Adsorbent/dose (mg/L)/contact time (h)	Influent (ng/L)	Removal (%)	References
Pharmaceutically active compounds				
Acetaminophen	PAC, 5, 4	25	69	(Westerhoff et al., 2005)
Carbamazepine	GAC, 5, 0.25	1000	100	(Ternes et al., 2002a)
	PAC, 5, 4	105-180	100	(Westerhoff et al., 2005)
Diclofenac	GAC, 5, 0.25	40	100	(Ternes et al., 2002a)
	GAC, 35 – 40, 0.25	60	100	(Ternes et al., 2002a)
	PAC, 5, 4	35	48	(Westerhoff et al., 2005)
	PAC, 5, 4	20	21	(Westerhoff et al., 2005)
Naproxen	PAC, 5, 4	20	47	(Westerhoff et al., 2005)
Primidone	GAC, 35 – 40, 0.25	15	67	(Ternes et al., 2002a)
Sulfamethazine	PAC, 5-50, 4	50000	35-90	(Adams et al., 2002)
Sulfamethoxazole	PAC, 5, 4	20	20	(Westerhoff et al., 2005)
Trimethoprim	PAC, 5-50 , 4	50000	35-90	(Adams et al., 2002)
	PAC, 5, 4	65	20	(Westerhoff et al., 2005)
Steroids hormones				
17 α -Ethinylestradiol	PAC, 5-15, 4	29620	50-100	(Yoon et al., 2003)
	PAC, 5-15, 4	160	88	(Westerhoff et al., 2005)
17 β -Estradiol	PAC, 5-15, 4	27230	87-100	(Yoon et al., 2003)
	PAC, 1, 1	6.8-1360	39-51	(Westerhoff et al., 2005)

2.3.1.3 Membrane filtration technology

The removal of TrOCs by nanofiltration (NF) and reverse osmosis (RO) membrane filtration has been investigated at lab, pilot and full scale implementations. The results highlighted great capacity of NF/RO membranes to remove TrOCs (Bellona et al., 2008; Al-Rifai et al., 2011). However, TrOC removal by NF/RO is also dependent on various factors including physicochemical properties of TrOCs (*e.g.*, molecular size, hydrophobicity and polarity) and feed solution compositions (Bellona et al., 2004). For example, Nghiem *et al.* (2004a) reported that the removal of some hydrophobic compounds can be lower than that is expected based on the size exclusion mechanism. Hydrophobic TrOCs can adsorb to NF/RO membranes and then diffuse through the membrane material, leading to considerable transport of these compounds into the permeate. **Table 2.4** summaries the removal of selected TrOCs by NF/RO membrane.

Table 2.4: Removal of selected TrOCs by NF/RO membrane

Compounds	Removal (%)	References
Pharmaceutically active compounds		
Acetaminophen	85	(Snyder et al., 2007)
Carbamazepine	95	(Alturki et al., 2010)
Diclofenac	90	(Xie et al., 2012)
Gemfibrozil	89	(Snyder et al., 2007)
Ibuprofen	91	(Snyder et al., 2007)
Ketoprofen	99	(Alturki et al., 2010)
Naproxen	72 - 90	(Snyder et al., 2007; Alturki et al., 2010)
Primidone	85-92	(Alturki et al., 2010)
Triclosan	84 -100	(Snyder et al., 2007; Xie et al., 2012)
Steroid hormones		
17 α -ethinylestradiol	99	(Snyder et al., 2007; Alturki et al., 2010)
17 β -estradiol	96-100	(Snyder et al., 2007)
17 β -estradiol17-acetate	95-100	(Snyder et al., 2007; Alturki et al., 2010)
Estriol	96	(Snyder et al., 2007)
Estrone	69 - 98	(Snyder et al., 2007)
Industrial chemicals		
4-n-nonyphenol	99	(Snyder et al., 2007)
4-tert-butylphenol	99	(Snyder et al., 2007)
4-tert-octylphenol	95-99	(Snyder et al., 2007)
Bisphenol A	75-90	(Xie et al., 2012)

2.3.2 Biological treatment processes

2.3.2.1 Conventional activated sludge

Elimination of TrOCs in conventional activated sludge (CAS) treatment processes is often incomplete, and the reported overall removal of TrOCs in CAS varies significantly (Clara et al., 2005; Oppenheimer et al., 2007). As a consequence, a significant fraction of the TrOCs is discharged with the final effluent into the aquatic environment. Two major mechanisms of removal of TrOCs during CAS processes are sorption and biodegradation (Radjenovic et al., 2007b). Biodegradation of TrOCs in CAS depends on the composition of biomass (*i.e.*, microbial community structure) and their associated enzyme systems (Tran et al., 2013). The microbial community is influenced by the operating conditions such as pH, temperature, concentration of growth substrates. For example, higher nitrifying activity of activated sludge has been observed in CAS system which showed high removal of TrOCs. In a study by Tran et al (2009), enriched nitrifying cultures achieved 10-30% improvement in the removal of 10 TrOCs in comparison to conventional activated sludge. Higher removal efficiency of some TrOCs has also been attributed to their adsorption to the activated sludge (Radjenovic et al., 2007a). Compounds which are relatively hydrophilic ($\log D < 3.2$) show limited sorption to sludge (Joss et al., 2006). However, hydrophilic compounds such as fluoroquinolone antibiotics may mainly be eliminated by sorption to sludge by electrostatic interactions with the cell membranes of the microorganisms (Golet et al., 2003; Lindberg 2006). Therefore, the physical and chemical properties of these compounds can greatly influence their fate and behaviour as well as the removal efficiency of TrOCs during CAS treatment. For example, Kimura et al. (2007) found that ketoprofen and naproxen were not eliminated at all in CAS treatment.

2.3.2.2 Membrane bioreactor

MBRs, which combines biological treatment and membrane separation in a single process, offers some benefits over CAS treatment. MBRs can operate at a biomass concentration as high as 20 g/L and at a prolonged sludge retention time (SRT). Currently MBRs for domestic wastewater treatment are operated within biomass concentration range of 8-12 g/L. The high sludge concentration in MBR is not only beneficial for biodegradation of TrOCs but it can also have a beneficial effect on the removal efficiency of TrOCs that can absorb to the sludge. This may

promote the degradation of persistent substances because of the improved adaptation of bacteria for TrOCs.

Considerable research efforts have been devoted to the assessment of TrOC removal by MBR treatment. The reported data ranges from nearly complete removal for some compounds to almost no removal for several others. Excellent removal of ibuprofen (up to 98%) was confirmed along with naproxen (84%) and erythromycin (91%) by Reif et al. (2008) in their pilot-scale MBR. However, sulfamethoxazole and musk fragrances (*i.e.*, galaxolide, tonalide, and celestolide) were moderately removed (>50%) probably due to partial adsorption on the biomass. On the other hand, carbamazepine, diazepam, diclofenac, and trimethoprim were poorly removed (<10%). Nghiem et al. (2009) also confirmed the possibility of achieving good treatment of bisphenol A (90%) due to both biodegradation and adsorption. On the contrary, sulfamethoxazole removal was solely attributed to biodegradation, which can explain the lower removal (50%) as this compound is rather hydrophilic (Nghiem et al., 2009).

Table 2.5: Removal of selected TrOCs by membrane bioreactor

Compounds	Removal (%)	References
Pharmaceutically active compounds		
Acetaminophen	40 – 100	(Radjenovic et al., 2007a; Hai et al., 2011)
Carbamazepine	0–35	(Bernhard et al., 2006; Kim et al., 2007; Reif et al., 2008; Abegglen et al., 2009)
Diclofenac	0 – 87	(Bernhard et al., 2006; Radjenovic et al., 2007a; Reif et al., 2008; Abegglen et al., 2009)
Gemfibrozil	90– 98	(Radjenovic et al., 2007a; Hai et al., 2011)
Ibuprofen	50 – 99	(Urase et al., 2005; Bernhard et al., 2006; Hai et al., 2011)
Ketoprofen	52 - 92	(Radjenovic et al., 2007a; Hai et al., 2011)
Metronidazole	36	(Hai et al., 2011; Nguyen et al., 2012b)
Naproxen	10 – 84	(Urase et al., 2005; Reif et al., 2008; Abegglen et al., 2009; Hai et al., 2011)
Primidone	10– 35	(Abegglen et al., 2009; Hai et al., 2011)
Salicylic acid	93	(Hai et al., 2011; Nguyen et al., 2012b)
Triclosan	70– 97	(Kim et al., 2007; Hai et al., 2011)
Steroid hormones		
17 α -ethinylestradiol	60– 98	(Urase et al., 2005; Bernhard et al., 2006; Hai et al., 2011)
17 β -estradiol	97– 99	(Radjenovic et al., 2007a; Hai et al., 2011)
17 β -estradiol 17-acetate	98	(Urase et al., 2005; Reif et al., 2008;

		Abegglen et al., 2009; Hai et al., 2011)
Estriol	83	(Hai et al., 2011; Nguyen et al., 2012b)
Estrone	99	(Urase et al., 2005; Hai et al., 2011)
Industrial chemicals		
4-n-nonyphenol	87–92	(Hai et al., 2011; Nguyen et al., 2012b)
4-tert-butylphenol	98	(Hai et al., 2011; Hernández-Leal et al., 2011)
4-tert-octylphenol	98	(Urase et al., 2005; Hai et al., 2011)
Bisphenol A	52–98	(Hai et al., 2011; Nguyen et al., 2012b)
Pesticides		
Fenoprop	10– 21	(Visvanathan et al., 2005; Hai et al., 2011)
Pentachlorophenol	82– 99	(Hai et al., 2011; Nguyen et al., 2012b)

2.3.3 Advanced oxidation processes

Advanced oxidation processes (AOPs) have been used to remove organic pollutants from reclaimed effluent and ground water (Azbar et al., 2004). Numerous studies in the literature have demonstrated the effectiveness as well as limitations of AOPs for the removal of TrOCs from wastewater (Esplugas et al., 2002; Contreras et al., 2003; Ternes et al., 2003; Andreozzi et al., 2004; Klavarioti et al., 2009). For example, oxidation using ozone can reach from 90% to complete removal of many pharmaceutical and pesticides such as ofloxacin, sulfamethoxazole, propranolol, carbamazepine, clofibric acid, diclofenac, atrazine and diuron (Ternes et al., 2003; Andreozzi et al., 2004; Maldonado et al., 2006), while only around 50% or less removal was achieved for ibuprofen, naproxen, caffeine, iodinated X-ray contrast medium, and tonalide (Ternes et al., 2003). Furthermore, the Fenton process has also been tested to treat TrOCs from wastewater, particularly from hospitals and pharmaceutical manufacturers (Klavarioti et al., 2009). For example, using the Fenton process at a concentration of 5.1 mg/L Fe^{+2} combined with 10 mg/L H_2O_2 , 75% removal of pesticides such as atrazine and 94% removal of herbicides from ground water was reported (Esplugas et al., 2007). Additionally, electrochemical oxidation using a TiO_2 anode has been shown to oxidise 80% of PhACs (ofloxacin, sulfamethoxazole, propranolol, carbamazepine, clofibric acid and diclofenac) (Andreozzi et al., 2004; Klavarioti et al., 2009).

UV oxidation is often used as a disinfection step in water and wastewater treatment processes. The capability of UV oxidation to treat TrOCs such as atrazine, diclofenac, ketoprofen, ibuprofen, clofibric acid and naproxen (Beltrain et al., 1994; Packer et al., 2003; Nguyen et al., 2013b) has been reported. Packer et al. (2003) reported that diclofenac is subjected to rapid

photodegradation, while ketoprofen and naproxen are mildly photodegraded. UV oxidation showed a significant variation in the removal efficiency of 22 TrOCs studied by Nguyen et al. (2013b). The authors suggested that physicochemical properties and molecular structures of TrOCs influence their removal by UV oxidation. For example, all the phenolic compounds were well removed (above 80%) by UV oxidation. Kim et al. (2009) reported that the removal efficiency of compounds which have similar molecular structures can be similar because of cleavage of the same covalent bond or molecular moiety. The influent properties (*i.e.*, high bulk organic content and high turbidity) could affect UV oxidation and sometime require high UV dosages (Wu et al., 2008; Basile et al., 2011). Chong et al. (2010) noted that to ensure rapid photocatalytic reaction rate, the turbidity of the target wastewater should be kept below 5 nephelometric turbidity units for optimal UV light utilisation and photocatalytic reaction.

Huber et al. (2003) reported that UV treatment alone could lead to 75 %, 13 %, and 7 % removal of diclofenac, iopromide, and sulfamethoxazole, respectively. Complete removal of several pharmaceuticals such as ofloxacin, sulfamethoxazole, propranolol, carbamazepine, clofibric acid and diclofenac was achieved using the combination of hydrogen peroxide and UV radiation (H_2O_2/UV) (Andreozzi et al., 2004). However, in another study, only 30-40% of ibuprofen, diphenhydramine, phenazone, and phenytoin can be removed using H_2O_2/UV (Yuan et al., 2009).

2.3.4 Combined treatment processes

The idea of combining two different processes in a hybrid system appears to be a promising technique as the process may lead to complementary advantages. To date, there have been a few studies on the application of integrated treatment processes for TrOC removal for example MBR-UV oxidation treatment process (Laera et al., 2011; Köhler et al., 2012), MBR-NF/RO membrane filtration (Alturki et al., 2010) and PAC-MBR (Li et al., 2011) or MBR – GAC (Nguyen et al., 2012a). Previously reported data have confirmed complementary effect of integrated system. For examples, Nguyen et al. (2013a) reported that >95% removal of all 22 TrOCs was achieved by MBR–GAC and PAC–MBR initially. Alturki et al. (2010) reported more than 95% removal (or removal to below the analytical detection limit) of all 40 TrOCs by a combination of MBR and a low pressure RO membrane. **Table 2.6** summarises the advantages and limitations of individual techniques for TrOC removal.

Table 2.6: Summary the advantages and disadvantages of current techniques for TrOCs removal (adapted from Alexander et al., 2012).

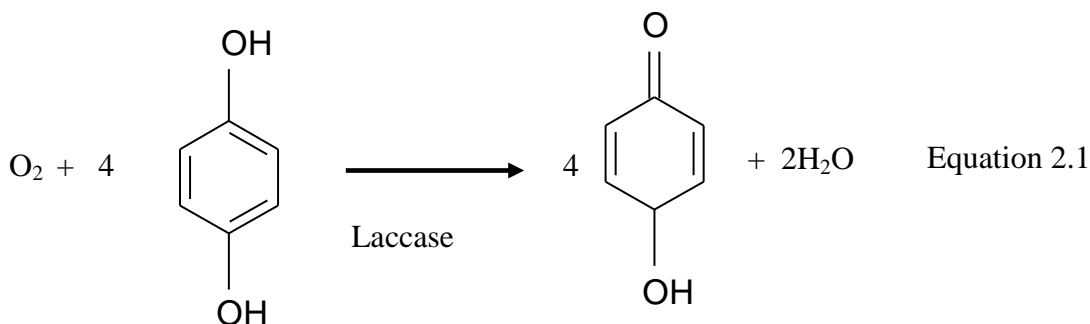
Process	Advantages	Disadvantages	Reference
Coagulation	<ul style="list-style-type: none"> - Simple process - Cost-effective - High removal of turbidity and colour 	<ul style="list-style-type: none"> - Large quantity of sludge - Ineffective in TrOC removal (0-10%) 	(Choi et al., 2006; Matilainen et al., 2010)
Electrocoagulation	<ul style="list-style-type: none"> - Adsorption and degradation - Removal of soluble and insoluble organics 	<ul style="list-style-type: none"> - Produce secondary pollutants - High cost - Compound-specific removal 	(Martins et al., 2011)
Activated carbon adsorption	<ul style="list-style-type: none"> - Well-established tertiary treatment - Removal of wide variety of TrOCs 	<ul style="list-style-type: none"> - Difficult regeneration - Costly disposal of potentially hazardous spent adsorbent 	(Ternes et al., 2002a; Grover et al., 2011; Nguyen et al., 2012a)
Nanofiltration/ reverse osmosis	<ul style="list-style-type: none"> - Removal of a number of TrOCs 	<ul style="list-style-type: none"> - Complexity associated with the separation process - Produce concentrated sludge - Low removal of hydrophobic compounds due to adsorption to membrane surface and subsequent diffusion through the membrane 	(Nghiem et al., 2004b; Alturki et al., 2010)
Advanced oxidation process	<ul style="list-style-type: none"> - Degradation of various TrOCs 	<ul style="list-style-type: none"> - Pre-treatment is required - May produce toxic by-products - Expensive 	(Belgiorno et al., 2007; Klavarioti et al., 2009)
Membrane bioreactor	<ul style="list-style-type: none"> - High removal of hydrophobic TrOCs - High quality of effluent 	<ul style="list-style-type: none"> - Inefficient removal of hydrophilic and persistent TrOCs - Post-treatment may be required 	(Hai et al., 2011; Tadkaew et al., 2011; Nguyen et al., 2012a)

2.4 WHITE-ROT FUNGI AND THEIR LIGNIN MODIFYING ENZYMES

WRF are organisms that are able to degrade lignin, the structural polymer found in woody plants, exposing the lighter-coloured cellulose. WRF produce extracellular lignin modifying enzymes (LMEs) such as laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP). These enzymes are capable of oxidising a wide variety of toxic xenobiotic. Laccase uses oxygen as an electron acceptor during the oxidation which makes it easier to use, whereas MnP and LiP require addition of H_2O_2 . WRF could be used in bioremediation of a variety of toxic chemical pollutants due to high tolerance to toxic substances in the environment. Further discussion on the application of WRF and their LMEs is presented in **Section 2.5**.

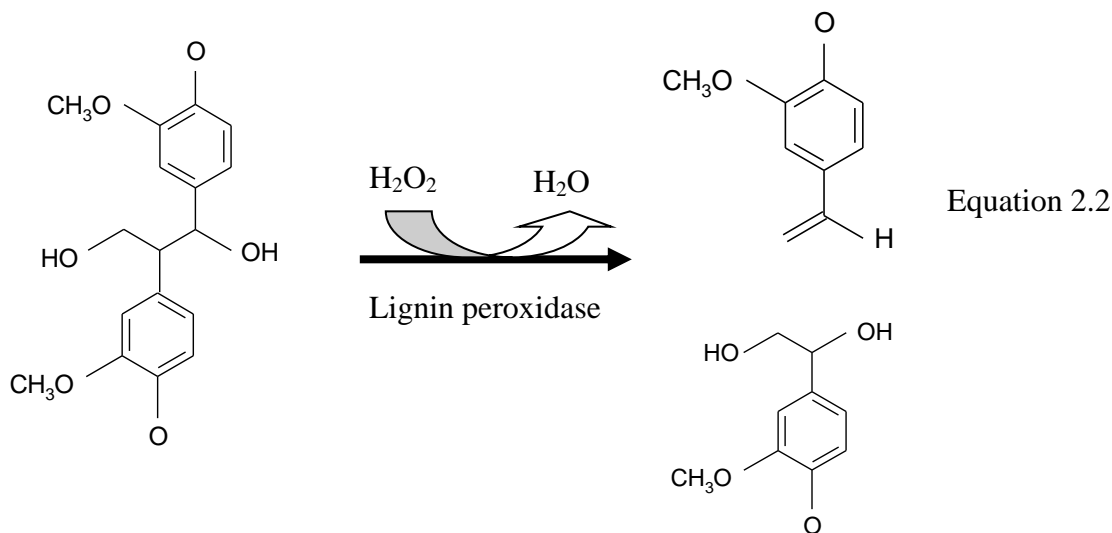
2.4.1 Laccase

Laccase (Benzenediol: oxygen oxidoreductase; EC 1.10.3.2) are ligninolytic enzymes that are secreted by white-rot fungi during the degradation of lignin (the hard cover protecting wood). They are multi-copper atom containing enzymes that can also catalyse the oxidation of a wide range of phenolic substrates using oxygen as an electron acceptor (Riva 2006). The oxidation of a substrate typically involves formation of a free (cation) radical after the transfer a single electron to laccase. The oxidative efficiency of laccases depends on the redox potential differences between the reducing substrate and type 1 Cu in laccase., Laccases generally show lower redox potential (0.5-0.8 V) (Xu 1997; Kumar et al., 2003) compared to other oxidoreductases such as peroxidases, and, therefore, can attack only the phenolic moieties in the lignin polymer (20% of lignin polymer). Example of the oxidation of 1-4 dihydroxy benzene in the presence of laccase is showed in Equation 2.1.



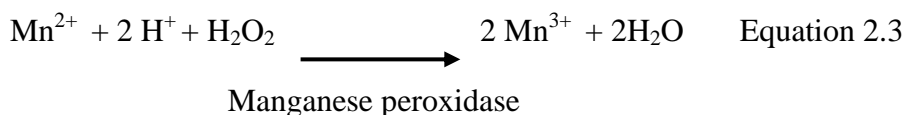
2.4.2 Lignin peroxidase

LiP (EC 1.11.1.14) is the first oxidative enzyme discovered in *Phanerochaete chrysosporium*. This enzyme belongs to the family of oxidoreductases which require peroxide as electron acceptor. It is a monomeric glycoprotein with a heme group in its active centre. LiP is capable of catalysing depolymerisation of a variety of non-phenolic lignin model compounds in the presence of H_2O_2 (Karam et al., 1997).



2.4.3 Manganese peroxidase

MnP (EC 1.11.1.13) is also a glycosylated heme protein secreted by ligninolytic fungi into their environment. MnP is capable of oxidizing Mn^{2+} which is often found in wood and soils, into highly reactive Mn^{3+} . Then, the highly reactive Mn^{3+} attacks phenolic lignin structures (Hofrichter et al., 2010).



2.4.4 Versatile peroxidase

Versatile peroxidase (VP) is a heme peroxidase produced by a few WRF. It is capable of oxidising Mn^{2+} to Mn^{3+} , which subsequently oxidises organic compounds and non-phenolic aromatic compounds in the presence of H_2O_2 (Palma et al., 2000). Versatile peroxidase can also

directly oxidise phenols and other aromatic compounds with high redox potentials, including dyes.

Although there are four main types of enzyme secreted by WRF, a particular strain may secrete one type of enzyme predominantly. The enzyme systems may vary from one WRF species to another. **Table 2.7** lists the enzymes described in selected fungus species and **Table 2.8** summaries typical properties of white-rot fungal enzymes.

Table 2.7: Typical LMEs from different species of WRF

WRF species	LMEs	References
<i>Trametes versicolor</i>	Laccase, MnP, LiP	(Bending et al., 2002; Cajthaml et al., 2009)
<i>Phanerochaete chrysosporium</i>	MnP, LiP	(Aitken et al., 1989; Hatakka 1994; Karam et al., 1997; Rodarte-Morales et al., 2012b)
<i>Bjerkandera adusta</i>	Laccase, MnP, LiP	(Cajthaml et al., 2009)
<i>Pleurotus ostreatus</i>	Laccase, MnP	(Hirai et al., 2004; Cajthaml et al., 2009; Golan-Rozen et al., 2011)
<i>Irpex lacteus</i>	Laccase, MnP, MnP	(Novotný et al., 2000; Cajthaml et al., 2009)
<i>Dichotomitius squalens</i>	Laccase, MnP	(Cajthaml et al., 2009)
<i>Pycnoporus cinnabarinus</i>	Laccase, MnP	(Cajthaml et al., 2009)
<i>Coriolopsis polyzona</i>	Laccase	(Cabana et al., 2007; Cabana et al., 2009)
<i>Dichomitius squalens</i>	Laccase, MnP	(Cajthaml et al., 2009)
<i>Phanerochaete sordida</i>	MnP	(Tamagawa et al., 2005; Lee et al., 2010)

Table 2.8: Typical properties of some white rot fungal enzymes

Enzyme	MW (kDa)	Optimal Ph	Optimal Temp °C	Co-factor	Reaction	Reference
Lac	50 -110	3.5-7	20 - 80	O ₂	- Phenols are oxidised to phenoxyl radicals-mediator radicals - Redox potential of around 0.4–0.8 V	(Katuri et al., 2009; Lloret et al., 2010)
MnP	38-50	4-4.5	30 - 60	H ₂ O ₂	- Mn ²⁺ are oxidised to Mn ³⁺ , Mn ³⁺ oxidises aromatic substrates, oxidation of phenolic compounds to phenoxyl radicals that cleave C α -C β and alkyl aryl bonds - Redox potential of 1.0–1.2 V	(Hofrichter 2002; Davila-Vazquez et al., 2005)
LiP	38-47	2.5-3	35 - 55	H ₂ O ₂	- Abstracts an electron from the substrate aromatic ring, generating an aryl cation radical which decomposes by enzymatic and non-enzymatic processes - Redox potential of 1.4–1.5 V	(Ruiz-Dueñas et al., 2009)
VP	Not known	pH 5 (Mn ²⁺) pH 3 (aromatic substrates)	Not known	H ₂ O ₂	- Same effect on aromatic compounds as MnP and LiP	(Ruiz-Dueñas et al., 2009; Hofrichter et al., 2010)

2.5 APPLICATION OF WRF AND LMEs FOR TrOC REMOVAL

Different WRF have been used for the removal of TrOCs, either by the whole fungal culture or by the oxidative action of the enzyme produced (crude solution and purified enzyme). This section presents a comprehensive literature review on the application of WRF and LMEs for TrOC removal. Factors affecting the performance are also discussed. The application of WRF and enzyme in fungal membrane bioreactor and EMR is critically reviewed.

2.5.1 Removal by whole-cell WRF

Considerable research has been devoted to test the performance of different WRF for the removal of TrOCs. Cajthaml et al. (2009) investigated the performance of eight different strains of WRF for the removal of some representative EDCs. Almost all tested fungal strains were able to degrade the selected EDCs to different extents. Of the fungal strains tested, *I. lacteus* and *P. ostreatus* provided the highest removal efficiency of EDCs (*i.e.*, 90% and 80%, respectively) after 7 days of incubation. Moreover, these strains could completely eliminate three phenolic compounds (*i.e.*, 4-nonylphenol, bisphenol A, and 17- α ethinylestradiol). Jelic et al. (2012) reported effective degradation of carbamazepine by *T. versicolor*. The degradation rate was 94% in the case of 9 mg/L carbamazepine addition after 6 days while it was only 61% in the case of 50 μ g/L after 7 days of incubation. This also revealed that TrOCs concentration may affect the degradation rate. Tran et al. (2010) assessed the degradation of a ten PhACs in their mixture (10 μ g/L each) by *T. versicolor* with and without the addition of a redox-mediator ABTS. Complete removal of some PhACs (*i.e.*, ibuprofen, naproxen, diclofenac, ketoprofen, indomethacin and fenoprop) and partial removal of others (*i.e.*, carbamazepine, clofibric acid, and propyphenazone) was achieved. Comparing the removal by whole cell and crude laccase solution, the authors suggested that the degradation of PhACs by WRF may be caused by the presence of laccase, MnP and Cytochrome P450. The results from a heat-killed sample also implied that biodegradation was the main mechanism accounting for the decrease in PhAC concentration. The removal of carbamazepine, ibuprofen and clofibric acid by *T. versicolor*, *I. lacteus*, *G. lucidum* and *P. chrysosporium* strains in batch mode was studied by Marco-Urrea et al. (2009). Ibuprofen could be effectively removed by all strains. Carbamazepine and clofibric acid were persistent to tested fungal strains except *T. versicolor*. In another study by Marco-Urrea et al. (2010b), diclofenac, which is a nonsteroidal anti-inflammatory drug was also well removed by

pellets of *T. versicolor*. The authors suggested that at least two different mechanisms were involved in the degradation of diclofenac; (i) cytochrome P450 system and (ii) laccase catalysis. The result confirmed that the removal of same TrOCs is WRF species dependent due to the different enzyme systems excreted by different fungi. Bending et al. (2002) compared the abilities of nine species of white-rot fungi on the degradation of pesticides. The authors reported considerable variation between removal efficiency of each fungus.

Apart from the application of WRF for the removal of TrOCs from liquid solution, a number of studies employed WRF in a solid-phase to remove TrOCs. Rodríguez-Rodríguez et al. (2011) reported that *T.versicolor* was an alternative treatment for TrOCs in sludge. Some compounds such as phenazone, bezafibrate, sulfamethazine and atenolol were completely removed, while a removal of 42 to 80% was achieved for some other compounds *e.g.*, carbamazepine, diazepam and diclofenac. The results also highlighted that *T.versicolor* reduced the ecotoxicological impact of TrOCs present in sewage sludge. A complete removal of sulfamethazine from sewage sludge was achieved by inoculation of *T. versicolor* (García-Galán et al., 2011).

2.5.2 Removal by mixed culture of WRF

TrOC biodegradation mainly depends on their enzyme systems. The enzyme systems vary on the fungi strain. For example, *Coriolopsis polyzona* produce mainly laccase (Cabana et al., 2009) while, *Phanerochaete sordida* produce mostly MnP (Tamagawa et al., 2007). The co-culture of WRF may stimulate both the production of enzyme and the degradation capacity. A few studies have focused on textile wastewater degradation and wood decay using co-culture of WRF. Chi et al. (2007) reported that, compared to single cultures, the co-culture of *Ceriporiopsis subvermispora* and *Pleurotus ostreatus* could significantly stimulate wood decay. Asgher et al. (2012) investigated a co-culture of *P. ostreatus* IBL-02 and *C. versicolor* IBL-04 to decolorise and detoxify dye wastewater. The decolourisation of dye after 6 days of incubation of *P. ostreatus* IBL-02, *C. versicolor* IBL-04 and their co-culture was 45, 53 and 57%, respectively. The co-culture also produced all three major enzymes, while *P. ostreatus* IBL-02 secreted high level of laccase and LiP and *C. versicolor* IBL-04 produced mainly MnP. The enhancement in decolourisation by co-culture was possibly due to the combined oxidation capacity of the

enzymes. Baldrian et al. (2004) observed an increase in laccase production in the co-culture of *P. ostreatus* and *T. versicolor*.

2.5.3 Removal by extracellular enzyme

Enzymatic tests employing crude or purified enzyme solutions reveal the capacity of biodegradation catalysed by different enzymes without the effect of sorption to fungal biomass. Direct use of enzymes harvested from microbial cultures for pollutant degradation may be advantageous in terms of separating the ‘growth of microbes’ and ‘biodegradation of pollutant’ stages. Both crude and purified enzyme solutions have been used for the removal of TrOCs.

2.5.3.1 Removal by crude enzyme solution

TrOC degradation by fungal extracellular extract (*i.e.*, crude enzyme) has been reported in some recent studies. The purpose is to investigate the role of extracellular enzymes in the degradation of TrOCs. Crude solution collected from fungi culture was used in both batch and continuous reactor modes. Wen et al. (2010) used crude MnP prepared from *P. chrysosporium* to test the degradation of tetracycline and oxytetracycline. 72.5% and 84.3% tetracycline and oxytetracycline (50 mg/L) was degraded by an enzyme solution with a MnP activity of 40 U/L. In another study by Tran et al. (2010) used a crude enzyme solution with an initial laccase and MnP activity of 1500 U/L and 30 U/L, respectively for the degradation of ten selected TrOCs (10 µg/L each). Complete removal of diclofenac, naproxen and indomethacin was observed within 12 h of incubation, while partial degradation of the rest of the compounds was observed. Zhang et al. (2010) used crude LiP collected from *P. chrysosporium* for degradation of carbamazepine and diclofenac. It was found that LiP completely degraded diclofenac at pH 3.0- 4.5 and 3 - 24 mg/L of H₂O₂ addition, while the degradation efficiency of carbamazepine was mostly below 10%. The performance of crude solutions from different fungi was also reported in a study by Wang et al. (2012). The removal of EDCs by LiP from WRF *P. sordida* YK-624 (YK-LiP1) and *P. chrysosporium* was investigated in that study. Five EDCs namely, p-t-octylphenol, bisphenol A, estrone, 17β-estradiol, and 17α-ethinylestradiol were degraded by LiP from *P. sordida* YK-624 more effectively than LiP from *P. chrysosporium*, and p-t-octylphenol and bisphenol A were removed almost completely from the reaction mixture containing LiP from *P. sordida* YK-624 after 24 h treatment. The benefit of using crude enzyme solution is that the crude solution may

contain some natural mediators which are produced during the growth of fungi. Tran et al. (2010) suggested that the complete removal of diclofenac, naproxen and indomethacin by the crude enzyme may be due to the combination of laccase and natural mediators present in crude enzyme. Moreover, the use of crude enzyme without any further enzyme purification may reduce the cost of the treatment process.

2.5.3.2 Removal by purified enzyme solution

Tran et al. (2010) used laccase purified from *T. versicolor* (23 U/mg proteins) for the degradation of ten selected TrOCs. The initial laccase activity and TrOC concentration was 6000 U/L and 10 µg/L each, respectively. The test was conducted in water buffered with 50 mM sodium acetate at pH 4.5. Consistent with the test using a crude solution, the author showed that diclofenac, naproxen and indomethacin were rapidly transformed by laccase (Tran et al., 2010). Complete removal of diclofenac (40 mg/L) was also reported in a study by Marco-Urrea et al. (2010b) who used a laccase solution with final enzyme activity of 2000 U/L. A commercial laccase purified from *M. thermophila* has been reported to remove 65% and 40% of 5 mg/L diclofenac at pH 4 and 5, respectively by Lloret et al. (2010). However, no significant removal of naproxen was observed. The observation of different removal percentage in the literature may be because of the use of different laccase from different species, concentrations, test condition and time of observation. Overall, the results from the available studies demonstrate that enzymes, either in a crude or purified form, could degrade a range of TrOCs.

2.5.4 Factors affecting the performance of whole cell WRF and enzymatic treatment

Using WRF for water and wastewater treatment can subject the fungi to environmental conditions far removed from their natural habitat. Physical and chemical properties of the water to be treated could adversely affect fungal growth. The physical and chemical properties of the water could also have an impact on the enzymatic activity, stability and substrate specificity of the employed enzyme. These features are important in process design and optimisation of the fungal and enzyme treatment process for TrOCs removal. This section discusses the effect of environmental parameters such as pH, temperature, enzyme dose, and metal ions on the performance of WRF and enzyme treatment process.

2.5.4.1 pH

The pH of the reaction environment can have an effect on both enzyme and substrate properties. Therefore, it may affect the performance of the enzyme in the removal of the pollutant. For example, 65% and 40% diclofenac was removed by a commercial laccase from *M. thermophila* at pH 4 and 5, respectively (Lloret et al., 2010). However, Zhang et al. (2010) reported that carbamazepine was recalcitrant to LiP degradation with degradation efficiencies around 7% at all studied pH values (*i.e.*, 2.5, 3, 4, 4.5 and 6). On the contrary, diclofenac was completely degraded in the pH range of 3.0 – 4.5. However, its degradation efficiency significantly decreased from 100% at pH 4.5 to 10% at pH 6.0. The reduction in diclofenac degradation was in accordance with the inactivation of LiP at higher pH values. Nguyen et al. (2014c) observed that pH 4.5 was optimal pH for the removal of diclofenac. Wen et al. (2010) used crude extracellular extract containing MnP and LiP for removal of two antibiotics (*i.e.*, tetracycline and oxytetracycline). They observed that the degradation rate was highly dependent on the pH. A pH of 5 appears to be the best for degradation of triclosan by laccase (*T. versicolor*) (Kim et al., 2006). The optimum pH for bisphenol A degradation by laccase was found to be 6.0 (Fukuda et al., 2001). Nguyen et al. (2014c) compared the removal of bisphenol A by laccase at five pH values (*i.e.*, 3, 4.5, 6, 7 and 9). The results revealed that bisphenol A was degraded almost completely (96%) within 4 h of incubation at a pH of 7, while over the same period a degradation of 56%, 61%, 88% and 74% was achieved at pH of 3, 4.5, 6, and 9, respectively. The optimal pH for laccase to degrade chlorophenols was around 5.5 (Zhang et al., 2008). The pH optimum for the pentachlorophenol reaction by laccase was 5.0 (Ullah et al., 2000).

pH did not have any remarkable effect on the removal of estrogen in a study by Lloret et al. (2010). A similar degradation result was observed at three pH value (*i.e.*, 4, 5 and 7) (Lloret et al., 2010). The reason may be because estrogen is easily removed by enzymatic treatment. However, considering the enzyme deactivation, Lloret et al. (2010) concluded that the optimal pH for the degradation of estrogen and anti-inflammatories (*i.e.*, naproxen and diclofenac) was pH 4. pH in the range of 5 - 9 was studied for estriol, estrone and estradiol removal by a commercial laccase preparation from *T. versicolour* (Auriol et al., 2007). They observed that the optimum pH for laccase-catalysed treatment was approximately 6 for each estrogen (Auriol et al., 2007).

2.5.4.2 Temperature

The optimal temperature for LME activity may differ greatly from one strain to another (Yang et al., 2013c). However, 25-30°C and 37-40°C are usually reported as the optimum temperature for laccase and peroxidases, respectively. For example, the removal efficiency of chlorophenols by laccase increased rapidly with a rise in temperature (20-25°C) (Zhang et al., 2008). The effect of temperature on the extent of pentachlorophenol removal by laccase showed that the reaction was optimal at 25°C, with a range of 10 - 45°C (Ullah et al., 2000). MnP has its highest activity at 37-40°C (Wen et al., 2010). LiP activity was found to be optimal at 37.5°C (Bosco et al., 2002). On the other hand, an increase in temperature was not found to enhance the degradation of carbamazepine (Zhang et al., 2010).

2.5.4.3 TrOC properties

The functional groups within the TrOC structure may have an effect on the extent of their degradation by WRF and their enzymes. Based on the functional groups, TrOCs can be divided into 3 different categories; (i) compounds containing electron-donating groups (EDGs) (*e.g.*, amine (-NH₂), hydroxyl (-OH), alkoxy (-OR), alkyl (-R), acyl (-COR) (ii) compounds containing electron -withdrawing groups (EWGs) (*e.g.*, amide (-CONR₂), carboxylic (-COOH), halogen (-X) and nitro (-NO₂) and iii) compounds containing both EDGs and EWGs.

Studies related to bacteria-dominated activated sludge have reported high removal of TrOCs with EDGs (Tadkaew et al., 2011). It is interesting to note that the TrOCs having EDGs have been reported to be effectively removed by WRF and their LME. For example, Lloret et al. (2010) reported that estrogens (*i.e.*, estrone, 17 β -estradiol and 17 α -ethinylestradiol) were significantly removed by purified laccase. Nonylphenol (3 mg/L) was completely removed by eight different WRF (*i.e.*, *I. lacteus*, *B. adusta*, *P. chrysosporium*, *P. magnoliae*, *P. ostreatus*, *P. cinnabarinus*, *T. versicolor* and *D. squalens*) within 3 days of incubation with 4-5 mg WRF (dry wt) (Cajthaml et al., 2009). Similar results can be found in other studies using WRF (Saito et al., 2004; Soares et al., 2005; Tamagawa et al., 2007; Mizuno et al., 2009a). Significant removal (66 to 100%) was also observed when crude laccase from *C. polyzona* was used (Cabana et al., 2007). Other EDG compounds such as bisphenol was also effectively removed by WRF and their enzyme (Saito et al., 2004; Cabana et al., 2007; Cajthaml et al., 2009; Wang et al., 2012).

Compounds containing EWGs have been found to be persistent to WRF and their enzyme. Lloret et al. (2010) reported that naproxen was not removed by direct action of laccase. Because of the carboxylic group present in its structure is not likely to be attached by laccase. Tran et al. (2010) used purified and crude laccase and MnP from *T. versicolor* and WRF in pellet form for the degradation of carbamazepine. Carbamazepine was not degraded by crude laccase and MnP resulted in 5% to 37% removal, while the WRF pellet showed 75% degradation. The author suggested that cytochrome P 450 played a role in the degradation. The low removal of carbamazepine has also been observed in other studies (Zhang et al., 2010; Eibes et al., 2011). A complete removal carbamazepine (1 mg/L) by three different WRF (*i.e.*, *B. adusta*, *P. chrysosporium* and *Bjerkandera sp. R1*) was reported by Rodarte-Morales et al. (2011).

For the compounds containing both EDGs and EWGs, significant variation in removal efficiency has been reported. Some compounds achieved a high degree of removal (*e.g.*, triclosan, diclofenac, ibuprofen) while no removal also was observed for some compounds (*e.g.*, clofibric acid, atrazine, metalaxyl and diuron). Cajthaml et al. (2009) studied the degradation of triclosan (a compound which has both EDG and EWG) by pellets of several different species (*P. cinnabarinus*, *P. chrysosporium*, *P. magnolia*, *P. ostreatus*). 85% of 2.5 mg/L triclosan was degraded after 14 days of incubation. An exception was *B. adusta* which showed no significant removal (Cajthaml et al., 2009). These results suggest that different species may have different abilities to degrade the same compound.

2.5.4.4 Metal ions

Some studies have reported the effect of the presence of metal ions in water or wastewater matrix on TrOC removal performance and stability of LME. For example, Mn^{2+} has been reported to promote the degradation of tetracycline and oxytetracycline by crude MnP from *P. chrysosporium*. The degradation percentage became stable at 78% tetracycline and 81% oxytetracycline with 0.1 mM Mn^{2+} (Wen et al., 2010). The presence of ions including SO_3^{2-} , S^{2-} , CN^- , Cl^- , Fe^{3+} and Cu^{2+} resulted in reduced treatment efficiency of triclosan by purified laccase from *T. versicolor* (Kim et al., 2006). In the presence of 1 mM CN^- , Cu^{2+} and Fe^{3+} significantly reduced conversion of triclosan was observed. These ions may interrupt the electron transport systems of laccase and then decrease the triclosan conversion. Kim et al. (2006) reported that

bisphenol A conversion was inhibited in the presence of anions such as SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$, S^{2-} , NO_2^- and CN^- but heavy metal ions such as Co^{2+} , Zn^{2+} , Ni^{2+} , and Mn^{2+} showed no significant difference in bisphenol A conversion relative to controls. The concentration of metal ions also effects enzyme activity. Laccase activity was found to be enhanced by metal ions such as Ca^{2+} , Co^{2+} , Cu^{2+} and Zn^{2+} at concentrations of 1 mM. However, except for Cu^{2+} and Zn^{2+} , increasing the concentration of metal ions up to 5 mM and above decreased the laccase activity (Murugesan et al., 2009). Some heavy metal such as Fe^{2+} highly inhibited laccase activity (Murugesan et al., 2009).

2.5.4.5 Salinity

Several studies have investigated the effect of salinity on fungal growth and the removal efficiency by WRF. A study on the impact of salinity on the growth of the white rot fungi *B. adusta*, *I. lacteus*, and *L. tigrinus* reported that *I. lacteus* and *L. tigrinus* were less affected by salinity, but the growth of *B. adusta* was inhibited when the salt concentration reached 32 % (Valentin et al., 2006). Kim et al. (2006) investigated the effect of different salts (*i.e.*, ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$), ammonium chloride (NH_4Cl), sodium chloride (NaCl), sodium sulphate (Na_2SO_4), calcium chloride (CaCl_2), magnesium chloride (MgCl_2), and magnesium sulphate (MgSO_4) ion. Results showed that ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$), sodium sulphate (Na_2SO_4), and magnesium sulphate (MgSO_4) did not exert a significant effect on enzymatic bisphenol A conversion. However, the chloride compounds (NaCl , NH_4Cl , CaCl_2 and MgCl_2) substantially inhibited bisphenol A conversion. For example, in the presence of 50 mM NaCl or CaCl_2 , the conversion achieved by a fixed dose of enzyme was reduced to one-half or one third, respectively as compared to conversion achieved in control.

2.5.4.6 Enzyme dose

Enzymes are involved in the degradation of TrOCs and the application of different enzyme dose may have an effect on TrOCs removal. There is an optimum relationship between the concentration of enzyme and substrate for achieving maximum activity (Katuri et al., 2009). However this relationship varies for different compounds and in different applied conditions. A study by Auriol et al. (2007) showed that 20 U/mL of initial laccase activity was required to completely remove each estrogen from synthetic water within an hour incubation. In the case of

17 α -ethinylestradiol, only 15 U/mL was required to achieve 100% removal. Kim et al. (2006) investigated the degradation of triclosan (20 μ M) at 3 different concentrations (0.3, 1.5 and 3 U/mL) of purified laccase from *T. versicolor*. The removal efficiency increased from 71% to complete removal under laccase activity of 0.3 and 3 U/mL, respectively. However, the entire enzyme was inactivated at the end of the period. As such if the enzyme dose is increased, there are diminishing returns in terms of the amount of triclosan transformed per quantity of enzyme supplied. The degree of bisphenol A degradation depended on laccase concentrations in the study by Fukuda et al. (2001). The test was conducted at pH 6.0 and 60°C using purified laccase from *T. villosa*. Zhang et al. (2008) observed improvement in chlorophenol degradation with increase in laccase concentration. However, when taking into account the degradation quantity per unit laccase, the lower laccase concentration appeared to be more effective. The authors suggested that a laccase dosage from 20 to 60 mg/L for removal of 10 mg/L of chlorophenols.

2.5.3.3.7 Enzyme immobilization

The idea of enzyme immobilisation appears to be a promising way to overcome some of the disadvantages associated with application of freely suspended enzymes. The idea is to ensure localisation of the biological catalysts in a definite space area and thus reduce its loss (Rios et al., 2004). Immobilisation of the enzyme may result in laccase stabilisation against thermal and chemical reaction. The methods for enzyme immobilisation include chemical and physical methods. Chemical methods include: (i) enzyme attachment to the matrix by covalent bonds and (ii) enzyme cross-linking by multifunctional reagents. Physical methods involve entrapment of enzyme molecules within different support materials such as a porous hollow fiber, spun fibers, insoluble gel matrix and a reverse micelle (Durán et al., 2002). Enzyme immobilization has been applied for removal of pollutants e.g., dyes and TrOCs (Corvini et al., 2010; Nicolucci et al., 2011). Results from these studies indicate that enzyme immobilization can enhance enzyme stability and enzymatic degradation compared to the use of free enzymes. Immobilisation can improve stability of laccase to temperature, pH, chemical inhibitors and storage time. The activity of immobilised enzyme is more resistant against high temperatures than the free enzyme (Nicolucci et al., 2011). In a study by Dodor et al. (2004) immobilised enzyme retained almost 100% of its initial activity, whereas the free laccase retained between 35 - 60% under the same conditions. Comparatively better performance of immobilised laccase in terms of decolourisation

(Peralta-Zamora et al., 2003) as well the laccase activity preservation has been reported in the literature. For example, the chitosan-immobilised laccase showed a lower activity than that of free laccase. However, the activity of chitosan-immobilised laccase remained stable as demonstrated by consistent removal of 2,4-dichlorophenol (above 65%) after six cycles of operation (Zhang et al., 2008). Nevertheless, because immobilisation can make the enzyme more stable, it is important to investigate better ways of enzyme immobilisation for larger scale operations.

2.5.5 Laccase-mediator system

The use of a mediator in the enzymatic reaction process allows maximisation of the efficacy of the enzyme by enhanced electron transfer. In such a case, the enzymatic reaction becomes a two-step process where the redox mediator firstly reacts with the enzyme and generates a strong oxidising intermediate which then reacts with the substrate (Burton 2003; Lloret et al., 2010). The range of substrate may be widened by means of the action of mediators that boost the oxidation capacity of the enzyme.



Figure 2.1: Schematic diagram of laccase mediator system

According to the above schematic, the role of enzyme is to oxidize the mediator, whereas the actual oxidation of the substrate takes place in a subsequent non-enzymatic step by the action of the radicals generated. There are three mechanisms by which mediators promote the oxidation of substrates: hydrogen atom transfer route, electron transfer and ionic oxidation. Mediators can be categorized based on the oxidation mechanism. The first group of mediators includes the compounds that have N-OH functional group in their chemical structure (*e.g.*, 1-hydroxylbenzotriazole, violuric acid, N-hydroxyphthalimide). Laccase oxidize these mediators to form aminoxyl radicals which target the substrate by hydrogen atom transfer route. The driving force for the oxidation of substrate is the energy difference between the dissociated bond

(C-H) in the target substrate and the forming bond (NO-H) in the mediator. This mechanism requires substrates with relatively weak C : H bonds (Xu et al., 2000). The mediator TEMPO follows ionic mechanism. It is oxidized by laccase forming an oxo ammonium ion ($\text{NN}=\text{O}^*$) (Christopher et al., 2014). The electron transfer route appears in the case of laccase-ABTS system (Baiocco et al., 2003). **Figure 2.2** presents the three laccase mediator mechanisms and **Table 2.9** summaries characteristics of mediators.

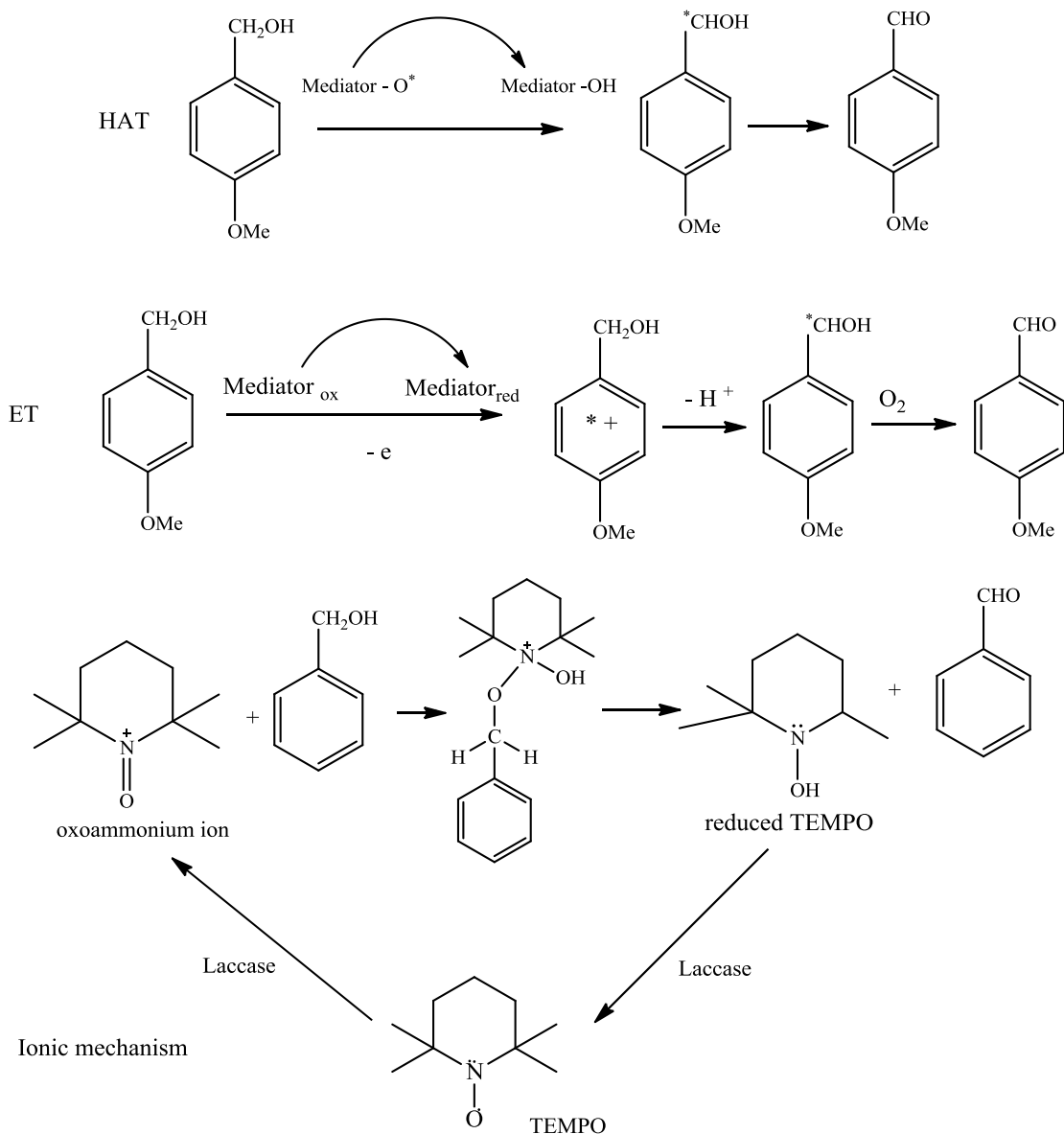
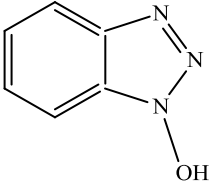
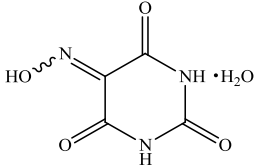
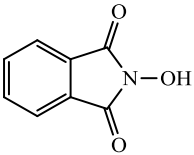
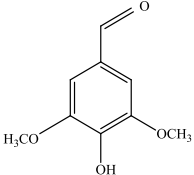
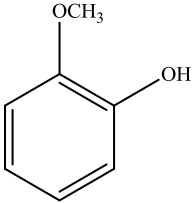
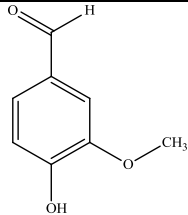
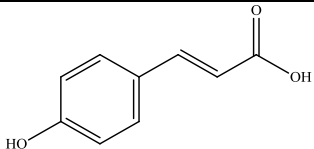
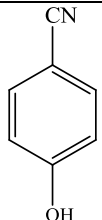
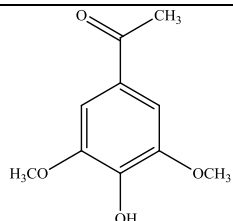
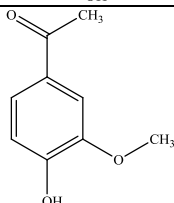
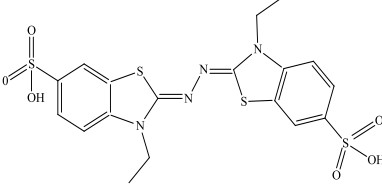
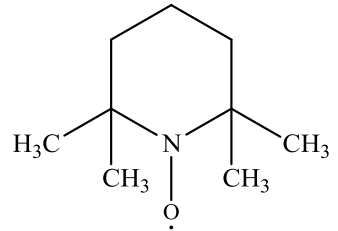


Figure 2.2: Laccase-mediator reaction mechanisms (adapted from Xu et al., 2000 and Christopher et al., 2014).

Table 2.9: Characteristics of mediators

Compound (Molecular weight (g/mol))	Chemicals structure	Free radicals generated	Oxidation mechanisms	Examples of substrates tested	Reference
1-hydroxibenzotriazole (C ₆ H ₅ N ₃ O) (135.12)		aminoxyl	HAT	Textile, dye or printing industries, phenols, non-phenolic lignin, steroid hormones	(Kurniawati et al., 2007; Hata et al., 2010a; Suda et al., 2012; Nguyen et al., 2014a)
Violuric acid (C ₄ H ₃ N ₃ O ₄) (157.09)		aminoxyl	HAT	Lignin, non-phenolic lignin dimer	(Kurniawati et al., 2007; Weng et al., 2012)
N-hydroxyphthalimide (C ₈ H ₅ NO ₃) (163)		aminoxyl	HAT	Phenol	(Baiocco et al., 2003; Azbar et al., 2004)
Syringaldehyde (C ₉ H ₁₀ O ₄) (182.17)		phenoxyl	HAT	Synthetic dyes, bisphenol A, EDC, Pharmaceutical	(Torres-Duarte et al., 2009; Nguyen et al., 2014a)
Guaiacol (C ₇ H ₈ O ₂) (124)		phenoxyl	HAT	TrOCs	(Murugesan et al., 2010)

Vanillin (C ₈ H ₈ O ₃) (152.15)		phenoxyl	HAT	Synthetic dyes, TrOCs	(Murugesan et al., 2010; Weng et al., 2012)
P-coumaric acid (C ₉ H ₈ O ₃) (164.16)		phenoxyl	HAT	Synthetic dyes, TrOCs	(Murugesan et al., 2010; Weng et al., 2012)
4-cyanophenol (C ₇ H ₅ NO) (119.3)		phenoxyl	HAT	TrOCs	(Weng et al., 2012)
Acetosyringone (C ₁₀ H ₁₂ O ₄) (196)		phenoxyl	HAT	Synthetic dyes, TrOCs	(Murugesan et al., 2010)
Acetovanillone (C ₉ H ₁₀ O ₃) (166)		phenoxyl	HAT	TrOCs	(Murugesan et al., 2010)

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (C ₁₈ H ₂₄ N ₆ O ₆ S ₄) (548.67)		ABTS ⁺ ABTS ⁺⁺	ET	Synthetic dyes, TrOCs, phenolic compounds	(Cabana et al., 2007; Kurniawati et al., 2007; Weng et al., 2012)
2,2,6,6-Tetramethylpiperidinyloxyl (TEMPO) (C ₉ H ₁₈ NO) (156)		N=O [•] Oxoammonium	Ionic mechanism	Phenolic compounds, synthetic dyes	(Murugesan et al., 2010; Weng et al., 2012)

Several studies have demonstrated improvement of TrOC removal by the addition of mediators. Hata et al. (2010a) reported that the aminoxyl radicals generated in the laccase – HBT system could eliminate carbamazepine, while laccase only could not. Marco-Urrea et al. (2010a) achieved 10% and 100% removal of naproxen in laccase and laccase + HBT, respectively. Lloret et al. (2010) compared the removal of naproxen and diclofenac by laccase and laccase –HBT system at pH 4 and 5. The results showed that laccase – HBT provided a better removal of naproxen and diclofenac at both pH (*i.e.*, improvement of removal of naproxen by 60% and 40% and of diclofenac by 30% and 25% at pH 4 and 5, respectively).

The presence of HBT and ABTS helped increase the removal of ketoprofen from 25% to 45% and 60%, respectively (Tran et al., 2010). Murugesan et al. (2010) observed a significant improvement of triclosan removal from 56% to 92 % and 84% after addition of HBT and SA to the laccase solution, respectively. The enhancement of TrOC removal by laccase mediator system may be due to the two factors attributable to radical species generated from mediator during the laccase-mediator reaction i) higher redox potential and ii) reduction of steric hindrance. Weng et al. (2012) observed an increase in the redox potential of laccase solution after addition of SA and thus, laccase-SA system could improve the removal of sulphonamide. In addition, low molecular weight mediators can interact with complex compounds that cannot directly access the enzyme active site (Xu et al., 2000).

The capacity of a certain mediator to improve TrOC degradation is compound-specific. For example, in the case of triclosan, addition of SA or TEMPO to laccase solution derived from a *T. versicolor* culture resulted in inferior performance than with no mediator present (Kim et al., 2006). Weng et al. (2012) investigated the effect of 6 mediators on degradation of sulfadimethoxine and sulfamonomethoxine by laccase. The authors reported that ABTS and violuric acid provided the fastest transformations, while relatively slower transformation was observed in presence of SA and 4-hydroxybenzyl alcohol. No significant impact of 4-hydroxyacetophenone and 4-cyanophenol was observed. Nguyen et al. (2014a) compared the TrOC removal performance of HBT and SA (1 mM). HBT showed better removal than SA for some compounds, namely salicylic acid, oxybenzone, pentachlorophenol, enterolactone, primidone, atrazine, and naproxen. On the other hand, SA led to better removal of formononetin and octocrylene.

The performance of laccase mediator system has also been found to be dependent on the mediator concentration. This may be because mediator concentration influences both the abundance and stability of oxidized intermediates (Murugesan et al., 2010). Removal improvement will reach saturation at a certain mediator concentration, beyond which further increase in mediator concentration cannot increase the removal. For example, Mizuno et al. (2009b) observed increase in the removal of iso-butylparaben and n-butylparaben when the mediator HBT was added to laccase (from *T. versicolor*) reaction over a concentration range of 0.2 – 2 mM, beyond which no further improvement was observed. Benzina et al. (2012) studied the effect of HBT and enzyme concentration on decolorization at a fixed dye concentration of 60 mg/L. Increase in the HBT and enzyme concentration led to the enhancement of decolorization but the enhancement reached its highest point at 0.9 mM HBT and 0.1 U/mL laccase. The threshold concentrations depend on the source of laccase, the target compound and the mediator used (Nguyen et al., 2014b).

The radicals generated from laccase-mediator reaction could inactivate laccase by oxidizing the aromatic amino acid residues on the proteinaceous enzyme surface (Khelifi-Slama et al., 2012). The extent of laccase denaturation somewhat depends on the type and mediator concentrations. The activity of *T. versicolor* laccase was observed during 40 days in the absence/presence of mediator. In the presence of 50 μ M SA, the laccase was deactivated within 30 min (Mendoza et al., 2011). However, reducing the mediator concentration to 25 μ M resulted in a similar deactivation profile as without the mediator (Mendoza et al., 2011). Kurniawati and Nicell (2007) observed only a slight inactivation (<5%) of laccase from *T. versicolor* when a range of different mediators was used at concentrations up to 0.5 mM. Nguyen et al. (2014a) observed 14 to 39% loss of laccase activity for HBT dosages of 0.1–1 mM.

Laccase-mediator systems have been used for removal of selected pollutants with promising results. However, reports on application of laccase and mediators systems in reactors are limited possibly due to the continuous addition of mediators which may increase the cost of treatment process or operational difficulty. A way of reusing the mediator could be to couple it to a large polymer such as polyethylene glycol to increase its molecular size, and then use it in a membrane reactor where both enzyme and mediator could be retained. Mendoza et al. (2011) confirmed that the membrane (MWCO of a kDa) could retain the mediator (TEMPO) (immobilized on PEG)

within the reactor and prevent mediator loss significantly. Thus, the authors could operate the membrane reactor up to 9 batches without replenishment of enzyme and mediator. However, the reaction time, which was 4.5 h in the first batch to obtain a complete decolorization, increased up to 27 h to achieve 78% color removal at the end of operation period. The decrease in removal efficiency and increase in time required for reaction is possibly due to the drop in the enzymatic activity. Thus, in a continuous system re-addition of both enzyme and mediator is required to maintain stable removal efficiency. In that context, recovery of mediator from the effluent and reuse in the reactor could be a potential solution. Chhabra et al. (2009) achieved 70% ABTS recovery from the membrane reactor effluent through ammonium sulphate precipitation. Further discussion on this aspect is presented in **Section 2.5.7**.

It is also noteworthy that the addition of mediators may increase the toxicity of the treated solution. Nguyen et al. (2014a) observed a significant increase in toxicity of treated effluent after addition of HBT and SA into laccase reaction. The toxicity increased with mediator concentration. It was hypothesized that the increase in toxicity may be due to the reactive radicals that are generated from mediators, which may conceivably attack and damage the cells of the bacteria used toxicity assay (Kim et al., 2006). Cortez et al. (2010) reported that SA inhibited the growth and xylitol production of *Candida guilliermondii* via respiration inhibition.

2.5.6 Continuous flow fungal reactor

WRF have been reported to effectively remove a wide range of TrOCs in small scale (few hundred millilitres) and pure culture batch tests under sterile conditions. However, studies applying fungus in larger scale bioreactors are very scarce in the literature. Moreover, most of these studies focused on dye removal (Borchert et al., 2001; Chhabra et al., 2009). Only a few studies have focused on TrOC removal by continuous flow fungal reactors (Blázquez et al., 2008; Jelic et al., 2012) and among these only one study has explored a fungal MBR using an MF membrane (Yang et al., 2013b). **Table 2.10** summarizes information from available studies on TrOC removal by fungal reactors. Conceptually, application of whole-cell fungi in the reactor may provide some advantages such as; (i) fungi may continuously produce enzymes, therefore keeping the enzyme level in the bioreactor stable, (ii) the intracellular enzymes can also play an important role in the removal of pollutants which will increase the TrOC removal and (iii) adsorption on fungal cell may also provide additional removal. Thus, TrOCs could be removed

simultaneously by degradation by extracellular and/or intracellular enzymes and adsorption on fungal mycelium. For example, in the presence of the cytochrome P-450 inhibitor 1-aminobenzotriazole (5 mM), Marco-Urrea et al. (2010c) reported a decrease in ketoprofen oxidation, suggesting that intracellular cytochrome P450 mediated degradation constitutes an important part in ketoprofen degradation. A notable removal of carbamazepine by the *T. versicolor* culture was attributed to the intracellular cytochrome P450 enzyme system by Zhang et al. (2010). A similar observation was reported in a study by Jelic et al. (2012). Blaquez et al. (2008) reported the removal of two estrogens by *T. versicolor* inoculated in an air fluidised bioreactor. Estrone and 17 β -estradiol were completely removed at a volumetric removal rate of 0.16 and 0.09 mg/L.h, respectively when fed at 18.8 and 7.3 mg/L, respectively over the operation period of 26 days. The degradation was observed to be due to the laccase excreted from the fungi. This study demonstrated fungal treatment of estrogens using continuous flow bioreactor with suspended fungal biomass. Rodarte-Morales et al. (2012a) used pellets of *Phanerochaete chrysosporium* in fed-batch bioreactors operated under continuous air supply or periodic pulsation of oxygen for the transformation of three anti-inflammatory drugs namely, diclofenac, ibuprofen and naproxen. The performance of the fungal reactor was steady over a period 30 day with a complete removal of ibuprofen and diclofenac and 77 - 99% removal of naproxen under both aeration conditions. Jelic et al. (2012) investigated the degradation of carbamazepine by *T. versicolor* in an air pulsed fluidised bed bioreactor and identified degradation by-products. The bioreactor was operated in continuous mode with a hydraulic retention time of 3 days. 54% of the influent concentration (200 μ g/L) was reduced at steady state (25 days) with a carbamazepine degradation rate of 11.9 μ g/g biomass (dry wt.).day (Jelic et al., 2012).

