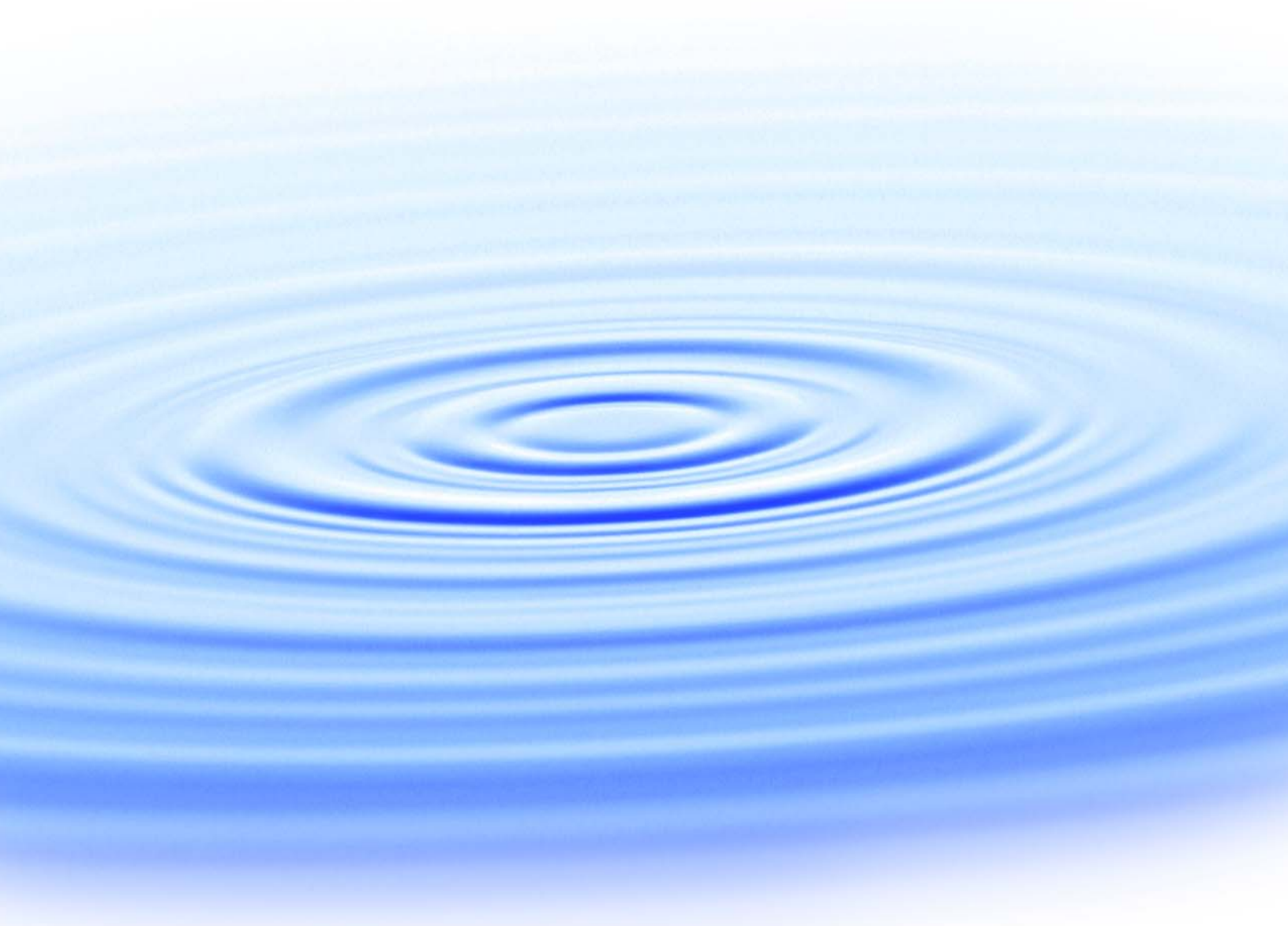
Enzymes: The New Wastewater Treatment Chemical for Water Reuse



WateReuse Research Foundation

Enzymes: The New Wastewater Treatment Chemical for Water Reuse

About the WateReuse Research Foundation

The mission of the WateReuse Research Foundation is to conduct and promote applied research on the reclamation, recycling, reuse, and desalination of water. The Foundation's research advances the science of water reuse and supports communities across the United States and abroad in their efforts to create new sources of high quality water for various uses through reclamation, recycling, reuse, and desalination while protecting public health and the environment.

