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Enzymatic treatment of organic micropollutants in municipal wastewater: treatment conditions and reaction kinetics

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Enzymatic treatment of organic micropollutants in municipal wastewater: treatment conditions and reaction kinetics

by

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Dedication

To my parents and my brothers.

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Many people have encouraged, helped, and supported me throughout my Ph.D. process. I am very grateful to you all.

Enzymatic treatment of organic micropollutants in municipal wastewater: treatment conditions and reaction kinetics

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Organic micropollutants enter natural waters via treated municipal wastewater discharges, threatening aquatic ecosystems and exposing humans to trace levels of these diverse chemicals through downstream drinking water supplies. Both of these concerns would be addressed simultaneously if micropollutants, which are often recalcitrant to conventional treatment processes, were removed in municipal wastewater treatment plants. Thus, the ultimate goal of this research was to advance the development of a sustainable and cost-effective enzymatic treatment process that oxidizes organic micropollutants in municipal wastewater. The enzymatic treatment process studied, laccase-catalyzed oxidation, involves the oxidation of mediator compounds by the enzyme laccase, generating mediator free radicals that can oxidize target micropollutants. The influences of key treatment conditions (pH, enzyme activity, mediator concentration, wastewater organic content) on the efficacy of the treatment process were evaluated, and the reaction kinetics of enzymatic treatment were investigated. The results demonstrated that enzymatic treatment can transform environmentally relevant concentrations of two representative micropollutants, oxybenzone and sulfamethoxazole, in a primary effluent wastewater matrix under realistic treatment conditions. Experiments with a set of structurally related chlorophenolic target compounds revealed several impacts of target

compound structure on the kinetics and mechanisms of the laccase-mediator-target compound reactions. The electronic properties of the target compounds, their acid-base speciation, and steric hindrance to coupling reactions by substitution of the aromatic ring were found to influence their relative reactivities and reaction mechanisms with the free radicals generated by the laccase-mediator system. A kinetic model of the reactions between the enzyme laccase, the mediator acetosyringone, and the chlorophenolic target compounds was developed and fit the experimental data quite well despite the complexity and non-linearity of the system. This model indicated that the rate limiting step was the oxidation of the mediator by the enzyme. The relative rate constants for the reactions of the resulting mediator radical represent the distribution of the radicals among several possible reaction pathways. Although still in its early stages of development, the results of this research indicate that enzymatic treatment could one day be implemented in wastewater treatment plants for the mitigation of micropollutant release into the environment.

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Chapter 1: Introduction

1.1. Research Motivation

Organic micropollutants, now prevalent in natural waters and throughout the urban water cycle, often enter the environment through municipal wastewater. The release of micropollutants into natural waters via treated wastewater discharges is a threat to aquatic ecosystems and exposes humans to trace levels of these chemicals through drinking water downstream. Both of these concerns would be addressed simultaneously if micropollutants were removed during municipal wastewater treatment. Their chemical diversity and recalcitrance to conventional wastewater treatment processes make effective treatment of micropollutants one of the major challenges of water and wastewater treatment today. Global consumption of pharmaceuticals and many products containing micropollutants is continually increasing, and therefore, so are the associated environmental repercussions. Thorough research of potential means to address this problem is imperative.

1.2. Research Objectives

The ultimate goal of this research is to advance the development of a sustainable and cost-effective enzymatic treatment process that oxidizes organic micropollutants in municipal wastewater. In this research, enzymatic treatment involved the oxidation of mediator compounds by the enzyme laccase, creating free radicals that could oxidize target micropollutants. This study aimed to address important questions about this treatment process by evaluating the influences of key process parameters, testing potential treatment configurations, and investigating the reaction kinetics of enzymatic treatment. Successful development and implementation of enzymatic treatment of municipal wastewater could significantly reduce the release of micropollutants into the

environment, and consequently reduce their presence in natural waters, including many drinking water sources. Thus, not only would the health of aquatic ecosystems be improved, but human exposure to these chemicals through drinking water would also be reduced.

The objectives of this research were addressed in four phases, as follows.

Phase 1: Evaluated the influences of key process and water quality parameters (*e.g.*, pH, enzyme activity) on the transformation of two representative micropollutants, oxybenzone and sulfamethoxazole, in municipal wastewater primary effluent in two possible treatment configurations.

Phase 2: Investigated the effectiveness of the laccase-mediator system for micropollutant degradation in a continuous flow, non-ideal plug flow reactor.

Phase 3: Studied the kinetics of the laccase-mediator-target compound reactions in a pure buffered system with a model target compound, 2-chlorophenol.

Phase 4: Investigated the influence of target compound structure on the enzymatic treatment reaction kinetics and mechanisms with a set of structurally related model chlorophenol compounds and the structurally related micropollutant chloroxylenol.

Phases 1 and 2 were designed to demonstrate that enzymatic treatment can transform micropollutants in municipal wastewater primary effluent under realistic treatment conditions, and to more narrowly define an optimal set of treatment conditions. These first two study phases focused on the feasibility of enzymatic treatment in municipal wastewater, whereas Phases 3 and 4 were designed to provide a more detailed picture of the reactions involved in this treatment process, which will be essential to the continuation of process development and design.

1.3. DISSERTATION STRUCTURE

This dissertation is organized into the following chapters.

Chapter 2: Background

Contains an overview of the occurrence and impacts of micropollutants and background information on enzymatic treatment processes.

Chapter 3: Materials and Methods

Details the chemicals, experimental procedures, analytical methods, laboratory equipment, and other materials used throughout this research.

Chapter 4: Phase 1 Results – Evaluation of Enzymatic Treatment Conditions in Batch Experiments

Discusses the results of enzymatic treatment of two representative micropollutants under varying treatment conditions and treatment configurations in batch experiments.

Chapter 5: Phase 2 Results – Enzymatic Treatment in a Continuous Flow Reactor

Discusses the results of enzymatic treatment of two representative micropollutants in a continuous flow setting.

Chapter 6: Phase 3 Results – Investigation of Enzymatic Treatment Reaction Kinetics

Discusses the results of a kinetic study of a model laccase-mediator-target compound system.

Chapter 7: Phase 4 Results – The Influence of Target Compound Structure and Speciation on Enzymatic Treatment Reaction Kinetics

Discusses the results of kinetic experiments with a set of structurally related target compounds.

Chapter 8: Kinetic Modeling

Discusses the development and results of a kinetic model for the laccase-mediator-target compound system.

Chapter 9: Conclusions and Recommendations

Outlines the major findings and conclusions of this research and provides recommendations for future enzymatic treatment research.

Chapter 2: Background

2.1. Occurrence and Impacts of Micropollutants

Micropollutants are now routinely detected in raw and treated municipal wastewater, the natural environment, and drinking water sources (Khetan & Collins, 2007; Kolpin *et al.*, 2002; Morasch *et al.*, 2010; Snyder *et al.*, 2005). Micropollutants are present in municipal wastewater due to human excretion, flushing of unused medications, and our daily use of personal care products. Many of these compounds are only partially removed, or not removed at all, in conventional wastewater treatment processes. As a result, micropollutants enter natural water bodies with treated wastewater effluent. Since the pathways between treated wastewater outfalls and raw drinking water intakes are shrinking, and many micropollutants persist for relatively long times in the environment, micropollutants ultimately reach our drinking water. Thus, in addition to threatening aquatic ecosystems, micropollutants in municipal wastewater potentially threaten human health through drinking water. A study by Vulliet *et al.* (2009) analyzed source surface waters and finished drinking waters of eight French drinking water treatment plants for 51 micropollutants. They detected 27 of the selected micropollutants in the source waters and 25 of them in the finished drinking waters. Every finished drinking water contained measurable concentrations of at least one micropollutant. In the United States, source water, finished drinking water, and tap water in 19 water utilities across the country were screened for 51 pharmaceuticals, potential endocrine disrupting compounds, and other unregulated organic contaminants in a study by Benotti *et al.* (2009). At least one contaminant was detected in every source water, and 11 of the 51 contaminants were detected in more than half of the source waters. Furthermore, 13 of the 15 tap waters tested contained detectable concentrations of at least one contaminant.

Many researchers have documented that exposure to micropollutants can have toxicological and endocrine disrupting effects on natural biota (Dussault *et al.*, 2008; Gagné *et al.*, 2006; Thorpe *et al.*, 2009; Vajda *et al.*, 2008). Additionally, the prolonged presence of trace levels of antibiotics in the environment may increase antibiotic resistance in natural bacterial populations (Jones *et al.*, 2003). Although human health effects due to chronic exposure to trace concentrations of micropollutants have not been documented, research by Pomati *et al.* (2006) indicates that there is reason for concern. They found that a mixture of 13 micropollutants, representative of combinations and concentrations found in northern Italian rivers, inhibited growth of human embryonic cells *in vitro* by as much as 30%. Thus, it is critical to prevent the release of micropollutants into the environment to protect the health of natural aquatic systems *and* human health.

Although micropollutants can enter surface waters in a variety of ways, municipal wastewater is the most important point source in developed countries (Moldovan *et al.*, 2009; Snyder *et al.*, 2005). As a point source, municipal wastewater is a convenient place to address micropollutant pollution. However, further investigation of suitable treatment technologies to remove the broad range of micropollutants present in municipal wastewater is still needed, particularly of technologies that could be implemented near the front end of a wastewater treatment plant to maximize the possibility of complete removal of the micropollutants and mitigate the release of residual micropollutants or micropollutant transformation products into the environment.

Existing treatment technologies outside of conventional wastewater treatment processes that have been investigated for micropollutant removal include activated carbon, advanced oxidation processes (*e.g.*, ultraviolet irradiation with hydrogen peroxide), membrane bioreactors, moving bed biofilm reactors, ozonation, and slow sand

filtration (Escolà Casas & Bester, 2015; Lundström *et al.*, 2010a; Lundström *et al.*, 2010b). The enzymatic treatment process studied in this research, laccase-catalyzed oxidation, is another potentially suitable technology for addressing micropollutant contamination in municipal wastewater.

2.2. TARGET MICROPOLLUTANTS SELECTED FOR STUDY

Two representative target micropollutants were selected for study based on their relatively high concentrations in treated municipal wastewater and potential environmental and health effects. One personal care product and one pharmaceutical, oxybenzone and sulfamethoxazole, respectively, were selected, and their molecular structures and relevant data are provided in Table 3-1.

Oxybenzone was selected for study for several reasons. Laccase-catalyzed oxidation of this compound had already been studied in our laboratory (Garcia *et al.*, 2011), and the first phase of this research built upon that prior knowledge. The previous research had shown that oxybenzone is not directly oxidized by the laccase enzyme and is therefore representative of micropollutants requiring the laccase-mediator system, the enzymatic treatment process focused on in this research, for effective treatment. High oxybenzone concentrations in treated municipal wastewater (up to 700 ng/L) have been reported (Balmer *et al.*, 2005), which is of concern because oxybenzone has the potential to disrupt endocrine and/or reproductive systems of fish (Coronado *et al.*, 2008).

Sulfamethoxazole was selected for study due to its widespread occurrence at relatively high concentrations in both municipal wastewater and natural waters. A 2011 study on the occurrence of sulfonamide antibiotics in wastewater treatment plants and the Ebro River basin in Spain by García-Galán *et al.* found sulfamethoxazole in the influents and effluents of all seven wastewater treatment plants studied. In one of two sampling

campaigns, sulfamethoxazole was detected at higher concentrations in the effluents (up to 650 ng/L) than in the influents of five of the seven plants. Sulfamethoxazole was detected in 100% and 69% of surface water samples during the two different sampling campaigns at average concentrations of 89.2 ng/L and 25.5 ng/L, respectively. Rainfall was significantly higher during the second campaign. In some cases, concentrations were found to be higher downstream (up to more than 150 ng/L) than upstream of the wastewater treatment plants. Furthermore, Vulliet *et al.* (2009) detected sulfamethoxazole in each of the eight surface drinking water sources they tested in France in at least one of three sampling campaigns. In some cases, sulfamethoxazole was detected in finished drinking waters (Stolker *et al.*, 2004; Vulliet *et al.*, 2009). The presence of this micropollutant in the environment is of concern due to the potential for antibiotic resistance to develop in natural bacterial populations.

2.3. LACCASES AND LACCASE-MEDIATOR SYSTEMS

Enzymatic treatment processes are inspired by processes that occur in nature. White rot fungi secrete oxidoreductase enzymes, including laccases, to degrade lignin and thereby access cellulose. White rot fungi also use oxidoreductases to degrade other complex natural polymers, such as humic acids. Laccases are members of a small group of enzymes called blue copper oxidases, which transfer one electron each from four substrate molecules to a molecule of oxygen, reducing it to two water molecules (Figure 2-1:a). This process is facilitated by four copper atoms; one copper atom is responsible for substrate oxidation, and the accepted electron is transferred to a trinuclear copper center, where dioxygen is bound and reduced. Laccases from different organisms can have structural variations that lead to differences in redox potential (typically 0.5 – 0.8 V), substrate specificity, and enzyme stability (Shleev *et al.*, 2005; Strong & Claus, 2011;

Xu *et al.*, 1996). Laccases and laccase-like enzymes have been found in a variety of organisms in addition to fungi, including plants, yeasts, insects, and prokaryotes (Strong & Claus, 2011).

Laccases process a wide range of substrates by direct oxidation (Figure 2-1:a), but can also oxidize compounds indirectly via mediator molecules (Figure 2-1:b). White rot fungi also secrete a variety of low molar mass mediator molecules that enhance the oxidative ability of the oxidoreductase enzymes. The mediators are oxidized by the enzymes, generating free radicals that can then oxidize other compounds, allowing oxidation of compounds not directly processed by the enzymes.

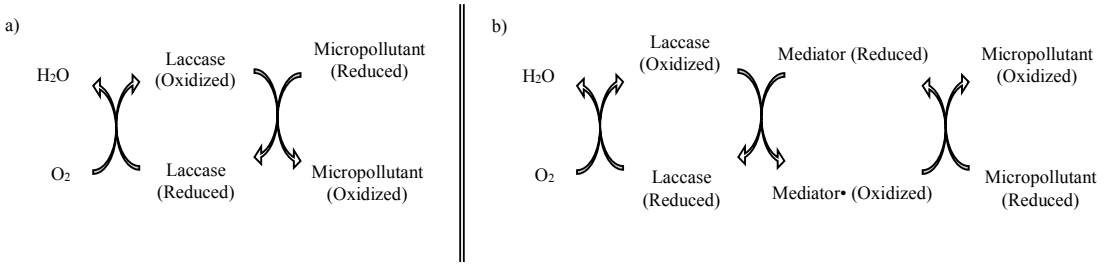


Figure 2-1: Schematic representation of ideal catalytic redox cycles for micropollutant oxidation: (a) without a mediator compound; (b) with the addition of a mediator compound

Direct oxidation of a substrate (mediator molecule or micropollutant) is determined by two properties: whether the substrate fits in the active site of the enzyme (adjacent to the copper cluster) and the substrate's redox potential. Some micropollutants do not fit in the active site of the enzyme and/or do not have a suitable redox potential, rendering the success of enzymatic treatment of these compounds reliant on both the laccase enzyme and the mediator molecule (Cañas & Camarero, 2010; Giardina *et al.*, 2010; Thurston, 1994). In this research, enzymatic treatment takes advantage of the expanded oxidative range of this laccase-mediator system.

2.4. ENZYMATIC TREATMENT

Laccases exhibit high specificity for a variety of substrates, including aromatic amines, aryl diamines, hydroxyindols, inorganic and organic metal compounds, methoxy substituted phenols, phenols, polyphenols, and thiophenols (Kunamneni *et al.*, 2008; Strong & Claus, 2011). They are able to oxidize phenolic compounds that are considered toxic or recalcitrant to conventional biological treatment systems (Strong & Claus, 2011). For these reasons, as well as its relatively simple requirements (naturally abundant oxygen and substrate), laccases have been studied extensively for the treatment of industrial wastewaters. Laccases and/or laccase-mediator systems have been studied for treating wastewaters from distilleries, olive oil mills, petroleum refineries, printing operations, pulp and paper mills, and textile industries (Garcia *et al.*, 2011; Strong & Claus, 2011).

Laccases have been studied much less extensively for the treatment of organic micropollutants in municipal wastewater. In buffered ultrapure water, laccases have been shown to directly oxidize bisphenol A and nonylphenol (Cabana *et al.*, 2007); triclosan (Kim & Nicell, 2006); diclofenac, estradiol, estrone, and ethinylestradiol (Lloret *et al.*, 2010; Lloret *et al.*, 2012); acetaminophen (Lu *et al.*, 2009); and mefenamic acid (Margot *et al.*, 2013a; Margot *et al.*, 2013b). In buffered ultrapure water, laccase-mediator systems have been shown to oxidize benzyl alcohols (Baiocco *et al.*, 2003) and isoproturon and sulfamethoxazole (Margot *et al.*, 2015). In a study by Torres-Duarte *et al.* (2009), a white rot fungal culture was able to transform nine halogenated pesticides, whereas a laccase-mediator system using purified laccase from the same fungus was only able to degrade four of the pesticides. Many of these studies were performed using high concentrations of the target micropollutants.

Relatively few published studies have investigated laccase-catalyzed oxidation for the degradation of low concentrations of micropollutants in actual municipal wastewater (Auriol *et al.*, 2007; Garcia *et al.*, 2011; Lloret *et al.*, 2013; Tran *et al.*, 2013). Auriol *et al.* (2007) demonstrated that steroid estrogens at an environmentally relevant concentration of 100 ng/L were degraded by free laccase from *Trametes versicolor* in filtered secondary effluent. Garcia *et al.* (2011) used the laccase-mediator (laccase from *Trametes versicolor*) system in filtered primary effluent to oxidize 10 µg/L oxybenzone, a micropollutant not directly oxidized by laccase. Although Auriol *et al.* (2007) also used the laccase-mediator system, mediators were used for the purpose of increasing reaction rates, not for the purpose of expanding the range of micropollutants treated. Lloret *et al.* (2013) utilized a laboratory-scale enzymatic membrane reactor to directly oxidize environmental concentrations (0.29 – 1.52 ng/L) of natural (estradiol and estrone) and synthetic (ethinylestradiol) estrogens in filtered secondary effluent with a laccase from *Myceliophthora thermophila*. Tran *et al.* (2013) examined the removal of the insect repellent *N,N*-diethyl-m-toluamide (DEET) by laccase from *Trametes versicolor* and two laccase-mediator systems in filtered raw wastewater. Experiments in this research were conducted with environmentally relevant micropollutant concentrations (10 µg/L) in unfiltered primary effluent and used the laccase-mediator system (laccase from *Myceliophthora thermophila*) to expand the oxidative range of laccase to compounds not directly oxidized by the enzyme.

The research by Garcia *et al.* (2011) indicated that laccase-catalyzed oxidation can degrade micropollutants in municipal wastewater primary effluent, but can also generate transformation products and oxidation byproducts, just as in ozonation and advanced oxidation processes. Implementation of enzymatic treatment in primary effluent would be a great advantage, as it would allow for further removal of

transformation products and oxidation byproducts in subsequent activated sludge treatment. This removal could occur by biodegradation or adsorption to the biomass present in secondary treatment. The biodegradation of laccase transformation products of bisphenol A was demonstrated by Nakamura & Mtui (2003). Several authors have also reported enzyme-catalyzed oxidative coupling of organic substrates, including laccase-catalyzed oxidative coupling of acetaminophen (Lu *et al.*, 2009; Dec & Bollag, 1990; Huang *et al.*, 2005). The products of oxidative coupling reactions are usually less hydrophilic, particularly as the molecules become larger (Lu *et al.*, 2009), and thus may be easier to remove from the aqueous phase by adsorption. As is the case for ozonation and advanced oxidation processes, implementation of enzymatic treatment in secondary effluent would require a subsequent biological treatment process, such as slow sand filtration.

2.5. LACCASE REDOX MEDIATORS

Laccase redox mediators are generally divided into two categories: natural and synthetic. Natural mediators are often phenolic compounds derived from oxidized lignin units, including syringyl type mediators like acetosyringone, methyl syringate, and syringaldehyde (Camarero *et al.*, 2005; Rosado *et al.*, 2012). Such naturally occurring mediators are likely to be true mediators of laccase oxidation of lignin polymers by white rot fungi in nature (Rosado *et al.*, 2012). Common synthetic mediators include ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), 1-hydroxybenzotriazole, and TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy free radical). Synthetic mediators, although in some cases more efficient, are expensive, toxic, and can inhibit enzyme activity at higher concentrations (Strong & Claus, 2011), and are therefore not suitable for municipal wastewater treatment applications. However, it is not safe to assume that

all natural mediators are nontoxic and have no negative effects on enzyme activity. A cytotoxicity study by Maruyama *et al.* (2007) provided evidence of high toxicity of the naturally occurring mediator 4-hydroxybenzoic acid. Nguyen *et al.* (2014) reported high cytotoxicity (1,200 – 2,200 times that of the control) of solutions of trace organic contaminants treated by the laccase-syringaldehyde system. Margot *et al.* (2015) tested the ecotoxicity (with green algae) of solutions of two micropollutants treated by three different laccase-mediator systems (ABTS, acetosyringone, and syringaldehyde). They found significantly lower algal toxicity of treated reaction mixtures as compared to the parent micropollutants. However, the residual ecotoxicity after enzymatic treatment, although largely reduced, was found to be due to mediator transformation products rather than micropollutant transformation products.

An ideal laccase redox mediator for enzymatic treatment would be efficiently oxidized by the laccase enzyme to a reactive, but relatively stable radical that is regenerated during the oxidation of target micropollutants and has no negative effects on the enzyme. The stability of the radical is important to allow for its diffusion away from the enzyme active site with limited self-reactions and radical decay. The parent mediator compound, as well as its transformation products, would be nontoxic. Such an ideal mediator has not yet been discovered, but the structurally similar mediators acetosyringone and syringaldehyde have been found to be some of the most efficient natural mediators, in some cases outperforming synthetic mediators as well (Camarero *et al.*, 2005; Torres-Duarte *et al.*, 2009). These two mediators are phenols with two methoxy groups in the *ortho* positions and an acetyl (acetosyringone) or aldehyde (syringaldehyde) group in the *para* position. Their molecular structures contribute to their efficiencies as mediators. The electron donating methoxy substituents enhance mediator oxidation by the laccase enzyme, and the mediator radicals are relatively stable

due to delocalization of the unpaired electron by the electron withdrawing acetyl or aldehyde substituent and steric hindrance to self-coupling reactions by the substituents. The half-life of the radical of acetosyringone, the mediator used in this research, was found to be three minutes at pH 4.5 in a study by Medina *et al.* (2013). They found that of the radical of syringaldehyde to be 98 minutes. Radical stability can be influenced by pH, and as reported by Fabbrini *et al.* (2002), the optimum pH for oxidation of target micropollutants by a laccase-mediator system might be more strongly influenced by the stability of the mediator radical than by the stability of the enzyme itself.

2.6. KINETICS AND MECHANISMS OF ENZYMATIC TREATMENT REACTIONS

Kinetic and mechanistic studies have been much more numerous regarding laccase oxidation of a substrate (*i.e.*, a mediator compound or direct oxidation of a target compound) (*e.g.*, Calcaterra *et al.*, 2008; Kulys *et al.*, 2000; Medina *et al.*, 2013; Rosado *et al.*, 2012; Xu, 1996) than regarding the laccase-mediator-target compound system (*e.g.*, Almansa *et al.*, 2004; Murugesan *et al.*, 2010; Torres-Duarte *et al.*, 2009). Such studies have often been more focused on reaction mechanisms and more focused on synthetic mediators.

For various laccase enzymes, pH and temperature influences on enzyme activity and stability, which would influence the kinetics of laccase oxidation, have been studied (*e.g.*, Kurniawati & Nicell, 2008; Lloret *et al.*, 2010; Margot *et al.*, 2013b). A kinetic model for laccase-catalyzed oxidation of phenol in an open system was developed by Kurniawati and Nicell (2009). Laccase oxidation of a substrate can occur via an electron transfer (ET) mechanism or via a hydrogen abstraction (HAT) mechanism, as shown in Figure 2-2 for acetosyringone, the mediator used in this research (Martorana *et al.*, 2013).

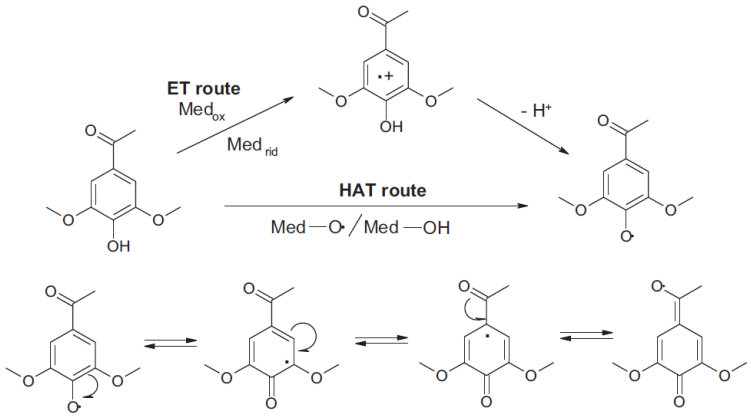


Figure 2-2: Mechanism of laccase oxidation of acetosyringone (top) and delocalization of resulting unpaired electron (bottom). Reproduced from Martorana *et al.* (2013).

Several mechanisms have been proposed for target compound oxidation by a mediator radical, with the reaction mechanism depending on the radical type. ABTS has been reported to oxidize target compounds via an electron transfer mechanism (Baiocco *et al.*, 2003; Fabbrini *et al.*, 2002). TEMPO and other *N*-oxyl type mediator radicals have been reported to oxidize target compounds via a hydrogen abstraction mechanism (Fabbrini *et al.*, 2002). Phenoxyl mediator radicals have been reported to oxidize target compounds via both electron transfer and hydrogen abstraction mechanisms. The mediator used in this research, acetosyringone, would be oxidized to a phenoxyl radical. In the study by Torres-Duarte *et al.* (2009), in which a laccase-syringaldehyde system (generating a phenoxyl radical) was used to transform halogenated pesticides at pH 4.5, transformation products were identified. They found evidence of an electron transfer mechanism resulting in dehalogenation of the pesticide, as well as a mediator-pesticide coupling mechanism. Murugesan *et al.* (2010) studied triclosan transformation by direct laccase oxidation and by a laccase-mediator system at pH 4.0. They found that the triclosan oxidation mechanism was different with and without a mediator. Without a mediator (*i.e.*, direct oxidation by laccase), dimers and trimers of triclosan were formed,

whereas ether bond cleavage followed by dechlorination was seen in the presence of redox mediators.