Recently, there have been some studies on the removal of TrOCs from real wastewater and/or under non-sterile conditions (Cruz-Morató et al., 2013a; Gros et al., 2014). Yang et al. (2013b) achieved stable removal of bisphenol A (80-90%) and diclofenac (55%) during the course of three months of operation by a membrane bioreactor which was inoculated with *T. versicolor*. The results from that study confirmed biodegradation as the main removal mechanism. A fluidized bed bioreactor inoculated with *T. versicolor* was operated to degrade TrOCs contained in urban wastewater under non sterilized condition (Cruz-Morató et al., 2013a). Ibuprofen and acetaminophen were completely removed despite of their high concentration. Ketoprofen was

completely removed after 2 days. The high removal of ibuprofen and ketoprofen were quite promising since these compounds have been reported to be resistant in activated sludge treatment. The result from this study demonstrated that fungus could be used to remove TrOCs which are present at low concentration in real and complex matrices. Performance of different species for the same TrOC removal may vary. Diverse enzyme systems harboured by different fungal species may lead to different levels of performance.

Table 2.10: Summary of current studies on fungal reactor for removal of TrOCs

TrOCs	Concentration in feed (mg/L)	Reactor type	HRT (h)	Biomass concentration (g/L)	Operation duration (day)	Removal (%)	Reference
Diclofenac	0.8	Fed batch stirred reactor	24	6.5	30	100	(Rodarte-Morales et al., 2011)
Diclofenac	0.4 ± 0.01	Whole-cell fungal membrane bioreactor	48	3	90	55	(Yang et al., 2013a)
Ibuprofen	0.8	Fed batch stirred reactor	24	6.5	30	100	(Rodarte-Morales et al., 2011)
Naproxen	0.8	Fed batch stirred reactor	24	6.5	30	100	(Rodarte-Morales et al., 2011)
Carbamazepine	1	Attached growth on polyether foam bioreactor	15.6	-	100	80	(Zhang et al., 2012)
Carbamazepine	0.2	Air fluidised bed bioreactor	3	-	25	54	(Jelic et al., 2012)
Pentachlorophenol	0.1	Rotating tube bioreactor	-	-	8	99	(Alleman et al., 1995)
Bisphenol A	0.5 ± 0.01	Whole-cell fungal membrane bioreactor	48	3	90	80-90	(Yang et al., 2013b)
Iopromide	0.19	Air pulsed fluidised bed bioreactor (sterile condition)	-	1.4	8	87	(Gros et al., 2014)
Iopromide	0.19	Air pulsed fluidised bed bioreactor (non-sterile condition)	-	1.4	8	65.4	(Gros et al., 2014)
Ofloxacin	0.024	Air pulsed fluidised bed	-	1.4	8	98.5	(Gros et al.,

		bioreactor (sterile condition)					2014)
Ofloxacin	0.024	Air pulsed fluidised bed bioreactor (non-sterile condition)	-	1.4	8	99	(Gros et al., 2014)
Clofibric acid	0.16	Air fluidised bed bioreactor	96	3.8	25	80	(Cruz-Morató et al., 2013b)
Estrone	18.8	Air fluidised bioreactor	120	-	26	100	(Blánquez et al., 2008)
17 α -ethinylestradiol	7.3	Air fluidised bioreactor	120	-	26	100	(Blánquez et al., 2008)

2.5.6.1 Limitations of current continuous flow whole-cell fungal reactors

The application of fungi under non-sterile conditions is a technical challenge. The main reason is that fungi are low-level eukaryotic microorganisms which grow slower than bacteria. Once bacteria invades into the reactor, they could compete for nutrients with the fungi. Bacterial contamination usually causes deterioration in the degradation performance of the fungi. For example, Heinfling et al. (1998) observed a high and stable decolourisation rate under sterile conditions but bacterial contamination led to a decline in dye decolourisation efficiency (Heinfling et al., 1998). Furthermore, it is obviously impractical to use the sterilisation means (which are used in laboratory) to prevent bacterial contamination to a full-scale reactor system. Another limitation of continuous fungal reactors is the loss of enzymes and mediators along with treated effluent (Hai et al., 2008; Hai et al., 2012b).

2.5.6.2 Strategies to improve continuous flow fungal reactor performance

Bacterial contamination and washout of extracellular enzymes with effluent are major limitations of a continuous flow whole-cell fungal reactor. In batch incubation tests Gao et al., (2008) observed that while a suspended culture was highly contaminated in non-sterile conditions, the immobilised fungal culture restrained bacterial growth. Consequently, the immobilised fungal culture produced higher MnP activity than the suspended one (*i.e.*, 690 U/L *vs.* 125 U/L). Results also showed higher decolourisation efficiency (93.5% *vs.* 15%) by the immobilised culture (Gao et al., 2008). However, this report was based on a short term observation, *i.e.*, the batch test was conducted over 3 and 6 days.

Zhang et al. (2000) entrapped PAC inside *T. versicolor* pellets. The results in comparison with fungi pellet only, fungi pellet with PAC (free) and PAC only demonstrated that the fungi pellet with entrapped PAC inside promoted the dye degradation. The enhanced decolouration ability of the fungi pellets entrapped PAC implies that the microenvironment around the fungi cell was changed. The PAC can absorb and concentrate the chemical thereby increasing the bioavailability of these compounds for microbial and enzyme attack. More interestingly, PAC can retain the extracellular enzyme and mediator that are necessary for degradation. Hai et al. (2012b) also proposed to prevent extracellular enzyme wash out by the addition of GAC to the reactor in order to adsorb the dye and enzyme simultaneously.

2.5.7 Effect of bio-augmentation of activated sludge with fungi

As discussed above, most of the studies with WRF were operated under sterile conditions in lab scale tests and the results demonstrated that WRF can be used for TrOC degradation. However, an important consideration for their use in treating wastewater containing TrOCs is whether similar degrees of treatment can be achieved under non-sterile conditions. A few available studies on TrOCs removal by WRF under non-sterile conditions report somewhat poorer removal by fungal reactor. For example, decrease in performance of fungal reactor under non-sterile conditions and/or in continuous flow mode has been reported in study by Jelic et al. (2012) and Zhang et al. (2012). Libra et al. (2003) also observed a significant decrease in decolorization of reactive black 5 by *T. versicolor* performance owing to bacterial contamination. Therefore, more systematic studies on the enhancement of fungal degradation of TrOCs in continuous reactors under non-sterile condition are necessary. It is important to investigate on bio-augmentation between bacteria and WRF in more details. Interestingly, in the soil environment, bacteria and fungi seem to adopt different but complementary metabolic pathways for the degradation of some recalcitrant pesticides (Rønhede et al., 2005). For instance, the contributions of both bacteria and fungi to the degradation of a chloroaromatic fungicide - chlorothalonil - in soil were confirmed by a selective inhibition method (Mori et al., 1998). Hai et al. (2012a) observed enhanced removal of three widely used recalcitrant pesticides from their liquid mixture by implementing a non-acclimated mixed culture of bacteria and white-rot fungus. The combination of activated sludge with *T. versicolor* for treatment of olive mill wastewater resulted in a significant reduction of phenolic monomers in olive mill wastewater has been reported by Dhouib et al. (2006). Utilisation of mixed microbial consortia dominated by WRF, instead of conventional bacterial consortia, led to excellent degradation of dye by an MBR (Hai et al., 2008).

2.5.8 Degradation products and toxicity of effluent

Metabolites arising from degradation of the parent compounds also need to be monitored to understand the degradation pathway and the toxicity of the metabolites compared to the parent compounds during the treatment with WRF and their LMEs. However, the majority of studies focused on the disappearance of parent compounds after treatment. There is only a limited number of studies on the degradation pathways and toxicity of treated effluent. Hata et al. (2010b)

reported that diclofenac was degraded via hydroxylation pathway by cytochrome P450 in *Phanerochaete sordida* YK-624. A hydroxyl group was first attached to diclofenac and formed hydroxy diclofenac which was more amenable to further degradation. In another study, Marco-Urrea et al. (2010b) observed a slight decrease in removal of diclofenac after addition of 1-aminobenzotriazole, a known inhibitor of cytochrome P450, into *T. versicolor* confirming the involvement of cytochrome P450 in the degradation of diclofenac. *In vivo* and *in vitro* experiments using purified laccase suggested another degradation pathway of diclofenac. The metabolite 4-(2,6 dichlorophenylamino)-1,3-benzenedimethanol was formed during the laccase oxidation of diclofenac (Marco-Urrea et al., 2010b). Different pathways namely, desmethylation by Cytochrome P450 and oxidation by laccase occurred during naproxen degradation by WRF treatment. The former pathway led to the formation of 6-desmethylnaproxen, while 1-(6-methoxynaphthalen-2-yl)ethanone was formed via the later pathway (Marco-Urrea et al., 2010a). Further analysis of naproxen and its metabolites revealed that these compounds were completely removed after 6 h incubation. Thus, it was also confirmed that the treated medium was non-toxic (Marco-Urrea et al., 2010a). Three metabolites of ibuprofen namely, 1-hydroxyibuprofen, 1,2-dihydroxyibuprofen and 2-hydroxyibuprofen were identified after WRF treatment. The appearance of these metabolites was accompanied by a corresponding progressive decrease in ibuprofen concentration (Marco-Urrea et al., 2009). Marco-Urrea et al. (2010c) elucidated three intermediates namely, 2-[3-(4-hydroxybenzoyl) phenyl]-propanoic acid, 2-[(3-hydroxy(phenyl)methyl)phenyl]-propanoic acid, and 2-(3-benzoyl-4-hydroxyphenyl)-propanoic acid from the oxidation of ketoprofen. Some metabolites (*i.e.*, acridone, acridine, 10,11-dihydro-10,11-dihydroxy-carbamazepine, and 10, 11-epoxy-carbamazepine) were identified after treatment of carbamazepine by *T. versicolor* (Jelic et al., 2012). Moreover, acute toxicity tests indicated that the final treated effluent was non-toxic. However, effluent toxicity may arise due to addition of redox-mediators to enhance laccase-catalysed degradation. This aspect has been detailed in the results and discussion related chapters within this thesis.

2.5.9 Enzymatic membrane reactors

2.5.9.1 EMR configurations

Enzymatic membrane reactors (EMRs) couple membrane separation with enzymatic degradation in a single unit. The selective membrane aims to separate the biocatalyst (enzyme) from the reaction products. The main objective of EMR is to ensure complete retention of the enzyme in order to maintain biodegradation inside the reactor. In this system, the membranes retain the enzyme by size exclusion, and freely circulate on the retentate side. However, in some cases such as when the degradation product is larger than the enzyme, enlargement of enzyme by cross-linked enzyme aggregates or binding them onto a carrier is required (Jochems et al., 2011). Another way is that the enzyme can be immobilised onto the membrane surface or inside its porous structure by physical or chemical interactions (Rios et al., 2004). Thus, the membranes can act as a support layer for enzyme immobilization. The configurations of enzymatic membrane reactor are presented in **Figure 2.3**. Each configuration has its own advantages. Enzyme use in suspension offers several advantages such as more effective retention of enzymes, spreading of enzymes in the reactor, and easy replenishment of fresh enzymes during long term operation. On the other hand, a key advantage of immobilising enzymes on the membrane material is to increase their stability and resistance towards chemical inhibitors. This is particularly important for wastewater treatment (Rios et al., 2004).

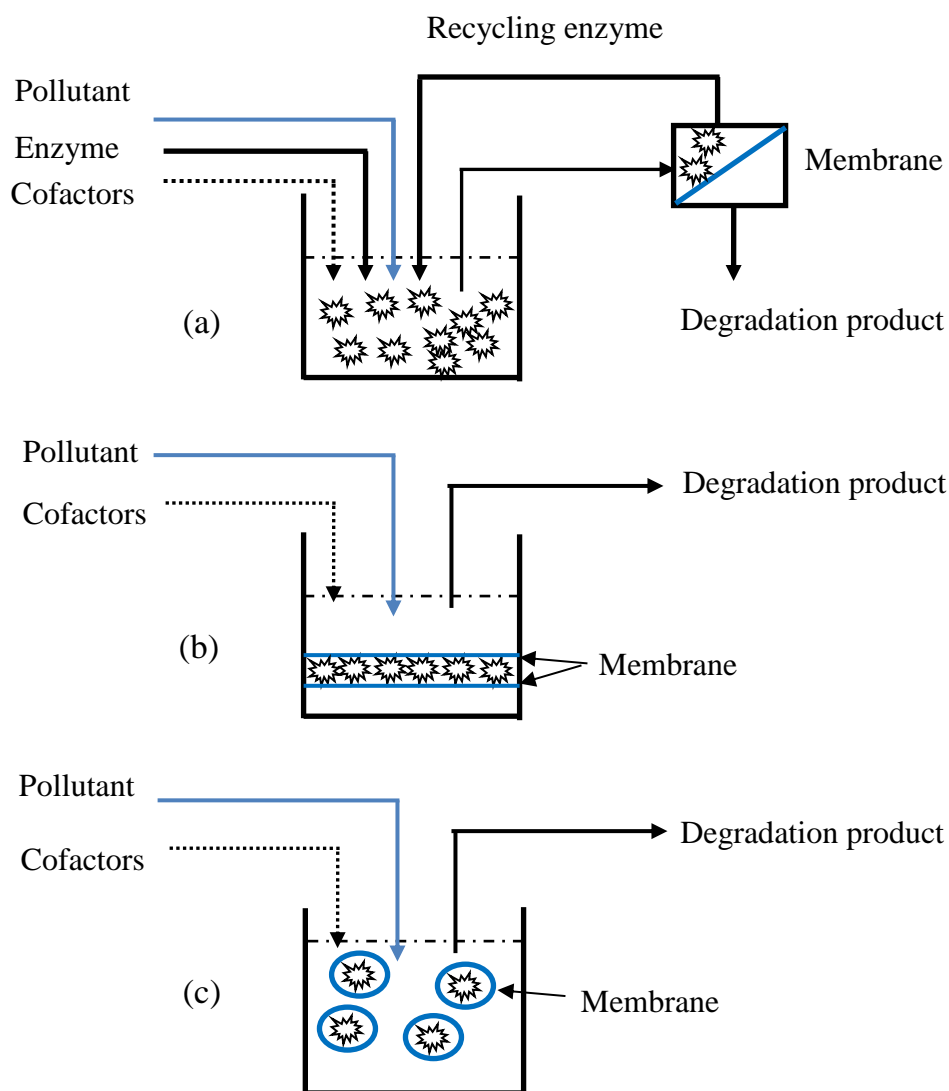


Figure 2.3: Schematic diagram of three different configurations of enzymatic membrane bioreactor (a) suspended enzyme in solution in a reactor coupled with membrane unit, (b) immobilised within the membrane matrix itself and (c) entrapped in gels or microcapsules (adapted from Rios et al., 2004).

2.5.9.2 Application of EMR for the removal of dyes

EMRs have been used for treatment of dyes in a few studies (Lopez et al., 2004; Katuri et al., 2009; Mendoza et al., 2011). Mendoza et al. (2011) investigated the removal of azo dyes by laccase/mediator in an EMR with polysulfonate UF membrane (MWCO of 10 kDa). The

decolourisation yields were 52% to 95% depending on the dye type. Another study by Chhabra et al. (2009) also used laccase with mediator in an EMR. Effective decolourisation (>95%) was achieved up to 25 days with a single dose of enzyme and more than 45% of enzyme activity remained at the end of operation. Katuri et al. (2009) operated different EMRs (*i.e.*, direct contact MBR, enzyme impregnated MR and immobilised EMR) for the removal of acid azo dye. The immobilized laccase showed high decolourization efficiency towards dye removal from aqueous solutions and laccase encapsulated in chitosan membranes presented several advantages such as requirement of short contact period and reusability. Decolourization of acid black with laccase obtained from *P. ostreatus* was effective even without presence of any mediators (Katuri et al., 2009). Lopez et al. (2004) also suggested that EMR can be proposed as a promising technology for continuous decolourisation of dye wastewater.

2.5.9.3 TrOC removal by EMR

Recently, few studies have focused on the removal of TrOCs by enzymes suspended in the reactor (Lloret et al., 2012a; Nguyen et al., 2014c; Nguyen et al., 2015) or immobilised on the membrane (Nicolucci et al., 2011; Lloret et al., 2012b) (**Table 2.11**). Lloret et al. (2012a) investigated the removal of two estrogens (estrone and 17 β -estradiol) by suspension of a commercial laccase from *M. thermophila* in the EMR. The UF membrane (MWCO of 10 kDa), which was submerged in the enzymatic reactor, completely retained the enzyme. The EMR effectively removed estrone and 17 β -estradiol to 64 – 100% at an enzymatic activity of 500 U/L. In addition to the high removal efficiency, the EMR effectively reduced estrogenic activity to 97%.

Denaturation of enzyme (*i.e.*, loss of enzymatic activity) has been observed in some EMR studies. Denaturation of enzyme may be due to various factors including physical, chemical and biological inhibitors and the effect of shear stress during filtration (Rios et al., 2004; Mendoza et al., 2011). Consequently, denaturation of enzyme leads to the decline in the performance of the EMR system. Reduction in enzymatic activity was observed in a study by Hata et al. (2010a). The authors reported a rapid decrease during the first 4 h (initial E = 10 nkat/mL drop to 3 nkat/mL) of treatment and the residual of laccase was below 10 % after 8 h of treatment. Thus, periodic enzyme addition may be necessary to maintain stable enzymatic activity and the EMP

performance. Hata et al. (2010a) re-injected laccase every 8 h to the reactor to enhance the performance of the system. Escalona et al. (2014) used laccase and peroxidase from horseradish to remove bisphenol A in an EMR. This system integrated an enzymatic reactor with a nanofiltration membrane (NF). As a result, bisphenol A was rejected by the membrane and degraded by the enzyme-catalysed reactions. The results revealed the efficiency of the enzyme treatment for bisphenol A degradation. However, the presence of enzyme reduced the membrane permeability (Escalona et al., 2014). Apart from laccase, other ligninolytic enzymes such as versatile peroxidase and manganese peroxidase have been also used for the removal of TrOCs. Méndez-Hernández et al. (2015) presented a two-stage membrane reactor based on the production and use of Mn^{3+} -malonate (chemical oxidant) which was produced in an enzymatic reactor (R1) by versatile peroxidase. The Mn^{3+} -malonate was transferred to oxidation reactor (R2) where the oxidation of one TrOC (nonylphenol) took place. A complete removal of nonylphenol was achieved at a hydraulic retention time of 20 min. The separation of these two reactors allowed constant production of Mn^{3+} -malonate in R1 with minimal deduction of enzymatic activity. This study also confirmed that the system could effectively remove nonylphenol in real wastewater (454 nM) up to 99% (Méndez-Hernández et al., 2015).

Immobilisation of enzyme on membrane or support materials is another potential application of enzyme in a continuous enzymatic reactor. Nicolucci et al. (2011) immobilised laccase on polyacrylonitrile beads in a fluidized bed reactor for removal of bisphenol. A complete removal of bisphenol was achieved. The author also reported that the immobilization of laccase improved its stability and resistance to pH and temperature changes. The removal efficiency of estrogens by immobilised laccase in a fluidized bed reactor was investigated in a study by Lloret et al. (2012b). High removals (between 76 and 90%) were obtained for 16 d in this study. Lante et al. (2000) immobilised laccase onto a spiral-wound asymmetric polyethersulphone membrane and applied for the degradation of 18 phenols. It was confirmed that the immobilised laccase on the membrane could oxidise the phenols. The enzyme immobilisation generally increase its stability compared to the free enzyme. Chea et al. (2014) prepared an enzymatic membrane by grafting laccase (*T. versicolor*) on a gelatine layer deposited onto α -alumina tubular membranes. The system was operated in a dead end mode. A phenolic compound 2,6-dimethoxyphenol was completely removed in that study (Chea et al., 2014). In another study, tetracycline, an antibiotic

was degraded by immobilized laccase (*T. versicolor*) in an EMR (De Cazes et al., 2014). The immobilized laccase-EMR achieved 56% removal of tetracycline as compared to only 30% with free laccase. These results highlighted the stability of the immobilized enzyme for the degradation of tetracycline (De Cazes et al., 2014). Hou et al. (2014) immobilized laccase on TiO₂ nanoparticles which were coated on the membrane surface and used for the removal of bisphenol A. Accordingly, an improvement in bisphenol A removal efficiency and stability of enzyme were achieved.

Table 2.11 EMR studies for TrOC removal

Membrane material (MWCO)	pH	Temp (°C)	Laccase (μM/min)	HRT (h)	Wastewater composition	Reactor volume (L)	Reference
UF polyethersulfone (10 kDa)	7	26	500, 1000	1, 4	TrOCs (4 mg/L each) in water buffered with 100 mM sodium phosphate	0.25	(Lloret et al., 2012a)
UF polyethersulfone (10 kDa)	4, 7	25	500	2, 4	TrOCs (4 mg/L each) in water buffered with 100 mM sodium acetate at pH 4 and with 100 mM sodium phosphate at pH 7	0.25	(Lloret et al., 2012c)
UF Polyacrylonitrile (6 kDa)	6.8	28	90	8	TrOCs (0.5 mg/L each) in Milli-Q water	1.5	(Nguyen et al., 2014c) ^a
UF Polyacrylonitrile (6 kDa)	6.8	28	180	8	TrOCs (0.005 mg/L each) in Milli-Q water	1.5	(Nguyen et al., 2015) ^a
UF Polyacrylonitrile (6 kDa)	6.8	28	90	8	TrOCs (0.25 and 0.5 mg/L each) in Milli-Q water	1.5	(Nguyen et al., 2014d) ^b
Laccase-grafted ceramic membrane (0.2 and 1.4 μm)	7	25	-	24	TrOCs (20 mg/L each) in deionized water	2	(de Cazes et al., 2015)
Laccase on TiO ₂ coated on PVDF membrane	5.5	22	0.42	24	TrOCs (35 mg/L each) in water buffered with 100 mM acetate	0.04	(Hou et al., 2014)
Enzymatic reactor -NF membrane	4.5	30	Versatile and MnP	0.8	TrOCs (100 mg/L each) in water buffered with 12.5 mM sodium malonate	0.27	(Méndez-Hernández et al., 2015)

^{a,b} Chapter 5, and 7 of this thesis

2.5.9.4 Enhancement of EMR performance

Reusability and stability of enzyme are key factor in the operation of EMR, because enzymes are responsible for the oxidation of pollutants. The membranes effectivity retains the enzymes to enhance the reusability. However, denaturation of enzyme occurs due to various factors including physical, chemical and biological inhibitors and the effect of shear stress during filtration (Rios et al., 2004). Apart from enzyme reinjection, the addition of preservative compounds to the enzymatic reactor could prevent or decrease the loss of enzymatic activity. Mendoza et al. (2011) reported that ethylenediaminetetraacetic acid and polyethylene glycol could protect the enzyme, especially under oxidative stress. Immobilisation of enzyme on the membrane may increase their stability in the EMR.

The performance of the EMR on TrOC removal could be enhanced by addition of redox-mediator. As mentioned in **Section 2.5.4**, mediator act as an electron shuttle between the oxidizing enzyme and target compounds. The addition of mediators (SA and HBT) was reported to extend the spectrum of efficiently degraded TrOCs (Chapter 5). Apart from the mediator addition strategy, addition of adsorbent (PAC or GAC) has recently been applied to the EMR. Because laccase can degrade TrOCs that are inefficiently degraded by conventional biological processes, PAC/GAC dosing to the EMR may lead to enhanced biodegradation. As mentioned previously, PAC/GAC addition to MBR has been shown to enhance the removal of TrOCs. This thesis demonstrates a 14 – 25% improvement in aqueous phase removal of four TrOCs (*i.e.*, carbamazepine, diclofenac, sulfamethoxazole and atrazine) by dosing 3 g/L GAC to EMR (Chapter 7).

2.6. REFERENCES

- Abegglen, C., A. Joss, C. S. McArdell, G. Fink, M. P. Schlüsener, T. A. Ternes and H. Siegrist (2009). The fate of selected micropollutants in a single-house MBR. *Water Res.* 43, 2036-2046.
- Adams, C., Y. Wang, K. Loftin and M. Meyer (2002). Removal of antibiotics from surface and distilled water in conventional water treatment processes. *J. Environ. Eng.*, 128, 253-260.
- Aitken, M. D., R. Venkatadri and R. L. Irvine (1989). Oxidation of phenolic pollutants by a lignin degrading enzyme from the white-rot fungus *Phanerochaete chrysosporium*. *Water Res.* 23, 443-450.
- Al-Rifai, J. H., H. Khabbaz and A. I. Schäfer (2011). Removal of pharmaceuticals and endocrine disrupting compounds in a water recycling process using reverse osmosis systems. *Sep Purif Technol.* 77, 60-67.

- Alexander, J. T., F. I. Hai and T. M. Al-aboud (2012). Chemical coagulation-based processes for trace organic contaminant removal: Current state and future potential. *J. Environ. Manage.*, 111, 195-207.
- Alleman, B. C., B. E. Logan and R. L. Gilbertson (1995). Degradation of pentachlorophenol by fixed films of white rot fungi in rotating tube bioreactors. *Water Res.* 29, 61-67.
- Alturki, A. A., N. Tadkaew, J. A. McDonald, S. J. Khan, W. E. Price and L. D. Nghiem (2010). Combining MBR and NF/RO membrane filtration for the removal of trace organics in indirect potable water reuse applications. *J. Membr. Sci.*, 365, 206-215.
- Alzieu, C. (2000). Impact of tributyltin on marine invertebrates. *Ecotoxicology*. 9, 71-76.
- Andreozzi, R., L. Campanella, B. Frayse, J. Garric, A. Gonnella, R. L. Giudice, R. Marotta, G. Pinto and A. Pollio (2004). Effects of advanced oxidation processes (AOPs) on the toxicity of a mixture of pharmaceuticals. *Water Sci. Technol.*, 50, 23-28.
- Angus, R. A., J. Stanko, R. L. Jenkins and R. D. Watson (2005). Effects of 17 α -ethynylestradiol on sexual development of male western mosquitofish (*Gambusia affinis*). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, 140, 330-339.
- Asgher, M., S. Noreen and H. M. N. Iqbal (2012). Investigation of the potential of a fungal co-culture for bioremediation of textile industry effluents. *Fresenius environmental bulletin*. 21, 1864-1872.
- Auriol, M., Y. Filali-Meknassi, R. D. Tyagi and C. D. Adams (2007). Laccase-catalyzed conversion of natural and synthetic hormones from a municipal wastewater. *Water Res.* 41, 3281-3288.
- Azbar, N., T. Yonar and K. Kestioglu (2004). Comparison of various advanced oxidation processes and chemical treatment methods for COD and color removal from a polyester and acetate fiber dyeing effluent. *Chemosphere*. 55, 35-43.
- Backhaus, T., J. Sumpter, H. Blanck and K. Kümmerer (2008). On the ecotoxicology of pharmaceutical mixtures in the environment, Springer Berlin Heidelberg: 257-276.
- Baiocco, P., A. M. Barreca, M. Fabbrini, C. Galli and P. Gentili (2003). Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase-mediator systems. *Organic & Biomolecular Chemistry*. 1, 191-197.
- Baldrian, P. (2004). Increase of laccase activity during interspecific interactions of white-rot fungi. *FEMS Microbio Ecol.* 50, 245-253.
- Ballard, B. and A. MacKay (2005). Estimating the removal of anthropogenic organic chemicals from raw drinking water by coagulation flocculation. *J. Environ. Eng.*, 131, 108-118.
- Basile, T., A. Petrella, M. Petrella, G. Boghetich, V. Petruzzelli, S. Colasuonno and D. Petruzzelli (2011). Review of endocrine disrupting compound removal technologies in water and wastewater treatment plants: An EU perspective. *Ind. Eng. Chem. Res.*, 50, 8389-8401.
- Belgiorno, V., L. Rizzo, D. Fatta, C. Della Rocca, G. Lofrano, A. Nikolaou, V. Naddeo and S. Meric (2007). Review on endocrine disrupting-emerging compounds in urban wastewater: occurrence and removal by photocatalysis and ultrasonic irradiation for wastewater reuse. *Desalination*. 215, 166-176.
- Bellona, C., J. E. Drewes, G. Oelker, J. Luna, G. Filteau and G. Amy (2008). Comparing nanofiltration and reverse osmosis for drinking water augmentation. *Journal - American Water Works Association*. 100, 102-116.
- Bellona, C., J. E. Drewes, P. Xu and G. Amy (2004). Factors affecting the rejection of organic solutes during NF/RO treatment—a literature review. *Water Res.* 38, 2795-2809.

- Beltrain, F. J., J. F. Garca-Araya and B. Acedo (1994). Advanced oxidation of atrazine in water II. Ozonation combined with ultraviolet radiation. *Water Res.* 28, 2165-2174.
- Bending, G. D., M. Friloux and A. Walker (2002). Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. *FEMS Microbiol. Lett.*, 212, 59-63.
- Benzina, O., F. Frikha, H. Zouari-Mechichi, S. Woodward, L. Belbahri, E. Mnif and T. Mechichi (2012). Enhanced decolourization of the azo dye Sirius rose BB by laccase–HBT system. *3 Biotech.* 2, 149-157.
- Bernhard, M., J. Maller and T. P. Knepper (2006). Biodegradation of persistent polar pollutants in wastewater: Comparison of an optimised lab-scale membrane bioreactor and activated sludge treatment. *Water Res.* 40, 3419-3428.
- Blázquez, P. and B. Guieysse (2008). Continuous biodegradation of 17 β -estradiol and 17 α -ethynylestradiol by *Trametes versicolor*. *J. Hazard. Mater.*, 150, 459-462.
- Bolong, N., A. F. Ismail, M. R. Salim and T. Matsuura (2009). A review of the effects of emerging contaminants in wastewater and options for their removal. *Desalination.* 239, 229-246.
- Borchert, M. and J. A. Libra (2001). Decolorization of reactive dyes by the white rot fungus *Trametes versicolor* in sequencing batch reactors. *Biotechnol. Bioeng.*, 75, 313-321.
- Bosco, F., A. Capolongo and B. Ruggeri (2002). Effect of temperature, pH, ionic strength, and sodium nitrate on activity of LiPs: Implications for bioremediation. *Bioremediat J.* 6, 65-76.
- Brennan, S. J., C. A. Brougham, J. J. Roche and A. M. Fogarty (2006). Multi-generational effects of four selected environmental oestrogens on *Daphnia magna*. *Chemosphere.* 64, 49-55.
- Bundy, M. M., W. J. Doucette, L. McNeill and J. F. Ericson (2007). Removal of pharmaceuticals and related compounds by a bench-scale drinking water treatment system. *Journal of Water Supply: Research & Technology-AQUA.* 56, 105-115.
- Burton, S. G. (2003). Laccases and phenol oxidases in organic synthesis - A review. *Curr. Org. Chem.*, 7, 1317-1331.
- Cabana, H., C. Alexandre, S. N. Agathos and J. P. Jones (2009). Immobilization of laccase from the white rot fungus *Coriolopsis polyzona* and use of the immobilized biocatalyst for the continuous elimination of endocrine disrupting chemicals. *Bioresource Technol.* 100, 3447-3458.
- Cabana, H., J.-L. H. Jiwan, R. Rozenberg, V. Elisashvili, M. Penninckx, S. N. Agathos and J. P. Jones (2007). Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using enzyme preparation from the white rot fungus *Coriolopsis polyzona*. *Chemosphere.* 67, 770-778.
- Cajthaml, T., Z. Křesinová, K. Svobodová and M. Möder (2009). Biodegradation of endocrine-disrupting compounds and suppression of estrogenic activity by ligninolytic fungi. *Chemosphere.* 75, 745-750.
- Carlsson, C., A.-K. Johansson, G. Alvan, K. Bergman and T. Kühler (2006). Are pharmaceuticals potent environmental pollutants?: Part II: Environmental risk assessments of selected pharmaceutical excipients. *Sci. Total Environ.*, 364, 88-95.
- Cater, S. R., M. I. Stefan, J. R. Bolton and A. Safarzadeh-Amiri (2000). UV/H₂O₂ Treatment of methyl tert-butyl ether in contaminated waters. *Environmental Science and Technology.* 34, 659-662.

- Chea, V., D. Paolucci-Jeanjean, J. Sanchez and M.-P. Belleville (2014). Potentialities of a Membrane Reactor with Laccase Grafted Membranes for the Enzymatic Degradation of Phenolic Compounds in Water. *Membranes*. 4, 678-691.
- Chhabra, M., S. Mishra and T. R. Sreekrishnan (2009). Laccase/mediator assisted degradation of triarylmethane dyes in a continuous membrane reactor. *J. Biotechnol.*, 143, 69-78.
- Chi, Y., A. Hatakka and P. Maijala (2007). Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin-degrading enzymes? *Int. Biodeterior. Biodegradation*. 59, 32-39.
- Choi, K., S. Kim, C. Kim and J. Park (2006). Removal efficiencies of endocrine disrupting chemicals by coagulation/flocculation, ozonation, powdered/granular activated carbon adsorption, and chlorination. *Korean Journal of Chemical Engineering*. 23, 399-408.
- Chong, M. N., B. Jin, C. W. K. Chow and C. Saint (2010). Recent developments in photocatalytic water treatment technology: A review. *Water Res.* 44, 2997-3027.
- Christensen, A. M., F. Ingerslev and A. Baun (2006). Ecotoxicity of mixtures of antibiotics used in aquacultures. *Environ. Toxicol. Chem.*, 25, 2208-2215.
- Christopher, L. P., B. Yao and Y. Ji (2014). Lignin biodegradation with laccase-mediator systems. *Front. Energy Res.*, 2.
- Clara, M., B. Strenn, O. Gans, E. Martinez, N. Kreuzinger and H. Kroiss (2005). Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. *Water Res.* 39, 4797-4807.
- Combalbert, S. and G. Hernandez-Raquet (2010). Occurrence, fate, and biodegradation of estrogens in sewage and manure. *Appl Microbiol Biotechnol.* 86, 1671-1692.
- Contreras, S., M. Rodríguez, F. A. Momani, C. Sans and S. Esplugas (2003). Contribution of the ozonation pre-treatment to the biodegradation of aqueous solutions of 2,4-dichlorophenol. *Water Res.* 37, 3164-3171.
- Cortez, D. V. and I. C. Roberto (2010). Individual and interaction effects of vanillin and syringaldehyde on the xylitol formation by *Candida guilliermondii*. *Bioresource Technol.* 101, 1858-1865.
- Corvini, P. X. and P. Shahgaldian (2010). Laccase-nanoparticle conjugates for the elimination of micropollutants (endocrine disrupting chemicals) from wastewater in bioreactors. *Rev Environ Sci Biotechnol.* 9, 23-27.
- Cruz-Morató, C., L. Ferrando-Climent, S. Rodriguez-Mozaz, D. Barceló, E. Marco-Urrea, T. Vicent and M. Sarrà (2013a). Degradation of pharmaceuticals in non-sterile urban wastewater by *Trametes versicolor* in a fluidized bed bioreactor. *Water Res.* 47, 5200-5210.
- Cruz-Morató, C., A. Jelić, S. Perez, M. Petrović, D. Barceló, E. Marco-Urrea, M. Sarrà and T. Vicent (2013b). Continuous treatment of clofibric acid by *Trametes versicolor* in a fluidized bed bioreactor: Identification of transformation products and toxicity assessment. *Biochem. Eng. J.*, 75, 79-85.
- Cunningham, V. L., S. P. Binks and M. J. Olson (2009). Human health risk assessment from the presence of human pharmaceuticals in the aquatic environment. *Regul. Toxicol. Pharm.*, 53, 39-45.
- Davila-Vazquez, G., R. Tinoco, M. A. Pickard and R. Vazquez-Duhalt (2005). Transformation of halogenated pesticides by versatile peroxidase from *Bjerkandera adusta*. *Enzyme Microb. Technol.*, 36, 223-231.

- de Cazes, M., M. P. Belleville, M. Mougél, H. Kellner and J. Sanchez-Marcano (2015). Characterization of laccase-grafted ceramic membranes for pharmaceuticals degradation. *J. Membr. Sci.*, 476, 384-393.
- De Cazes, M., M. P. Belleville, E. Petit, M. Llorca, S. Rodríguez-Mozaz, J. De Gunzburg, D. Barceló and J. Sanchez-Marcano (2014). Design and optimization of an enzymatic membrane reactor for tetracycline degradation. *Catalysis Today*. 236, 146-152.
- Dhouib, A., M. Ellouz, F. Aloui and S. Sayadi (2006). Effect of bioaugmentation of activated sludge with white-rot fungi on olive mill wastewater detoxification. *Letters in Applied Microbiology*. 42, 405-411.
- Dodor, D. E., H.-M. Hwang and S. I. N. Ekunwe (2004). Oxidation of anthracene and benzo[a]pyrene by immobilized laccase from *Trametes versicolor*. *Enzyme Microb. Technol.*, 35, 210-217.
- Durán, N., M. A. Rosa, A. D'Annibale and L. Gianfreda (2002). Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. *Enzyme Microb. Technol.*, 31, 907-931.
- Eibes, G., G. Debernardi, G. Feijoo, M. T. Moreira and J. M. Lema (2011). Oxidation of pharmaceutically active compounds by a ligninolytic fungal peroxidase. *Biodegradation*. 22, 539-550.
- Escalona, I., J. de Grooth, J. Font and K. Nijmeijer (2014). Removal of BPA by enzyme polymerization using NF membranes. *J. Membr. Sci.*, 468, 192-201.
- Esplugas, S., D. M. Bila, L. G. T. Krause and M. r. Dezotti (2007). Ozonation and advanced oxidation technologies to remove endocrine disrupting chemicals (EDCs) and pharmaceuticals and personal care products (PPCPs) in water effluents. *J. Hazard. Mater.*, 149, 631-642.
- Esplugas, S., J. Giménez, S. Contreras, E. Pascual and M. Rodríguez (2002). Comparison of different advanced oxidation processes for phenol degradation. *Water Res.* 36, 1034-1042.
- Fent, K., A. A. Weston and D. Caminada (2006). Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.*, 76, 122-159.
- Fu, T.-T., J.-T. Liu and J. K. Hammitt (1999). Consumer willingness to pay for low-pesticide fresh produce in Taiwan. *J. Agric. Econ.*, 50, 220-233.
- Fukuda, T., H. Uchida, Y. Takashima, T. Uwajima, T. Kawabata and M. Suzuki (2001). Degradation of bisphenol a by purified laccase from *Trametes villosa*. *Biochem. Biophys. Res. Commun.*, 284, 704-706.
- Gao, D., Y. Zeng, X. Wen and Y. Qian (2008). Competition strategies for the incubation of white rot fungi under non-sterile conditions. *Process Biochem.* 43, 937-944.
- García-Galán, J., C. E. Rodríguez-Rodríguez, T. Vicent, G. Caminal, M. S. Díaz-Cruz and D. Barceló (2011). Biodegradation of sulfamethazine by *Trametes versicolor*: Removal from sewage sludge and identification of intermediate products by UPLC–QqTOF-MS. *Sci. Total Environ.*, 409, 5505-5512.
- Golan-Rozen, N., B. Chefetz, J. Ben-Ari, J. Geva and Y. Hadar (2011). Transformation of the recalcitrant pharmaceutical compound carbamazepine by *Pleurotus ostreatus*: Role of Cytochrome P450 monooxygenase and manganese peroxidase. *Environ Sci Technol.* 45, 6800-6805.
- Golet, E. M., I. Xifra, H. Siegrist, A. C. Alder and W. Giger (2003). Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. *Environmental Science and Technology*. 37, 3243 - 3249.

- Gómez, M. J., M. M. Gómez-Ramos, A. Agüera, M. Mezcua, S. Herrera and A. R. Fernández-Alba (2009). A new gas chromatography/mass spectrometry method for the simultaneous analysis of target and non-target organic contaminants in waters. *J. Chromatogr. A.* 1216, 4071-4082.
- Gros, M., C. Cruz-Morato, E. Marco-Urrea, P. Longrée, H. Singer, M. Sarra, J. Hollender, T. Vicent, S. Rodriguez-Mozaz and D. Barceló (2014). Biodegradation of the X-ray contrast agent iopromide and the fluoroquinolone antibiotic ofloxacin by the white rot fungus *Trametes versicolor* in hospital wastewaters and identification of degradation products. *Water Res.* 60, 228-241.
- Grover, D. P., J. L. Zhou, P. E. Frickers and J. W. Readman (2011). Improved removal of estrogenic and pharmaceutical compounds in sewage effluent by full scale granular activated carbon: Impact on receiving river water. *J. Hazard. Mater.*, 185, 1005-1011.
- Guillette, L. J., D. A. Crain, M. P. Gunderson, S. A. E. Kools, M. R. Milnes, E. F. Orlando, A. A. Rooney and A. R. Woodward (2000). Alligators and endocrine disrupting contaminants: A current perspective. *Am. Zool.*, 40, 438-452.
- Hai, F. I., O. Modin, K. Yamamoto, K. Fukushi, F. Nakajima and L. D. Nghiem (2012a). Pesticide removal by a mixed culture of bacteria and white-rot fungi. *J Taiwan Inst Chem Eng.* 43, 459-462.
- Hai, F. I., K. Tessmer, L. N. Nguyen, J. Kang, W. E. Price and L. D. Nghiem (2011). Removal of micropollutants by membrane bioreactor under temperature variation. *J. Membr. Sci.*, 383, 144-151.
- Hai, F. I., K. Yamamoto, F. Nakajima and K. Fukushi (2008). Removal of structurally different dyes in submerged membrane fungi reactor—Biosorption/PAC-adsorption, membrane retention and biodegradation. *J. Membr. Sci.*, 325, 395-403.
- Hai, F. I., K. Yamamoto, F. Nakajima and K. Fukushi (2012b). Application of a GAC-coated hollow fiber module to couple enzymatic degradation of dye on membrane to whole cell biodegradation within a membrane bioreactor. *J. Membr. Sci.*, 389, 67-75.
- Hall, W. S., J. B. Patoczka, R. J. Mirenda, B. A. Porter and E. Miller (1989). Acute toxicity of industrial surfactants to *Mysidopsis bahia*. *Arch Environ Conta Toxicol.* 18, 765-772.
- Halling-Sørensen, B., S. Nors Nielsen, P. F. Lanzky, F. Ingerslev, H. C. Holten Lützhøft and S. E. Jørgensen (1998). Occurrence, fate and effects of pharmaceutical substances in the environment- A review. *Chemosphere.* 36, 357-393.
- Hata, T., H. Shintate, S. Kawai, H. Okamura and T. Nishida (2010a). Elimination of carbamazepine by repeated treatment with laccase in the presence of 1-hydroxybenzotriazole. *J. Hazard. Mater.*, 181, 1175-1178.
- Hata, T., S. Kawai, H. Okamura and T. Nishida (2010b). Removal of diclofenac and mefenamic acid by the white rot fungus *Phanerochaete sordida* YK-624 and identification of their metabolites after fungal transformation. *Biodegradation.* 21, 681-689.
- Hatakka, A. (1994). Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. *FEMS Microbi Rev.* 13, 125-135.
- Heinfling, A., M. J. Martínez, A. T. Martínez, M. Bergbauer and U. Szewzyk (1998). Transformation of industrial dyes by manganese peroxidases from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese -independent reaction. *Appl. Environ. Microbiol.*, 64, 2788-2793.

- Hernández-Leal, L., H. Temmink, G. Zeeman and C. J. N. Buisman (2011). Removal of micropollutants from aerobically treated grey water via ozone and activated carbon. *Water Res.* 45, 2887-2896.
- Hirai, H., S. Nakanishi and T. Nishida (2004). Oxidative dechlorination of methoxychlor by ligninolytic enzymes from white-rot fungi. *Chemosphere*. 55, 641-645.
- Hoeger, B., B. Köllner, D. R. Dietrich and B. Hitzfeld (2005). Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta f. fario*). *Aquat. Toxicol.*, 75, 53-64.
- Hofrichter, M. (2002). Review: lignin conversion by manganese peroxidase (MnP). *Enzyme Microb. Technol.*, 30, 454-466.
- Hofrichter, M., R. Ullrich, M. Pecyna, C. Liers and T. Lundell (2010). New and classic families of secreted fungal heme peroxidases. *Appl Microbiol Biotechnol.* 87, 871-897.
- Holbech, H., K. Kinnberg, G. I. Petersen, P. Jackson, K. Hylland, L. Norrgren and P. Bjerregaard (2006). Detection of endocrine disruptors: Evaluation of a fish sexual development test (FSDT). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, 144, 57-66.
- Hou, J., G. Dong, Y. Ye and V. Chen (2014). Enzymatic degradation of bisphenol-A with immobilized laccase on TiO₂ sol-gel coated PVDF membrane. *J. Membr. Sci.*, 469, 19-30.
- Huber, M. M., S. Canonica, G.-Y. Park and U. von Gunten (2003). Oxidation of pharmaceuticals during ozonation and advanced oxidation processes. *Environ Sci Technol.* 37, 1016-1024.
- Ishido, M., Y. Masuo, M. Terasaki and M. Morita (2011). Rat hyperactivity by bisphenol A, but not by its derivatives, 3-hydroxybisphenol A or bisphenol A 3,4-quinone. *Toxicol. Lett.*, 206, 300-305.
- Iso, T., T. Watanabe, T. Iwamoto, A. Shimamoto and Y. Furuichi (2006). DNA damage caused by bisphenol A and estradiol through estrogenic activity. *Biol. Pharm. Bull.*, 29, 206- 210.
- Jaser, W., G. F. Severin, U. Jütting, I. Jüttner, K.-W. Schramm and A. Kettrup (2003). Effects of 17[a]-ethinylestradiol on the reproduction of the cladoceran species *Ceriodaphnia reticulata* and *Sida crystallina*. *Environ. Int.*, 28, 633-638.
- Jelic, A., C. Cruz-Morató, E. Marco-Urrea, M. Sarra, S. Perez, T. Vicent, M. Petrović and D. Barcelo (2012). Degradation of carbamazepine by *Trametes versicolor* in an air pulsed fluidized bed bioreactor and identification of intermediates. *Water Res.* 46, 955-964.
- Jobling, S., M. Nolan, C. R. Tyler, G. Brighty and J. P. Sumpter (1998). Widespread sexual disruption in wild fish. *Environ Sci Technol.* 32, 2498-2506.
- Jochems, P., Y. Satyawali, L. Diels and W. Dejonghe (2011). Enzyme immobilization on/in polymeric membranes: Status, challenges and perspectives in biocatalytic membrane reactors (BMRs). *Green Chem.*, 13, 1609-1623.
- Johnson, A. C. and R. J. Williams (2004). A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works. *Environ Sci Technol.* 38, 3649-3658.
- Joss, A., S. Zabczynski, A. Gobel, B. Hoffmann, D. Löffler, C. S. McArdell, T. A. Ternes, A. Thomsen and H. Siegrist (2006). Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. *Water Res.* 40, 1686-1696.
- Kaiser, D., A. Sieratowicz, H. Zielke, M. Oetken, H. Hollert and J. Oehlmann (2012). Ecotoxicological effect characterisation of widely used organic UV filters. *Environ. Pollut.*, 163, 84-90.
- Kang, J. and W. E. Price (2009). Occurrence of phytoestrogens in municipal wastewater and surface waters. *J. Environ. Monit.*, 11, 1477-1483.

- Karam, J. and J. A. Nicell (1997). Potential applications of enzymes in waste treatment. *J Chem Technol Biotechnol.* 69, 141-153.
- Katuri, K. P., S. Venkata Mohan, S. Sridhar, B. R. Pati and P. N. Sarma (2009). Laccase-membrane reactors for decolorization of an acid azo dye in aqueous phase: Process optimization. *Water Res.* 43, 3647-3658.
- Khlifi-Slama, R., T. Mechichi, S. Sayadi and A. Dhouib (2012). Effect of natural mediators on the stability of *Trametes trogii* laccase during the decolourization of textile wastewaters. *The Journal of Microbiology.* 50, 226-234.
- Kim, I. and H. Tanaka (2009). Photodegradation characteristics of PPCPs in water with UV treatment. *Environ. Int.*, 35, 793-802.
- Kim, S. D., J. Cho, I. S. Kim, B. J. Vanderford and S. A. Snyder (2007). Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water Res.* 41, 1013-1021.
- Kim, Y.-J. and J. A. Nicell (2006). Laccase-catalysed oxidation of aqueous triclosan. *J Chem Technol Biotechnol.* 81, 1344-1352.
- Kimura, K., H. Hara and Y. Watanabe (2007). Elimination of selected acidic pharmaceuticals from municipal wastewater by an activated sludge system and membrane bioreactors. *Environ Sci Technol.* 41, 3708-3714.
- Klaus, K. (2009). The presence of pharmaceuticals in the environment due to human use - present knowledge and future challenges. *J. Environ. Manage.*, 90, 2354-2366.
- Klavarioti, M., D. Mantzavinos and D. Kassinos (2009). Removal of residual pharmaceuticals from aqueous systems by advanced oxidation processes. *Environ. Int.*, 35, 402-417.
- Köhler, C., S. Venditti, E. Igos, K. Klepiszewski, E. Benetto and A. Cornelissen (2012). Elimination of pharmaceutical residues in biologically pre-treated hospital wastewater using advanced UV irradiation technology: A comparative assessment. *J. Hazard. Mater.*, In press.
- Kolpin, D. W., E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber and H. T. Buxton (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999 - 2000: A national reconnaissance. *Environ Sci Technol.* 36, 1202-1211.
- Kumar, S. V. S., P. S. Phale, S. Durani and P. P. Wangikar (2003). Combined sequence and structure analysis of the fungal laccase family. *Biotechnol. Bioeng.*, 83, 386-394.
- Kurniawati, S. and J. A. Nicell (2007). Efficacy of mediators for enhancing the laccase-catalyzed oxidation of aqueous phenol. *Enzyme Microb. Technol.*, 41, 353-361.
- Laera, G., M. N. Chong, B. Jin and A. Lopez (2011). An integrated MBR-TiO₂ photocatalysis process for the removal of carbamazepine from simulated pharmaceutical industrial effluent. *Bioresource Technol.* 102, 7012-7015.
- Lante, A., A. Crapisi, A. Krastanov and P. Spettoli (2000). Biodegradation of phenols by laccase immobilised in a membrane reactor. *Process Biochem.* 36, 51-58.
- Le-Minh, N., S. J. Khan, J. E. Drewes and R. M. Stuetz (2010). Fate of antibiotics during municipal water recycling treatment processes. *Water Res.* 44, 4295-4323.
- Lee, H., Y. s. Choi, M. j. Kim, N. y. Huh, G. h. Kim, Y. W. Lim, S. m. Kang, S. t. Cho and J. j. Kim (2010). Degrading ability of oligocyclic aromates by *Phanerochaete sordida* selected via screening of white rot fungi. *Folia Microbiologica.* 55, 447-453.

- Li, X., F. I. Hai and L. D. Nghiem (2011). Simultaneous activated carbon adsorption within a membrane bioreactor for an enhanced micropollutant removal. *Bioresource Technol.* 102, 5319-5324.
- Libra, J. A., M. Borchert and S. Banit (2003). Competition strategies for the decolorization of a textile-reactive dye with the white-rot fungi *Trametes versicolor* under non-sterile conditions. *Biotechnol. Bioeng.*, 82, 736-744.
- Lindberg, R. H., Olofsson, U., Rendahl, P., Johansson, M.I., Tysklind, M., Andersson, B.A.V. (2006). Behavior of fluoroquinolones and trimethoprim during mechanical, chemical, and active sludge treatment of sewage water and digestion of sludge. *Environmental Science and Technology.* 40, 1042-1048.
- Liu, Z.-h., Y. Kanjo and S. Mizutani (2010). A review of phytoestrogens: Their occurrence and fate in the environment. *Water Res.* 44, 567-577.
- Lloret, L., G. Eibes, G. Feijoo, M. T. Moreira and J. M. Lema (2012a). Continuous biotransformation of estrogens by laccase in an enzymatic membrane reactor. *Chem. Eng. Trans.*, 27, 31-36.
- Lloret, L., G. Eibes, G. Feijoo, M. T. Moreira and J. M. Lema (2012b). Continuous operation of a fluidized bed reactor for the removal of estrogens by immobilized laccase on Eupergit supports. *J. Biotechnol.*, 162, 404-406.
- Lloret, L., G. Eibes, G. Feijoo, M. T. Moreira and J. M. Lema (2012c). Degradation of estrogens by laccase from *Myceliophthora thermophila* in fed-batch and enzymatic membrane reactors. *J. Hazard. Mater.*, 213–214, 175-183.
- Lloret, L., G. Eibes, T. A. Lú-Chau, M. T. Moreira, G. Feijoo and J. M. Lema (2010). Laccase-catalyzed degradation of anti-inflammatories and estrogens. *Biochem. Eng. J.*, 51, 124-131.
- Loos, R., B. M. Gawlik, G. Locoro, E. Rimaviciute, S. Contini and G. Bidoglio (2009). EU-wide survey of polar organic persistent pollutants in European river waters. *Environ. Pollut.*, 157, 561-568.
- Lopez, C., M. T. Moreira, G. Feijoo and J. M. Lema (2004). Dye decolorization by manganese peroxidase in an enzymatic membrane bioreactor. *Biotechnol. Prog.* 20, 74-81.
- Lyssimachou, A. and A. Arukwe (2007). Alteration of brain and interrenal StAR protein, P450scc, and Cyp11b, mRNA levels in *Atlantic Salmon* after nominal waterborne exposure to the synthetic pharmaceutical estrogen ethynylestradiol. *J Toxicol Environ Health.* 70, 606 - 613.
- Maldonado, M. I., S. Malato, L. A. Pérez-Estrada, W. Gernjak, I. Oller, X. Doménech and J. Peral (2006). Partial degradation of five pesticides and an industrial pollutant by ozonation in a pilot-plant scale reactor. *J. Hazard. Mater.*, 138, 363-369.
- Marco-Urrea, E., M. Pérez-Trujillo, P. Blánquez, T. Vicent and G. Caminal (2010a). Biodegradation of the analgesic naproxen by *Trametes versicolor* and identification of intermediates using HPLC-DAD-MS and NMR. *Bioresource Technology.* 101, 2159-2166.
- Marco-Urrea, E., M. Pérez-Trujillo, C. Cruz-Morató, G. Caminal and T. Vicent (2010b). Degradation of the drug sodium diclofenac by *Trametes versicolor* pellets and identification of some intermediates by NMR. *J. Hazard. Mater.*, 176, 836-842.
- Marco-Urrea, E., M. Pérez-Trujillo, C. Cruz-Morató, G. Caminal and T. Vicent (2010c). White-rot fungus-mediated degradation of the analgesic ketoprofen and identification of intermediates by HPLC–DAD–MS and NMR. *Chemosphere.* 78, 474-481.

- Marco-Urrea, E., M. Perez-Trujillo, T. Vicent and G. Caminal (2009). Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. *Chemosphere*. 74, 765-772.
- Martins, A. F., A. F. Martins, C. A. Mallmann, D. R. Arsand and F. M. Mayer (2011). Occurrence of the antimicrobials sulfamethoxazole and trimethoprim in hospital effluent and study of their degradation products after electrocoagulation. *CLEAN – Soil, Air, Water*. 39, 21-27.
- Matilainen, A., M. Vepsäläinen and M. Sillanpää (2010). Natural organic matter removal by coagulation during drinking water treatment: A review. *Advances in Colloid and Interface Science*. 159, 189-197.
- Méndez-Hernández, J. E., G. Eibes, A. Arca-Ramos, T. A. Lú-Chau, G. Feijoo, M. T. Moreira and J. M. Lema (2015). Continuous Removal of Nonylphenol by Versatile Peroxidase in a Two-Stage Membrane Bioreactor. *Appl Biochem Biotechnol*. 175, 3038-3047.
- Mendoza, L., M. Jonstrup, R. Hatti-Kaul and B. Mattiasson (2011). Azo dye decolorization by a laccase/mediator system in a membrane reactor: Enzyme and mediator reusability. *Enzyme and Microbial Technology*. 49, 478-484.
- Mizuno, H., H. Hirai, S. Kawai and T. Nishida (2009a). Removal of estrogenic activity of isobutylparaben and butylparaben by laccase in the presence of 1-hydroxybenzotriazole. *Biodegradation*. 20, 533-539.
- Mizuno, H., H. Hirai, S. Kawai and T. Nishida (2009b). Removal of estrogenic activity of isobutylparaben and n-butylparaben by laccase in the presence of 1-hydroxybenzotriazole. *Biodegradation*. 20, 533-539.
- Mompelat, S., B. Le Bot and O. Thomas (2009). Occurrence and fate of pharmaceutical products and by-products, from resource to drinking water. *Environment International*. 35, 803-814.
- Mori, T., K. Fujie and A. Katayama (1998). Bacterial and fungal contributions to chlorothalonil degradation in soil. *Soil Science and Plant Nutrition*. 44, 297-304.
- Murugesan, K., Y.-Y. Chang, Y.-M. Kim, J.-R. Jeon, E.-J. Kim and Y.-S. Chang (2010). Enhanced transformation of triclosan by laccase in the presence of redox mediators. *Water Res*. 44, 298-308.
- Murugesan, K., Y.-M. Kim, J.-R. Jeon and Y.-S. Chang (2009). Effect of metal ions on reactive dye decolorization by laccase from *Ganoderma lucidum*. *J. Hazard. Mater.*, 168, 523-529.
- Nghiem, L. D., A. I. Schafer and M. Elimelech (2004a). Removal of natural Hormones by nanofiltration membranes: measurement, modeling, and mechanisms. *Environ Sci Technol*. 38, 1888-1896.
- Nghiem, L. D., A. I. Schafer and M. Elimelech (2004b). Removal of natural hormones by nanofiltration membranes: measurement, modeling, and mechanisms. *Environ. Sci. Technol*. 38, 1888-1896.
- Nghiem, L. D., N. Tadkaew and M. Sivakumar (2009). Removal of trace organic contaminants by submerged membrane bioreactors. *Desalination*. 236, 127-134.
- Nguyen, L. N., F. I. Hai, J. Kang, F. D. L. Leusch, F. Roddick, S. F. Magram, W. E. Price and L. D. Nghiem (2014a). Enhancement of trace organic contaminant degradation by crude enzyme extract from *Trametes versicolor* culture: Effect of mediator type and concentration. *Journal of the Taiwan Institute of Chemical Engineers*. 45, 1855-1862.
- Nguyen, L. N., F. I. Hai, J. Kang, F. D. L. Leusch, F. Roddick, S. F. Magram, W. E. Price and L. D. Nghiem (2014b). Enhancement of trace organic contaminant degradation by crude

- enzyme extract from *Trametes versicolor* culture: Effect of mediator type and concentration. J. Taiwan Inst. Chem. Eng., 45, 1855-1862.
- Nguyen, L. N., F. I. Hai, J. Kang, L. D. Nghiem, W. E. Price, W. Guo, H. H. Ngo and K.-L. Tung (2013a). Comparison between sequential and simultaneous application of activated carbon with membrane bioreactor for trace organic contaminant removal. Bioresource Technol. 130, 412-417.
- Nguyen, L. N., F. I. Hai, J. Kang, W. E. Price and L. D. Nghiem (2012a). Removal of trace organic contaminants by a membrane bioreactor–granular activated carbon (MBR–GAC) system. Bioresource Technology. 113, 169-173.
- Nguyen, L. N., F. I. Hai, J. Kang, W. E. Price and L. D. Nghiem (2012b). Removal of trace organic contaminants by a membrane bioreactor - granular activated carbon (MBR-GAC) system. Bioresource Technol. 113, 169-173.
- Nguyen, L. N., F. I. Hai, J. Kang, W. E. Price and L. D. Nghiem (2013b). Removal of emerging trace organic contaminants by MBR-based hybrid treatment processes. Int. Biodeterior. Biodegradation. 85, 474-482.
- Nguyen, L. N., F. I. Hai, W. E. Price, J. Kang, F. D. L. Leusch, F. Roddick, J. P. van de Merwe, S. F. Magram and L. D. Nghiem (2015). Degradation of a broad spectrum of trace organic contaminants by an enzymatic membrane reactor: Complementary role of membrane retention and enzymatic degradation. Int. Biodeterior. Biodegradation. 99, 115-122.
- Nguyen, L. N., F. I. Hai, W. E. Price, F. D. L. Leusch, F. Roddick, E. J. McAdam, S. F. Magram and L. D. Nghiem (2014c). Continuous biotransformation of bisphenol A and diclofenac by laccase in an enzymatic membrane reactor. Int. Biodeterior. Biodegradation. 95, Part A, 25-32.
- Nguyen, L. N., F. I. Hai, W. E. Price, F. D. L. Leusch, F. Roddick, H. H. Ngo, W. Guo, S. F. Magram and L. D. Nghiem (2014d). The effects of mediator and granular activated carbon addition on degradation of trace organic contaminants by an enzymatic membrane reactor. Bioresource Technology. 167, 169-177.
- Nicolucci, C., S. Rossi, C. Menale, T. Godjevargova, Y. Ivanov, M. Bianco, L. Mita, U. Bencivenga, D. Mita and N. Diano (2011). Biodegradation of bisphenols with immobilized laccase or tyrosinase on polyacrylonitrile beads. Biodegradation. 22, 673-683.
- Novotný, Č., P. Erbanová, T. Cajthaml, N. Rothschild, C. Dosoretz and V. Šašek (2000). Irpex lacteus: A white-rot fungus applicable to water and soil bioremediation. Appl Microbiol Biotechnol. 54, 850-853.
- Oppenheimer, J., R. Stephenson, A. Burbano and L. Liu (2007). Characterizing the passage of personal care products through wastewater treatment processes. Water Environ Res. 79, 2564-2577.
- Packer, J. L., J. J. Werner, D. E. Latch, K. McNeill and W. A. Arnold (2003). Photochemical fate of pharmaceuticals in the environment: Naproxen, diclofenac, clofibric acid, and ibuprofen. Aquatic Science. 65, 342-351.
- Pal, A., K. Y.-H. Gin, A. Y.-C. Lin and M. Reinhard (2010). Impacts of emerging organic contaminants on freshwater resources: Review of recent occurrences, sources, fate and effects. Sci. Total Environ., 408, 6062-6069.

- Palma, C., A. T. Martínez, J. M. Lema and M. J. Martínez (2000). Different fungal manganese-oxidizing peroxidases: a comparison between *Bjerkandera sp.* and *Phanerochaete chrysosporium*. *J. Biotechnol.*, 77, 235-245.
- Peralta-Zamora, P., C. M. Pereira, E. R. L. Tiburtius, S. G. Moraes, M. A. Rosa, R. C. Minussi and N. Durán (2003). Decolorization of reactive dyes by immobilized laccase. *Appl Catal B*. 42, 131-144.
- Poiger, T., H.-R. Buser, M. E. Balmer, P.-A. Bergqvist and M. D. Müller (2004). Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. *Chemosphere*. 55, 951-963.
- Radjenovic, J., M. Petrovic and D. Barceló (2007a). Analysis of pharmaceuticals in wastewater and removal using a membrane bioreactor. *Analytical and Bioanalytical Chemistry*. 387, 1365-1377.
- Radjenovic, J., M. Petrovic and D. Barceló (2007b). Analysis of pharmaceuticals in wastewater and removal using a membrane bioreactor *J Anal Bioanly Chem*. 387, 1365-1377
- Reif, R., S. Suairez, F. Omil and J. M. Lema (2008). Fate of pharmaceuticals and cosmetic ingredients during the operation of a MBR treating sewage. *Desalination*. 221, 511-517.
- Rios, G. M., M. P. Belleville, D. Paolucci and J. Sanchez (2004). Progress in enzymatic membrane reactors – a review. *J. Membr. Sci.*, 242, 189-196.
- Riva, S. (2006). Laccases: blue enzymes for green chemistry. *Trends Biotechnol.*, 24, 219-226.
- Rodarte-Morales, A., G. Feijoo, M. Moreira and J. Lema (2011). Degradation of selected pharmaceutical and personal care products (PPCPs) by white-rot fungi. *World J. Microbiol. Biotechnol.*, 27, 1839-1846.
- Rodarte-Morales, A. I., G. Feijoo, M. T. Moreira and J. M. Lema (2012a). Biotransformation of three pharmaceutical active compounds by the fungus *Phanerochaete chrysosporium* in a fed batch stirred reactor under air and oxygen supply. *Biodegradation*. 23, 145-156.
- Rodarte-Morales, A. I., G. Feijoo, M. T. Moreira and J. M. Lema (2012b). Evaluation of two operational regimes: Fed-batch and continuous for the removal of pharmaceuticals in a fungal stirred tank reactor. *Chem. Eng. Trans.*, 27, 151-156.
- Rodgers-Gray, T. P., S. Jobling, S. Morris, C. Kelly, S. Kirby, A. Janbakhsh, J. E. Harries, M. J. Waldoock, J. P. Sumpter and C. R. Tyler (2000). Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. *Environ Sci Technol*. 34, 1521-1528.
- Rodríguez-Rodríguez, C. E., A. Jelić, M. Llorca, M. Farré, G. Caminal, M. Petrović, D. Barceló and T. Vicent (2011). Solid-phase treatment with the fungus *Trametes versicolor* substantially reduces pharmaceutical concentrations and toxicity from sewage sludge. *Bioresource Technol*. 102, 5602-5608.
- Rønhede, S., B. Jensen, S. Rosendahl, B. B. Kragelund, R. K. Juhler and J. Aamand (2005). Hydroxylation of the Herbicide Isoproturon by Fungi Isolated from Agricultural Soil. *Appl. Environ. Microbiol.*, 71, 7927-7932.
- Ruiz-Dueñas, F. J., M. Morales, E. García, Y. Miki, M. J. Martínez and A. T. Martínez (2009). Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. *J. Exp. Bot.*, 60, 441-452.
- Saito, T., K. Kato, Y. Yokogawa, M. Nishida and N. Yamashita (2004). Detoxification of bisphenol A and nonylphenol by purified extracellular laccase from a fungus isolated from soil. *J. Biosci. Bioeng.*, 98, 64-66.