The Foundation sponsors research on all aspects of water reuse, including emerging chemical contaminants, microbiological agents, treatment technologies, reduction of energy requirements, concentrate management and desalination, public perception and acceptance, economics, and marketing. The Foundation's research informs the public of the safety of reclaimed water and provides water professionals with the tools and knowledge to meet their commitment of providing a reliable, safe product for its intended use.

The Foundation's funding partners include the supporters of the California Direct Potable Reuse Initiative, Water Services Association of Australia, Pentair Foundation, and Bureau of Reclamation. Funding is also provided by the Foundation's Subscribers, water and wastewater agencies, and other interested organizations.

Enzymes: The New Wastewater Treatment Chemical for Water Reuse

Principal Investigators
Desmond F. Lawler
Kerry A. Kinney
The University of Texas at Austin

Cosponsors
Bureau of Reclamation

WateReuse Research Foundation
Alexandria, VA

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For more information, contact:

WateReuse Research Foundation
1199 North Fairfax Street, Suite 410
Alexandria, VA 22314
703-548-0880
703-548-5085 (fax)
www.WateReuse.org/Foundation

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Acronyms

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AOPs	advanced oxidation processes
BOD	biological oxygen demand
CFSTR	continuous flow stirred-tank reactor
DA	direct addition
DBPs	disinfection byproducts
DO	dissolved oxygen
EPA	Environmental Protection Agency
FRG	free radical generator
GAE	gallic acid equivalents
LC/MS	liquid chromatography-mass spectrometry
LC/MS/MS	liquid chromatography-tandem mass spectrometry
PFR	plug flow reactor
PPCPs	pharmaceuticals and personal care products
SAR	South Austin Regional (wastewater treatment plant)
TSS	total suspended solids
U	unit of enzyme activity
UV	ultraviolet
WC	Walnut Creek (wastewater treatment plant)

Foreword

The WateReuse Research Foundation, a nonprofit corporation, sponsors research that advances the science of water reclamation, recycling, reuse, and desalination. The Foundation funds projects that meet the water reuse and desalination research needs of water and wastewater agencies and the public. The goal of the Foundation's research is to ensure that water reuse and desalination projects provide sustainable sources of high-quality water, protect public health, and improve the environment.

An Operating Plan guides the Foundation's research program. Under the plan, a research agenda of high-priority topics is maintained. The agenda is developed in cooperation with the water reuse and desalination communities including water professionals, academics, and Foundation subscribers. The Foundation's research focuses on a broad range of water reuse and desalination research topics including:

- Defining and addressing emerging contaminants, including chemicals and pathogens
- Determining effective and efficient treatment technologies to create 'fit for purpose' water
- Understanding public perceptions and increasing acceptance of water reuse
- Enhancing management practices related to direct and indirect potable reuse
- Managing concentrate resulting from desalination and potable reuse operations
- Demonstrating the feasibility and safety of direct potable reuse

The Operating Plan outlines the role of the Foundation's Research Advisory Committee (RAC), Project Advisory Committees (PACs), and Foundation staff. The RAC sets priorities, recommends projects for funding, and provides advice and recommendations on the Foundation's research agenda and other related efforts. PACs are convened for each project to provide technical review and oversight. The Foundation's RAC and PACs consist of experts in their fields and provide the Foundation with an independent review, which ensures the credibility of the Foundation's research results. The Foundation's Project Managers facilitate the efforts of the RAC and PACs and provide overall management of projects.