In general, the kinetics and mechanisms of laccases and laccase-mediator-target compound systems are difficult to assess and compare because of the heterogeneity of all system components (laccases, mediators, and target compounds), the strong influences of certain reaction conditions (*e.g.*, pH), and the resulting heterogeneity of research studies performed. Moving forward, for applications of enzymatic treatment in municipal wastewater, an enzyme that is relatively active and stable near neutral pH is essential, assuming process implementation in a treatment plant's main flow. Even with this restriction, many different laccases could be employed. However, the reactions of generated mediator radicals with target compounds can be compared among systems with different laccases. Thus, more thorough research of mediator radical and target compound reactions, specifically the influences of their respective characteristics and molecular structures on reaction kinetics and mechanisms, could be more broadly applicable.

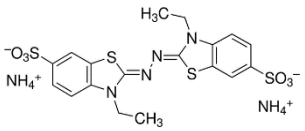
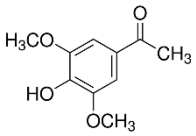
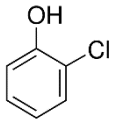
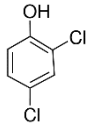
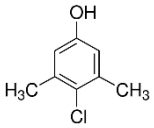
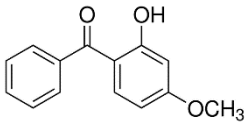
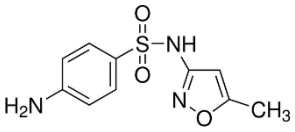
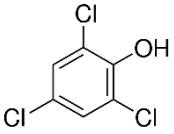
Chapter 3: Materials and Methods

The chemicals, experimental procedures, analytical methods, laboratory equipment, and other materials used throughout this research are described in this chapter.

3.1. CHEMICALS

The laccase enzyme used in this study is Novozym® 51003 (Novozymes A/S, Bagsvaerd, Denmark). This commercially available laccase from *Myceliophthora thermophila* is produced in genetically modified *Aspergillus* sp. by submerged fermentation, and two different molecular weights, 56 and 85 kDa, have been reported in the literature (Brinch & Pedersen, 2002; Lloret *et al.*, 2012; Novozym® 51003 Product Data Sheet; Wellington & Kolesnikova, 2012). The enzyme protein itself is not genetically modified. Other chemicals used in this study are listed, along with relevant data and their molecular structures, in Table 3-1.

Table 3-1: Chemicals used and relevant data

Chemical, purity	Chemical formula and CAS registry number	Molar mass (g/mol) and pK_a	Molecular structure	Supplier
ABTS diammonium salt, ≥98.0%	$C_{18}H_{24}N_6O_6S_4$ 30931-67-0	548.68 --		Sigma-Aldrich
Acetosyringone, 97%	$C_{10}H_{12}O_4$ 2478-38-8	196.20 7.8 ± 0.2		Indofine Chemical Company, Inc. and Alfa Aesar
2-Chlorophenol, analytical standard	C_6H_5OCl 95-57-8	128.56 8.3		Sigma-Aldrich (Fluka)
2,4-Dichlorophenol, analytical standard	$C_6H_4OCl_2$ 120-83-2	163.00 7.8		Sigma-Aldrich (Fluka)
Chloroxylenol, 99%	C_8H_9OCl 88-04-0	156.61 9.0		Sigma-Aldrich
Oxybenzone, 98%	$C_{14}H_{12}O_3$ 131-57-7	228.24 7.6		Sigma-Aldrich
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$ 723-46-6	253.28 5.7		Sigma-Aldrich (Fluka)
2,4,6-Trichlorophenol, analytical standard	$C_6H_3OCl_3$ 88-06-2	197.45 6.1		Sigma-Aldrich (Fluka)

3.2. EXPERIMENTAL PROCEDURES

3.2.1. Phase 1 Experiments

In Phase 1 experiments, the abatement of two representative micropollutants in municipal wastewater primary effluent was measured under various treatment conditions to evaluate the influences of key process and water quality parameters. The effectiveness of the laccase-mediator system for micropollutant degradation was compared between two different treatment configurations that could be practical from a wastewater treatment utility standpoint. The two different treatment configurations, referred to as the single oxidation reactor (SOR) treatment configuration and the free radical generator (FRG) treatment configuration, are shown in Figure 3-1. Phase 1 experiments were conducted with environmentally relevant concentrations of the micropollutants oxybenzone (10 μg/L = 43.8 nM) and sulfamethoxazole (10 μg/L = 39.5 nM) in municipal wastewater primary effluent collected from Walnut Creek and South Austin Regional Wastewater Treatment Plants in Austin, Texas, USA. In all experiments, laccase was added at time zero, starting the experiment. At selected reaction times, samples were collected for analysis of micropollutant concentration(s) and enzyme activity. Micropollutant concentrations were determined as described in Section 3.3.2. Enzyme activity samples were analyzed as described in Section 3.3.1. The pH was measured at the beginning and end of each experiment.

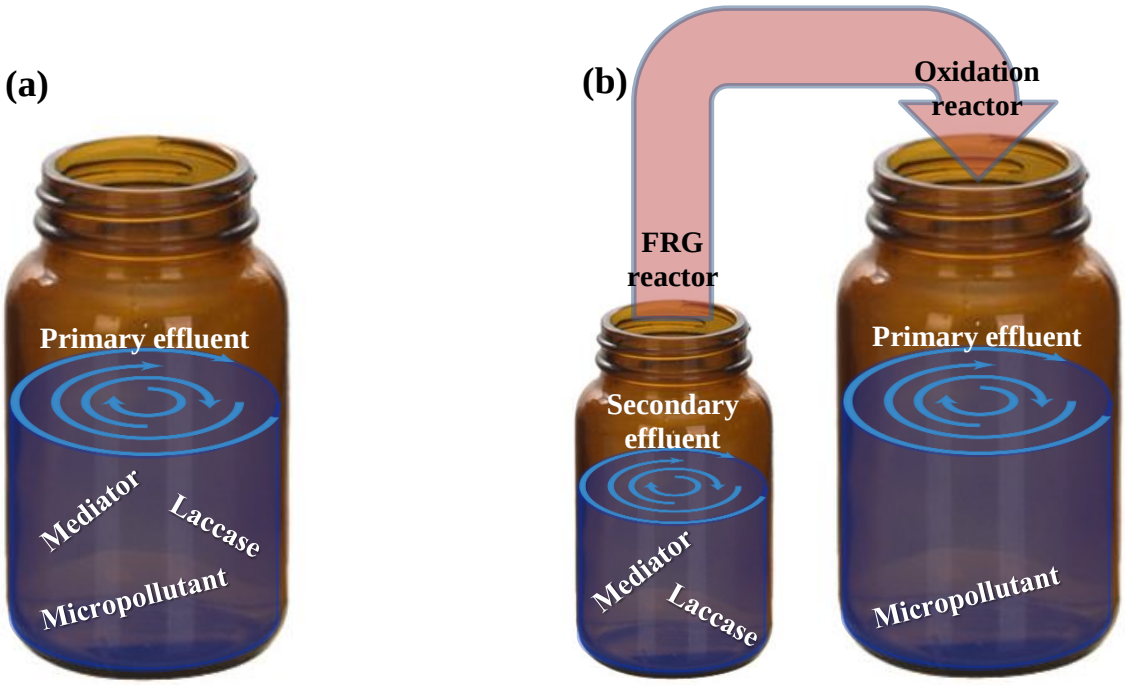


Figure 3-1: Schematic representation of (a) the single oxidation reactor (SOR) treatment configuration and (b) the free radical generator (FRG) treatment configuration

In experiments with the single oxidation reactor (SOR) treatment configuration (Figure 3-1:a), the laccase enzyme was added directly to the oxidation reactor, which contained primary effluent spiked with the mediator acetosyringone and the micropollutant oxybenzone. In experiments with the free radical generator (FRG) configuration (Figure 3-1:b), the laccase enzyme and the mediator acetosyringone first reacted in a separate, smaller "FRG reactor," and after a designated detention time, an aliquot from the FRG reactor was added to the "oxidation reactor" containing primary effluent spiked with the micropollutant oxybenzone.

In both treatment configurations, the influences of several key process and water quality parameters were investigated. For each treatment configuration, a series of experiments was conducted, varying one treatment parameter at a time. If a set of experimental conditions resulted in oxybenzone abatement greater than 90% in the two

hours of reaction time, those conditions were used in the next set of experiments varying a different treatment parameter. In cases where equivalent oxybenzone abatement occurred for more than one value of a given parameter, the more economically favorable value was selected. The treatment parameters tested for the SOR treatment configuration included the initial pH of the reaction mixture (adjusted with hydrochloric acid), the initial enzyme activity, and the initial mediator concentration. The treatment parameters tested for the FRG treatment configuration included the initial pH of the FRG reactor, the initial enzyme activity, the initial mediator concentration, and the detention time of the FRG reactor.

The treatment conditions (*i.e.*, pH and laccase/acetosyringone ratio in the FRG reactor) in the first FRG experiments were based on successful conditions from the SOR experiments. The reaction mixture in the FRG reactor was prepared in 0.1 M sodium phosphate buffer in some experiments, and then in secondary effluent from both wastewater treatment plants in later experiments. In FRG experiments, the pH of the oxidation reactor was not adjusted. The aliquot from the FRG reactor that was added to the oxidation reactor was always 5% of the oxidation reactor volume. FRG experiments were conducted at two different scales, with a reaction mixture volume of either 100 mL or 1 L in the oxidation reactor. Therefore, 5 and 50 mL of the FRG reaction mixture were delivered to the oxidation reactor, respectively.

3.2.2. Phase 2 Experiments

Based on the results of Phase 1, experimental conditions were selected to test the effectiveness of enzymatic treatment under the more realistic setting of a continuous flow reactor. A bench-scale continuous flow reactor with six baffled chambers was constructed of glass fiber (Polyfibre, Birmingham, United Kingdom). Drawings of the

reactor are shown in Figure 3-2 and Figure 3-3. The baffled reactor design was intended to encourage plug flow, and the hydraulic flow through the reactor was characterized by a tracer test. Initially, a pulse input tracer test was performed, but it was determined that a pulse input was not appropriate because the injection of the pulse itself changed the flow pattern in the reactor. During the few seconds of pulse injection of red food coloring (the tracer), the flow rate entering the reactor increased significantly, disrupting the steady-state flow that had been established. Therefore, a step input tracer test was performed using the experimental setup shown in Figure 3-4. A peristaltic pump fed distilled water from a large reservoir into a small reservoir, which overflowed back into the large reservoir. This small reservoir provided a constant head for a second peristaltic pump, which pumped from the small reservoir to the reactor. The flow entered the reactor via tubing that was secured to the wall of the reactor with the opening pointed downward at a depth of half the water depth. Just before the tubing entered the reactor, there was a T-connection with a septum, which allowed for the injection of the tracer into the main flow via the needle of a syringe on a syringe pump. Water was pumped through the reactor at approximately 40 mL/min for at least two hours (approximately one detention time) before time zero to establish steady-state flow. At time zero, the syringe pump was turned on and started injecting tracer at 100 μL/min, a negligible flow rate compared to the main flow. Samples were collected from the reactor effluent just before time zero, at 3 minute intervals for the first 90 minutes, at 5 minute intervals from 90 to 180 minutes, at 10 minute intervals from 180 to 240 minutes, and at 15 minute intervals from 240 to 375 minutes, at which point it was determined that the effluent tracer concentration was essentially the same as the influent concentration. Samples were analyzed for tracer (red food coloring) concentration using an ultraviolet-visible spectrophotometer (Agilent

Technologies, Waldbronn, Germany). The absorbance was measured at a wavelength of 500 nm, which is the wavelength with the maximum molar absorptivity for the red tracer.

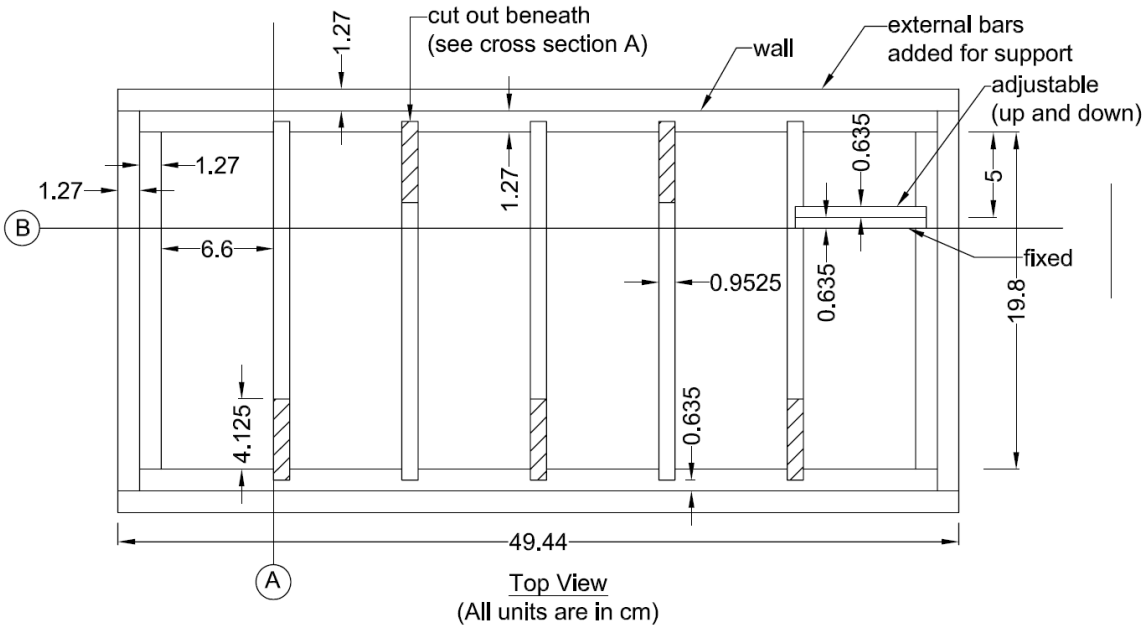


Figure 3-2: Plan view of the continuous flow reactor

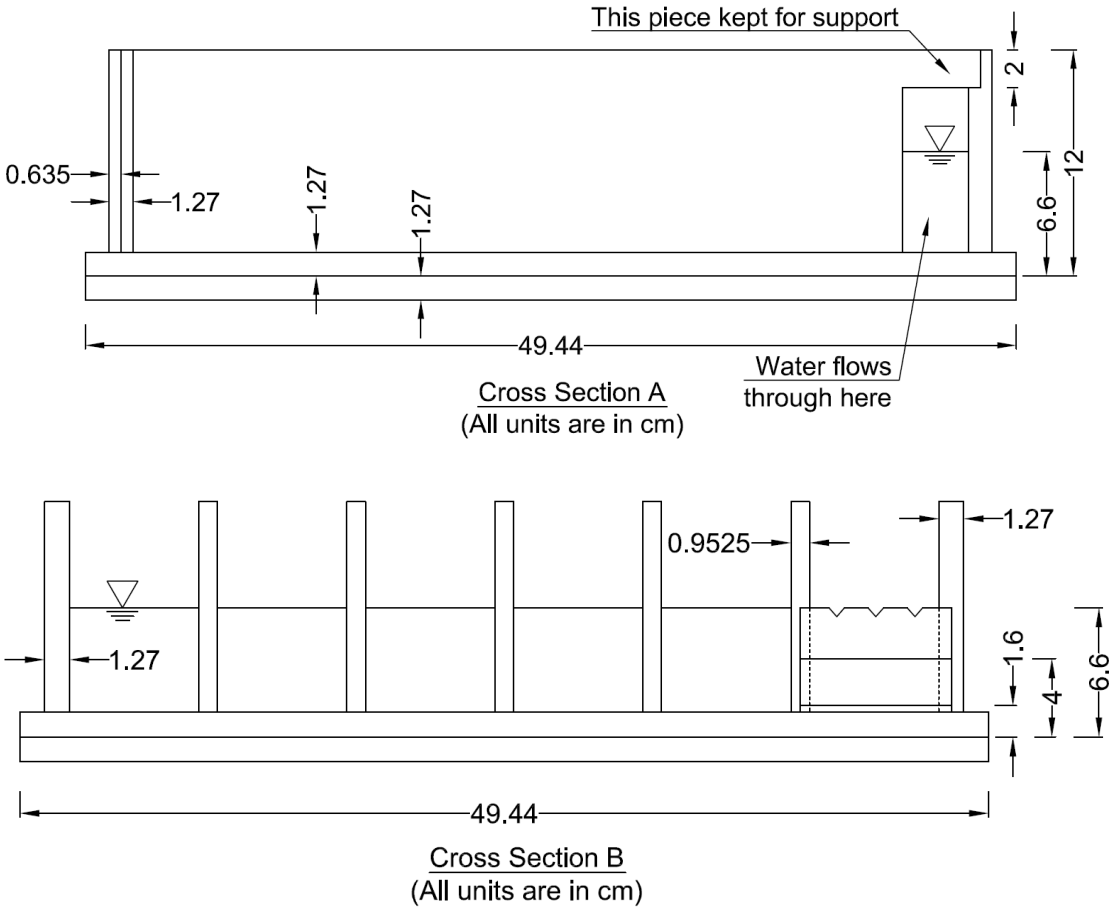


Figure 3-3: Vertical cross sections of the continuous flow reactor

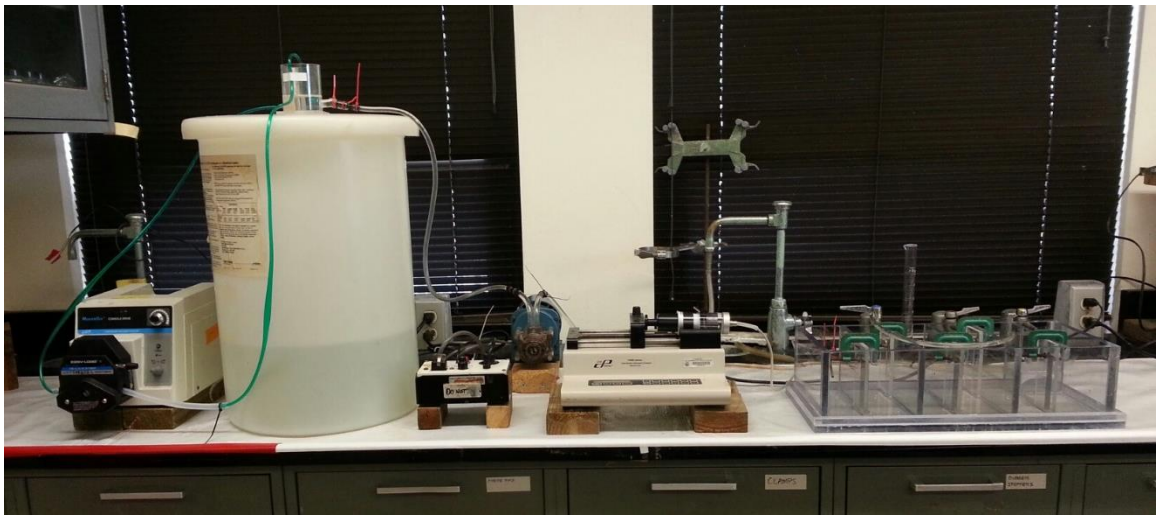


Figure 3-4: Continuous flow experimental setup

Continuous flow experiments for the enzymatic treatment of primary effluent containing micropollutants were conducted with the same experimental setup as the tracer test. Instead of distilled water, the large reservoir contained primary effluent spiked with 10 µg/L oxybenzone, 10 µg/L sulfamethoxazole, and 20.63 mg/L acetosyringone to yield an acetosyringone/oxybenzone molar ratio of 2,400. Instead of red dye, the syringe pump contained laccase diluted in distilled water to an activity that would yield a steady-state enzyme activity of 0.5 U/mL in the reactor. Prior to time zero, primary effluent was pumped through the reactor at approximately 40 mL/min for two hours (approximately one detention time) to establish steady-state flow. At time zero, the syringe pump was turned on and started delivering laccase at 100 µL/min, a negligible flow rate compared to the main flow. Samples were collected from the reactor effluent just before time zero, and then at 50 minute intervals. At each sampling time, the flow rate and pH were measured, and samples were collected for analysis of micropollutant concentrations and enzyme activity.

3.2.3. Phase 3 and 4 Experiments

Phase 3 and 4 experiments investigated the kinetics of the laccase-mediator-target compound reactions in a pure buffered system. Phase 3 experiments were performed with one model target compound (2-chlorophenol). In Phase 4 experiments, the reaction rates of enzymatic treatment of a set of structurally related model compounds (chlorophenols) and a micropollutant (chloroxylenol) were measured and compared to examine the effect of target structure on the reaction kinetics. To allow for comparison of the reaction rates of different target compounds, Phase 4 experiments were conducted with multiple target compounds simultaneously. Phase 3 and 4 experiments were performed with the same experimental setup and with very similar methods. Phase 3 experiments were conducted in 150 mL glass beakers containing 122 mL of reaction mixture. Phase 4 experiments were conducted in 120 mL glass beakers containing 99 mL of reaction mixture. A sample dispenser (Brand Dispensette®, Wertheim, Germany) and an air diffuser were placed in the reaction mixture, and the reactor was placed on a stir plate. Prior to each experiment, two separate solutions of equivalent volume (62 mL in Phase 3 and 50 mL in Phase 4) were prepared. Concentrated stock solutions of phosphate buffer, mediator, and target compound(s) were diluted in Milli-Q water to make the first solution, which contained twice the desired initial concentrations of mediator (0 – 300 μM) and target compound (0 – 900 μM) in 5 mM phosphate buffer. Concentrated stock solutions of phosphate buffer and laccase were diluted in Milli-Q water to make the second solution, which contained twice the desired initial laccase activity (0.1 – 1 U/mL) in 5 mM phosphate buffer. All stock solutions were prepared in Milli-Q water. The phosphate buffer stock solution was prepared with NaH_2PO_4 and Na_2HPO_4 salts. Prior to starting an experiment, samples of each solution were taken, and each solution's pH was measured and adjusted, if necessary. Phase 3 experiments were all conducted at pH 7.

Phase 4 experiments were conducted at pH 5, 7, and 9.3. The two solutions, now 61 mL each in Phase 3 and 49.5 mL each in Phase 4, were mixed at time zero, starting the experiment. After allowing the solutions to mix for 20 to 45 seconds, the sample dispenser was pumped several times to fill it with the reaction mixture. At designated reaction times, 1 mL samples were dispensed directly into amber HPLC vials containing 25 μL of 5 N HCl, which would decrease the pH of the sample to below 2, stopping the enzymatic reaction. These samples were analyzed for mediator and target compound concentrations by HPLC, as described in Section 3.3.3. Samples for enzyme activity measurements were also taken at designated reaction times. These samples were diluted in Milli-Q water to activities of approximately 0.01 U/mL and immediately stored in the refrigerator at 4°C for later analysis, as described in Section 3.3.1. Throughout an experiment, the reaction mixture was aerated with humidified air and stirred at 500 rpm. At the end of an experiment, the pH of the reaction mixture was measured. The samples for HPLC analysis were stored at 4°C for up to 24 hours prior to launching the HPLC, and the samples for enzyme activity analysis were stored at 4°C for up to one week.

Some of the earlier experiments were conducted without aeration and with measurement of dissolved oxygen concentration using a dissolved oxygen probe (Mettler-Toledo, Greifensee, Switzerland). Those experiments were only stirred for the first 45 seconds to allow the two prepared solutions to mix. The dissolved oxygen meter recorded the dissolved oxygen concentration every minute.

3.3. MEASUREMENTS AND SAMPLE ANALYSES

The following parameters were measured during all or some experiments: pH, dissolved oxygen (DO) concentration, enzyme activity, mediator concentration, and target compound concentration. DO concentration was measured by a Mettler-Toledo

DO meter. The methods for quantification of enzyme activity, mediator concentration, and target compound concentration are described below.

3.3.1. Enzyme Activity Assay

Enzyme activity was quantified by a colorimetric assay similar to the methods of Auriol *et al.* (2007) and Garcia *et al.* (2011). This assay measures the rate at which the enzyme catalyzes the oxidation of a specific substrate, ABTS, to ABTS$\bullet^{+}$, which absorbs light at 420 nm with an extinction coefficient of 3.6×10^{4} $M^{-1}cm^{-1}$. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of ABTS per minute. During Phases 1 and 2, the assay mixture (250 μL), which contained 25 μL of an enzyme solution and 5 mM ABTS in 0.1 M sodium acetate buffer at pH 5, was placed in a 300 μL well of a microplate. A microplate reader (BioTek, Winooski, VT, USA) was then used to read the absorbance every 1.5 minutes for 15 minutes, from which the oxidation rate could be calculated. The microplate reader incubated the microplate at 37°C, and shook it before each reading. Each enzyme activity measurement was performed in triplicate with three wells, each containing 250 μL of assay mixture. The enzyme solutions were diluted to obtain activities of approximately 1×10^{-3} U/mL in assay mixtures.

During Phase 3, the enzyme activity assay was performed using a different spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The assay mixture (3 mL), which contained 300 μL of an enzyme solution and 5 mM ABTS in 0.1 M sodium acetate buffer at pH 5, was placed in a quartz cuvette with a path length of 1 cm. The spectrophotometer read the absorbance every 30 seconds for 5 minutes at room temperature. The enzyme solutions were diluted to obtain activities of approximately 1×10^{-3} U/mL in assay mixtures.

During Phase 4, the enzyme activity assay was performed using a different microplate reader (BioTek, Winooski, VT, USA). Each enzyme activity measurement was performed in triplicate with three 300 µL wells, each containing 250 µL of assay mixture, which consisted of 25 µL of an enzyme solution and 5 mM ABTS in 0.1 M sodium acetate buffer at pH 5. The absorbance was read every 30 seconds for 5 minutes at room temperature. The enzyme solutions were diluted to obtain activities of approximately 1×10^{-3} U/mL in assay mixtures.

3.3.2. Phase 1 and 2 Micropollutant Concentrations: LC/MS/MS

Environmentally relevant micropollutant concentrations were determined by liquid chromatography / tandem mass spectrometry (LC/MS/MS), preceded by solid phase extraction (SPE) of the analytes in acidified samples with 3-cm^3, 60-mg Oasis HLB SPE cartridges (Waters, Milford, MA, USA). SPE was used for sample purification and concentration (by a factor of eight). Oxybenzone and sulfamethoxazole were isolated using a Shimadzu 150×4.6 mm Premier C18 column (particle diameter = 5 µm, pore diameter = 120 Å; Kyoto, Japan) and a binary gradient of LC/MS grade methanol and water. The instrument configuration included a Finnigan Surveyor autosampler, a Finnigan Surveyor mass spectrometer pump, and a TSQuantum mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA). Electrospray ionization in the positive mode was used.