- Schwaiger, J., H. Ferling, U. Mallow, H. Wintermayr and R. D. Negele (2004). Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout. *Aquat. Toxicol.*, 68, 141-150.
- Schwarzenbach, R. P., B. I. Escher, K. Fenner, T. B. Hofstetter, C. A. Johnson, U. von Gunten and B. Wehrli (2006). The Challenge of Micropollutants in Aquatic Systems. *Science*, 313, 1072-1077.
- Scott-Fordsmand, J. J. and P. H. Krogh (2004). The influence of application form on the toxicity of nonylphenol to *Folsomia fimetaria* (Collembola: Isotomidae). *Ecotoxicol Environ Safety*, 58, 294-299.
- Snyder, S. A., S. Adham, A. M. Redding, F. S. Cannon, J. DeCarolis, J. Oppenheimer, E. C. Wert and Y. Yoon (2007). Role of membranes and activated carbon in the removal of endocrine disruptors and pharmaceuticals. *Desalination*, 202, 156-181.
- Soares, A., K. Jonasson, E. Terrazas, B. Guieysse and B. Mattiasson (2005). The ability of white-rot fungi to degrade the endocrine-disrupting compound nonylphenol. *Appl Microbiol Biotechnol*, 66, 719-725.
- Soderstrom, J., L. Murray, M. Little and F. F. F. S. Daly. (2006). Toxicology case of the month: carbamazepine overdose. *Emerg. Med. J.*, 23, 869-871.
- Song, Y., S. B. Subramanian, R. D. Tyagi, Y. S. Rao and C. Z. Tian (2010). Emerging contaminants of environmental concern: source, transport, fate and treatment. *J Haza Toxic and Radioa Waste Mang*, 14, 2-20.
- Suda, T., T. Hata, S. Kawai, H. Okamura and T. Nishida (2012). Treatment of tetracycline antibiotics by laccase in the presence of 1-hydroxybenzotriazole. *Bioresource Technol*, 103, 498-501.
- Tadkaew, N., F. I. Hai, J. A. McDonald, S. J. Khan and L. D. Nghiem (2011). Removal of trace organics by MBR treatment: The role of molecular properties. *Water Research*, 45, 2439-2451.
- Tamagawa, Y., H. Hirai, S. Kawai and T. Nishida (2005). Removal of estrogenic activity of endocrine-disrupting genistein by ligninolytic enzymes from white rot fungi. *FEMS Microbiol. Lett.*, 244, 93-98.
- Tamagawa, Y., H. Hirai, S. Kawai and T. Nishida (2007). Removal of estrogenic activity of 4-tert-octylphenol by ligninolytic enzymes from white rot fungi. *Environ. Toxicol.*, 22, 281-286.
- Ternes, T. A., M. Meisenheimer, D. McDowell, F. Sacher, H.-J. Brauch, B. Haist-Gulde, G. Preuss, U. Wilme and N. Zulei-Seibert (2002a). Removal of pharmaceuticals during drinking water treatment. *Environ Sci Technol*, 36, 3855-3863.
- Ternes, T. A., M. Meisenheimer, D. McDowell, F. Sacher, H.-J. r. Brauch, B. Haist-Gulde, G. Preuss, U. Wilme and N. Zulei-Seibert (2002b). Removal of pharmaceuticals during drinking water treatment. *Environ. Sci. Technol*, 36, 3855-3863.
- Ternes, T. A., J. Stüber, N. Herrmann, D. McDowell, A. Ried, M. Kampmann and B. Teiser (2003). Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? *Water Res*, 37, 1976-1982.
- Thoma, K. and N. Kübler (1997). Photostability of antifungal agents. Part 2: Photostability of polyene antibiotics. *Pharmazie*, 52, 294-302.
- Torres-Duarte, C., R. Roman, R. Tinoco and R. Vazquez-Duhalt (2009). Halogenated pesticide transformation by a laccase–mediator system. *Chemosphere*, 77, 687-692.

- Tran, N. H., T. Urase and O. Kusakabe (2009). The characteristics of enriched nitrifier culture in the degradation of selected pharmaceutically active compounds. *Journal of Hazardous Materials*. 171, 1051-1057.
- Tran, N. H., T. Urase and O. Kusakabe (2010). Biodegradation characteristics of pharmaceutical substances by whole fungal culture *Trametes versicolor* and its laccase. *J Water Environ Technol*. 8, 125-140.
- Tran, N. H., T. Urase, H. H. Ngo, J. Hu and S. L. Ong (2013b). Insight into metabolic and cometabolic activities of autotrophic and heterotrophic microorganisms in the biodegradation of emerging trace organic contaminants. *Bioresour. Technol*. 146, 721-731.
- Tran, N. H., T. Urase and T. T. Ta (2014). A Preliminary Study on the Occurrence of Pharmaceutically Active Compounds in Hospital Wastewater and Surface Water in Hanoi, Vietnam. *CLEAN – Soil, Air, Water*. 42, 267-275.
- Ullah, M. A., C. T. Bedford and C. S. Evans (2000). Reactions of pentachlorophenol with laccase from *Coriolus versicolor*. *Appl Microbiol Biotechnol*. 53, 230-234.
- Urase, T., C. Kagawa and T. Kikuta (2005). Factors affecting removal of pharmaceutical substances and estrogens in membrane separation bioreactors. *Desalination*. 178, 107-113.
- Valentín, L., G. Feijoo, M. T. Moreira and J. M. Lema (2006). Biodegradation of polycyclic aromatic hydrocarbons in forest and salt marsh soils by white-rot fungi. *Int. Biodeterior. Biodegradation*. 58, 15-21.
- Veldhoen, N., R. C. Skirrow, H. Osachoff, H. Wigmore, D. J. Clapson, M. P. Gunderson, G. Van Aggelen and C. C. Helbing (2006). The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. *Aquat. Toxicol.*, 80, 217-227.
- Vieno, N., T. Tuhkanen and L. Kronberg (2006). Removal of pharmaceuticals in drinking water treatment: effect of chemical coagulation. *Environ. Technol.*, 27, 183-192.
- Visvanathan, C., L. N. Thu, V. Jegatheesan and J. Anotai (2005). Biodegradation of pentachlorophenol in a membrane bioreactor. *Desalination*. 183, 455-464.
- Wang, J., N. Majima, H. Hirai and H. Kawagishi (2012). Effective removal of endocrine-disrupting compounds by lignin peroxidase from the white-rot fungus *Phanerochaete sordida* YK-624. *Curr. Microbiol.*, 64, 300-303.
- Wen, X., Y. Jia and J. Li (2010). Enzymatic degradation of tetracycline and oxytetracycline by crude manganese peroxidase prepared from *Phanerochaete chrysosporium*. *J. Hazard. Mater.*, 177, 924-928.
- Weng, S.-S., K.-L. Ku and H.-T. Lai (2012). The implication of mediators for enhancement of laccase oxidation of sulfonamide antibiotics. *Bioresource Technol*. 113, 259-264.
- Westerhoff, P., Y. Yoon, S. Snyder and E. Wert (2005). Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. *Environ Sci Technol*. 39, 6649-6663.
- Wilson, C. and C. Tisdell (2001). Why farmers continue to use pesticides despite environmental, health and sustainability costs. *Ecological Economics*. 39, 449-462.
- Wu, C. and K. G. Linden (2008). Degradation and byproduct formation of parathion in aqueous solutions by UV and UV/H₂O₂ treatment. *Water Res*. 42, 4780-4790.
- Xie, M., L. D. Nghiem, W. E. Price and M. Elimelech (2012). Comparison of the removal of hydrophobic trace organic contaminants by forward osmosis and reverse osmosis. *Water Res*. 46, 2683-2692.

- Xu, F. (1997). Effects of Redox Potential and Hydroxide Inhibition on the pH Activity Profile of Fungal Laccases. *J Bio Chem.* 272, 924-928.
- Xu, F., J. J. Kulys, K. Duke, K. Li, K. Krikstopaitis, H.-J. W. Deussen, E. Abbate, V. Galinyte and P. Schneider (2000). Redox Chemistry in Laccase-Catalyzed Oxidation of N-Hydroxy Compounds. *Appl. Environ. Microbiol.*, 66, 2052-2056.
- Yang, S., F. I. Hai, L. D. Nghiem, L. N. Nguyen, F. Roddick and W. E. Price (2013a). Removal of bisphenol A and diclofenac by a novel fungal membrane bioreactor operated under non-sterile conditions. *International Biodeterioration & Biodegradation*.
- Yang, S., F. I. Hai, L. D. Nghiem, L. N. Nguyen, F. Roddick and W. E. Price (2013b). Removal of bisphenol A and diclofenac by a novel fungal membrane bioreactor operated under non-sterile conditions. *Int. Biodeterior. Biodegradation*. Accepted.
- Yang, S., F. I. Hai, L. D. Nghiem, W. E. Price, F. Roddick, M. T. Moreira and S. F. Magram (2013c). Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: A critical review. *Bioresource Technol.*
- Yoon, Y., P. Westerhoff, S. A. Snyder and M. Esparza (2003). HPLC-fluorescence detection and adsorption of bisphenol A, 17 β -estradiol, and 17 α -ethynyl estradiol on powdered activated carbon. *Water Res.* 37, 3530-3537.
- Yuan, F., C. Hu, X. Hu, J. Qu and M. Yang (2009). Degradation of selected pharmaceuticals in aqueous solution with UV and UV/H₂O₂. *Water Res.* 43, 1766-1774.
- Zhang, F. and J. Yu (2000). Decolourisation of acid violet 7 with complex pellets of white rot fungus and activated carbon. *Bioprocess Engineering*. 23, 295-301.
- Zhang, J., X. Liu, Z. Xu, H. Chen and Y. Yang (2008). Degradation of chlorophenols catalyzed by laccase. *Int. Biodeterior. Biodegradation*. 61, 351-356.
- Zhang, Y. and S.-U. Geißen (2012). Elimination of carbamazepine in a non-sterile fungal bioreactor. *Bioresource Technol.* 112, 221-227.
- Zhang, Y. and S.-U. Geissen (2010). In vitro degradation of carbamazepine and diclofenac by crude lignin peroxidase. *J. Hazard. Mater.*, 176, 1089-1092.

CHAPTER 3: Trace organic contaminant removal by whole-cell and extracellular enzyme of *Trametes versicolor*

The chapter has been published as:

Luong N Nguyen, Faisal I Hai, Shufan Yang, Jinguo Kang, Frederic DL Leusch, Felicity Roddick, William E Price, Long D Nghiem. (2014) Removal of pharmaceuticals, steroid hormones, phytoestrogens, UV-filters, industrial chemicals and pesticides by *Trametes versicolor*: Role of biosorption and biodegradation. *International Biodeterioration & Biodegradation*, 88, 169-175

Luong N Nguyen, Faisal I Hai, Jinguo Kang, Frederic DL Leusch, Felicity Roddick, Saleh F Magram, William E Price, Long D Nghiem. (2014) Enhancement of trace organic contaminant degradation by crude enzyme extract from *Trametes versicolor* culture: Effect of mediator type and concentration. *Journal of the Taiwan Institute of Chemical Engineers*. 45, 1855-1862

3.1. INTRODUCTION

Trace organic contaminants (TrOCs) consist of both naturally occurring and anthropogenic compounds and are frequently detected in sewage-impacted water bodies. These include pharmaceuticals, steroid hormones, pesticides and industrial chemicals. The resistance of certain TrOCs to conventional wastewater treatment and their potential adverse effects on human and ecological health raise significant concerns (Snyder 2008). This has prompted research on TrOC degradation by white-rot fungi (Yang et al., 2013a).

White-rot fungi (WRF) can efficiently degrade a wide range of organic compounds including TrOCs that are resistant to bacterial degradation via one or more extracellular enzymes including lignin peroxidases, manganese-dependent peroxidases and laccase. Apart from the extracellular enzymes, intracellular enzyme systems, such as cytochrome P450, have been reported to play important roles in the removal of some TrOCs (Hata et al., 2010). In addition to whole-cell preparations, removal of TrOCs has been investigated either by employing crude culture extract ('crude enzyme') or by purified enzymes (Marco-Urrea et al., 2009; Zhang et al., 2010). Enzyme-catalysis has been shown to be enhanced by the addition of low molecular weight mediator compounds that act as an 'electron shuttle' between the oxidizing enzyme and target compounds (Kim et al., 2006).

Several studies, as noted above, have investigated the TrOC removal capacity by different fungal species. The removal efficiency has been observed to depend on fungal species, their specific enzyme systems and the TrOC characteristics. However, with a few exceptions (Tran et al., 2010; Rodarte-Morales et al., 2011), individual studies to date have focused mostly on a single or only a few TrOCs at a time. This has hindered the establishment of a uniform database on white-rot fungal degradation of a broad spectrum of TrOCs, particularly because of the differences in experimental conditions in individual studies. Furthermore, some TrOC categories such as UV filters (active ingredients of sunscreens) and phytoestrogens have not been investigated by the previous studies.

Enzymatic degradation of a range of TrOCs has been investigated previously (Nguyen et al., 2013). However, because of the synergistic effect of intracellular, mycelium-bound and extracellular enzymes as well as sorption of TrOCs on the biomass, whole-cell white-rot fungal

treatment may cover a wider spectrum of TrOCs compared with enzymatic treatment. A few studies have separately compared the performance of whole-cell and extracellular extract (Auriol et al., 2007; Tran et al., 2010) or have studied the role of intracellular enzyme systems (Marco-Urrea et al., 2009; Hata et al., 2010). However, systematic studies on the relative contribution of biosorption and various modes of biodegradation (*e.g.*, extracellular enzyme dependent/independent) during fungal removal of TrOCs remain scarce. Elucidation of such mechanisms is a prerequisite to formulation of strategies to ensure stable operation of continuous flow fungal reactors (Yang et al., 2013a).

A series of batch tests was conducted in this study to address the research gaps outlined above. The removal of 30 TrOCs belonging to diverse groups in terms of applications or origins and chemical structures by a whole-cell pure fungus culture (*Trametes versicolor* (*T. versicolor*)) and the fungal extracellular enzyme extract has been systematically compared. In discussing the removal efficiency, the relative contribution of biosorption and biodegradation is addressed, as well as potential catalytic modes (extracellular enzyme dependent/independent) responsible for fungal degradation of TrOCs. The effect of augmenting enzymatic transformation with a redox mediator on the removal performance and the treated media toxicity was also studied.

3.2. MATERIALS AND METHODS

3.2.1 Trace organic contaminants and mediators

A mixture of 30 TrOCs, including 11 pharmaceuticals, six pesticides, five steroid hormones, three industrial precursors and products, three UV filters and two phytoestrogens was used in this study. Key properties of these compounds are listed in (**Appendix Table A-1**). These were selected in view of their widespread occurrence in wastewater and wastewater-impacted water bodies and their diverse physicochemical properties (*e.g.* hydrophobicity and molecular weight). Analytical grade chemicals were purchased from Sigma-Aldrich (Australia). A mixed stock solution of the TrOCs was prepared at a concentration of 1 g/L (each) in pure methanol, stored at -18°C and used within one month.

Two redox mediators, namely 1-hydroxybenzotriazole (HBT) and syringaldehyde (SA), were used in this study. HBT is a =N-OH type mediator, while SA is a phenolic mediators (**Appendix**

Table A-2). The oxidation of these mediators by laccase generates highly reactive aminoxyl ($=N-O^{\cdot}$) and phenoxyl ($C_6H_5O^{\cdot}$) radicals, respectively, both of which follow the HAT pathway during substrate (TrOCs) degradation (d'Acunzo et al., 2006). These mediators were also purchased from Sigma-Aldrich (Australia). The mediators were prepared as a stock solution at a concentration of 50 mM and stored at 4 °C.

3.2.2 Fungus culture and preparation of crude enzyme extract

The white-rot fungus *T. versicolor* ATCC 7731 (USA) was used in this study. Malt extract broth (Merck, Germany) was used as both the growth and the test medium at concentrations of 5 g/L and 1 g/L, respectively. The pH of the growth medium as well as the batch test medium was adjusted to 4.5 with 4 M HCl. Refrigerated stock fungal suspension was grown on a rotary shaker at 28 °C in sterilized growth medium for a week (Yang et al., 2013a). Freshly grown fungal pellets and the supernatant of the fungal culture ('crude enzyme extract') were then harvested and subsequently used for whole-cell and crude enzyme batch tests.

3.2.3 Batch test protocol

Batch tests were conducted under three broad categories to assess: i) removal by live and 'chemically inactivated' (biosorption only) whole-cell preparations; ii) performance of the live whole-cell in the presence of an intracellular cytochrome P450 inhibitor; and iii) degradation by the crude enzyme extract with/without addition of a redox-mediator. During the whole-cell test, sterile test medium (50 mL), in 400 mL sterile beakers, was inoculated with approximately 0.4 g/L freshly grown fungus culture. TrOCs were added to the test solution at final concentrations of 50 µg/L (each). Separate beakers were incubated to assess the removal by sodium azide (0.1 mM)-inactivated fungus culture (Yang et al., 2011). The intracellular enzyme inhibition test followed a similar procedure by adding 1-aminobenzotriazole (1mM) (Hata et al., 2010) to inactivate intracellular cytochrome P450 enzyme systems. The containers were incubated in the same fashion mentioned above. All experiments were conducted in triplicate. At the end of the incubation period, the whole test medium was harvested. The samples were diluted to 500 mL with deionised (Milli-Q) water and filtered through 0.45 µm glass fibre filter (Filtech, Australia). The pH of the sample was adjusted to 2 by using 4 M H₂SO₄ before refrigerating until the solid-

phase extraction (for GC/MS analysis, see **Section 3.2.4.1**) within 2 d. For toxicity testing, undiluted samples were kept at 4 °C until analysis.

In the enzymatic degradation test, the stock TrOC solution was added to 25 mL crude enzyme extract in 100 mL beakers to give a final concentration of 100 µg/L of each TrOC. The crude enzyme extract demonstrated a laccase activity of 35 µM_(DMP) /min.

To investigate the effect of mediator addition, each mediator was added separately to obtain solutions with final mediator concentration of 0.1, 0.5 and 1 mM. Control samples comprised TrOCs in deionised (Milli-Q) water. The beakers were covered with aluminium foil and incubated on a rotary shaker at 70 rpm and 25 °C for 24 h. The experiments were conducted in triplicate and a sampling method similar to that in the tests with whole-cell preparations was adopted.

3.2.4 Analytical methods

3.2.4.1 TrOC analysis

The TrOCs in feed and permeate samples were extracted using 6 mL 200 mg Oasis hydrophilic-lipophilic-balanced (HLB) cartridges (Waters, Milford, MA, USA). The cartridges were pre-conditioned with 7 mL dichloromethane and methanol (1:1, v/v), 7 mL methanol, and 7 mL reagent water, respectively. The feed and permeate samples (500 mL each) were adjusted with H₂SO₄ 0.4 M to pH 2–3, then loaded onto the cartridges at a flow rate of 15 mL/min, after which the cartridges were rinsed with 20 mL Milli-Q water and dried with a stream of nitrogen for 30 min (see **Figure 3.1**). The TrOCs were eluted from the cartridges with 7 mL methanol followed by 7 mL dichloromethane and methanol (1:1, v/v) at a flow rate of 1–5 mL/min, and the eluents were evaporated to dryness under a gentle stream of nitrogen in a water bath at 40 °C. The extracted residues were then dissolved with 200 µL methanol solution which contained 5 µg bisphenol A-d₁₆ and transferred to 1.5 mL vials, and further evaporated to dryness under a gentle nitrogen stream. Finally, the dry residues in the vials were derivatized by addition of 100 µL of BSTFA (1% TMCS) plus 100 µL of pyridine (dried with KOH solid), which were then heated in a heating block at 60 – 70°C for 30 min. The derivatives were cooled to room temperature and subjected to GC-MS analysis (Liu et al., 2004). The SPE recovery of TrOCs was above 80% in all analysis.

The TrOCs were analysed using a Shimadzu GCMS-QP5000 system, equipped with a Shimadzu AOC 20i autosampler. A Phenomenex Zebron ZB-5 (5% diphenyl – 95% dimethylpolysiloxane) capillary column (30 m \times 0.25 mm ID, d_f = 0.25 μ m) was used. Helium carrier gas was maintained at a constant flow rate of 1.3 mL/min. The GC column temperature was programmed from 100 °C (initial equilibrium time 1 min) to 175°C via a ramp of 10 °C/min and maintained 3 min, 175 – 210°C via a ramp of 30°C, 210 – 228°C via a ramp of 2 °C/min, 228 – 260°C via a ramp of 30 °C, 260 – 290°C via a ramp of 3 °C/min and maintained 3 min. The injector port and the interface temperature were maintained at 280 °C. Sample injection (1 μ L) was in splitless mode.

For qualitative analysis, the MS full-scan mode from m/z , 50 – 600 was used, apart from the mass spectrum, the relative retention times of each compound was used for confirmation of the compound. Quantitative analysis was carried out using selected ion monitoring (SIM) mode. For each compound, the most abundant characteristic ions were selected for quantification. The selected ions of the analysed compounds after silyl derivatization are in agreement with those reported elsewhere (Gatidou et al., 2007; Zhang et al., 2007).

Standard solutions of the analytes were prepared at 1, 10, 50, 100, 500 and 1000 ng/mL, and an internal instrument calibration was carried out with bisphenol A- d_{16} as internal standard. The calibration curves for all the analytes had a correlation coefficient of 0.99 or better. Detection limits were defined as the concentration of an analyte giving a signal to noise (s/n) ratio greater than 3, where, ‘noise’ is the fluctuation in the instrument background signal and generally measured as the standard deviation of the background signal and, analyte ‘signal’ is the change in instrument response to the presence of a target chemical. The limit of reporting was determined using an s/n ratio of greater than 10.

Statistical analysis of data was performed using the Student *t*-test function. Values of $p < 0.05$ were considered to indicate statistical significance.

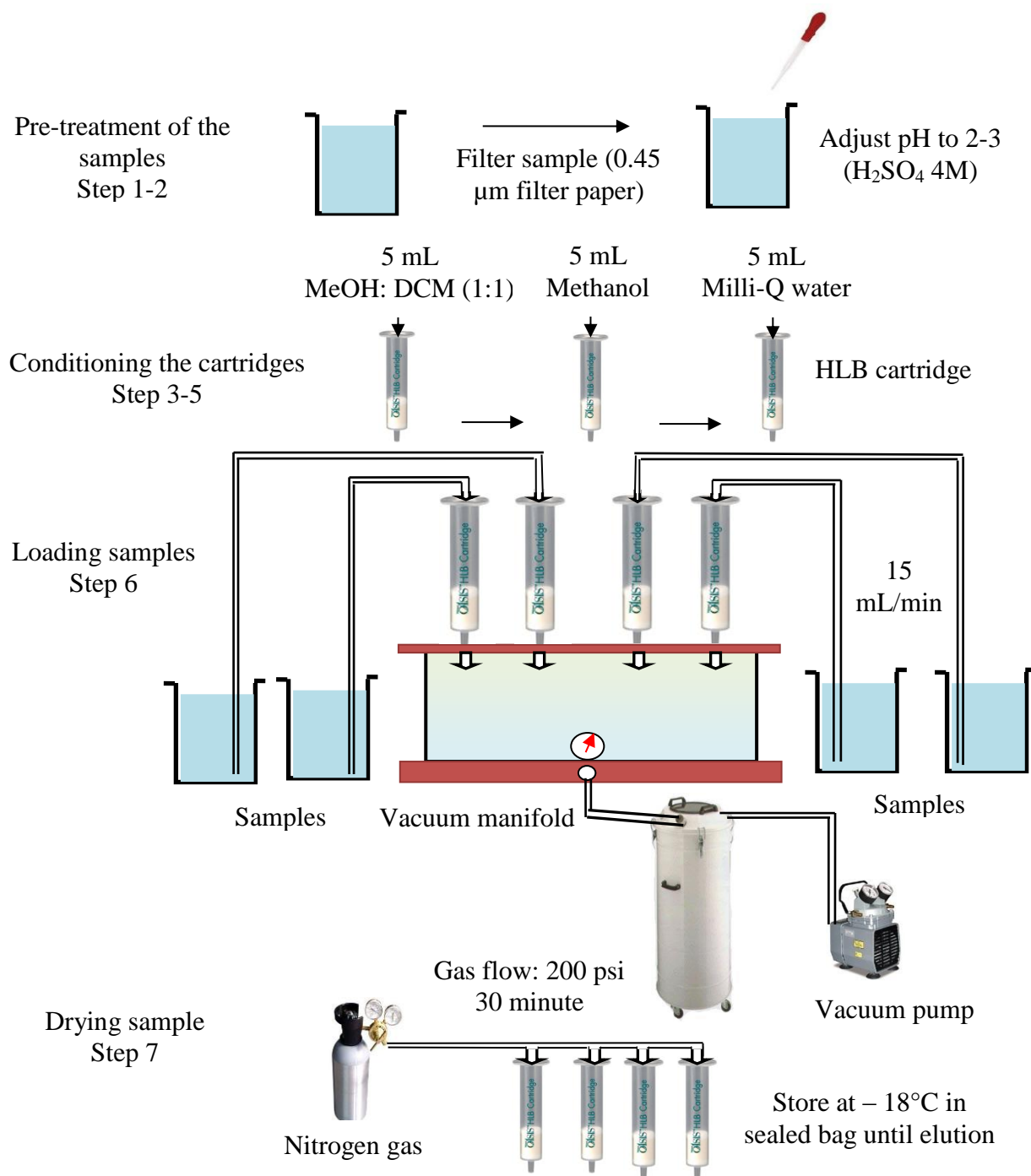


Figure 3.1: Schematic of sample preparation for GC-MS measurement of TrOCs (MeOH - methanol, DCM – dichloromethane, SPE – solid phase extraction, HLB -Hydrophilic-lipophilic-balanced)

3.2.4.2 Enzymatic activity and toxicity assay

Under the experimental conditions used, the fungus predominantly secreted the extracellular enzyme laccase. Laccase activity was measured by monitoring the change in absorbance at 468 nm due to the oxidation of 2,6-dimethoxy phenol (DMP) at room temperature over 2 min using a spectrophotometer (PharmaSpec UV-1700, Shimadzu, Kyoto, Japan). Laccase activity was calculated from the molar extinction coefficient $\epsilon = 49.6$ (mM/cm) and expressed in μM substrate/min. The toxicity of the treated solution was tested in duplicate by measuring bioluminescence inhibition in *Photobacterium leiognathi* (ToxScreen3 assay, CheckLight Ltd, Israel) as described previously (Nguyen et al., 2013). Toxicity was expressed as Relative Toxic Unit (rTU) calculated as $\text{rTU} = 1/\text{IC}_{20}$, with IC_{20} (concentration of the sample required to kill 20% of the bacteria) determined by linear regression of the toxicity response vs. relative sample enrichment within the linear range ($\text{IC}_{00} - \text{IC}_{40}$).

3.3. RESULTS AND DISCUSSION

3.3.1 Removal performance of live and chemically inactivated whole-cell

Biodegradation, biosorption, photodegradation and volatilization are the possible removal mechanisms of TrOCs. Photodegradation of TrOCs was minimized by covering the test containers (**Section 3.2.3**), while the volatilization of the selected compounds was deemed negligible given their low vapour pressure (**Appendix Table A-1**). Therefore, biodegradation and biosorption were considered as potential removal mechanisms under the tested conditions.

Hydrophobic interactions play a very important role in sorption of TrOCs to biomass. According to a definition widely accepted in the literature, an organic compound is hydrophobic when its pH-dependent effective octanol water partitioning coefficient (measured by $\log D$) is 3 or higher (Hai et al., 2013). In this study, for almost all significantly hydrophobic compounds ($\log D \geq 4$) similar levels ($> 50\%$) of removal were achieved by the live and sodium azide-inactivated (biosorption only) cultures (**Figure 3.2**). In particular, over 80% removal of 17 β -estradiol 17-acetate, pentachlorophenol, 4-*tert*-octylphenol and triclosan was achieved by the live and inactivated cultures. No previous study of the removal of 17 β -estradiol 17-acetate by whole-cell white-rot fungi could be found. However, the results in the current study are in good agreement with previous studies reporting the significant removal of 4-*tert*-octylphenol (Tamagawa et al.,

2007), triclosan (Cajthaml et al., 2009) and pentachlorophenol (Ryu et al., 2000). Because a similar removal was attained of these significantly hydrophobic TrOCs by both live and inactivated cultures, the governing mode of removal (biodegradation or biosorption) could not be ascertained from their aqueous phase concentration change. Nevertheless further insight into this aspect is provided in the following section while comparing the performance of whole-cell and crude enzyme extract.

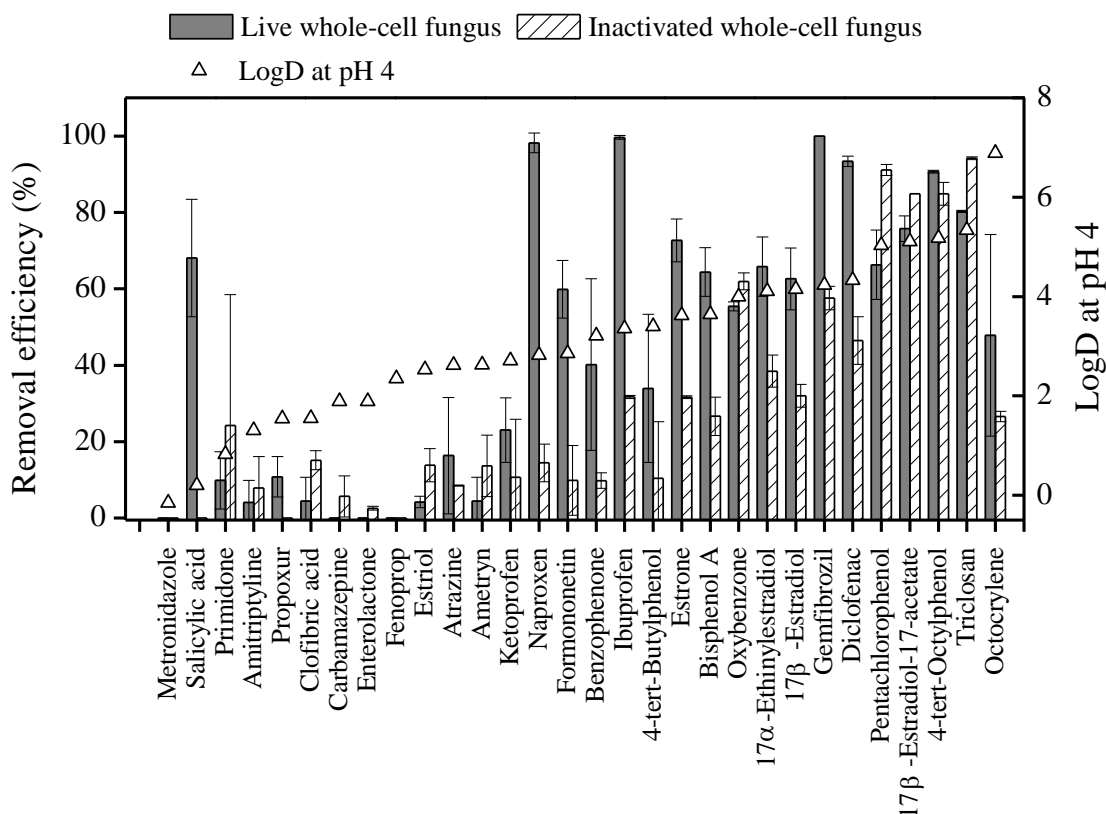


Figure 3.2: Removal of 30 TrOCs from aqueous phase after 24 h of incubation of live and inactivated white-rot fungus in malt extract broth. The initial pH, fungus concentration and the concentration of each of the TrOCs were 4.5, 0.4 g/L and 50 μ g/L, respectively. Error bars indicate average \pm standard deviation (n=3). Fungus was inactivated by adding 0.1 mM sodium azide to the test solution.

Of particular interest was the high (60-99%) removal of five hydrophobic ($\log D=3-4$) compounds, namely, naproxen, formononetin, ibuprofen, estrone and bisphenol A by the live culture, but less removal by the inactivated culture ($p < 0.05$, **Appendix Table A-3**). This observation suggests that biodegradation (and not biosorption) was the main mechanism of removal of these compounds. No previous reports regarding the removal of formononetin by white-rot fungi could be identified. However, previous studies have revealed high removal of naproxen (Marco-Urrea et al., 2010; Rodarte-Morales et al., 2010; Tran et al., 2010; Rodarte-Morales et al., 2011), ibuprofen (Marco-Urrea et al., 2009), estrone (Tamagawa et al., 2005) and bisphenol A (Cajthaml et al., 2009; Yang et al., 2013c) by whole-cell white-rot fungi treatment. The high removal of these TrOCs is also in good agreement with a TrOC classification proposed by Yang et al. (2013a) based on their degradation by white-rot fungi.

As expected, 12 hydrophilic compounds possessing a $\log D < 3$ showed negligible removal by the inactivated culture, confirming their negligible biosorption (**Figure 3.2**). However, their removal by the live culture was also generally low or negligible, indicating their resistance to degradation by the *T. versicolor* strain used in this study. These hydrophilic TrOCs include some highly resistant compounds (showing low or variable removal) reported in the literature (Yang et al., 2013a). For instance, carbamazepine has been shown to be resistant to fungal treatment by whole-cell culture (Marco-Urrea et al., 2009) and enzymatic treatment (Marco-Urrea et al., 2009; Zhang et al., 2010; Eibes et al., 2011), although certain species were able to degrade significantly (Rodarte-Morales et al., 2010; Rodarte-Morales et al., 2011). Marco-Urrea et al. (2009) observed 97% removal of clofibric acid by a different strain of *T. versicolor* than that used in this study, however, they reported lower removal (0-24%) by *Irpex lacteus* and *Phanerochaete chrysosporium*. Atrazine was found to be resistant to treatment by *Dichotomitus squalens*, *Phanerochaete velutina*, and *Hypholoma fasciculare* (Bending et al., 2002); however, a different strain of *T. versicolor* was shown to achieve a better removal (Bending et al., 2002). There is further discussion on the behavior of the resistant TrOCs in regards to their molecular structures in the next section.

3.3.2 Comparison of removal by whole-cell and crude enzyme extract

3.3.2.1 Removal mode

Highly hydrophobic compounds may be removed solely due to biosorption. However, when the performance of live/inactivated whole-cell culture (**Figure 3.2**) and that of crude enzyme extract (**Figure 3.3**) is taken into consideration, it can be confirmed that most of the very hydrophobic TrOCs ($\log D > 3$) in this study were in fact biodegraded. Performance comparison between whole-cell and crude enzyme extract also helped identify the TrOCs for which low or negligible removal by the latter was observed ($p < 0.05$, **Appendix Table A-3**). These include: three very hydrophobic TrOCs (oxybenzone, gemfibrozil, and pentachlorophenol), two moderately hydrophobic TrOCs (formononetin and ibuprofen), and two significantly hydrophilic TrOCs (salicylic acid and naproxen). Better performance of the whole-cell culture for these compounds indicated three possibilities: i) biosorption in fact was the main mechanism (*i.e.*, resistant to biodegradation); ii) intracellular cytochrome P450 enzyme system was significantly involved in degradation by the whole-cell; or iii) mycelium-associated enzyme played a significant role in biodegradation.

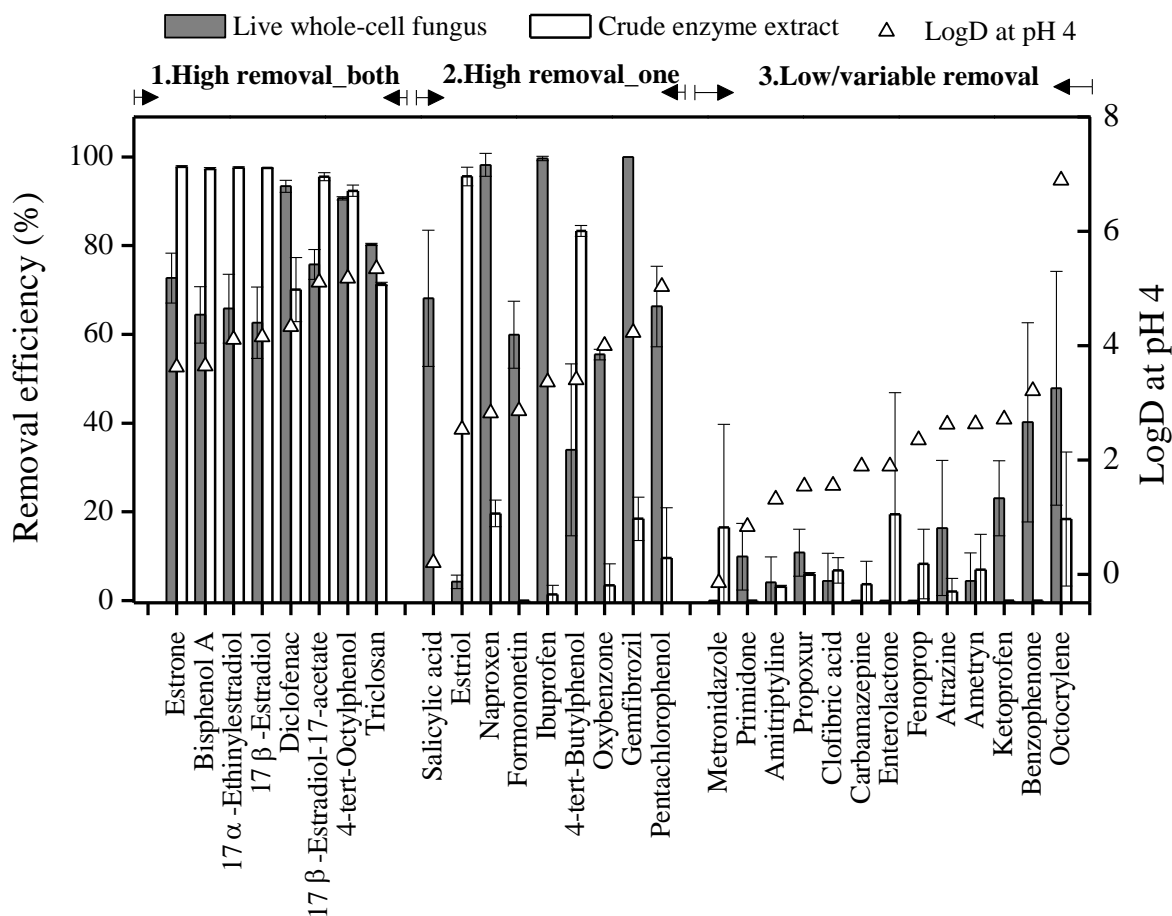


Figure 3.3: Removal of 30 TrOCs from aqueous phase after 24 h of incubation of live fungus and crude enzyme extract. Live fungus was incubated in malt extract broth (1 g/L), and the initial pH, fungus concentration and the concentration of each of the TrOCs were 4.5, 0.4 g/L and 50 μ g/L, respectively. The initial laccase activity of the crude enzyme extract was 35 μ M_(DMP)/ min. TrOCs were added to the crude enzyme extract at a concentration of 100 μ g/L. Error bars indicate average \pm standard deviation (n=3).

The role of intracellular cytochrome P450 was assessed by comparing the removal by whole-cell culture in the presence and absence of 1-aminobenzotriazole (a cytochrome P450 inhibitor). The cytochrome P450 inhibitor was found to have a negligible effect on the degradation of all except seven TrOCs, namely, salicylic acid, ibuprofen, gemfibrozil, naproxen, bisphenol A, diclofenac and 17 β -estradiol (**Figure 3.4**). The role of intracellular enzymes in the fungal degradation of salicylic acid, bisphenol A and 17 β -estradiol has not been studied before. However, the data in

the current study is consistent with that from other studies reporting the negative impact of cytochrome P450 inhibition on the degradation of naproxen (Marco-Urrea et al., 2010), ibuprofen (Marco-Urrea et al., 2009), gemfibrozil (Tran et al., 2010) and diclofenac (Hata et al., 2010). Previous reports have also demonstrated the important role of mycelium-associated enzymatic activity in the degradation of resistant compounds such as dye (Svobodova et al., 2008) and TrOC. Considering the data in **Figure 3.2**, **Figure 3.3** and **Figure 3.4** collectively, in this study, the reason for lower removal by the crude enzyme extract compared to that by the whole-cell culture can be considered compound-specific. For salicylic acid, naproxen, formononetin, ibuprofen and gemfibrozil, the role of cytochrome P450 and/or mycelium-associated enzymes was critical, while for oxybenzone and pentachlorophenol the role of biosorption was significant in their removal. Further clarification on this aspect is presented in **Section 3.3.3**. The TrOC adsorption capacity of crude enzyme has not been tested in this study. It is recommended that the potential adsorption of TrOCs on the protein molecules or other biopolymers in the inactivated crude enzyme is investigated in future studies.

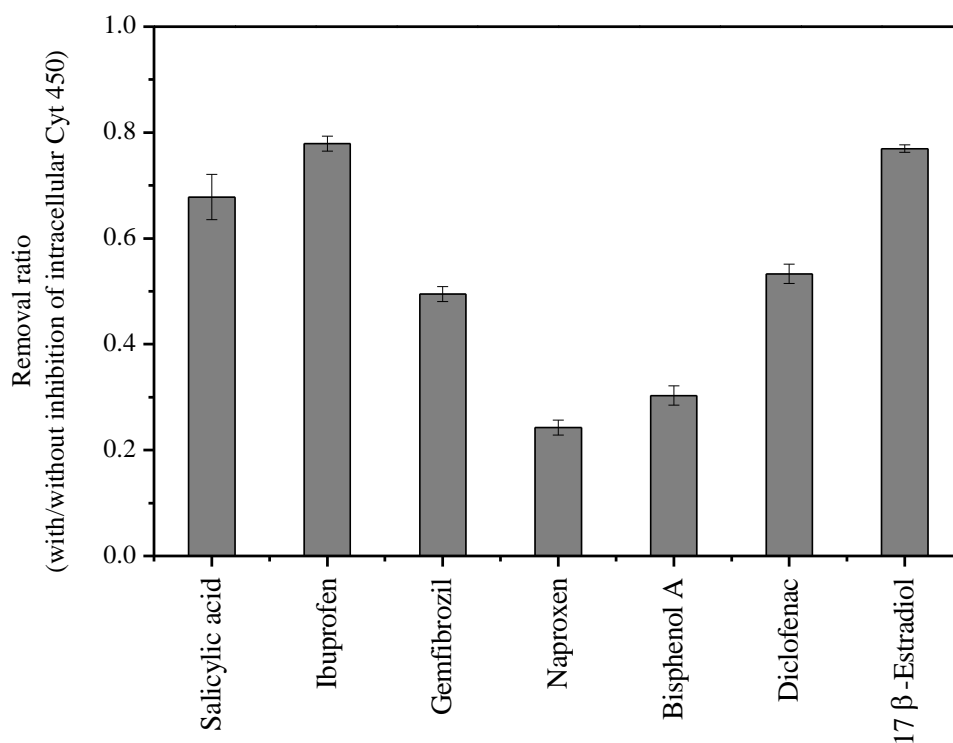


Figure 3.4: Removal of seven affected TrOCs in presence and absence of an intracellular cytochrome P450 inhibitor (1-aminobenzotriazole) at a concentration of 1mM. Error bars indicate average \pm standard deviation (n=3).

3.3.2.2 Impact of enzymatic activity

It is noted that similar removal by whole-cell and crude enzyme extract was obtained for bisphenol A and diclofenac (**Figure 3.3**) although a significant (but not exclusive) role of intracellular enzymes in the degradation of these TrOCs was evident (**Figure 3.4**). Furthermore, for two hydrophilic compounds, namely, estriol and 4-*tert*-butylphenol, the extracellular enzyme extract achieved better and/or more stable removal. This was despite the fact that a higher initial concentration was applied during the test with crude enzyme extract (100 $\mu\text{g/L}$ of each compound, compared to 50 $\mu\text{g/L}$ of each in the whole-cell test). This may be attributed to the fact that the laccase activity in the whole-cell culture gradually increased from zero to approximately 20 $\mu\text{M}_{(\text{DMP})}/\text{min}$ within 24 h, while that in the extracellular extract remained at 35 $\mu\text{M}_{(\text{DMP})}/\text{min}$ from the start of the test (**Figure 3.5**). Auriol et al. (2007) previously observed the removal of synthetic hormones including estriol to depend on the level of laccase activity in the test media. In that study, estriol was completely removed by a laccase solution exhibiting an activity of 20 U/ mL, while the removal corresponding to a laccase activity of 5 U/mL was 88%. Tran et al. (2010) also reported increased removal of pharmaceuticals such as naproxen (from 30 to 100%), ibuprofen (from 10 to 40%) and ketoprofen (from 25 to 50%) with the increase of laccase activity from 2 to 6 U/mL. The potential effect of level of laccase activity on the removal of the above TrOCs in this study is also indicative of their relatively resistant structure against fungal degradation.

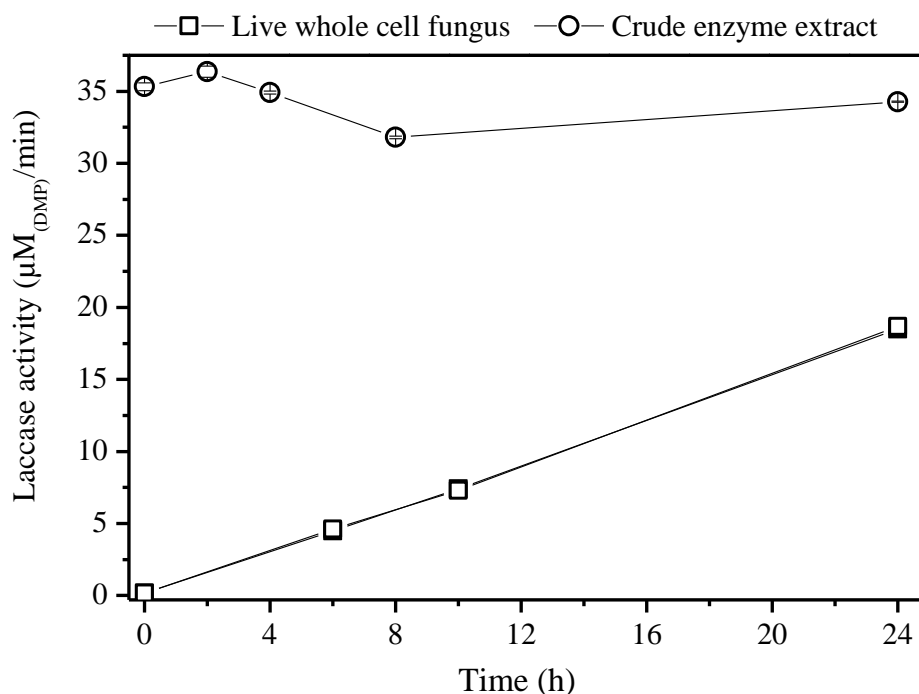


Figure 3.5: Level of laccase activity in the media during the test with the whole-cell culture and the crude enzyme extract. Error bars indicate average \pm standard deviation ($n=3$).

3.3.2.3 Resistant TrOC

Under the tested conditions, little or no (<30%) removal of 15 TrOCs, namely, metronidazole, primidone, amitriptyline, propoxur, clofibric acid, carbamazepine, enterolactone, fenoprop, atrazine, ametryn, ketoprofen, benzophenone and octocrylene (Category 3, **Figure 3.3**) and estriol and 4-*tert*-butylphenol (Category 2, **Figure 3.3**) was achieved by the whole-cell culture. The crude enzyme extract could substantially degrade estriol and 4-*tert*-butylphenol but little or no degradation of an additional six TrOCs including salicylic acid, naproxen, formononetin, ibuprofen, gemfibrozil and pentachlorophenol was obtained (**Figure 3.3**). This section particularly discusses the case of 13 TrOCs for which treatment by neither whole-cell nor crude enzyme preparations was found effective (Category 3, **Figure 3.3**). It is noteworthy that the removal of only a few of these 13 TrOCs (*e.g.*, clofibric acid, carbamazepine, atrazine and ketoprofen) by whole-cell culture has been studied before; however, the existing literature confirms the resistance of these TrOCs to fungal degradation (Yang et al., 2013a).

The resistance of the aforementioned TrOCs can be attributed to their chemical structures. The presence of electron withdrawing groups (EWG) generates an electron deficiency and thus renders the compounds less susceptible to oxidative catabolism (Tadkaew et al., 2011). The low removal of the chlorinated TrOCs clofibric acid and fenoprop can be explained by the fact that chlorine is a strong EWG. Treatment by laccase from *T. versicolor* was reported to inefficiently remove clofibric acid (Tran et al., 2010). Atrazine and ametryn are two triazine compounds. Atrazine additionally contains a chloride group. Despite having the weak electron donating groups (EDG) methyl and secondary amine, atrazine and ametryn were poorly removed. This is consistent with the report of Mougin et al. (1997) who found that triazine pesticides are resistant to fungal treatment. The insignificant removal of metronidazole, propoxur and carbamazepine can be attributed to the presence of strong EWG nitro, carbamate and amide groups, respectively in their structures. The presence of these groups has been reported to cause higher resistance to biodegradation (Tadkaew et al., 2011; Yang et al., 2013a). In a previous study, at an initial concentration of 10 µg/L, complete removal of ketoprofen was reported by the whole-cell of a different strain of *T. versicolor*, while only 12% removal was achieved by the extracellular extract (Tran et al., 2010). Notably, ketoprofen contains a carboxylic acid group which is strongly electron withdrawing. Its degradation pathway may be limited to being via hydroxylation of the vicinal unsubstituted aromatic fragment and the mono-carbon-substituted benzenoid (Quintana et al., 2005), and this pathway may have been unavailable to the *T. versicolor* strain utilized in this study.

3.3.3 Effect of mediator addition on the removal by crude enzyme extract

3.3.3.1 Overall improvement in TrOC degradation

As noted in **Section 3.3.2**, of the 30 TrOCs tested, the crude enzyme extract could attain significant degradation of only ten compounds. Two mediators were added to the crude enzyme extract to test if any improvement in the remaining TrOC removal could be achieved.

In a laccase-mediator system, the role of the enzyme is to oxidize the mediator, while the actual oxidation of the substrate takes place in a subsequent non-enzymatic step by the action of the oxidized mediator species. Therefore, the reaction mechanisms by which the radicals generated from the laccase-mediator system oxidize a substrate are important (Fabbrini et al., 2002; Astolfi

et al., 2005). The oxidation of HBT and SA by laccase generates highly reactive aminoxyl ($=N-O^*$) and phenoxyl ($C_6H_5O^*$) radicals, respectively. The aminoxyl radical oxidizes the target substrate by the HAT mechanism (Xu et al., 2000; Fabbrini et al., 2002). Therefore, the enthalpic balance between the dissociated bond (C-H) in the target substrate and the forming bond (NO-H) in the mediator is the driving force of this mechanism (d'Acunzo et al., 2003; Cañas et al., 2010). It has been reported that phenoxyl radicals act analogously to aminoxyl radicals (Cañas et al., 2010). The HAT mechanism has been implicated in the oxidation of resistant compounds such as non-phenolic compounds (Fabbrini et al., 2002; Astolfi et al., 2005). For instance, Bernini et al. (2011) reported that the laccase-mediator system can effectively trigger H-abstraction from benzylic carbons of non-phenolic compounds.

Figure 3.6 shows the effect of adding the mediators separately (1 mM) to the crude enzyme extract (containing predominantly laccase) on the removal of the selected TrOCs. Mediator addition extended the spectrum of efficiently degraded TrOCs to 13 phenolic and three non-phenolic compounds (*i.e.*, atrazine, naproxen and diclofenac), with moderate improvements in the removal of a few other non-phenolics. For example, the degradation rate of atrazine increased from negligible to over 50 ng/L.h. Similarly the degradation rate of naproxen improved from 20 to over 80 ng/L.h, while the degradation rate of diclofenac, which was already well removed without mediator addition, improved by only around 10 ng/L.h (**Appendix Table A-4**)

The superior performance of the mediator-amended crude enzyme extract may be explained by two factors attributable to the aminoxyl and phenoxyl radical species generated from HBT and SA by laccase, respectively: i) higher redox potential, and ii) reduction of steric hindrance. The oxidation of even some phenolic compounds by laccase may be hindered by solubility or steric issues. Low molecular weight mediators can interact with complex compounds that cannot directly access the enzyme active site. Moreover, compounds with high redox potential can be oxidized by radical mediators (*e.g.*, aminoxyl and phenoxyl) through the operation of H-abstraction mechanism. The laccase-mediator system can achieve better removal of compounds with high redox potential as the oxidized mediator species have redox potentials higher than that of laccase only (Klonowska et al., 2002). **Figure 3.7** indeed demonstrates that in our study, the redox potential of the crude enzyme extract increased significantly due to the addition of the mediators. The redox potential of the crude enzyme extract with SA and HBT was 540 and 441

mV, respectively, which were significantly higher than that for the crude enzyme extract, SA solution and HBT solution (207, 249 and 251 mV, respectively) (**Figure 3.6**). Our results are consistent with those of Weng et al. (2012) who reported that the redox potential of a range of mediators including SA was higher than that of laccase or the mediator separately, and thus higher degradation of sulfonamide antibiotics was achieved.

The factors and mechanisms discussed in this section may explain the significantly improved degradation of the phenolic TrOCs (*i.e.*, formononetin, salicylic acid, oxybenzone, pentachlorophenol and enterolactone) and the non-phenolic TrOCs (*i.e.*, primidone, atrazine, amitriptyline, octocrylene, naproxen, and diclofenac) in our study in the presence of mediators (**Figure 3.7**). Aminoxyl radicals generated from oxidation of HBT by laccase from the fungus *T. villosa* were previously shown to carry abstract hydrogen from certain amide substrates (Coniglio et al., 2008). However, the lack of improvement in the removal of the amide compound carbamazepine observed in this study is probably due to the difference in redox potential of laccase from different *Trametes* species as well as the specific structure of the compounds.

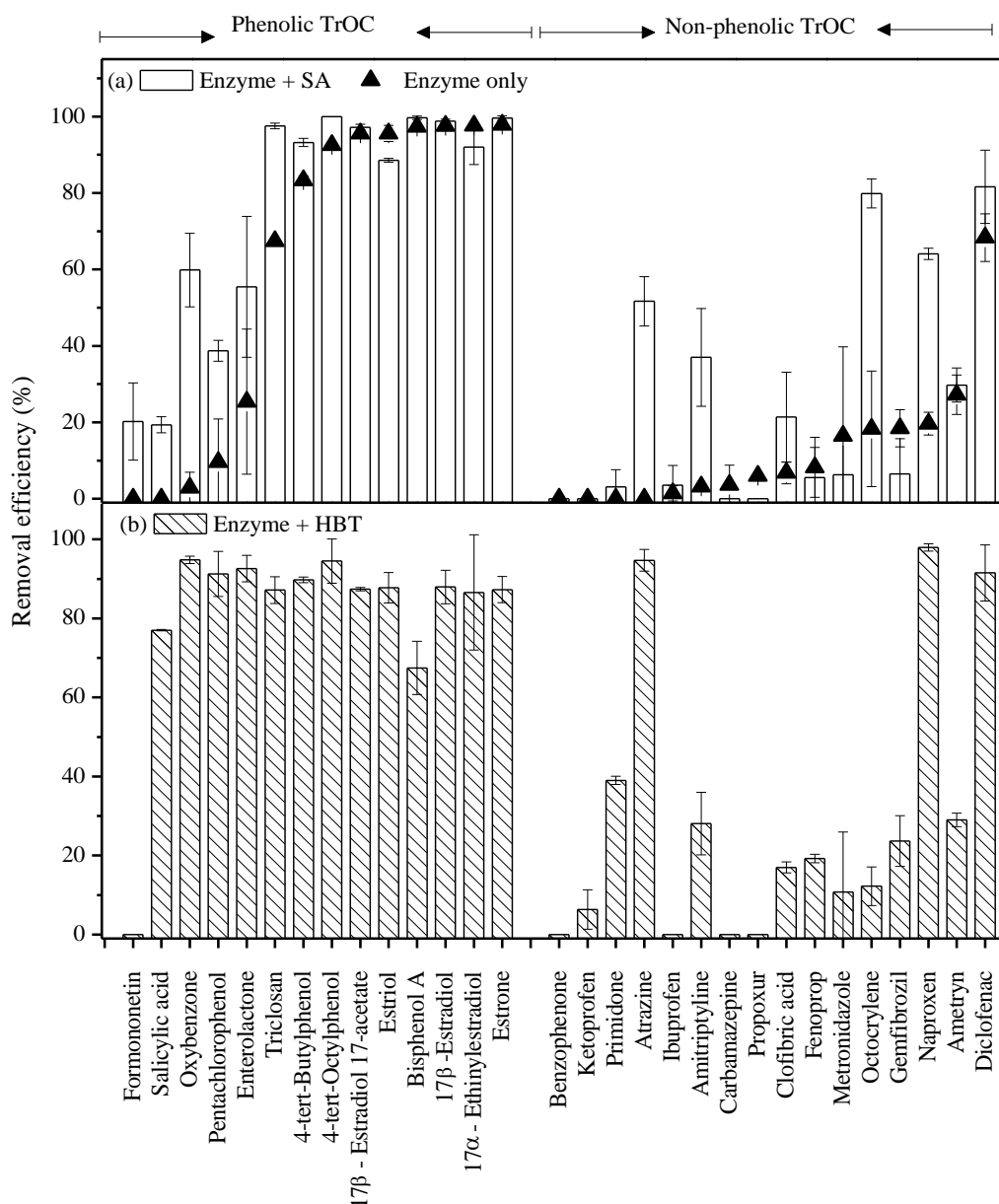


Figure 3.6: Removal of 30 TrOCs by crude enzyme extract from *T. versicolor* (ATCC 7731) in the presence of two mediators (SA and HBT) at a concentration of 1 mM. The error bars represent the standard deviation of three replicates.

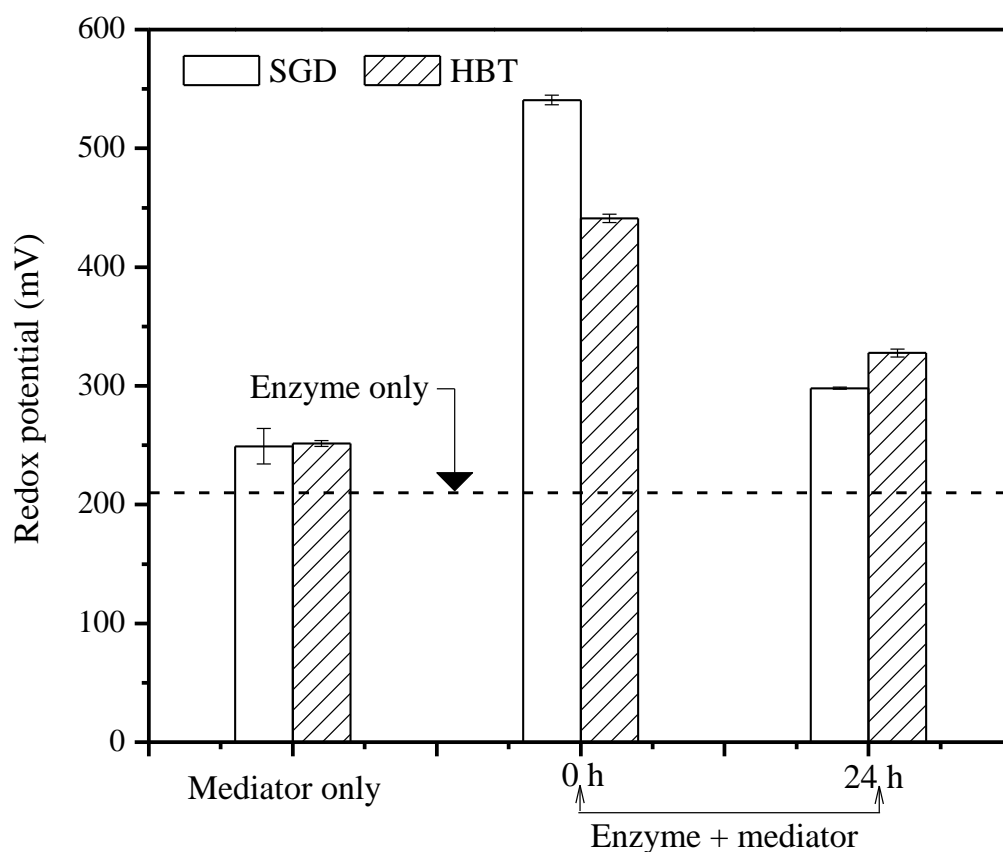


Figure 3.7: Redox potential of enzyme, mediator and mediator-amended enzyme solutions at a mediator concentration of 1 mM. The error bars represent the standard deviation of three replicates.

3.3.3.2 Mediator performance

In this study, HBT facilitated better removal than SA for four phenolic compounds, namely salicylic acid, oxybenzone, pentachlorophenol and enterolactone, and three non-phenolic compounds, namely primidone, atrazine, and naproxen (**Figure 3.6**). By contrast, SA led to better removal of the phenolic compound formononetin and the non-phenolic compound octocrylene, while similar removal of the non-phenolic compound amitriptyline was achieved by the mediators. No relevant data could be retrieved from the literature for a direct comparison with our data. However, in general, there appears to be no clear consensus on the comparative performance of SA and HBT. For example, Khlifi-Slama et al. (2010) reported that a laccase-

HBT system achieved the best decolorization of a textile industry effluent among the nine mediators tested including SA. On the other hand, Camarero et al. (2005) reported better degradation using SA than HBT for a range of phenolic and non-phenolic dyes possessing strong EWG.

The performance of the laccase-mediator systems depends on the electrochemical potential of the radical generated, redox reversibility of the reaction of the radical with the substrate, as well as on the balance between the stability and reactivity of the mediator radical (Camarero et al., 2005). As noted in **Section 3.3.3.1**, both SA and HBT act via the HAT mechanism. In addition, in this study, the redox potential values of the crude enzyme solution amended with the mediators separately were not significantly different (**Figure 3.7**). Therefore, the difference in the performance of the two mediators is due to the relatively higher stability of aminoxyl radicals compared to phenoxyl radicals. In general, both radicals (phenoxyl and aminoxyl) have short half-lives due to their poor thermostability. For example, aminoxyl radicals have a half-life of 120 s (Coniglio et al., 2009), and convert rapidly to benzotriazole and other inactive compounds. Phenoxyl radicals (from SA) have often been reported to rapidly turn into quinone, which is much less active (Xu et al., 2000). Nonetheless, Xu et al. (2000) hypothesizes that the half-life of aminoxyl radicals is long enough to allow diffusion to the substrate. In our study, this is further evidenced by the fact that at the end of the incubation period, the redox potential of the SA-amended solution demonstrated a 45% reduction from the initial value as compared to a 25% reduction in case of the HBT-amended solution (**Figure 3.7**). This may explain the better performance of HBT observed in our study.

3.3.3.3 Effect of mediator concentration on TrOC removal and enzymatic stability

The abundance and stability of the oxidized intermediates from the mediators affect TrOC degradation. Mediator concentration can influence both the abundance and stability of oxidized intermediates. Here, the effect of three different concentrations (0.1, 0.5, and 1 mM) of the mediators (SA and HBT) on TrOC removal is discussed focusing on the five phenolic and five non-phenolic compounds for which the mediators produced significant improvement in removal. The complete data set is presented in (**Appendix Figure A-1 and 2**).

In this study, TrOC degradation improved by up to 60 and 95% using SA and HBT, respectively (**Figure 3.8**). The redox potential of the crude enzyme extract was not significantly different between different dosages of SA and HBT (**Figure 3.9**). In accordance with that, approximately similar removal efficiencies were achieved for all three tested concentrations of SA (**Figure 3.8**). On the other hand, the removals on addition of HBT at 0.5 and 1 mM were similar but significantly better than that at 0.1 mM. These results are in line with the general trend observed in the literature that pollutant removal profiles may reach a plateau beyond a certain mediator concentration, and that such threshold concentrations depend on the source of laccase, the target compound and the mediator used. For example, Mizuno et al. (2009) found a gradual increase in iso-butylparaben and n-butylparaben removal due to addition of HBT to laccase (from *T. versicolor*) over a concentration range of 0.2 to 2 mM, beyond which no further improvement was observed. By contrast, Lloret et al. (2010) obtained diclofenac removal in a range from 40 to 80% for the SA concentrations ranging from 0.1 to 0.5 mM, while complete removal was achieved at 1 mM.

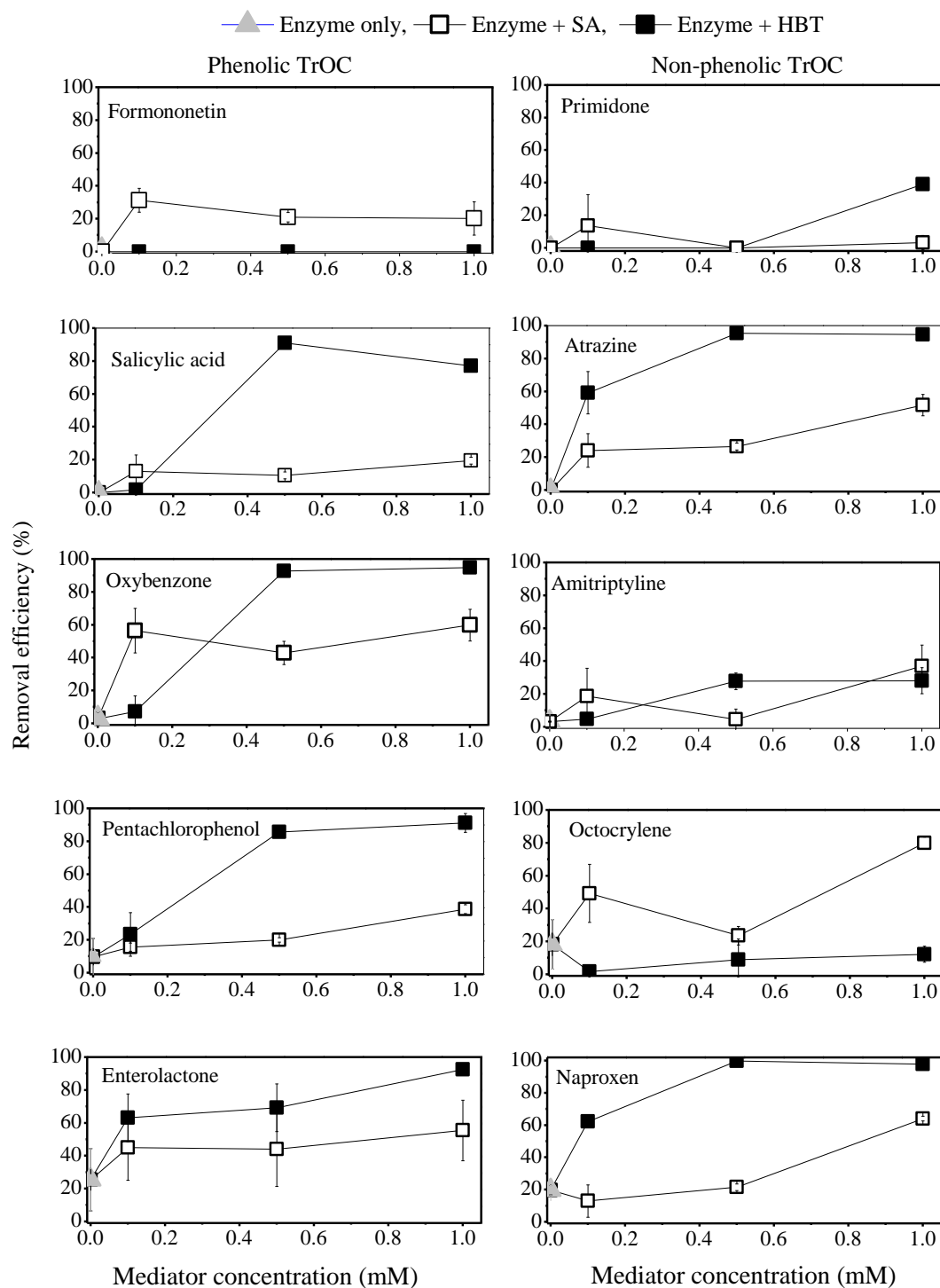


Figure 3.8: Effect of mediator concentrations on phenolic and non-phenolic TrOC removal improvement. The error bars represent the standard deviation of three replicates.