This project investigated the possibility of using laccase enzymes to initiate the oxidation of pharmaceuticals and personal care products (PPCPs) in the context of wastewater treatment. The specific compounds investigated were oxybenzone (a sunscreen ingredient) and sulfamethoxazole (an antibiotic), both of which have been found in natural waters downstream of wastewater treatment plant discharges. Oxybenzone could only be removed with the addition of another mediator compound, whereas sulfamethoxazole could be attacked directly by the laccase enzyme. Treatment was successful for these two compounds in primary effluent and was equally successful when the compounds were present together or individually. Although far more research would be needed before enzymatic treatment could be instituted at wastewater treatment plants, the results are quite promising in showing that the enzymatic treatment is virtually as successful in primary effluent as in uncontaminated water and in the finding that the use of a mediator can extend the possibility of this treatment methodology to compounds that cannot be removed by enzymes alone.

Doug Owen
Chair
WateReuse Research Foundation

Melissa Meeker
Executive Director
WateReuse Research Foundation

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Dr. Hector Garcia initiated the work that led to this project when he was a doctoral student in the Environmental and Water Resources Engineering (EWRE) program at the University of Texas, and we are particularly grateful for his creativity and leadership; Dr. Garcia is now on the faculty at IHE-UNESCO in Delft, Netherlands. Consultation by the principal investigators and project team with EWRE faculty members Lynn Katz, Mary Jo Kirisits, and Howard Liljestrand was helpful throughout the project. Laboratory manager and instrument specialist Charlie Perego was helpful in many ways throughout the project. Several graduate students in the EWRE program were also helpful with various details, particularly Syed Yasir Husain, Ijung Kim, Amanda van Epps, Arielle Mimouni, and Austin Weidner. Johnny Kim did his undergraduate thesis on the free radical generator concept and performed preliminary experiments on that idea. The project team thanks engineers and plant operators at the City of Austin Water and Wastewater Utility for their cooperation and help throughout the project.

Principal Investigators
Desmond F. Lawler
Kerry A. Kinney
The University of Texas at Austin

Project Team
Catherine M. Hoffman, *The University of Texas at Austin*
Margaret E. Sharkey, *The University of Texas at Austin*

Participating Agencies
City of Austin Water and Wastewater Utility

Project Advisory Committee
Bob Bastian, *U.S. EPA*
David Jenkins, *David Jenkins & Associates, Inc.*
Marco Aieta, *Carollo Engineers, Inc.*
Yuliana Porras, *Bureau of Reclamation*

Executive Summary

Conventional wastewater treatment plants do not effectively remove all pharmaceuticals and personal care products (PPCPs). As a result, some PPCPs enter the environment via treated wastewater discharge. Enzymatic treatment using the laccase-mediator system is a novel biochemical process that has been shown to effectively treat some PPCPs. This study investigates the efficacy of the laccase-mediator system to treat PPCPs using a process that can be easily implemented at an existing wastewater treatment plant. Enzymatic treatment will be most beneficial after primary sedimentation and before conventional biological treatment; unoxidized PPCPs and byproducts could have the opportunity for further degradation during biological treatment.

In this work, two enzymatic treatment configurations were studied, focused on the removal of two representative PPCPs—oxybenzone and sulfamethoxazole. A step-wise optimization process was used that alternately varied treatment conditions: pH, enzyme activity, mediator concentration, and reactor detention time. In the optimization process of each configuration, successful oxybenzone removal (~90%) was achieved in municipal primary effluent. In a direct comparison of treatment configurations, both resulted in similar percentage removals of oxybenzone. Therefore, the configuration with the simpler operation and reactor design was chosen for further study.

During the optimization process, several noteworthy conclusions were made that might have full-scale enzymatic treatment implications. Specifically, successful oxybenzone removal occurred at unadjusted pH and without aeration, but higher biological oxygen demand of the wastewater required higher mediator concentrations. Whereas the first finding would decrease enzymatic treatment costs, the latter would increase the costs associated with the mediator. Thus, an alternative mediator source, specifically one high in phenolic compounds, is desired. The use of wine as a surrogate of winery wastewater (which contains high concentrations of phenolics) was investigated and proved ineffective. Further investigation of alternative mediator sources is required.