3.3.3. Phase 3 and 4 Mediator and Target Compound Concentrations: HPLC/UV

Mediator and target compound concentrations during kinetic experiments were determined by high performance liquid chromatography / ultraviolet spectrophotometry (HPLC/UV). A Macherey-Nagel 125×3 mm Nucleosil® C18 column (particle diameter = 5 µm, pore diameter = 100 Å; Düren, Germany) provided the stationary phase, and the

mobile phase consisted of 10 mM phosphate buffer at pH 2.3 and HPLC grade methanol. The phosphate buffer was prepared by diluting 674 μL of phosphoric acid in 1 L of Milli-Q water. The instrument configuration included an UltiMate 3000 autosampler, an UltiMate 3000 pump, and an UltiMate 3000 diode array detector (Dionex, Thermo Scientific, Sunnyvale, CA, USA).

Chapter 4: Phase 1 Results – Evaluation of Enzymatic Treatment Conditions in Batch Experiments

4.1. INTRODUCTION

The principle objectives of Phase 1 of this research were to demonstrate that enzymatic treatment can transform micropollutants in municipal wastewater under realistic treatment conditions and to evaluate the influences of key process parameters in two different treatment configurations. The abatement of two representative micropollutants, oxybenzone and sulfamethoxazole, in municipal wastewater primary effluent was measured under varying conditions of pH, enzyme activity, mediator concentration, and wastewater characteristics. Two possible configurations for the enzymatic treatment process, shown in Figure 3-1, were considered.

Experiments in this research phase were conducted in amber glass batch reactors to simulate ideal plug flow conditions, with reaction times in the batch reactors representing detention times in an ideal plug flow reactor. The single oxidation reactor (SOR) treatment configuration (Figure 3-1:a), consisted of a single reactor containing a well-mixed reaction solution of all reactants (the laccase enzyme, the mediator acetosyringone, and the target micropollutant(s)) in a background matrix of unfiltered primary effluent. The free radical generator (FRG) treatment configuration (Figure 3-1:b) consisted of two reactors, the FRG reactor and the oxidation reactor. The FRG reactor was smaller and contained a cleaner background matrix of phosphate buffer or secondary effluent. The laccase enzyme and the mediator acetosyringone were added to the FRG reactor, and after a designated detention time, a small aliquot from the FRG reactor was transferred to the oxidation reactor containing primary effluent spiked with the target micropollutant oxybenzone.

As making certain changes, such as drastically altering the pH of the entire flow through a wastewater treatment plant would not be realistic, the FRG treatment configuration was considered since it might allow more freedom in optimizing reactor conditions for the laccase-mediator reaction. Additionally, this separate reactor would allow the enzyme and mediator to react in a cleaner background matrix prior to coming in contact with primary effluent. It was hypothesized that the addition of the FRG reactor to the treatment configuration would allow for more efficient radical production due to the more optimal reactor conditions and cleaner background matrix, and that the radicals were stable enough for a significant portion of them to still be reactive upon introduction to the oxidation reactor, thus providing increased micropollutant abatement. It should be noted that in these experiments, the enzyme was not immobilized or retained in the FRG reactor. Therefore, laccase and potentially unreacted acetosyringone were also transferred to the oxidation reactor.

4.2. SINGLE OXIDATION REACTOR (SOR) TREATMENT CONFIGURATION

Experiments with the single oxidation reactor (SOR) treatment configuration were designed to assess the effects of pH, enzyme activity, mediator concentration, and wastewater characteristics on enzymatic treatment of the target micropollutant oxybenzone. Sets of experiments were performed varying one of the four treatment parameters at a time to isolate the influence of each parameter. Experimental conditions tested and their associated relative residual oxybenzone concentrations after two hours of treatment are shown in Table 4-1.

Table 4-1: Experimental conditions and resulting relative residual oxybenzone concentrations after two hours of treatment with the single oxidation reactor (SOR) treatment configuration

Experiment #	Primary effluent source	Reactor volume (L)	Initial pH	Initial enzyme activity (U/mL)	Initial [aceto-syringone] (μM)	Relative residual [oxybenzone] (%)
SOR-1	WC	0.1	5, 6, 7	1	87.6	21, 9, 13
SOR-2	WC	1	6, 6.5, 7	1	87.6	10, 20, 15
SOR-3	WC (aerated), WC	1	6.5	1	87.6	24, 15
SOR-4	WC	1	7	0.1	26.3, 87.6	79, 23
SOR-5	WC	1	7	1	26.3, 87.6	71, 8
SOR-6	WC	1	7	0.3, 1	87.6	28, 19
SOR-7	SAR	0.1	7	1	87.6, 105	34, 20
SOR-8	SAR	0.1	7, unadjusted	1	105	20, 24
SOR-9	SAR	1	unadjusted	0.5	105	38

Note: Experiments were conducted in primary effluent from Walnut Creek (WC; lower organic content) and South Austin Regional (SAR; higher organic content) wastewater treatment plants with an initial oxybenzone concentration of 10 μg/L = 43.8 nM.

4.2.1. Influence of pH

Experiments in our laboratory preceding this study were performed with a laccase from the Basidiomycota white rot fungus *Trametes versicolor*. That particular laccase exhibits its highest activity under acidic conditions (pH 4 to 6) and little to no activity at neutral or alkaline pH (Margot *et al.*, 2013a). Therefore, those earlier experiments were

conducted in filtered primary effluent adjusted to an initial pH of 6, which is not representative of most wastewaters. In part to address this pH issue, the laccase source was changed for this study to a commercially available laccase produced by Novozymes, which comes from the Ascomycota fungus *Myceliophthora thermophila*. This laccase retains its activity over a wide pH range (pH 4 to 9) and is quite stable at pH 7 (Babot *et al.*, 2011; Lloret *et al.*, 2012). As shown in Figure 4-1, the Novozymes laccase showed significant removal of oxybenzone at neutral pH, as well as at the alkaline pH of the primary effluent used in the experiment.

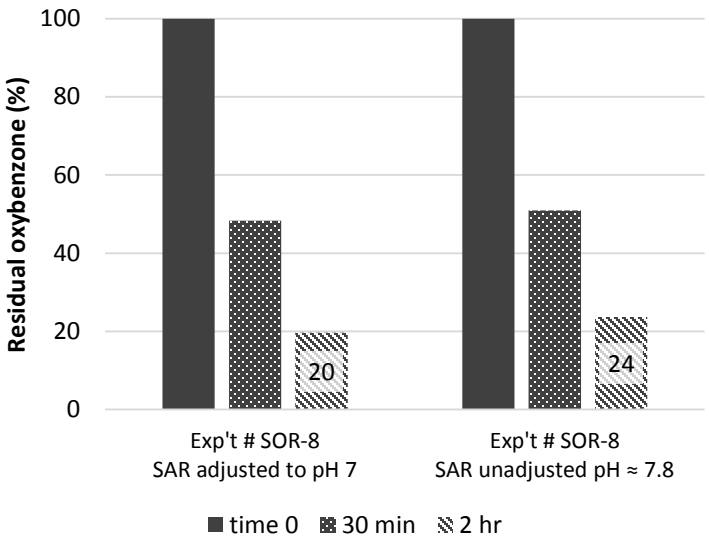


Figure 4-1: Relative residual oxybenzone concentrations after 0, 0.5, and 2 hours of treatment

Note: Experiments were conducted in primary effluent from South Austin Regional (SAR) wastewater treatment plant with the following initial conditions: pH 7 or ≈ 7.8, 1 U/mL enzyme activity, 105 µM acetosyringone, 10 µg/L = 43.8 nM oxybenzone.

When the pH of the reaction solution was not buffered, which was the case in all Phase 1 experiments, significant increases in pH (sometimes more than one pH unit) over the course of experiments were often observed. These pH rises were most likely due to

Austin's high alkalinity wastewater equilibrating with the atmosphere, as reaction mixture volumes were small and mixed. Similar changes in pH were observed in control experiments without the laccase enzyme, but such drastic increases in pH would not be expected to occur in most wastewaters.

To examine the impact of pH on enzymatic treatment of the representative target micropollutant oxybenzone, Experiments SOR-1 and SOR-2 consisted of a total of six reactors with four different initial pH values ranging from 5 to 7. These pH values rose as high as 8.31 over the course of these experiments. Therefore, these experiments were considered to cover typical pH values of municipal wastewater. As is evident in Figure 4-2, within this pH range, there was no substantial difference in oxybenzone abatement after two hours of treatment due to the initial pH of the reaction solution. This was a promising result, indicating that enzymatic treatment with this Novozymes laccase might be applicable in wastewaters with differing pH values within a normal range of pH 6 to 8. However, an effect of initial pH on residual enzyme activity at the end of the experiments was observed. After two hours of treatment, more of the initial enzyme activity was retained in the reactors with an initial pH of 7 (residual enzyme activity = 61%) than in the reactors with initial pH values of 5 (residual enzyme activity = 43%) and 6 (residual enzyme activity = 48%). This finding is consistent with reports that this particular laccase enzyme is very stable at pH 7 and unstable at acidic pH, despite higher activities (Lloret *et al.*, 2012). Using this same laccase for direct oxidation of estrogens, Lloret *et al.* (2012) found that degradation was higher at pH 7 than at pH 4.

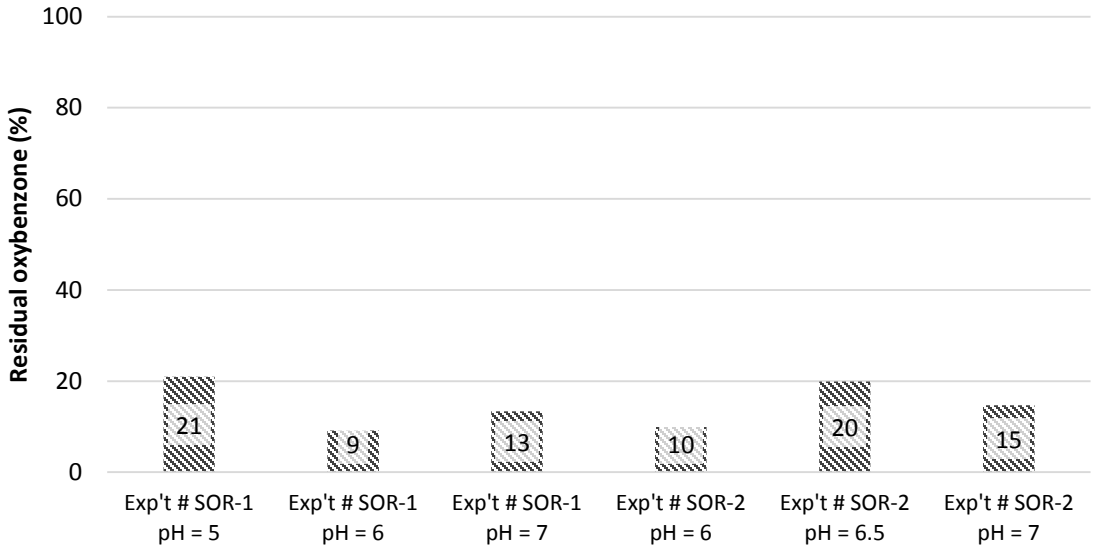


Figure 4-2: Relative residual oxybenzone concentrations after 2 hours of treatment

Note: Experiments were conducted in primary effluent from Walnut Creek (WC) wastewater treatment plant with the following initial conditions: 1 U/mL enzyme activity, 87.6 μM acetosyringone, 10 μg/L = 43.8 nM oxybenzone.

4.2.2. Influence of enzyme activity

Normally, it would be expected for an increase in enzyme activity to result in an increased rate of radical production, which should lead to an increased rate of target transformation if radical production is the rate limiting step in this system. Although the rate of target transformation was not followed over time in these experiments, an increased rate of target transformation would be expected to lead to a lower target residual after a given reaction time. As can be seen in Figure 4-3, for the range of initial enzyme activities tested in this set of experiments, there was not a substantial increase in oxybenzone abatement efficiency with increasing initial enzyme activity when other experimental conditions were the same (*i.e.*, at the same initial mediator concentration). It is possible that the initial rates of oxybenzone transformation were different, but were not captured in these experiments, and that not all of the oxybenzone was oxidized for

other reasons. It is also possible that under these conditions, radical production (controlled by enzyme activity) was not the rate limiting step.

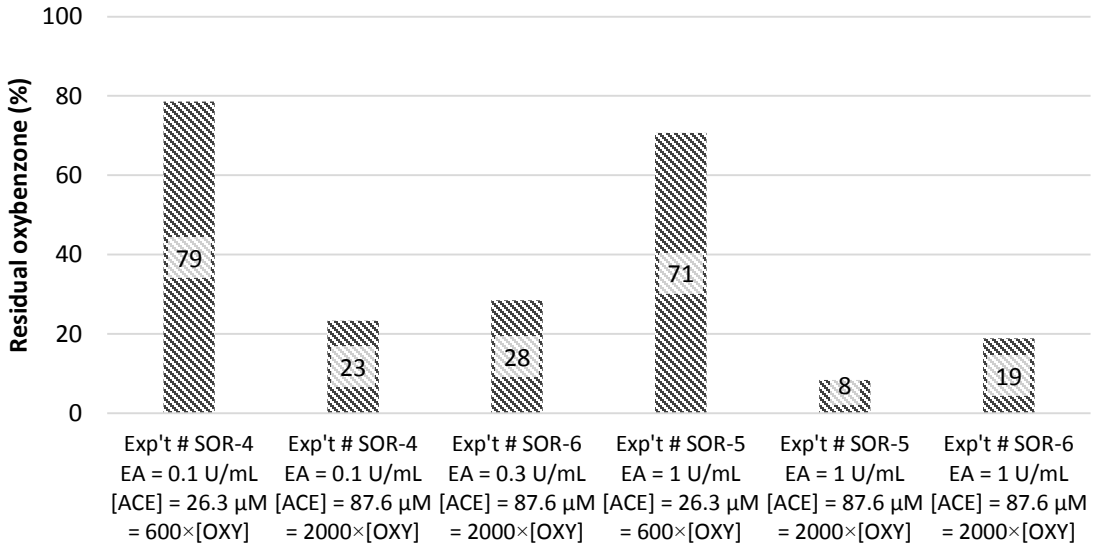


Figure 4-3: Relative residual oxybenzone concentrations after 2 hours of treatment

Note: Experiments were conducted in primary effluent from Walnut Creek (WC) wastewater treatment plant with the following initial conditions: pH 7, 26.3 or 87.6 µM acetosyringone, 10 µg/L = 43.8 nM oxybenzone.

A slightly larger impact on oxybenzone abatement after two hours of treatment was observed in primary effluent from a different wastewater treatment plant in Experiments SOR-8 (initial enzyme activity = 1 U/mL; residual oxybenzone = 24%) and SOR-9 (initial enzyme activity = 0.5 U/mL; residual oxybenzone = 38%). The slightly greater influence of enzyme activity in these experiments could be related to the higher organic content of the primary effluent.

4.2.3. Influence of mediator concentration

Under the conditions tested, initial mediator concentration proved to be a very influential parameter on oxybenzone transformation. As can be seen in Figure 4-3, the

treatment efficiency of oxybenzone was significantly higher with an initial acetosyringone concentration of 87.6 μM (initial enzyme activity = 0.1 U/mL; residual oxybenzone = 23%) as compared to 26.3 μM (initial enzyme activity = 0.1 U/mL; residual oxybenzone = 79%). The fact that initial mediator concentration was much more influential than initial enzyme activity indicates that the total quantity of radicals produced was more important than the rate at which they were produced, potentially because of radical scavenging by the wastewater matrix.

4.2.4. Influence of wastewater characteristics

To investigate the effect of wastewater composition on treatment efficiency, Experiments SOR-6 and SOR-7 were conducted in primary effluent collected from two different wastewater treatment plants in Austin, Texas. Walnut Creek (WC) and South Austin Regional (SAR) wastewater treatment plants had significantly different wastewaters in terms of biological oxygen demand (BOD) and total suspended solids (TSS), as shown in Table 4-2. Overall, the WC wastewater was less concentrated than the SAR wastewater, containing lower levels of BOD and TSS.

Table 4-2: Ten-year average (2003-2013) biological oxygen demand (BOD) and total suspended solids (TSS) of the primary effluent of Walnut Creek (WC) and South Austin Regional (SAR) wastewater treatment plants

Wastewater treatment plant	BOD (mg/L)	TSS (mg/L)
WC	101	66
SAR	139	117

As shown in Figure 4-4, higher mediator concentrations were required in SAR wastewater than in WC wastewater to achieve equivalent oxybenzone abatement, presumably because of the higher concentration of organic matter in the SAR wastewater.

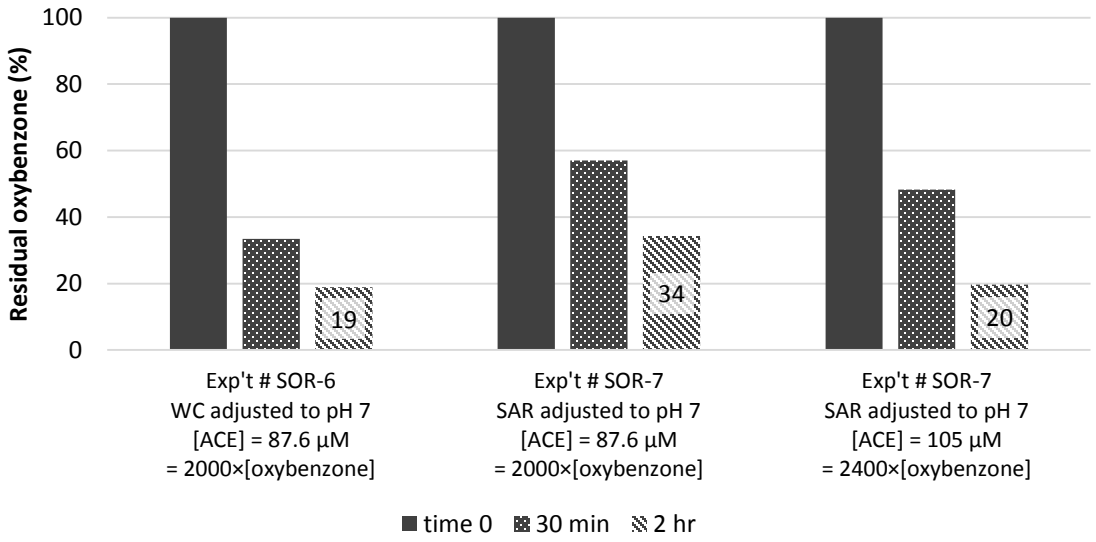


Figure 4-4: Relative residual oxybenzone concentrations after 0, 0.5, and 2 hours of treatment

Note: Experiments were conducted in primary effluent from Walnut Creek (WC; lower organic content) and South Austin Regional (SAR; higher organic content) wastewater treatment plants with the following initial conditions: pH 7, 1 U/mL enzyme activity, 87.6 or 105 µM acetosyringone, 10 µg/L = 43.8 nM oxybenzone.

4.2.5. Simultaneous enzymatic treatment of oxybenzone and sulfamethoxazole

Another key finding in the results from Phase 1 was that the laccase-mediator system is capable of treating a mixture of micropollutants in unfiltered primary effluent. As shown in Figure 4-5, although sulfamethoxazole transformation (≈65%) was not as substantial as oxybenzone transformation (≈90%), the transformation achieved during individual treatments was maintained when the two micropollutants were treated together.

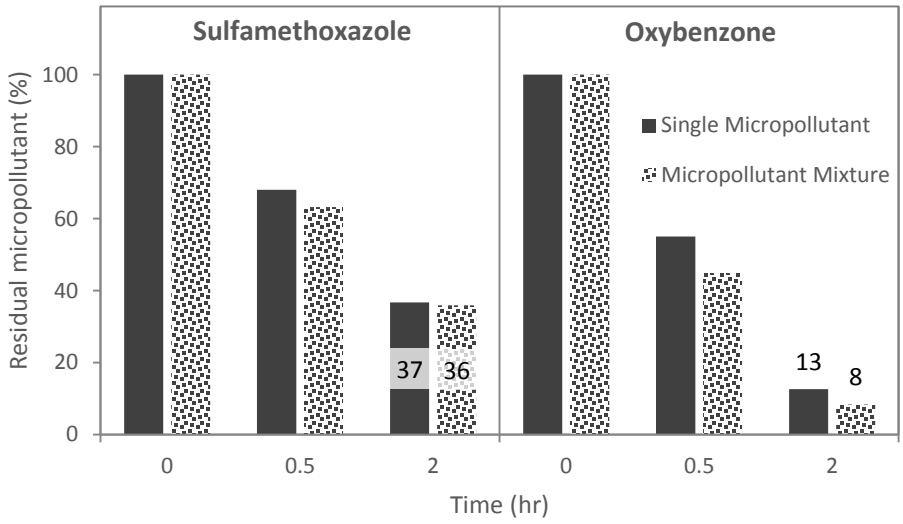


Figure 4-5: Relative residual micropollutant concentrations after 0, 0.5, and 2 hours of treatment

Note: Experiments were conducted in primary effluent from South Austin Regional (SAR) wastewater treatment plant with the following initial conditions: pH ≈ 7.8, 0.5 U/mL enzyme activity, 105 µM acetosyringone, 10 µg/L micropollutant = 43.8 nM oxybenzone = 39.5 nM sulfamethoxazole.

The difference in treatment efficiency for the two representative micropollutants highlights the fact that different micropollutants will not necessarily be treated at the same rate by the laccase-mediator system. As enzymatic treatment is a treatment process meant to transform many different compounds, in order to assess its effectiveness, it is essential to know how long different compounds will need to be treated to meet treatment objectives. These results were part of the motivation for Phases 3 and 4 of this research, which investigated the kinetics of enzymatic treatment and the influences of certain target properties on oxidation rates.

4.3. FREE RADICAL GENERATOR (FRG) TREATMENT CONFIGURATION

Experiments with the free radical generator (FRG) treatment configuration were designed to assess the effects of pH, enzyme activity, mediator concentration, background matrix, and detention time of the FRG reactor on oxybenzone transformation in the oxidation reactor. The oxidation reactor conditions remained constant, consisting of the target micropollutant oxybenzone spiked in otherwise unaltered primary effluent. Sets of experiments were performed varying one of the treatment parameters at a time to isolate the influence of each parameter. Experimental conditions tested and their associated relative residual oxybenzone concentrations after two hours of treatment are shown in Table 4-3.

Table 4-3: Experimental conditions and resulting relative residual oxybenzone concentrations after two hours of treatment with the free radical generator (FRG) treatment configuration

Experiment #	FRG background matrix	Oxidation reactor volume (L)	FRG detention time (min)	FRG initial pH	Initial enzyme activity (U/mL)	Initial [acetosyringone] (μM)	Relative residual [oxybenzone] (%)
FRG-1	Buffer	0.1	2.5	6	10	700, 1050, 1400, 1750	46, 25, 13, 9
FRG-2	Buffer	0.1	0.5, 2, 3.5, 5	6	2.5	1400	14, 13, 18, 16
FRG-3	Buffer	0.1	0.5, 2, 3.5, 5	6	5	1400	13, 12, 11, 13
FRG-4	Buffer	0.1	0.5, 1.5, 2.5, 3.5	6	10	1400	7, 9, 9, 9
FRG-5	Buffer	0.1	2	5, 6, 7	5	1400	14, 14, 17
FRG-6	Buffer	0.1, 1	2	6	5	1400	14, 9
FRG-7	WC 2°	0.1	2	6	5	1400	32
FRG-8	WC 2°	0.1	0.5, 2, 3.5, 5	6	10	2100	5, 6, 6, 10
FRG-9	SAR 2°	1	2	6	10	2100	37

Note: The FRG reactor contained the laccase enzyme and the mediator acetosyringone at the initial conditions listed in the table in phosphate buffer or secondary effluent from either Walnut Creek (WC; lower organic content) or South Austin Regional (SAR; higher organic content) wastewater treatment plant. The oxidation reactor contained an initial oxybenzone concentration of 10 μg/L = 43.8 nM in primary effluent from WC wastewater treatment plant, except for in Experiment FRG-9, in which it contained primary effluent from SAR wastewater treatment plant.

As was the case with the SOR treatment configuration, the most influential treatment parameters with the FRG treatment configuration were mediator concentration and background matrix. Within the ranges of values tested, pH, enzyme activity, and detention time of the FRG reactor did not have a large influence on oxybenzone transformation in the oxidation reactor. A clear increase in oxybenzone transformation was observed with increasing initial mediator concentration, as shown in Figure 4-6.

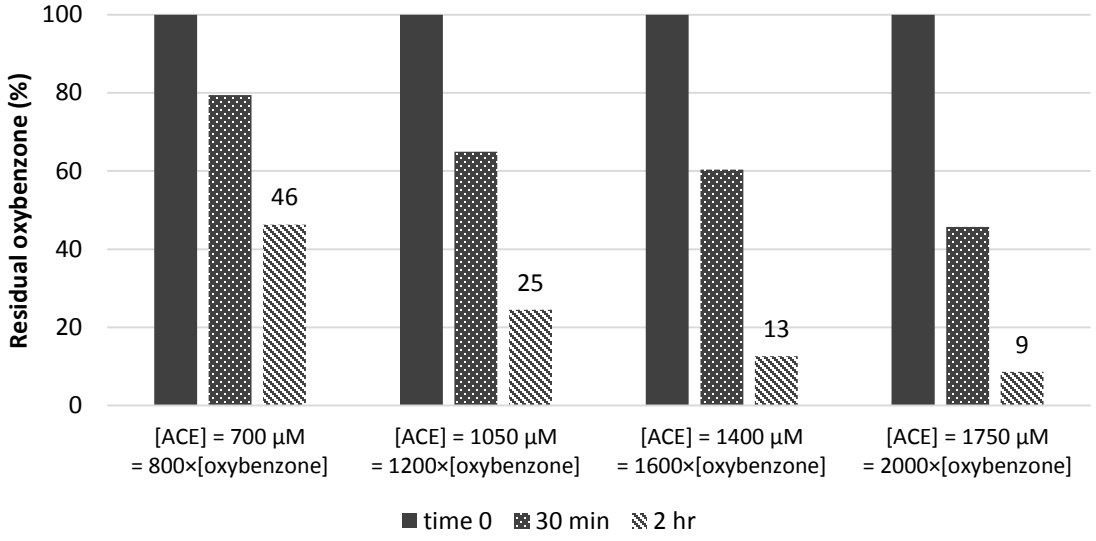


Figure 4-6: Relative residual oxybenzone concentrations after 0, 0.5, and 2 hours of treatment

Note: Experiments were conducted using the free radical generator (FRG) treatment configuration. The FRG reactor contained phosphate buffer at pH 6 with an initial enzyme activity of 10 U/mL. The oxidation reactor contained WC primary effluent with the following initial conditions: pH 7.74, 10 µg/L = 43.8 nM oxybenzone.