The free radicals generated from laccase-mediator systems that can improve pollutant degradation may inactivate laccase by oxidizing the aromatic amino acid residues on the proteinaceous enzyme surface (Khlifi-Slama et al., 2012). However, the range of mediator concentrations beyond which fast and significant laccase inactivation occurs tends to vary depending on the source of laccase and the type of the mediator. For example, in a study by Khlifi-Slama et al. (2012), a gradual increase in the degree of inactivation of laccase from *T. trogl* was observed for HBT concentrations from 0.1 to 10 mM. On the other hand, Kurniawati and Nicell (2007) observed only a slight inactivation (< 5%) of laccase from *T. versicolor* when a range of different mediators was used at concentrations up to 0.5 mM. In this study, when no mediators were added, the loss of laccase activity was 8 %, possibly due to the rotational shaking (agitation) during incubation (Hai et al., 2012). In contrast, depending on the mediator concentration (0.1 to 1 mM), the loss of laccase activity ranged from 14 to 39% (**Figure 3.9**). This gradual inactivation may be a contributing factor to the lack of increase in TrOC degradation beyond a dosage of 0.1 mM for SA and 0.5 mM for HBT.

Cost is an important consideration for practical implementation of a treatment process. While this study provides unique insights on the effect of augmenting enzymatic transformation with different dosage of HBT and SA on the TrOC removal performance, scale up of the system is a critical prerequisite to a meaningful economical assessment. Malt extract broth was used to obtain crude enzyme extract in this study. However, practical implementation would require selection of a more inexpensive medium such as agricultural residues. Given the potential of use of such renewable inexpensive growth medium, it appears that mediator cost will comprise a major fraction of the material cost. While the importance of feasibility assessment of the process cannot be overlooked, further detailed coverage of the cost analysis is beyond the scope of this study.

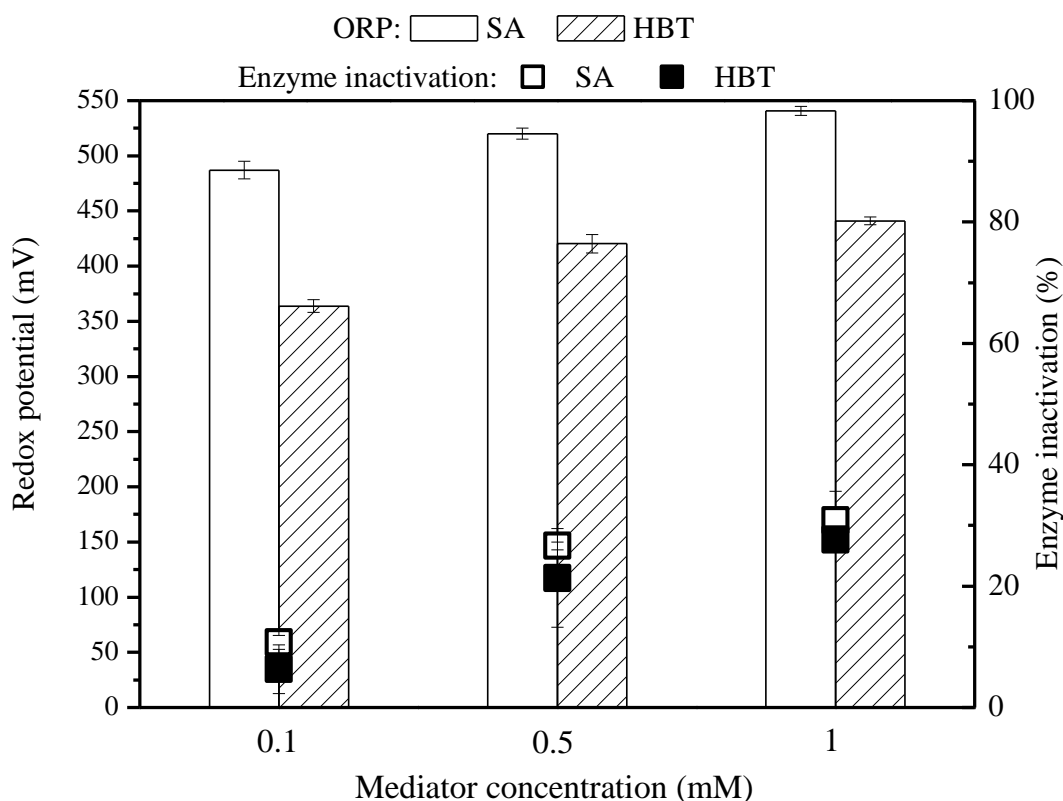


Figure 3.9: Impact of mediator concentration on the redox potential and the enzyme inactivation. The error bars represent the standard deviation of three replicates. Enzyme inactivation (%) = (Initial – Final enzymatic activity) / Initial enzymatic activity. Enzyme inactivation for the enzyme solution without mediators was 8%.

3.3.4 Effect of treatment mode on effluent toxicity

While TrOC transformation via fungal treatment may often lead to detoxification (Jelic et al., 2012), increase in toxicity of white-rot fungi-treated media containing TrOCs has also been reported. For example, Kim and Nicell (2006) reported that the addition of the mediator 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid enhanced the laccase catalysed oxidation of aqueous triclosan, but negatively affected the residual toxicity. A recent study has reported increased effluent phytotoxicity following laccase-HBT catalysed treatment of a dye solution (Daassi et al., 2013). However, there appears to be no specific literature regarding toxicity

following laccase-HBT catalysed treatment of TrOCs. In this study, a ToxScreen3 assay (**Section 3.2.4.2**) revealed a slight decrease in toxicity after treatment by whole-cell or crude enzyme extract. For instance, the toxicity of the TrOC solution in Milli-Q water was 14.7 ± 1.0 rTU, which reduced to 10.7 ± 1.1 rTU after the treatment by crude enzyme extract.

The toxicity of the test media treated with laccase-HBT was comparable at HBT doses of 0.1 and 0.5 mM, but increased at the higher HBT concentration of 1 mM (88.6 ± 3.9 rTU vs. 6.2 ± 1.1 rTU of control). The toxicity of the laccase-HBT solution was however much lower than the laccase-SA solution (8240 ± 1336 , 14202 ± 5894 , and 15250 ± 4359 rTU for the SA doses of 0.1, 0.5 and 1 mM, respectively) (**Figure 3.10**). The higher toxicity of SA is consistent with a study by Fillat et al. (2010), who reported that under the same mediator concentration, the toxicity of laccase-SA treated flax pulp effluent was 10 to 20-fold higher than that of laccase-HBT treated effluent, but did not offer any explanation behind this observation. It is possible that radicals formed due to oxidation of SA interact with the vitally important biomolecules in a different manner. For example, Cortez et al. (2010) reported inhibition of growth of *Candida guilliermondii* and its xylitol production due to SA, and noted that unlike some other phenolic compounds the microbial activity inhibition mechanism of SA was not directly related to damaging the integrity of the cell membrane but comprised respiration inhibition. Lack of relevant information in the literature restricts offering further insight to the difference between the behavior of SA and HBT.

Whilst the observations made in this study are generally consistent with the available information, elucidation of the critical influence of the type and the dose of the mediators is a novel outcome of this study. The toxicity of the treated media due to the addition of mediator requires further research: screening of non-toxic mediator and optimizing the mediator concentration are potential approaches to addressing this issue. Furthermore, investigations specifically aimed at identification of metabolites are needed to clarify the relative contribution of TrOC-metabolites and mediator to treated media toxicity. However, these aspects are beyond the scope of this study.

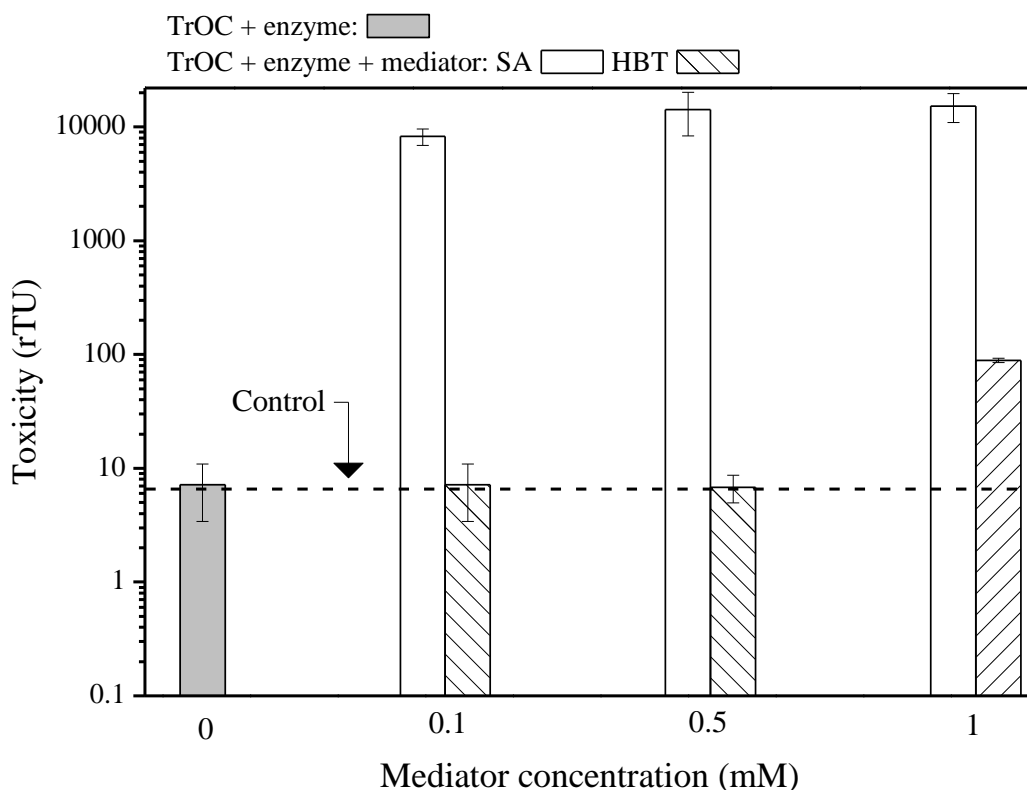


Figure 3.10: Comparison of toxicity following enzymatic treatment with and without the mediators. TrOC solution in Milli-Q water served as the control. The error bars represent the standard deviation of two replicates.

3.3.5 Insight into overall removal of TrOC classes

Generalizations regarding TrOC removal based on the intended applications or origins can be difficult as compounds within the same TrOC class can have vastly different chemical structures (Hai et al., 2013). Nevertheless, some interesting trends regarding fungal degradation of different TrOC classes can be observed in this study (**Table 3.1**). In line with several previous studies (Tamagawa et al., 2005; Cajthaml et al., 2009; Yang et al., 2013b) all hormones and the industrial chemicals were well degraded. Among these, the degradation of estriol (which is a hormone) and 4-*tert*-butylphenol (which is an industrial chemical) appeared to be more sensitive to the level of enzymatic activity (**Section 3.3.2.2**).

The removal of the pharmaceutically active compounds by whole-cell culture varied over a wide range: no removal (metronidazole, amitriptyline, primidone, carbamazepine and ketoprofen) to over 80% removal (salicylic acid, naproxen, ibuprofen, gemfibrozil, diclofenac and triclosan). The removal of salicylic acid, primidone and naproxen by crude enzyme extract was significantly improved by HBT addition. SA addition enhanced the removal of salicylic acid and naproxen.

In good agreement with the literature (Mougin et al., 1997; Tran et al., 2010; Yang et al., 2013a), pesticides demonstrated particular resistance to degradation. Among the six pesticides, no degradation was observed for clofibric acid, propoxur and fenoprop. HBT addition was essential for the degradation of ametryn (30%), atrazine (>90%) and pentachlorophenol (>90%). SA addition also improved the removal but lower extent of ametryn (30%), atrazine (>50%) and pentachlorophenol (>38%) compared to that of HBT addition.

Degradation of UV filters and phytoestrogens by white-rot fungi has not been studied before. In this study, among the UV filters, high removal of oxybenzone was achieved by the live whole-cell culture, but degradation by the crude enzyme required HBT or SA addition. Benzophenone and octocrylene were removed only by the live whole-cell culture. Of the two phytoestrogens, formononetin was degraded only by whole-cell preparations, while enterolactone degradation was only possible by amending the extracellular extract with HBT or SA.

Comparing the performance of the fungal strain used in this study with that of conventional bacterial activated sludge was not the aim of this study. However, it is worth noting that the results suggest differences in the mode of removal and the spectrum of TrOC covered by these options. The white-rot fungi strain achieved significant degradation of some pharmaceuticals such as naproxen and diclofenac, and the pesticide atrazine, which are usually reported to be highly resistant to bacterial degradation (Hai et al., 2013). In addition, the significantly hydrophobic TrOCs such as many of the hormones, personal care products and alkyl phenolic industrial compounds are well removed from the aqueous phase during conventional (bacterial) wastewater treatment. However, some of these TrOC such as 17 β -estradiol, 4-*tert*-octyl phenol and triclosan exhibit significant resistance to bacterial degradation (Hai et al., 2013). The high biosorption of these resistant TrOC in conventional treatment poses a risk of leaching of these

compounds during sludge processing and disposal. By contrast, the data reported in this study confirm fungal degradation of these TrOC (at least the disappearance of the parent molecules).

Table 3.1: Comparative removal of TrOC classes by different treatment options.

Category	Compound	Phenolic moiety	Log <i>D</i> (pH 4)	Removal (%) by different options				
				I	II	III	IV	V
Pharmaceuticals	Metronidazole		-0.2	0	0	17 ±23	10 ±15	6 ±8
	Salicylic acid	Yes	0.2	0		0		19 ±2
	Primidone		0.8	24 ±34	10 ±7	0		3 ±4
	Amitriptyline		1.3	8 ±8	5 ±4	4±1		
	Carbamazepine		1.9	6±5	0	4 ±5	0	0
	Ketoprofen		2.7	10 ±15	24 ±8	0	7 ±5	0
	Naproxen		2.8	14±5		20 ±3		
	Ibuprofen		3.4	32 ±1		2 ±2	0	3 ±5
	Gemfibrozil		4.2			18 ±4	24 ±3	7 ±9
	Diclofenac		4.3					
	Triclosan	Yes	5.3					
Pesticides	Propoxur		1.5	0	10 ±5	7 ±1	0	0
	Fenoprop		2.4	0	0	9 ±7	1 ±1	6 ±7
	Clofibric acid		1.6	15 ±3	4 ±5	8 ±2	7 ±8	21 ±11
	Atrazine		2.6	9 ±1	16±15	3 ±2		
	Ametryn		2.6	14 ±8	5 ±6	7 ±7	29 ±1	29 ±4
	Pentachlorophenol	Yes	5.0			10 ±11		
Industrial products	4-tert-Butylphenol	Yes	3.4	10 ±14				
	Bisphenol A	Yes	3.6	26 ±5				
	4-tert-Octolphenol	Yes	5.2					
	Estriol	Yes	2.5	13 ±4	5 ±2			
Steroid hormones	Estrone	Yes	3.6					
	17 α -Ethinylestradiol	Yes	4.1					
	17 β -Estradiol	Yes	4.2					
	17 β -Estradiol-17-acetate	Yes	5.2					
	Enterolactone	Yes	1.9	3 ±1	0	19 ±27		
Phytoestrogens	Formononetin	Yes	2.9	15 ±5		0	0	20 ±10
UV filters	Benzophenone		3.2	10 ±2		0	0	0
	Oxybenzone	Yes	4.0			4 ±5		
	Octocrylene		6.9	27 ±2		18 ±14	13 ±5	

Treatment options: ^IWhole- cell (inactivated), ^{II}Whole-cell (live), ^{III}Crude enzyme extract, ^{IV}Crude enzyme extract with 1 mM HBT, and ^V Crude enzyme extract with 1 mM SA.

□ 0 ~ 30% ▨ >60%

3.4. CONCLUSION

In this study, all 14 phenolic TrOCs but only a half of the tested non-phenolic TrOCs were effectively degraded. All hydrophobic ($\log D > 3$) TrOCs were well removed. By comparing the performance of the whole-cell preparations and the extracellular extract (no biosorption), it was confirmed that biodegradation was the main mechanism of removal. However, the fact that the hydrophilic compounds were poorly removed may indicate the importance of biosorption in subsequent degradation by the whole-cell. In addition, the lower degradation of some hydrophobic compounds by the extracellular extract, and the impact of intracellular cytochrome P450 system inhibition on the degradation of some TrOCs by the whole-cell culture indicated the importance of extracellular enzyme-independent catalytic pathways.

This is also the first study to reveal the dosage-specific comparative enhancement of enzymatic degradation of a set of 30 TrOCs by two redox-mediators, namely HBT and SA. HBT and SA, which produce aminoxyl and phenoxyl radicals, respectively, achieved significant removal of additional five phenolic and two non-phenolic TrOCs, with moderate removal of a few other non-phenolics. The improved removal may be attributed to the increase in redox potential of the enzyme solution following mediator addition. However, despite the similar redox potentials of the enzyme-mediator cocktail for HBT and SA, SA achieved less efficient degradation and did not exhibit significant improvement in degradation at concentrations higher than 0.1 mM, indicating the imbalance between the stability and reactivity of the radicals generated. Addition of SA at all concentrations (0.1 – 1 mM) also resulted in a >1000-fold increase in bacterial cytotoxicity. In contrast, only the higher HBT dose (1 mM) produced measurable toxicity, with no detectable increase in toxicity at lower doses (0.1-0.5 mM). Overall, addition of HBT at a concentration of 0.5 mM achieved the best removal without raising any increase in toxicity of the treated media.

3.5. REFERENCES

Astolfi, P., P. Brandi, C. Galli, P. Gentili, M. F. Gerini, L. Greci and O. Lanzalunga (2005). New mediators for the enzyme laccase: mechanistic features and selectivity in the oxidation of non-phenolic substrates. *New J. Chem.*, 29, 1308-1317.

- Auriol, M., Y. Filali-Meknassi, R. D. Tyagi and C. D. Adams (2007). Laccase-catalyzed conversion of natural and synthetic hormones from a municipal wastewater. *Water Res.* 41, 3281-3288.
- Bending, G. D., M. Friloux and A. Walker (2002). Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. *FEMS Microbiol. Lett.*, 212, 59-63.
- Bernini, R., F. Crisante, P. Gentili, F. Morana, M. Pierini and M. Piras (2011). Chemoselective C-4 aerobic oxidation of catechin derivatives catalyzed by the *Trametes villosa* laccase/1-hydroxybenzotriazole system: Synthetic and mechanistic aspects. *J Org Chem.* 76, 820-832.
- Cajthaml, T., Z. Křesinová, K. Svobodová and M. Möder (2009). Biodegradation of endocrine-disrupting compounds and suppression of estrogenic activity by ligninolytic fungi. *Chemosphere.* 75, 745-750.
- Camarero, S., D. Ibarra, M. J. Martínez and Á. T. Martínez (2005). Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Appl. Environ. Microbiol.*, 71, 1775-1784.
- Cañas, A. I. and S. Camarero (2010). Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. *Biotechnol. Adv.* 28, 694-705.
- Coniglio, A., C. Galli, P. Gentili and R. Vadala (2009). Hydrogen atom abstraction from C-H bonds of benzylamides by the aminoxyl radical BTNO: A kinetic study. *Org. Biomol. Chem.*, 7, 155-160.
- Coniglio, A., C. Galli, P. Gentili and R. Vadalà (2008). Oxidation of amides by laccase-generated aminoxyl radicals. *J.Mol. Catal. B. Enzym.*, 50, 40-49.
- Cortez, D. V. and I. C. Roberto (2010). Individual and interaction effects of vanillin and syringaldehyde on the xylitol formation by *Candida guilliermondii*. *Bioresource Technol.* 101, 1858-1865.
- d'Acunzo, F., P. Baiocco and C. Galli (2003). A study of the oxidation of ethers with the enzyme laccase under mediation by two N-OH-type compounds. *New J. Chem.*, 27, 329-332.
- d'Acunzo, F., C. Galli, P. Gentili and F. Sergi (2006). Mechanistic and steric issues in the oxidation of phenolic and non-phenolic compounds by laccase or laccase-mediator systems. The case of bifunctional substrates. *New J. Chem.*, 30, 583-591.
- Daassi, D., H. Zouari-Mechichi, F. Frikha, M. Martinez, M. Nasri and T. Mechichi (2013). Decolorization of the azo dye Acid Orange 51 by laccase produced in solid culture of a newly isolated *Trametes trogii* strain. *3 Biotechnology.* 3, 115-125.
- Eibes, G., G. Debernardi, G. Feijoo, M. Moreira and J. Lema (2011). Oxidation of pharmaceutically active compounds by a ligninolytic fungal peroxidase. *Biodegradation.* 22, 539-550.
- Fabbrini, M., C. Galli and P. Gentili (2002). Comparing the catalytic efficiency of some mediators of laccase. *J.Mol. Catal. B. Enzym.*, 16, 231-240.
- Fillat, A., J. F. Colom and T. Vidal (2010). A new approach to the biobleaching of flax pulp with laccase using natural mediators. *Bioresource Technol.* 101, 4104-4110.
- Gatidou, G., N. S. Thomaidis, A. S. Stasinakis and T. D. Lekkas (2007). Simultaneous determination of the endocrine disrupting compounds nonylphenol, nonylphenol ethoxylates, triclosan and bisphenol A in wastewater and sewage sludge by gas chromatography-mass spectrometry. *J. Chromatogr. A.* 1138, 32-41.

- Hai, F. I., L. D. Nghiem, S. J. Khan, W. E. Price and K. Yamamoto (2013). Wastewater reuse: Removal of emerging trace organic contaminants. *Membrane Biological Reactors*. F. I. Hai, K. Yamamoto and C.-H. Lee. London (ISBN: 9781780400655), IWA publishing: 163-203.
- Hai, F. I., K. Yamamoto, F. Nakajima and K. Fukushi (2012). Application of a GAC-coated hollow fiber module to couple enzymatic degradation of dye on membrane to whole cell biodegradation within a membrane bioreactor. *J. Membr. Sci.*, 389, 67-75.
- Hata, T., S. Kawai, H. Okamura and T. Nishida (2010). Removal of diclofenac and mefenamic acid by the white rot fungus *Phanerochaete sordida* YK-624 and identification of their metabolites after fungal transformation. *Biodegradation*. 21, 681-689.
- Jelic, A., C. Cruz-Morató, E. Marco-Urrea, M. Sarra, S. Perez, T. Vicent, M. Petrović and D. Barcelo (2012). Degradation of carbamazepine by *Trametes versicolor* in an air pulsed fluidized bed bioreactor and identification of intermediates. *Water Res.* 46, 955-964.
- Khlifi-Slama, R., T. Mechichi, S. Sayadi and A. Dhouib (2012). Effect of natural mediators on the stability of *Trametes troglit* laccase during the decolourization of textile wastewaters. *J. Microbiol.* 50, 226-234.
- Khlifi, R., L. Belbahri, S. Woodward, M. Ellouz, A. Dhouib, S. Sayadi and T. Mechichi (2010). Decolourization and detoxification of textile industry wastewater by the laccase-mediator system. *J. Hazard. Mater.*, 175, 802-808.
- Kim, Y. J. and J. A. Nicell (2006). Laccase catalysed oxidation of aqueous triclosan. *J. Chem. Technol. Biotechnol.*, 81, 1344-1352.
- Klonowska, A., C. Gaudin, A. Fournel, M. Asso, J. Le Petit, M. Giorgi and T. Tron (2002). Characterization of a low redox potential laccase from the basidiomycete C30. *Eur. J. Biochem.*, 269, 6119-6125.
- Kurniawati, S. and J. A. Nicell (2007). Efficacy of mediators for enhancing the laccase-catalyzed oxidation of aqueous phenol. *Enzyme Microb. Technol.*, 41, 353-361.
- Liu, R., J. L. Zhou and A. Wilding (2004). Simultaneous determination of endocrine disrupting phenolic compounds and steroids in water by solid-phase extraction-gas chromatography-mass spectrometry. *J. Chromatogr. A*. 1022, 179-189.
- Lloret, L., G. Eibes, T. A. Lú-Chau, M. T. Moreira, G. Feijoo and J. M. Lema (2010). Laccase-catalyzed degradation of anti-inflammatories and estrogens. *Biochem. Eng. J.*, 51, 124-131.
- Marco-Urrea, E., M. Pérez-Trujillo, P. Blázquez, T. Vicent and G. Caminal (2010). Biodegradation of the analgesic naproxen by *Trametes versicolor* and identification of intermediates using HPLC-DAD-MS and NMR. *Bioresource Technol.* 101, 2159-2166.
- Marco-Urrea, E., M. Pérez-Trujillo, T. Vicent and G. Caminal (2009). Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. *Chemosphere*. 74, 765-772.
- Mizuno, H., H. Hirai, S. Kawai and T. Nishida (2009). Removal of estrogenic activity of isobutylparaben and n-butylparaben by laccase in the presence of 1-hydroxybenzotriazole. *Biodegradation*. 20, 533-539.
- Mougin, C., C. Laugero, M. Asther and V. Chaplain (1997). Biotransformation of s-Triazine Herbicides and Related Degradation Products in Liquid Cultures by the White Rot Fungus *Phanerochaete chrysosporium*. *Pestic Sci.* 49, 169-177.

- Nguyen, L. N., F. I. Hai, S. Yang, J. Kang, F. D. L. Leusch, F. Roddick, W. E. Price and L. D. Nghiem (2013). Removal of trace organic contaminants by an MBR comprising a mixed culture of bacteria and white-rot fungi. *Bioresource Technol.* 143, 234-241.
- Quintana, J. B., S. Weiss and T. Reemtsma (2005). Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. *Water Res.* 39, 2654-2664.
- Rodarte-Morales, A., G. Feijoo, M. Moreira and J. Lema (2011). Degradation of selected pharmaceutical and personal care products (PPCPs) by white-rot fungi. *World J Microbio Biotechnol.*, 27, 1839-1846.
- Rodarte-Morales, A. I., M. T. Moreira, G. Feijoo and J. M. Lema (2010). Evaluation of two fungal strains for the degradation of pharmaceutical and personal care products (PPCPs). *Chem. Eng. Trans.*, 20, 31-36.
- Ryu, W. R., S. H. Shim, M. Y. Jang, Y. J. Jeon, K. K. Oh and M. H. Cho (2000). Biodegradation of pentachlorophenol by white rot fungi under ligninolytic and nonligninolytic conditions. *Biotechnol. Bioprocess Eng.*, 5, 211-214.
- Snyder, S. A. (2008). Occurrence, Treatment, and Toxicological Relevance of EDCs and Pharmaceuticals in Water. *Ozone Sci. Eng.*, 30, 65-69.
- Svobodova, K., A. Majcherczyk, C. Novotny and U. Kues (2008). Implication of mycelium-associated laccase from *Irpex lacteus* in the decolorization of synthetic dyes. *Bioresource Technol.* 99, 463-471.
- Tadkaew, N., F. I. Hai, J. A. McDonald, S. J. Khan and L. D. Nghiem (2011). Removal of trace organics by MBR treatment: The role of molecular properties. *Water Res.* 45, 2439-2451.
- Tamagawa, Y., H. Hirai, S. Kawai and T. Nishida (2005). Removal of estrogenic activity of endocrine-disrupting genistein by ligninolytic enzymes from white rot fungi. *FEMS Microbiol. Lett.*, 244, 93-98.
- Tamagawa, Y., H. Hirai, S. Kawai and T. Nishida (2007). Removal of estrogenic activity of 4-tert-octylphenol by ligninolytic enzymes from white rot fungi. *Environ. Toxicol.*, 22, 281-286.
- Tran, N. H., T. Urase and O. Kusakabe (2010). Biodegradation characteristics of pharmaceutical substances by whole fungal culture *Trametes versicolor* and its laccase. *J Water Environ Technol.*, 8, 125-140.
- Weng, S.-S., K.-L. Ku and H.-T. Lai (2012). The implication of mediators for enhancement of laccase oxidation of sulfonamide antibiotics. *Bioresource Technol.* 113, 259-264.
- Xu, F., J. J. Kulys, K. Duke, K. Li, K. Krikstopaitis, H.-J. W. Deussen, E. Abbate, V. Galinyte and P. Schneider (2000). Redox chemistry in laccase-catalyzed oxidation of N-hydroxy compounds. *Appl. Environ. Microbiol.*, 66, 2052-2056.
- Yang, S.-F., C.-F. Lin, A. Yu-Chen Lin and P.-K. Andy Hong (2011). Sorption and biodegradation of sulfonamide antibiotics by activated sludge: Experimental assessment using batch data obtained under aerobic conditions. *Water Res.* 45, 3389-3397.
- Yang, S., F. I. Hai, L. D. Nghiem, L. N. Nguyen, F. Roddick and W. E. Price (2013a). Removal of bisphenol A and diclofenac by a novel fungal membrane bioreactor operated under non-sterile conditions. *Int. Biodeterior. Biodegradation.* 85, 483-490.
- Yang, S., F. I. Hai, L. D. Nghiem, W. E. Price, F. Roddick, M. T. Moreira and S. F. Magram (2013b). Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: a critical review. *Bioresource Technol.* 141, 97-108.

- Yang, S., I. F. Hai, D. L. Nghiem, F. Roddick and W. E. Price (2013c). Removal of trace organic contaminants by nitrifying activated sludge and whole-cell and crude enzyme extract of *Trametes versicolour*. *Water Sci. Technol.*, 67, 1216-1223.
- Zhang, S. Y., Q. A. Zhang, S. Darisaw, O. Ehie and G. D. Wang (2007). Simultaneous quantification of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and pharmaceuticals and personal care products (PPCPs) in Mississippi river water, in New Orleans, Louisiana, USA. *Chemosphere*. 66, 1057-1069.
- Zhang, Y. and S.-U. Geißen (2010). In vitro degradation of carbamazepine and diclofenac by crude lignin peroxidase. *J. Hazard. Mater.*, 176, 1089-1092.

CHAPTER 4: Removal of trace organic contaminants by an MBR comprising a mixed culture of bacteria and white-rot fungi

This chapter has been published as:

Luong N Nguyen, Faisal I Hai, Shufan Yang, Jinguo Kang, Frederic DL Leusch, Felicity Roddick, William E Price, Long D Nghiem. (2013) Removal of trace organic contaminants by an MBR comprising a mixed culture of bacteria and white-rot fungi, *Bioresource technology*, 148, 234-241

4.1. INTRODUCTION

Trace organic contaminants (TrOCs) comprise a wide range of naturally occurring and synthetic compounds including pharmaceutically active compounds (PhACs), steroid hormones, pesticides and industrial chemicals. TrOCs have been detected in raw sewage, secondary treated effluents, surface water bodies, and even in drinking water (Schwarzenbach et al., 2010). Given the potential adverse effects of TrOCs on aquatic organisms and human health, recent studies have been devoted to improving current processes or developing new technologies for the removal of these compounds from wastewater (Bourbonnais et al., 1998; Tadkaew et al., 2011). The conventional activated sludge (CAS) treatment process (that is widely employed for wastewater treatment) can effectively remove bulk organic matter and nutrients (nitrogen and phosphorus). However, the CAS process was not specifically designed to remove TrOCs. Thus, the removal efficiency of TrOCs by CAS can vary significantly (Hai et al., 2013). Due to the limitations of TrOC removal by the bacteria-dominated CAS process, several small-scale batch studies have been conducted to explore the application of white-rot fungal bioremediation (Yang et al., 2013a). White-rot fungi possess one or more extracellular enzymes including lignin peroxidases (LiP), manganese-dependent peroxidases (MnP) and laccases. They can efficiently degrade a wide range of persistent organic compounds including TrOCs that are resistant to bacterial degradation (Marco-Urrea et al., 2009; Tran et al., 2010; Yang et al., 2013a).

Despite the significant number of small-scale sterile batch test studies, only a few studies have been conducted on continuous flow fungal reactors (Blázquez et al., 2008b; Jelic et al., 2012; Rodarte-Morales et al., 2012). Furthermore, to date, only two studies have investigated the removal of TrOCs under non-sterile conditions, which are more realistic for a practical application (Zhang et al., 2012; Yang et al., 2013a). Compared to the performance in pure culture batch tests, that of a continuous flow fungal reactor can be adversely affected due to two major phenomena: i) continuous washout of fungal extracellular enzyme with treated effluent (Hai et al., 2012a; Yang et al., 2013a), and ii) bacterial destabilization of fungal activity (Libra et al., 2003; Hai et al., 2009). On the other hand, combination of white-rot fungi with activated sludge may bring about synergistic degradation of TrOCs. For example, based on a series of batch tests, Hai et al. (2012b) reported enhanced removal of three widely used resistant pesticides from their mixed solution by implementing a mixed culture of bacteria and white-rot fungus. However, a

systematic assessment of the relative performance of fungus and activated sludge, and the implications of combining them in a continuous flow system is absent from the literature.

Fungal enzyme-catalysis can be enhanced by adding low molecular weight mediators that act as an ‘electron shuttle’ between the oxidizing enzyme and target compounds (Kim et al., 2006a). The degree of enhancement depends predominantly on the type of mediator and the TrOC structure. Apart from a few short-term studies on enzymatic membrane reactors (EMR) (e.g. Lloret et al. (2012)), most of the studies on mediator-enhanced enzyme system have been carried out in batch mode. Even with the EMR configuration, gradual loss of enzymatic activity due to various physical, chemical and biological inhibitors under wastewater conditions is inevitable. Thus, it is necessary to periodically replenish the reactor with fresh enzyme. By contrast, a whole-cell reactor may bring about the added advantage of continuous enzyme production. In this context, continuous dosing of a mediator to a whole-cell reactor may achieve enhanced removal of TrOCs. However, no work in this line could be identified in the literature.

This study aims to evaluate the removal of TrOCs by a reactor containing the white-rot fungus *Trametes versicolor* (ATCC 7731) and activated sludge. To prevent the washout of the fungus, a microfiltration membrane was coupled with the mixed culture bioreactor. A control activated sludge reactor was also operated to assess the implications of the combination explored. To further facilitate the comparison between fungus and activated sludge, the TrOC degradation capacity of the fungal enzyme (laccase) was assessed in a series of batch tests. The effect of continuous dosing of a mediator (1- hydroxy benzotriazole, HBT) to the fungus-augmented MBR on TrOC removal was also studied.

4.2. MATERIALS AND METHODS

Two identical MBR systems, one with activated sludge and the other inoculated with fungus-augmented sludge (details follow in **Section 4.2.4**), were operated under similar conditions to enable assessment of the effect of white-rot fungus (*T. versicolor*) augmentation with conventional activated sludge. Because of the practical difficulties in maintaining sterility (Libra et al., 2003), operation of a control MBR with a pure culture of *T. versicolor* was not attempted. However, in order to shed light on the probable differences between the performances of the

mixed culture and the pure fungus culture, the results of the batch (fungal) enzymatic tests (**Section 4.2.3**) were taken into account.

4.2.1 Trace organic contaminants

A set of 30 TrOCs was selected for investigation in this study based on their widespread occurrence in raw sewage and sewage-impacted water bodies and their diverse physicochemical properties (*e.g.*, hydrophobicity and molecular weight) as presented in (**Appendix Table A-1**). These compounds represent six major groups of TrOCs including eleven PhACs, five steroid hormones, six pesticides, three industrial chemicals, three UV filters and two phytoestrogens. A stock solution of all selected TrOCs was prepared in pure methanol at a concentration of 25 mg/L of each compound on a monthly basis and stored at -18°C prior to use with dilution.

4.2.2 White-rot fungi, growth medium, synthetic wastewater and mediator

The white-rot fungus *T. versicolor* ATCC 7731 (USA) was used in this study. The fungus was maintained on malt extract agar (Oxoid, England). A liquid culture was obtained by inoculating pieces of fungus grown on agar into malt extract broth (5 g/L) (MEB) (Merck, Germany). Refrigerated stock fungal suspension was poured into sterilised growth medium (pH 4.5) and incubated in a temperature-controlled (28°C) shaker for 7 d in order to obtain fresh batches of fungus. The supernatant of the same fungal culture was harvested as crude enzyme extract and refrigerated (4°C) in sterilized bottles.

A malt-based synthetic wastewater consisting of MEB (0.5 g/L), KH_2PO_4 (0.2 g/L), MgSO_4 (0.05 g/L), FeSO_4 (0.01 g/L) and thiamine (0.001 g/L) (Yang et al., 2013a) was used for the operation of the fungus-augmented MBR as described in **Section 4.2.4**. All chemicals used for preparing the synthetic wastewater were dissolved in Milli-Q water. The pH of the synthetic wastewater was adjusted to 4.5 with HCl (4 M). The MBR containing activated sludge (see **Section 4.2.4**), on the other hand, was fed with a synthetic wastewater validated previously for activated sludge (Nguyen et al., 2012). The stock TrOC solution (**Section 4.2.1**) was introduced to the respective synthetic wastewater to obtain a concentration of approximately 5 $\mu\text{g/L}$ of each TrOC and then fed continuously to the MBRs.

HBT (Sigma-Aldrich, Australia), which is a widely studied redox mediator for fungal laccase (Coniglio et al., 2008; Hata et al., 2010), was used in this study. A stock solution of the mediator was prepared at a concentration of 50 mM and stored at 4 °C.

4.2.3. Protocol of batch enzymatic degradation tests

See Section 3.2.3, Chapter 3.

4.2.3 MBR systems and operating protocol

As noted earlier, two identical MBR systems, one inoculated with fungus-augmented sludge (details follow) and the other with activated sludge, were operated under similar conditions. Each MBR comprised of a 5.5 L (active volume) glass reactor and housed a PVDF hollow fiber membrane module (SADF0790M mini module, Mitsubishi Rayon Engineering, Japan) with a nominal pore size of 0.4 μm and a total effective membrane surface area of 0.074 m^2 . The membrane was operated on a 8 min “suction” and 2 min “relaxation” cycle under an average flux of 1.54 $\text{L}/\text{m}^2\text{h}$, resulting in a hydraulic retention time (HRT) of 2 d. The focus of this study was on the removal performance of the MBR. Therefore the reactor was designed as such that frequent membrane fouling, requiring periodic cleaning, could be avoided by maintaining a low average membrane flux. A vacuum gauge (Model 840064, Sper Scientific Ltd., USA), which was connected to a data logging computer, was used to monitor transmembrane pressure (TMP) as an indicator of membrane fouling. Aeration pumps (ACO-002, Zhenjiang Sensen Industry Co. Ltd, China) supplied air to the reactor through diffusers. Peristaltic pumps (Master-Flex, Cole-Parmer, Australia) were used to supply influent and draw effluent. The MBRs were placed in a water bath to maintain the mixed liquor temperature at $28.0 \pm 0.2^\circ\text{C}$ by a heating immersion circulator (Julabo, Germany). The dissolved oxygen (DO) concentration in the reactors was monitored daily by a DO meter (YSI, USA). Aeration was provided to avoid settling of the mixed liquor in the corners of the reactor, leading to a consistently high DO concentration of approximately 5 mg/L within the MBRs.

The fungus-augmented MBR in the current study was seeded with sludge from a previous lab-scale MBR (Yang et al., 2013a) which was originally inoculated with a pure culture of *T. versicolor* (ATCC 7731) and operated for 90 d to treat a synthetic wastewater containing two model TrOCs (bisphenol A and diclofenac). Because the original MBR (Yang et al., 2013a) was

operated under non-sterile conditions, a mixed culture of fungi and bacteria gradually developed in the reactor. The mixed sludge, however, continued to exhibit laccase (a white-rot fungal enzyme) activity — a characteristic uncommon to activated sludge. After seeding with this fungus-augmented sludge, the MBR in the current study was operated for 110 d, of which the investigation over the last 30 d was conducted with continuous dosing of 5 μ M of HBT (redox mediator) to the MBR. HBT was added continuously via a peristaltic pump at a flow rate of 0.5 mL/min to achieve a mediator concentration of 5 μ M in the reactor. The conventional MBR, on the other hand, was inoculated with activated sludge from another lab-scale MBR operated for over 3 years for TrOC removal (Tadkaew et al., 2011). The initial mixed liquor suspended solid concentration in both the MBRs was 3 g/L. Sludge was withdrawn from the MBRs only during sampling. As noted earlier, the MBRs were fed continuously with respective synthetic wastewater (**Section 4.2.2**) spiked with the selected 30 TrOC each with a concentration of approximately 5 μ g/L.

4.2.4 Analytical methods

4.2.4.1 Basic parameters and trace organic contaminants

Total organic carbon (TOC) and total nitrogen (TN) concentrations were measured simultaneously by a Shimadzu TOC/TN-VCSH analyser (Japan). The determination of mixed liquor suspended solid (MLSS) and mixed liquor volatile suspended solid (MLVSS) concentration (Method 2540E) and enumeration (colony forming unit, CFU/mL) of fungi (Method 9610 C) and bacteria (Method 9215) followed Standard Methods for the Examination of Water and Wastewater (Eaton et al., 2005).

TrOCs adsorbed on the MBR biomass was extracted by a previously developed solvent extraction method (Wijekoon et al., 2013). Freeze-dried (0.5 g) sludge was ground to powder and subjected to repeated solvent extraction (methanol and dichloromethane) via sonication. The extracted TrOCs in the solution was diluted to 500 mL with Milli-Q water. Then, the samples were subjected to SPE and finally to GC-MS analysis as outlined above. The concentration was expressed as ng TrOC per g of MLSS (dry wt).

The TrOC concentrations in all the samples were measured using a Shimadzu GC/MS (QP5000) system (see **Section 3.2.4.1 Chapter 3**).

4.2.4.2 Enzymatic activity and toxicity assay

See Section 3.2.4.2, Chapter 3.

4.3. RESULTS AND DISCUSSION

4.3.1 Performance stability of MBR

The seed sludge for the lab-scale MBRs in the current study was sourced from MBRs treating TrOCs, and the MBRs were operated under stable temperature, pH, DO and organic loading conditions. Under the applied organic loading and MLSS sampling pattern (for weekly determination of MLSS/MLVSS, fungal/bacterial count and biomass laccase activity, and determination of amount of TrOC adsorbed on biosolids), the MLSS in the MBRs remained stable around 3 g/L. Under the applied low flux and vigorous aeration, severe membrane fouling, causing TMP build-up, did not occur (data not shown), confirming stable hydraulic performance of the membrane. Accordingly, the fungus-augmented MBR showed good and stable performance with respect to the basic water quality parameters TOC and TN (**Table 4.1**). The addition of the mediator (HBT) appeared not to adversely affect the biological stability (e.g. in terms of MLVSS/MLSS ratio and TOC/TN removal). This confirms that the TrOC removal performance of the MBR was evaluated under stable conditions. Notably, in good agreement with a previous study (Nguyen et al., 2012), the basic operating and water quality parameters for the conventional MBR in the current study were also stable (data not shown).

Table 4.1: Basic biological performance of the fungus-augmented MBR with/without continuous dosing of a redox mediator (1-hydroxy benzotriazole) (5 μ M).

Process	MLVSS/MLSS ratio	Fungal count (10^6 CFU/mL)	Bacterial count (10^6 CFU/mL)	Biomass laccase activity (μ M/min/g MLSS)	TOC removal (%)	TN removal (%)
Without mediator	0.67 \pm 0.1 (n = 15)	125 \pm 71 (n=15)	122 \pm 69 (n=15)	45 \pm 23 (n=15)	97 \pm 2 (n=17)	75 \pm 12 (n=15)
With mediator	0.75 \pm 0.1 (n = 6)	100 \pm 38 (n=6)	125 \pm 54 (n=6)	59 \pm 8 (n=6)	97 \pm 2 (n=11)	79.0 \pm 12.5 (n=11)

Consistent with previous work (Yang et al., 2013a), the viable fungal and bacterial count in the fungus-augmented MBR supernatant varied over the operating period (**Table 4.1**). It is noted that fungi prefer to grow in pellet form in suspension, and, thus, monitoring the fungal count in the mixed liquor supernatant sample may not accurately reflect the fungal concentration in the MBR. Nevertheless, monitoring the fungal count serves the important purpose of confirming the presence of viable fungi in the MBR throughout the operating period.

Consistent with the observations made in previous studies on continuous flow fungal reactors (Blázquez et al., 2008a; Yang et al., 2013a), laccase activity was only occasionally detected in the mixed liquor supernatant (data not shown). This could be attributed to the previously reported factors such as: i) bacterial denaturation (Libra et al., 2003), as would be expected in real wastewater conditions, and ii) passage of laccase (59-110 kDa) through the microfiltration membrane along with permeate (Hai et al., 2012a; Yang et al., 2013a). Nevertheless, the consistent detection of laccase activity in the MBR biomass (**Table 4.1**) confirmed the active presence of the white-rot fungi *T. versicolor* that was originally inoculated into the seed sludge source MBR.

4.3.2 TrOC removal by fungus-augmented MBR

4.3.2.1 Batch enzymatic test vs. MBR operation

Figure 4.1 presents the removal of 30 TrOCs by the fungus-augmented MBR. For the TrOCs that were degraded with an efficiency of over 50% by crude enzyme solution in batch tests, the fungus-augmented MBR maintained high removal (80% - complete) of all of them (except diclofenac with a removal of *ca.* 50%). Furthermore, of the 18 TrOCs showing limited enzymatic degradation (negligible - 50%), ten were removed with an efficiency of over 80% by the fungus-augmented MBR (**Figure 4.1**). Degradation of some compounds, namely, gemfibrozil (24%), amitriptyline (28%), primidone (40%), salicylic acid (77%), enterolactone (93%) and oxybenzone (95%) in the batch enzymatic tests was only obtained with the addition of the mediator, while the fungus-augmented MBR achieved a high removal (> 90%) without mediator dosing (**Figure 4.1**).

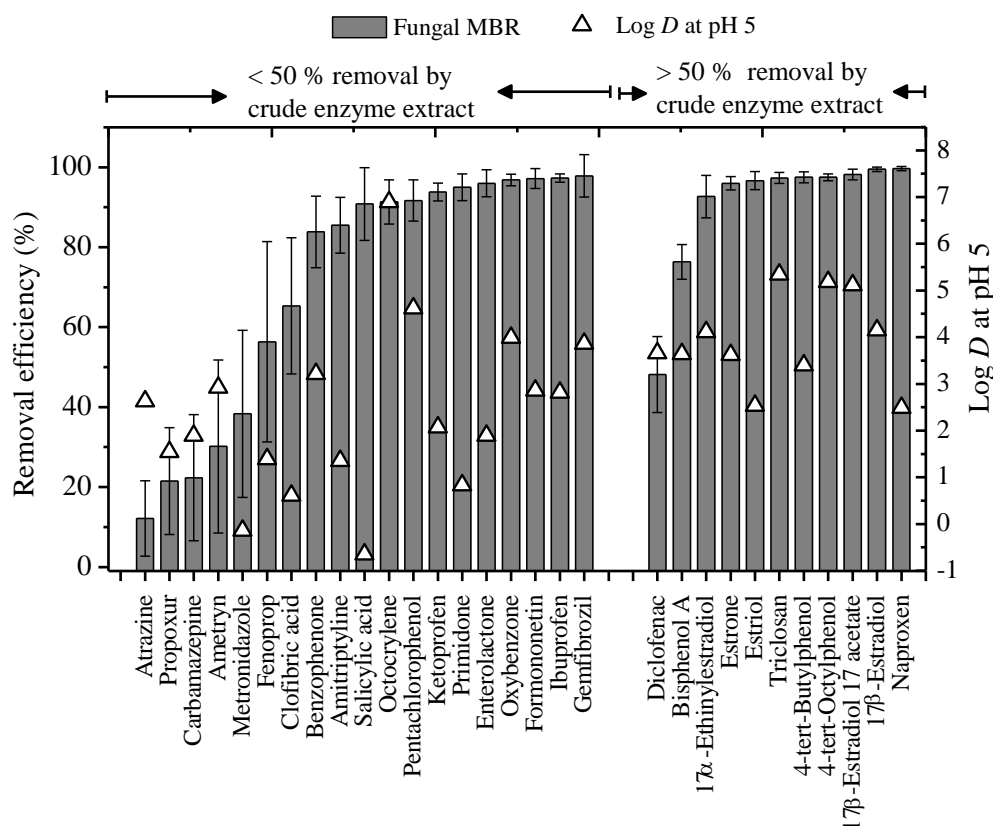


Figure 4.1: Removal of 30 TrOCs by the fungus-augmented MBR. Error bars represent the standard deviation of 14 samples.

Data from this study also highlighted the better removal performance of a fungus-augmented MBR (white-rot fungus + bacteria) compared to a conventional MBR. As shown in **Table 4.2**, the fungus-augmented MBR achieved better removal of six compounds, namely, fenoprop, clofibric acid, pentachlorophenol, ketoprofen, diclofenac and naproxen that are all well-known compounds resistant to bacterial degradation (Tadkaew et al., 2011; Hai et al., 2012a; Nguyen et al., 2012). Of further interest is the fact that the removal of fenoprop, clofibric acid, pentachlorophenol and ketoprofen, as obtained by the fungus-augmented MBR, was better than that by both batch fungal enzymatic tests and conventional (bacterial) MBR (**Table 4.2** and **Figure 4.1**). This observation points to a synergistic effect achievable by combining a mixed culture of bacteria and white-rot fungus. This is in keeping with another study where enhanced removal of three pesticides from their mixture was demonstrated by a mixed culture of bacteria

and white-rot fungus as compared to bacteria or white-rot fungus -only cultures (Hai et al., 2012b).

Table 4.2: Removal efficiency (average \pm standard deviation, n =14) of six TrOCs consistently showing better aqueous phase removal by the fungus-augmented MBR than the conventional MBR.

TrOCs	Removal efficiency (%)	
	Fungus-augmented MBR	Conventional MBR
Fenoprop	57 \pm 25	20 \pm 15
Clofibric acid	65 \pm 17	25 \pm 3
Pentachlorophenol	92 \pm 5	60 \pm 10
Ketoprofen	94 \pm 2	65 \pm 13
Diclofenac	50 \pm 9	15 \pm 10
Naproxen	>99	45 \pm 15

4.3.3.2 Role of biosorption and biodegradation

Given the very low vapour pressure or Henry's constant (H) and low H/log D ratio of the TrOCs selected in this study (**Appendix Table A-1**), their removal by volatilization is expected to be negligible. Therefore, biosorption and biodegradation/transformation can be considered as the major mechanisms of TrOC removal by MBR. One may wonder whether the improved TrOC removal by the fungus-augmented MBR as compared to that in the batch enzymatic test was due mainly to the adsorption of the compounds on biomass in the former. However, for several of these compounds (e.g. fenoprop, clofibric acid, amitriptyline, salicylic acid, ketoprofen, and enterolactone) biosorption is likely to have played a limited role as they are hydrophilic (log D <3 (pH=5), as marked on **Figure 4.1**). Indeed, the dominant role of biodegradation can be demonstrated by **Figure 4.2** which plots the adsorption of the 30 TrOCs on fungus-augmented MBR biomass at the mid-point and the end of the operating period. **Figure 4.2** confirms the gradual reduction of the concentration of the TrOCs on fungus-augmented MBR biomass. The

role of biodegradation is also supported by **Figure 4.4** which depicts the overall fate of the selected TrOCs during fungus-augmented MBR treatment. The total loading of each TrOCs and the amount passing through the system untransformed along with permeate was calculated considering the feed/permeate volume, TrOC concentration in each and the experimental duration. The difference between the amounts fed and lost with permeate indicated the amount ‘retained’, *i.e.*, that went through transformation or simply remained adsorbed on biomass. The residual amount of untransformed TrOCs on biomass was calculated considering the MLSS concentration, reactor volume and the residual TrOC concentration on biomass at the end of the experiment, and finally, the percentage of biodegradation/transformation was estimated.

Biosorption may facilitate further biodegradation (Tadkaew et al., 2011; Wijekoon et al., 2013). However, in this study, the improved removal by the fungus-augmented MBR, as compared to *in vitro* enzymatic degradation, was not merely due to adsorption on biomass but due to progressively fortified biodegradation. This is confirmed by the data regarding the concentration of the TrOCs on MBR biomass, coupled with the fact that no biomass was withdrawn from the system except for sampling. It is noted that, irrespective of the hydrophobicity, the TrOCs for which the MBR achieved better aqueous phase removal than that observed during batch enzymatic degradation tests, biosorption accounted for less than 5% of the overall fate (**Figure 4.3**). This observation regarding the major role of fungal biodegradation in TrOC removal is in good agreement with previous reports (García-Galán et al., 2011; Zhang et al., 2012; Yang et al., 2013b).

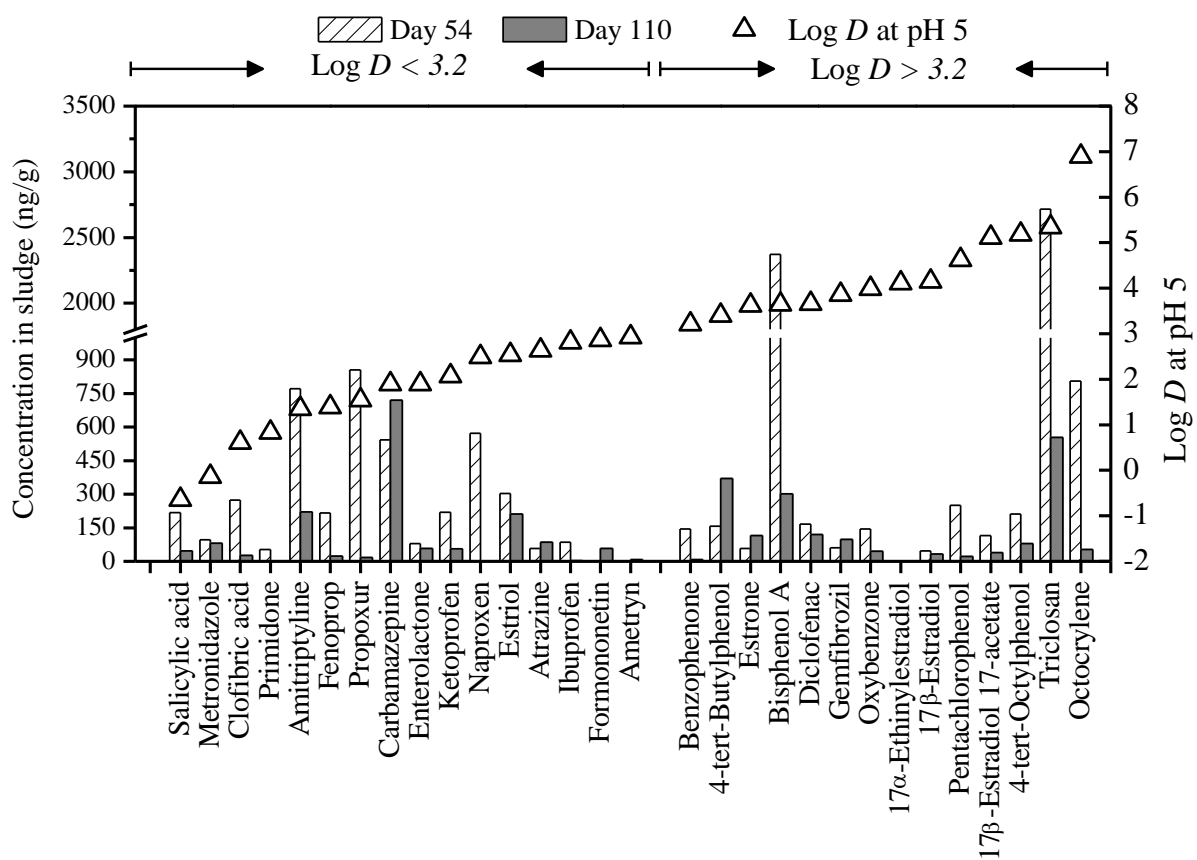


Figure 4.2: Adsorption of 30 TrOCs on fungus-augmented MBR biomass at the mid-point and the end of the operating period.

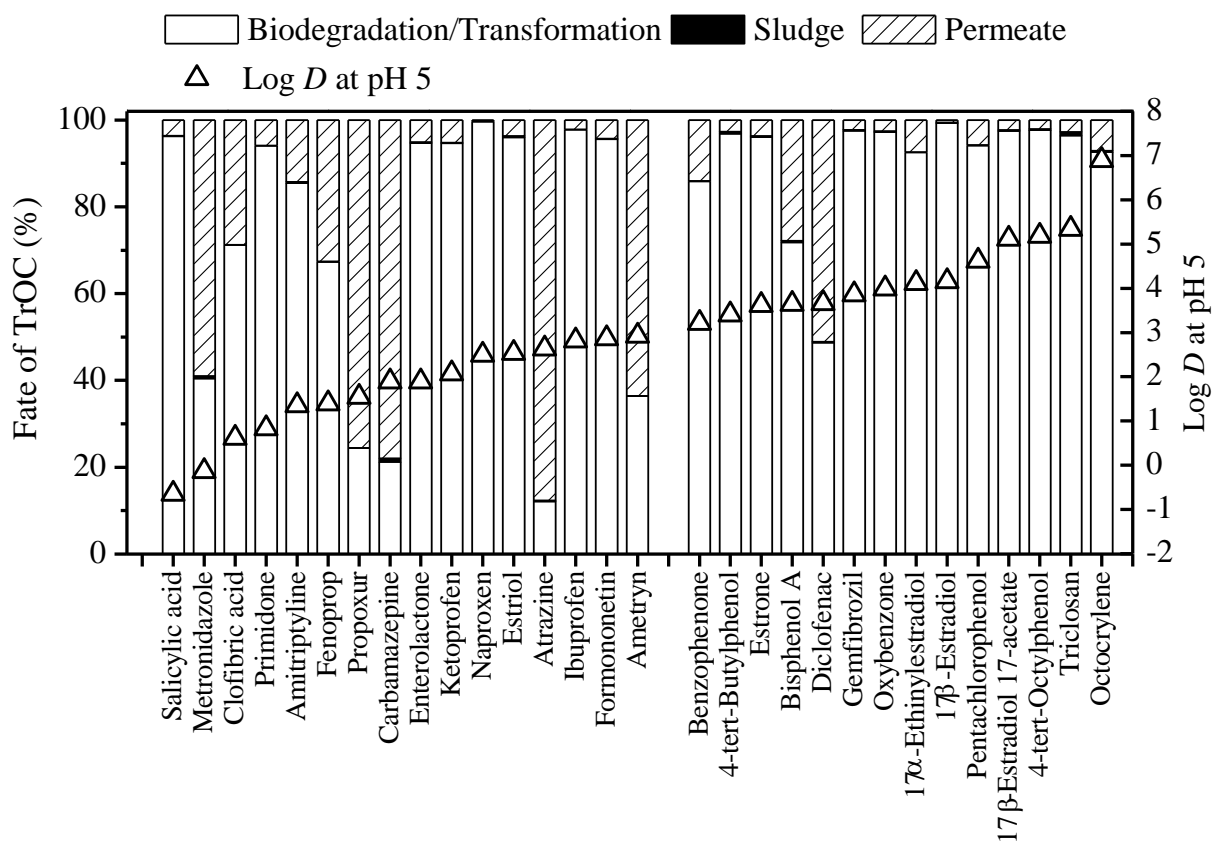


Figure 4.3: Fate of the selected TrOCs during fungus-augmented MBR treatment. Operating conditions of MBR are presented in **Section 4.2.4**.

4.3.3 Effect of continuous dosing of mediator to fungus-augmented MBR

Different levels of improvement (22% (ametryn) to 93% (atrazine)) in degradation were observed for 11 TrOCs during batch tests due to the addition of the mediator in crude enzyme solution (see **Section 3.3.2.1, Chapter 3**). Of them, all TrOCs except atrazine, ametryn and diclofenac were already well removed by the fungus-augmented MBR without mediator dosing (**Figure 4.1**), indicating the fact that fungal removal was enhanced due to bacterial presence (**Section 4.3.2.1**). The investigation with continuous dosing of mediator to fungus-augmented MBR was conducted to check whether any improvement in the remaining TrOC removal could be achieved.

No significant difference in removal by the MBR with/without mediator dosing was observed for other TrOCs (data not shown); therefore discussion here will focus on the removal of the aforementioned three compounds, namely, atrazine, ametryn and diclofenac. Only the removal of diclofenac was significantly improved by mediator dosing to MBR (**Figure 4.4**). The removal of atrazine and ametryn did not improve even after doubling the mediator dose to 10 μM (data not shown). The improvement in enzymatic degradation of ametryn as achieved during batch tests due to the mediator addition was limited ($\sim 20\%$ improvement with the final level of degradation not exceeding 30%). However, as high as 93% improvement in enzymatic degradation of atrazine was achieved due to mediator addition during batch tests (see **Section 3.3.2.1, Chapter 3**). Therefore, no increase in atrazine removal by the MBR after mediator addition necessitates further discussion.

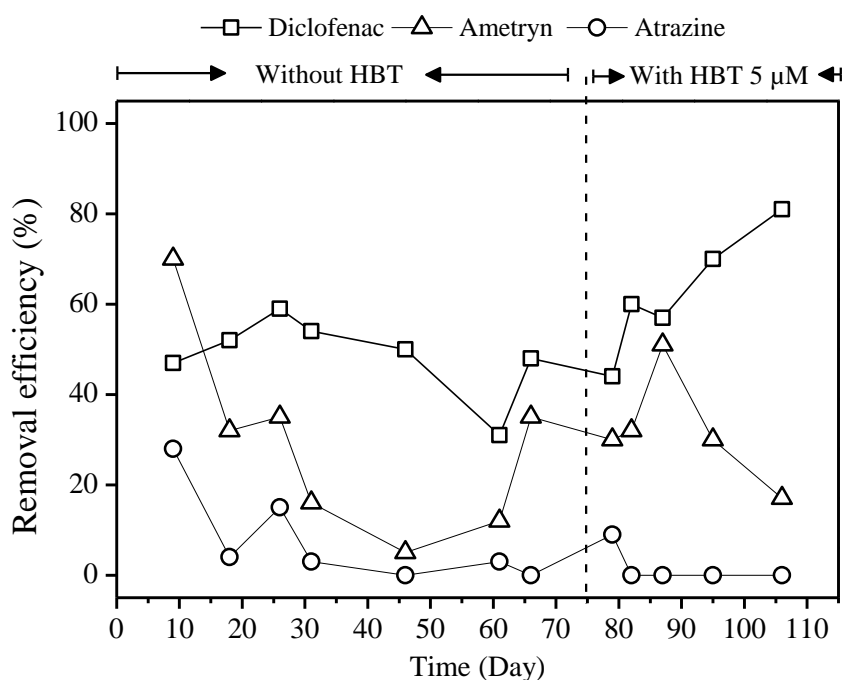


Figure 4.4: Removal of diclofenac, ametryn and atrazine by the fungus-augmented MBR with and without addition of a mediator (1-hydroxy benzotriazole) at a concentration of 5 μM . Each data point corresponds to the average of duplicate samples with less than 5% variation.

No study has systematically compared the performance of mediator-enhanced laccase system in sterile/non-sterile and batch/continuous flow systems. However, some inferences regarding the

cause of inefficient removal of atrazine by the MBR can be drawn from the available batch studies. Mediators such as HBT can cause inactivation of laccase by free radical attack on the catalytic site of the enzyme and consequently slow down the compound conversion rate, thus reducing the positive impact of mediator addition on enzymatic degradation (Kim et al., 2006b). In this study, a higher atrazine removal was achieved due to HBT addition during batch tests (**Figure 4.1**) despite the fact that the laccase activity of the batch test solution within 24 h of HBT addition dropped by *ca.* 50% (as compared to no drop in the test solution without HBT) (data not shown). However, this laccase activity lowering effect of HBT addition may have been more significant in case of the MBR where the supernatant laccase activity was mostly at a non-detectable level (although the laccase activity in biomass was always detectable, **Table 4.1**), while during batch tests the initial laccase activity of the test solution was 35 $\mu\text{M}_{(\text{DMP})}/\text{min}$. Another possible reason may be the difference between the wastewater matrix in the batch test and MBR. It is worth reiterating that, in this study, the crude enzyme solution was obtained by growing the pure culture of *T. versicolor* in MEB, while the MBR was continuously fed with a wastewater composed of MEB along with thiamine and some salts, namely, KH_2PO_4 , MgSO_4 and FeSO_4 (**Section 4.2.2**). Furthermore, in contrast to the enzyme solution used in the batch tests, the bioreactor mixed liquor was characterized by higher suspended solids. In this context, it is notable that the effect of wastewater constituents on the performance of laccase-mediator system has been reported previously in the literature. For example, the presence of humic acid in the media containing laccase and the mediator syringaldehyde reduced the degradation of the fungicide cyprodinil by 12 % (Kang et al., 2002). The presence of ions such as Cu^{2+} and Fe^{3+} in the laccase solution caused significant reduction in the conversion of triclosan (Kim et al., 2006b). These ions may interrupt the electron transport between laccase and mediator resulting in reduction of laccase-mediator performance.