Treatment of another PPCP, sulfamethoxazole, was less efficient (65% removal) than that of oxybenzone, but nevertheless, the considerable removal of this substantially different compound suggests that the laccase-mediator system might be suitable for other PPCPs. The most promising result of this work was the simultaneous treatment of both PPCPs, oxybenzone and sulfamethoxazole. Simultaneous treatment proved to be as effective as the treatment of each PPCP individually.

Chapter 1

Introduction

1.1 Background

Pharmaceuticals and personal care products (PPCPs) are chemical compounds used in medications and cosmetic goods. They have been detected in surface water, in groundwater, and even in drinking water throughout the world. The most common pathway in which PPCPs reach the environment is through treated wastewater discharge. PPCPs enter wastewater via human excretion, washing, and improper disposal and, depending on the chemical characteristics of the compound, can be resistant to degradation during conventional wastewater treatment. Common PPCPs detected in the environment include, but are not limited to, antibiotics, steroids, antidepressants, sunscreens, and fragrances.

PPCPs are engineered to be stable over time to ensure adequate dosage and to maintain their physical, chemical, and microbiological properties during usage and storage. Even though some PPCPs do not have significantly long half-lives, their constant loading into the sewage system, and therefore into the aquatic environment, maintains their constant presence. PPCPs are therefore defined as "pseudo-persistent" (Daughton, 2004) and have potential ecological impacts and even human health risks.

Several studies have looked at the risks that PPCPs pose to bacteria, algae, amphibians, and fish and have found evidence of toxic effects including antibiotic-resistant bacteria, compromised functions of algal cells, and possible developmental and reproductive effects in fish (Costanzo et al., 2005; Al Aukidy et al., 2012; Harada et al., 2008; Coronado et al., 2008; Dévier et al., 2011). Unfortunately, determining risk is difficult to do when considering effects on an entire ecosystem. The synergistic effects of multiple PPCPs and the broader impacts to human health are largely unknown. To reduce these risks to the aquatic environment and human health, advanced technology is needed to remove PPCPs from wastewater before their discharge to the natural environment and the contamination of drinking water sources.

The ubiquitous presence of PPCPs also deters public acceptance of water reuse for potable purposes. Water reuse is the process of reclaiming wastewater and making beneficial use of it. Reuse is becoming a reality for many municipalities that face increased water demands from a growing population, changes in weather patterns that are due to climate change, and the depletion of pristine water sources. If direct water reuse for potable purposes is to become a reality, municipal wastewater must be rigorously treated to remove PPCPs and other xenobiotics.

Current research is focused on finding a safe, efficient, and cost-effective method of removing PPCPs in wastewater treatment. Moreover, an effective treatment technology needs to be easily applicable to a wide range of existing wastewater treatment plants. Several technologies (e.g., ozonation, photocatalysis) have been proposed but do not provide degradation without producing potentially harmful byproducts. Activated carbon can remove PPCPs, but it only transfers them from one phase to another, leaving the question of ultimate disposal unanswered. These proposed treatment technologies, which are often used in

drinking water treatment as a polishing step, are remarkably expensive when considering the treatment of lower-quality wastewater.

However, a biochemical process using enzymes has recently been proposed to oxidize PPCPs. This novel enzymatic process might provide a feasible and efficient treatment of PPCPs that can be easily implemented at existing treatment plants. This research focuses on the use of the laccase enzyme in the presence of a mediator compound to degrade PPCPs in wastewater. The laccase-mediator system mimics the natural behavior of the white rot fungi: the fungi excrete the laccase enzyme and low-molecular-weight mediator compounds to begin a lignin degradation process. Lignin, a complex chemical polymer derived from wood, has similar chemical properties and structure to many PPCPs, specifically its resistance to degradation and its numerous aromatic functional groups. In nature, the laccase and mediator interact to generate free radicals. Free radicals are highly reactive chemical species with unpaired valence electrons that indiscriminately react with surrounding molecules to obtain stability. The generated free radicals then go on to oxidize lignin to begin the degradation process.

The goal of enzymatic treatment is to mimic the laccase-mediator process to degrade PPCPs in wastewater. If successful, enzymatic treatment will reduce the environmental impacts of PPCPs and also improve the safety and public acceptance of indirect and direct water reuse.