Treatment efficiency also clearly improved with decreasing organic content of the background matrix in the FRG reactor. Experiments FRG-6 and FRG-7 were performed under the same conditions except for the background matrix of the FRG reactor. With a FRG reactor containing phosphate buffer at pH 6 (FRG-6), the residual oxybenzone was

14% after two hours of treatment. With a FRG reactor containing WC secondary effluent adjusted to an initial pH of 6 (FRG-7), the residual oxybenzone was 32% after two hours of treatment. Similarly, Experiments FRG-8 and FRG-9 were performed under the same conditions except for the background matrices of both reactors. With a FRG reactor containing the lower organic content WC secondary effluent and an oxidation reactor containing WC primary effluent (FRG-8), the residual oxybenzone was 6% after two hours of treatment. With a FRG reactor containing the higher organic content SAR secondary effluent and an oxidation reactor containing SAR primary effluent (FRG-9), the residual oxybenzone was 37% after two hours of treatment.

In general, the FRG treatment configuration did provide higher treatment efficiencies than the SOR treatment configuration. However, when comparing the two treatment configurations on the basis of chemical usage (*i.e.*, enzyme and mediator used per volume of treated primary effluent), the improvement in treatment efficiency was not very impressive. The added complication and cost of operating a second reactor and pumping secondary effluent back to the FRG reactor in a real system would have to be weighed against the marginal improvement in treatment efficiency. Since the enzyme was not immobilized or retained in the FRG reactor, laccase and potentially unreacted acetosyringone were also transferred to the oxidation reactor. Therefore, an important remaining question is whether free radicals generated in the FRG reactor were actually transferred to the oxidation reactor, or if the oxybenzone transformation was due to the transfer of enzyme and unreacted acetosyringone. Further experiments should be performed in which the enzyme is not transferred to the oxidation reactor to answer this question.

4.4. CONCLUSIONS

The results from this first study phase demonstrate that enzymatic treatment can transform micropollutants in municipal wastewater primary effluent under realistic treatment conditions. However, high mediator concentrations were required to achieve significant micropollutant transformation. Presumably, a large portion of the mediator radicals generated are scavenged by the matrix, but a better understanding of the kinetics and mechanisms of the reactions involved is essential for further development and improvement of the enzymatic treatment process. These results were a first step towards finding a way to implement enzymatic treatment in a wastewater treatment plant prior to biological treatment. However, these experiments were performed in batch reactors, which represent ideal plug flow conditions, whereas a real application of this treatment process would occur in a continuous flow reactor with non-ideal plug flow conditions. Therefore, the subsequent research phase looked at the efficacy of enzymatic treatment in a continuous flow, non-ideal plug flow reactor. Furthermore, these results also demonstrate the need for a deeper understanding of the kinetics of enzymatic treatment and how the kinetics, and therefore the efficiency of the treatment process, will change for various compounds. These points were considered in the development of Phases 3 and 4 of this research.

Note: The experimental work presented in this chapter was performed jointly by Catherine M. Hoffman and Margaret E. Sharkey. These results are also discussed in the Master's thesis of Margaret E. Sharkey, entitled "Enzymatic Treatment of Pharmaceuticals and Personal Care Products (PPCPs) in Municipal Wastewater," as well as in the report on WateReuse Research Foundation Project Number WateReuse–10–16, entitled "Enzymes: The New Wastewater Treatment Chemical for Water Reuse."

Chapter 5: Phase 2 Results – Enzymatic Treatment in a Continuous Flow Reactor

5.1. INTRODUCTION

A bench-scale continuous flow reactor was constructed to investigate the effectiveness of enzymatic treatment in this more applied setting. The reactor design, consisting of six baffled chambers, was intended to encourage plug flow, and a tracer test was performed to model the hydraulic characteristics of the reactor. Subsequently, enzymatic treatment conditions were selected based on the results of Phase 1 batch experiments, and the abatement of the two representative micropollutants from Phase 1, oxybenzone and sulfamethoxazole, was measured in municipal wastewater primary effluent in a continuous flow experiment.

5.2. HYDRAULIC FLOW MODELING

The hydraulic flow through the reactor was characterized by a step tracer test, which yielded the exit age distribution shown in Figure 5-1 and the cumulative age distribution shown in Figure 5-2. In both cases, the experimental data are shown as the points in the figures. The fact that the exit age distribution has two peaks indicates that the hydraulic flow through the reactor was made up of two separate flow patterns.

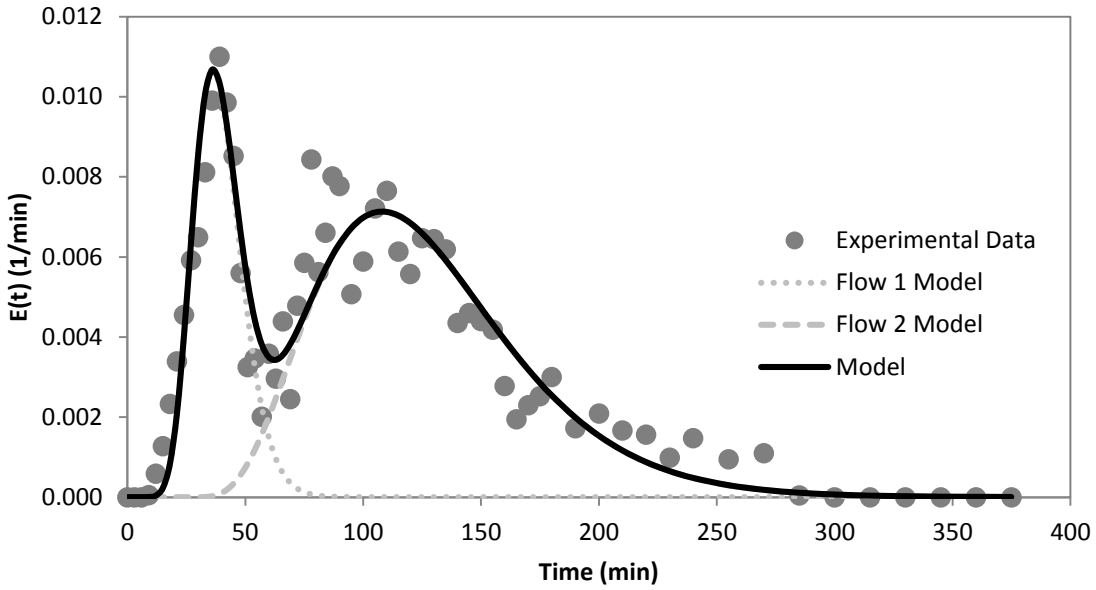


Figure 5-1: Exit age distribution (E(t)) of the continuous flow reactor, as determined by the step tracer test

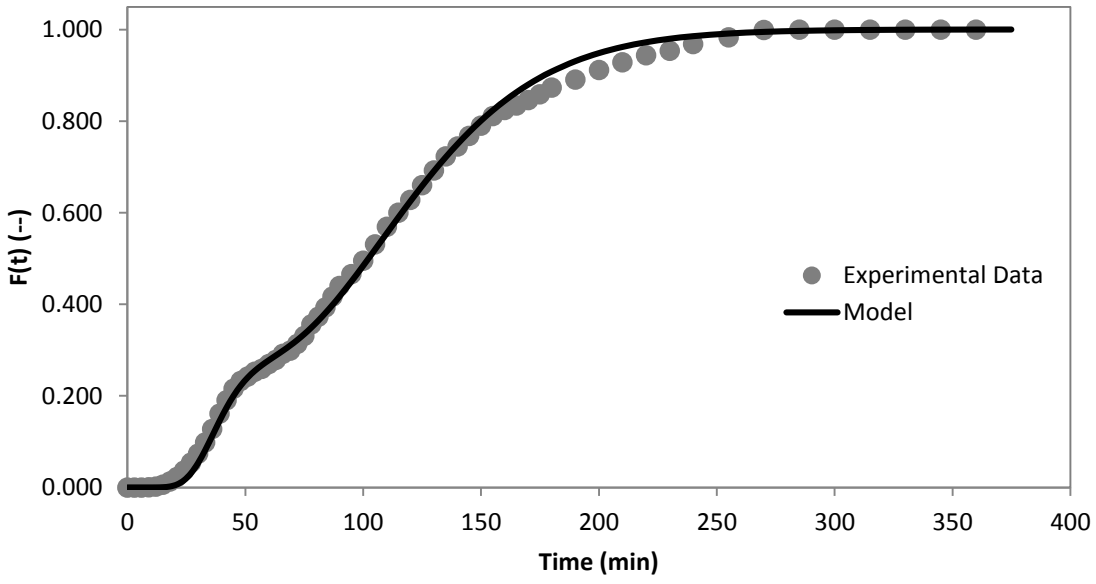


Figure 5-2: Cumulative age distribution (F(t)) of the continuous flow reactor, as determined by the step tracer test

To model this phenomenon, two conceptual reactors operating in parallel within the real reactor can be imagined, with each conceptual reactor representing one of the flow patterns. The first conceptual reactor was modeled as a series of equally sized continuous flow stirred tank reactors (CFSTRs). The second conceptual reactor was modeled as two reactors in series: a plug flow reactor (PFR) followed by a series of CFSTRs. The sum of these two models is the exit age distribution model for the real reactor. The mathematical model equations are shown in Equation 5-1, Equation 5-2, and Equation 5-3 (Benjamin & Lawler, 2013; Lawler, 2010).

Equation 5-1: Exit age distribution model for the first conceptual reactor (Reactor 1)

$$E_1(t) = f_1 \frac{1}{\bar{t}_1} \frac{{N_1}^{N_1}}{(N_1 - 1)!} \left(\frac{t}{\bar{t}_1}\right)^{N_1 - 1} \exp\left(-\frac{N_1 t}{\bar{t}_1}\right) t$$

Equation 5-2: Exit age distribution model for the second conceptual reactor (Reactor 2)

$$E_2(t) = (1 - f_1) \frac{1}{\bar{t}_2} \frac{{N_2}^{N_2}}{(N_2 - 1)!} \left(\frac{t - t_{pfr}}{\bar{t}_2}\right)^{N_2 - 1} \exp\left(-\frac{N_2\left(t - t_{pfr}\right)}{\bar{t}_2}\right)$$

Equation 5-3: Exit age distribution model for the real reactor

$$E_{tot}(t) = E_1(t) + E_2(t)$$

Here, the subscripts 1 and 2 refer to the two (imaginary) reactors that operate in parallel and that constitute the overall (real) reactor. Reactor 1 receives a fraction f_1 of the total flow, has a theoretical detention time of $\bar{t}_1$, and is envisioned to operate as a series of N_1 equally sized CFSTRs in series. Reactor 2 receives the rest of the flow (1-f_1)

and is envisioned to be composed of two reactors in series; the first is a PFR with a detention time of t_{pfr}, and the second is modeled as N_2 equally sized CFSTRs in series with a total theoretical detention time of $\bar{t}_2$. In essence, Reactor 1 is responsible for the first peak in the exit age distribution of the real reactor, Reactor 2 is responsible for the second peak, and the offset between them is due to a combination of the plug flow portion of Reactor 2 and the fact that $\bar{t}_2$ is larger than $\bar{t}_1$. In terms of the cumulative age distribution, the shoulder in the experimental results at approximately 50 minutes is accounted for in the model by the plug flow portion of Reactor 2. Hence, the model has six parameters: the value of f_1, the two values of $\bar{t}$, the two values of N, and the value of t_{pfr}. The cumulative age distribution is the running sum of the area under the exit age distribution from time zero to the time of interest; although this distribution could be determined analytically from the previous equations, it was done numerically in this research.

The model was programmed into a spreadsheet, and the values of all of the parameters were determined by a best fit by eye of the resulting E(t) and F(t) curves. In doing so, it was useful to be cognizant of a few characteristics of the N CFSTRs in series model (Benjamin & Lawler, 2013; Lawler, 2010). The spread of the model curve is dependent on the value of N, with a narrower curve resulting from a higher value of N, and the peak value in the exit age distribution occurs at a value of $t = \left((N-1)\bar{t}\right)/N$. Also, as noted previously, the PFR portion of the second conceptual reactor is the primary cause of the shoulder in the cumulative age distribution. Thus, the length of time associated with the shoulder in the experimental results shown in Figure 5-2 could be used to estimate t_{pfr}. With these concepts in mind, the first model parameter values tried were reasonably constrained, and further fitting of the model to the experimental results was straightforward.

The selected values for all of the model parameters are shown in Table 5-1, and the model results are shown along with the experimental data in Figure 5-1 for the exit age distribution and Figure 5-2 for the cumulative age distribution. For both distributions, the model fits the experimental data quite well, with some deviation at the later time points.

Table 5-1: Hydraulic flow model parameters

Parameter	Reactor 1 model	Reactor 2 model
f	0.27	0.73
$\bar{t}$ (min)	39	100
N	14	5
t_{pfr} (min)	N/A	28

5.3. CONTINUOUS FLOW ENZYMATIC TREATMENT EXPERIMENT

A continuous flow enzymatic treatment experiment with both oxybenzone and sulfamethoxazole was performed using the same experimental setup as the tracer test. Prior to time zero, primary effluent was pumped through the reactor at 38 mL/min for two hours (approximately one detention time) to establish steady state hydraulic flow. At time zero, the syringe pump was turned on and started delivering laccase at 100 µL/min (a negligible flow rate compared to the total flow rate) into the main influent flow just before it entered the reactor. Samples were collected from the reactor effluent just before time zero, and then at 50 minute intervals, for analysis of micropollutant concentrations

and enzyme activity, the results of which are shown in Figure 5-3. The effluent pH and flow rate were also measured at each sampling time. The effluent pH rose from 7.57 to 7.68 over the course of the experiment. The flow rate also increased slightly over the course of the experiment, from 38 mL/min to 39.8 mL/min. It was expected that the reactor would reach a steady state within 400 minutes (more than three detention times), but the results show that a steady state was not reached during the experiment. Nevertheless, several observations can be made based on these results.

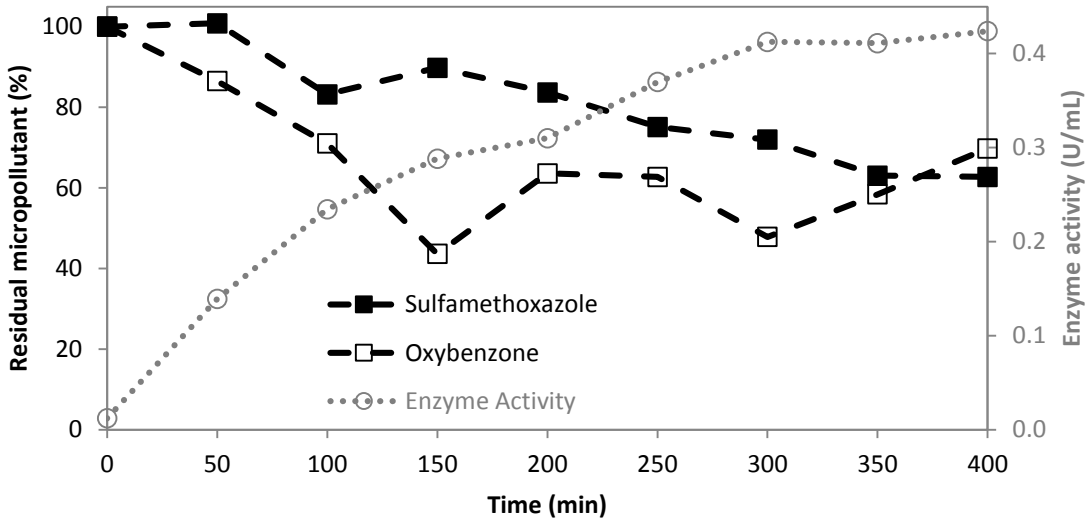


Figure 5-3: Left axis: Relative residual micropollutant concentrations in the continuous flow reactor effluent. Right axis: Effluent enzyme activity over the course of the experiment.

Note: The experiment was conducted in primary effluent from South Austin Regional (SAR) wastewater treatment plant with the following influent conditions: pH 7.57, 0.46 U/mL enzyme activity, 105 µM acetosyringone, 10 µg/L micropollutant = 43.8 nM oxybenzone = 39.5 nM sulfamethoxazole.

This first enzymatic treatment experiment in a continuous flow setting yielded much lower micropollutant removals than those measured in batch experiments. This

finding can be partly or wholly explained by the hydraulic characteristics of the continuous flow reactor. The batch experiments previously conducted simulated an ideal PFR with a detention time of two hours, and as the tracer test results revealed, the continuous flow reactor does not behave like an ideal PFR. The cumulative age distribution (Figure 5-2) shows that 63% of the flow through the reactor has a detention time of less than two hours, and that 25% has a detention time of less than one hour. Thus, in 63% of the flow, there was less treatment time than in the batch experiments, in which 100% of the reaction mixture was treated for two hours. In addition, batch reactors were mixed throughout the two hours of reaction time, whereas there was no mechanical mixing in the continuous flow reactor. This lack of mechanical mixing might explain the fluctuating oxybenzone trend after 150 minutes.

For a first order reaction in a continuous flow reactor, the steady state effluent concentration (or residual micropollutant) can be predicted from the exit age distribution by Equation 5-4 (Benjamin & Lawler, 2013; Lawler, 2010).

Equation 5-4: Steady state effluent concentration for a first order reaction in a continuous flow reactor

$$\frac{C_{out}}{C_{in}} = \sum_{all\ t} E(t) e^{-kt}\, \Delta t$$

The expected steady state oxybenzone and sulfamethoxazole treatment efficiencies under the conditions of the continuous flow experiment were calculated using the exit age distribution model from the tracer test and estimates of the apparent first order rate constants of enzymatic treatment of oxybenzone and sulfamethoxazole. The micropollutant concentration data from the batch experiment shown in Figure 4-5, in

which oxybenzone and sulfamethoxazole were treated simultaneously under conditions very similar to those in the continuous flow reactor experiment, were used to estimate the apparent first order rate constants under this specific set of conditions. The estimated apparent first order rate constants for enzymatic treatment of oxybenzone and sulfamethoxazole are 0.02022 min^{-1} and 0.00801 min^{-1}, respectively. Combining these estimated apparent rate constants with the exit age distribution model yielded predicted steady state transformation efficiencies of 80% and 52% for oxybenzone and sulfamethoxazole, respectively. The maximum transformation efficiencies achieved in this experiment were 56% and 37% for oxybenzone and sulfamethoxazole, respectively. Since steady state was not reached during this experiment, it is not possible to tell whether the predicted steady state removal efficiencies would have been reached.

Based on the measured enzyme activity of the enzyme solution in the syringe and on the flow rates, the influent enzyme activity was calculated to be 0.46 U/mL and 0.44 U/mL at the beginning and end of the experiment, respectively. The variation in calculated influent enzyme activity is due to the slight increase in the flow rate through the reactor over the course of the experiment. At the end of the experiment, the effluent enzyme activity was 0.42 U/mL. Very little (if any, as a steady state had not been reached) enzyme activity was lost in the reactor.

5.4. CONCLUSIONS

The tracer test in the continuous flow reactor revealed that the hydraulic flow through the reactor was far from ideal plug flow, and therefore more difficult to compare to the batch experiments. Nevertheless, a continuous flow enzymatic treatment experiment demonstrated that environmentally relevant concentrations of oxybenzone and sulfamethoxazole can be simultaneously treated by the laccase-acetosyringone

system in municipal wastewater primary effluent under realistic treatment conditions. However, these representative micropollutants were not oxidized to the extent that was expected based on the results of the batch experiments in Phase 1 and the hydraulic flow model developed for the continuous flow reactor. These first experiments in the continuous flow reactor exposed several issues that should be addressed prior to further continuous flow experiments. The reactor should be replaced or modified to more closely produce plug flow conditions. Also, better kinetic data are needed to properly predict micropollutant effluent concentrations based on the exit age distribution. Finally, future experiments should be conducted for longer periods of time to ensure that a steady state is reached during the experiments.

Chapter 6: Phase 3 Results – Investigation of Enzymatic Treatment Reaction Kinetics

6.1. INTRODUCTION

The results from Phases 1 and 2 indicated the need for a better understanding of the reaction kinetics and mechanisms involved in enzymatic treatment. The first two research phases focused on the feasibility of enzymatic treatment of organic micropollutants in primary effluent (*i.e.*, prior to activated sludge treatment), and while the results demonstrated the potential for successful development of an enzymatic treatment process, many fundamental questions remained. Consequently, study Phases 3 and 4 were completed to provide a more detailed picture of the chemical reactions involved in this treatment process. As a first step towards describing the kinetics of the laccase-mediator-target compound system, the kinetics of the oxidation of a model target compound, 2-chlorophenol, by the laccase-acetosyringone system were investigated in Phase 3. 2-Chlorophenol was selected as a model phenol because it is not directly oxidized (to an appreciable extent) by the laccase enzyme. It therefore represents target micropollutants containing an electron-rich phenolic functional group that require the presence of a mediator for effective enzymatic treatment.

To eliminate matrix and variable pH effects on the measured reaction kinetics, the Phase 3 experiments were performed in a simplified, well-mixed buffered solution containing a sodium phosphate buffer, the laccase enzyme, the mediator acetosyringone (ACE), and the model target compound 2-chlorophenol (2-CP) in Milli-Q water. The 5 mM sodium phosphate buffer held the reaction mixture pH at 7 in all Phase 3 experiments. The initial reactant concentrations were varied systematically to delineate their impacts on the oxidation rates of both acetosyringone and 2-chlorophenol. The oxidation of acetosyringone and 2-chlorophenol were measured as a function of reaction

time. Enzyme activity and pH were measured at the beginning and end of each experiment.

Based on the earlier results of this study and the findings of Margot *et al.* (2015), the following conceptual reaction model was assumed to represent the principal reactions occurring in the experimental system. A slightly expanded kinetic model including these reactions was later fit to experimental data gathered in this research phase; the modeling is discussed in Chapter 8.

Equation 6-1: $laccase + ACE + \frac{1}{4}O_2 \leftrightarrow complex \rightarrow laccase + ACE\bullet + \frac{1}{2}H_2O$

Equation 6-2: $ACE\bullet + 2 - CP \rightarrow transformation\ products$

Equation 6-3: $ACE\bullet + ACE\bullet \rightarrow mediator\ couple$

Equation 6-4: $ACE\bullet \rightarrow decay\ products$

Equation 6-5: $laccase + 2 - CP + \frac{1}{4}O_2 \leftrightarrow complex \rightarrow laccase + 2 - CP\bullet + \frac{1}{2}H_2O$

It was assumed that the rate of mediator (ACE) disappearance equals the rate of mediator radical (ACE•) production (Equation 6-1). Since it was not feasible to measure the concentration of ACE•, the only evidence of the oxidation rate of the target compound (2-CP) by ACE• (Equation 6-2) was the disappearance of 2-chlorophenol. It was confirmed in control experiments that the mediator acetosyringone is only transformed in the presence of laccase. It was also confirmed that direct oxidation of 2-chlorophenol by the laccase enzyme (Equation 6-5) is relatively slow. Therefore, 2-chlorophenol transformation in experiments with the laccase-acetosyringone system can primarily be attributed to the presence of the mediator acetosyringone (*i.e.*, oxidation by the mediator radical ACE•). Experimental conditions tested and the associated relative

residual enzyme activities at the conclusion of each experiment are presented in Table 6-1.

Table 6-1: Phase 3 experimental conditions and associated relative residual enzyme activities at the conclusion of each experiment

Experiment #	Initial enzyme activity (U/mL)	Approximate initial [laccase] (µM)	Initial [ACE] (µM)	Initial [2-CP] (µM)	Relative residual enzyme activity (%)	Aeration	Experiment duration (min)
1	1	0.3	300	0	9	no	60
2	1	0.3	300	15	8	no	60
3	1	0.3	300	100	10	no	60
4	1	0.3	300	300	98	no	60
5	1	0.3	300	30*	5	no	60
6	0.2	0.06	300	300	101	no	60
7	0.2	0.06	300	100	14	no	60
8	0.2	0.06	60	60	102	no	60
9	0.1	0.03	30	30	105	yes	60
10	1	0.3	300	300	109	yes	20
11	1	0.3	300	600	97	yes	20
12	1	0.3	300	900	96	yes	20

Note: Experiments were conducted in 5 mM sodium phosphate buffer at pH 7.
*2-CP added at t = 30 minutes

6.2. INFLUENCE OF 2-CHLOROPHENOL CONCENTRATION

The first set of experiments (Experiments 1 – 4) was performed with varying initial 2-chlorophenol concentration (from 15 to 300 μM) while keeping the other initial conditions the same (1 U/mL enzyme activity, 300 μM acetosyringone). The acetosyringone concentration was set to be 1,000 times the approximate molar concentration of enzyme, which was estimated based on the density and composition of the enzyme solution provided in the Novozym® 51003 Product Data Sheet (Novozymes A/S, Bagsvaerd, Denmark) and the reported molar mass of the enzyme. The measured concentrations of acetosyringone and 2-chlorophenol over the course of these experiments are presented in Figure 6-1 and Figure 6-2, respectively.