4.3.4 Effluent toxicity

TrOC transformation via white-rot fungi often leads to detoxification (Jelic et al., 2012). On the other hand, increase in toxicity of white-rot fungi-treated media containing TrOCs has also been reported in the literature. For example, 1,2- hydroxy ibuprofen, the main metabolite during ibuprofen degradation by *T. versicolor*, was more toxic than ibuprofen (Marco-Urrea et al., 2009). Increased toxicity of the treated media is a particular concern associated with the addition

of mediators (Kim et al., 2006b). Therefore, the toxicity of the MBR permeate relative to the feed wastewater was tested. A ToxScreen3 assay (**Section 4.2.5.2**) revealed no significant increase in the toxicity of the effluent during MBR treatment of the synthetic wastewater (relative toxic unit of 4.1 ± 0.3 and 4.6 ± 1.1 ($n=2$), respectively). Fungus-augmented MBR, therefore, can be recommended to more efficiently remove TrOCs without the production of toxic metabolites; however, a risk assessment should be performed for each specific process (*e.g.*, wastewater matrix and TrOC types and concentrations) given the contrasting data in the literature.

4.4. CONCLUSION

Results from this study highlight that a mixed culture of bacteria and a white-rot fungus in a fungus-augmented MBR can achieve better TrOC removal than a system containing fungus or bacteria alone. The major role of biodegradation was confirmed for all TrOC for which the MBR system achieved high aqueous phase removal. Redox mediators can enhance the performance of fungal enzyme (laccase); however, compared to batch tests, due to lower enzymatic activity level and more complex wastewater matrix, limited improvement in removal due to mediator dosing may be achieved by the MBR for compounds originally highly resistant to fungal degradation.

In this study, viable fungal count in the fungus-augmented MBR and consistent detection of laccase activity in the MBR biomass confirmed the active presence of the white-rot fungi *T. versicolor* that was originally inoculated into the MBR. Nevertheless, unlike the pure culture laccase activity was only occasionally detected in the mixed liquor supernatant of the MBR. Bacterial growth is often faster than fungi and it may negatively affect the fungal growth and enzymatic activity. Further studies will need to be carried out to ensure fungal dominance and stability of fungal enzymatic activity in such MBRs.

4.5. REFERENCES

- Blázquez, P. and B. Guieysse (2008a). Continuous biodegradation of 17β -estradiol and 17α -ethynylestradiol by *Trametes versicolor*. J. Hazard. Mater., 150, 459-462.
- Blázquez, P., M. Sarraà and T. Vicent (2008b). Development of a continuous process to adapt the textile wastewater treatment by fungi to industrial conditions. Process Biochem. 43, 1-7.
- Bourbonnais, R., D. Leech and M. G. Paice (1998). Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochimica et Biophysica Acta (BBA) - General Subjects. 1379, 381-390.

- Coniglio, A., C. Galli, P. Gentili and R. Vadalà (2008). Oxidation of amides by laccase-generated aminoxyl radicals. *J. Mol. Catal., B Enzym.*, 50, 40-49.
- Eaton, A. D., L. S. Clescerl, E. W. Rice and A. E. Greenberg, Eds. (2005). *Standard Methods for Examination of Water & Wastewater*. Washington, D.C., American Public Health Association.
- García-Galán, J., C. E. Rodríguez-Rodríguez, T. Vicent, G. Caminal, M. S. Díaz-Cruz and D. Barceló (2011). Biodegradation of sulfamethazine by *Trametes versicolor*: Removal from sewage sludge and identification of intermediate products by UPLC–QqTOF-MS. *Sci. Total Environ.*, 409, 5505-5512.
- Hai, F. I., L. D. Nghiem, S. J. Khan, W. Price and K. Yamamoto (2013). Wastewater reuse: Removal of Emerging Trace Organic Contaminants. *Membrane Biological reactors*. F. I. Hai, K. Yamamoto and C.-H. Lee. UK, IWA publishing.
- Hai, F. I., K. Yamamoto, F. Nakajima and K. Fukushima (2009). Factors governing performance of continuous fungal reactor during non-sterile operation – The case of a membrane bioreactor treating textile wastewater. *Chemosphere*. 74, 810-817.
- Hai, F. I., K. Yamamoto, F. Nakajima and K. Fukushima (2012a). Application of a GAC-coated hollow fiber module to couple enzymatic degradation of dye on membrane to whole cell biodegradation within a membrane bioreactor. *J Mem Sci*. 389, 67-75.
- Hai, F. I., K. Yamamoto, F. Nakajima and K. Fukushima (2012b). Application of a GAC-coated hollow fiber module to couple enzymatic degradation of dye on membrane to whole cell biodegradation within a membrane bioreactor. *J. Membr. Sci.*, 389, 67-75.
- Hata, T., H. Shintate, S. Kawai, H. Okamura and T. Nishida (2010). Elimination of carbamazepine by repeated treatment with laccase in the presence of 1-hydroxybenzotriazole. *J. Hazard. Mater.*, 181, 1175-1178.
- Jelic, A., C. Cruz-Morató, E. Marco-Urrea, M. Sarra, S. Perez, T. Vicent, M. Petrović and D. Barcelo (2012). Degradation of carbamazepine by *Trametes versicolor* in an air pulsed fluidized bed bioreactor and identification of intermediates. *Water Res.* 46, 955-964.
- Kang, K.-H., J. Dec, H. Park and J.-M. Bollag (2002). Transformation of the fungicide cyprodinil by a laccase of *Trametes villosa* in the presence of phenolic mediators and humic acid. *Water Res.* 36, 4907-4915.
- Kim, Y., Jin and J. A. Nicell (2006a). Laccase catalysed oxidation of aqueous triclosan. *J. Chem. Technol. Biotechnol.*, 81, 1344-1352.
- Kim, Y. J. and J. A. Nicell (2006b). Impact of reaction conditions on the laccase-catalyzed conversion of bisphenol A. *Bioresource Technol.* 97, 1431-1442.
- Libra, J. A., M. Borchert and S. Banit (2003). Competition strategies for the decolorization of a textile-reactive dye with the white-rot fungi *Trametes versicolor* under non-sterile conditions. *Biotechnol. Bioeng.*, 82, 736-744.
- Lloret, L., G. Eibes, G. Feijoo, M. T. Moreira and J. M. Lema (2012). Degradation of estrogens by laccase from *Myceliophthora thermophila* in fed-batch and enzymatic membrane reactors. *J. Hazard. Mater.*, 213–214, 175-183.
- Marco-Urrea, E., M. Perez-Trujillo, T. Vicent and G. Caminal (2009). Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. *Chemosphere*. 74, 765-772.
- Nguyen, L. N., F. I. Hai, J. Kang, W. E. Price and L. D. Nghiem (2012). Removal of trace organic contaminants by a membrane bioreactor–granular activated carbon (MBR–GAC) system. *Bioresource Technol.* 113, 169-173.

- Rodarte-Morales, A. I., G. Feijoo, M. M.Teresa and J. Lema (2012). Evaluation of two operational regimes fed-batch and continuous for the removal of pharmaceuticals in a fungal stirred tank reactor. *Chem. Eng. Trans.*, 27, 151-156.
- Schwarzenbach, R. P., E. Thomas, H. Thomas, G. Urs Von and W. Bernhard (2010). Global Water Pollution and Human Health. *Annual Review of Environment and Resources*. 35, 109-136.
- Tadkaew, N., F. I. Hai, J. A. McDonald, S. J. Khan and L. D. Nghiem (2011). Removal of trace organics by MBR treatment: The role of molecular properties. *Water Res.* 45, 2439-2451.
- Tran, N. H., T. Urase and O. Kusakabe (2010). Biodegradation characteristics of pharmaceutical substances by whole fungal culture *Trametes versicolor* and its laccase. *Journal of Water and Environment Technology*. 8, 125-140.
- Wijekoon, K. C., T. Fujioka, J. A. McDonald, S. J. Khan, F. I. Hai, W. E. Price and L. D. Nghiem (2013). Removal of N-nitrosamines by an aerobic membrane bioreactor. *Bioresource Technol.*
- Yang, S., F. I. Hai, L. D. Nghiem, L. N. Nguyen, F. Roddick and W. E. Price (2013a). Removal of bisphenol A and diclofenac by a novel fungal membrane bioreactor operated under non-sterile conditions. *Int. Biodeterior. Biodegradation*. 85, 483-490.
- Yang, S., F. I. Hai, L. D. Nghiem, W. E. Price, F. Roddick, M. T. Moreira and S. F. Magram (2013b). Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: a critical review. *Bioresource Technol.* 141, 97-108.
- Zhang, Y. and S.-U. Geißen (2012). Elimination of carbamazepine in a non-sterile fungal bioreactor. *Bioresource Technol.* 112, 221-227.

CHAPTER 5: Trace organic contaminant removal by an enzymatic membrane reactor: Complementary role of membrane retention and enzymatic degradation

This chapter has been published as:

Luong N Nguyen, Faisal I Hai, William E Price, Frederic DL Leusch, Felicity Roddick, Ewan J McAdam, Saleh F Magram, Long D Nghiem. (2014) Continuous biotransformation of bisphenol A and diclofenac by laccase in an enzymatic membrane reactor. *International Biodeterioration & Biodegradation*. 95, 25-32.

Luong N Nguyen, Faisal I Hai, William E Price, Jinguo Kang, Frederic DL Leusch, Felicity Roddick, Jason P van de Merwe, Saleh F Magram, Long D Nghiem. (2014) Degradation of a broad spectrum of trace organic contaminants by an enzymatic membrane reactor: Complementary role of membrane retention and enzymatic degradation. *International Biodeterioration & Biodegradation*, 99, 115-122.

5.1. INTRODUCTION

Trace organic contaminants (TrOCs) are ubiquitous in wastewater, and water sources polluted by wastewater. Conventional wastewater treatment plants do not effectively remove TrOCs (Luo et al., 2014). Thus for both safe discharge into the environment and wastewater reuse it is important to develop effective wastewater treatment processes.

Enzymatic degradation of wastewater-borne resistant pollutants has gained much attention in recent years. Compared to conventional chemical oxidation, enzymatic degradation can be achieved under milder conditions, while realizing higher rates and reaction specificity. Laccases (EC 1.10.3.2) are copper-containing oxidoreductase enzymes that can use atmospheric oxygen as the terminal oxidant. Laccase has been used in various industrial biotechnology processes such as denim bleaching and pulp delignification. It has also been reported to efficiently degrade resistant compounds including aromatic hydrocarbons and dyes (Modin et al., 2014). Recent studies demonstrate that laccase can efficiently degrade a broad spectrum of TrOCs that are hardly degradable by conventional biological processes (Cabana et al., 2007; Yang et al., 2013a). Notably, most of the available studies on enzymatic degradation of resistant compounds in general and TrOC in particular have been conducted in small scale and batch mode.

Enzymatic TrOC degradation may depend on various factors such as chemical structure of the TrOCs, chemistry of the reaction media (*i.e.*, pH, temperature and ionic strength), and the characteristics of the enzyme applied (Yang et al., 2013a). Laccase can efficiently degrade compounds with phenolic moiety including diphenols, methoxy-substituted monophenols as well as aromatic/aliphatic amines. For compounds which possess higher redox potential than laccase, or are too large to gain access to the active sites of the enzyme, addition of redox-mediators may facilitate their oxidation. Mediators are low-molecular weight substrates of laccase which can act as “electron carrier” between the enzyme and the target pollutant. However, the efficiency of a laccase-mediator system depends largely on mediator type and the molecular structure of TrOCs (Yang et al., 2013a).

Enzyme-washout with treated effluent is a critical problem encountered during their application in continuous systems such as wastewater treatment plants. By using a membrane with an appropriate pore size relative to the enzyme molecule, an enzymatic membrane reactor (EMR)

can prevent enzyme washout. This approach offers several advantages over other alternatives: (i) EMR retains enzymes more effectively than conventional packed bed reactors, (ii) operation with free enzyme avoids limitation of mass transfer associated with immobilization on carriers, and (iii) enzyme can be easily replenished during long term operation (Modin et al., 2014). To date, only a few studies have explored continuous biotransformation of TrOCs by EMR (Lloret et al., 2012; Nguyen et al., 2014d). Lloret et al. (2012) reported high removal of phenolic TrOCs, namely estradiol and estrone; however, that study was conducted for only 8 h. High and stable biotransformation of both bisphenol A and diclofenac by an EMR was demonstrated by Nguyen et al. (2014c). Nguyen et al. (2014d) investigated the removal of four non-phenolic compounds, namely diclofenac, carbamazepine, sulfamethoxazole, and atrazine, and proposed simultaneous dosing of mediator and activated carbon to enhance their removal efficiencies.

Most, if not all, studies to date on TrOC removal by EMR have focused on a few compounds at a time. For the establishment of a uniform database regarding EMR performance, investigation of a broader spectrum of TrOCs is imperative. Another aspect that requires further systematic investigation is the role of the membrane in enzymatic degradation of TrOCs in an EMR. Ultrafiltration membranes typically used in EMRs cannot retain TrOCs. However, enzyme gel layer, which typically forms on membrane surface, can adsorb the TrOCs. Thus the membrane may facilitate enzymatic degradation of TrOCs. A few studies have alluded to this aspect (Nguyen et al., 2014d), however, any systematic study elucidating the phenomena involved, particularly the extent of adsorption and biodegradation during prolonged operation, has not been reported.

The objective of this study was to assess the performance of an EMR utilizing a commercially available laccase from *Aspergillus oryzae* for the removal of 30 chemically diverse TrOCs (*e.g.*, phenolic/non-phenolic moieties and electron releasing/demanding substituent groups). Baseline batch tests provided valuable insight into the EMR performance. The effect of addition of redox-mediators, namely 1-hydroxybenzotriazole (HBT) or syringaldehyde (SA), on the enzymatic TrOC degradation was highlighted. Particularly, the complementary role of TrOC retention by the gel layer on the membrane and their enzymatic degradation was systematically elucidated.

5. 2. MATERIALS AND METHODS

5.2.1 Trace organic contaminants, laccase and mediators

Firstly, the EMR was operated for a preliminary assessment of the removal of two model compounds namely bisphenol A (BPA) and diclofenac (DCF). Key properties of these compounds are summarised in (**Appendix Table A-1**). A stock solution of the TrOCs was prepared at a concentration of 1 g/L of each compound in pure methanol, stored at -18 °C and used within one month. Based on the preliminary data, the EMR was operated for the removal of a broad spectrum of 30 TrOCs. The details of TrOCs have been presented in **Section 3.2.1, Chapter 3**.

A commercially available laccase (Novozym 51003) purified from the culture of genetically modified *A. oryzae* was supplied by Novozymes (Novozymes Australia Pty. Ltd). A genomic DNA segment encoding an extracellular laccase was isolated from the thermophilic fungus *Myceliophthora thermophila*, and the nucleotide sequence of this gene was determined. A vector containing the *M. thermophila* laccase coding region was constructed for heterologous expression in *A. oryzae* (Berka et al., 1997). The enzyme had a molecular weight of 56 kDa, and the enzyme stock solution had a density, purity and activity of 1.12 g/mL, approximately 10% (w/w) and 150,000 $\mu\text{M}_{(\text{DMP})}/\text{min}$, respectively.

Properties of the mediators are summarized in **Section 3.2.1, Chapter 3**.

5.2.2 Batch test description

5.2.2.1 Effect of pH

In this study, enzymatic degradation of BPA and DCF was investigated under a range of pH from acidic to basic (*i.e.*, 3, 4.5, 6, 7 and 9). The experiments were conducted in screw-capped test tubes (13 mL). All laboratory apparatus was sterilized by autoclaving before use to maintain aseptic conditions. The concentrated stock solution (3 μL) of laccase was diluted to a final volume of 5 mL in the test tubes by Milli-Q water to obtain an initial enzymatic activity of approximately 180 $\mu\text{M}_{(\text{DMP})}/\text{min}$. The TrOCs were added at an initial nominal concentration of 1000 $\mu\text{g}/\text{L}$ into test tubes (actual concentrations of 1100 ± 10 and 860 ± 10 $\mu\text{g}/\text{L}$ ($n=3$) for BPA and DCF, respectively). Sodium citrate at a concentration of 100 mM was used to control the pH

of the reaction media. All test tubes were incubated in a rotary shaker at 70 rpm and 28 °C (Bioline Shaker Incubator BL 8600, Edwards Group Pty. Ltd, NSW, Australia), and samples were collected at 2, 4, 8 and 22 h for TrOC analysis and enzymatic activity measurement. To verify that degradation took place only due to enzymatic oxidation, control samples containing only TrOCs in Milli-Q water (without enzyme) were incubated in parallel.

5.2.2.2 Effect of redox-mediator addition

The effect of addition of the redox-mediator SA on the degradation of BPA and DCF as well as the stability of enzymatic activity was tested. Based on the results from the tests described in **Section 5.3.1.1**, the effect of SA concentration was studied at a pH of 7. SA concentrations of 5, 100, 500, and 1000 µM were selected based on the concentration-range used in other batch test studies (Mizuno et al., 2009; Mendoza et al., 2011). All other steps were similar to those described in **Section 5.2.2.1**.

5.2.2.3 Batch degradation of a broader set of TrOCs

The degradation capacity of laccase utilized in this study was further tested for the degradation of 30 diverse TrOCs. The test solution contained an enzymatic activity of *ca.* 180 µM_(DMP)/min (dilution of 30 µL stock laccase solution by Milli-Q water to 25 mL). An aliquot of the stock TrOC cocktail was added to the test solution to obtain an initial nominal concentration of 100 µg/L of each TrOCs. The impact of redox mediator addition on laccase degradation of TrOC was assessed by adding SA or HBT (10 µM) to the test solution. The mediator concentration was selected following a baseline investigation with bisphenol A and diclofenac (Nguyen et al., 2014d). TrOCs in Milli-Q water (without laccase) served as control. The pH of the test solution was 6.8 ± 0.2. All the containers were covered and incubated at 25 °C for 24 h in a rotary shaker (70 rpm). The experiments were conducted in triplicate. The whole test solution was collected for TrOC analysis at the end of the incubation period. The samples were diluted to 500 mL, filtered through 0.45 µm glass fiber filter, and pH immediately adjusted to 2 by adding H₂SO₄ (4 M).

5.2.3 EMR system and operation protocol

5.2.3.1 EMR setup

A laboratory scale EMR system consisting of a 1.5 L (active volume) glass reactor was used (**Figure 5.1**). An ultrafiltration (UF), hollow fiber membrane module made of polyacrylonitrile was submerged in the reactor. The membrane was supplied by Microza Membranes (Pall Corporation, NSW, Australia). It had a molecular weight cut off (MWCO), surface area and clean water flux of 6 kDa, 0.19 m², and 10 L/h.bar, respectively.

Stock solution (1 mL) of laccase was diluted to a final volume of 1.5 L in the reactor by Milli-Q water to obtain an initial enzymatic activity of approximately 180 $\mu\text{M}_{(\text{DMP})}/\text{min}$. The membrane was operated at a flux of 1.1 L/m².h via a peristaltic pump (Masterflex L/S, USA) with a 8 min on and 1 min off cycle, and the permeate was returned to the reactor. The reactor was placed in a water bath with a temperature control unit (Julabo, Germany) to maintain the temperature at 28 °C. Dissolved oxygen (DO) concentration was maintained at above 3 mg/L via an air pump (ACO-002, Zhejiang Sensen Industry Co. Ltd, Zhejiang, China) connected to a diffuser placed at the bottom of the reactor. The pH of the reactor contents was 6.8 ± 0.2 . Transmembrane pressure (TMP) was continuously monitored using a high-resolution (± 0.1 kPa) pressure sensor (SPER scientific 840064, Extech equipment Pty. Ltd., Victoria, Australia) connected to a computer for data logging.

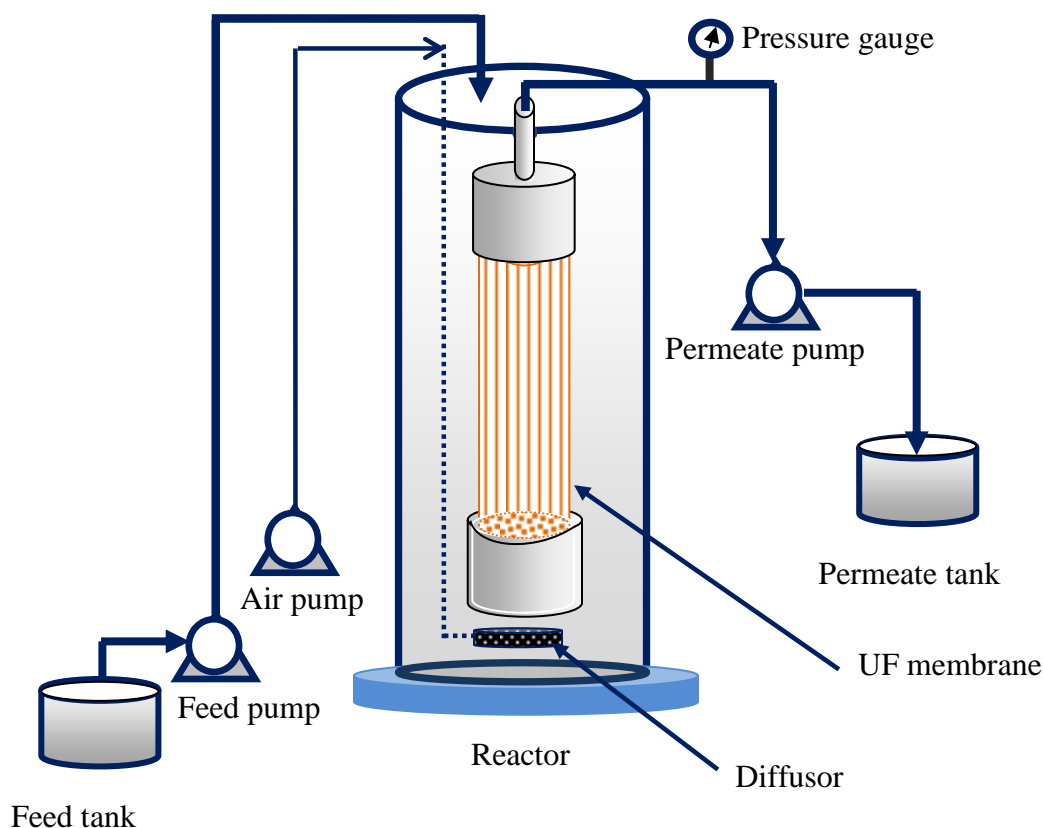


Figure 5.1: Schematic diagram of the enzymatic membrane reactor

5.2.3.2 Retention of soluble enzyme by UF membrane and enzymatic activity

The EMR was first operated to confirm retention of the enzyme by the UF membrane. Permeate and reactor supernatant samples were taken every 2 h for enzymatic activity measurement over a period of 24 h for this specific part of the experiment.

Once enzyme retention by the membrane was confirmed, the stability of enzymatic activity under the applied hydraulic conditions was tested. Feed containing TrOCs in Milli-Q water (4.5 L) was prepared daily. The EMR was operated at a hydraulic retention time (HRT) of 8 h, and the TrOC concentration in the feed water was maintained such that a TrOC loading rate of approximately 500 $\mu\text{g/L.d}$ (for each TrOC) was applied. Operation was initiated with an enzymatic activity of 90 $\mu\text{M}_{(\text{DMP})}/\text{min}$. Permeate and reactor supernatant samples were taken

every 5 h for TrOC removal and enzymatic activity measurement until no enzymatic activity was detected in the reactor (72 h). Following this, another dose of enzyme was added to the EMR to reinstate the enzymatic activity to the initial level ($90 \mu\text{M}_{(\text{DMP})}/\text{min}$), and the above run was repeated.

5.2.3.3 Preliminary assessment of TrOC degradation by EMR

The EMR was continuously operated to investigate TrOC degradation. Laccase solution ($200 \mu\text{L}$ laccase/L reactor volume) was re-injected every 12 h into the EMR to maintain the enzymatic activity within a range of $70\text{--}100 \mu\text{M}_{(\text{DMP})}/\text{min}$. This run was conducted at an HRT of 8 h, and the TrOC loading rates were $570 \pm 70 \mu\text{g/L d}$ and $480 \pm 40 \mu\text{g/L d}$ for BPA and DCF, respectively. Permeate and reactor supernatant samples were taken every 5 h for TrOC concentration and enzymatic activity measurements over a period of 132 h.



Figure 5.2: Experimental set-up of EMR

5.2.3.4 Degradation of a diverse set of TrOCs

In this test, the removal of broad spectrum (30 compounds) TrOC was investigated in the EMR. To start up the EMR, stock solution (2 mL) of laccase was added to 1.5 L Milli-Q water. This resulted in an enzymatic activity of *ca.* $180 \mu\text{M}_{(\text{DMP})}/\text{min}$ in the reactor. The enzymatic activity in this study was double of the preliminary test. A peristaltic pump was used to operate the membrane intermittently (8 min on and 1 min off) at an average flux of $1.1 \text{ L/m}^2 \text{ h}$. This resulted in a hydraulic retention time (HRT) of 8 h. Feed solution containing TrOCs at a concentration of $5 \mu\text{g/L}$ in Milli-Q water was continuously fed to the reactor. The EMR was first operated for 72 h

(*i.e.*, $9 \times \text{HRT}$) without any mediator. After operating the EMR for a period of $4 \times \text{HRT}$, collection of TrOC samples was initiated, and three sets of feed, supernatant and permeate samples were collected at equal intervals over the rest of the operation period.

5.2.3.5 Effect of dosing mediator

The mediator (SA or HBT) was continuously fed to the reactor, obtaining a final mediator concentration of $10 \mu\text{M}$, to study the effect of mediator addition on enzymatic degradation of TrOCs. To avoid any possible interaction between the mediators and TrOCs during the storage time in the feed tank, feed containing the TrOCs and mediators was prepared daily and added separately by two different pumps.

5.2.3.6 Role of membrane and enzymatic degradation

In an EMR, a gel layer can form on the membrane surface due to retention of the enzyme by the membrane. Such a gel layer may facilitate TrOC retention and possibly their subsequent enzymatic degradation. In order to clarify the role of the membrane gel layer, TrOC removal performance of the EMR with addition of active and heat-inactivated laccase (laccase incubated at 80°C for 10 min) was compared. Four TrOCs (*i.e.*, pentachlorophenol, oxybenzone, ibuprofen and benzophenone) which represented a group of compounds showing high removal by the EMR were selected for this investigation. To reach the adsorption capacity of the enzyme gel layer, TrOC concentration in feed was increased to $100 \mu\text{g/L}$ of each compound for this investigation. All other parameters were kept the same as described in **Section 5.2.3.4**.

5.2.4 Analytical methods

5.2.4.1 TrOC analysis

The concentration of bisphenol A, diclofenac, pentachlorophenol, oxybenzone, benzophenone and ketoprofen was measured by a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a Supelco Drug Discovery C-18 column (with diameter, length and pore size of 4.6 mm, 150 mm, and $5 \mu\text{m}$, respectively) and a UV-Vis detector. The column temperature was set at 20°C . A sample injection volume of $50 \mu\text{L}$ was used. The mobile phase composed of acetonitrile and Milli-Q grade deionized water buffered with $25 \text{ mM KH}_2\text{PO}_4$. Two eluents, namely, eluent A (80 % acetonitrile + 20% buffer, v/v) and eluent B (20 % acetonitrile + 80 % buffer, v/v) were

used. Eluent A and B were delivered through the column in time-dependent gradient proportions (**Table 5.1**). Calibration always yielded standard curves with coefficients of determination (R^2) greater than 0.98 within the range of experimental concentrations used. The quantification limit for the analytes under investigation using these conditions was approximated at 10 µg/L.

The concentration of 30 TrOCs was measured by a GC-MS system. The method has been described in **Section 3.2.4.1** of **Chapter 3**.

Table 5.1: Gradient eluent profiles used in HPLC-UV analyses

For bisphenol A and diclofenac (wavelength: 280 nm, eluent flow rate: 1 mL/min)								
Time (min)	0	12	20	25				
Eluent B, %	80	80	0	80				
For pentachlorophenol, oxybenzone and benzophenone (wavelength: 280 nm, eluent flow rate: 0.7 mL/min)								
Time (min)	0	12	20	25	25			
Eluent B, %	85	80	0	0	80			
For ketoprofen (wavelength: 225 nm, eluent flow rate: 0.7 mL/min)								
Time (min)	0	7	19	20	35			
Eluent B, %	50	50	20	50	50			

5.2.4.2 Enzymatic activity and toxicity assay

Laccase activity and toxicity assay are summarized in **Section 3.2.4.2**, **Chapter 3**.

5.3. RESULTS AND DISCUSSION

5.3.1 TrOC removal in batch tests

5.3.1.1 Effect of pH

BPA was degraded almost completely (96%) within 4 h of incubation at a pH of 7, while over the same period a degradation of 56%, 61%, 88% and 74% was achieved at a pH of 3, 4.5, 6 and 9, respectively (**Table 5.2**). However, irrespective of the media pH, at least 80% removal of BPA was observed after 22 h (**Figure 5.3**). The overall high degradation of BPA can be attributed to the strong electron donating group (EDG) –OH in its structure which renders it a good substrate of laccase (Yang et al., 2013b). This observation is consistent with that in the available literature. For example, a complete degradation of three different bisphenols (bisphenol

A, bisphenol B and bisphenol F) by laccase within 1.5 h of incubation was reported by Nicolucci et al. (2011). Furthermore, the slightly lower removal of BPA at highly acidic (pH 3) and alkaline (pH 9) conditions may be attributed to the significant (*i.e.*, 46% and 53%) inactivation of the laccase activity within 2 h of incubation. Kim and Nicell (2006b) also reported a complete removal of BPA in the pH range of 5 - 7, which decreased only slightly in the acidic regime (pH 3 - 4).

Table 5.2: Effect of incubation pH and duration on TrOC degradation by laccase (mean \pm standard deviation from triplicate experiments).

pH	BPA removal (%)				DCF removal (%)			
	Incubation Time (h)							
	2	4	8	22	2	4	8	22
3	43 ± 5.0	56 ± 3.5	77 ± 0.5	89 ± 3.0	36 ± 1.6	57 ± 3.5	69 ± 2.3	91 ± 4.6
4.5	51 ± 0.5	61 ± 3.5	82 ± 3.2	94 ± 1.5	44 ± 7.2	50 ± 1.0	82 ± 0.5	98 ± 2.0
6	79 ± 0.5	88 ± 9.4	92 ± 1.8	95 ± 0.5	42 ± 3.5	51 ± 2.8	65 ± 1.7	71 ± 4.5
7	87 ± 4.0	96 ± 1.5	100 ± 0	100 ± 0	29 ± 8.4	33 ± 2.5	40 ± 2.8	59 ± 7.8
9	69 ± 4.5	74.0 ±	84 ± 3.2	83 ± 0.7	11 ± 3.4	30 ± 5.9	48 ± 2.8	61 ± 1.7

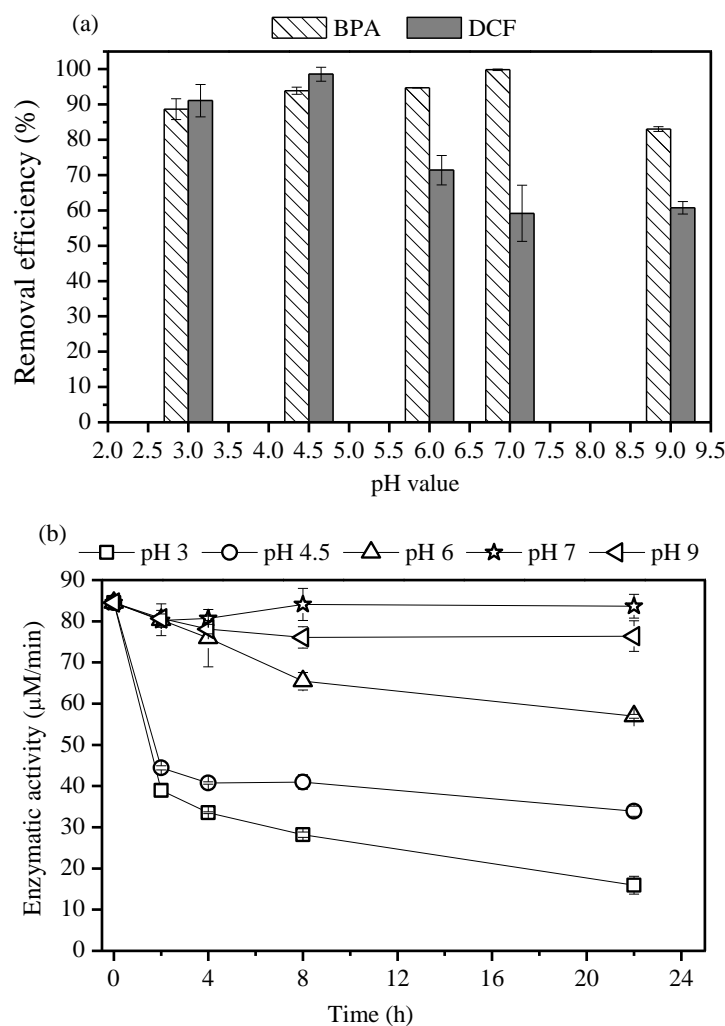


Figure 5.3: Removal of BPA and DCF (a), and the change in enzymatic activity (b) during treatment by laccase at different pH. Control samples contained TrOC in Milli-Q water in absence of laccase. Error bars represent the standard deviation of three samples.

Unlike BPA, DCF degradation was significantly better at the acidic pH (3 and 4.5), and the removal consistently decreased irrespective of the incubation period with the increase in media pH beyond a value of 4.5 (**Table 5.2**). For example, compared to a complete removal after 22 h of incubation at a pH of 4.5, DCF removal reached only 61% at pH 7 over the same period (**Figure 5.3**). Lloret et al. (2010) also reported high removal of DCF by laccase at acidic pH. According to Sathishkumar et al. (2014), the drop in laccase mediated degradation at alkaline pH is probably due to the combination of hydroxide ion inhibition and its binding with the T2/T3

site of laccase (Sathishkumar et al., 2014). Instances of significant effect of pH on the degradation of DCF by other enzymes can also be found in the literature. For example, Zhang and Geissen (2010) reported that DCF was completely degraded by lignin peroxidase (LiP) in the pH range of 3 - 4.5. However, its degradation by LiP significantly decreased from complete removal at a pH of 4.5 to 10% at a pH of 6.

Not only the extent of removal but also the stability of enzymatic activity is important, particularly in case of a continuous flow reactor. In this study, significant inactivation of laccase activity at acidic pH was observed. In contrast, almost no enzyme inactivation was found at the neutral pH (**Figure 5.3**). As a compromise between treatment efficiency and stability of enzymatic activity, and considering the ease of operation under neutral pH (avoiding the requirement of pH adjustment), further investigation was conducted at a pH of 7.

5.3.1.2 Effect of redox-mediator addition

BPA was completely removed within 4 h of incubation irrespective of the mediator concentration. This was expected as BPA was completely removed even without mediator addition. However, DCF removal was significantly improved after SA addition. For example, over 80% removal was achieved within 8 h at all mediator concentrations tested (**Figure 5.4**). This compares favourably with a 40% removal of DCF in the absence of SA over the same period. Furthermore, the addition of SA (at all concentrations) achieved a complete removal of DCF within 22 h (end of observation), while the removal in the absence of SA was only 60%. This observation is in line with the literature. For example, Lloret et al. (2010) observed an enhancement of DCF removal from less than 5% to complete removal due to the addition of SA at a concentration of 100 μ M. The better performance of the mediator-amended enzyme may be explained by two factors attributable to the radical species generated from SA by laccase: i) higher redox potential, and ii) reduction of steric hindrance. Compounds with high electrochemical potential can be oxidized by radical mediators (*e.g.*, phenoxyl produced from SA) through the operation of H-abstraction mechanism. The laccase-mediator system can achieve better removal of compounds with high electrochemical potential as the oxidized mediator species have redox potentials higher than that of laccase only (Nguyen et al., 2014a). In this study, tests with TrOCs and SA (without laccase) yielded no TrOC degradation (data not shown).

Therefore, the observed improvement in degradation was due to the enhancement of enzymatic degradation by SA, not due to direct degradation by SA.

Overall, the batch test data reveals two options for a continuous flow reactor to achieve high removal of the TrOCs, particularly DCF: i) to operate at acidic pH with frequent replenishment of enzyme (due to aggravated enzyme deactivation under this pH), or ii) to operate at neutral pH with mediator dosing. The latter option was chosen in this study from the point of view of enzymatic stability.

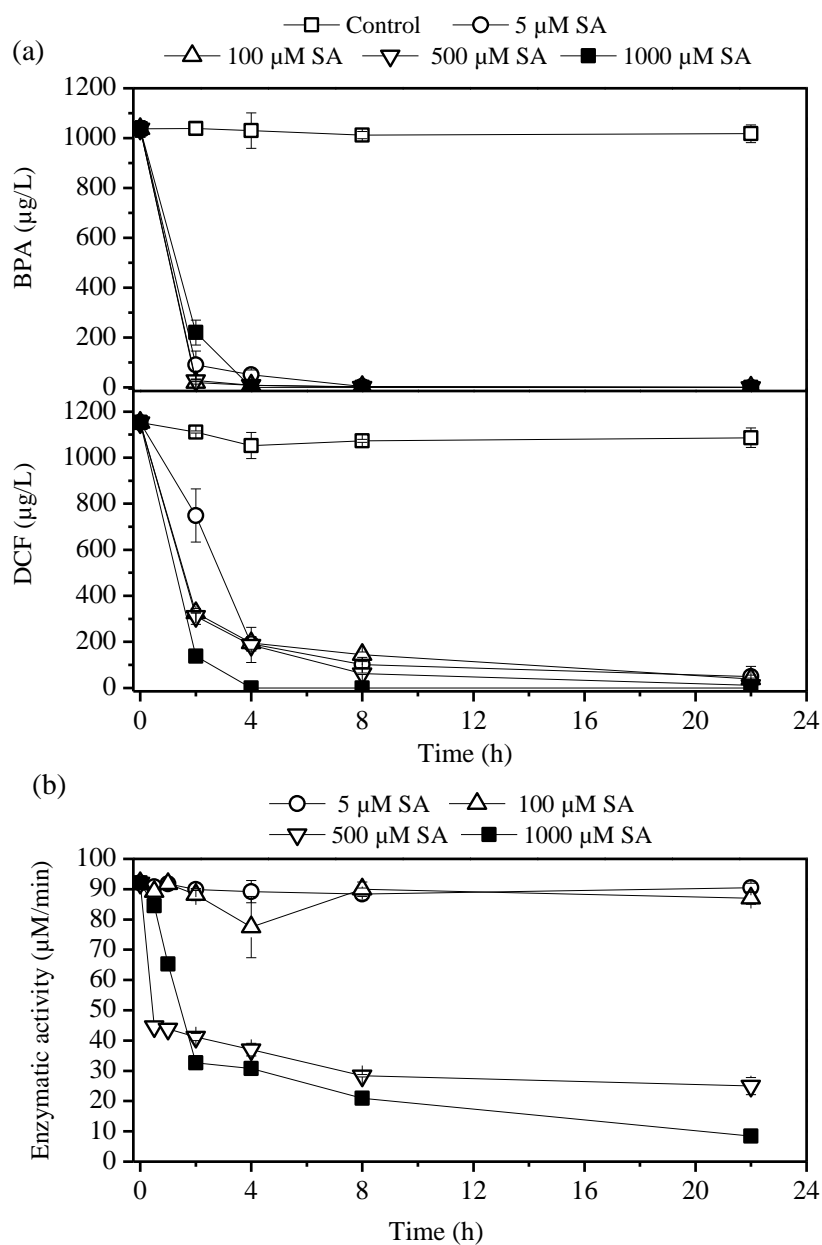


Figure 5.4: Concentration of BPA and DCF (a), and the change in enzymatic activity (b) during treatment by laccase in the presence of a redox-mediator (syringaldehyde) at different concentrations (5, 100, 500, and 1000 μM). Control samples contained TrOC and mediator in Milli-Q water in absence of laccase. Error bars represent the standard deviation of three samples.

5.3.1.3 Impact of mediator dose on performance

The effect of mediator concentration could be clearly observed at shorter incubation periods (**Figure 5.4**). For example, for an incubation period of 2 h, DCF was removed by 90% at an SA concentration of 1000 μM . In comparison, the removal of DCF for the same period ranged between 30 - 70% for SA concentrations between 5 - 500 μM . Similarly, Lloret et al. (2010) observed complete degradation of DCF by laccase within 1 h in presence of 1000 μM SA, while the removal ranged from 40 - 80% for the lower SA concentrations (*i.e.*, 100 - 500 μM). The effect of mediator concentration has also been reported for other combinations of resistant pollutants and mediators. A notable example is the enhanced degradation of iso-butylparaben and n-butylparaben due to the dosing of a mediator (1-hydroxybenzotriazole, HBT) in the range of 200-2000 μM (Mizuno et al., 2009).

In contrast to better DCF removal, higher SA concentrations (*i.e.*, 500 and 1000 μM) resulted in a remarkable decrease in laccase activity (**Figure 5.4**). For example, within 4 h of incubation, enzymatic activity of the test media reduced by 60% for an SA dose of 500 μM . Mediator-induced inactivation of enzyme has been previously reported (Kurniawati et al., 2007; Khlifi-Slama et al., 2012). Loss of enzymatic activity has been attributed to the degradation of essential amino acid residues or the glycosyl moieties at the surface of the enzyme (Khlifi-Slama et al., 2012). However, the range of mediator concentrations beyond which a fast and significant laccase inactivation occurs tends to vary depending on the source of laccase and the type of the mediator. For example, in the study by Khlifi-Slama et al. (2012), the inactivation of laccase from *Trametes troglia* was proportional to the mediator (HBT) concentrations in the range from 100 - 10,000 μM . The authors explained that free radicals generated from HBT inactivated the laccase. On the other hand, Kurniawati and Nicell (2007) observed that up to a mediator concentration of 500 μM , there was only a slight inactivation (< 5%) of laccase (from a white-rot fungus - *Trametes versicolor*). However, when the mediator concentration was increased to 10,000 μM , the inactivation was remarkable (90%). In another study, in the presence of 50 μM SA, laccase from *Trametes versicolor* was inactivated within 30 min (Mendoza et al., 2011). However, reducing the SA concentration to 25 μM resulted in a similar inactivation profile as without the mediator. In general the use of mediators can enhance TrOC transformation;

however, in certain instances their practical application may be limited since they lead to significant inactivation of laccase.

Overall, in this study, it has been demonstrated that laccase offers greater stability at neutral pH (**Figure 5.3**) and at an SA concentration of up to 100 μM (**Figure 5.4**). Because there was virtually no difference in removal of TrOCs between SA concentrations of 5 and 100 μM , a SA concentration of 10 μM was selected for the operation of the EMR.

5.3.1.4 Laccase degradation of a diverse set of TrOCs

Together with a few compounds (*i.e.*, bisphenol A, estradiol, estrone, diclofenac, atrazine and carbamazepine) that have been recently investigated (Lloret et al., 2012; Nguyen et al., 2014d), this study included a broad spectrum of TrOCs to establish a comprehensive understanding of the degradation capacity of the laccase preparation used. The batch test data regarding laccase catalysed degradation of the selected TrOCs provided important baseline information to explain EMR performance. Given the difference in the chemical structures of the TrOCs in this study (phenolic/non-phenolic moieties and the presence of electron releasing/demanding functional groups), as expected, a significant variation in their enzymatic degradation was observed (**Table 5.3**). Laccase efficiently degrades compounds with phenolic moiety. Thus high degradation of all phenolic TrOCs except salicylic acid, formononetin, pentachlorophenol, enterolactone and oxybenzone was achieved. In contrast, laccase could not efficiently remove non-phenolic compounds except octocrylene and diclofenac (31 and 41% removal, respectively).

All steroid hormones and the industrial chemicals tested were phenolic compounds and thus well degraded. Similarly, the phenolic personal care product triclosan was efficiently degraded. However, despite being phenolic compounds, none of the phytoestrogens (*i.e.*, enterolactone and formononetin) were degraded by laccase. The low enzymatic degradation of a few of the phenolic compounds identified in **Table 5.3** will be discussed later in this section. With the exception of octocrylene and diclofenac, none of the non-phenolic compounds were enzymatically degraded. Indeed, consistent with the literature (Hai et al., 2012a; Tran et al., 2013; Yang et al., 2013a), all pesticides (including pentachlorophenol, which is a phenolic compound) demonstrated particular resistance to degradation. All the pharmaceuticals, except salicylic acid, were non-phenolic compounds and thus underwent negligible enzymatic

degradation. A similar observation could be made with all of the UV-filters (personal care product).

Table 5.3: Assessment of TrOC degradation capacity of the laccase in batch tests. Values listed indicate average \pm standard deviation (n=3).

Category	Compound	Phenolic moiety	Enzymatic removal (%)
Pharmaceuticals	Salicylic acid	Yes	0
	Ibuprofen		0
	Metronidazole		0
	Naproxen		0
	Primidone		1.2 \pm 0.9
	Amitriptyline		8.5 \pm 8.0
	Carbamazepine		2.8 \pm 3.9
	Ketoprofen		0
	Gemfibrozil		0
	Diclofenac		41.5 \pm 1.7
Personal care products	Triclosan	Yes	88.7 \pm 3.9
	Benzophenone		11.7 \pm 8.2
	Oxybenzone	Yes	6.1 \pm 8.5
	Octocrylene		31.6 \pm 3.2
Pesticides	Propoxur		0
	Fenoprop		0
	Clofibric acid		0
	Atrazine		0
	Ametryn		0
	Pentachlorophenol	Yes	0
Industrial chemicals	4-tert-Butylphenol	Yes	95.5 \pm 2.5
	Bisphenol A	Yes	97.0 \pm 4.2
	4-tert-Octolphenol	Yes	93.3 \pm 6.6
Steroid hormones	Estriol	Yes	98.5 \pm 0.5
	Estrone	Yes	94.8 \pm 3.1
	17 α -Ethinylestradiol	Yes	98.2 \pm 0.3
	17 β -Estradiol	Yes	98.5 \pm 0.1
	17 β -Estradiol-17-	Yes	93.0 \pm 2.5
Phytoestrogens	Enterolactone	Yes	4.2 \pm 5.8
	Formononetin	Yes	0

The low or negligible removal of non-phenolic compounds reported in **Table 5.3** is consistent with a previous study where the crude enzyme extract from *Trametes versicolor* was used (see **Chapter 3**). The observed low removal may be explained by the presence of strong electron demanding groups or absence of strong electron releasing groups in the TrOC structure. Tadkaew et al. (2011) reported that the presence of electron demanding groups (*e.g.*, amide (-CONR₂), carboxylic (-COOH), and halogen (-X)) renders the compounds resistant to oxidation. The high removal of the non-phenolic TrOC diclofenac may have been due to the electron releasing functional group aromatic amine in its structure. Similarly, direct oxidation of diclofenac by laccase was reported by Lloret et al. (2012), who anticipated that laccase degradation of diclofenac is initiated with the aniline group.

Despite the usual high amenability of phenolic compounds to laccase degradation, in this study, a few phenolic TrOCs, namely salicylic acid, formononetin, pentachlorophenol, enterolactone and oxybenzone were degraded inefficiently. The observed low removal of these compounds may be due to deficient oxidative capacity of the laccase, which depends on the ORP difference between the TrOC and laccase, and/or steric hindrance. For example, while testing the electrochemical behaviour of natural phenolics, Simić et al. (2007) reported that the ORP of salicylic acid is 0.94 V, which is significantly higher than the ORP of the laccase used in this study (0.28 V). In contrast, d'Acunzo et al. (2006) observed that some phenolic compounds such as β -naphthols and 2,4,6-trichlorophenol were not oxidized by laccase due to steric hindrance. They explained that the presence of electron demanding groups (sterically demanding groups) at *ortho* position to the -OH group can hinder the approach of the substrate to the active sites of laccase. Consistent with that explanation, in the current study, among the poorly removed phenolic TrOCs, salicylic acid, pentachlorophenol and oxybenzone contain EWG at the *ortho* position. Notably, similar to this study, Jeon et al. (2008) observed poor degradation of pentachlorophenol by laccase (from *Ganoderma lucidum*). Conversely, Ullah et al. (2000) reported complete degradation of pentachlorophenol by laccase (from *Coriolus versicolor*). This contradiction may be explained by the difference in ORP of laccase derived from different sources (Xu et al., 2000).

5.3.2 Enzymatic activity within EMR

5.3.2.1 Enzyme retention by the UF membrane

In the context of continuous operation, enzyme retention and enzymatic stability within an EMR is particularly important. López et al. (2004) confirmed the retention of manganese peroxidase (MnP) within the reactor by a polyethersulfone UF membrane (MWCO of 10 kDa). Laccase from *Cyathus bulleri* (molecular weight of 53 kDa) was retained by a polyacrylonitrile UF membrane (MWCO of 20 kDa) (Chhabra et al., 2009). Considering the molecular weight of the enzyme (56 kDa) and the MWCO of the membrane (6 kDa) used in this study, laccase was expected to be retained. But enzyme retention and activity can be influenced by factors such as membrane material, wastewater matrix and other operational parameters (Rios et al., 2004). Thus, the suitability of the selected membrane was tested for the retention of laccase under the operating conditions used in this study (**Section 5.2.3.2**). The enzymatic activity in the permeate and in the reactor-supernatant was monitored during continuous filtration over a period of 24 h. No enzymatic activity in the permeate was observed, while that in the reactor supernatant remained stable (**Figure 5.5**), confirming that the membrane effectively retained the enzyme.

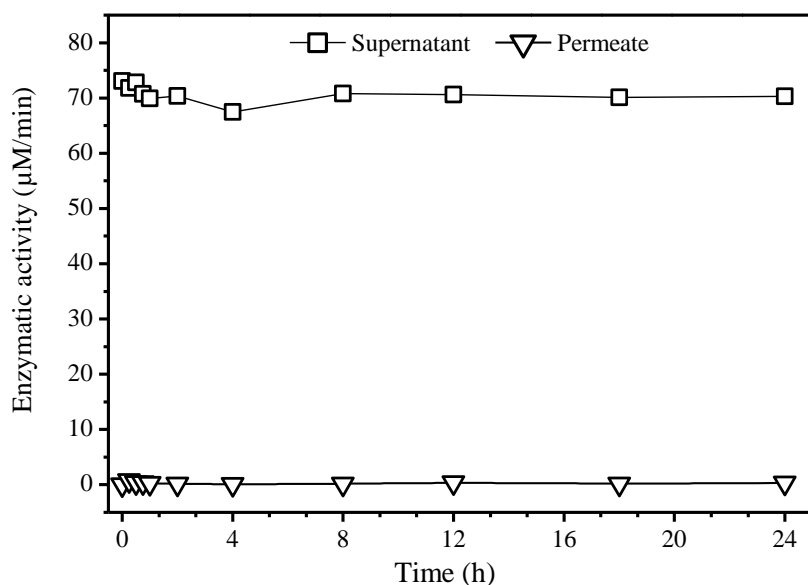


Figure 5.5: Enzyme retention by the UF membrane

5.3.2.2 Maintenance of enzymatic activity during EMR operation

The maintenance of enzymatic activity is an important parameter for long term stable operation of an EMR. As mentioned in **Section 5.3.2.1**, the enzyme was completely retained within EMR. However, to assess the probable change of enzymatic activity during continuous operation of the EMR, the enzymatic activity was measured periodically. As presented in **Figure 5.6**, the enzymatic activity gradually dropped to undetectable levels within 72 h of continuous operation. This was accompanied by a significant decrease in the removal of BPA and DCF. Denaturation of an enzyme can occur for various reasons including physical, chemical and biological inhibitors, and the effect of shear stress (Rios et al., 2004). Depending on the shear rate and flow geometry, deformation of the enzyme molecules during filtration could cause enzyme inactivation. There is only one study on continuous TrOC (*i.e.*, estrone and estradiol) degradation by an EMR (Lloret et al., 2012). The authors of that study observed no enzyme denaturation within the short observation period of 8 h. Nevertheless, inactivation of enzyme has been reported in a few studies investigating the removal of other resistant groups of compounds. For example, Mendoza et al. (2011) observed complete inactivation of laccase within 180 h during repeated batch decolorization of Remazol Blue RR.

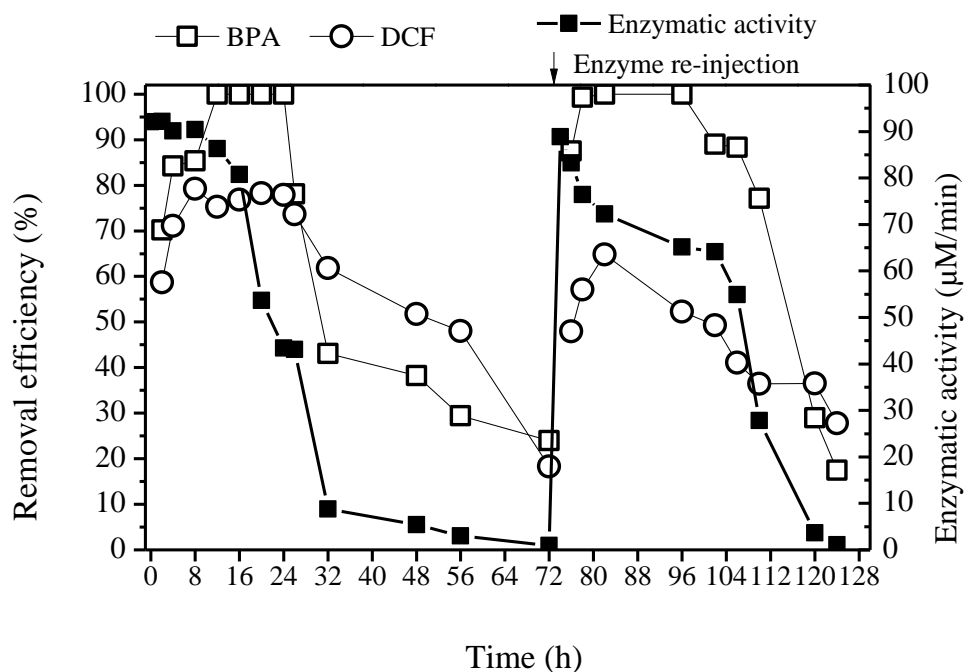


Figure 5.6: Enzymatic activity decrease during continuous operation of the EMR following addition of a single dose of laccase, and concomitant reduction in removal of BPA and DCF.

In this study, the UF membrane completely retained laccase within the EMR, but the enzymatic activity and TrOC removal decreased gradually (**Figure 5.6**). Therefore, periodic replenishment of laccase was deemed to be required to keep the enzymatic activity and TrOC removal stable. The enzymatic activity was maintained by the addition of 200 μ L of the commercial laccase solution (see **Section 5.2.3.3**) per L of the reactor volume every 12 h (equivalent to a laccase dose of 23 mg/L.d). Apart from enzyme reinjection, various additives, *e.g.*, ethylenediaminetetraacetic acid and polyethylene glycol, which are believed to possess a protecting role for proteins, especially under oxidative stress, may be added to an EMR (Mendoza et al., 2011). However, this aspect was beyond the scope of the current study.

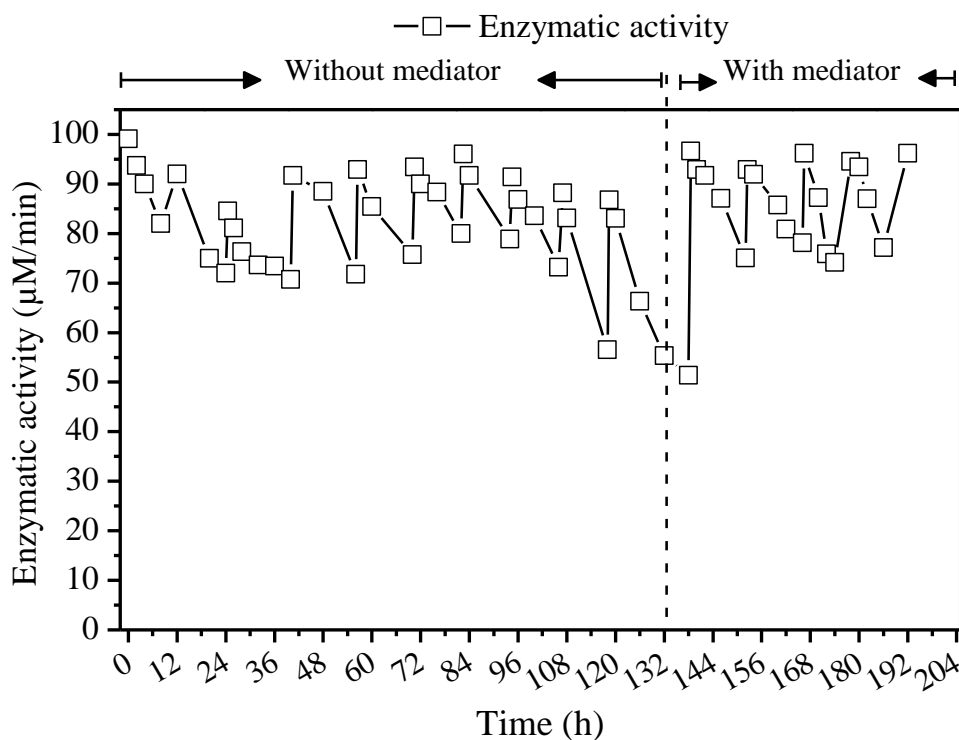


Figure 5.7: Denaturation of the enzyme within the reactor and maintenance of stable enzymatic activity over long-term operation by the addition of 200 μ L of the commercial laccase solution per L of the reactor volume every 12 h (equivalent to a laccase dose of 23 mg/L.d).

5.3.3 TrOC removal performance of the laccase-EMR

5.3.3.1 Overall TrOC removal and the role of membrane

TrOC degradation data from batch and continuous EMR experiments cannot be directly compared due to the difference in the operation modes. However, two distinct patterns in the TrOC degradation profile for the EMR (**Figure 5.8**) are worth noting: (i) significantly higher removal of some phenolic and non-phenolic TrOCs, with low but discernible improvement in removal of some other non-phenolic compounds, (ii) lower removal (although still maintaining at 40-90%) of some phenolic compounds which were well degraded in batch tests (**Table 5.3**).

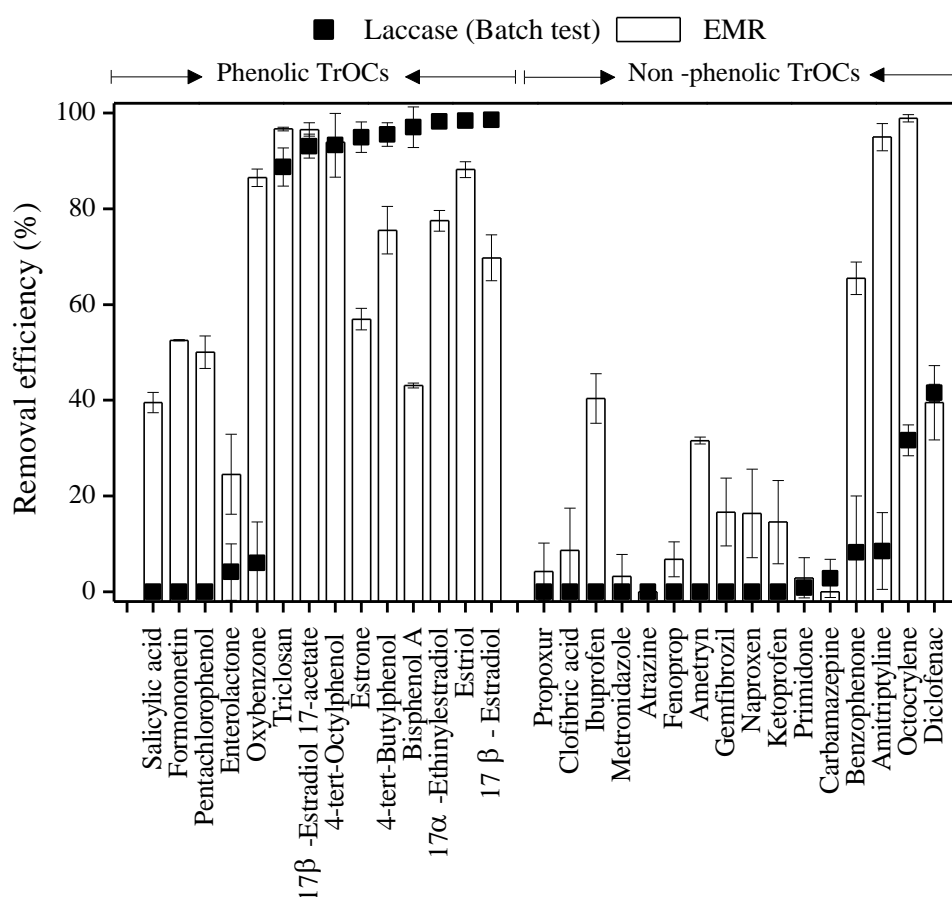


Figure 5.8: Removal efficiency of 30 TrOCs by the laccase-EMR as compared to batch test data. Laccase activity was maintained by the addition of 400 μ L of the commercial laccase solution per liter of the reactor volume every 12 h (equivalent to a laccase dose of 46 mg/L d). The EMR was operated for 72 h (*i.e.*, 9 x HRT). Data presented as average \pm standard deviation (n=3) in both batch and the EMR studies.

Compared to the batch test results, lower (although significant) removal of some compounds (*i.e.*, estrone, 4-tert-butylphenol, bisphenol A, 17 α -ethinylestradiol, estriol and 17 β -estradiol) during the continuous operation of the EMR may be attributed to the sustained TrOC-loading to the EMR, and is not entirely unexpected (Lloret et al., 2012). Of particular interest was the better removal ($p < 0.05$) achieved by the EMR for five phenolic and five non-phenolic TrOCs. The UF membrane used in this study was not expected to retain any TrOC by size exclusion. The EMR was operated in the absence of the enzyme to determine TrOC adsorption directly on the membrane surface. Results show that TrOC removal by adsorption on the membrane was negligible (<5%). However, when an enzyme solution is flushed through an UF membrane that rejects the enzyme molecules, the enzyme can form a porous, thin gel layer ('secondary membrane') on the membrane (Modin et al., 2014). Wastewater-borne suspended and colloidal particles as well as water soluble macromolecules may co-deposit on the gel layer and enhance removal of pollutants (Hai et al., 2012b). Therefore, it was hypothesized that the improved removal was due to the retention of TrOCs by an enzyme gel layer dynamically formed on the membrane, followed possibly by enzymatic degradation. Indeed high laccase activity was detected in the cleaning solution from *ex situ* Milli-Q water backwashing. A litre of cleaning solution was generated in each Milli-Q backwash, and the enzymatic activity in that solution was 60 $\mu\text{M}_{(\text{DMP})}/\text{min}$. This translates to an accumulation of *ca.* 0.24 g laccase/m² membrane surface, evidencing formation of a laccase gel layer on the membrane surface. Furthermore, for the TrOCs which received significantly higher removal by the EMR (*i.e.*, salicylic acid, formononetin, pentachlorophenol, enterolactone and oxybenzone), the ratio of the concentration in permeate and supernatant (P/S ratio) was significantly below 1 (**Figure 5.9**), confirming that TrOCs were indeed retained by the gel layer of laccase on the membrane.

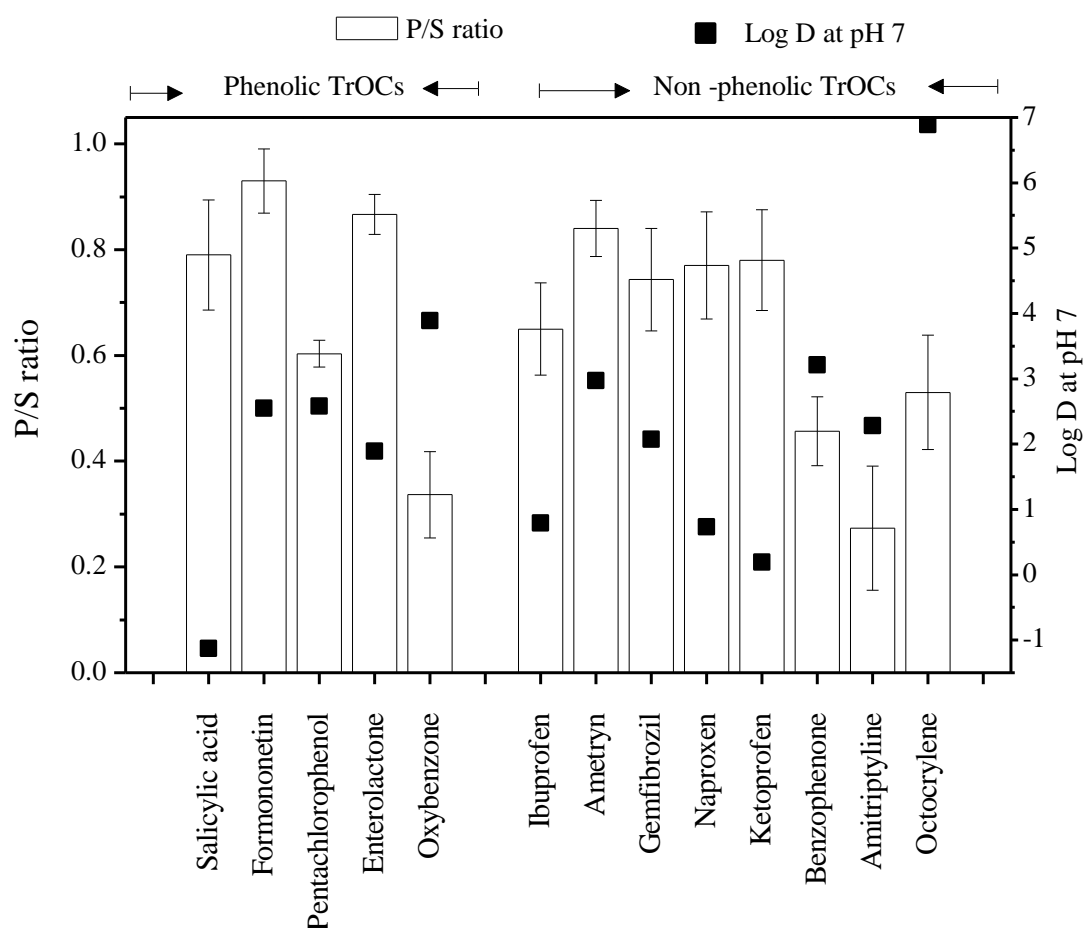


Figure 5.9: Concentration ratio in membrane permeate and reactor supernatant (P/S ratio) for TrOCs by laccase-EMR (compared to that in batch test). Data presented as average \pm standard deviation ($n=3$).