1.2 Objectives

The main objective of this research is to capitalize on the natural laccase-mediator system in the treatment of PPCPs in wastewater. Enzymatic treatment was simulated at the bench scale by treating representative PPCPs in real wastewater. To optimize an economically viable enzymatic treatment process, the research involved specific goals as outlined here:

- evaluate and compare two enzyme oxidation treatment configurations for removing PPCPs in municipal primary effluent wastewater by investigating a range of treatment parameters including pH, enzyme and mediator concentrations, and treatment time;
- investigate the efficacy of using an inexpensive mediator source to reduce the cost of enzymatic treatment;
- study the simultaneous removal of multiple PPCPs; and
- test enzymatic treatment conditions in a continuous flow regime.

To familiarize the reader with PPCPs and the current state of the art, a comprehensive literature review is provided in Chapter 2. Contextual support of PPCPs in the environment, the limitations of treatment technologies, and an introduction to enzymatic treatment are covered. Specific attention is given to the mechanism of the laccase-mediator system. To investigate enzymatic treatment, a pseudo-optimization process was employed in this work. The approach, methods, and materials used are discussed in Chapter 3. The experimental results are presented in Chapter 4, and a summary of the work and conclusions are presented in Chapter 5.

Chapter 2

Literature Review

Daughton and Ternes (1999) first recognized PPCPs as a new class of contaminant with significant environmental impacts. This class encompasses a wide variety of compounds including antibiotics, estrogens and steroids, antiepileptics, anti-inflammatories, antidepressants, analgesics, lipid regulators, beta-blockers, sunscreens, musks and fragrances, iodinated X-ray contrast media, and numerous others. In a study conducted 10 years ago, 95 of these various PPCPs were detected in more than 80% of U.S. streams receiving wastewater treatment discharge (Kolpin et al., 2002). It is apparent that conventional wastewater treatment plants are doing little to remove a large number of PPCPs before discharge, causing yet unknown environmental and ecological risks.

This literature review is meant to acquaint the reader with the latest research concerning the presence of PPCPs in the environment from ineffective treatment in conventional wastewater treatment plants. In addition, this review presents several studies that demonstrate the effects of PPCPs and their potential risk to aquatic life. Numerous treatment technologies have been proposed to address the problem of PPCP removal from wastewater, and a brief survey of their limitations is provided. Finally, a mechanistic background of enzymatic treatment is given.

2.1 PPCPs in the Natural Environment

PPCPs enter the natural environment via point and nonpoint sources. The most common point source is wastewater treatment plants. Conventional wastewater treatment aims to remove "priority pollutants" such as solids, organic matter, and pathogens but not necessarily the numerous micropollutants that exist at low concentrations (Daughton and Ternes, 1999). PPCPs enter the sewage system via human excretion, showering, and improper disposal of excess prescriptions and eventually reach these treatment plants (Al Aukidy et al., 2012; Batt et al., 2006; Hedgespeth et al., 2012). Nonpoint sources include runoff from animal production lots, leaching from domestic septic systems, and land applications of sludge (Westerhoff et al., 2005; García-Galán et al., 2012; Kolpin et al., 2002; Wu et al., 2012).

Although aquatic monitoring of PPCPs has become more common around the world, far more compounds are potentially present than are actually detected in the environment or among the number of regularly monitored pollutants (Dévier et al., 2011). Moreover, given the large temporal and spatial variability of many compounds, it is likely impossible to measure all PPCPs and determine an effective means of treatment.

2.1.1 PPCPs in Conventional Wastewater Treatment

Although environmental contamination originates from both point and nonpoint sources, a significant fraction come from wastewater treatment plants. Many studies have reported detection of PPCPs in plant effluents as well as downstream of effluent discharge sites (Spongberg and Witter, 2008). Although complete PPCP degradation is not achieved during the wastewater treatment process, it is important to understand which compounds are removed using conventional processes and with what efficiency. Recent studies have

attempted to track specific compounds through the treatment process. The studies have shown that partial degradation commonly occurs during secondary treatment, filtration, and disinfection.