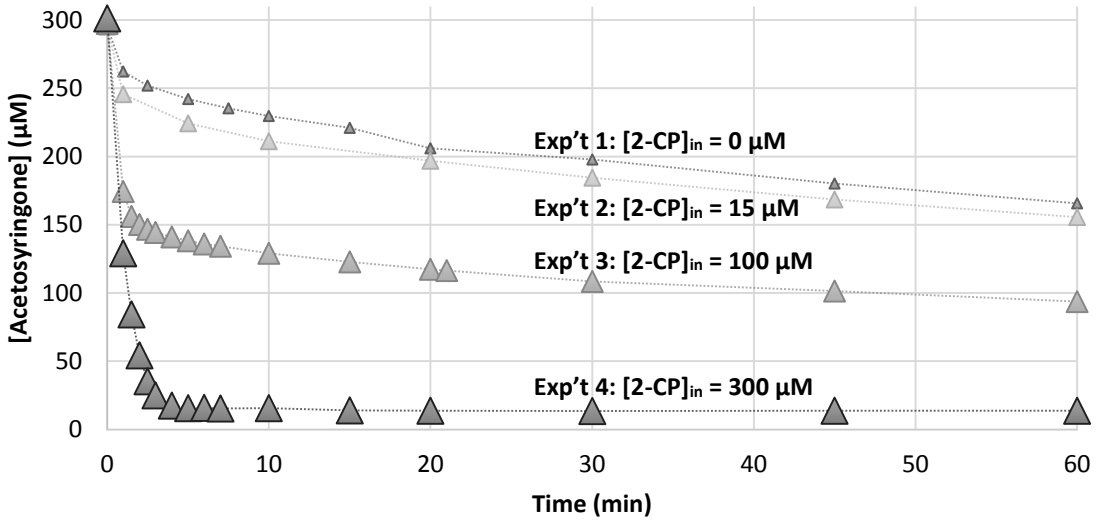


Figure 6-1: Acetosyringone (ACE) concentration over time in Experiments 1 – 4, in which the initial 2-chlorophenol (2-CP) concentration was varied

Note: Experiments were conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 μM acetosyringone, 0, 15, 100, or 300 μM 2-chlorophenol.

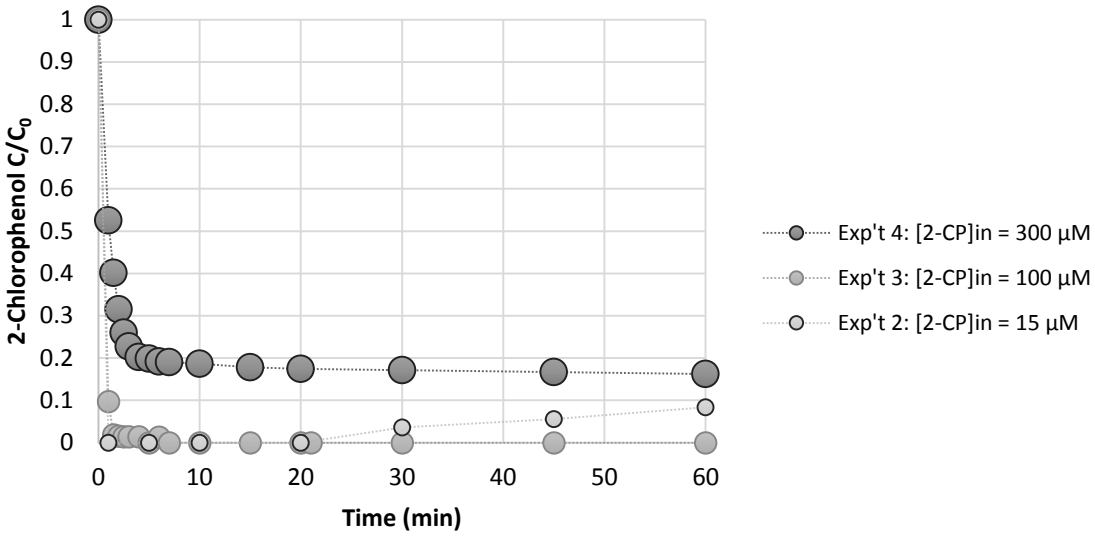


Figure 6-2: 2-chlorophenol (2-CP) concentration, expressed as C/C_0, over time in Experiments 2 – 4, in which the initial 2-CP concentration was varied

Note: Experiments were conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone, 15, 100, or 300 µM 2-chlorophenol.

Two interesting behaviors were observed in the results of these first kinetic experiments. First, the mediator radical (ACE•) was consumed in the reaction with 2-chlorophenol (Equation 6-2) rather than being reduced back to ACE, as it would be in a true catalytic cycle. A true mediator would be recycled; a single mediator molecule would accordingly be able to oxidize more than one target molecule. In these experiments, similar amounts of acetosyringone and 2-chlorophenol were consumed, suggesting that each ACE• oxidized one target molecule, perhaps via a coupling mechanism. Using a different laccase (from *Trametes versicolor*), Margot *et al.* (2015) also reported mediator consumption for three different mediators, including acetosyringone, with the micropollutant sulfamethoxazole.

The second behavior observed was that the oxidation rate of acetosyringone increased as the initial 2-chlorophenol concentration increased. The enzyme activity assay results, presented in Table 6-1, point to one possible explanation for this trend; that an excess of acetosyringone radicals (ACE•) (due to the lack of 2-chlorophenol to react with) leads to inactivation or inhibition of the laccase enzyme, which would in turn lead to a slower acetosyringone oxidation rate. Most of the enzyme activity was lost over the course of all experiments with an acetosyringone:2-chlorophenol molar ratio greater than one. These enzyme activity losses are discussed further in Section 6.3.

In the Margot *et al.* (2015) study, the mediator acetosyringone was still consumed completely in experiments without sulfamethoxazole (*i.e.*, without a target compound), which were also performed in a pure buffered system. Thus, the *Trametes versicolor* laccase did not exhibit the dependence of the acetosyringone oxidation rate on the target compound concentration that was observed in this research with the Novozymes laccase. This difference between the two laccases could be due to different products being formed, or more likely, to differing characteristics of the *Trametes versicolor* laccase that protect it from the inactivation or inhibition affecting the Novozymes laccase.

Another possible, although less likely explanation for the observed dependence of the acetosyringone oxidation rate on the target compound concentration is that an additional reaction to those in the assumed conceptual reaction model was occurring, in which the acetosyringone (not the ACE•) and 2-chlorophenol were involved in the same reaction. If such a reaction was occurring, both oxidation rates (that of acetosyringone and that of 2-chlorophenol) would depend on the concentrations of both acetosyringone and 2-chlorophenol. This theory could be supported by the results of Experiment 5 (shown in Figure 6-3), in which the 2-chlorophenol was added 30 minutes after the

laccase and acetosyringone were mixed. As soon as the 2-chlorophenol was added, it was quickly oxidized, and the acetosyringone oxidation rate increased dramatically.

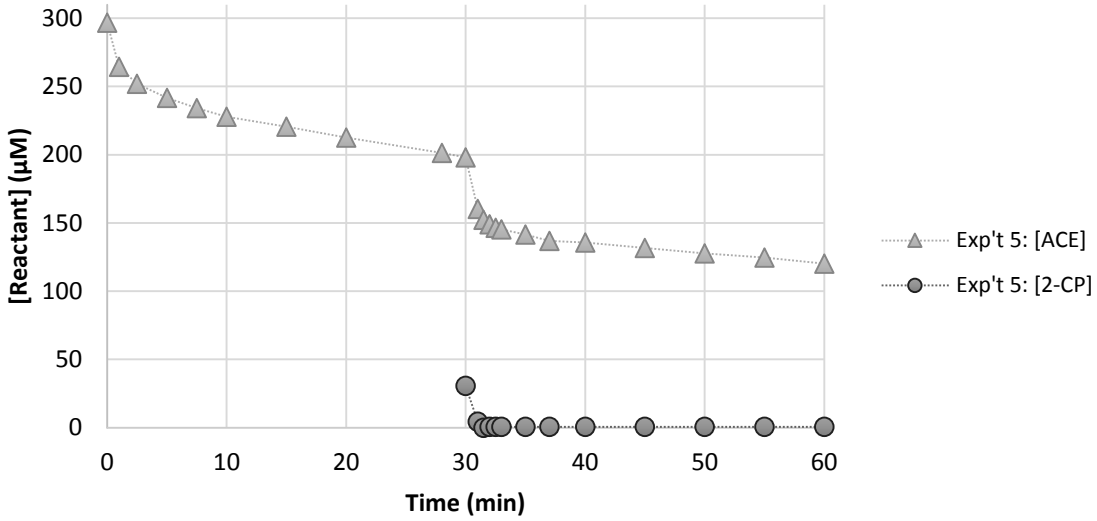


Figure 6-3: Acetosyringone (ACE) and 2-chlorophenol (2-CP) concentrations over time in Experiment 5, in which the 2-CP was added after 30 minutes

Note: The experiment was conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone, 30 µM 2-chlorophenol.

Experiments were also performed with 2-chlorophenol concentrations greater than the acetosyringone concentration. A set of experiments (Experiments 10 – 12) was performed in which the initial 2-chlorophenol concentration was varied from 300 to 900 µM, while keeping the other initial conditions the same as those of Experiments 1 – 4 (1 U/mL enzyme activity, 300 µM acetosyringone). As can be seen in Figure 6-5, the 2-chlorophenol oxidation rates were very similar for the three different initial 2-chlorophenol concentrations. Furthermore, the extent of 2-chlorophenol oxidation was limited by the acetosyringone concentration, further supporting the earlier evidence that acetosyringone is not recycled, as a true mediator would be, in this system. The

acetosyringone oxidation rates, shown in Figure 6-4, appeared to be slightly faster in the experiments with higher initial 2-chlorophenol concentrations when the reactant concentrations were getting low (*i.e.*, around t = 2 to 3 minutes). This is consistent with the theory that an excess of ACE• inactivates or inhibits the enzyme, slowing down the reaction system. In Experiments 11 and 12, there was always an excess of 2-chlorophenol to quench ACE•.

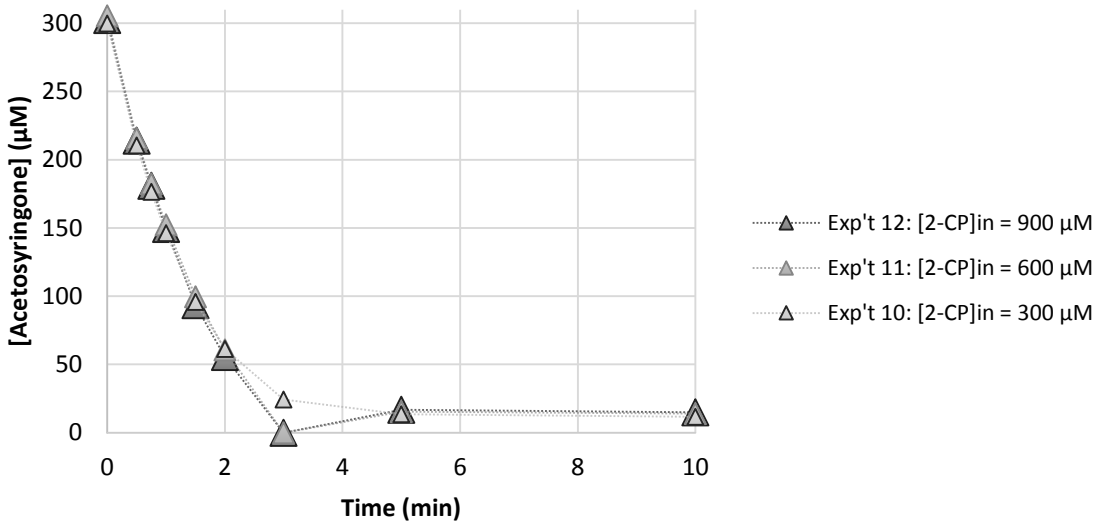


Figure 6-4: Acetosyringone (ACE) concentration over time in Experiments 10 – 12, in which the initial 2-chlorophenol (2-CP) concentration was varied

Note: Experiments were conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone, 300, 600, or 900 µM 2-chlorophenol.

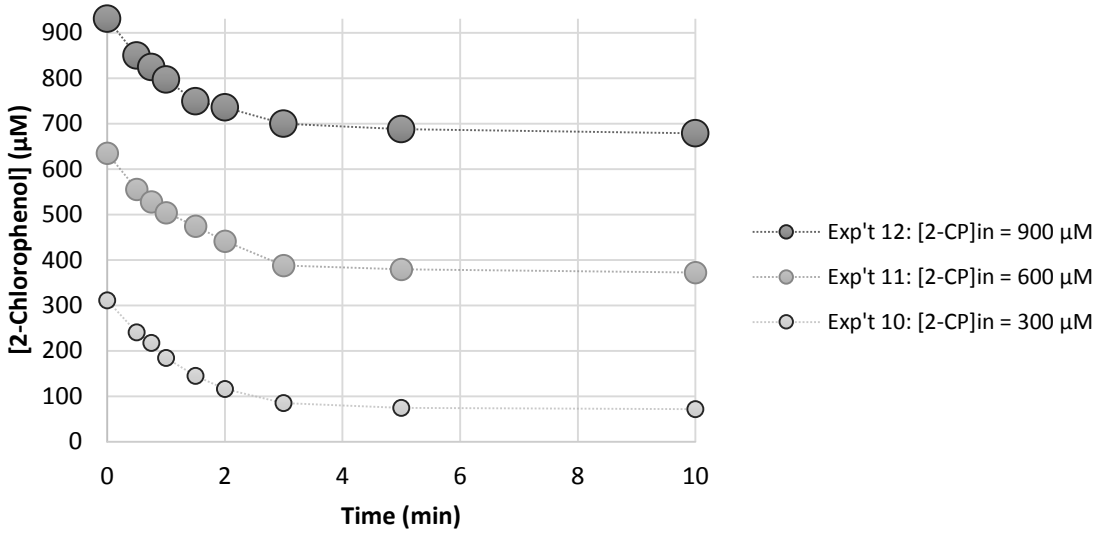


Figure 6-5: 2-chlorophenol (2-CP) concentration over time in Experiments 10 – 12, in which the initial 2-CP concentration was varied

Note: Experiments were conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone, 300, 600, or 900 µM 2-chlorophenol.

6.3. INFLUENCE OF ENZYME ACTIVITY

Experiments 6 – 8 were performed at a lower initial enzyme activity of 0.2 U/mL. Experiments 6 and 7 were conducted with the same initial acetosyringone and 2-chlorophenol concentrations as Experiments 4 and 3, respectively. In Experiments 6 and 7, the initial acetosyringone concentration was 5,000 times the approximated molar concentration of enzyme, whereas this ratio was 1,000:1 in all other experiments. Experiment 8 was conducted with the same initial reactant ratios as Experiment 4 (*i.e.*, [2-CP] = [ACE] = 1000×[laccase]. These results, shown in Figure 6-6, are consistent with all experiments with the laccase-acetosyringone-2-chlorophenol system in that the rate of 2-chlorophenol oxidation closely followed the rate of acetosyringone oxidation, which is assumed to be the rate of ACE• production. This suggests that either (1) the 2-

chlorophenol reacts very quickly with the produced ACE•, in which case the system might be limited by ACE• production or (2) a reaction involving both acetosyringone (not the ACE•) and 2-chlorophenol is occurring, as discussed previously. Additionally, more acetosyringone than 2-chlorophenol was always consumed. This difference could be accounted for by other ACE• reaction pathways (*e.g.*, Equation 6-3 and Equation 6-4).

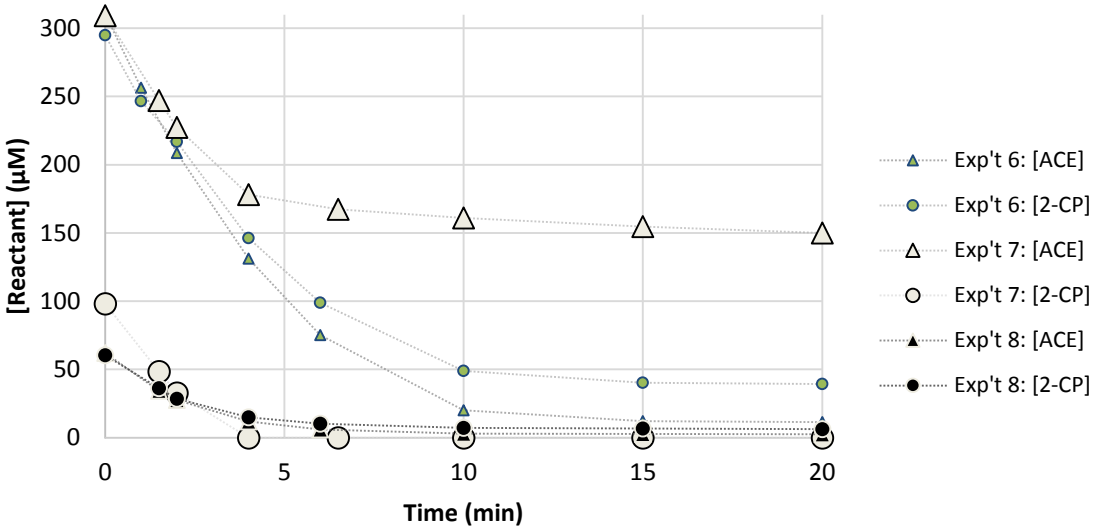


Figure 6-6: Acetosyringone (ACE) and 2-chlorophenol (2-CP) concentrations over time in Experiments 6 – 8

Note: The experiments were conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 0.2 U/mL enzyme activity; Experiment 6: 300 µM acetosyringone, 300 µM 2-chlorophenol; Experiment 7: 300 µM acetosyringone, 100 µM 2-chlorophenol; Experiment 8: 60 µM acetosyringone, 60 µM 2-chlorophenol.

To further investigate the enzyme activity loss observed in the first set of experiments, intermediate enzyme activity measurements were taken during these experiments. These results, shown in Figure 6-7, further supported the observation that enzyme activity was lost only in experiments with ACE:2-CP ratios of greater than one.

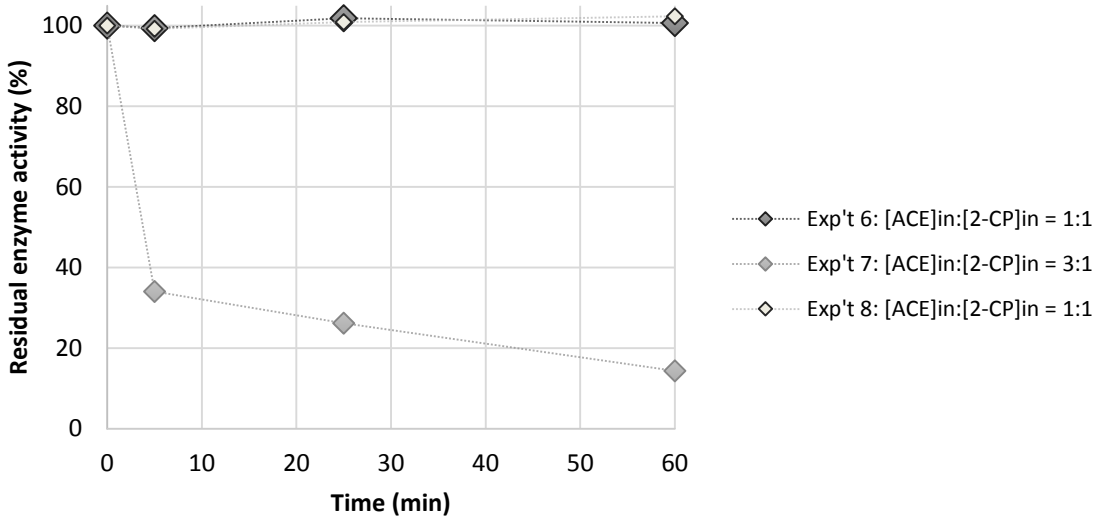


Figure 6-7: Percent residual enzyme activity over time in Experiments 6 – 8

Note: The experiments were conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 0.2 U/mL enzyme activity; Experiment 6: 300 μM acetosyringone, 300 μM 2-chlorophenol; Experiment 7: 300 μM acetosyringone, 100 μM 2-chlorophenol; Experiment 8: 60 μM acetosyringone, 60 μM 2-chlorophenol.

It was initially hypothesized that the observed enzyme activity losses were due to ACE• or H_2O_2 produced by incomplete reduction of O_2 to H_2O (Equation 6-1) oxidizing the enzyme itself. Alternatively, higher concentrations of ACE• could promote polymerization reactions like Equation 6-3, and the enzyme could adhere to these polymers. Dasgupta *et al.* (2007) found that laccase adhered to precipitated polymers of phenol, which had a significant effect on the enzyme activity and subsequent removal of phenol. Such inactivation or inhibition problems due to excess ACE• would most likely be of much less concern in a real wastewater matrix, which would quench excess radicals.

Earlier experiments in this research phase were not aerated, and the dissolved oxygen concentration was followed over the course of each experiment. It was observed that more oxygen was consumed than stoichiometrically required for the amount of

acetosyringone oxidized, which suggested that some of the oxygen was not completely reduced to water, and that hydrogen peroxide was likely forming. To rule out hydrogen peroxide as the cause of the observed enzyme activity losses, two experiments (Experiments 4 and 8) were repeated with the addition of 250 μM H_2O_2 to check if the additional H_2O_2 affected the oxidation rates, and enzyme activity assays were performed on enzyme solutions exposed to varying concentrations of H_2O_2 for periods of time up to one hour. These experiments did not show a negative effect of hydrogen peroxide on enzyme activity or the oxidation rates, indicating that hydrogen peroxide formation, while possibly occurring, was likely not the cause of the observed enzyme activity losses.

6.4. CONCLUSIONS

The Phase 3 experimental results demonstrated that the kinetics of the laccase-acetosyringone-2-chlorophenol system are complex and non-linear. The mediator radical (ACE•) was consumed in the reaction with 2-chlorophenol rather than being reduced back to acetosyringone, as it would be in a true catalytic cycle. This type of behavior is consistent with the very high acetosyringone concentrations required in Phase 1 and 2 experiments. Moreover, acetosyringone and 2-chlorophenol concentrations decreased at nearly 1:1 molar ratios, indicating a 1:1 reaction between ACE• and 2-chlorophenol. The similar oxidation rates of acetosyringone and 2-chlorophenol suggest that the system might have been limited by ACE• production.

In experiments with acetosyringone:2-chlorophenol molar ratios greater than one, significant enzyme activity was lost. The exact mechanism of this enzyme activity loss is unknown, but appears to be related to excess ACE•. In a real system, enzyme activity loss by this same mechanism might not occur, as there would be other matrix components to quench excess ACE•, preventing possible attack of the enzyme by the ACE• and

limiting the formation of polymerization products formed by radical-radical interactions (*e.g.*, Equation 6-3) that might inactivate or inhibit the enzyme.

Phase 3 experiments were performed with one specific laccase-mediator-target compound system, which raises the question if the observed behavior is typical of all such systems. These results do exhibit some differences from the results of Margot *et al.* (2015), most likely because of the different laccases used. To expand the investigation to other laccase-mediator-target compound systems and to investigate the effects of target compound structure on reaction kinetics, the next research phase looked at the oxidation kinetics of several other target compounds structurally related to 2-chlorophenol.

Chapter 7: Phase 4 Results – The Influence of Target Compound Structure and Speciation on Enzymatic Treatment Reaction Kinetics

7.1. INTRODUCTION

For the development of an efficient treatment process that is able to remove mixtures of diverse micropollutants simultaneously, it is important to know how fast the enzymatic treatment reactions can be expected to occur for a wide variety of micropollutants in a complex background matrix. Since measuring the enzymatic treatment rates individually for thousands of micropollutants currently known is not feasible, it would be useful to be able to make predictions about the reactivity of a compound with the laccase-mediator system based on its structure. To investigate how the kinetics of the laccase-mediator-target compound system are affected by target compound structure, Phase 4 experiments studied the kinetics of the oxidation of a group of structurally related target compounds by the laccase-acetosyringone system. The selected target compounds, the structures for which are shown in Table 3-1, included three model chlorophenols and the micropollutant chloroxylenol, which contains a chlorophenol group.

The three model chlorophenols are electron-rich aromatic rings with differing numbers of electron withdrawing substituents. Based on their electronic properties (*i.e.*, electron density in the aromatic ring), the model chlorophenols were expected to exhibit increasing reactivity in the following order: 2,4,6-trichlorophenol < 2,4-dichlorophenol < 2-chlorophenol. If their oxidation rates were to fit this predicted trend, it would suggest that the rate of oxidation of a target compound by the acetosyringone mediator radical is related to the electronic properties of the target.

As the previous kinetic experiments demonstrated, the kinetics of the laccase-mediator-target compound system are complex and non-linear. Due to the complex

kinetics, it was not possible to keep reaction mixture conditions constant over the course of an experiment. For example, the mediator acetosyringone could not be added in excess of the target compound 2-chlorophenol, or a significant portion of the enzyme activity would be lost. Therefore, competition kinetics were used to compare the reactivities of the target compounds to ensure they were being compared under identical conditions. In addition, the Phase 3 results indicated that the enzyme oxidation of acetosyringone was the rate limiting step in the system. Thus, if the rate constants of all model target compounds were fast enough, it would be impossible to distinguish the differences in their reactivities in individual kinetic experiments. With competition kinetics, even if their absolute rate constants cannot be determined, the ratios of their reaction rates can be related to the ratios of their rate constants.

7.2. Influence of Steric Effects

The first experiments with the three model chlorophenols were performed at pH 7. Each chlorophenol was oxidized individually, and an experiment was performed with the three in competition. The results of the experiments in which each chlorophenol was treated individually are shown in Figure 7-1, Figure 7-2, and Figure 7-3. The first finding that stands out in examining these results is that different amounts of the mediator acetosyringone were consumed with each of the three chlorophenols. In Phase 3, it was observed that the acetosyringone was consumed at nearly 1:1 molar ratios with 2-chlorophenol, and was therefore not regenerated; these results are repeated in Figure 7-1. Conversely, the acetosyringone was clearly regenerated in its oxidation of 2,4-dichlorophenol and 2,4,6-trichlorophenol. With 2,4-dichlorophenol, some acetosyringone was consumed (approximately 25%) while the dichlorophenol was still present, as opposed to essentially 100% consumption with 2-chlorophenol. With 2,4,6-

trichlorophenol, very little acetosyringone was consumed (approximately 8%) while the trichlorophenol was still present. Of course, after the chlorophenols were completely oxidized, the acetosyringone oxidation behaved as in experiments without a target compound; it continued to be oxidized, but enzyme activity loss slowed down the reaction.