Of the TrOCs shown in **Figure 5.9**, pentachlorophenol, oxybenzone, benzophenone and octocrylene were all significantly hydrophobic (as indicated by $\log D_{\text{pH}=7}$ values greater than 3) and showed high retention on the membrane (P/S ratios 0.44 ± 0.15 , $n=15$). These compounds also showed high improvement (49 – 84%) in removal by the EMR compared to batch test removal (**Figure 5.8**). In contrast, enterolactone, gemfibrozil, naproxen and ketoprofen, which are hydrophilic ($\log D_{\text{pH}=7}$ values ranging from 0.19 – 1.89), had P/S ratios of 0.79 ± 0.1 ($n=12$), and were still better removed by the EMR compared to the batch test removal, but to a lesser extent (10 – 17%). These observations strongly point to the importance of hydrophobicity for retention of these TrOCs on the membrane gel layer, which consequently governed their overall

removal by the EMR. However, a few of the TrOCs plotted in **Figure 5.8** (*i.e.*, salicylic acid, formononetin, ibuprofen, ametryn and amitriptyline) as well as the TrOCs that showed somewhat lower removal by the EMR than in the batch tests, did not conform to the trend of high hydrophobicity effecting high membrane retention (low P/S ratio) and high EMR removal, or vice versa. For example, compared to batch tests, high improvement in removal of salicylic acid was observed by the EMR although it was highly hydrophilic ($\log D_{\text{pH}=7} = -1.13$). Conversely, despite having a high $\log D_{\text{pH}=7}$ of 4.11, 17 α -ethinylestradiol was removed with an efficiency of around 80% by the EMR as compared to complete removal in batch tests. Taken together, these observations indicate the following possibilities: (i) TrOC retention on membrane gel layer was governed not only by hydrophobic partitioning but also by non-hydrophobic interactions such as electrostatic interactions (Luo et al., 2014), (ii) both retention on membrane and enzymatic degradation was important.

The formation of the enzyme gel layer on the membrane may lead to gradual fouling and drop in permeate flux (Lloret et al., 2012; Modin et al., 2014). In order to keep the focus of this study on assessment of TrOC removal mode, a low flux of 1.1 L/m² h was applied. Under the operating conditions in this study, a TMP increase rate of 3.3 kPa/d was observed, and a mild *in situ* backwash on every third day as per the protocol described in Section 2.3 was adequate to reinstate the TMP to its original value. Although beyond the scope of this study, further work to establish the applicable flux-range as a function of feed wastewater characteristics would be interesting.

5.3.3.2 Fate of TrOC following membrane retention

The data presented in **Figure 5.8** and **Figure 5.9** show a complementary role of TrOC retention by the membrane gel layer and enzymatic degradation. In order to provide further evidence, EMR performance with active laccase and heat-inactivated laccase was compared. For this set of experiments, four TrOCs (two phenolics, *i.e.*, pentachlorophenol and oxybenzone, and two non-phenolics *i.e.*, ibuprofen and benzophenone), which showed significant retention on the membrane (low P/S ratio) were selected.

When the EMR was operated with inactivated laccase, a significant amount of TrOCs was initially adsorbed on the laccase gel layer as evidenced by stable removal of TrOCs for nearly 2 d (**Figure 5.10**). However, thenceforth, the removal efficiency gradually diminished. The

saturation of the adsorption capacity of the layer of enzyme (inactivated) on the membrane was evidenced by the P/S ratio gradually approaching unity. In contrast, a significantly high and stable removal of these compounds was sustained by the EMR when operated with the addition of active laccase (**Figure 5.10**). Thus it was concluded that TrOCs retained by the enzyme gel layer were eventually degraded.

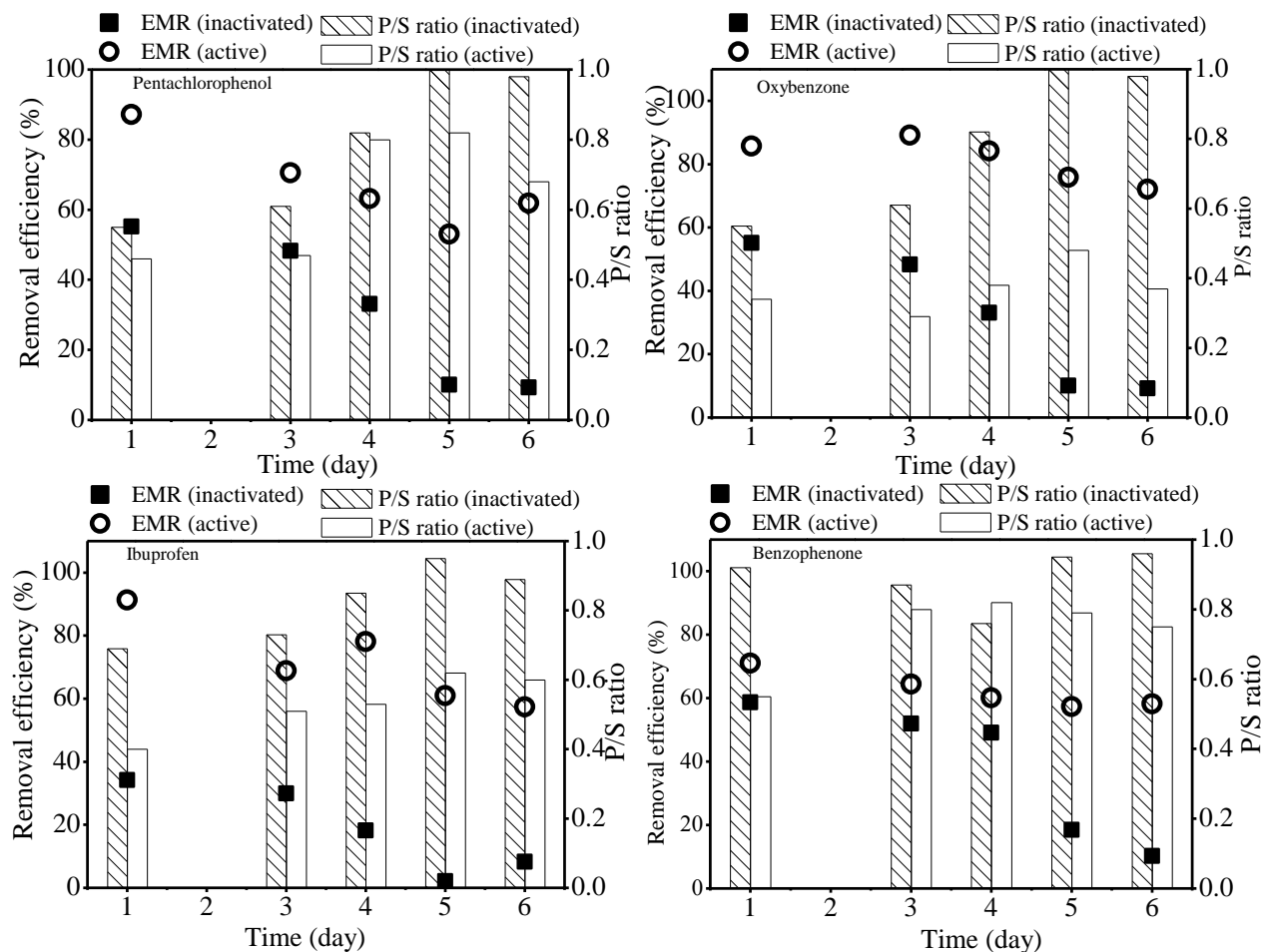


Figure 5.10: Removal efficiency and concentration ratio (P/S ratio) profiles of four selected TrOCs during the operation of the laccase-EMR with heat-inactivated or active laccase. The EMRs were operated for a period of 18 x HRT to confirm the dominant role of enzymatic degradation.

Previous studies have reported short-term increase in organics removal due to pre-formed adsorbent-coating on membrane, or dynamically formed adsorbent layer on membrane, although the role of biodegradation was not addressed (Heijman et al., 2009; Löwenberg et al., 2014). By contrast, a few studies have shown the advantages of adding adsorbents to membrane bioreactors to effect simultaneous adsorption, retention on membrane cake layer and biodegradation (Hai et al., 2012b; Nguyen et al., 2014b). Li and Loh (2007) developed a hollow fibre membrane reactor wherein microbes were entrapped within the fibres. They achieved improved removal of phenol when granular activated carbon too was incorporated into the membrane structure. Conversely, various enzymes can be immobilized on membranes, thereby facilitating conversion of resistant chemicals concurrent with the retention of the insoluble metabolites (Gasser et al., 2014; Hou et al., 2014). The uniqueness of this study is that it systematically demonstrates improved degradation of a range of TrOCs by dynamically immobilized laccase on the UF membrane.

5.3.4 EMR performance with mediator addition

Laccase-mediator systems can generate free radicals having higher redox potential than laccase itself. Furthermore, these radical species can act as a carrier of electrons between laccase and the substrate, thereby overcoming the steric hindrance that may exist between them (Xu et al., 2000). In a batch test study by Weng et al. (2012), dosing of SA to laccase led to concomitant increase in ORP of the solution and degradation of sulphonamide antibiotics. In the current study, the ORP of the laccase solution increased significantly from 0.27 V to 0.53 and 0.48 V, due to addition of SA and HBT, respectively. Accordingly the substrate spectrum was observed to be broadened and higher TrOC removal by the EMR could be obtained due to mediator dosing to the EMR (**Figure 5.11**).

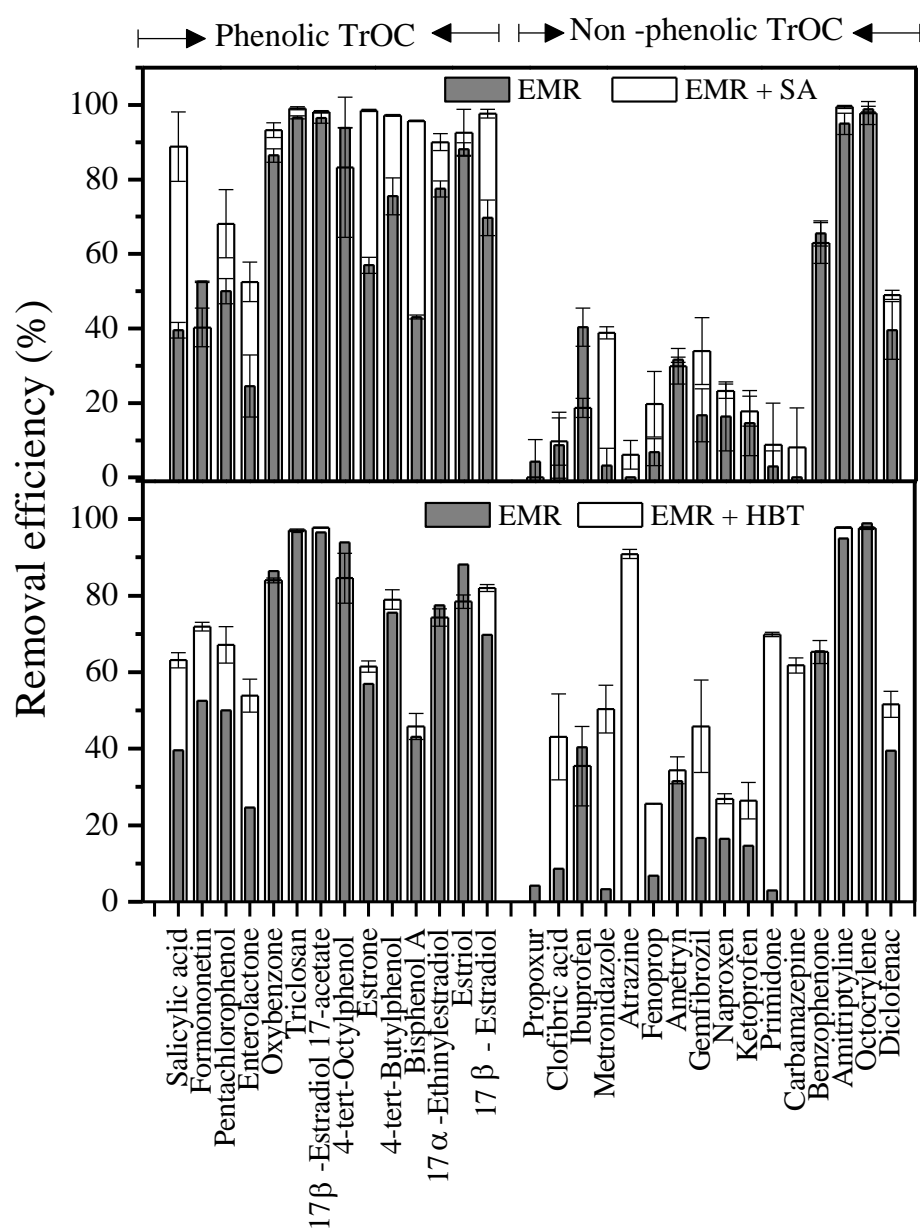


Figure 5.11: Enhancement of TrOC removal by the EMR due to the addition of mediators (SA or HBT) at a concentration of 10 μ M to the laccase-EMR. Laccase activity was maintained by the addition of 400 μ L of the commercial laccase solution per liter of the reactor volume every 12 h (equivalent to a laccase dose of 46 mg/L d). The EMR was operated for 72 h (*i.e.*, 9 x HRT). Data presented as average \pm standard deviation (n=3).

5.3.4.1 Substrate specificity

The aspect of mediator addition to improve TrOC degradation by EMR has been communicated in a limited number of studies (Nguyen et al., 2014d). In our previous study involving four non-phenolic TrOCs, SA addition to an EMR resulted in a significant improvement of TrOC removal depending on the properties and loading rates of the TrOCs (Nguyen et al., 2014d). The current study compares performance of SA and HBT for a larger set of TrOCs. A notable observation was the substrate-specificity demonstrated by the mediators. For example, SA addition achieved better removal of phenolic compounds namely, estrone, 4-tert-butylphenol and bisphenol A ($p < 0.05$, **Appendix Table A-6**). Conversely HBT achieved better removal of non-phenolic compounds, namely clofibric acid, atrazine, primidone, and carbamazepine ($p < 0.05$, **Appendix Table A-6**). Overall, HBT appeared to offer more versatility *i.e.*, effective degradation of both phenolic and non-phenolic TrOCs.

Substrate specificity of mediators has been reported before, but only in conjunction with batch tests (Camarero et al., 2005). The efficiency of a laccase-mediator system depends not only on the ORP of the radicals generated, but also on reversibility of the reaction of the radicals with the substrate, and the stability of the radicals generated (Camarero et al., 2005). However, both SA and HBT act via the same oxidation mechanism (*i.e.*, hydrogen atom transfer), and the ORP of the laccase preparation with addition of the mediators (separately) were similar. Thus the performance difference between the mediators may be explained by the overall stability of the highly reactive radicals formed. Phenoxyl radicals are generated due to laccase oxidation of SA, and these radicals have been reported to be more active than aminoxyl radicals generated from HBT. Conversely, phenoxyl radicals are extremely unstable and convert to much less active quinone (Xu et al., 2000). While the aminoxyl radicals generated from HBT also turn rapidly to benzotriazole and other inactive compounds, their reactivity and stability appears to be better balanced than that of phenoxyl radicals (Xu et al., 2000).

5.3.4.2 Laccase-mediator system: Batch vs. EMR operation

An ideal redox-mediator must not be consumed during the reaction. However, as noted above, phenoxyl and aminoxyl radicals can convert to quinone and benzotriazole, respectively (Xu et al., 2000), which are far less efficient as mediators. Thus, it is necessary to continuously add the mediators to maintain a stable removal. For example, Hata et al. (2010) achieved 40% increase in

the removal of carbamazepine by adding HBT every 8 h to the reactor. In the current study, a marked additional improvement in TrOC removal by the EMR (continuous mediator dosing) was achieved (**Figure 5.12**) when compared with the improvement achieved during batch incubation tests. This is evidenced by the distinct removal-improvement profiles (batch vs. EMR) of seven highly resistant TrOCs shown in **Figure 5.12** for HBT as an example.

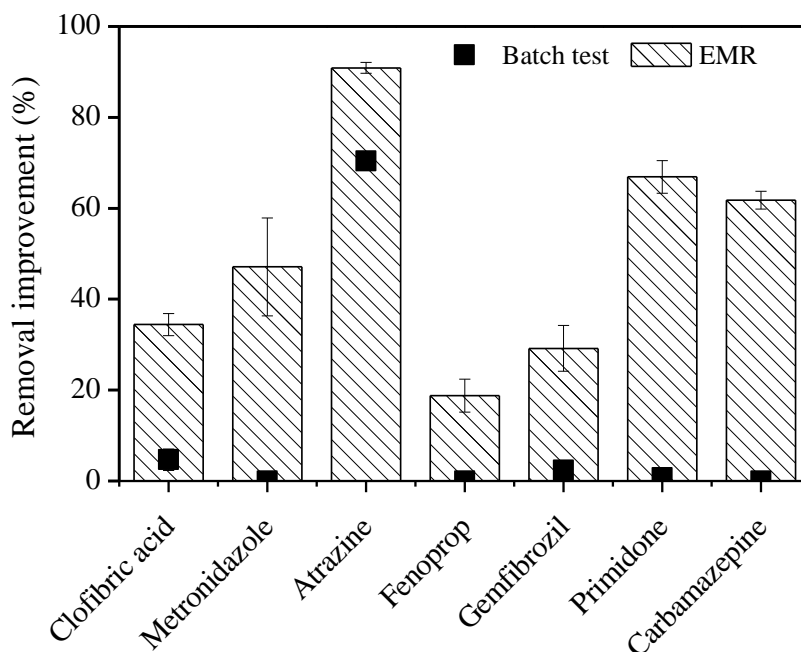


Figure 5.12: Comparison of the TrOC removal improvement (%) due to HBT addition in batch tests and continuous operation of EMR demonstrating that mediator addition was more effective in case of the EMR operation. Data presented as average \pm standard deviation (n=3).

Figure 5.13 provides further unique insight to the plausible reasons for the better removal by the EMR. Of the seven TrOCs plotted in **Figure 5.13**, for atrazine and carbamazepine, significant improvement in removal due to HBT dosing to EMR was accompanied by a significant drop in their reactor-supernatant concentrations, evidencing better degradation capacity of the laccase-HBT system. For the rest of the TrOCs, however, little difference in reactor-supernatant concentration was observed (*i.e.*, less amenable to laccase-HBT system than atrazine or carbamazepine), although significant improvement in their removal by the EMR was obtained when HBT was added. Consistent with the discussion in **Section 5.3.3**, the observations made

here strongly point to a complementary role of enhanced degradation by the laccase-mediator system and membrane retention, and also that for certain compounds which are less amenable to mediator-enhanced enzymatic degradation (*i.e.*, clofibric acid, metronidazole, fenoprop, gemfibrozil and primidone) among the compounds shown in **Figure 5.13**) membrane retention has a more profound impact on the overall removal.

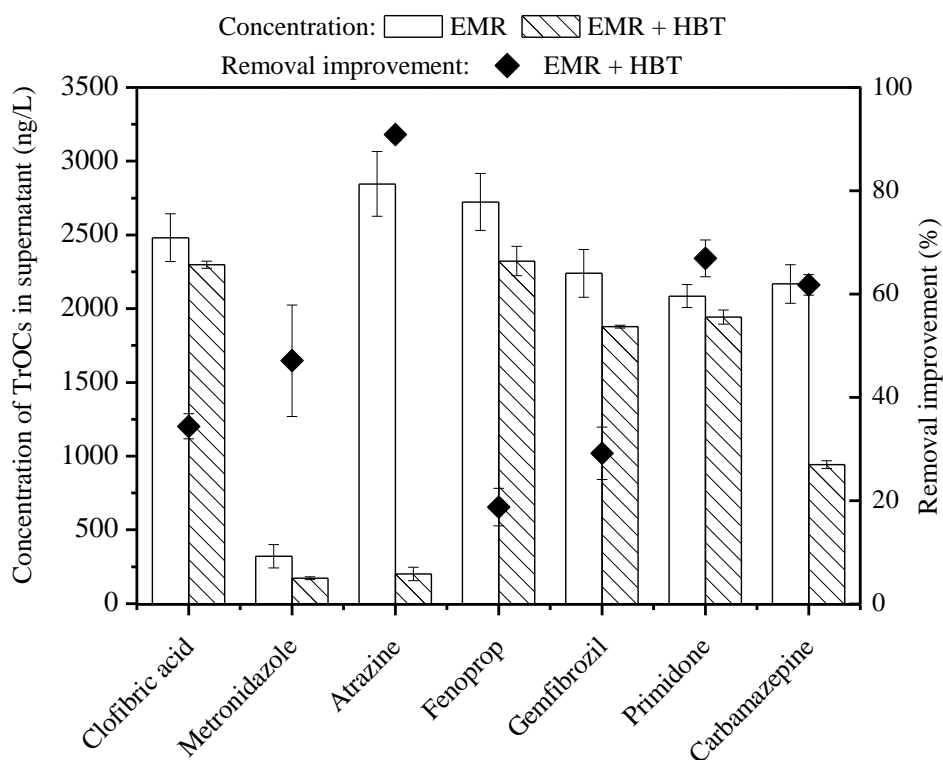


Figure 5.13: Supernatant concentration of resistant non-phenolic TrOCs during the operation of the laccase-EMR with and without HBT dosing, and corresponding improvement (%) in TrOC removal during HBT dosing. Data presented as average \pm standard deviation (n=3).

5.3.5 Effluent toxicity

Despite efficient degradation of the target compounds, increased effluent toxicity may be observed following enzymatic treatment (Marco-Urrea et al., 2009; Nguyen et al., 2014a). This has been attributed to metabolites produced during TrOC degradation and/or highly reactive radical species generated from oxidation of mediators (Kim et al., 2006a). Because a set of 30 TrOCs was used in this study, it was not possible to relate the metabolites to their parent

compounds. Thus, the toxicity of the effluent to a bacterial system was quantified as a measure of the overall toxicity of metabolites and reactive radical species generated via the mediator-enhanced laccase system.

There was a small but insignificant increase in toxicity following treatment by the laccase-EMR (feed toxicity 4.1 ± 0.43 rTU vs. permeate toxicity 7.0 ± 1.8 rTU, $n=2$), indicating that the laccase-treatment did not generate toxic by-products. Furthermore, there was no detectable increase in toxicity in the treated solution after addition of 10 μ M of either SA or HBT to the EMR. This is consistent with HBT results from previous batch tests (Nguyen et al., 2014a), but suggests that SA addition to EMR may cause slightly less toxicity than previously thought (Nguyen et al., 2014d). The slight discrepancy in case of SA may be due to the fact that in the previous EMR studies fewer numbers of TrOCs but with higher dosage were tested (Nguyen et al., 2014d). However, the investigations conducted within the scope of this study confirm efficient degradation of a range of TrOCs by the EMR without causing significantly elevated toxicity in treated effluent.

5.4. CONCLUSION

Preliminary investigation confirmed the impact of pH on the degradation of BPA and DCF by the laccase preparation used. BPA was almost completely removed at neutral pH (pH 7), while DCF degradation was significantly better at the acidic pH (pH 4.5). Moreover, laccase was more stable at neutral pH. The addition of mediator SA enhanced the removal of DCF, which was originally resistant to degradation by laccase.

Batch tests with a diverse set of 30 TrOCs revealed laccase-catalysed degradation of nine out of 14 phenolic and only two out of 16 non-phenolic compounds. The ORP of the laccase solution almost doubled due to addition of the mediators (SA or HBT); however, this led to better degradation of only one phenolic and three non-phenolic TrOCs. Compared to the batch tests, the EMR was observed to facilitate degradation of a number of TrOCs originally showing resistance to enzymatic degradation. This was attributed to the formation of a dynamic layer of laccase over the membrane surface which retained TrOCs and facilitated their subsequent enzymatic degradation. A complementary role of enhanced degradation by the laccase-mediator system and retention by the membrane gel layer was revealed. Membrane retention particularly enhanced the

degradation of the compounds which are less amenable to mediator-enhanced enzymatic degradation.

5.5. REFERENCES

- Berka, R. M., P. Schneider, E. J. Golightly, S. H. Brown, M. Madden, K. M. Brown, T. Halkier, K. Mondorf and F. Xu (1997). Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae*. *Appl. Environ. Microbiol.*, 63, 3151-3157.
- Cabana, H., J. P. Jones and S. N. Agathos (2007). Elimination of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: A review. *Eng. Life Sci.*, 7, 429-456.
- Camarero, S., D. Ibarra, M. J. Martínez and Á. T. Martínez (2005). Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Appl. Environ. Microbiol.*, 71, 1775-1784.
- Chhabra, M., S. Mishra and T. R. Sreekrishnan (2009). Laccase/mediator assisted degradation of triarylmethane dyes in a continuous membrane reactor. *J. Biotechnol.*, 143, 69-78.
- d'Acunzo, F., C. Galli, P. Gentili and F. Sergi (2006). Mechanistic and steric issues in the oxidation of phenolic and non-phenolic compounds by laccase or laccase-mediator systems. The case of bifunctional substrates. *New J. Chem.*, 30, 583-591.
- Gasser, C., L. Yu, J. Svojitka, T. Wintgens, E. Ammann, P. Shahgaldian, P. X. Corvini and G. Hommes (2014). Advanced enzymatic elimination of phenolic contaminants in wastewater: a nano approach at field scale. *Appl. Microbiol. Biotechnol.*, 98, 3305-3316.
- Hai, F. I., O. Modin, K. Yamamoto, K. Fukushima, F. Nakajima and L. D. Nghiem (2012a). Pesticide removal by a mixed culture of bacteria and white-rot fungi. *J Taiwan Inst Chem Eng.* 43, 459-462.
- Hai, F. I., K. Yamamoto, F. Nakajima and K. Fukushima (2012b). Application of a GAC-coated hollow fiber module to couple enzymatic degradation of dye on membrane to whole cell biodegradation within a membrane bioreactor. *J Mem Sci.* 389, 67-75.
- Hata, T., H. Shintate, S. Kawai, H. Okamura and T. Nishida (2010). Elimination of carbamazepine by repeated treatment with laccase in the presence of 1-hydroxybenzotriazole. *J. Hazard. Mater.*, 181, 1175-1178.
- Heijman, S. G. J., J. Z. Hamad, M. D. Kennedy, J. Schippers and G. Amy (2009). Submicron powdered activated carbon used as a pre-coat in ceramic micro-filtration. *Desalination Water Treat.*, 9, 86-91.
- Hou, J., G. Dong, Y. Ye and V. Chen (2014). Enzymatic degradation of bisphenol-A with immobilized laccase on TiO₂ sol-gel coated PVDF membrane. *J Mem Sci.* 469, 19-30.
- Jeon, J.-R., K. Murugesan, Y.-M. Kim, E.-J. Kim and Y.-S. Chang (2008). Synergistic effect of laccase mediators on pentachlorophenol removal by *Ganoderma lucidum* laccase. *Appl. Microbiol. Biotechnol.*, 81, 783-790.
- Khelifi-Slama, R., T. Mechichi, S. Sayadi and A. Dhouib (2012). Effect of natural mediators on the stability of *Trametes trogii* laccase during the decolourization of textile wastewaters. *The Journal of Microbiology.* 50, 226-234.
- Kim, Y.-J. and J. A. Nicell (2006a). Laccase-catalysed oxidation of aqueous triclosan. *J Chem Technol Biotechnol.* 81, 1344-1352.

- Kim, Y. J. and J. A. Nicell (2006b). Impact of reaction conditions on the laccase-catalyzed conversion of bisphenol A. *Bioresource Technol.* 97, 1431-1442.
- Kurniawati, S. and J. A. Nicell (2007). Efficacy of mediators for enhancing the laccase-catalyzed oxidation of aqueous phenol. *Enzyme Microb. Technol.*, 41, 353-361.
- Li, Y. and K. Loh (2007). Hybrid-Hollow-Fiber Membrane Bioreactor for Cometabolic Transformation of 4-Chlorophenol in the Presence of Phenol. *J. Environ. Eng.*, 133, 404-410.
- Lloret, L., G. Eibes, G. Feijoo, M. T. Moreira and J. M. Lema (2012). Degradation of estrogens by laccase from *Myceliophthora thermophila* in fed-batch and enzymatic membrane reactors. *J. Hazard. Mater.*, 213–214, 175-183.
- Lloret, L., G. Eibes, T. A. Lú-Chau, M. T. Moreira, G. Feijoo and J. M. Lema (2010). Laccase-catalyzed degradation of anti-inflammatories and estrogens. *Biochem. Eng. J.*, 51, 124-131.
- López, C., M. T. Moreira, G. Feijoo and J. M. Lema (2004). Dye decolorization by manganese peroxidase in an enzymatic membrane bioreactor. *Biotechnol. Prog.* 20, 74-81.
- Löwenberg, J., A. Zenker, M. Baggenstos, G. Koch, C. Kazner and T. Wintgens (2014). Comparison of two PAC/UF processes for the removal of micropollutants from wastewater treatment plant effluent: Process performance and removal efficiency. *Water Res.* 56, 26-36.
- Luo, Y., W. Guo, H. H. Ngo, L. D. Nghiem, F. I. Hai, J. Zhang, S. Liang and X. C. Wang (2014). A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Sci. Total Environ.*, 473–474, 619-641.
- Marco-Urrea, E., M. Pérez-Trujillo, T. Vicent and G. Caminal (2009). Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. *Chemosphere.* 74, 765-772.
- Mendoza, L., M. Jonstrup, R. Hatti-Kaul and B. Mattiasson (2011). Azo dye decolorization by a laccase/mediator system in a membrane reactor: Enzyme and mediator reusability. *Enzyme Microb. Technol.*, 49, 478-484.
- Mizuno, H., H. Hirai, S. Kawai and T. Nishida (2009). Removal of estrogenic activity of isobutylparaben and butylparaben by laccase in the presence of 1-hydroxybenzotriazole. *Biodegradation.* 20, 533-539.
- Modin, O., F. I. Hai, L. D. Nghiem and A. Basile (2014). Gas-diffusion, extractive, and biocatalytic membrane biological reactors. *Membrane Biological reactors.* F. I. Hai, K. Yamamoto and C.-H. Lee. UK (ISBN:971780400655), IWA publishing: 299-333.
- Nguyen, L. N., F. I. Hai, J. Kang, F. D. L. Leusch, F. Roddick, S. F. Magram, W. E. Price and L. D. Nghiem (2014a). Enhancement of trace organic contaminant degradation by crude enzyme extract from *Trametes versicolor* culture: Effect of mediator type and concentration. *J Taiwan Inst Chem Eng.* 45, 1855-1862.
- Nguyen, L. N., F. I. Hai, L. D. Nghiem, J. Kang, W. E. Price, C. Park and K. Yamamoto (2014b). Enhancement of removal of trace organic contaminants by powdered activated carbon dosing into membrane bioreactors. *J Taiwan Inst Chem Eng.* 45, 571-578.
- Nguyen, L. N., F. I. Hai, W. E. Price, F. D. L. Leusch, F. Roddick, E. J. McAdam, S. F. Magram and L. D. Nghiem (2014c). Continuous biotransformation of bisphenol A and diclofenac by laccase in an enzymatic membrane reactor. *Int. Biodeterior. Biodegradation.* 95, 25-32.
- Nguyen, L. N., F. I. Hai, W. E. Price, F. D. L. Leusch, F. Roddick, H. H. Ngo, W. Guo, S. F. Magram and L. D. Nghiem (2014d). The effects of mediator and granular activated

- carbon addition on degradation of trace organic contaminants by an enzymatic membrane reactor. *Bioresource Technol.* 167, 169-177.
- Nicolucci, C., S. Rossi, C. Menale, T. Godjevargova, Y. Ivanov, M. Bianco, L. Mita, U. Bencivenga, D. Mita and N. Diano (2011). Biodegradation of bisphenols with immobilized laccase or tyrosinase on polyacrylonitrile beads. *Biodegradation*. 22, 673-683.
- Rios, G. M., M. P. Belleville, D. Paolucci and J. Sanchez (2004). Progress in enzymatic membrane reactors – a review. *J Mem Sci.* 242, 189-196.
- Sathishkumar, P., A. Mythili, T. Hadibarata, R. Jayakumar, M. S. Kanthimathi, T. Palvannan, M. Ponraj, M. R. Salim and A. R. Mohd Yusoff (2014). Laccase mediated diclofenac transformation and cytotoxicity assessment on mouse fibroblast 3T3-L1 preadipocytes. *RSC Advances*. 4, 11689-11697.
- Simić, A., D. Manojlović, D. Šegan and M. Todorović (2007). Electrochemical behavior and antioxidant and prooxidant activity of natural phenolics. *Molecules*. 12, 2327-2340.
- Tadkaew, N., F. I. Hai, J. A. McDonald, S. J. Khan and L. D. Nghiem (2011). Removal of trace organics by MBR treatment: The role of molecular properties. *Water Res.* 45, 2439-2451.
- Tran, N. H., J. Hu and T. Urase (2013). Removal of the insect repellent N,N-diethyl-m-toluamide (DEET) by laccase-mediated systems. *Bioresource Technol.* 147, 667-671.
- Ullah, M. A., C. T. Bedford and C. S. Evans (2000). Reactions of pentachlorophenol with laccase from *Coriolus versicolor*. *Appl. Microbiol. Biotechnol.*, 53, 230-234.
- Weng, S.-S., K.-L. Ku and H.-T. Lai (2012). The implication of mediators for enhancement of laccase oxidation of sulfonamide antibiotics. *Bioresource Technol.* 113, 259-264.
- Xu, F., J. J. Kulys, K. Duke, K. Li, K. Krikstopaitis, H.-J. W. Deussen, E. Abbate, V. Galinyte and P. Schneider (2000). Redox Chemistry in Laccase-Catalyzed Oxidation of N-Hydroxy Compounds. *Appl. Environ. Microbiol.*, 66, 2052-2056.
- Yang, S., F. I. Hai, L. D. Nghiem, L. N. Nguyen, F. Roddick and W. E. Price (2013a). Removal of bisphenol A and diclofenac by a novel fungal membrane bioreactor operated under non-sterile conditions. *Int. Biodeterior. Biodegradation*. 85, 483-490.
- Yang, S., F. I. Hai, L. D. Nghiem, W. E. Price, F. Roddick, M. T. Moreira and S. F. Magram (2013b). Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: A critical review. *Bioresource Technol.* 141, 97-108.
- Zhang, Y. and S.-U. Geissen (2010). In vitro degradation of carbamazepine and diclofenac by crude lignin peroxidase. *J. Hazard. Mater.*, 176, 1089-1092.

CHAPTER 6: Trace organic contaminant removal by an enzymatic membrane reactor: Effects of redox-mediator concentration

This chapter has been published as:

Luong N. Nguyen, Jason P. van de Merwe, Faisal I. Hai, Frederic D.L. Leusch, Jinguo Kang, William E. Price, Felicity Roddick, Saleh F. Magram, and Long D. Nghiem. (2015) Laccase – syringaldehyde (SA) – mediated degradation of trace organic contaminants (TrOCs) in an enzymatic membrane reactor (EMR): Removal efficiency and effluent toxicity. *Bioresource Technology* (accepted on Oct 6, 2015).

6.1. INTRODUCTION

Laccases (EC 1.10.3.2) are oxidoreductase enzymes that can effectively oxidize a range of aromatic compounds such as phenols and aromatic amines (d'Acunzo et al., 2006; Cañas et al., 2010) using atmospheric oxygen as the terminal oxidant. Detection of trace organic contaminants (TrOCs) including pharmaceutically active compounds, industrial chemicals, pesticides, and natural and artificial hormones in the aquatic environment has raised considerable concern due to their potential effects on human health and the ecosystem (Schwarzenbach et al., 2006). Biodegradation of TrOCs by means of enzymes such as laccases has recently attracted much attention (Hai et al., 2013). A significant barrier against the application of laccase in a continuous flow wastewater treatment process, however, is the wash out of the enzyme along with the treated effluent. The loss of laccase in the treated effluent may be effectively prevented by coupling a membrane to the bioreactor, thus forming an enzymatic membrane reactor (EMR). Only a handful of studies have assessed TrOC removal performance in continuous flow EMRs (Ba et al., 2014; Escalona et al., 2014; Nguyen et al., 2015). TrOC degradation performance of EMRs depends on various factors including the chemical structure of TrOCs. For example, in a previous study, phenolic compounds were more effectively removed than the non-phenolic TrOCs (Nguyen et al., 2015).

Addition of a redox mediator may broaden the substrate spectrum of laccase. Mediators are low-molecular weight substrates of laccase that can act as “electron carriers” between the enzyme and the target pollutant. These include both synthetic (*e.g.*, 1-hydroxybenzotriazole (HBT) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS)) and natural (*e.g.*, syringaldehyde (SA) and *P*-coumaric acid) mediators. Owing to the cost and potential toxicity of the synthetic mediators, the use of laccase in combination with natural mediators has been proposed as an alternative (Xu et al., 2000; Camarero et al., 2007). While the natural mediators have shown case-specific efficiency in enhancing TrOC degradation (Yang et al., 2013; Nguyen et al., 2014), certain studies have raised concern about elevated toxicity in the effluent even when natural mediators are used to enhance laccase-catalyzed degradation of pollutants. For example, Fillat et al. (2010) observed increased toxicity in effluent when they used the natural mediator SA to enhance the laccase-catalyzed bleaching of flax pulp. Although there is a lack of consensus regarding the mechanisms of such toxicity, it has been proposed that the highly reactive radical

species generated via the laccase – SA system, which aid in enhancing pollutant degradation, may cause cytotoxicity (Fillat et al., 2010; Khelifi et al., 2010). In addition, previous studies employing laccase - SA combination to degrade resistant compounds including TrOCs seem to have overlooked the reported inhibitory impact of SA itself (in absence of laccase) on microbes, for example, those capable of biofuel production from lignocellulosic biomass (Richmond et al., 2012; Yu et al., 2014). It is also important to take into consideration the fact that the laccase system forms a part of the defense system in certain eukaryotes – a notable example being the expression of antibacterial activity of some white-rot fungi (de Boer et al., 2010). Although probably not to the same extent as SA, laccase preparations may also contribute to effluent toxicity as assessed by common single bacterium based aquatic toxicity assays. Notably, most of the available studies on TrOC degradation by laccase – SA system has been carried out in batch mode. Moreover, to date no study has systematically compared the toxicity of laccase and SA preparations separately or in mixture. Particularly, the impact of SA-dose on TrOC removal efficiency by EMR and effluent toxicity remains to be elucidated.

The objective of this study was to assess the performance of an EMR at degrading 31 selected TrOCs at different TrOC and SA loadings, with a special focus on the toxicity of the treated effluent. In discussing the TrOC degradation profiles, due consideration has been provided to the phenolic moiety and other important features of TrOC structure. Effluent toxicity following EMR treatment may be due to generation of toxic by-products and/or due to the afore-mentioned laccase – SA mechanisms. A series of batch tests assessing the toxicity of pure solutions of laccase and SA preparations as well their mixture helped to provide insights into this aspect. Through a systematic consideration of both TrOC removal efficiency and effluent toxicity, an optimal SA dosage range was recommended.

6.2. MATERIALS AND METHODS

6.2.1 Trace organic contaminants

See **Section 3.2.1, Chapter 3.**

6.2.2 Enzyme solution and mediator

See **Section 5.3.5.3, Chapter 5.**

6.2.3 Assessment of laccase and SA toxicity

The bacterial luminescence toxicity screen (BLT-Screen) method described by van de Merwe and Leusch (2015) was used to investigate the individual and interactive toxicity of laccase and SA. SA was serially diluted (1:2) across the columns of a 96-well plate with phosphate buffered saline assay media to obtain concentrations ranging from 4 to 1000 μM . Similarly, laccase was serially diluted (1:2) down the rows of a separate 96-well plate to obtain concentrations ranging from 12 to 750 $\mu\text{M}_{(\text{DMP})/\text{min}}$. A volume of 100 μL from each of the aforementioned plates (final volume of 200 μL per well) was then added to a white 96-well microplate (Greiner Bio-One, Austria), resulting in different combinations of laccase and SA concentrations ranging from 2 to 500 μM for SA and 6 to 375 $\mu\text{M}_{(\text{DMP})/\text{min}}$ for laccase. Serially diluted (1:5) standard curves of pentachlorophenol (which was used as the reference compound) and Milli-Q water (negative control) in phosphate buffered saline assay media were included in duplicate for quality control. Five microliters of the luminescent bacteria, *Photobacterium leiognathi* (from a cryopreserved aliquot) was then added to each well using a multi-channel pipette. Exactly 30 mins later the luminescence of each well was measured on a Fluostar plate reader (BMG Labtech, Germany).

The experiment was run on two separate occasions and the mean % inhibition of luminescence was calculated for each combination of laccase and SA, using following equation from van de Merwe and Leusch (2015).

$$\% \text{ Inhibition} = \left[1 - \left(\frac{\text{luminescence}_{\text{sample}}}{\text{luminescence}_{\text{control}}} \right) \right] \times 100 \quad \text{Equation 6.1}$$

For each SA: laccase ratio (which ranged from 0.01 to 21.3) the inhibition of luminescence (regarded as ‘toxicity’) was plotted against the sum of SA and laccase concentrations, and the IC_{50} (concentration that causes 50% inhibition of bacterial luminescence) was calculated from the straight line regression (note that all data was below 70%, and thus within the linear phase of the dose-response curve). The % inhibition was also plotted against SA only and laccase only concentrations and the IC_{50} values for SA and laccase were calculated from the straight line regressions (see Section 6.3.1). These IC_{50} values were further used in the assessment of interactive toxicity. The interactive toxicity of laccase and SA was analysed using an isobologram, following methods originally described by Gaddum (1949) and Loewe (1953), and

implemented widely in mixture toxicity research (*e.g.*, (Sørensen et al., 2007; Mori et al., 2015). The EC₅₀ (concentration required to inhibit 50% of bacterial luminescence) for each SA : laccase ratio (which ranged from 0.01 to 21.3) was derived by linear interpolation from the dataset, and individual toxic units (TUs) at each ratio were calculated in Microsoft Excel (Microsoft Corp, Redmond, WA, USA) for both laccase and SA as **Equation 6.2**:

$$TU_i = (p_i \times IC_{50i}) / IC_{50} \quad \text{Equation 6.2}$$

where

i = a specific laccase:SA ratio,

p_i = the proportion of the total concentration due to the compound (*e.g.*, $p_{i\text{laccase}} = C_{\text{laccase}} / (C_{\text{laccase}} + C_{\text{SA}})$, where C_{laccase} and C_{SA} are the concentration of laccase and SA in the mixture, respectively),

IC_{50i} = the IC_{50} at the particular laccase:SA ratio i , and

IC_{50} = the IC_{50} of each compound individually determined from the concentration-effect curve of single compounds (*i.e.*, 380 μM and 370 $\mu\text{M}_{(\text{DMP})/\text{min}}$ for SA and laccase, respectively; see Section 3.1). (see **Section 6.3.1**).

Laccase and SA interactive toxicity was analysed by plotting TU_{laccase} vs. TU_{SA} and examining the position of the resulting isobole (see **Section 6.3.1**). In addition, a contour map of toxicity, plotting laccase activity vs. SA concentration was generated in SigmaPlot (Systat Software Inc, San Jose, CA, USA).

6.2.4 Enzymatic membrane reactor

The lab scale EMR set up has been described in a previous study (Nguyen et al., 2015). Briefly, a hollow fiber membrane module (Microza Membranes, Pall Corporation, NSW, Australia) with a molecular weight cut off of 6 kDa was submerged in a 1.5 L (active volume) glass reactor. This membrane was selected based on initial trials confirming complete retention of the enzyme. The membrane module was made of polyacrylonitrile, and had a surface area of 0.19 m². Laccase (2 mL) was added from stock solution to 1.5 L Milli-Q water. This resulted in an enzymatic activity of 180 $\mu\text{M}_{(\text{DMP})/\text{min}}$ in the reactor. Laccase activity was maintained in the range of 160 to 180 $\mu\text{M}_{(\text{DMP})/\text{min}}$ by adding 400 μL of the commercial laccase solution per liter of the reactor volume every 12 h (equivalent to a laccase dose of 46 mg/L.d) following a previously developed protocol (Nguyen et al., 2015). A peristaltic pump was used to operate the membrane intermittently

(8 min on and 1 min off) at an average flux of 1.1 L/ m² h. This resulted in a hydraulic retention time (HRT) of 8 h. In this study, the EMR was operated under six different combinations of TrOC and SA concentrations (Table 6.1) to assess their impacts on both the removal efficiency and effluent toxicity. Feed solutions containing TrOCs at concentrations of 5, 50 and 100 µg/L in Milli-Q water were prepared daily and fed continuously to the reactor. SA solutions were continuously fed to the reactor at a final concentrations of 10, 50 or 100 µM. A low flux was applied to minimize membrane fouling, thus allowing the focus of the study to remain on the removal performance. The EMR was operated for a period of 9 x HRT (i.e. 72 h) in each run. Duplicate samples of influent and permeate were collected at three sampling events over the operation period. The samples were collected after a period of 3 x HRT (i.e. 24 h) following the start of operation to ensure that steady removal performance was established. At the end of each run, duplicate samples were collected to quantify TrOC concentration in the reactor supernatant. Additionally, the membrane module was taken out of the reactor and subjected to backwashing with 1 L Milli-Q water at a flux of 5 L/m² h. The backwash solution was collected to measure any TrOC released during the backwashing. Milli-Q water backwashing was adequate to retrieve the original transmembrane membrane pressure (1 kPa) before a new run. There was negligible increase in TMP during each run.

Table 6.1: Sequence of EMR experiment

Experiment ID	SA concentration (µM)	TrOC concentration (µg/L)	Duration (x HRT)
1	0	5	9
2	10	5	9
3	10	50	9
4	10	100	9
5	50	100	9
6	100	100	9

6.2.5 Analytical methods

6.2.5.1 Trace organic contaminants

The concentrations of the TrOCs were measured by a previously reported analytical technique involving solid phase extraction, derivatisation and quantitative determination by a Shimadzu GC/MS (QP5000) system (**Section 3.2.4.1, Chapter 3**). The quantitative detection limits of this analytical method were compound specific and in the range 1 to 20 ng/L (**Appendix Table A-1**).

The removal efficiency was calculated as shown in Equation 6.3:

$$R=100 \times \left(1 - \frac{C_{eff}}{C_{inf}}\right) \quad \text{Equation 6.3}$$

where C_{inf} and C_{eff} are influent and effluent (permeate) concentrations of the TrOCs, respectively. TrOC biodegradation during EMR treatment was calculated using the following mass balance equation:

$$C_{inf} \times V_{inf} = C_{eff} \times V_{eff} + C_{ads} \times V_{ads} + C_{sup} \times V_{sup} + \text{biodegradation} \quad \text{Equation 6.4}$$

where C_{inf} and V_{inf} are influent concentration (ng/L) and volume (L), respectively; C_{eff} and V_{eff} are effluent concentration (ng/L) and volume (L), respectively; C_{ads} and V_{ads} correspond to TrOCs adsorbed on membrane and denote the concentration of TrOCs (ng/L) in and volume (L) of cleaning solution during the membrane backwashing with Milli-Q water at the end of operation, respectively; C_{sup} and V_{sup} are reactor supernatant concentration (ng/L) and volume (L), respectively. The mass of TrOCs adsorbed on membrane was estimated from 1 L of cleaning solution during the membrane backwashing with Milli-Q water.

6.2.5.2 Enzymatic activity and toxicity assay

See **Section 3.3.4.2, Chapter 3**.

6.3. RESULTS AND DISCUSSION

6.3.1 Toxicity of utilized laccase and mediator

6.3.1.1 Toxicity of the individual solutions

Pure solutions of laccase and SA were both toxic to the luminescent bacteria, *Photobacterium leiognathi* (**Figure 6.1**). Using the regression equations presented in Figure 1, the IC_{20} values

(limit of quantification of the BLT-Screen, van de Merwe and Leusch 2015) were 147 $\mu\text{M}_{(\text{DMP})/\text{min}}$ for laccase and 153 μM for SA. In addition, more relevant to the calculations of interactive toxicity, the IC_{50} (*i.e.*, concentration required to inhibit 50% of bacterial luminescence) for laccase and SA was 370 $\mu\text{M}_{(\text{DMP})/\text{min}}$ (**Figure 6.1a**) and 380 μM (**Figure 6.1b**), respectively. In nature, the laccase system has been previously described to protect plant-pathogen fungi from the toxic phytoalexins and tannins in the host plant (Pipe et al., 2000) or to protect invertebrates (*e.g.*, crustaceans) from invading microorganisms (Luna-Acosta et al., 2011). However, this is the first report regarding aquatic toxicity (measured in terms of inhibition of bacterial luminescence) of a purified laccase preparation. Notably, Kim et al. (2006b) reported negligible toxicity of a laccase purified from *T. versicolor*. They, however, carried out the toxicity assay with a much lower laccase activity of 1.5 $\mu\text{M}_{(\text{ABTS})/\text{min}}$, and also used a different luminescent bacterium (*i.e.*, *Photobacterium phosphoreum*). The laccase preparation used in this study contained preservatives such as propylene glycol, glucose and glycine (25%, 4% and 2%, w/w, respectively), which are generally considered non-toxic. The toxicity of laccase observed in the present study is therefore most likely attributed to the damage to bacterial cell via laccase-catalyzed reactions as further discussed in Section 6.3.1.2.

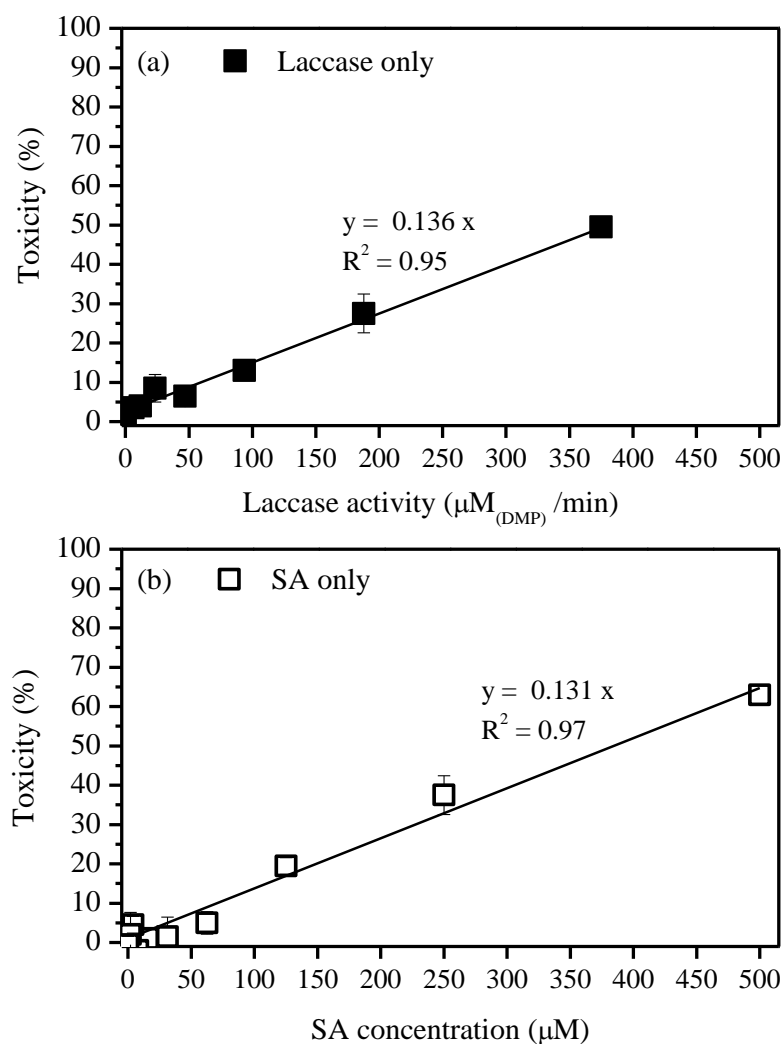


Figure 6.1: Toxicity (%) *i.e.*, inhibition of bacterial luminescence (%) of: (a) laccase ($\mu\text{M}_{(\text{DMP})}/\text{min}$) and (b) SA (μM) in batch tests. Data presented as average \pm standard deviation ($n=2$ independent measurements). Data below the detection limit of the BLT-Screen (10% inhibition of luminescence) have been included to highlight the low toxicity at low SA and laccase concentrations. Exclusion of these data makes results in identical slope and R^2 values of these regressions.

SA is a naturally occurring lignocellulose degradation product, which is thought to inhibit growth of a range of microbes including yeast, bacteria and algae (Richmond et al., 2012; Yu et al., 2014). For example, Richmond et al. (2012) reported that SA hampers metabolism of *Clostridium beijerinckii* (NCIMB 8052) by inhibiting the expression and activity of coenzyme A

transferase during its growth. However, as a notable omission, most available studies investigating pollutant removal via SA-enhanced laccase systems did not investigate SA toxicity, and assumed that SA was non-toxic due to its natural origin (Camarero et al., 2007; Cañas et al., 2010). The results in this study confirm the toxicity of the pure solution of SA at concentrations above 125 μM .

6.3.1.2 Toxicity of laccase-SA mixture

Oxidation of mediators (in this case SA) generates highly reactive radical species, which can degrade target pollutants (Kim et al., 2006a; Fillat et al., 2010). Kim and Nicell (2006a) suggested that radicals formed due to oxidation of mediators may also interact with vitally important biomolecules and result in cytotoxic effects. Thus, it was anticipated that the laccase - SA mixtures prepared here would exhibit increased toxicity. Indeed a strong synergism between laccase and SA in terms of the toxicity of the mixture was observed (**Figure 6.2**). As noted in **Section 6.2.3**, the interactive toxicity of laccase and SA was analysed using an isobologram *i.e.*, $\text{TU}_{\text{laccase}}$ vs. TU_{SA} plot (**Figure 6.2a**). Chemicals with common modes of action can act jointly to produce combination effects that are larger than the effects of each mixture component applied individually. These effects can be described by ‘dose or concentration addition’. In this study, all the points in the isobologram stayed below the ‘concentration addition line’ or in the ‘zone of synergism’ (**Figure 6.2a**), confirming that the combination of laccase and SA had a synergistic effect on the tested microorganism (*i.e.*, *Photobacterium leiognathi*). Through a Microtox assay using *Vibrio fischeri*, Fillat et al. (2010) observed an 18 fold increase in effluent toxicity due to laccase – SA interaction during bleaching of flax pulp by laccase (the pulp contains SA). Maruyama et al. (2007) observed effective degradation of imazalil (a postharvest fungicide) by laccase in the presence of 4-hydroxybenzoic acid (a natural mediator) but cytotoxicity of the effluent against mouse fibroblast L929 cells revealed toxicity of 4-hydroxybenzoic acid. These previous investigations were conducted in batch mode and under selected dosages of laccase and the mediator. The data reported in the current study demonstrates this phenomenon in a more comprehensive manner *i.e.*, over a broader range of concentrations of laccase and SA, and in both batch (**Section 6.3.1.2**) and continuous flow (**Section 6.3.6**) modes. The toxicity contour plot shown in **Figure 6.2b** demonstrates further the strong impact of the presence of laccase on

SA toxicity. Additional discussion on the effluent toxicity during the treatment of TrOCs by EMR has been presented in **Section 6.3.6**.

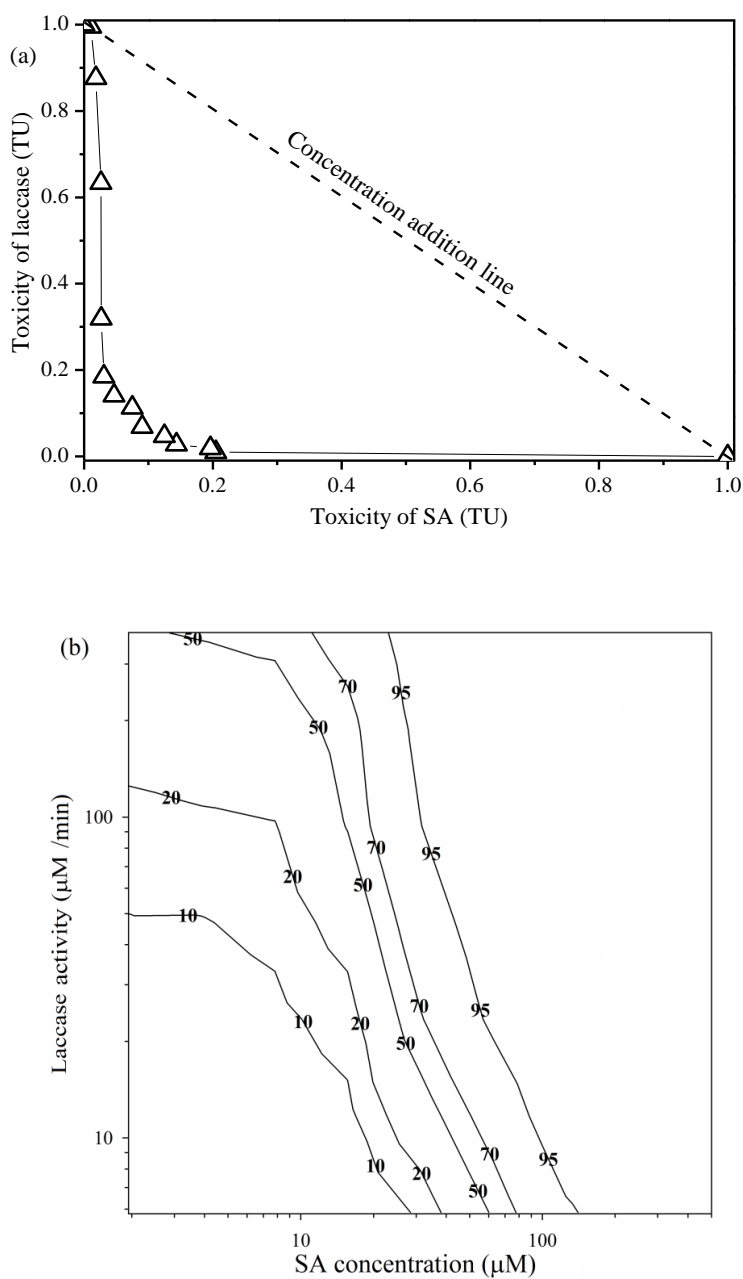


Figure 6.2: Synergistic effect between laccase and SA toxicity (a) isobologram and (b) toxicity contour map showing 10, 20, 50, 70 and 95% toxicity profiles at varying concentrations of laccase activity vs. SA concentration.

6.3.2 Overall TrOC removal by EMR

The performance of the EMR in removing TrOCs was evaluated at different combinations of TrOC concentration (from 5 - 100 µg/L) and SA concentration (from 0 - 100 µM) (**Table 6.1**). TrOC removal performance at a TrOC concentration of 5 µg/L and SA concentration of 0 and 10 µM is first discussed to illustrate the general trend (**Figure 6.3**). Laccase can efficiently degrade phenolic compounds, particularly the polyphenols (Yang et al., 2013), and thus it was not a surprise that, even in the absence of SA, the EMR achieved efficient removal of all but a few phenolic TrOCs, namely salicylic acid, formononetin, pentachlorophenol and enterolactone (below 40% removal). The low removal efficiency of these compounds may be due to steric hindrance which prevents the reaction between laccase and the compounds. For example, d'Acunzo et al. (2006) observed that β -naphthols and 2,4,6-trichlorophenol were not oxidized by laccase due to steric hindrance. The presence of electron withdrawing group at the *ortho* position to the –OH group hinders the approach of the substrate to the active sites of laccase (d'Acunzo et al., 2006), which may explain the low removal of salicylic acid and pentachlorophenol in the current study. On the other hand, except for benzophenone, amitriptyline, octocrylene and diclofenac, the non-phenolic TrOCs were poorly removed by the EMR. The efficient removal of diclofenac could be due to its direct oxidation by laccase via the aniline group in its structure (Lloret et al., 2010). Laccase could mediate oxidative cleavage of carbonyl group in ketone such as [N,N-dimethylaminophenyl][N-methylaminophenyl] benzophenone (Parshetti et al., 2011), which may explain the high removal of benzophenone in this study. Amitriptyline and octocrylene were previously noted to be persistent to laccase oxidation in batch tests (Nguyen et al., 2014; Nguyen et al., 2015). However, their high removal by the EMR in this study may be due to their retention by a laccase gel layer (formed on the membrane) and subsequent degradation, as further discussed in **Section 6.3.5**. Moreover, the oxidation of phenolic compounds may produce radicals which may react with non-phenolic TrOCs (Tran et al., 2010). However, the assessment of the fact whether the selected phenolic TrOCs acted as laccase mediator is beyond the scope of this study.

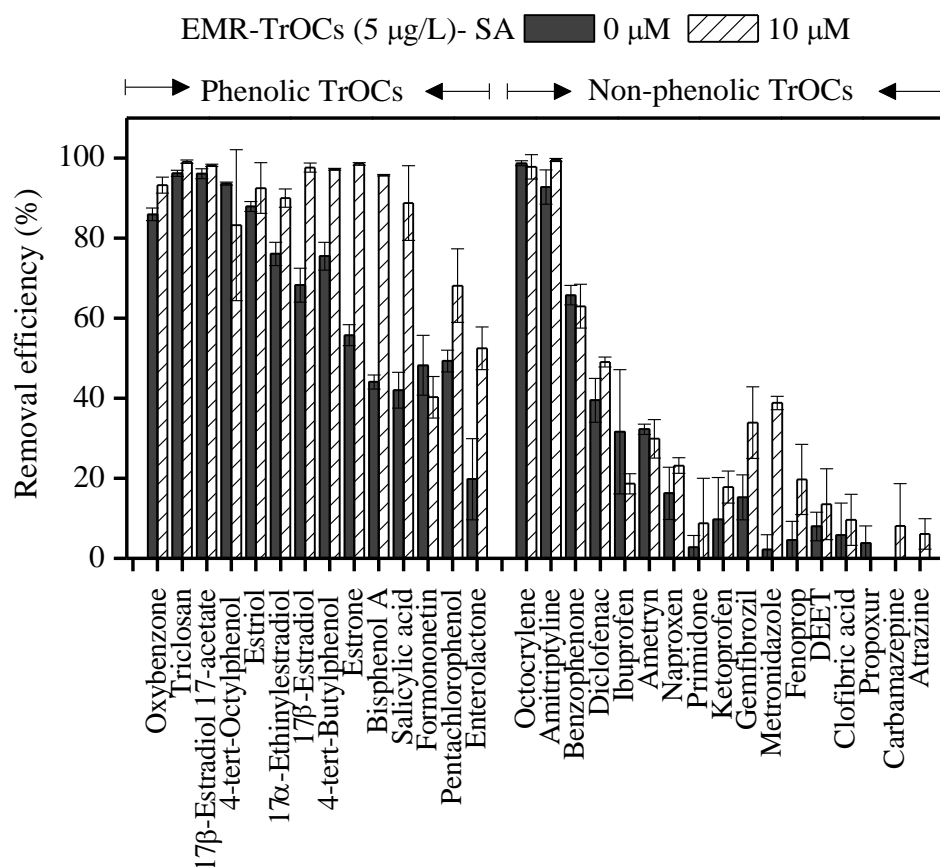


Figure 6.3: Removal efficiency of 31 TrOCs by the laccase-EMR. Laccase activity was maintained at the range from 160 to 180 $\mu\text{M}_{(\text{DMP})}/\text{min}$ by the addition of 400 μL of the commercial laccase solution per liter of the reactor volume every 12 h (equivalent to a laccase dose of 46 mg/ L.d). The EMR was operated for 72 h (*i.e.*, 9 x HRT). Data presented as average \pm standard deviation ($n=6$).

Addition of SA at 10 μM led to the following changes in TrOC removal (**Figure 6.3**): i) higher removal of previously well-degraded TrOCs (*e.g.*, estriol, 17 α -ethinylestradiol, 17 β -estradiol, and 4-tert-butylphenol), ii) improved degradation of some phenolic TrOCs (*e.g.*, estrone, bisphenol A, salicylic acid, formononetin, pentachlorophenol, and enterolactone) originally resistant to laccase-EMR treatment, and iii) degradation of a few additional non-phenolic TrOCs (*e.g.*, metronidazole, gemfibrozil, and ketoprofen). The phenoxyl radicals formed during the oxidation of SA by laccase may act as ‘electron shuttles’ between laccase and the target compound (Xu et al., 2000). These radical species may have higher redox potential than laccase

and/or overcome the steric hindrance due to their small size, and thus improve TrOC removal. Indeed the redox-potential of the laccase solution increased significantly (from 270 mV to 530 mV) due to SA addition at 10 μ M in this study. This is consistent with the observation of Weng et al. (2012), who reported only batch test data and monitored removal of a few TrOCs.