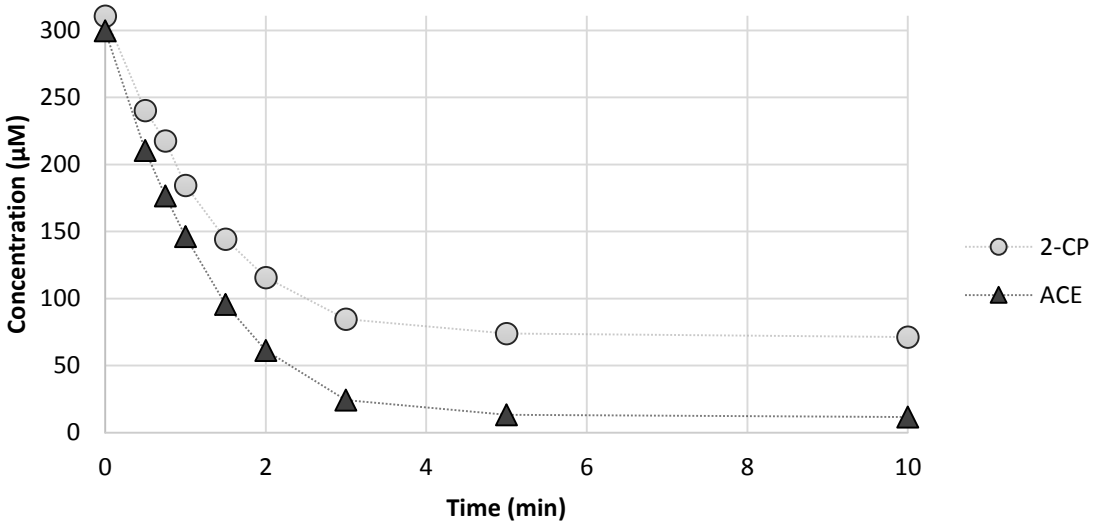


Figure 7-1: Individual treatment of 2-CP by the laccase-ACE system at pH 7

Note: Experiment was conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone (ACE), 300 µM 2-chlorophenol (2-CP).

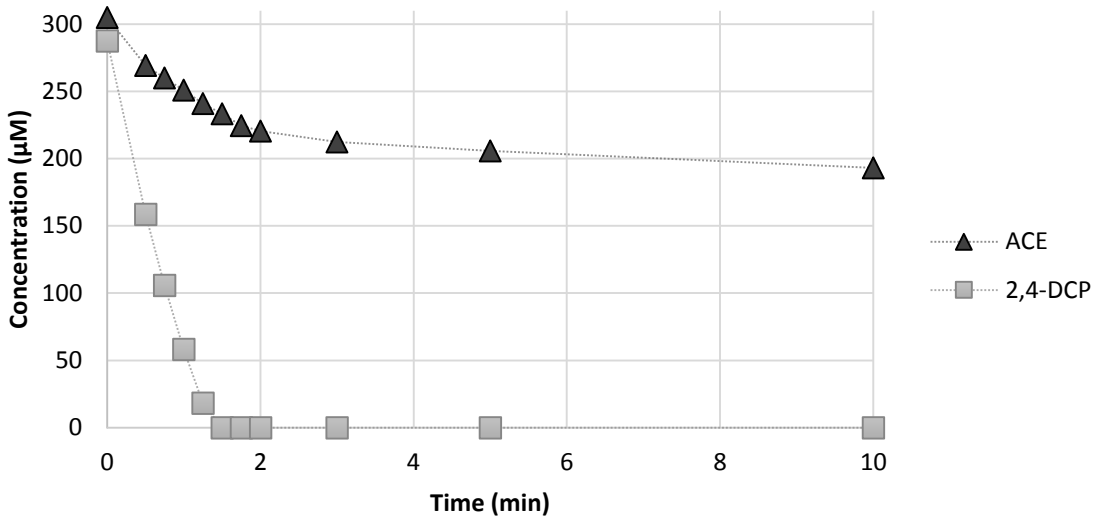


Figure 7-2: Individual treatment of 2,4-DCP by the laccase-ACE system at pH 7

Note: Experiment was conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone (ACE), 300 µM 2,4,-dichlorophenol (2,4-DCP).

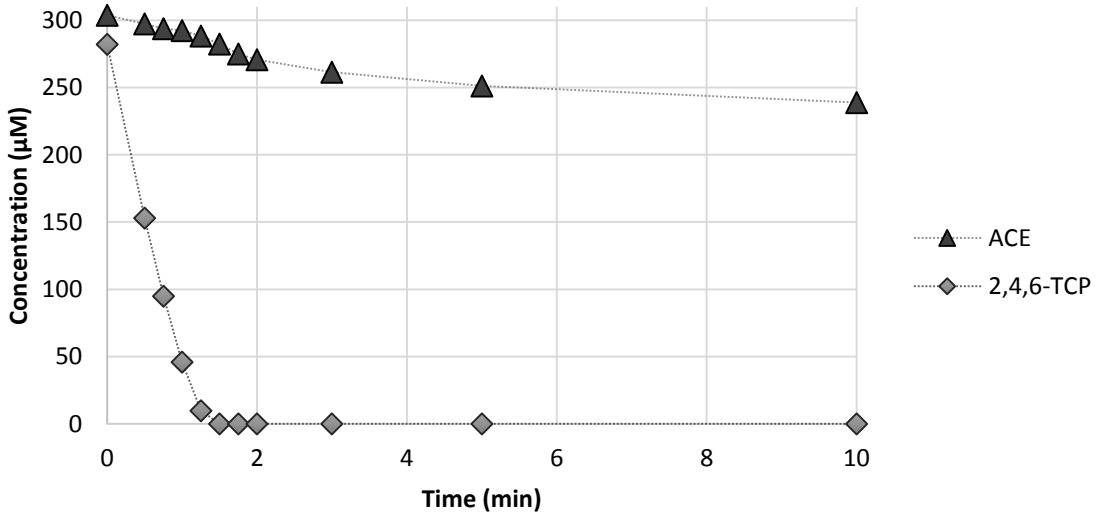


Figure 7-3: Individual treatment of 2,4,6-TCP by the laccase-ACE system at pH 7

Note: Experiment was conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 μM acetosyringone (ACE), 300 μM 2,4,6-trichlorophenol (2,4,6-TCP).

These results highlight an important finding of this research. One major influence of target compound structure on the enzymatic treatment process is its effect on the mechanism of oxidation by the mediator radical, and hence the regeneration of the mediator. The target compound structure, in this case, seemed to determine whether the ACE• coupled with the target compound or oxidized the target compound by an electron transfer mechanism, and was itself reduced back to acetosyringone. Specifically, the increasing substitution of the aromatic ring in the chlorophenols apparently made the coupling reaction route less favorable. Both of these reaction mechanisms have been observed or proposed by other researchers with various laccase-mediator-target compound systems. Some researchers (*e.g.*, Baiocco *et al.*, 2003; Fabbrini *et al.*, 2002) have proposed that the reaction in which a mediator is regenerated could take place via an electron transfer mechanism or via a hydrogen abstraction mechanism, although both would result in the same transformation product. Torres-Duarte *et al.* (2009) found evidence of an electron transfer mechanism between the radical of the mediator syringaldehyde, which has a similar structure to acetosyringone, and the target compound dichlorophen, a pesticide containing two chlorophenol groups. It is therefore likely that the reaction in which the acetosyringone was regenerated in this research was occurring via an electron transfer mechanism.

In the experiments where the chlorophenols were treated individually, the steric effects on the reaction mechanism and the resulting impact on mediator regeneration could be observed. In the experiment with the three chlorophenols in competition, however, the expected order of reactivities based on the electronic properties of the three chlorophenols was not observed. These results are shown in Figure 7-4. The differing

importance of the two reaction mechanisms among the three different chlorophenols likely played a role in their apparent reactivities, but there also seemed to be an influence of the acid-base speciation of the chlorophenols. This competition kinetics experiment was performed at pH 7, which is below the pK_a values of 2-chlorophenol (8.3) and 2,4-dichlorophenol (7.8) and above the pK_a of 2,4,6-trichlorophenol (6.1). 2,4-dichlorophenol exhibited a higher reactivity than 2-chlorophenol despite having a lower electron density in its aromatic ring. With other oxidants (*e.g.*, ClO_2, HOCl, ozone), phenolate species are known to be more reactive than their protonated phenol counterparts. Assuming a higher reactivity of phenolate species, this result could be explained by the higher fraction of deprotonation of 2,4-dichlorophenol at this pH (13.7% vs. 4.8% for 2-chlorophenol), by its additional and presumably faster electron transfer reaction mechanism, or by some combination of these two effects. 2,4,6-trichlorophenol exhibited a reactivity between those of 2-chlorophenol and 2,4-dichlorophenol despite having the lowest electron density in its aromatic ring, which could be explained similarly. 2,4,6-trichlorophenol is 88.8% deprotonated at pH 7, and it is feasible that the reactivity of its phenolate species is competitive with the reactivities of the protonated species of the other chlorophenols. Also, 2,4,6-trichlorophenol was shown to react primarily by the electron transfer mechanism, which is presumed to be faster than the coupling mechanism.

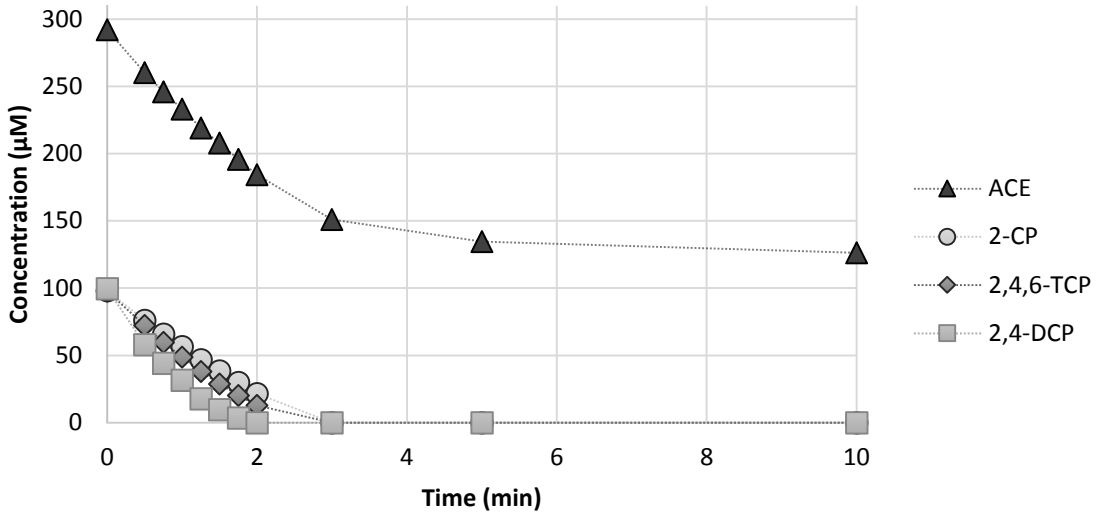


Figure 7-4: Competition kinetics experiment with the three model chlorophenols at pH 7

Note: Experiment was conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 μM acetosyringone (ACE), 100 μM 2-chlorophenol (2-CP), 100 μM 2,4-dichlorophenol (2,4-DCP), 100 μM 2,4,6-trichlorophenol (2,4,6-TCP).

7.3. INFLUENCE OF PH

To further explore the impact of target compound acid-base speciation on its oxidation by the mediator radical, additional competition kinetics experiments were performed at pH 5 and pH 9.3. These pH values were selected to be at least one log unit below and above the lowest and highest pK_a values of the target model chlorophenols, respectively.

As can be seen in Figure 7-5, despite an apparently significant reduction in enzyme activity at pH 9.3, the expected order of reactivity (2,4,6-trichlorophenol < 2,4-dichlorophenol < 2-chlorophenol) was observed at this pH above the pK_a values of all three chlorophenols. At pH 9.3, all three chlorophenols are primarily in their phenolate forms, with 2-chlorophenol having the highest fraction of protonation (9.1%). This result

confirms that the electronic properties of the target compound play at least some role in its reactivity with the mediator radical. However, the results showed that at pH values more relevant to water and wastewater treatment, the influence of the electronic properties of the model chlorophenols is eclipsed by the influence of a pronounced difference in the reactivities of the protonated and deprotonated chlorophenol species. Although this result is important for the target compounds examined in these experiments, for target compounds that do not speciate, or that have pK_a values well outside of the range of normal treatment pH values, their electronic properties would likely be important in determining their reactivities with the mediator radical.

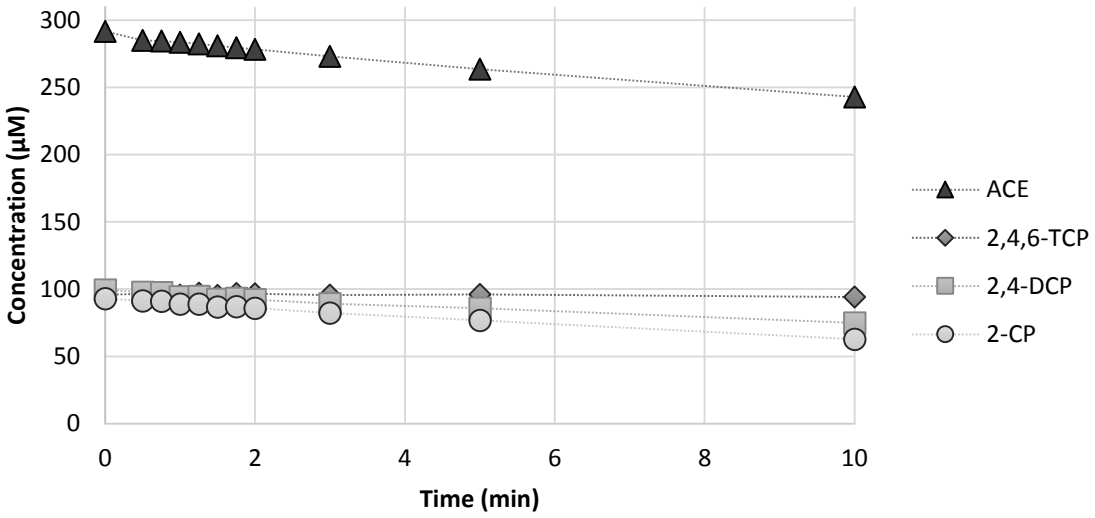


Figure 7-5: Competition kinetics experiment with the three model chlorophenols at pH 9.3

Note: Experiment was conducted in 5 mM borate buffer at pH 9.3 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone (ACE), 100 µM 2-chlorophenol (2-CP), 100 µM 2,4-dichlorophenol (2,4-DCP), 100 µM 2,4,6-trichlorophenol (2,4,6-TCP).

At pH 5, which is below the pKa values of all three chlorophenols, the compounds are primarily in their protonated forms, with 2,4,6-trichlorophenol being the most deprotonated (7.4%). In the competition kinetics experiment at pH 5, the results of which are shown in Figure 7-6, 2,4-dichlorophenol exhibited a higher reactivity than 2-chlorophenol despite its lower electron density in the aromatic ring, as was the case at pH 7. This result could still be explained by the higher fraction of deprotonation of 2,4-dichlorophenol at this pH (0.16% vs. 0.05% for 2-chlorophenol), but the extent of deprotonation is small for both compounds, and it therefore seems likely that 2,4-dichlorophneol's dominant and presumably faster electron transfer reaction mechanism plays a significant role in this result. Despite having the lowest electron density in its aromatic ring, 2,4,6-trichlorophenol exhibited the highest reactivity at pH 5, which was not surprising given its significantly higher deprotonated fraction (7.4%).

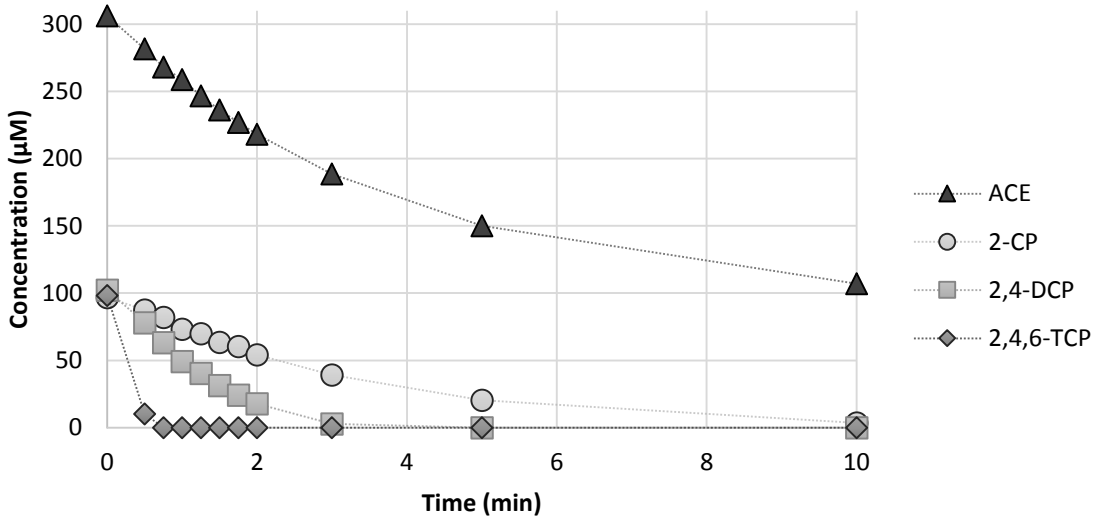


Figure 7-6: Competition kinetics experiment with the three model chlorophenols at pH 5

Note: Experiment was conducted in 5 mM sodium acetate buffer at pH 5 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone (ACE),

100 μM 2-chlorophenol (2-CP), 100 μM 2,4-dichlorophenol (2,4-DCP), 100 μM 2,4,6-trichlorophenol (2,4,6-TCP).

Since pH also affects the activity and stability of the laccase enzyme, enzyme activity assays were performed at each of the experimental pH values. Normally, the enzyme activity was measured by a standardized assay at pH 5, as described in Section 3.3.1, which served as a consistent method for measuring the amount of "potentially active" enzyme present in a solution. However, the standardized enzyme activity assay does not account for variations in the enzyme activity based the enzyme's environment outside of the assay mixture, such as in the experimental reaction mixture. In the standardized enzyme activity assay, a sample from a reaction mixture would be diluted in Milli-Q water, and this diluted sample would make up only 10% of the assay mixture. Thus, the enzyme's environment in the assay mixture is standardized, but not representative of the reactor conditions. To measure how the actual reactor environment at different pH values was influencing the activity and stability of the enzyme, assays were performed with the same procedure as the standardized enzyme activity assay, but in the same buffer and buffer concentration as in each reactor experiment. The conditions tested included 5 mM sodium acetate buffer at pH 5, 5 mM sodium phosphate buffer at pH 7, and 5 mM borate buffer at pH 9.3. For each condition, an assay was performed in which the laccase was exposed to the condition just before the assay began (prior to which it was diluted in Milli-Q water), and a second assay was performed in which the laccase was exposed to the condition for an hour before the assay began. This second assay was performed to check the stability of the enzyme activity at each condition. The results of these assays at pH 5 and pH 7 are presented in Figure 7-7. With this assay procedure, no enzyme activity could be measured at pH 9.3, but some

small amount of activity must have been present as some oxidation of the model chlorophenols did occur in the competition kinetics experiment at pH 9.3.

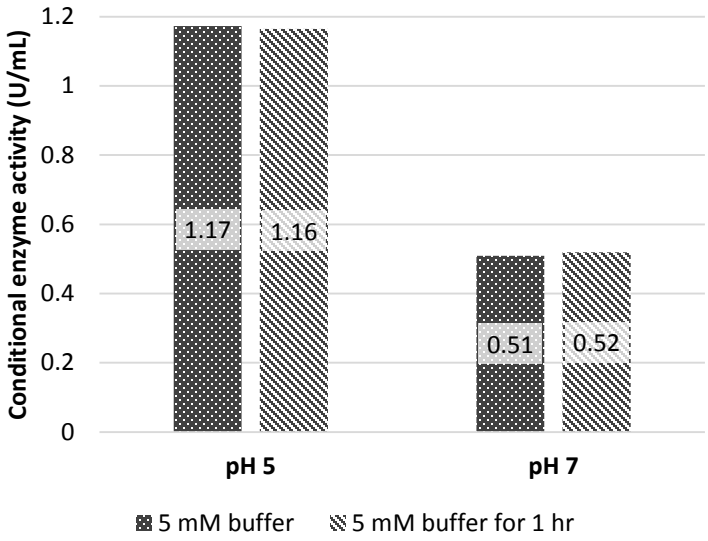


Figure 7-7: Results of the reactor condition-specific enzyme activity assays

Control experiments without the mediator acetosyringone were also performed at each pH to verify that direct oxidation of the chlorophenols by the laccase enzyme was negligible. The results of these control experiments at pH values of 5, 7, and 9.3 are shown in Figure 7-8, Figure 7-9, and Figure 7-10, respectively. At each of the pH values tested, direct laccase oxidation of the model chlorophenols was insignificant compared to the oxidation by the laccase-acetosyringone system.

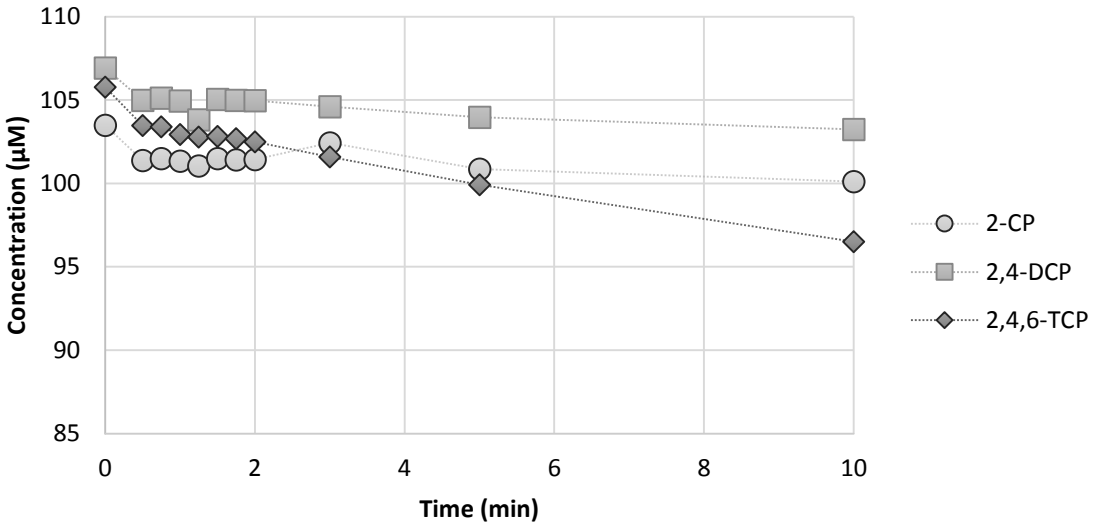


Figure 7-8: Control experiment without the mediator acetosyringone at pH 5

Note: Experiment was conducted in 5 mM sodium acetate buffer at pH 5 with the following initial conditions: 1 U/mL enzyme activity, 100 µM 2-chlorophenol (2-CP), 100 µM 2,4-dichlorophenol (2,4-DCP), 100 µM 2,4,6-trichlorophenol (2,4,6-TCP).

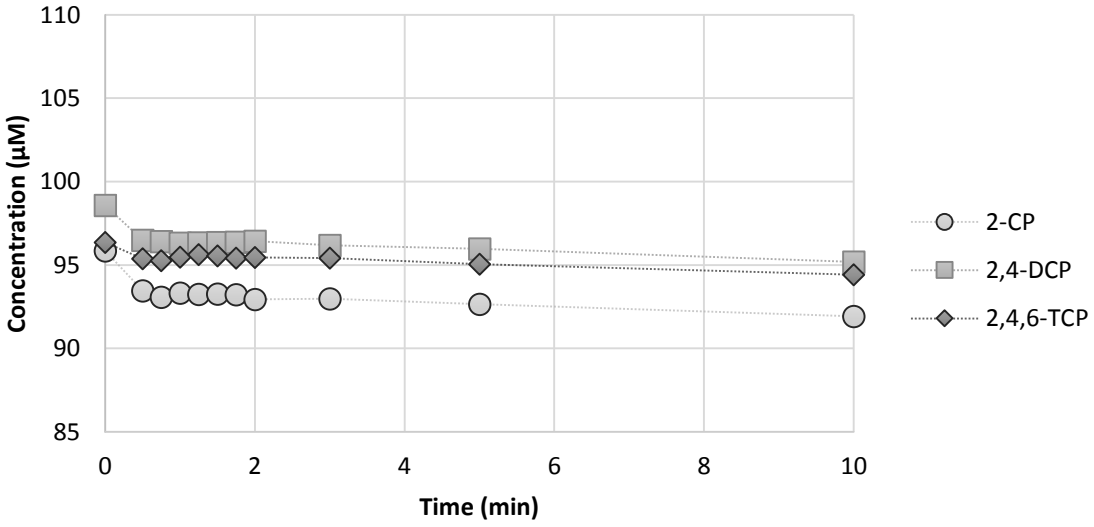


Figure 7-9: Control experiment without the mediator acetosyringone at pH 7

Note: Experiment was conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 100 µM 2-chlorophenol (2-CP), 100 µM 2,4-dichlorophenol (2,4-DCP), 100 µM 2,4,6-trichlorophenol (2,4,6-TCP).

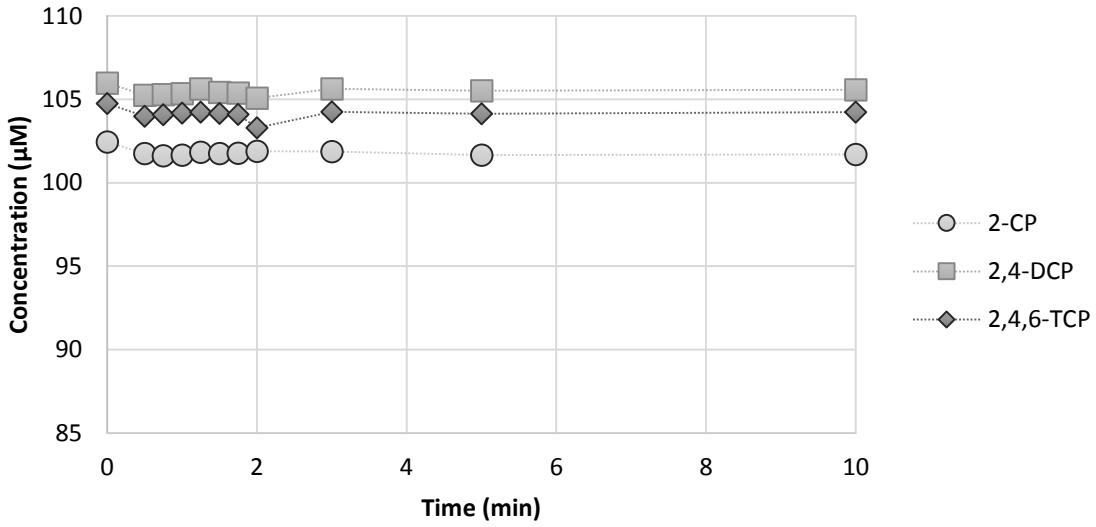


Figure 7-10: Control experiment without the mediator acetosyringone at pH 9.3

Note: Experiment was conducted in 5 mM borate buffer at pH 9.3 with the following initial conditions: 1 U/mL enzyme activity, 100 µM 2-chlorophenol (2-CP), 100 µM 2,4-dichlorophenol (2,4-DCP), 100 µM 2,4,6-trichlorophenol (2,4,6-TCP).

As is the case in most water treatment processes, pH is a parameter of great importance in enzymatic treatment. It is well known and well documented in the literature that pH has a large influence on the activity and stability of laccase enzymes (Almansa *et al.*, 2004; Camarero *et al.*, 2005; Kurniawati & Nicell, 2008), and different laccases have different optimum pH ranges. In some cases, laccase might not exhibit its highest activity and highest stability within the same pH range, which are important factors to consider in selecting a treatment pH. As mentioned above, the acid-base speciation of a phenolic mediator might influence its reactivity with the enzyme. It has also been documented that phenoxyl radicals, such as that of acetosyringone, are more or less stable as a function of pH (Camarero *et al.*, 2005). Although to a lesser extent than

with other oxidants, such as ozone and chlorine, this research shows that acid-base speciation affects the reactivities of target compounds in enzymatic treatment. Thus, for a target compound that speciates, pH plays an important role in essentially every reaction leading up to and including the oxidation of the target compound.

7.4. RELATIVE REACTIVITY OF CHLOROXYLENOL

As discussed previously, the electronic properties of the three model chlorophenols influenced their reactivities, and the effect was most evident in the competition kinetics experiment at pH 9.3. The antimicrobial chemical chloroxylenol was also investigated, serving as an example of a micropollutant with a chlorophenol group and as a compound structurally similar to the model chlorophenols, but with slightly different substitution of its aromatic ring (Table 3-1). It has one electron withdrawing chlorine atom and two electron donating methyl groups, making it more activated than the three model chlorophenols. Thus, chloroxylenol was expected to be more reactive than the model chlorophenols with ACE•.

Figure 7-11 shows the results of an experiment in which chloroxylenol was treated individually. Similarly to 2,4-dichlorophenol and 2,4,6-trichlorophenol, much less acetosyringone was consumed (approximately 19%) than chloroxylenol (100%), indicating regeneration of the mediator. This result is consistent with a steric effect on the reaction mechanism, and the resulting impact on mediator regeneration. The results suggest that with increasing substitution of the aromatic ring (*i.e.*, increasing steric hindrance to coupling), there is an increasing tendency for an electron transfer reaction mechanism between the phenolic target compound and phenoxyl mediator radical. Evidence supporting this pattern includes decreasing mediator consumption with increasing substitution of the phenol. 2,4-dichlorophenol, with two substituents,

exhibited higher mediator consumption (25%) than chloroxylenol (19%) and 2,4,6-trichlorophenol (8%), each with three substituents. The results indicate that the positions of the 2,4,6-trichlorophenol substituents were even more hindering to a coupling reaction than the positions of the chloroxylenol substituents (3,4,5).

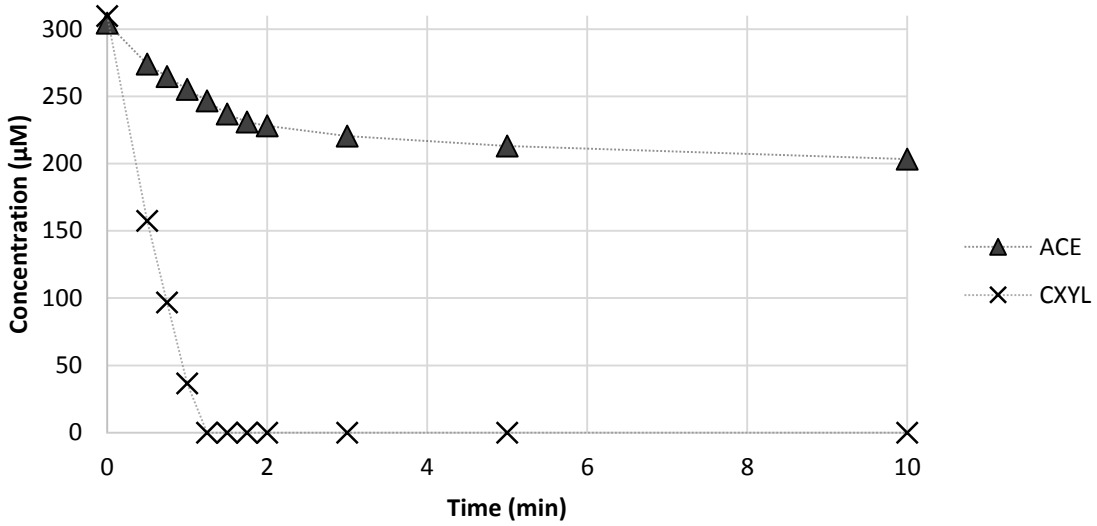


Figure 7-11: Individual treatment of CXYL by the laccase-ACE system at pH 7

Note: Experiment was conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone, 300 µM chloroxylenol (CXYL).

Figure 7-12 shows the results of an experiment in which chloroxylenol was treated in competition with 2-chlorophenol at pH 7, which is below both of their pK_a values. At pH 7, chloroxylenol (pK_a = 9.0) is 99.0% protonated, and 2-chlorophenol (pK_a = 8.3) is 95.2% protonated. Chloroxylenol exhibited a reactivity approximately three times that of 2-chlorophenol, and when compared to the competition kinetics experiment at pH 7 with the three model chlorophenols (Figure 7-4), chloroxylenol has the highest reactivity at pH 7. The primarily protonated (99.0%) chloroxylenol was more

reactive with ACE• than the primarily deprotonated (88.8%) 2,4,6-trichlorophenol that reacted to a larger extent by electron transfer. This can most likely be attributed to the electron donating methyl groups and resulting higher electron density of the chloroxylenol.

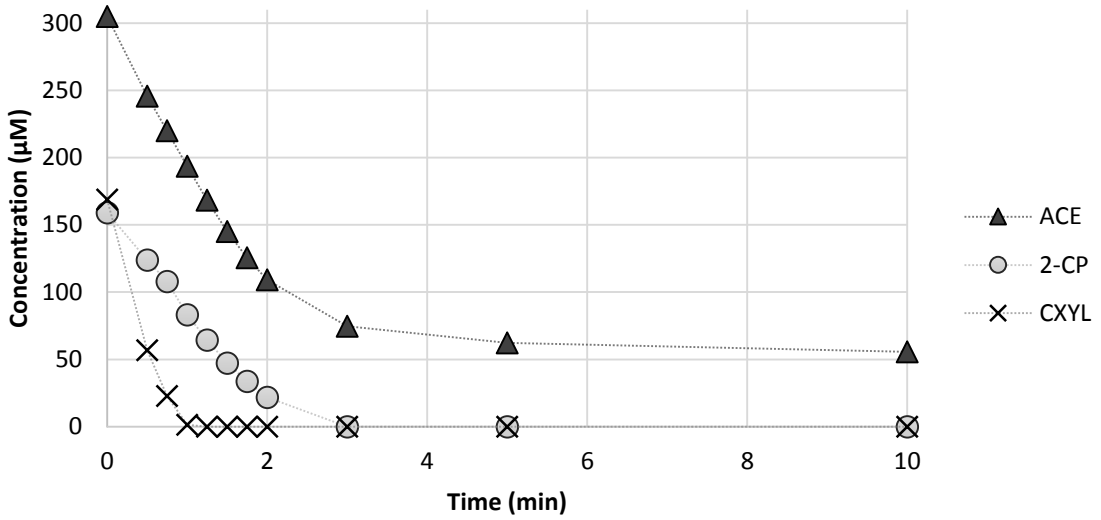


Figure 7-12: Competition kinetics experiment with CXYL and 2-CP at pH 7

Note: Experiment was conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone, 150 µM 2-chlorophenol (2-CP), 150 µM chloroxylenol (CXYL).

A control experiment without the mediator acetosyringone was also performed with chloroxylenol and 2-chlorophenol in competition at pH 7, the results of which are shown in Figure 7-13. Direct laccase oxidation of the two compounds was insignificant compared to the oxidation by the laccase-acetosyringone system. The direct oxidation of chloroxylenol seemed to be slightly faster than the oxidation of 2-chlorophenol, which could be due to its higher electron density. A correlation between the rate of laccase

oxidation of a substrate and the substrate's reduction potential has been reported by many researchers (*e.g.*, Tadesse *et al.*, 2008; Xu, 1996).

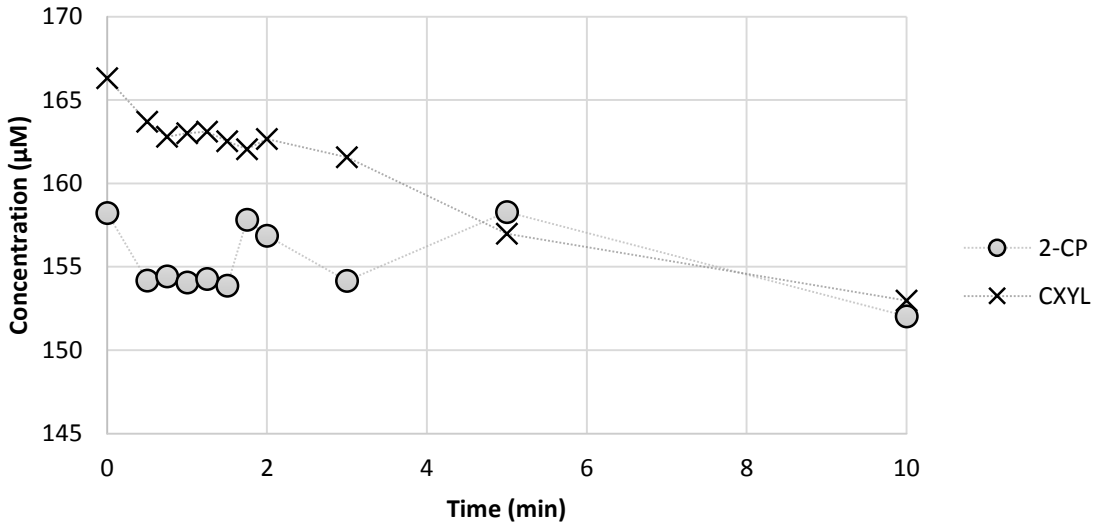


Figure 7-13: Control experiment without the mediator acetosyringone at pH 7

Note: Experiment was conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 150 µM 2-chlorophenol (2-CP), 150 µM chloroxylenol (CXYL).

7.5. CONCLUSIONS

It was hypothesized that the relative reactivities of the three model chlorophenol target compounds and the micropollutant chloroxylenol with the mediator radical ACE• would depend on their electronic properties. Specifically, they were expected to exhibit decreasing reactivity with decreasing electron density in their aromatic rings (*i.e.*, with increasing chlorination of the ring). Although some influence of their electronic properties was evident, other factors were also very influential in determining the relative reactivities of this set of compounds. The mechanism by which the chlorophenol reacted with ACE•, which appeared to be determined by the presence or absence of sterically

hindering substituents on the aromatic ring, as well as the acid-base speciation of the chlorophenol, seemed to be important factors in determining a compound's reactivity with ACE•. The influence of acid-base speciation would not be of importance for all potential target compounds, as many organic micropollutants do not have acid-base properties, or are acids that do not speciate near the pH range relevant in water and wastewater treatment. When considering the suitability of enzymatic treatment with a laccase-phenolic mediator system for a particular target compound or class of compounds, the likely reaction mechanisms, electronic properties, and acid-base properties of the target compounds are all factors that must be taken into account.

Chapter 8: Kinetic Modeling

8.1. INTRODUCTION

In Phase 3, qualitative comparison of the observed oxidation rates of both the mediator acetosyringone and the target compound 2-chlorophenol revealed several kinetic and mechanistic behaviors of the laccase-acetosyringone-2-chlorophenol system. In Phase 4, observed differences in the reactivities of the chlorophenolic target compounds with the laccase-acetosyringone system could be explained quite well qualitatively. However, to further probe the reaction kinetics and mechanisms of this system and verify the conclusions drawn to this point, a more quantitative description of the reaction kinetics is necessary. Thus, a set of chemical reactions was hypothesized based on the understanding of the experimental results, and the software KinTek Explorer (Version 5.0, KinTek Corporation) was used to fit the kinetic rate constants of the hypothesized reactions using selected experimental data.

8.2. MODEL DEVELOPMENT

The kinetic model consists of a series of reactions and their rate constants, which are shown in Table 8-1. The conceptual reaction model for enzymatic treatment outlined in Equation 6-1 through Equation 6-5 was used as a starting point for model development (Reactions 1, 2, 3, 13, and 15 in Table 8-1), but several reactions were added to better represent the behaviors observed in the experiments. Specifically, Reaction 16 was added to account for the enzyme activity losses observed in experiments with acetosyringone:chlorophenol molar ratios greater than one. Although the mechanism of enzyme activity loss has not been confirmed, it appeared to be related to excess acetosyringone radical (ACE•), and it was therefore modeled as a reaction between the enzyme and ACE•. Based on observed mediator regeneration in Phase 4 experiments

with 2,4-dichlorophenol (2,4-DCP) and 2,4,6-trichlorophenol (2,4,6-TCP), two possible reaction pathways for each of these compounds with ACE• were included, one in which ACE• was consumed (Reactions 4 and 6), and one in which ACE• was reduced back to the mediator ACE (Reactions 5 and 7). Separate reactions were input for ACE• oxidation of the chlorophenols at pH 7 and at pH 5 to fit apparent rate constants at each pH. Reaction 14 was added to the model because of reported coupling between ACE• and neutral ACE by Martorana *et al.* (2013). It has also been reported that the first electron transfer from a phenol is the rate limiting step in laccase oxidation of a phenolic substrate (Khlifi-Slama *et al.*, 2012; Tadesse *et al.*, 2008; Xu, 1996), meaning that the binding and reduction of oxygen by the laccase enzyme are not rate limiting. The model therefore assumes that the enzyme always has oxygen bound and ready to accept electrons. It was also assumed that all of the reactions, except for the enzyme binding a substrate (Reaction 1), were essentially irreversible. Hence, their reverse rate constants were constrained to zero.

The kinetic model should theoretically consist of only elementary reactions, but some mechanistic information is still missing, making some assumptions and simplification necessary. Accordingly, some of the included reactions are not elementary reactions, but most likely reflect a more complex set of reactions that might involve several steps. This situation applies to the coupling reactions (Reactions 3, 4, and 6). The exact mechanism of coupling between ACE• and the target chlorophenols is unknown and could possibly happen via (at least) two different routes. The coupling could occur between ACE• and the neutral target chlorophenol, as currently written in the model, forming a coupling product that is also a radical, which would then undergo further transformation to a stable product via steps that are not included in the model. Similarly, the electron transfer reactions included in the model (Reactions 5 and 7) show

the formation of the regenerated mediator ACE and a transformation product. In reality, the transformation product would first be a chlorophenol radical, which would then undergo further transformation to a stable product via steps that are not included in the model.

Alternatively, the coupling could occur in two steps, with the first step being an electron transfer from the target chlorophenol to the ACE•, creating a chlorophenol radical that then couples with another ACE•. In this case, an electron transfer reaction would always occur between ACE• and the target chlorophenol, after which two possible further reaction pathways could be followed by the generated chlorophenol radical. It could react via a coupling pathway, in which it would couple with an ACE•, resulting in ACE• consumption. It could otherwise further react with other reaction mixture components, such as oxygen, to stable products without consuming ACE•. Further research would be necessary to determine the mechanism of coupling between ACE• and the target chlorophenols, but the current model formulation still represents the distribution of ACE• between the two oxidation pathways (coupling resulting in ACE• consumption and electron transfer without further coupling or ACE• consumption).

Reaction 15 was intended to represent intramolecular rearrangement of ACE• to form a stable transformation product such as 2,6-dimethoxybenzoquinone. This pathway has been proposed by several researchers (*e.g.*, Rosado *et al.*, 2012) based on the measurement of this transformation product in the laccase oxidation of acetosyringone and other syringyl type mediators. However, other unknown mechanisms of radical loss that are not currently included in the kinetic model could also be included in the rate constant fit for Reaction 15.

Table 8-1: Kinetic model reactions and fitted relative rate constants

Reaction #	Reaction	k_f ($\mu M^{-1}s^{-1}$)	k_r ($\mu M^{-1}s^{-1}$)
1	$EO + ACE \leftrightarrow EOACE$	1	165
2	$EOACE \leftrightarrow EO + ACE\bullet$	21.7	0
3	$ACE\bullet + MCP \xleftrightarrow{k_{app-pH\,7}} P_1$	100	0
4	$ACE\bullet + DCP \xleftrightarrow{k_{app-pH\,7}} P_2$	93.6	0
5	$ACE\bullet + DCP \xleftrightarrow{k_{app-pH\,7}} P_3 + ACE$	414	0
6	$ACE\bullet + TCP \xleftrightarrow{k_{app-pH\,7}} P_4$	0	0
7	$ACE\bullet + TCP \xleftrightarrow{k_{app-pH\,7}} P_5 + ACE$	332	0
8	$ACE\bullet + MCP \xleftrightarrow{k_{app-pH\,5}} P_1$	19.7	0
9	$ACE\bullet + DCP \xleftrightarrow{k_{app-pH\,5}} P_2$	1.13	0
10	$ACE\bullet + DCP \xleftrightarrow{k_{app-pH\,5}} P_3 + ACE$	42.6	0
11	$ACE\bullet + TCP \xleftrightarrow{k_{app-pH\,5}} P_4$	0	0
12	$ACE\bullet + TCP \xleftrightarrow{k_{app-pH\,5}} P_5 + ACE$	145	0
13	$ACE\bullet + ACE\bullet \leftrightarrow P_6$	27.1	0
14	$ACE\bullet + ACE \leftrightarrow P_7$	0	0
15	$ACE\bullet \leftrightarrow P_8$	1320	0
16	$ACE\bullet + EO \leftrightarrow EO_{inactivated}$	197	0

Note: EO = enzyme with bound oxygen; ACE = acetosyringone; EOACE = enzyme-acetosyringone complex; ACE• = acetosyringone radical; MCP = 2-chlorophenol; DCP = 2,4-dichlorophenol; TCP = 2,4,6-trichlorophenol; P_n = transformation product

Fitting of the model rate constants was performed with the experimental data from five of the Phase 4 experiments, including the three experiments in which each of the three model chlorophenols was treated individually at pH 7 (Figure 8-1) and the competition kinetics experiments at pH 7 and pH 5 (Figure 8-2). Although the same amount of laccase was added to the experiments at pH 7 and pH 5, the enzyme was more active at pH 5, as shown in Figure 7-7. This difference in apparent enzyme activity was accounted for by a proportional increase in the initial enzyme concentration entered in the model at pH 5. The rate constants of the first two reactions (the binding and oxidation of ACE by laccase) were manually adjusted to a best fit by eye; the program displays the experimental data and the simulated model curves simultaneously and allows the user to scroll any rate constant and immediately see the changes to the simulated model curves. Then, the rate constants of these first two reactions were constrained, and the remaining unconstrained rate constants were fit to the selected data using the fitting tool of the software, which is based on simultaneous numerical integration of all of the rate equations (Johnson *et al.*, 2009a; Johnson *et al.*, 2009b). All of the data selected for fitting was weighted equally.

The kinetic model indicates that the enzyme oxidation of acetosyringone is the rate limiting step at the enzyme activities tested. Thus, the other rate constants seem to represent the distribution of the radicals generated, but not the true rate constants of these reactions in absolute terms. This conclusion was established by proportionally increasing the rate constants of Reactions 3 through 16. As long as the proportionality among them is conserved, these radical reaction rate constants can be increased to the upper limit of rate constants (diffusion controlled) without any effect on the fit of the model to the data.

8.3. SPECIES SPECIFIC RATE CONSTANTS

The relative apparent rate constants (k_{app}) fit at pH 5 and pH 7 and shown in Table 8-1 were used to calculate second order species specific rate constants for the reactions of ACE• with the protonated (k_{PhOH}) and deprotonated (k_{PhO^-}) model chlorophenols. As discussed previously, these rate constants cannot be compared in absolute terms, but can be compared in relative terms. The calculated values are shown in Table 8-2 and were calculated using the rate expression shown below, where Ph represents a phenolic compound.

$$\frac{dC_{Ph}}{dt} = -k_{app}C_{Ph}C_{ACE\bullet} = -k_{PhOH}C_{PhOH}C_{ACE\bullet} - k_{PhO^-}C_{PhO^-}C_{ACE\bullet}$$

$$\therefore k_{app} = k_{PhOH}\alpha + k_{PhO^-}(1-\alpha)$$

Table 8-2: Calculated relative species specific rate constants in generic units of concentration^{-1}time^{-1}

Compound	Reaction mechanism	k_{PhOH} ($C^{-1}t^{-1}$)	k_{PhO^-} ($C^{-1}t^{-1}$)
2-CP	Coupling	18	1727
2,4-DCP	Coupling	0	685
2,4-DCP	Electron transfer	37	2788
2,4-DCP	Both	37	3473
2,4,6-TCP	Electron transfer	128	358

These calculated relative species specific rate constants reinforce in a more quantitative way some of the observations regarding the Phase 4 experimental results. For example, it can be clearly seen that the expectation and assumption that the phenolate

species are more reactive than the protonated phenols was correct. As is also evident in the apparent rate constants, the electron transfer mechanism is much faster than the coupling mechanism. The existence of these two possible reaction pathways and their differing importance for each of the three model chlorophenols confounds the relationship between the electronic properties of the chlorophenols and their apparent reaction rates. For instance, 2-CP is the least deactivated phenol, but appears to be less reactive than 2,4-DCP because 2,4-DCP reacts primarily by the faster electron transfer mechanism. When comparing the rate constants for reactions that occur via the same mechanism, the expected effect of the target compounds' electronic properties can be observed. For reactions via the coupling mechanism, 2-CP has higher rate constants than 2,4-DCP, and for reactions via the electron transfer mechanism, 2,4-DCP has higher rate constants than 2,4,6-TCP.

The differences between the calculated rate constants for the protonated and deprotonated species are less pronounced than those found for the reactions of other common oxidants with phenolic compounds. For the oxidation of phenol or phenolate by chlorine, chlorine dioxide, or ozone, the species specific rate constants differ by five to eight orders of magnitude (Lee & von Gunten, 2012). It is typical that radical oxidation reactions are less sensitive to acid-base speciation, and the smaller observed differences between k_{PhOH} and k_{PhO^-} could indicate that the rate constants are quite high. If the rate constant for the oxidation of the protonated phenol is already high, the oxidation of the phenolate species can only be so much faster, with diffusion limitation providing an upper limit. When comparing the k_{PhO^-} of two of the model chlorophenols reacting via the same mechanism, those of 2-CP and 2,4-DCP differ by an order of magnitude, as do those of 2,4-DCP and 2,4,6-TCP, which is comparable to the differences observed between the k_{PhO^-} for chlorine oxidation of these compounds (Lee & von Gunten, 2012).

8.4. MODEL FIT AND VALIDATION

Figure 8-1 and Figure 8-2 show the experimental data and simulated model curves for the experiments used to fit the kinetic rate constants in the model. The model generally fit these experimental data quite well, especially the individual treatments of 2,4-DCP and 2,4,6-TCP. Figure 8-3 shows the experimental data and simulated model curves for a second competition kinetics experiment performed at pH 7 with higher reactant concentrations than the experiments used in the model parameter fitting. The model captures the behavior of this system, successfully simulating the correct order of chlorophenol reactivities.