A notable observation made during the initial run was that even with the addition of SA (10 μ M), the laccase-EMR could degrade only a limited number of non-phenolic compounds (**Figure 6.3**). Previous batch studies have reported that both abundance and stability of the oxidized intermediates from the mediators affect TrOC degradation. Mediator concentration can influence these (Camarero et al., 2007; Nguyen et al., 2014). Thus, there may be scope of optimizing SA dosages. **Sections 6.3.3** and **6.3.4** present further discussion on this aspect.

6.3.3 Impact of TrOC concentrations on EMR performance

TrOC removal by the EMR was further evaluated at higher influent TrOC concentrations (*i.e.*, 50 and 100 μ g/L) but with the same SA dose of 10 μ M and laccase activity of 180 μ M_(DMP)/min as in the first trial (**Section 6.3.2**). Except pentachlorophenol, formononetin and enterolactone, which showed 25 to 40% improvement in removal, a similarly high removal efficiency of the phenolic TrOCs was noted irrespective of their concentration in the influent (**Figure 6.4**). Overall 80-100% removal of the phenolic TrOCs was achieved by the EMR. Therefore, further discussion in this section focuses on the removal of the non-phenolic TrOCs.

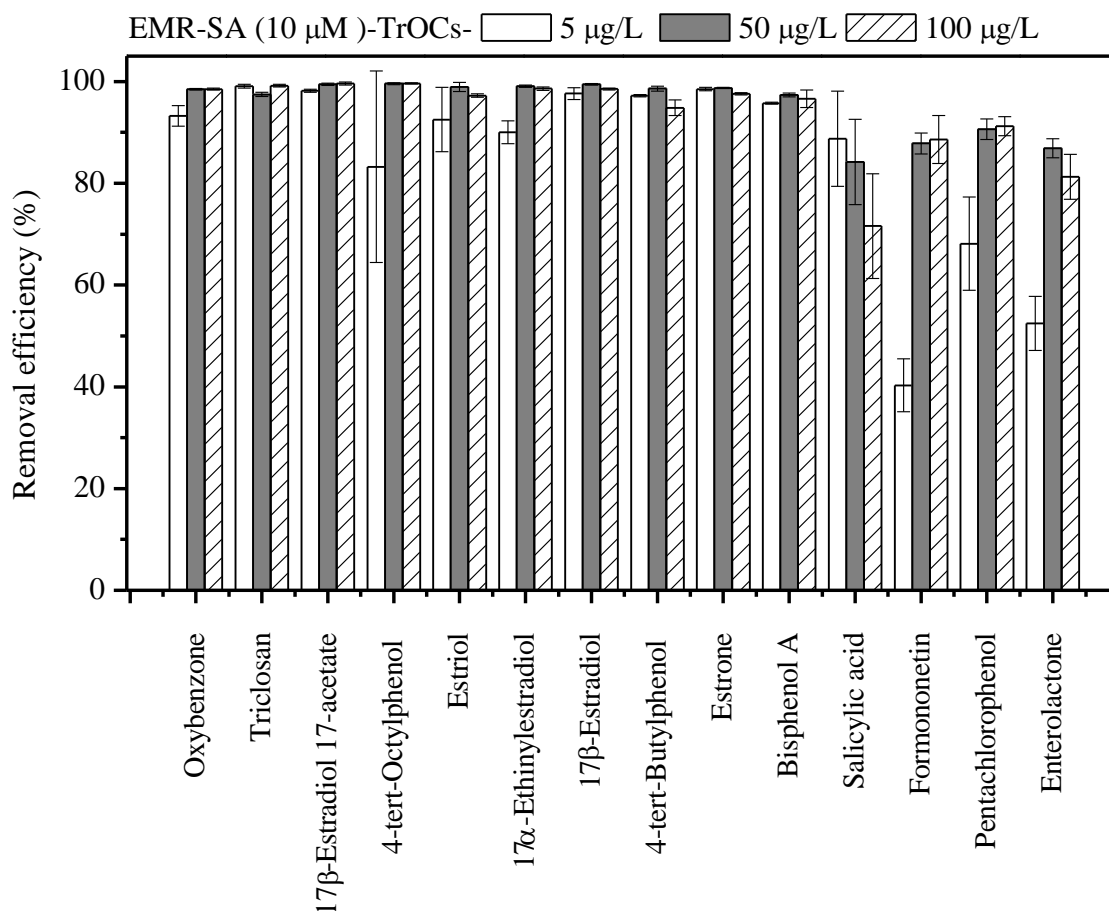


Figure 6.4: Removal efficiency of phenolic TrOCs by laccase-EMR with SA addition at a concentration of 10 µM. The feed contained 31 TrOCs at a concentration of 5, 50 and 100 µg/L of each compound. Laccase activity was maintained at the range from 160 to 180 µM_(DMP)/min by the addition of 400 µL of the commercial laccase solution per liter of the reactor volume every 12 h (equivalent to a laccase dose of 46 mg/Ld). The EMR was operated for 72 h (*i.e.*, 9 x HRT). Data presented as average ± standard deviation (n=6).

The removal efficiency of a number of non-phenolic TrOCs increased when influent TrOC concentration was increased from 5 to 50 µg/L (**Figure 6.5**). However, the removal of a few compounds, namely, ametryn, naproxen and primidone decreased when the influent TrOC concentration was further increased to 100 µg/L, probably due to kinetic limitations. In general, the rate of an enzymatic reaction increases with substrate concentration, but eventually reaches a

plateau as all the active sites of the enzyme become occupied (Cristóvão et al., 2008). Thus beyond a certain substrate concentration, the percentage removal efficiency drops. The drop in removal of ametryn, naproxen and primidone at their higher influent concentrations signifies particular resistance of these TrOCs to degradation by laccase-SA system. This is the first study to assess the impact of influent concentration on the removal of the non-phenolic TrOCs.

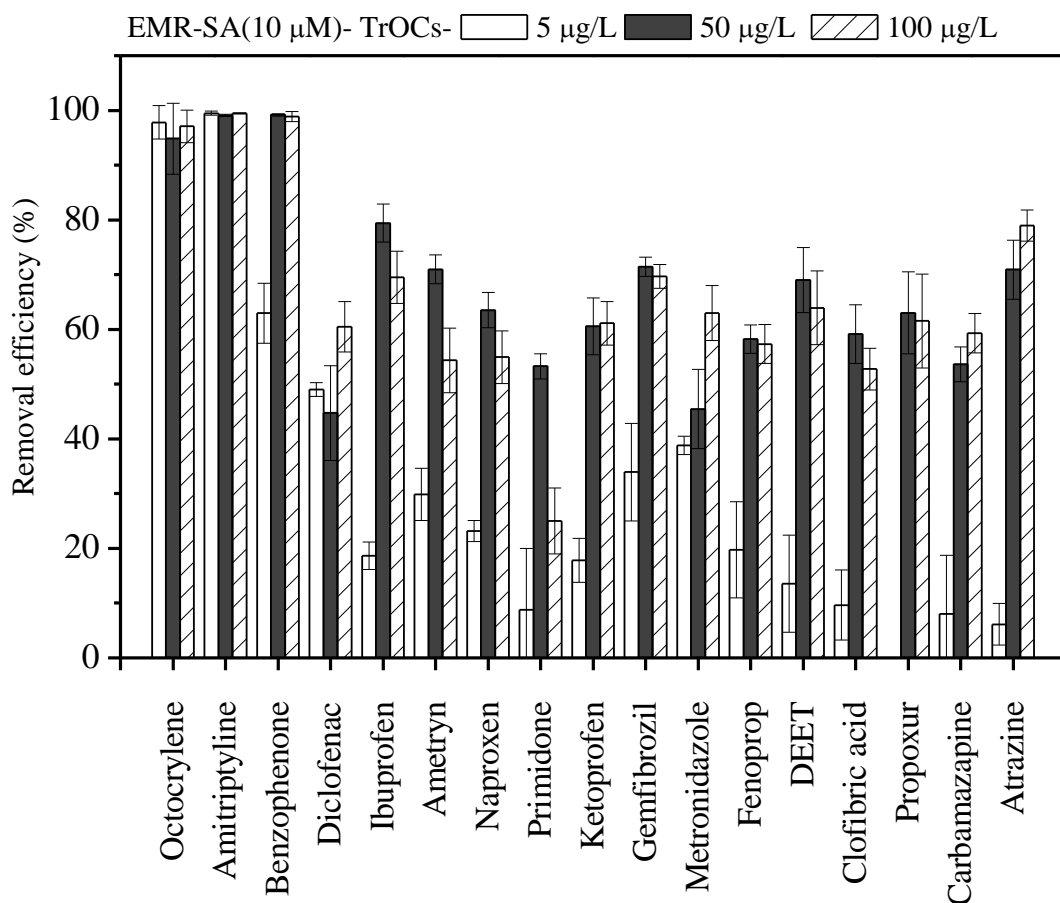


Figure 6.5: Removal efficiency of non-phenolic compounds by the laccase-EMR with SA addition at a concentration of 10 µM. The feed contained 31 TrOCs at a concentration of 5, 50 and 100 µg/L of each compound. Laccase activity was maintained at the range from 160 to 180 µM_(DMP)/min by the addition of 400 µL of the commercial laccase solution per liter of the reactor volume every 12 h (equivalent to a laccase dose of 46 mg/L d). The EMR was operated for 72 h (*i.e.*, 9 x HRT). Data presented as average ± standard deviation (n=6).

6.3.4 Impact of SA concentrations on EMR performance

High removal of the phenolic TrOCs was already obtained at an SA dose of 10 μM (**Figure 6.4**), and the removal efficiencies remained largely unaltered at the higher SA dosages of 50 and 100 μM (data not shown). Similarly, except for 20 - 35% improvement in the removal of diclofenac, metronidazole and primidone, virtually no change in the removal of the non-phenolic TrOCs was observed after increasing SA concentration from 10 to 50 μM (**Figure 6.6**). Furthermore, 10-20% decrease in the removal of a few TrOCs was noted when the SA concentration was increased from 50 to 100 μM . Our results are in line with the general trend observed in the available batch studies (Mizuno et al., 2009; Nguyen et al., 2014) that pollutant degradation by laccase may reach a saturation point beyond a certain mediator concentration, but shows this systematically for the first time in the context of a continuous flow EMR.

In the current study, the laccase activity in the EMR was maintained at the same level during operations with different SA dosages (10, 50 and 100 μM). Rate limitation of SA oxidation by laccase may have had occurred under higher SA concentrations because the rate of the reaction would not increase with the mediator concentration when it is already present at sufficiently high levels to saturate all the reactive sites of the enzyme. Thus, increasing SA concentration beyond 50 μM did not improve TrOC removal efficiency (**Figure 6.6**). Furthermore, reduced degradation of a few target compounds at an SA dose of 100 μM may be because, at excessive mediator concentrations, high levels of reactive radicals are quickly produced, which have a tendency to react with each other rather than with the pollutant (Margot, 2015). Two points are conceivable from the information depicted in **Figure 6.5** and **Figure 6.6**: (i) an SA dose greater than 10 μM is not required for influent TrOC concentrations not exceeding 50 $\mu\text{g/L}$, and (ii) even at an influent TrOC concentration of 100 $\mu\text{g/L}$, improvement in removal of a limited number of TrOCs would be achieved if the SA dose was increased to 50 μM . Applying the lowest possible SA dose would be economical, and additionally it may avoid SA-induced toxicity of treated effluent (discussed in **Section 6.3.6**).

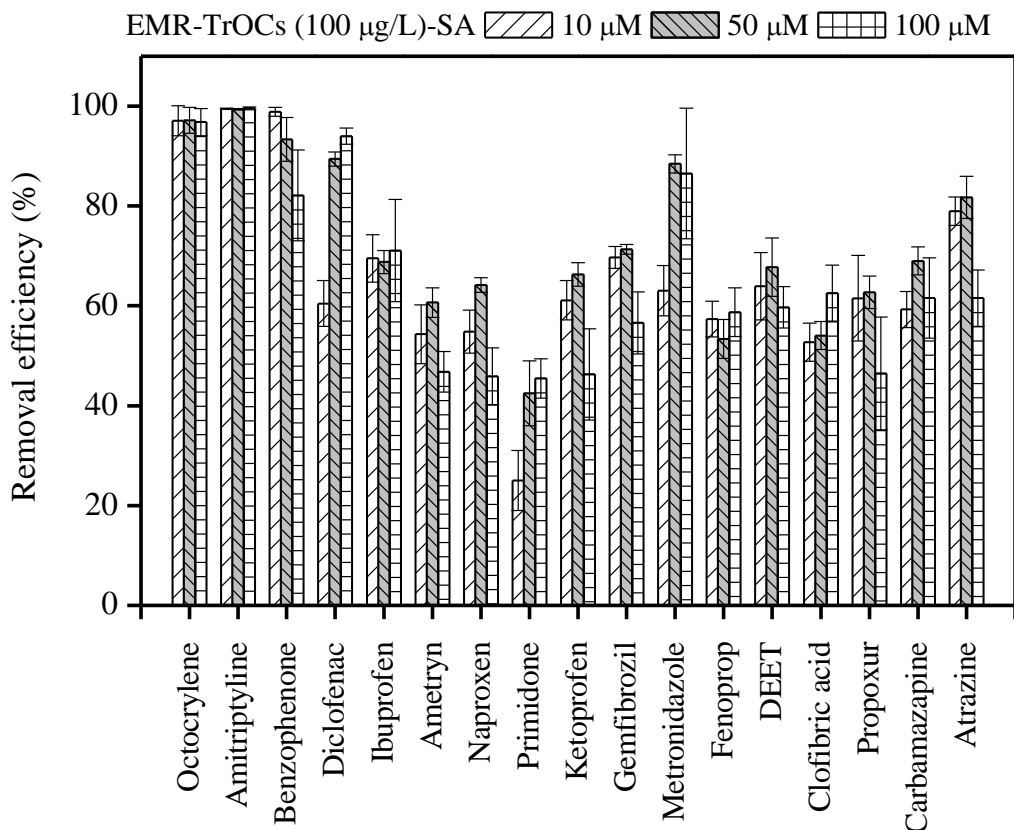


Figure 6.6: Removal efficiency of non-phenolic compounds by the laccase-EMR with SA addition at a concentration of 10, 50 and 100 μM . The feed contained 31 TrOCs at a concentration of 100 $\mu\text{g/L}$ of each compound. Laccase activity was maintained at a range of 160 to 180 $\mu\text{M}_{(\text{DMP})}/\text{min}$ by the addition of 400 μL of the commercial laccase solution per liter of the reactor volume every 12 h (equivalent to a laccase dose of 46 mg/L d). The EMR was operated for 72 h (*i.e.*, 9 x HRT). Data presented as average \pm standard deviation ($n=6$).

6.3.5 Fate of TrOCs

TrOC removal during EMR treatment may occur due to enzymatic degradation, adsorption on and subsequent rejection by the membrane, volatilization and photolysis. Volatilization was expected to be negligible because of the very low vapor pressure or Henry's constant (H) and low ' $H/\log D$ ratio' (**Appendix Table A-1**) of the selected TrOCs. TrOC photolysis was avoided by covering the EMR with aluminum foil. On the other hand, given the relative sizes, the ultrafiltration membrane used in this study was not capable of directly retaining any TrOCs.

However, the laccase molecules retained by a membrane can form a gel layer on it (Modin et al., 2014), which may retain the TrOCs in the reactor and facilitate their further degradation. The following observations made in the current study validate this hypothesis: (i) a laccase gel layer on the membrane was evident by a significant laccase activity in the membrane backwash solution (the measured laccase activity indicated an accumulation of approximately 0.24 g active laccase m² membrane surface *i.e.*, roughly 40% of the total laccase dosed during a run), (ii) TrOCs were detected at low but discernible concentrations in the backwash solution (**Figure 6.7**), evidencing TrOC retention by the laccase layer, and (iii) the ratio of concentration of TrOCs in effluent and supernatant was below 1 for a number of TrOCs (data not shown), indicating an additional ‘removal’ by the laccase gel layer. Overall, a mass balance analysis confirmed that, irrespective of the TrOC and SA concentrations, TrOC removal by the EMR was mainly due to biodegradation (**Figure 6.8**). It is noteworthy that the formation of an enzyme gel layer could lead to the membrane fouling (Nguyen et al., 2014b) requiring periodic cleaning. However, under the operating conditions (*i.e.*, low flux) in this study, no rise in TMP was observed following the development of an enzyme gel layer on the membrane.

The observations regarding the prime role of biodegradation in TrOC removal by EMR is generally consistent with a previous study (Nguyen et al., 2015), but the current study confirms this trend over a range of TrOC and SA concentrations. An additional aspect captured by the current investigation is that higher SA concentration did not necessarily correspond to the higher percentage of biodegradation. For example, most non-phenolic TrOCs were detected in higher concentrations in reactor supernatant for an SA dose of 100 µM than for 50 µM (**Figure 6.8**). This further strengthens the point highlighted in the previous section regarding the importance of mediator dose selection.

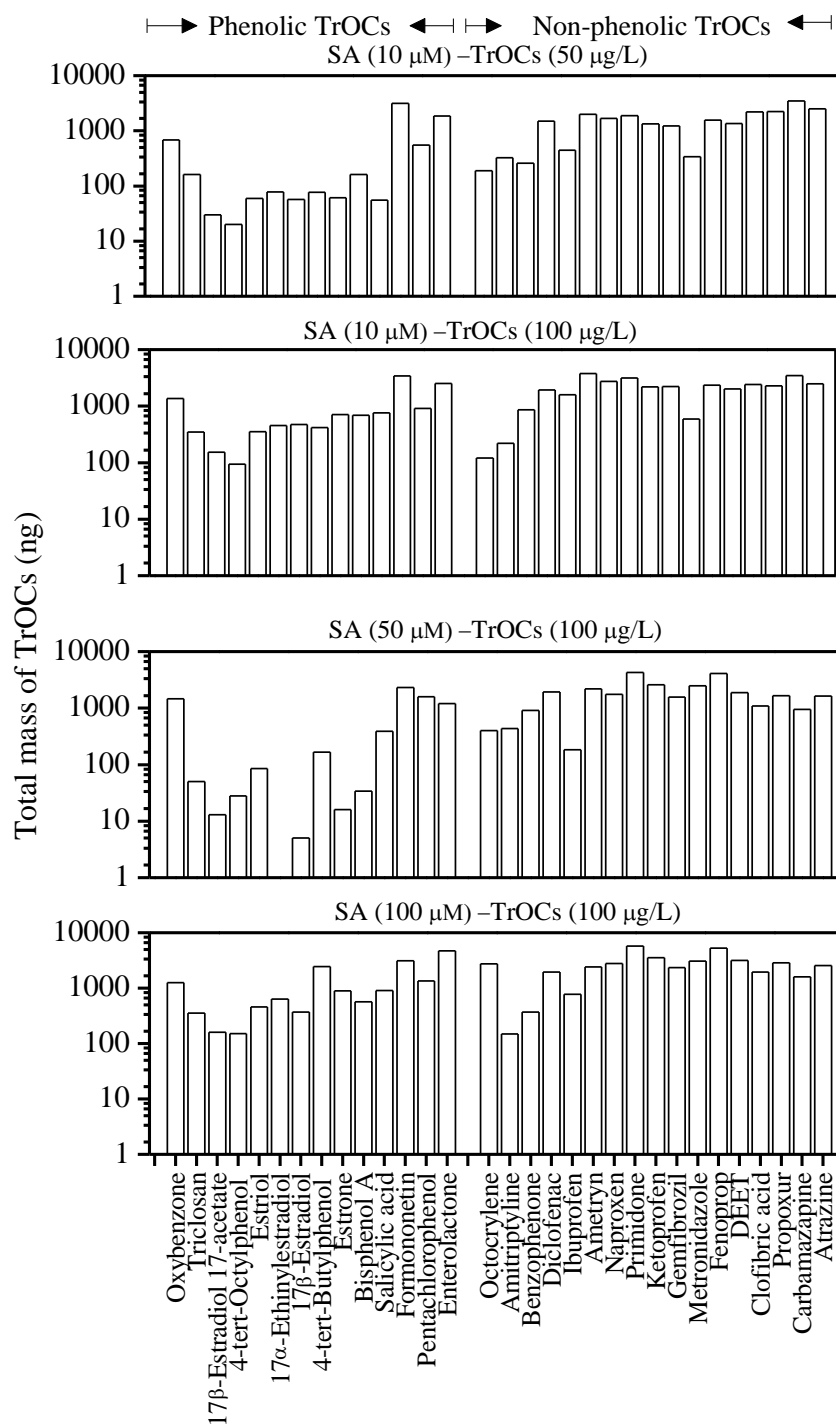


Figure 6.7: Adsorption of TrOCs on the membrane. TrOC extraction was conducted by membrane backwash with 1 L of Milli-Q water at a flux of 5 L/m²h.

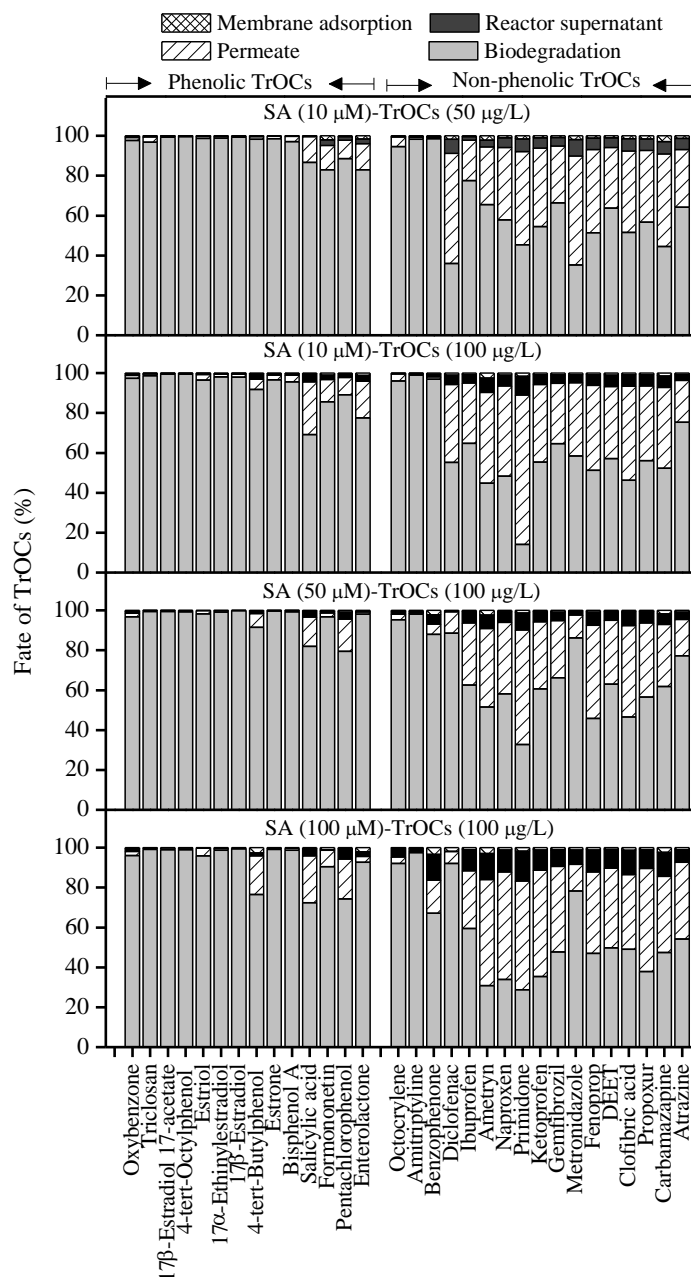


Figure 6.8: Fate of TrOCs (%) during EMR operation with SA addition at a concentration of 10, 50 and 100 μM and TrOC concentration of 50 and 100 μg/L.

6.3.6 Toxicity of EMR-treated effluent

At the concentrations tested, TrOC solutions (*i.e.*, 31 TrOCs in Milli-Q water) showed negligible toxicity, and no increase in toxicity was also observed in EMR effluent in the absence of SA (data not shown). On the other hand, consistent with the batch test data showing SA-induced

toxicity (**Figure 6.1b**), the toxicity of the influent to the EMR during the laccase-SA investigations (*i.e.*, TrOC and SA in Milli-Q) increased with SA concentration. For example, a toxicity of 11.1 rTU was recorded in case of a SA dose of 100 μ M, compared to a toxicity of 2.5 rTU at a SA dose of 10 μ M (**Figure 6.9**). Two notable observations were made regarding the toxicity of the EMR effluent: (i) the toxicity of the influent containing 10 and 50 μ M SA was significantly decreased after the EMR treatment (2.5 *vs.* below detection limit, and 10.5 *vs.* 7.0 rTU, respectively), but (ii) an increase in the toxicity of the effluent was observed when SA was added at a concentration of 100 μ M (11.1 *vs.* 19.9 rTU) (**Figure 6.9**). SA concentration – specific toxicity was also reported in a previous batch study wherein a crude enzyme extract (mainly laccase from *T.versicolor*) and SA were used (Nguyen et al., 2014). Highly reactive phenoxyl radicals are produced due to oxidation of SA by laccase. These radical species can be consumed as they react with TrOCs (Margot, 2015). However, due to continuous addition of SA in excess, copious amount of reactive radical species are likely to be produced. The unconsumed radicals and SA can permeate through the membrane to the treated effluent and increase its toxicity. Furthermore, although in this study the degradation products arising from laccase-only treatment appeared non-toxic, it is uncertain whether the toxicity of the laccase + SA treated effluent (**Figure 6.9**, “effluent”) is due only to the production of reactive radicals from the interaction between the enzyme and the mediator (**Figure 6.2**) or whether it is also caused by the generation of toxic TrOC by-products. Despite this uncertainty, the results indicate that to avoid heightened toxicity in the effluent, the SA dose must be carefully controlled, particularly because higher SA dose did not necessarily achieve better TrOC removal (**Figure 6.4**)

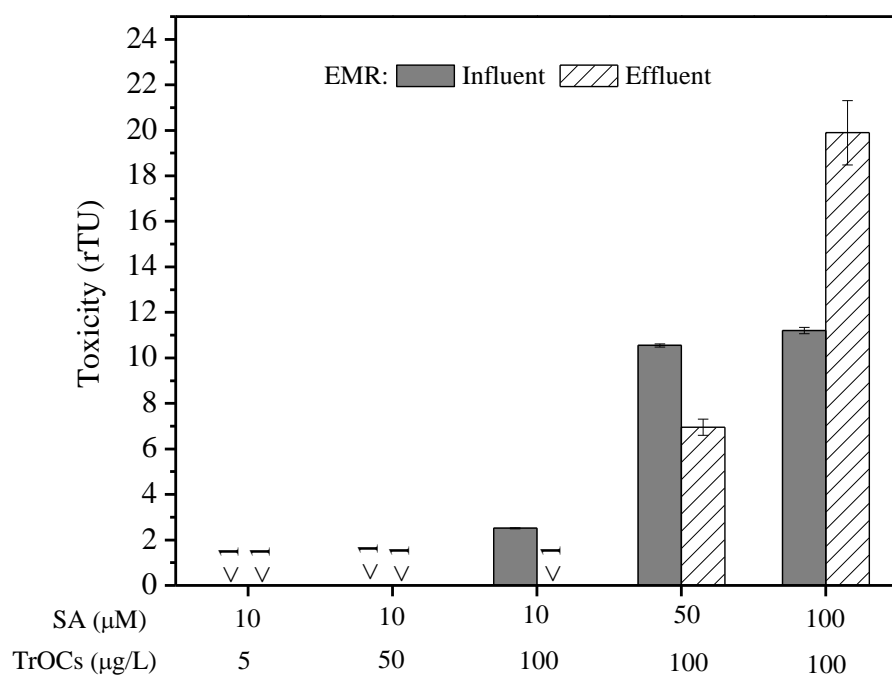


Figure 6.9: Comparison of toxicity following the EMR treatment at different TrOC and SA concentrations. The limit of detection of the toxicity assay was generally below 10% of inhibition values. A 20% inhibition of luminescence was considered to be a conservative value of the minimum response that could be quantified in the assay. The detection limit was 1 rTU. Data presented as average \pm standard deviation (n=2 independent measurements).

6.4. CONCLUSION

With increase in TrOC concentration from 50 - 100 $\mu\text{g/L}$, 8-28% reduction in removal of some resistant TrOCs was observed due to kinetic limitations. SA addition at a dose of 10 μM significantly increased TrOC removal. Elevated dosages of SA (50 and 100 μM) could not improve TrOC removal efficiency further, but increased effluent toxicity, which may be attributed to the passage of unconsumed SA and phenoxyl radicals (highly reactive radicals generated from SA-oxidation by laccase) through the membrane to the effluent. Overall, the TrOC removal and treated effluent toxicity data suggested that a high dose of SA should be avoided.

6.5. REFERENCES

- Ba, S., J. P. Jones and H. Cabana (2014). Hybrid bioreactor (HBR) of hollow fiber microfilter membrane and cross-linked laccase aggregates eliminate aromatic pharmaceuticals in wastewaters. *J. Hazard. Mater.*, 280, 662-670.
- Camarero, S., D. Ibarra, Á. T. Martínez, J. Romero, A. Gutiérrez and J. C. del Río (2007). Paper pulp delignification using laccase and natural mediators. *Enzyme Microb. Technol.*, 40, 1264-1271.
- Cañas, A. I. and S. Camarero (2010). Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. *Biotechnol. Adv.*, 28, 694-705.
- Cristóvão, R. O., A. P. M. Tavares, A. S. Ribeiro, J. M. Loureiro, R. A. R. Boaventura and E. A. Macedo (2008). Kinetic modelling and simulation of laccase catalyzed degradation of reactive textile dyes. *Bioresour. Technol.*, 99, 4768-4774.
- d'Acunzo, F., C. Galli, P. Gentili and F. Sergi (2006). Mechanistic and steric issues in the oxidation of phenolic and non-phenolic compounds by laccase or laccase-mediator systems. The case of bifunctional substrates. *New J. Chem.*, 30, 583-591.
- de Boer, W., L. B. Folman, P. J. A. Klein Gunnewiek, T. Svensson, D. Bastviken, G. Öberg, J. C. del Río and L. Boddy (2010). Mechanism of antibacterial activity of the white-rot fungus *Hypholoma fasciculare* colonizing wood. *Can. J. Microbiol.*, 56, 380-388.
- Escalona, I., J. de Grooth, J. Font and K. Nijmeijer (2014). Removal of BPA by enzyme polymerization using NF membranes. *J. Membr. Sci.*, 468, 192-201.
- Fillat, A., J. F. Colom and T. Vidal (2010). A new approach to the biobleaching of flax pulp with laccase using natural mediators. *Bioresour. Technol.*, 101, 4104-4110.
- Gaddum, J. H. (1949). *Pharmacology*, Oxford University Press, London.
- Hai, F. I., L. D. Nghiem and O. Modin (2013). Biocatalytic membrane reactors: Removal of recalcitrant and emerging pollutants. *Handbook of membrane reactors* (ISBN: 9780857094155). A. Basile. Cambridge, UK, Woodhead publishing. 2: 763-807(<http://dx.doi.org/10.1533/9780857097347.9780857097344.9780857097763>).
- Khelifi, R., L. Belbahri, S. Woodward, M. Ellouz, A. Dhoubi, S. Sayadi and T. Mechichi (2010). Decolourization and detoxification of textile industry wastewater by the laccase-mediator system. *Journal of Hazardous Materials*. 175, 802-808.
- Kim, Y.-J. and J. A. Nicell (2006a). Laccase-catalysed oxidation of aqueous triclosan. *J. Chem. Technol. Biotechnol.*, 81, 1344-1352.
- Kim, Y.-J. and J. A. Nicell (2006b). Laccase-catalyzed oxidation of bisphenol A with the aid of additives. *Process Biochem.*, 41, 1029-1037.
- Lloret, L. (2013). *Enzymatic bioreactors for the oxidation of estrogenic and anti-inflammatory compounds by laccases*. PhD, University of Santiago de Compostela.
- Lloret, L., G. Eibes, T. A. Lú-Chau, M. T. Moreira, G. Feijoo and J. M. Lema (2010). Laccase-catalyzed degradation of anti-inflammatories and estrogens. *Biochem Eng J.* 51, 124-131.
- Loewe, S. (1953). The problem of synergism and antagonism of combined drugs. *Arzneim-Forsch.* 3, 285-290.
- Luna-Acosta, A., D. Saulnier, M. Pommier, P. Haffner, S. De Decker, T. Renault and H. Thomas-Guyon (2011). First evidence of a potential antibacterial activity involving a laccase-type enzyme of the phenoloxidase system in Pacific oyster *Crassostrea gigas* haemocytes. *Fish Shellfish Immunol.*, 31, 795-800.

- Margot, J. (2015). Micropollutant removal from municipal wastewater-From conventional treatments to advanced biological processes. PhD, Ecole Polytechnique Fédérale de Lausanne.
- Maruyama, T., C. Komatsu, J. Michizoe, S. Sakai and M. Goto (2007). Laccase-mediated degradation and reduction of toxicity of the postharvest fungicide imazalil. *Process Biochem.*, 42, 459-461.
- Mizuno, H., H. Hirai, S. Kawai and T. Nishida (2009). Removal of estrogenic activity of isobutylparaben and n-butylparaben by laccase in the presence of 1-hydroxybenzotriazole. *Biodegradation*. 20, 533-539.
- Modin, O., F. I. Hai, L. D. Nghiem and A. Basile (2014). Gas-diffusion, extractive, and biocatalytic membrane biological reactors. *Membrane Biological reactors*. F. I. Hai, K. Yamamoto and C.-H. Lee. UK (ISBN:971780400655), IWA publishing: 299-333.
- Mori, I. C., C. R. Arias-Barreiro, A. Koutsafitis, A. Ogo, T. Kawano, K. Yoshizuka, S. H. Inayat-Hussain and I. Aoyama (2015). Toxicity of tetramethylammonium hydroxide to aquatic organisms and its synergistic action with potassium iodide. *Chemosphere*. 120, 299-304.
- Nguyen, L. N., F. I. Hai, J. Kang, F. D. L. Leusch, F. Roddick, S. F. Magram, W. E. Price and L. D. Nghiem (2014). Enhancement of trace organic contaminant degradation by crude enzyme extract from *Trametes versicolor* culture: Effect of mediator type and concentration. *J. Taiwan Inst. Chem. Eng.*, 45, 1855-1862.
- Nguyen, L. N., F. I. Hai, W. E. Price, J. Kang, F. D. L. Leusch, F. Roddick, J. P. van de Merwe, S. F. Magram and L. D. Nghiem (2015). Degradation of a broad spectrum of trace organic contaminants by an enzymatic membrane reactor: Complementary role of membrane retention and enzymatic degradation. *Int. Biodeterior. Biodegrad.*, 99, 115-122.
- Parshetti, G. K., S. G. Parshetti, A. A. Telke, D. C. Kalyani, R. A. Doong and S. P. Govindwar (2011). Biodegradation of Crystal Violet by *Agrobacterium radiobacter*. *J. Environ. Sci.* 23, 1384-1393.
- Pipe, N. D., C. M. Brasier and K. W. Buck (2000). Evolutionary Relationships of the Dutch Elm Disease Fungus *Ophiostoma novo-ulmi* to Other *Ophiostoma* Species Investigated by Restriction Fragment Length Polymorphism Analysis of the rDNA Region. *J. Phytopathol.*, 148, 533-539.
- Richmond, C., V. Ujor and T. C. Ezeji (2012). Impact of syringaldehyde on the growth of *Clostridium beijerinckii* NCIMB 8052 and butanol production. *3 Biotech*. 2, 159-167.
- Schwarzenbach, R. P., B. I. Escher, K. Fenner, T. B. Hofstetter, C. A. Johnson, U. von Gunten and B. Wehrli (2006). The Challenge of Micropollutants in Aquatic Systems. *Science*. 313, 1072-1077.
- Sørensen, H., N. Cedergreen, I. Skovgaard and J. Streibig (2007). An isobole-based statistical model and test for synergism/antagonism in binary mixture toxicity experiments. *Environ. Ecol. Stat.* 14, 383-397.
- van de Merwe, J. and F. Leusch (2015). A sensitive and high throughput bacterial luminescence assay for assessing aquatic toxicity - the BLT-Screen. *Environ. Sci. Processes Impacts*. In press.
- Weng, S.-S., K.-L. Ku and H.-T. Lai (2012). The implication of mediators for enhancement of laccase oxidation of sulfonamide antibiotics. *Bioresour. Technol.*, 113, 259-264.
- Xu, F., J. J. Kulys, K. Duke, K. Li, K. Krikstopaitis, H.-J. W. Deussen, E. Abbate, V. Galinyte and P. Schneider (2000). Redox chemistry in laccase-catalyzed oxidation of N-Hydroxy compounds. *Appl. Environ. Microbiol.*, 66, 2052-2056.

- Yang, S., F. I. Hai, L. D. Nghiem, W. E. Price, F. Roddick, M. T. Moreira and S. F. Magram (2013). Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: A critical review. *Bioresour. Technol.*, 141, 97-108.
- Yu, X., J. Zeng, Y. Zheng and S. Chen (2014). Effect of lignocellulose degradation products on microbial biomass and lipid production by the oleaginous yeast *Cryptococcus curvatus*. *Process Biochem.*, 49, 457-465.

CHAPTER 7: Trace organic contaminant removal by an enzymatic membrane reactor: Effects of simultaneous addition of mediator and granular activated carbon

This chapter has been published as:

Luong N Nguyen, Faisal I Hai, William E Price, Frederic DL Leusch, Felicity Roddick, Hao H Ngo, Wenshan Guo, Saleh F Magram, Long D Nghiem. (2014) The effects of mediator and granular activated carbon addition on degradation of trace organic contaminants by an enzymatic membrane reactor. *Bioresource Technology*, 167, 169-177

7.1. INTRODUCTION

Enzymatic transformation of organic contaminants that are otherwise resistant to conventional activated sludge treatment is a promising eco-friendly concept (Jochems et al., 2011). Enzymatic treatment can be accomplished under mild conditions, achieves high reaction specificity and rates, and generally only requires a small dosage. However, the application of enzymatic treatment in a continuous system remains a challenge due to enzyme washout with the treated effluent (Hai et al., 2012). The use of membranes with pore size smaller than the enzyme molecule presents an elegant approach to preventing enzyme wash out (Lloret et al., 2012). In an enzymatic membrane reactor (EMR), the enzyme remains within the reactor allowing operation with continuous feeding and treated effluent withdrawal. Compared to the conventional approach of enzyme immobilization on a suitable support material (Cabana et al., 2009), the EMR system offers several advantages such as more effective retention of enzymes, dispersion of enzymes in the reactor (eliminating the mass transfer limitations typically associated with attachment on supports), and easy replenishment of fresh enzymes during long term operation.

Recent studies have explored EMRs for the treatment of pollutants such as dyes and aromatic hydrocarbons (Chhabra et al., 2009; Mendoza et al., 2011). Trace organic contaminants (TrOCs) is a group of emerging pollutants that have been routinely detected in sewage and sewage-impacted natural water at concentrations ranging from ng/L to µg/L (Boonyaroj et al., 2012; Navaratna et al., 2012). TrOCs can be classified into several groups including pharmaceuticals and personal care products, pesticides, steroid hormones, phytoestrogens, and industrial chemicals. Many of these TrOCs can cause adverse physiological changes in aquatic organisms and can potentially affect human health after prolonged exposure. To date, the enzymatic removal of TrOCs has been mostly investigated in batch tests (Tran et al., 2010; Yang et al., 2013a) using different enzymes such as laccase, lignin peroxidase, and manganese peroxidase. Among these enzymes, laccase (Benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is of particular interest as it only requires molecular oxygen as a co-substrate. Recent studies have demonstrated that laccase can effectively degrade a range of TrOCs that are otherwise resistant to conventional biological processes (Marco-Urrea et al., 2009; Tran et al., 2010; Yang et al., 2013a). TrOC removal by an enzymatic system is dependent on various factors including the molecular structure of the TrOCs, pH, temperature, and properties of the specific enzyme.

Phenolic substrates are particularly amenable to degradation by laccase. The substrate range of laccase can be expanded in the presence of small molecular weight redox-mediators that act as an ‘electron shuttle’ between the enzyme and the target compounds. The degree of enhancement depends predominantly on the type of mediator and TrOC structure (Yang et al., 2013a). However, TrOC degradation by the mediator-enhanced laccase system has been studied almost exclusively in batch tests. To date, only a few studies have investigated continuous TrOC degradation by EMR with continuous dosing of a redox-mediator (Nguyen et al., 2014; Nguyen et al., 2015).

Adsorption onto powdered activated carbon (PAC) or granular activated carbon (GAC) can efficiently remove TrOCs from water. In addition to the hydrophobic partitioning, a range of other mechanisms may govern the adsorption of a compound onto a specific adsorbent. The additional mechanisms include hydrogen bonding, π - π interaction between aromatic rings, and van der Waals forces (*e.g.*, dipole-dipole interaction and London dispersion force). Limited adsorption of ionic compounds, particularly of those containing electron-withdrawing functional groups, has been reported by Nguyen et al. (2012). In this connection, the concept of combined processes such as coupling of membrane bioreactors with PAC/GAC has been explored in a few recent studies (Li et al., 2011; Nguyen et al., 2013). Available data confirms short-term enhancement in removal of resistant TrOCs but highlights the requirement of periodic withdrawal and replenishment of activated carbon. Because laccase can degrade TrOCs that are inefficiently degraded by conventional biological processes, activated carbon dosing to an EMR may lead to enhanced biodegradation rather than only temporary improvement in aqueous phase removal. However, no previous attempts have been made to validate this hypothesis.

This study aims to investigate the removal of four selected TrOCs, known to be resistant to conventional activated sludge treatment, by an EMR using laccase. The two hypotheses noted above to enhance the degradation of TrOCs, namely (i) continuous addition of a redox-mediator, and (ii) simultaneous addition of a redox mediator and GAC, have been systematically validated. Implications of GAC addition on TrOC removal, membrane fouling and the toxicity of the treated effluent are also discussed.

7.2. MATERIALS AND METHODS

7.2.1 Trace organic contaminants

Three pharmaceutically active compounds, namely carbamazepine (CBZ), diclofenac (DCF), and sulfamethoxazole (SMX) and the pesticide atrazine (ATZ) were selected based on their widespread occurrence in raw sewage and sewage-impacted water bodies as well as their resistance to conventional biological treatment processes. These are non-phenolic compounds with molecular weights ranging from 215 (ATZ) to 296 (DCF) Da and with $\log D_{(pH=6)}$ (indicating hydrophobicity) ranging from 0.43 (SMX) to 2.72 (DCF). Analytical grade (> 98%) standards of these TrOCs were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). A stock solution containing 1 g/L of each compound was prepared in pure methanol. This stock solution was stored at -18°C in the dark and was used within one month.

7.2.2 Enzyme solution, mediator and granular activated carbon

A commercially available laccase (Novozym 51003) purified from genetically modified *Aspergillus oryzae* was supplied by Novozymes Australia Pty Ltd. This enzyme has a molecular weight of 56 kDa. The enzyme stock solution had a density, purity and activity of 1.12 g/mL, approximately 10% (w/w) and 150,000 $\mu\text{M}_{(\text{DMP})}/\text{min}$, respectively.

The radicals generated due to oxidation of syringaldehyde (SA) by laccase have been reported to be more stable than those of other common redox-mediators (e.g. HBT) (González Arzola et al., 2009). Therefore, SA obtained from Sigma-Aldrich (NSW, Australia) was used in this study as the redox- mediator. A stock solution of SA was prepared in Milli-Q water at a concentration of 50 mM and stored at 4°C .

GAC-1200 (Activated Carbon Technologies Pty Ltd, Victoria, Australia) was chosen as adsorbent because of its high TrOC adsorption capacity (Nguyen et al., 2013). The physicochemical characteristics of this GAC are summarized in **Table 7.1**. Prior to the experiment, the GAC was washed with Milli-Q water to remove fine particles, dried at 105°C for 24 h and stored at room temperature until use.

Table 7.1: Physicochemical characteristics of the GAC-1200

Parameter	Values
Apparent density (g/mL)	0.42-0.50 ^a
Surface area (MultiPoint BET m ² /g)	1121 ^b
Ash content (%)	3 ^a
Iodine number (mg of I ₂ /g)	>1200 ^a
Carbon mesh size	6 x 12 mesh (1.6-2.0 mm) ^a
Pore volume (cc/g)	0.043 ^b
Pore diameter (nm)	3.132 ^b

^a Data from Activated Carbon Pty Ltd, Australia.

^b Data obtained from a nitrogen adsorption/desorption measurement using an Autosorb iQ. The measurement was conducted at the Australian Nuclear Science and Technology Organisation. Pore volume and pore diameter were calculated based on the Barret-Joyner-Halenda method.

7.2.3 Batch test description

The impact of GAC and/or SA addition on enzymatic degradation of TrOCs was first investigated in batch tests. Parallel tests on different combinations (*i.e.*, enzyme only, GAC only, enzyme + GAC, enzyme + SA, and enzyme + GAC + SA) were conducted with 200 mL test media in 400 mL beakers. In all tests, each TrOC was added at an initial nominal concentration of 5000 µg/L (actual concentrations of 5600 ± 110, 4900 ± 160, 4700 ± 100, and 4300 ± 400 µg/L for CBZ, DCF, SMX and ATZ, respectively). The initial enzymatic activity of the test solution was 90 µM_(DMP)/min. The enzymatic activity was selected based on a preliminary experiment (data not shown) showing that the enzymatic activity in the range of 60 to 90 µM/min resulted in approximately 80% removal of DCF at an initial concentration of 1000 µg/L. The enzymatic assay has been described in **Section 7.2.5.2**. SA was added at a concentration of 10 µM and a GAC dosage of 20 mg/L was used. The initial concentrations of the TrOCs and GAC were selected such that the TrOC loading exceeded the maximum adsorption capacity of

the GAC (250 and 94.3 mg/g GAC for CBZ and DCF, respectively (Nguyen et al., 2013)) allowing the effect of enzyme and mediators to be clearly observed. Triplicate samples were collected after 24 h of incubation to measure TrOC removal from the aqueous phase. The samples were diluted two-fold in methanol to immediately stop any residual enzyme activity. TrOC adsorbed on GAC was measured by a solvent extraction method (Wijekoon et al., 2013). Freeze-dried GAC was thoroughly mixed with 5 mL of methanol. The mixture was then sonicated for 10 min and the supernatant was collected. The remaining solid mass was subjected to further extraction using 5 mL methanol and dichloromethane (1:1 v/v) and the supernatant was collected. The extracted TrOC in the solution was measured by HPLC analysis as described in **Section 7.2.5.1**. The extraction efficiency of CBZ, DCF, SMX and ATZ from GAC was 64, 98, 82 and 79%, respectively.

7.2.4 EMR system and operation protocol

7.2.4.1 EMR setup

A laboratory scale EMR system has been described in **Section 5.2.3, Chapter 5**.

7.2.4.2 EMR operation

The EMR was continuously operated for 66 d to investigate TrOC degradation by laccase alone or laccase in the presence of GAC and/or SA. Based on the observed enzymatic stability (see **Section 5.3.2.2, Chapter 5**), laccase was injected (200 μ L laccase/ L reactor volume) every 12 h to the EMR to maintain the enzymatic activity within a range of 70 to 100 μ M_(DMP)/min. The whole experimental sequence has been detailed in **Table 7.2**. Under each operation regime, the EMR was run for at least a period of $6 \times$ HRT up to $45 \times$ HRT, and samples for measuring TrOC concentration were collected at $2 \times$ HRT following the operation condition change.

The effect of SA addition at different concentrations (*i.e.*, 5, 10 and 20 μ M) was investigated under two nominal TrOC loadings (1600 and 830 μ g/L.d). Mediator concentrations were selected based on the concentration range used in previous EMR studies targeting recalcitrant compounds such as dyes (Chhabra et al., 2009; Mendoza et al., 2011) . A single dose of GAC was added to the EMR on Day 30 to obtain a final GAC concentration of 3 g/L. The effect of GAC addition was assessed for 35 d.

Table 7.2: Sequence of the experiments and the relevant operating conditions of the enzymatic membrane reactor

Day	Experiment			TrOC loading rate				Sampling interval (h)
				(μg/L.day)				
	TrOC conc., (mg/L)	SA (μM)	GAC (g/L)	Compounds				
				CBZ	DCF	SMX	ATZ	
0 – 4	0.5	-	-	2060 ± 60	1860 ± 60	1700 ± 30	1660 ± 60	5
5 – 8	0.5	5	-	1830 ± 160	1730 ± 130	1660 ± 60	1660 ± 60	5
9 – 13	0.5	10	-	2030 ± 60	1800 ± 60	1760 ±30	1900 ± 100	5
14 – 18	0.5	20	-	2130 ± 160	1800 ± 30	1800 ± 60	1860 ± 130	5
19 – 23	0.25	-	-	930 ± 30	900 ± 160	850 ± 30	880 ± 160	8
24 – 26	0.25	5	-	930 ± 30	900 ± 50	860 ± 30	860 ± 30	4
27 – 29	0.25	10	-	930 ± 50	860 ± 30	900 ± 50	860± 30	4
30 – 46	0.5	5	3	2230 ± 160	1800 ± 100	1700 ± 60	1830 ± 130	11
47 – 55	0.25	5	3	1000 ± 160	100 ± 30	930 ± 60	760 ± 60	10
56 – 61	0.25	10	3	1060 ± 60	960 ± 30	960 ± 60	830 ± 100	8
62 – 66	0.25	20	3	1060 ± 50	1000 ±30	960 ± 30	900 ± 100	6

7.2.4.3 Membrane cleaning protocol

The UF membrane was cleaned by *in situ* Milli-Q water backwash at a flux of 24 L/m².h (5 seconds) when the TMP exceeded 40 kPa during any particular run. Additionally, the membrane was cleaned by *ex situ* backwash with 1 L of Milli-Q water followed by 1 L of NaOCl (500 mg/L active chlorine) under a flux of 5 L/m².h at the end of each run. To monitor the formation of an enzyme gel layer on the membrane surface, the enzymatic activity of the cleaning solution was measured after each *ex situ* Milli-Q water backwash.

7.2.5 Analytical methods

7.2.5.1 Trace organic contaminants

A HPLC system (Shimadzu, Kyoto, Japan), equipped with a 300 × 4.6 mm (5 µm pore size) C-18 column (Supelco Drug Discovery, Sigma-Aldrich, Australia) and an UV-vis detector, was used to measure TrOC concentrations. The detection wavelength, column temperature, and sample injection volume were 280 nm, 20°C, and 50 µL, respectively. The mobile phase comprised of acetonitrile and Milli-Q water buffered with 25 mM KH₂PO₄. Two eluents, A (80% acetonitrile and 20% buffer, v/v) and B (20% acetonitrile and 80% buffer, v/v) were delivered at 0.7 mL/min through the column for 30 min in a time-dependent gradient proportions [Time (min), B (%): [0, 85], [5, 40], [8, 0], [22, 85] (Hai et al., 2011). The limit of quantification for the analytes under investigation using these conditions was approximately 10 µg/L. The removal efficiency was calculated as $R = 100 \times \left(1 - \frac{C_{Eff}}{C_{Inf}} \right)$, where C_{Inf} and C_{Eff} are influent and effluent (permeate) concentrations of the TrOC, respectively.

7.2.5.2 Enzymatic activity and toxicity assay

Laccase activity and toxicity assay are summarized in **Section 3.2.4.2, Chapter 3**.

7.3. RESULTS AND DISCUSSION

7.3.1 Batch test: assessment of treatment capacity of each option studied

Although phenols are typical laccase substrates, laccase can directly oxidize some non-phenolic compounds if the electrochemical potential of the laccase used is sufficiently high (González Arzola et al., 2009). However, the laccase preparation used in this study did not efficiently

degrade the tested non-phenolics (**Table 7.3**), with the highest removal of 21% being achieved for DCF. Similarly, even after SA addition, except for DCF (64%) all compound removals ranged between 16 and 31%. The recalcitrance of the selected TrOCs in this study can be attributed to their chemical structures. According to Tadkaew et al. (2011) the presence of electron withdrawing groups (EWG) in TrOCs imparts resistance to oxidative catabolism. Therefore the low removal of CBZ can be attributed to the presence of strong EWG amide in its structure (Yang et al., 2013a). The low removal of the chlorinated TrOCs DCF and ATZ can similarly be explained by the fact that chloride is a strong EWG. This is consistent with a report by Tran et al. (2010) who also observed the resistance of chlorinated TrOCs to laccase treatment. Resistance of ATZ is also consistent with previous reports on inherent resistance of triazine pesticides (Navaratna et al., 2012). Among the sulfonamide antibiotics, thus far, only limited information on the enzymatic degradation of SMX is available. Consistent with this study, Yang et al. (2013b) reported negligible removal of SMX by a crude extracellular extract preparation (mainly laccase) from *Trametes versicolor*.

Table 7.3: Aqueous phase TrOC removal efficiencies by different options in batch tests

Compounds	Aqueous phase removal (%)				
	Enzyme only	Enzyme + SA	GAC only	Enzyme + GAC	Enzyme + SA + GAC
Carbamazepine	10	16	49	52	71
Diclofenac	21	64	60	63	67
Sulfamethoxazole	9	17	53	58	64
Atrazine	14	31	57	75	76

Standard deviation of 3 replicate experiments was less than 5%. Initial enzymatic activity was 90 $\mu\text{M}/\text{min}$. Carbamazepine, diclofenac, sulfamethoxazole and atrazine concentrations of 5600 ± 110 , 4900 ± 160 , 4700 ± 100 , and 4300 ± 400 $\mu\text{g}/\text{L}$, respectively were applied. SA and GAC concentrations were 10 μM and 20 mg/L , respectively.

Only DCF degradation was significantly improved by SA addition (**Table 7.3**). This may be due to the presence of the aromatic amine group (an electron donating group, EDG) in its structure, which possibly lowers its redox potential and makes it amenable to oxidation by laccase preparation when amended with SA. However, it could not be clarified why despite having an amine group, SMX removal efficiency improved by only 8% compared to 43% for DCF. This observation, however, is consistent with reports from other studies investigating the electrochemical behavior of DCF (Blanco-Lopez et al., 2005) and SMX (Msagati et al., 2002) at carbon electrodes: DCF appears to be electrochemically irreversibly oxidized at lower electric potential than SMX. It is also interesting to note that unlike in this study, Weng et al. (2012) observed significant improvement in the removal of sulfadimethoxine and sulfmanomethoxine, which have a similar backbone structure to SMX. This may be explained by the fact that the oxidation reduction potential of the SA-amended laccase solution in this study (540 mV) was lower than that in the study (626 mV) by Weng et al. (2012).

When only GAC was added to the test solution, 49-60% TrOC removal from the aqueous phase was achieved (**Table 7.3**). Therefore, it was not a surprise that the aqueous phase removal ranged from 64-76% with the simultaneous addition of laccase, SA and GAC. However, it is interesting to note that this improvement in aqueous phase removal (compared with that achieved by the laccase—SA system) was not only due to adsorption on GAC, but also due to enhanced enzymatic degradation. The fate of TrOCs (*i.e.*, residual amount in liquid phase, adsorbed onto GAC and biodegraded) was assessed by extracting residual TrOC from the GAC at the end of the incubation period. The adsorbed amount onto GAC was calculated taking the extraction efficiency into account. It was revealed that a 12-45% increase in the extent of enzymatic degradation occurred in presence of GAC (**Figure 7.1**). Apparently co-adsorption of enzyme and TrOC onto GAC facilitated enhanced TrOC degradation. Zille et al. (2003) observed that compared to dye adsorption on alumina, immobilization of enzyme on alumina led to a more consistent dye removal performance due to combined adsorption and biodegradation. Enhanced enzymatic degradation could be further advantageous in a continuous process wherein TrOCs are constantly loaded to the system.

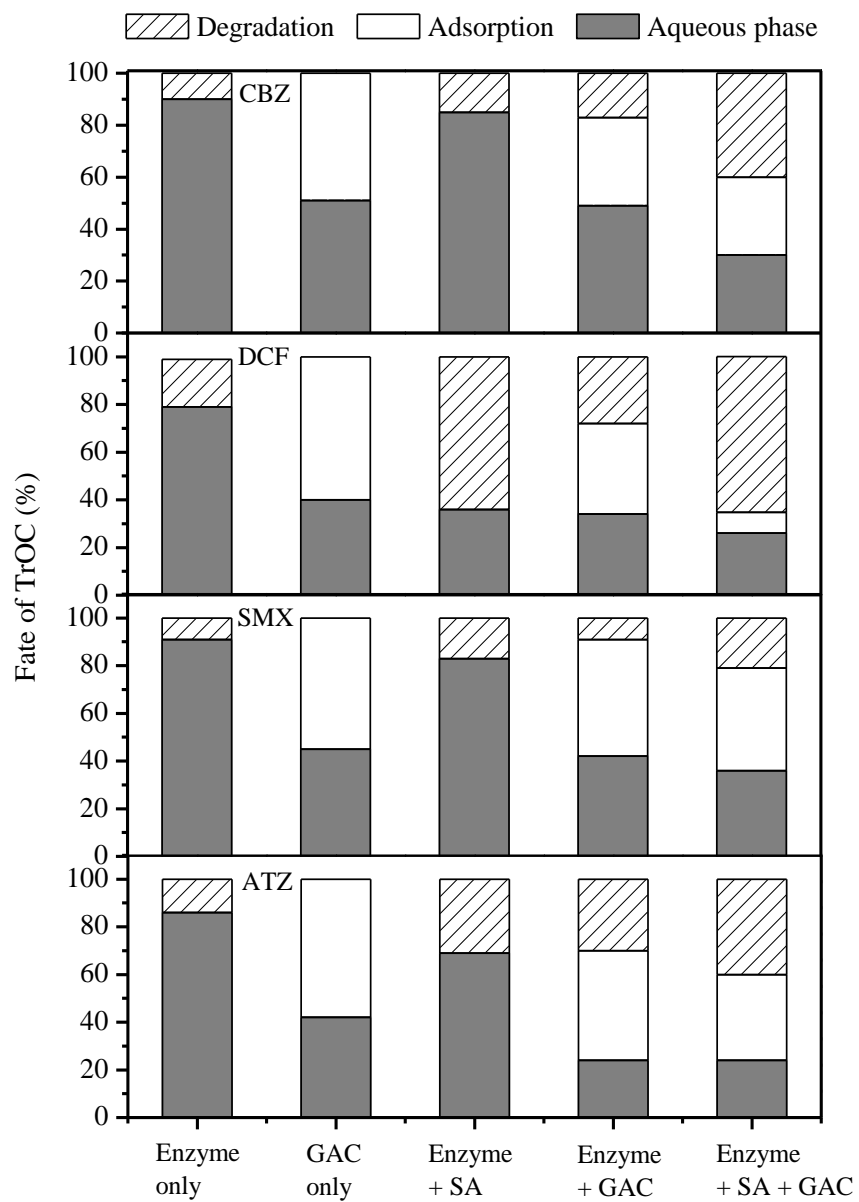


Figure 7.1: Overall fate of TrOCs following 24 h treatment via different combinations of enzyme, SA and GAC.

7.3.2 Performance of EMR

7.3.2.1 Maintenance of enzymatic activity during EMR operation

See Section 5.3.2.2, Chapter 5.

7.3.2.2 Continuous removal of TrOC by EMR

The removal efficiency of the compounds by the EMR followed the same order as in batch tests, *i.e.*, DCF>ATZ>CBZ>SMX. Although a direct comparison of removal efficiency between two operation modes (*i.e.*, batch *vs.* continuous) may not be valid, it is notable that the removal efficiency of DCF by the EMR was around 60%, which was three times that achieved during batch tests. The removal of the other compounds by EMR was slightly higher as well. The improved removal by the EMR despite continuous dosing of TrOCs can be attributed to the fact that in order to assess the contribution of adsorption and biodegradation, the batch tests (**Section 7.2.3**) were conducted under an initial TrOC concentration (5000 µg/L) 10 times that fed to the EMR. The effect of TrOC loading on the performance of the EMR was directly evidenced by a compound-specific improvement (3 to 12%) in removal efficiency when the TrOC loading was reduced to approximately half from 1660 µg/L.d (**Figure 7.2** and **Table 7.4**). The effect of TrOC loading on the removal of these non-phenolic TrOCs by EMR has not been reported before. However, the effect of TrOC loading was also demonstrated in a study by Lloret et al. (2012), who reported that the removal of estrone (a phenolic TrOC) increased from 64 to 73% when the loading decreased from 4 mg/L.h to 1 mg/L.h.

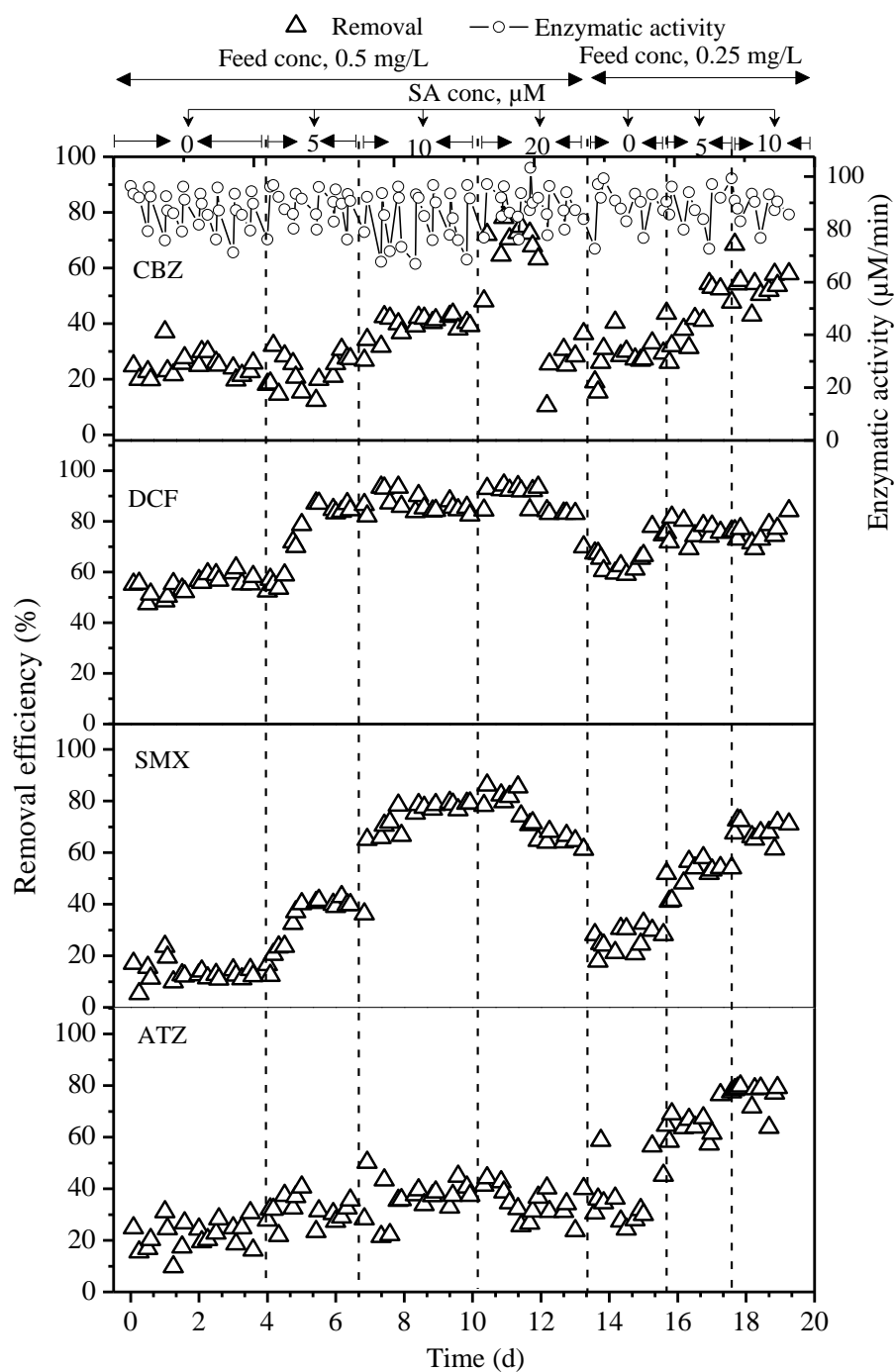


Figure 7.2: TrOC removal efficiencies by the EMR during operation under different mediator concentrations (5, 10 and 20 μM) and TrOC concentrations (*i.e.*, 0.5 and 0.25 mg/L corresponding to loadings of 1660 and 830 $\mu\text{g/L.d}$, respectively).

Table 7.4: Summary of the TrOC removal efficiencies by the treatment options studied.

Removal efficiency (%)									
Compounds	TrOC conc., (mg/L)	TrOC loading (µg/L.d)	Enzyme only	Enzyme + SA		Enzyme + SA + GAC			
				SA concentration (µM)					
			0	5	10	20	5	10	20
CBZ	0.5	1660	25 ± 4 (n = 20)	23 ± 6 (n =15)	40 ± 4 (n = 17)	53 ± 24 (n = 15)	50 ± 9 (n = 34)		
	0.25	830	28 ± 5 (n = 11)	41 ± 9 (n =11)	55 ± 6 (n = 11)		66 ± 8 (n = 20)	74 ± 4 (n = 16)	34 ± 7 (n = 16)
DCF	0.5	1660	55 ± 4 (n = 20)	75 ± 13 (n =15)	87 ± 4 (n = 17)	88 ± 5 (n= 15)	78 ± 14 (n = 34)		
	0.25	830	64 ± 3 (n = 11)	76 ± 5 (n =11)	82 ± 2 (n = 11)		78 ± 5 (n = 20)	75 ± 2 (n = 16)	73 ± 6 (n = 16)
SMX	0.5	1660	13 ± 4 (n = 20)	34 ± 9 (n =15)	75 ± 5 (n = 17)	73 ± 9 (n = 15)	48 ± 8 (n = 34)		
	0.25	830	25 ± 5 (n = 11)	51 ± 6 (n =11)	67 ± 4 (n = 11)		65 ± 6 (n = 20)	68 ± 3 (n = 16)	64 ± 4 (n = 16)
ATZ	0.5	1660	22 ± 6 (n = 20)	31 ± 5 (n =15)	37 ± 7 (n =17)	35 ± 6 (n = 15)	38 ± 8 (n = 34)		
	0.25	830	34 ± 9 (n = 11)	61 ± 6 (n =11)	78 ± 8 (n = 11)		79 ± 7 (n = 20)	89 ± 6 (n = 16)	56 ± 13 (n = 16)

7.3.3 Impact of mediator addition on EMR performance

In a laccase—mediator system, the role of laccase is to oxidize the mediator, while the actual oxidation of the substrate takes place in a subsequent non-enzymatic step by the action of the oxidized mediator species. The oxidation of SA by laccase generates highly active phenoxyl ($C_6H_5O^*$) radicals, which then target substrate via a hydrogen atom transfer (HAT) route (Fabbrini et al., 2002). This HAT mechanism has been implicated for the oxidation of resistant species such as non-phenolic compounds. However, to date the use of mediators to improve TrOC removal has been reported in mostly batch test studies (Yang et al., 2013a) and a few EMR studies that this thesis comprises (Nguyen et al., 2014; Nguyen et al., 2015) .