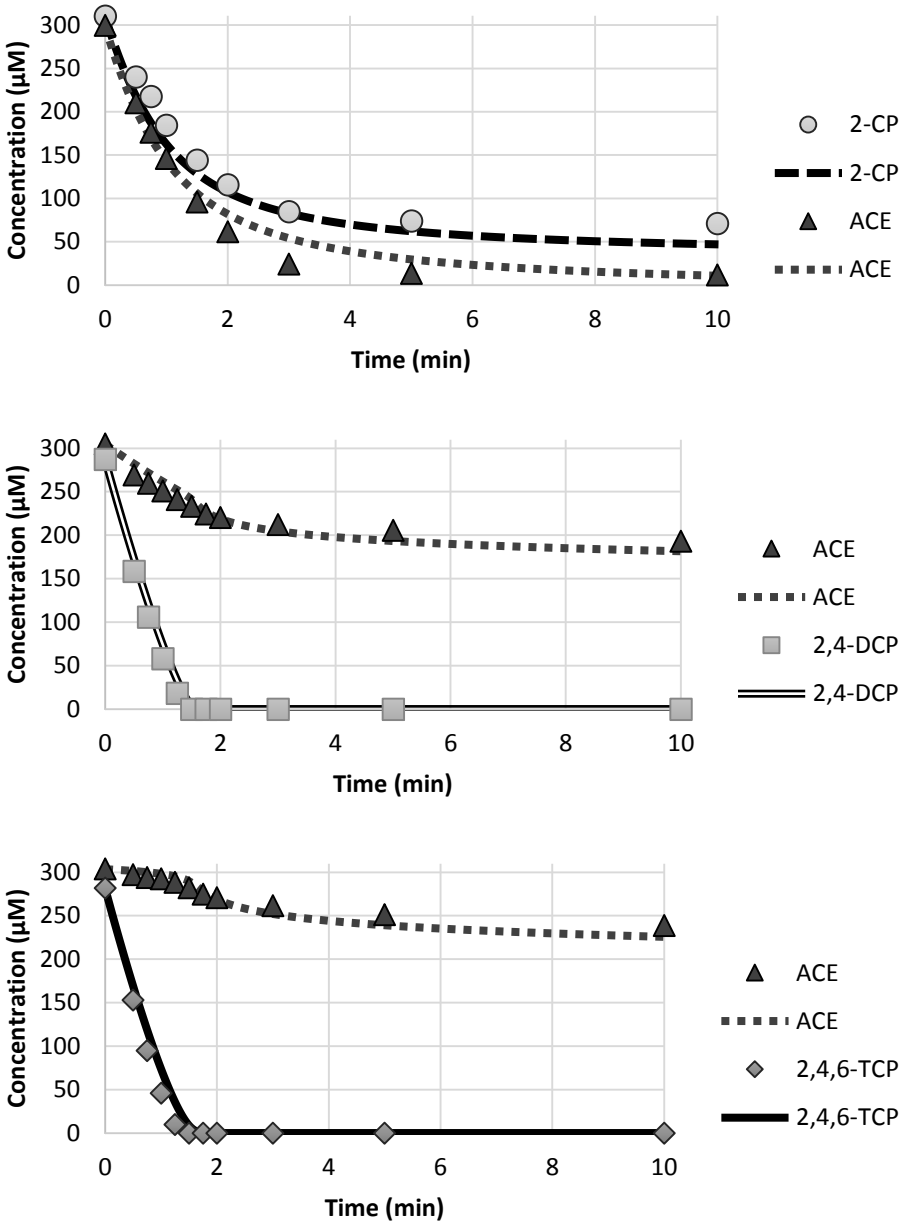


Figure 8-1: Experimental data (points) and model simulations (lines) of individual chlorophenol experiments at pH 7 used in kinetic model rate constant fitting

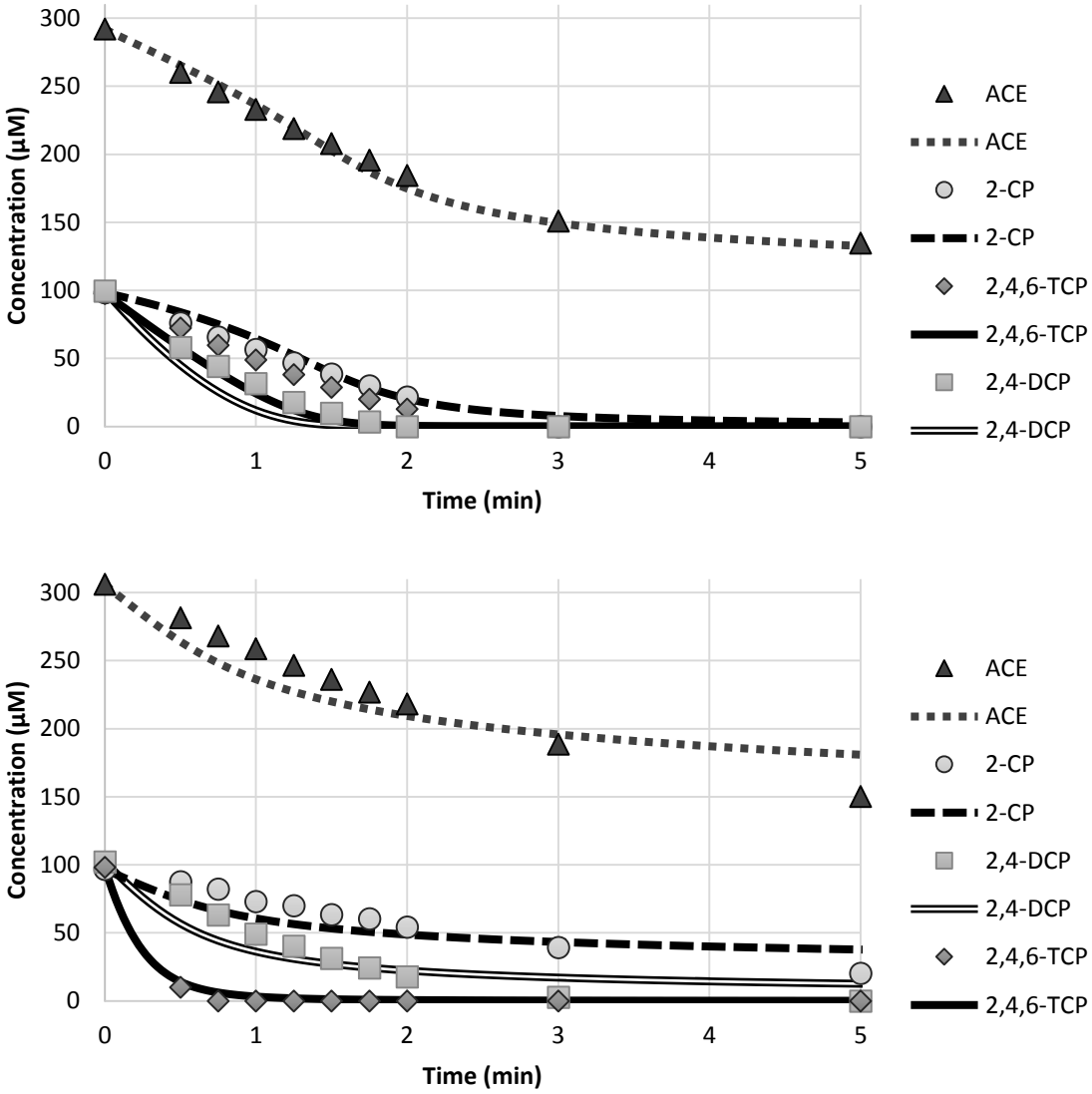


Figure 8-2: Experimental data (points) and model simulations (lines) of competition kinetics experiments at pH 7 (above) and pH 5 (below) used in kinetic model rate constant fitting

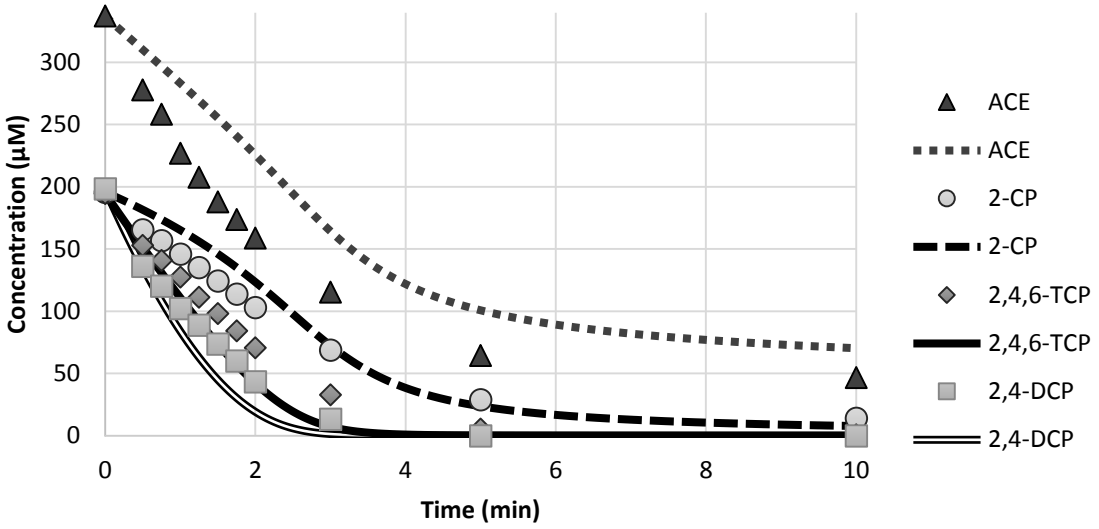


Figure 8-3: Experimental data (points) and model simulations (lines) of a second competition kinetics experiment at pH 7

Note: Experiment was conducted in 5 mM sodium phosphate buffer at pH 7 with the following initial conditions: 1 U/mL enzyme activity, 300 µM acetosyringone (ACE), 200 µM 2-chlorophenol (2-CP), 200 µM 2,4-dichlorophenol (2,4-DCP), 200 µM 2,4,6-trichlorophenol (2,4,6-TCP).

Although the model does a fairly good job of predicting the oxidation rates observed in Phase 3 experiments, it slightly underestimates the ACE oxidation rates (as in Figure 8-1:a), slightly overestimates the 2-CP oxidation rates with 1 U/mL enzyme activity (Figure 8-4), and significantly underestimates both oxidation rates at enzyme activities of less than 1 U/mL (Figure 8-5). The distribution of radicals generated, primarily represented by the rate constants for Reactions 3, 15, and 16 in Table 8-1 (*i.e.*, ACE• reacting with 2-CP, decaying, or causing enzyme activity loss), does not fit the data as well in the experiments performed with enzyme activities of less than 1 U/mL and proportionately lower concentrations of ACE and 2-CP. The results of two experiments performed at a lower enzyme activity (0.2 U/mL), but with higher initial ACE and 2-CP

concentrations (Experiments 6 and 7 in Phase 3), are fit better by the model, as can be seen in Figure 8-6.

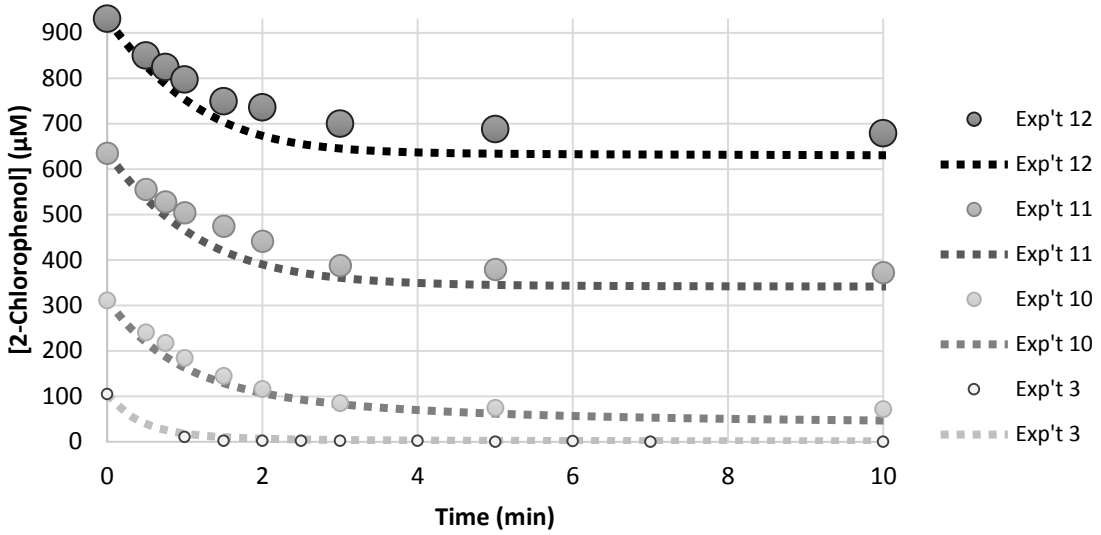


Figure 8-4: Experimental data (points) and model simulations (lines) of 2-chlorophenol oxidation in Experiments 3, 10, 11, and 12 from Phase 3

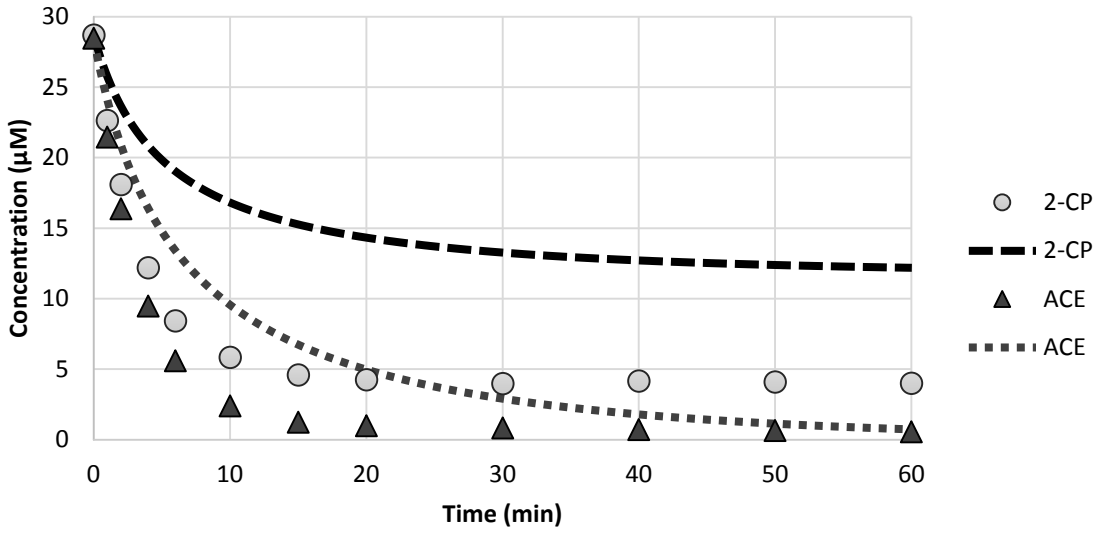


Figure 8-5: Experimental data (points) and model simulations (lines) of acetosyringone (ACE) and 2-chlorophenol (2-CP) oxidation in Experiment 9 from Phase 3

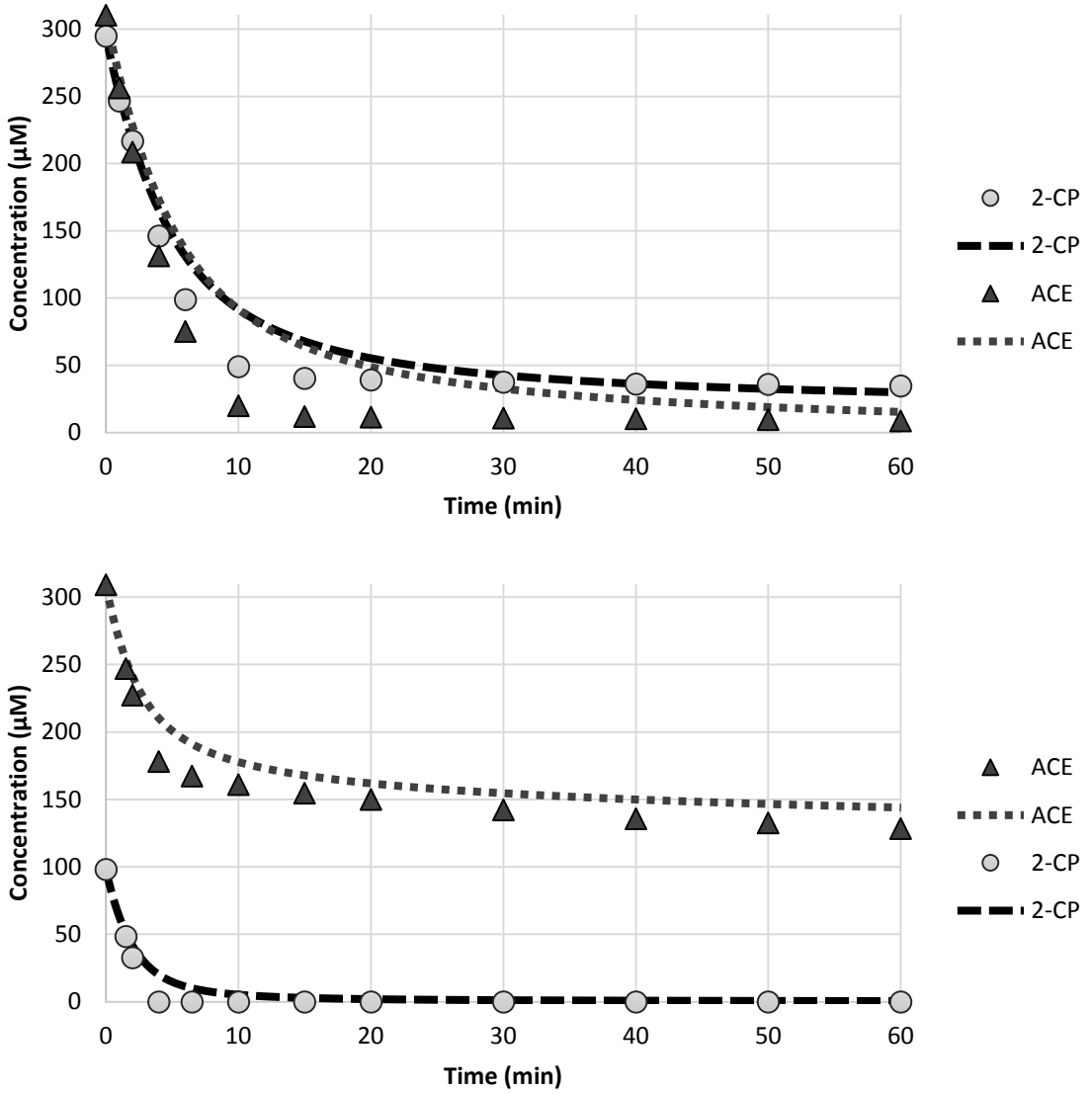


Figure 8-6: Experimental data (points) and model simulations (lines) of acetosyringone (ACE) and 2-chlorophenol (2-CP) oxidation in Experiments 6 (above) and 7 (below) from Phase 3

The problems with the model fit in experiments with lower initial enzyme activities and reactant concentrations are most likely related to Reaction 16 in the model, which accounts for the observed enzyme activity losses. It appears that this reaction does not represent the true mechanism of enzyme activity loss. Although the way this reaction

is modeled does a good job of accounting for the effect of enzyme activity loss on the oxidation rates (*e.g.*, in Experiments 1, 2, and 3 in Phase 3), it does not correctly account for the actual enzyme activity losses observed. In fact, it simulates that enzyme activity was lost in every experiment, which could explain the underestimation of acetosyringone oxidation rates. Modeling enzyme activity loss as a reaction between the enzyme and the coupling product of Reaction 13 in the model, or as a reaction between the enzyme and the product of Reaction 15 (radical decay), did not produce better results.

8.5. CONCLUSIONS

For the laccase-acetosyringone-chlorophenolic target compound system, the rate limiting step was found to be the oxidation of the mediator by the enzyme. The relative rate constants fit for the reactions of the resulting mediator radical, although not rate limiting, were important in determining the distribution of the radicals among several possible reaction pathways. The species specific rate constants calculated for the oxidation of the target chlorophenols confirmed that the acid-base speciation of a target compound is a critical factor in determining its reactivity with the mediator radical at a given pH. The deprotonated forms of the three chlorophenol target compounds tested were more reactive than their protonated counterparts, a pattern which has also been observed with many other oxidants (*e.g.*, chlorine, chlorine dioxide, and ozone). Such acid-base speciation would not be important for all potential target compounds, as many organic micropollutants do not have acid-base properties, or are acids that do not speciate near the pH range relevant to water and wastewater treatment.

The proposed kinetic model explains the experimental results to date quite well, suggesting that the set of posited reactions must capture the primary reaction pathways of the system. However, more reactions are likely to be occurring in this system than are

currently included in the model, such as the oxidation of transformation products by the laccase enzyme and reactions of the resulting radicals. Further validation of the model will be required, especially regarding the mechanism of enzyme activity loss (Reaction 16 in Table 8-1), which is currently not well understood. Identification of the transformation products and a kinetic study of their formation would be useful steps for validating and expanding the kinetic model.

Perhaps the most notable revelation of these kinetic experiments is the difference in the length of reaction time between experiments in a pure buffered system and experiments in primary effluent. In the Phase 1 experiments with an initial enzyme activity of 1 U/mL and an initial ACE concentration of 87.6 μM, oxybenzone oxidation continued for up to two hours in primary effluent with an initial pH of 6, whereas the kinetic experimental results and model show that that amount of acetosyringone would be completely depleted in a few minutes in the pure buffered system, assuming there was no enzyme activity loss. Of course, the rate constant for oxybenzone oxidation would be different than that for 2-CP oxidation, but for the transformation to have continued for hours, there must have been oxidants present in the reaction mixture for far longer than a few minutes. The question remains whether it was ACE• or some other oxidant that was transforming the micropollutants at the later time points in the experiments in primary effluent. If it was ACE•, then the matrix must have had a significant impact on the enzyme kinetics determining its rate of production, and/or the mediator ACE must have been regenerated. If it was not ACE•, then components of the matrix must have participated in radical cascade reactions. Investigation of this phenomenon should be a priority in future enzymatic treatment research.

Chapter 9: Conclusions and Recommendations

This research investigated the application of enzymatic treatment of organic micropollutants in primary effluent and the kinetics and mechanisms of the reactions involved in this treatment process. The enzyme-mediator system employed in this research included a commercially available laccase from the Ascomycota fungus *Myceliophthora thermophila* and the naturally occurring phenolic mediator acetosyringone.

In the first two study phases, enzymatic treatment of environmentally relevant concentrations (10 μg/L) of two micropollutants, oxybenzone and sulfamethoxazole, was tested in batch and continuous flow experiments in a primary effluent matrix to investigate the influence of treatment conditions on treatment efficiency. The results demonstrated that enzymatic treatment can transform low concentrations of micropollutants in a complex background matrix with high organic content. Many laccases require acidic conditions and are not very active or stable at the circumneutral pH values relevant to water and wastewater treatment, but this particular laccase was able to transform oxybenzone and sulfamethoxazole in primary effluent at slightly basic pH values. Mediator concentration and the organic content of the wastewater matrix were found to have a significant influence on enzymatic treatment efficiency. Relatively high mediator concentrations were required to achieve significant micropollutant transformation, and treatment in a primary effluent with higher organic content required a higher concentration of mediator, indicating that mediator radicals generated are (not surprisingly) scavenged by the matrix. While the addition of mediators to the enzymatic treatment process expands the range of micropollutants that can be treated, this expansion is a result of the much lower degree of specificity of the mediator radical as compared to

the active site of the enzyme, which naturally leads to unintended side reactions with matrix components.

A better understanding of the kinetics and mechanisms of the reactions involved in enzymatic treatment was deemed essential for several reasons. First, to be able to further investigate the matrix effects observed, a well-defined kinetic and mechanistic understanding of the system of interest, the laccase-mediator-target compound system, is necessary. Furthermore, an understanding of how the reaction kinetics, and therefore the efficiency of the treatment process, can be expected to change for various target compounds would be useful for further development of this treatment process intended to transform a wide variety of organic micropollutants. Thus, Phases 3 and 4 of this research focused on the kinetics of enzymatic treatment by investigating a model laccase-mediator-target compound system with structurally related chlorophenolic target compounds in a simplified buffer solution.

The results of the kinetic experiments demonstrated that the kinetics of the laccase-acetosyringone-chlorophenol system are complex and non-linear. Despite the complexity of the system, a kinetic model was developed and fit the experimental data reasonably well. The experimental and modeling results revealed several findings regarding the kinetics and mechanisms of enzymatic treatment reactions and the influence of target compound structure. Based on the experimental observations and kinetic model results, the following conclusions can be drawn:

- In experiments with molar ratios of acetosyringone to target compound greater than one, significant enzyme activity was lost. The exact mechanism of this enzyme activity loss has not been confirmed, but appears to be related to excess acetosyringone radicals. In a real treatment system, enzyme activity loss by the mechanism occurring in these kinetic experiments might not be of concern, as

other matrix components present would likely quench excess radicals relatively quickly. Under similar experimental conditions, but with a different laccase enzyme, such enzyme activity losses were not observed in a related study by Margot *et al.* (2015), indicating that excess radicals might not have the same effect on all laccase enzymes.

- For the laccase-acetosyringone-chlorophenolic target compound system, the rate limiting step was the oxidation of the mediator by the enzyme. The relative rate constants for the reactions of the resulting mediator radical, although not rate limiting, were important in determining the distribution of the radicals among several possible reaction pathways.
- The structure of the target compound, specifically its steric hindrance to coupling with the acetosyringone phenoxyl radical, determined the relative influences of two potential reaction mechanisms: one in which the mediator radical and target compound cross-couple, thereby consuming the mediator; and one in which an electron is transferred to the mediator radical, thereby regenerating the mediator and oxidizing the target compound.
- As was expected, the electronic properties of the target compounds influenced their relative reactivities with the mediator radical. Increasing electron density in the aromatic ring of the target chlorophenol, as determined by the presence of electron donating or electron withdrawing substituents on the ring, correlated with increasing reactivity via a given reaction mechanism.
- The acid-base speciation of a target compound was an important factor in determining its reactivity with the mediator radical at a given pH. The deprotonated forms of the three chlorophenol target compounds tested were more reactive than their protonated counterparts, a pattern which has also been

observed with many other oxidants (*e.g.*, chlorine, chlorine dioxide, and ozone). Such acid-base speciation would not be important for all potential target compounds, as many organic micropollutants do not have acid-base properties, or are acids that do not speciate near the pH range relevant to wastewater treatment.

- For the chlorophenolic target compounds investigated in this research, the influence of their electronic properties was less important than the influences of their reaction mechanisms with the mediator radical and their acid-base speciation at neutral pH. When considering the suitability of enzymatic treatment with a laccase-phenolic mediator system for a particular target compound or class of compounds, the likely reaction mechanisms, electronic properties, and acid-base properties of the target compounds are all important factors that must be considered.
- The proposed kinetic model fit the experimental results to date reasonably well, suggesting that the set of posited reactions must capture the primary reaction pathways of the system. However, further development and validation of the model will be required, especially regarding the mechanisms of coupling between the mediator radical and target chlorophenols and enzyme activity loss, which are currently not well understood. Identification of the transformation products and a kinetic study of their formation would be useful steps for validating and expanding the kinetic model.
- In experiments with similar enzyme activity, initial mediator concentration, and pH, micropollutant transformation was observed to continue for much longer periods of time in primary effluent (two hours) than in the pure buffered kinetic experiments (two minutes). This observation indicates that (1) the primary effluent matrix significantly impacted the enzyme kinetics determining the rate of

mediator radical production, and mediator radicals were generated slowly for two hours, (2) the mediator was regenerated despite the high organic content of the primary effluent, or (3) the primary effluent matrix facilitated radical cascade reactions. A systematic and quantitative investigation of the effects of wastewater matrices on enzymatic treatment reactions should be a priority in future enzymatic treatment research.

This research provided significant insights that will guide further development and improvement of enzymatic treatment processes for the oxidation of organic micropollutants in municipal wastewater. The transformation of environmentally relevant concentrations of organic micropollutants in unaltered, unfiltered primary effluent by the laccase-mediator system was a novel demonstration, and the examination of the influences of treatment conditions highlighted areas where improvements could be made and where future research should focus. In particular, matrix effects, other mediator loss mechanisms, and mediator selection should be explored further. This research subsequently investigated the kinetics and mechanisms of the reactions involved in enzymatic treatment. The experimental evidence of several influences of target compound structure on target reactivity with the laccase-mediator system and the development of a kinetic model are significant steps towards understanding the complex and non-linear kinetics of this system. Further confirmation and expansion of the kinetic model, especially with respect to reactions with an organic matrix, would provide additional insight to allow optimization of the enzymatic treatment process. Enzymatic treatment for micropollutant abatement is still in its early stages of development, but the results indicate that this process could one day be implemented in wastewater treatment plants. Once optimized, it should be compared to other available micropollutant treatment technologies in terms of energy consumption, placement within a treatment

plant, and reduction of estrogenicity and toxicity (accounting for oxidation byproduct and transformation product formation) to determine its viability for this application. Thus far, enzymatic treatment has shown promise as a potentially suitable technology for mitigating micropollutant release into the environment via treated wastewater discharges.

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