In this study the addition of SA to EMR resulted in significant improvement in TrOC removal depending on the mediator dose as well as the TrOC type and loading (**Figure 7.2** and **Table 7.4**). Two notable observations were made: (i) achievement of significant improvements in

removal up to a mediator dose of 10 μM , and (ii) the impact of TrOC loading on the removal performance by the laccase—SA system.

In general, the better performance of the laccase—SA system may be explained by two factors attributable to the radical species generated from SA by laccase: i) higher redox potential, and ii) reduction of steric hindrance. Low molecular weight mediators can interact with complex compounds that cannot access the active sites of the enzyme directly. Moreover, compounds with high electrochemical potential can be oxidized by radical mediators (*i.e.*, phenoxyl) through the operation of H-abstraction mechanism.

Our results are in line with the general trend observed in the literature that pollutant removal profile may reach a plateau beyond a certain mediator concentration (10 μM in this study) (Mizuno et al., 2009). This occurs because the free radicals generated from laccase—mediator systems that can improve pollutant degradation may also inactivate laccase by oxidizing the aromatic amino acid residues on the proteinaceous enzyme surface (Khelifi-Slama et al., 2012). Another notable observation in this study was the impact of TrOC loading on the performance of the laccase-SA system. During EMR operation, the effect of TrOC loading was particularly significant for ATZ (**Figure 7.2**). Furthermore, compared with batch tests, which were conducted under higher (10 times) TrOC concentrations, better performance during EMR operation with the same SA dose (10 μM) was observed for all tested TrOCs except DCF, which was well removed in both cases. This observation once again illustrates the significant impact of TrOC loading on enzymatic removal of the resistant TrOCs. At high substrate concentrations, when almost all of the enzyme molecules are bound to the substrate, the reaction rate is dependent solely on the amount of enzyme and, therefore, occurs with zero-order kinetics.

In this study, continuous dosing of the mediator was required because it was not retained by the membrane utilized. It is noted that continuous dosing of mediators to EMR may hinder its applicability due to mediator cost and complexity of operation. In this context, immobilization of the mediator or its recovery from the treated effluent may be applied. For example, Mendoza et al. (2011) confirmed that a membrane with a MWCO of 10 kDa could retain the mediator TEMPO which was immobilized on polyethylene glycol. The EMR was operated up to nine batches to treat a dye wastewater with a single addition of the mediator. In another study by

Chhabra et al. (2009), a recovery of 70% of ABTS was achieved from the treated effluent using ammonium sulphate precipitation method. It would be interesting to observe TrOC removal with the incorporation of such strategies. However, it is beyond the scope of the current study.

7.3.4 Effect of GAC addition on EMR performance

Following the single dose addition of GAC, the EMR was operated for 35 d with continuous feeding and periodic injection of laccase. The TrOC loading and SA concentration were varied systematically over 830 – 1660 $\mu\text{g/L.d}$ and 5—20 μM , respectively. A stable and improved removal of all three resistant TrOCs (*i.e.*, CBZ, SMX and ATZ) was observed for SA concentrations of 5 and 10 μM . For example, with a SA dose of 5 μM and a TrOC loading of 830 $\mu\text{g/L.d}$ (each), 14-25% improvement in removal was observed following GAC addition (**Figure 7.3** and **Table 7.4**). Notably, irrespective of TrOC loading and SA dose, the removal of DCF remained around 80%, which was already achieved without GAC addition.

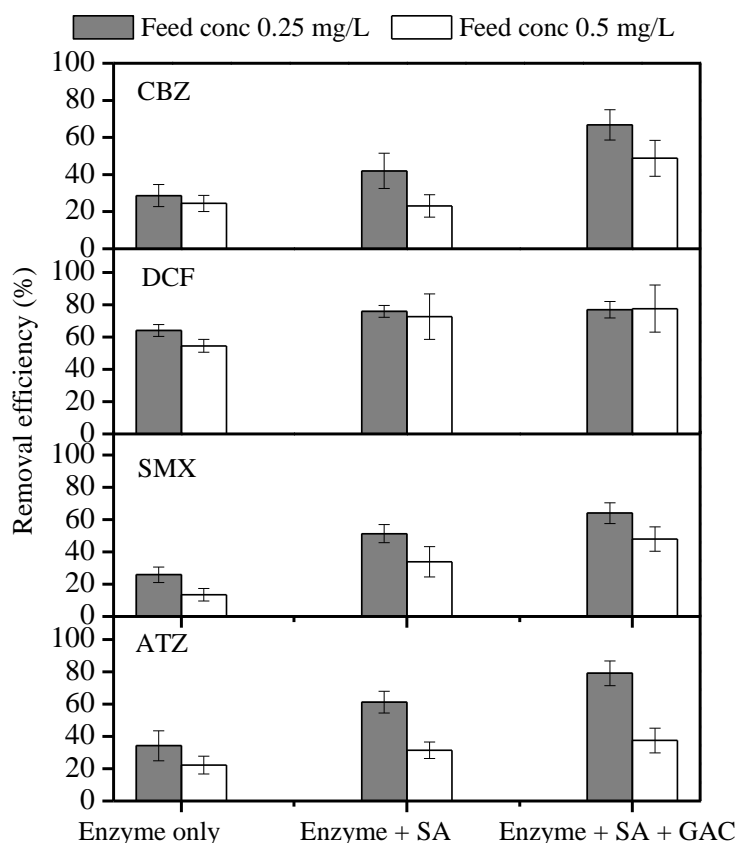


Figure 7.3: Enhancement of enzymatic degradation due to addition of GAC (3 g/L) and/or mediator (5 μ M) under different TrOC concentrations (*i.e.*, 0.5 and 0.25 mg/L corresponding to loadings of 1660 and 830 μ g/L.d, respectively). The error bars represent the standard deviation of available data points ($n = 11-34$, depending on the option).

Over the period of continuous operation of the EMR following GAC addition, the total mass of TrOCs fed to the reactor did not exceed the maximum adsorption capacity of the GAC added to the EMR. However, data from EMR operation, in line with the batch test data, indicates that the improved and stable aqueous phase removal achieved by the GAC-amended EMR was not only due to adsorption of TrOCs but also due to subsequent enzymatic degradation of the adsorbed amount. At the end of the EMR operation, residual amounts of TrOCs on GAC were measured and a mass balance was conducted which revealed that a major portion of the TrOCs retained within the EMR (as indicated by the difference in TrOC concentration in feed and permeate) was

biodegraded. Removal of the selected TrOCs in this study by volatilization was expected to be negligible because of the very low vapour pressure or Henry's constant (H) and low H/log D ratio (**Appendix Table A-1**) of the TrOCs investigated, and photolysis was avoided by covering the EMR. Thus, enzymatic degradation appeared to be the major mechanism of TrOC removal by the GAC-amended EMR system.

Table 7.5: Fate of TrOCs retained within EMR

Compounds	Total mass of TrOC (mg) ^a			Biodegradation (%) ^b
	In (I)	Out (O)	Residual on GAC (R)	
Carbamazepine	94	45	8	84
Diclofenac	85	19	1	99
Sulfamethoxazole	76	34	1	97
Atrazine	76	35	7	82

^a At the end of the 35 d operation period (with GAC addition) during which the EMR was operated under different TrOC loadings and SA concentrations as detailed in **Table 7.2**.

^b Biodegradation of the amount retained within the reactor was calculated as **Equation 7.1**.

$$\text{Biodegradation (\%)} = \frac{(I-O)-R}{I-O} \times 100 \quad \text{Equation 7.1}$$

The results reported here indicate that in a GAC-amended EMR system, simultaneous adsorption of TrOCs and enzyme on GAC can enable enhanced enzymatic degradation of TrOCs bound on GAC, and lead to overall improvement of TrOC degradation, possibly because it promotes the interaction of TrOCs with the active sites of enzyme. Similar phenomena have been observed when activated carbon (GAC or PAC) was added to conventional membrane bioreactors (Li et al., 2011) or fungal reactors (Zhang et al., 2000; Hai et al., 2008). For example, Zhang and Yu (2000) encapsulated PAC inside fungal mycelium pellets and applied them for the removal of dye. It was shown that the PAC facilitated co-adsorption of the dye molecules and the extracellular enzyme secreted by fungal cells and achieved enhanced dye degradation. Co-adsorption of enzyme and dye on PAC and subsequent enzymatic dye degradation was also confirmed in a study by Hai et al. (2008). Moreover, it is possible that the adsorption of enzyme

on GAC stabilizes enzymatic activity. This is supported by the observations made in studies involving enzyme immobilization on activated carbon (Jochems et al., 2011). The adsorption of TrOCs on GAC can also enhance degradation by increasing the contact time between the TrOCs and the enzyme.

7.3.5 Membrane performance

7.3.5.1 Role of the membrane in TrOC removal

A significant level of enzymatic activity in the membrane cleaning solution from *ex situ* Milli-Q backwashing was observed in this study. The enzymatic activity in 1 L of the cleaning solution was $60 \mu\text{M}_{(\text{DMP})\mu}/\text{min}$, which was equivalent to an accumulation of at least 0.24 g active laccase per m^2 of membrane surface. This demonstrated that an enzyme gel layer was formed on the membrane surface. Therefore, the TrOC concentration in permeate and supernatant of the reactor was measured periodically to investigate if there was any additional removal by the membrane and/or enzyme layer on the membrane. The ratio of concentration of TrOCs in permeate and supernatant (P/S ratio) was indeed below 1 for all TrOCs (**Figure 7.4**). Without addition of SA or GAC, the lowest P/S ratio was observed in case of DCF (0.52 ± 0.02 , $n=11$). Interestingly, the P/S ratio of DCF did not change significantly following addition of SA or GAC, which is consistent with its high removal by laccase alone. Conversely, for a TrOC loading of $830 \mu\text{g/L.d}$, the P/S ratios of CBZ and ATZ dropped significantly following GAC addition. It is noted that GAC particles did not significantly accumulate on the membrane surface, rather remained mainly in suspension by aeration within the reactor. However, the drop in P/S ratio indicates that adsorption on the suspended GAC facilitated retention of TrOCs within the reactor.

No EMR study could be found to directly compare these results, but the observations made in this study are in line with that by Li et al. (2011) who reported significant additional removal of CBZ and SMX by a microfiltration membrane submerged in a conventional activated sludge bioreactor to which PAC was added. A stable TrOC removal was achieved throughout this study, and there was no accumulation of TrOCs within the EMR (as evidenced by a stable concentration in the reactor supernatant). It can therefore be said that TrOCs adsorbed on the enzyme gel layer over the membrane were subsequently degraded. Dosing of GAC to an EMR can realize advantages additional to improved TrOC degradation, and these aspects have been discussed in **Sections 7.3.5.2 and 7.3.6**.

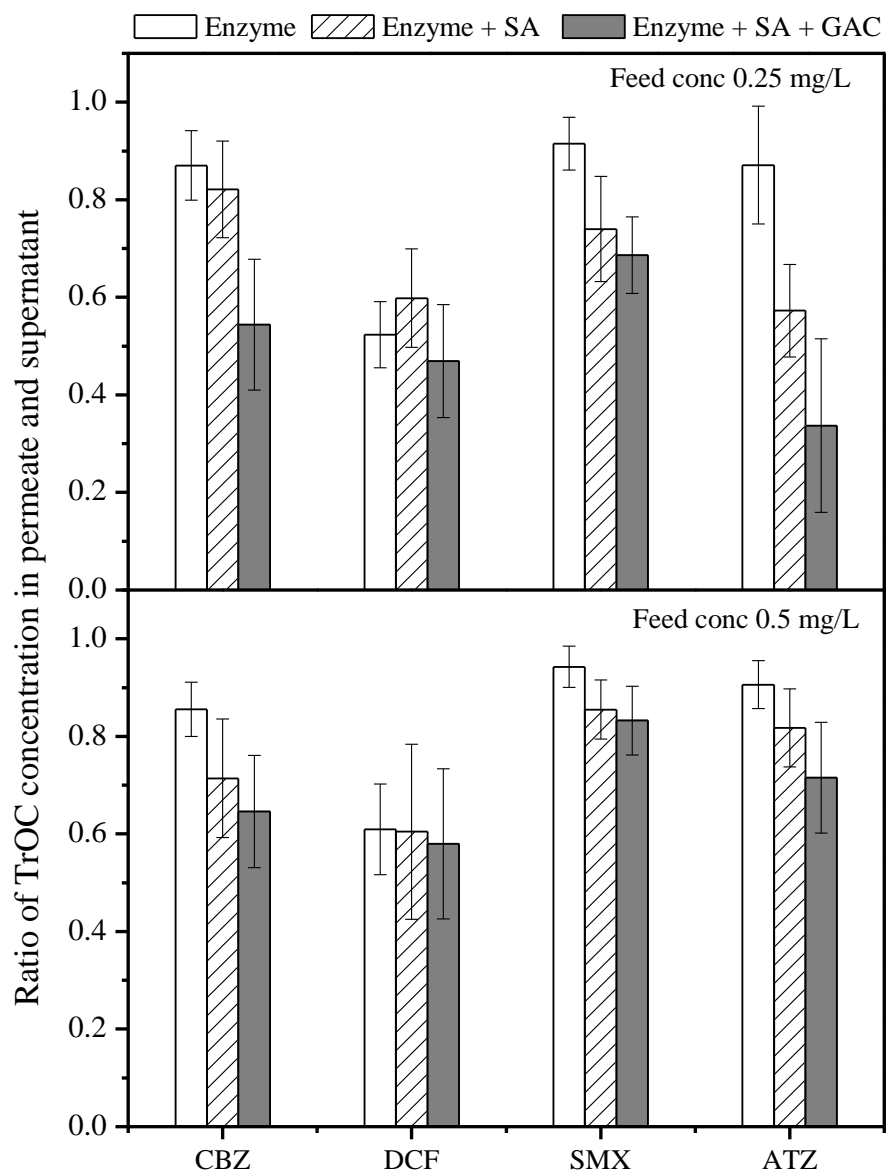


Figure 7.4: Ratio of TrOC concentration in membrane permeate and reactor supernatant during different runs. The error bars represent the standard deviation of available data (n =11-34, depending on the option).

7.3.5.2 Membrane fouling

Membrane fouling is an important aspect that requires due consideration when operating a continuous flow EMR. The formation of an enzyme gel layer on the membrane (**Section 7.3.5.1**) can lead to its gradual fouling (Jochems et al., 2011). In this study, during EMR operation without GAC, a TMP build up rate of approximately 9 kPa/d was observed (**Figure 7.5**). By contrast, following GAC addition, an initial TMP build up rate of approximately 3.3 kPa/d was noted, which further dropped to 2.5 kPa/d within a month of operation after the single dose addition of GAC.

The data reported here suggests that a dynamic layer of adsorbent over the membrane can be beneficial for long-term restoration of the membrane permeability. It is also likely that the scouring action of the GAC particles helped to restrict the overgrowth of the enzyme gel layer on the membrane surface, thus reducing the overall hydrodynamic boundary layer thickness on the membrane compared with operation without GAC. Previous studies have demonstrated that the addition of adsorbents such as activated carbon (PAC or GAC) can mitigate membrane fouling in membrane bioreactors (Ng et al., 2013). This study, however, is possibly the first to demonstrate the advantage of GAC dosing for mitigation of membrane fouling in an EMR.

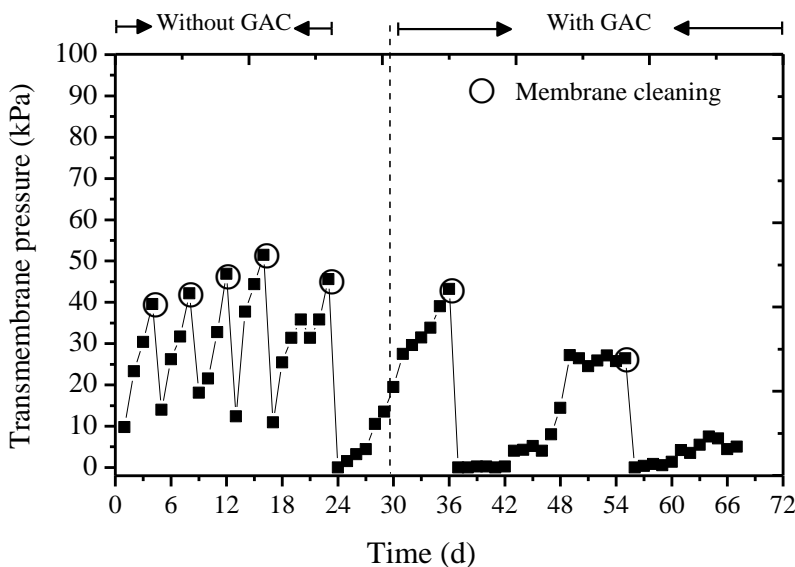


Figure 7.5: Variation of transmembrane pressure (TMP) as a function of operating time showing the impact of GAC addition to EMR.

7.3.6 Toxicity of treated effluent

A few recent batch test studies have shown increased toxicity of enzyme-treated media despite efficient degradation of the target pollutant (Marco-Urrea et al., 2009). In this study, a ToxScreen3 assay (**Section 7.2.5.2**) revealed no significant toxicity in the feed irrespective of the feed concentration (6.6 ± 0.3 rTU; $n=4$) indicating that the tested TrOCs were not particularly toxic to the indicator bacterium (*P. leiognathi*) used in this study. There was also no appreciable increase in toxicity in the EMR permeate (7.0 ± 1.7 rTU; $n=2$), suggesting that treatment did not produce toxic by-products. On the other hand, addition of the redox-mediator SA to the EMR considerably increased the toxicity of the permeate. The toxicity of treated effluent with SA dosage of 5 μ M and 10 μ M was 20.1 ± 5 ($n=2$) and 41.7 ± 2.1 rTU ($n=2$), respectively. This corresponds to 2.8 \times and 6.0 \times increase in toxicity, respectively.

The observed toxicity of treated effluent after SA addition is consistent with a report by Fillat et al. (2010) who studied the treatment of flax pulp. The toxicity of the treated medium, despite efficient TrOC removal after SA addition, may be due to the phenoxyl radicals formed in the laccase—SA system and/or the metabolites produced during the TrOC degradation. However, under the tested conditions, the toxicity test for SA itself (incubation with SA solution in the absence of laccase) revealed no toxicity (data not shown). Therefore, the toxicity of the media after enzymatic treatment with or without SA and the negligible toxicity of SA itself indicate that the effluent toxicity is most likely due to the radicals formed in the presence of both laccase and SA. This observation is in accordance with that by Kim and Nicell (2006) who suggested that radicals formed due to oxidation of mediators can interact with vitally important biomolecules and result in cytotoxic effects.

Nevertheless, bacterial assay can only provide a limited measure of ecotoxicity, and while the toxicity results clearly indicate that the effluents are toxic to bacteria, further tests using other organisms are needed to more thoroughly appreciate the potential ecological risks. Moreover, further work is required to screen mediators which enhance enzymatic degradation without raising the toxicity of the treated media. In this context, it is interesting to note that a significant reduction in effluent toxicity originating from SA dosing was observed after the addition of GAC to the EMR (**Figure 7.6**). In fact, following GAC addition, the treated effluent demonstrated no elevated toxicity with a SA dose of 5 μ M. It has been previously reported that the effects of

shock loads or toxic concentrations of pollutants/chemical species can be buffered as a result of their adsorption onto activated carbon. For example, PAC addition to an MBR treating tannery wastewater was shown to reduce the negative effects of natural and synthetic tannins that impart toxicity (Munz et al., 2007). Systematic demonstration of the problem of effluent toxicity originating from mediator dosing to EMR and proposal of a solution is a unique contribution of the current study.

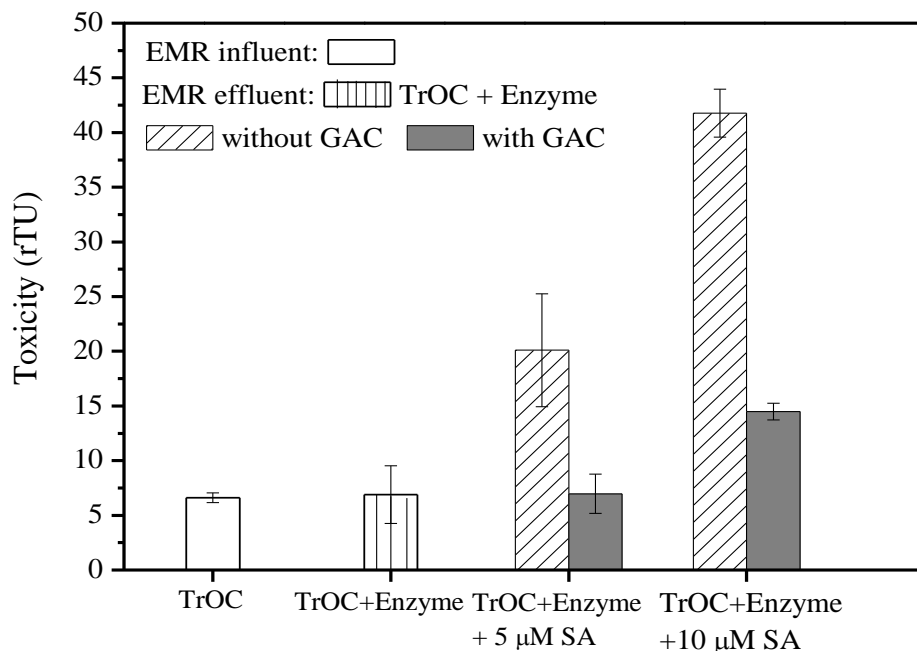


Figure 7.6: Toxicity of EMR effluent depending on the mediator concentration and the impact of GAC addition. The error bars represent the standard deviation of two samples.

7.4. CONCLUSION

This is the first report on the multiple advantages of GAC dosing to an EMR: (i) enhancement of TrOC degradation by a laccase—mediator (syringaldehyde) system, (ii) reduction of mediator-induced effluent toxicity, and (iii) mitigation of fouling by an enzyme gel layer over the membrane. TrOCs adsorbed onto the enzyme layer, and GAC addition further facilitated such TrOC retention on the membrane cake layer; nevertheless, subsequent TrOC degradation was confirmed. At a TrOC loading, mediator concentration and GAC dose of 830 µg/L.d, 5 µM, and 3 g/L, respectively, removal efficiency ranged from 65% (sulfamethoxazole) to 80% (diclofenac).

7.5. REFERENCES

- Blanco-Lopez, M. Carmen, L. Fernandez-Llano, M. J.-. Lobo-Castanon, A. J. Miranda-Ordieres and P. Tunon-Blanco (2005). Voltammetry of diclofenac at Graphite, Carbon Composites, and Molecularly Imprinted Polymer-Composite Electrodes. *Analytical Letters*. 37, 915-927.
- Boonyaroj, V., C. Chiemchaisri, W. Chiemchaisri, S. Theepharaksapan and K. Yamamoto (2012). Toxic organic micro-pollutants removal mechanisms in long-term operated membrane bioreactor treating municipal solid waste leachate. *Bioresource Technol.* 113, 174-180.
- Cabana, H., J. P. Jones and S. N. Agathos (2009). Utilization of cross-linked laccase aggregates in a perfusion basket reactor for the continuous elimination of endocrine-disrupting chemicals. *Biotechnol. Bioeng.*, 102, 1582-1592.
- Chhabra, M., S. Mishra and T. R. Sreekrishnan (2009). Laccase/mediator assisted degradation of triarylmethane dyes in a continuous membrane reactor. *J. Biotechnol.*, 143, 69-78.
- Fabbrini, M., C. Galli and P. Gentili (2002). Comparing the catalytic efficiency of some mediators of laccase. *Journal of Molecular Catalysis B: Enzyme*. 16, 231-240.
- Fillat, A., J. F. Colom and T. Vidal (2010). A new approach to the biobleaching of flax pulp with laccase using natural mediators. *Bioresource Technol.* 101, 4104-4110.
- González Arzola, K., M. C. Arévalo and M. A. Falcón (2009). Catalytic efficiency of natural and synthetic compounds used as laccase-mediators in oxidising veratryl alcohol and a kraft lignin, estimated by electrochemical analysis. *Electrochimica Acta*. 54, 2621-2629.
- Hai, F. I., X. Li, W. E. Price and L. D. Nghiem (2011). Removal of carbamazepine and sulfamethoxazole by MBR under anoxic and aerobic conditions. *Bioresource Technol.* 102, 10386-10390.
- Hai, F. I., K. Yamamoto, F. Nakajima and K. Fukushima (2008). Removal of structurally different dyes in submerged membrane fungi reactor—Biosorption/PAC-adsorption, membrane retention and biodegradation. *J Mem Sci.* 325, 395-403.
- Hai, F. I., K. Yamamoto, F. Nakajima and K. Fukushima (2012). Application of a GAC-coated hollow fiber module to couple enzymatic degradation of dye on membrane to whole cell biodegradation within a membrane bioreactor. *J. Membr. Sci.*, 389, 67-75.
- Hata, T., H. Shintate, S. Kawai, H. Okamura and T. Nishida (2010). Elimination of carbamazepine by repeated treatment with laccase in the presence of 1-hydroxybenzotriazole. *J. Hazard. Mater.*, 181, 1175-1178.
- Jochems, P., Y. Satyawali, L. Diels and W. Dejonghe (2011). Enzyme immobilization on/in polymeric membranes: status, challenges and perspectives in biocatalytic membrane reactors (BMRs). *Green Chem.*, 13, 1609-1623.
- Khlifi-Slama, R., T. Mechichi, S. Sayadi and A. Dhouib (2012). Effect of natural mediators on the stability of *Trametes troglia* laccase during the decolourization of textile wastewaters. *J. Microbiol.* 50, 226-234.
- Kim, Y.-J. and J. A. Nicell (2006). Laccase-catalysed oxidation of aqueous triclosan. *J Chem Technol Biotechnol.* 81, 1344-1352.
- Li, X., F. I. Hai and L. D. Nghiem (2011). Simultaneous activated carbon adsorption within a membrane bioreactor for an enhanced micropollutant removal. *Bioresource Technol.* 102, 5319-5324.

- Lloret, L., G. Eibes, G. Feijoo, M. T. Moreira and J. M. Lema (2012). Continuous biotransformation of estrogens by laccase in an enzymatic membrane reactor. *Chem. Eng. Trans.*, 27, 31-36.
- Marco-Urrea, E., M. Perez-Trujillo, T. Vicent and G. Caminal (2009). Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. *Chemosphere*. 74, 765-772.
- Mendoza, L., M. Jonstrup, R. Hatti-Kaul and B. Mattiasson (2011). Azo dye decolorization by a laccase/mediator system in a membrane reactor: Enzyme and mediator reusability. *Enzyme Microb. Technol.*, 49, 478-484.
- Mizuno, H., H. Hirai, S. Kawai and T. Nishida (2009). Removal of estrogenic activity of isobutylparaben and n-butylparaben by laccase in the presence of 1-hydroxybenzotriazole. *Biodegradation*. 20, 533-539.
- Msagati, T. A. M. and J. C. Ngila (2002). Voltammetric detection of sulfonamides at a poly(3-methylthiophene) electrode. *Talanta*. 58, 605-610.
- Munz, G., R. Gori, G. Mori and C. Lubello (2007). Powdered activated carbon and membrane bioreactors (MBR PAC) for tannery wastewater treatment: long term effect on biological and filtration process performances. *Desalination*. 207, 349-360.
- Navaratna, D., J. Elliman, A. Cooper, L. Shu, K. Baskaran and V. Jegatheesan (2012). Impact of herbicide Ametryn on microbial communities in mixed liquor of a membrane bioreactor (MBR). *Bioresource Technol.* 113, 181-190.
- Ng, C. A., D. Sun, M. J. K. Bashir, S. H. Wai, L. Y. Wong, H. Nisar, B. Wu and A. G. Fane (2013). Optimization of membrane bioreactors by the addition of powdered activated carbon. *Bioresource Technol.* 138, 38-47.
- Nguyen, L. N., F. I. Hai, J. Kang, W. E. Price and L. D. Nghiem (2013). Coupling granular activated carbon adsorption with membrane bioreactor treatment for trace organic contaminant removal: Breakthrough behaviour of persistent and hydrophilic compounds. *J. Environ. Manage.*, 119, 173-181.
- Nguyen, L. N., F. I. Hai, W. E. Price, J. Kang, F. D. L. Leusch, F. Roddick, J. P. van de Merwe, S. F. Magram and L. D. Nghiem (2015). Degradation of a broad spectrum of trace organic contaminants by an enzymatic membrane reactor: Complementary role of membrane retention and enzymatic degradation. *Int. Biodeterior. Biodegradation*. 99, 115-122.
- Nguyen, L. N., F. I. Hai, W. E. Price, F. D. L. Leusch, F. Roddick, E. J. McAdam, S. F. Magram and L. D. Nghiem (2014). Continuous biotransformation of bisphenol A and diclofenac by laccase in an enzymatic membrane reactor. *Int. Biodeterior. Biodegradation*. 95, Part A, 25-32.
- Tadkaew, N., F. I. Hai, J. A. McDonald, S. J. Khan and L. D. Nghiem (2011). Removal of trace organics by MBR treatment: The role of molecular properties. *Water Res.* 45, 2439-2451.
- Tran, N. H., T. Urase and O. Kusakabe (2010). Biodegradation characteristics of pharmaceutical substances by whole fungal culture *Trametes versicolor* and its laccase. *J Water Environ Technol.* 8, 125-140.
- Weng, S.-S., K.-L. Ku and H.-T. Lai (2012). The implication of mediators for enhancement of laccase oxidation of sulfonamide antibiotics. *Bioresource Technol.* 113, 259-264.
- Wijekoon, K. C., T. Fujioka, J. A. McDonald, S. J. Khan, F. I. Hai, W. E. Price and L. D. Nghiem (2013). Removal of N-nitrosamines by an aerobic membrane bioreactor. *Bioresource Technol.* 141, 41-45.

- Yang, S., F. I. Hai, L. D. Nghiem, W. E. Price, F. Roddick, M. T. Moreira and S. F. Magram (2013a). Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: A critical review. *Bioresource Technol.* 141, 97-108.
- Yang, S., F. I. Hai, L. D. Nghiem, F. Roddick and W. E. Price (2013b). Removal of trace organic contaminants by nitrifying activated sludge and whole-cell and crude enzyme extract of *Trametes versicolor*. *Water Sci. Technol.*, 67, 1216-1223.
- Zhang, F. and J. Yu (2000). Decolourisation of Acid Violet 7 with complex pellets of white rot fungus and activated carbon. *Bioprocess Eng.* 23, 295-301.
- Zille, A., T. Tzanov, G. M. Gübitz and A. Cavaco-Paulo (2003). Immobilized laccase for decolourization of reactive Black 5 dyeing effluent. *Biotechnol. Lett.*, 25, 1473-1477.

CHAPTER 8: Conclusions and Recommendations for Further Work

8.1. CONCLUSIONS

This thesis systematically investigates the removal of a broad spectrum of trace organic contaminants (TrOCs) by whole-cell white-rot fungi (WRF) and their extracellular enzyme (*i.e.*, laccase). Results have been discussed under two broad sections. The first section reports investigations involving a white-rot fungus *Trametes versicolor*. The roles of extracellular and intracellular enzyme systems (laccase and CytP450, respectively) as well as adsorption on fungal mycelium have been elucidated. In addition to batch reactors, a continuous flow membrane bioreactor was utilized to assess the TrOC removal performance. The second section reports a series of investigations involving a continuous flow enzymatic membrane reactor (EMR) system. A commercially available laccase preparation (Novozymes Australia) was used for this part of the investigations, and the impact of type and concentration of TrOCs as well as redox-mediators (low molecular weight chemicals added to enhance enzymatic degradation) on the removal and fate of TrOCs and the effluent toxicity were revealed.

In chapter 3, a series of batch tests was conducted to address the relative contribution of biosorption and various modes of biodegradation (*e.g.*, extracellular enzyme dependent/independent pathways) during fungal removal of TrOCs. Biodegradation was confirmed as the main mechanism of removal. However, results indicate that biosorption may aid in subsequent degradation by the whole-cell fungus. The extracellular enzyme laccase achieved significant degradation of the TrOCs, although the role of extracellular enzyme-independent catalytic pathways (*i.e.*, intracellular cytochrome P450 system and/or mycelium-associated enzymes) in degradation of some TrOCs was evident. The performance of laccase was further improved by the addition of redox-mediators. 1-hydroxybenzotriazole (HBT) and syringaldehyde (SA), which produce aminoxyl and phenoxy radicals during the oxidation by laccase, respectively, achieved significant removal of additional five phenolic and two non-phenolic TrOCs, with moderate removal of a few other non-phenolics. The improved removal may be attributed to the increase in redox potential of the enzyme solution following mediator

addition. However, despite the similar redox potentials of the enzyme-mediator cocktail for HBT and SA, SA achieved less efficient degradation and did not exhibit significant improvement in degradation at concentrations higher than 0.1 mM, indicating the imbalance between the stability and reactivity of the radicals generated. Addition of SA at all concentrations (0.1 – 1 mM) also resulted in a >1000-fold increase in bacterial cytotoxicity. In contrast, only the higher HBT dose (1 mM) produced measurable toxicity, with no detectable increase in toxicity at lower doses (0.1-0.5 mM). Overall, addition of HBT at a concentration of 0.5 mM achieved the best removal without raising any increase in toxicity of the treated media.

In chapter 4, the removal of TrOCs by a membrane bioreactor containing the WRF (*Trametes versicolor* (ATCC 7731) and activated sludge was evaluated. Results in this chapter highlight that a mixed culture of bacteria and *Trametes versicolor* can achieve better TrOC removal than a system containing fungus or bacteria alone. The major role of biodegradation was confirmed for all TrOCs for which the MBR system achieved high aqueous phase removal. Redox mediators can enhance the performance of fungal enzyme (laccase); however, compared to batch tests, due to lower enzymatic activity level and more complex wastewater matrix, limited improvement in removal due to mediator dosing may be achieved by the MBR for compounds originally highly resistant to fungal degradation.

In chapter 5, an EMR was developed to facilitate retention of laccase and its separation from treated effluent. The enzyme was completely retained by the membrane allowing continuous operation. However, denaturation of enzyme occurred due to physical, chemical and hydraulic conditions during EMR operation. The enzymatic activity was maintained by the addition of 200 µL of the commercial laccase solution per L of the reactor volume every 12 h (equivalent to a laccase dose of 23 mg/L.d). The EMR was observed to facilitate degradation of a number of TrOCs originally showing resistance to enzymatic degradation. This was attributed to the formation of a dynamic layer of laccase over the membrane surface which retained TrOCs and facilitated their subsequent enzymatic degradation. A complementary role of enhanced degradation by the laccase-mediator system and retention by the membrane gel layer was revealed. Membrane retention particularly enhanced the degradation of the compounds which are less amenable to mediator-enhanced enzymatic degradation.

In chapter 6, the performance of the EMR was evaluated in terms of the removal efficiency and toxicity of treated effluent under different TrOCs and redox-mediator (SA) concentrations. The removal of TrOCs increased with influent TrOC concentration-increase from 5 to 50 µg/L. Further increase in influent TrOC concentration to 100 µg/L resulted in a drop in the removal efficiency, indicating kinetic limitations. The addition of mediator SA at a dose of 10 µM to the EMR increased TrOC removal. Elevated concentrations of SA (50 and 100 µM) could not improve the removal efficiency, but it increased treated effluent toxicity. The toxicity may be attributed predominantly to the highly active radicals produced during the laccase—SA reaction, but also to the toxicity of SA itself. Under the same SA and enzyme loading, the treated effluent from the EMR showed lower toxicity than that observed during the batch tests. Overall, the TrOC removal and treated effluent toxicity data suggested that the lower possible dose to sustain the required level of TrOC removal must be used.

Chapter 7 reports the multiple advantages of granular activated carbon (GAC) dosing to the EMR: (i) enhancement of TrOC degradation by a laccase—mediator (SA) system, (ii) reduction of mediator-induced effluent toxicity, and (iii) mitigation of fouling by an enzyme gel layer over the membrane. TrOCs adsorbed onto the enzyme layer, and GAC addition further facilitated such TrOC retention on the membrane cake layer; nevertheless, subsequent TrOC degradation was confirmed. At a TrOC loading, mediator concentration and GAC dose of 830 µg/L.d, 5 µM, and 3 g/L, respectively, removal efficiency ranged from 65% (sulfamethoxazole) to 80% (diclofenac).

8.2. RECOMMENDATIONS FOR FURTHER RESEARCH

This thesis work provides comprehensive understanding of the TrOC removal performance of WRF and laccase in membrane reactors. However, some of the new ideas emerging during this research are worth to explore in the future.

The performance of EMR in this thesis was investigated mainly using a synthetic wastewater (*i.e.*, TrOC in ultrapure water). Before implementation of EMRs at an industrial scale, performance assessment using real wastewater would be required. The impact of common organics (*e.g.*, humic acid and chelating agents) or inorganics (*e.g.*, metals and salts) in wastewater on enzymatic degradation needs to be addressed in the future studies.

Enzyme replenishment was necessary to maintain the enzymatic activity level in the EMR. Thus, this may increase operation cost. Therefore, further research on the stabilization of enzyme during EMR operation is recommended. Immobilization of enzyme on carriers appears to be a potential alternative to improve the enzyme stability. The use of crude enzyme, which is collected directly from the culture of WRF without any further purification, may decrease the cost of enzyme and provide more flexible operation.

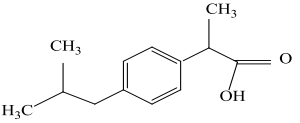
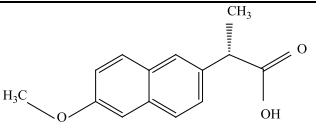
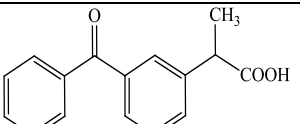
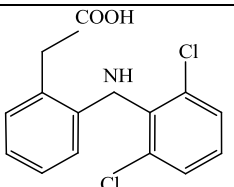
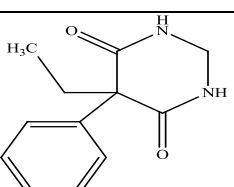
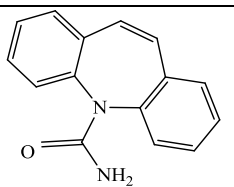
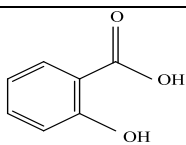
The toxicity of treated effluent was investigated in this work via bioluminescence assay. Monitoring the degradation by-products during the treatment is also important to understand the degradation pathway and fate of TrOCs. Data on the degradation pathway and the fate of the compounds would facilitate the assessment and explanation of the potential risks of TrOCs.

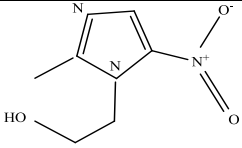
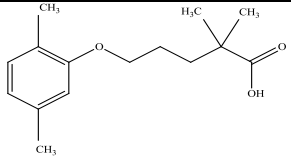
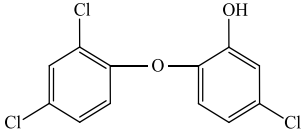
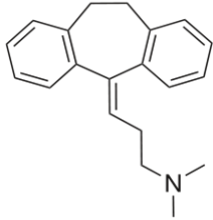
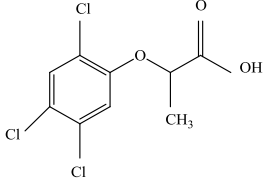
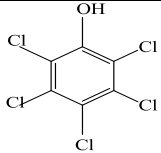
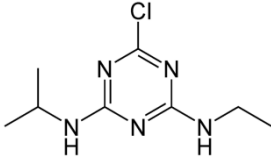
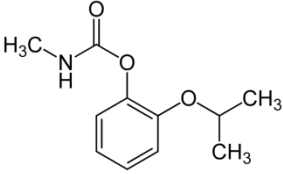
In this study, the addition of redox mediators has been demonstrated to improve the degradation and also extend the spectrum of compounds degraded by laccase. It is recommended that different groups of mediators are compared in the future studies..

Immobilization of laccase on supports could enhance its potential for industrial use due to the improvement in enzyme stability and reusability. This can be an important aspect to explore in the future studies.

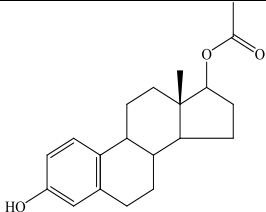
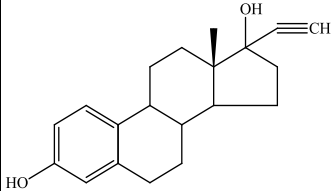
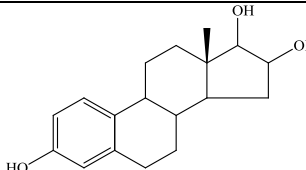
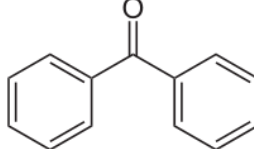
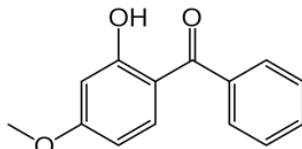
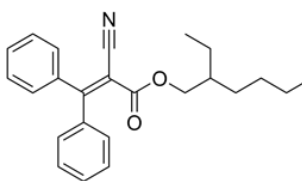
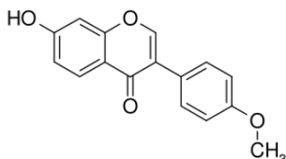
APPENDIX

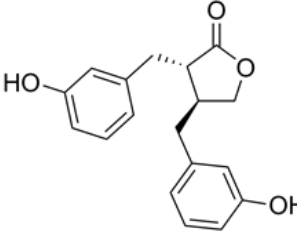
Table A-1: Physicochemical properties of the selected trace organic contaminants (TrOCs).

Category	Compound (CAS number)	Molecular weight (g mol ⁻¹)	Log D (pH 4) _a	Vapor pressure (mm Hg), at 25°C _a	Limit of detection (ng L ⁻¹) ^b	Chemical structure
Pharmaceuticals	Ibuprofen (C ₁₃ H ₁₈ O ₂) (5687-27-1)	206.28	2.81	1.39E-4	20	
	Naproxen (C ₁₄ H ₁₄ O ₃) (22204-53-1)	230.26	2.49	3.01E-7	1	
	Ketoprofen (C ₁₆ H ₁₄ O ₃) (22071-15-4)	254.28	2.07	3.32E-8	20	
	Diclofenac (C ₁₄ H ₁₁ Cl ₂ NO ₂) (15307-86-5)	296.15	3.66	1.59E-7	5	
	Primidone (C ₁₂ H ₁₄ N ₂ O ₂) (125-33-7)	218.25	0.83	6.08E-11	10	
	Carbamazepine (C ₁₅ H ₁₂ N ₂ O) (298-46-4)	236.27	1.89	5.78E-7	10	
	Salicylic acid (C ₇ H ₆ O ₃) (69-72-7)	138.12	-0.65	4.45E-5	1	

Pesticides	Metronidazole (C ₆ H ₉ N ₃ O ₃) (443-48-1)	171.15	-0.14	2.67E-7	20	
	Gemfibrozil (C ₁₅ H ₂₂ O ₃) (25812-30-0)	250.33	3.86	6.13E-7	1	
	Triclosan (C ₁₂ H ₇ Cl ₃ O ₂) (3380-34-5)	289.54	5.34	3.36E-5	1	
	Amitriptyline C ₂₀ H ₂₃ N (50-48-6)	277.40	2.28	1.50E-6	1	
	Fenoprop (C ₉ H ₇ Cl ₃ O ₃) (93-72-1)	269.51	1.39	2.13E-6	20	
	Pentachloro-phenol (C ₆ HCl ₅ O) (87-86-5)	266.34	4.62	3.49E-4	1	
	Atrazine (C ₈ H ₁₄ ClN ₅) (1912-24-9)	215.68	2.63	1.27E-5	10	
	Propoxur (C ₁₁ H ₁₅ NO ₃) (114-26-1)	209.24	1.54	1.53E-3	1	

	Ametryn (C ₉ H ₁₇ N ₅ S) (843-12-8)	227.33	2.92	1.72E-6	10	
	Clofibric acid (C ₁₀ H ₁₁ ClO ₃) (882-09-7)	214.65	0.61	1.03E-4	1	
Industrial chemicals and their metabolites	4-tert-butylphenol (C ₁₀ H ₁₄ O) (98-54-4)	150.22	3.40	0.0361	1	
	4-tert-octylphenol (C ₁₄ H ₂₂ O) (140-66-9)	206.32	5.18	1.98E-3	1	
	Bisphenol A (C ₁₅ H ₁₆ O ₂) (80-05-7)	228.29	3.64	5.34E-7	1	
Steroid hormones	Estrone (C ₁₈ H ₂₂ O ₂) (53-16-7)	270.37	3.62	1.54E-8	5	
	17β-estradiol (C ₁₈ H ₂₄ O ₂) (50-28-2)	272.38	4.15	9.82E-9	5	

	17 β -estradiol – 17 acetate (C ₂₀ H ₂₆ O ₃) (1743-60-8)	314.42	5.11	9.88E-9	5	
	17 α ethinylestradio l (C ₂₀ H ₂₄ O ₂) (57-63-6)	269.40	4.11	3.74E-9	10	
	Estriol (E3) (C ₁₈ H ₂₄ O ₃) (50-27-1)	288.38	2.53	1.34E-9	10	
UV filters	Benzophenone C ₁₃ H ₁₀ O (119-61-9)	182.22	3.21	8.23E-4	10	
	Oxybenzone C ₁₄ H ₁₂ O ₃ (131-57-7)	228.24	3.89	5.26E-6	10	
	Octocrylene C ₂₄ H ₂₇ N O ₂ (6197-30-4)	361.48	6.89	2.56E-9	10	
	Formononetin C ₁₆ H ₁₂ O ₄ (485-72-3)	268.26	2.86	8.17E-10	5	

Phytoestrogens	Enterolactone C ₁₈ H ₁₈ O ₄ (78473-71-9)	298.33	1.89	3.29E-13	5	
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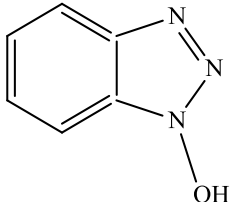
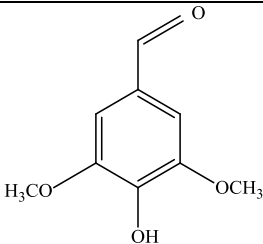
^a Source: SciFinder database <https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf>

Log *D* is logarithm of the distribution coefficient which is the ratio of the sum of concentrations of all forms of the compound (ionised and unionised) in octanol and water at a given pH.

na: data not available

^bLimit of detection (LOD) of the compounds during GC-MS analysis as described in Section 2.4. LOD is defined as the concentration of an analyte giving a signal to noise (S/N) ratio greater than 3. The limit of reporting was determined using an S/N ration of greater than 10.

Table A-2: Physicochemical properties of the selected mediators

Compounds	Molecular weight (g/mol)	Molecular formula (CAS number)	Dissociation constant (pKa) ^a	Chemical structure
1-Hydroxybenzotriazole (HBT)	135.12	C ₆ H ₅ N ₃ O (2592-95-2)	7.39 ± 0.58 0.48 ± 0.30	
Syringaldehyde (SA)	182.17	C ₉ H ₁₀ O ₄ (134-96-3)	7.80 ± 0.23	

^a Source: SciFinder database <https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf>

Table A-3: Statistical analysis of data

Compounds	<i>p</i> value		
	Live whole cell vs. inactivated whole cell fungus	Live whole cell vs. crude enzyme extract	Crude enzyme extract vs. mediator-enhanced crude enzyme extract
Metronidazole	-	0.225	0.225
Salicylic acid	0.008	0	0
Primidone	0.489	0.084	0.001
Amitriptyline	0.57	0.75	0.002
Propoxur	0.038	0.141	0.001
Clofibric acid	0.02	0.588	0.037
Carbamazepine	0.122	0.225	0.225
Enterolactone	0.005	0.225	0.018
Fenoprop	-	0.123	0.111
Estriol	0.014	0	0.094
Atrazine	0.333	0.194	0.001
Ametryn	0.255	0.711	0.024
Ketoprofen	0.329	0.022	0.089
Naproxen	0	0	0
Formononetin	0	0.003	-
Benzophenone	0.068	0.048	-
Ibuprofen	0	0	0.225
4-tert-Butylphenol	0.234	0.028	0.009
Estrone	0.004	0.008	0.006
Bisphenol A	0	0.006	0.004
Oxybenzone	0.043	0.002	0.01
17 α - Ethinylestradiol	0.003	0.01	0.056
17 β -Estradiol	0.004	0	0.03
Gemfibrozil	0.001	0.001	0.011
Diclofenac	0.002	0.001	0.021
Pentachlorophenol	0.029	0.021	0.007
17 β -Estradiol-17-acetate	0.022	0	0.014
4-tert-Octylphenol	0.052	0.124	0.418
Triclosan	0	0.001	0.111
Octocrylene	0.173	0.223	0.533

Table A-4: Comparative degradation rates of TrOCs by different treatment options (based on removal over 24 h)

TrOC degradation rate (ng/L.h)								
Category	Compounds	Laccase	Laccase- HBT			Laccase-SA		
			HBT concentration (mM)			SA concentration (mM)		
			0.1	0.5	1	0.1	0.5	1
Phenolic	Formononetin	0.0	0.0	0.0	0.0	37.7 ± 9.5	25.4 ± 4.0	24.2 ± 11.7
	Salicylic acid	0.0	0.0	66.2 ± 12.2	50.2 ± 16	10.4 ± 8.3	15.7 ± 4.5	15.2 ± 3.0
	Oxybenzone	0.0	6.6 ± 4.5	80.1 ± 16.7	68.5 ± 13.4	72.0 ± 5.4	31.2 ± 9.3	56.7 ± 11.2
	Pentachlorophenol	8.5 ± 5.0	19.9 ± 1	70.2 ± 12.4	67.8 ± 11.7	12.8 ± 2.4	9.7 ± 1.5	31.7 ± 1.4
	Enterolactone	16.1 ± 5.7	61.0 ± 31.2	69.8 ± 18.8	78.8 ± 12.8	48.8 ± 16.0	50.6 ± 12.3	63.1 ± 18.0
	Triclosan	62.5 ± 18.7	90.6 ± 14.7	91.8 ± 13.3	71.6 ± 15.4	85.8 ± 4.0	84.7 ± 2.7	84.8 ± 3.0
	4-tert-Butylphenol	62.2 ± 11.4	68.2 ± 14.2	65.8 ± 15.6	60.0 ± 13.6	51.6 ± 0.9	51.7 ± 0.5	51.5 ± 0.6
	4-tert-Octylphenol	59.2 ± 13.2	61.5 ± 12.4	62.3 ± 17.0	67.5 ± 16.2	67.2 ± 5.2	67.2 ± 8.5	67.2 ± 3.0
	17β-Estradiol 17-acetate	83.1 ± 17.9	84.1 ± 2.6	84.9 ± 11.0	86.7 ± 5.6	92.6 ± 3.8	93.0 ± 4.0	91.8 ± 2.8
	Estrinol	99.0 ± 5.6	99.6 ± 8.3	98.8 ± 5.2	93.6 ± 3.5	83.6 ± 4.5	86.7 ± 3.7	81.7 ± 2.3
	Bisphenol A	96.7 ± 3.6	99.3 ± 4.5	98.3 ± 3.5	96.1 ± 2.6	98.5 ± 2.8	98.5 ± 3.7	98.2 ± 2.5
	17β-Estradiol	99.0 ± 2.4	100.5 ± 2.8	101.2 ± 2.3	105.2 ± 3.0	98.4 ± 2.6	100.1 ± 3.5	99.2 ± 2.4
	17α-Ethinylestradiol	91.9 ± 2.4	92.0 ± 2.6	93.5 ± 1.8	91.6 ± 2.4	77.4 ± 1.3	78.2 ± 1.4	75.3 ± 1.8
Non-phenolic	Estrone	104.0 ± 17.5	105.4 ± 18.6	106.3 ± 13.6	106.0 ± 7.2	107.9 ± 6.6	108.5 ± 6.3	108.5 ± 5.6
	Benzophenone	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Ketoprofen	0.0	1.5 ± 2.8	2.5 ± 3.6	7.8 ± 2.8	0.0	0.0	0.0
	Primidone	0.0	0.0	0.0	29.2 ± 3.8	16.7 ± 28.0	0.0	0.0
	Atrazine	0.0	43.2 ± 15.8	69.1 ± 16.0	53.8 ± 13.9	23.9 ± 10.1	25.6 ± 4.1	51.2 ± 6.4
	Ibuprofen	0.0	0.0	0.0	0.0	10.9 ± 1.0	2.0 ± 5.2	0.9 ± 1.8
	Amitriptyline	2.8 ± 1.3	4.3 ± 1.6	24.8 ± 4.8	18.2 ± 3.4	18.9 ± 7.4	7.4 ± 2.4	34.8 ± 12.0
	Carbamazepine	3.9 ± 10.6	0.0	3.0 ± 11.7	0.0	0.0	0.0	0.0
	Propoxur	5.5 ± 2.8	5.5 ± 2.3	6.5 ± 9.2	8.8 ± 11.7	0.0	0.0	0.0
	Clofibric acid	6.2 ± 4.0	8.0 ± 3.5	17.3 ± 9.6	10.4 ± 4.7	16.4 ± 4.2	14.2 ± 8.7	22.3 ± 14.1
	Fenoprop	8.2 ± 1.3	13.8 ± 4.8	17.9 ± 3.5	18.6 ± 1.7	3.2 ± 12.8	4.6 ± 15.4	3.7 ± 13.5
	Metronidazole	2.6 ± 0.8	3.3 ± 0.8	6.3 ± 1.1	8.7 ± 0.5	7.9 ± 1.2	1.2 ± 4.5	1.0 ± 4.7
	Octocrylene	13.9 ± 26.8	1.1 ± 2.3	1.4 ± 2.8	1.9 ± 0.7	34.4 ± 14.5	16.3 ± 7.5	53.3 ± 9.8
	Gemfibrozil	18.0 ± 7.0	19.8 ± 12.0	32.6 ± 14.4	13.8 ± 5.4	12.8 ± 6.4	4.0 ± 4.5	6.6 ± 2.8
	Naproxen	19.0 ± 14.3	59.6 ± 9.0	95.6 ± 15.1	81.8 ± 12.4	12.1 ± 6.2	10.6 ± 0.8	57.6 ± 6.3
	Ametryn	24.0 ± 8.1	18.7 ± 5.3	29.0 ± 9.4	23.5 ± 4.3	24.4 ± 5.1	25.3 ± 2.8	19.0 ± 2.4
	Diclofenac	62.0 ± 17.3	57.0 ± 12.5	79.7 ± 14.5	70.1 ± 11.6	72.0 ± 16.5	85.6 ± 2.1	74.3 ± 13.2

Table A-5: Statistical analysis of data

		<i>p</i> value			
Category	Compounds	‘Laccase-EMR’ <i>versus</i> ‘Laccase-Batch test’	Laccase-EMR <i>versus</i> ‘Laccase-EMR-HBT’	‘Laccase-EMR’ <i>versus</i> ‘Laccase-EMR-SA’	‘Laccase-EMR-HBT’ <i>versus</i> ‘Laccase-EMR-SA’
Phenolic-TrOCs	Salicylic acid	0.004	0.009	0.012	0.031
	Formononetin	0.008	0.033	0.236	0.003
	Pentachlorophenol	0.001	0.004	0.477	0.07
	Enterolactone	0.174	0.04	0.048	0.225
	Oxybenzone	0.003	0.193	0.047	0.016
	Triclosan	0.055	0.225	0.035	0.074
	17 β -Estradiol 17-acetate	0.02	0.296	0.128	0.225
	4-tert-Octylphenol	0.904	0.073	0.318	0.912
	Estrone	0.001	0.225	0.247	0.004
	4-tert-Butylphenol	0.022	0.38	0.009	0.003
	Bisphenol A	0.002	0.443	0	0.001
	17 α -Ethinylestradiol	0.006	0.504	0.01	0.023
	Estriol	0.004	0.02	0.28	0.019
	17 β - Estradiol	0.008	0.038	0.009	0
Non-phenolic-TrOCs	Propoxur	0.267	0.267	0.267	0.267
	Clofibric acid	0.138	0.001	0.87	0.046
	Ibuprofen	0.002	0.743	0.283	0.089
	Metronidazole	0.423	0.01	0.002	0.073
	Atrazine	-	0	0.06	0
	Fenoprop	0.233	0.015	0.106	0.249
	Ametryn	0	0.317	0.734	0.248
	Gemfibrozil	0.044	0.007	0.115	0.296
	Naproxen	0.05	0.082	0.302	0.147
	Ketoprofen	0.252	0.152	0.374	0
	Primidone	0.561	0.001	0.398	0.006
	Carbamazepine	0.238	0	0.207	0.004
	Benzophenone	0.003	0.829	0.043	0.49
	Amitriptyline	0.003	0.124	0.094	0
	Octocrylene	0	0.057	0.478	0.84
	Diclofenac	0.701	0.02	0.115	0.271

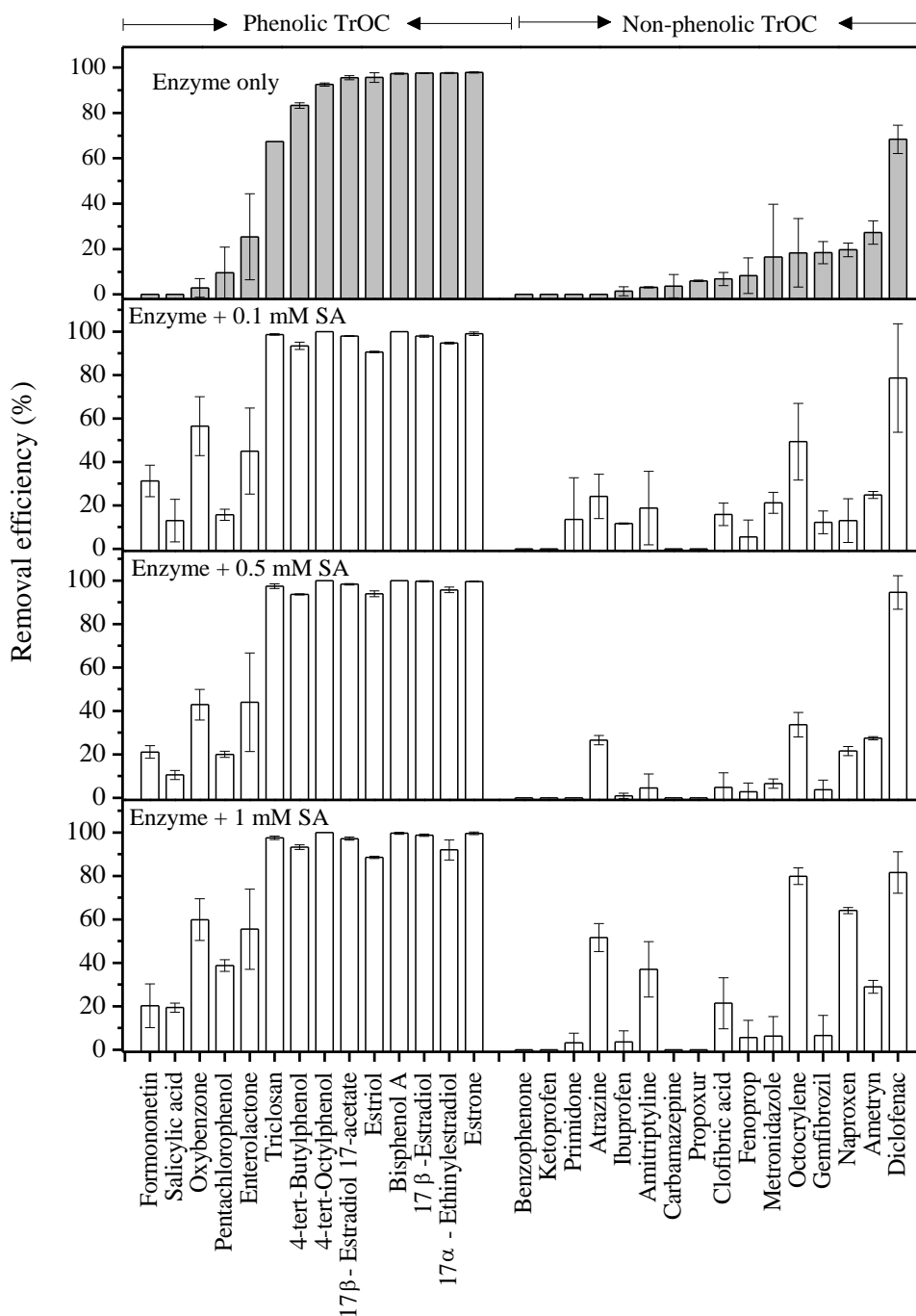


Figure A-1: Removal of 30 TrOC by crude enzyme extract from *T. versicolor* (ATCC 7731) in the absence and presence of SA at concentrations of 0.1, 0.5, and 1 mM. The error bars represent the standard deviation of three replicates.

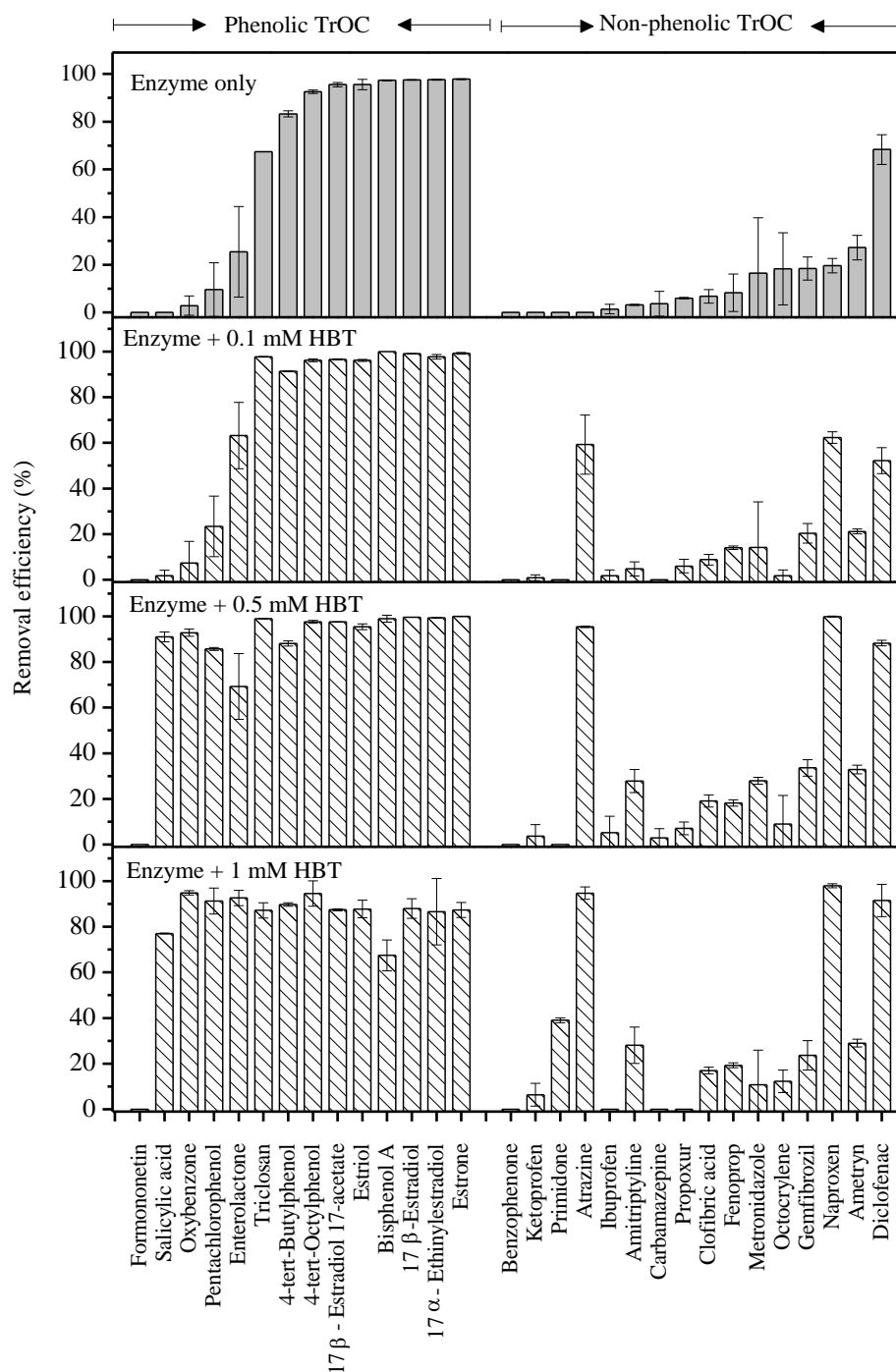


Figure A-2: Removal of 30 TrOC by crude enzyme extract from *T. versicolor* (ATCC 7731) in the absence and presence of HBT at concentrations of 0.1, 0.5, and 1 mM. The error bars represent the standard deviation of three replicates.