PPCP removal during secondary treatment has been studied for activated sludge, membrane bioreactors, and biofilms (Miège et al., 2009; Hijosa-Valsero et al., 2010; Salgado et al., 2012). Removal rates are significantly dependent on plant operating conditions, specifically the solids retention time. Greater PPCP removal was observed with increased sludge age (Oppenheimer et al., 2007), a case in which high retention times allow biodegradation and adsorption to occur. However, although PPCP removal is achieved from the liquid phase, stable and pharmacologically active compounds remain in the settled sludge (Harrison et al., 2006). Moreover, further degradation of PPCPs in the anaerobic digestion of settled sludge is minimal. The primary mechanism of removal of PPCPs in anaerobic digestion is abiotic, or further adsorption (Musson et al., 2010). The treated sludge, or biosolids, then becomes a nonpoint source of PPCPs if it is used in widespread land applications as a soil amendment. Although biological treatment partially removes PPCPs from wastewater, it does not guarantee the complete degradation or inactivation of many compounds.

Biofiltration, used in tertiary wastewater treatment as a polishing step, has also been studied for PPCP removal. Partial removal is possible, but the removal rates of specific PPCP compounds vary widely, typically ranging from 20 to 50%, although higher removal rates occur in some cases (e.g., with sulfamethoxazole; Reungoat et al., 2011). Furthermore, Onesios and Bouwer (2012) observed suppressed biofilm growth in sand columns receiving wastewater spiked with a mixture of PPCPs. The results imply the toxicity of at least one of the PPCPs; suppressed biofilm growth might limit the biofilter's capacity to remove other traditionally targeted pollutants. Ideally, PPCPs should be removed before this treatment step to ensure optimal performance.

PPCP degradation has been reported to occur during disinfection. Chlorine disinfection is often used as a polishing step at wastewater plants, and free chlorine has been shown to oxidize PPCPs containing phenolic groups, for example, estrogen hormones (Westerhoff et al., 2005). However, operators must be cautious if ammonia is present because free chlorine will be converted to monochloramine (Snyder et al., 2003), which can react with PPCPs to form carcinogenic nitrosamine disinfection byproducts (DBPs; Shen and Andrews, 2011). To prevent the formation of such DBPs, PPCPs should be removed before any chlorination.

A short synopsis of multiple studies that have examined the removal and occurrence of five PPCPs is presented in Table 2.1. Carbamazepine, ibuprofen, and sulfamethoxazole are perhaps the most frequently studied PPCPs, whereas estrone and estriol have been identified by the Environmental Protection Agency (EPA) for potential regulatory consideration (Kaplan, 2013). The removal data show variability between compounds and among treatment processes, highlighting the fact that no existing treatment process, other than expensive reverse osmosis, adequately removes a majority of these specific micropollutants. The brief dataset also shows that activated sludge, common to almost all wastewater treatment plants, is capable of removing PPCPs to a certain extent.

Table 2.1. Comparison of Removal and Occurrence Data for Five PPCPs

	Carbama-zepine	Estriol	Estrone	Ibuprofen	Sulfameth-oxazole	Sources
Wastewater Removal Rates (%)						
Conventional Activated Sludge	-8	NA	NA	77	41	Carballa et al., 2004; Sipma et al., 2010
Membrane Bioreactor	0	NA	NA	97	73	Sipma et al., 2010
Constructed Wetland	39	NA	NA	96	NA	Matamoros et al., 2008
Reverse Osmosis	>99	NA	>97	NA	99	Snyder et al., 2003
Anaerobic Sludge	0	NA	NA	41	99	Carballa et al., 2004
Conventional Activated Sludge Wastewater Effluent Concentrations (µg/L)						
Mean	0.674	0.013	0.014	1.172	0.115	Lishman et al., 2006; Miège et al., 2009
Environmental Occurrence (µg/L)						
Median	NA	0.019	0.027	0.200	0.150	Kolpin et al., 2002

Notes: NA=Not applicable

Source: Adapted from Kaplan, 2013

2.1.2 Ecotoxicology and Health Risks

The constant discharge of these compounds and their engineered stability has led many scientists to define PPCPs as "pseudo-persistent" (Daughton, 2004). The risk they pose to the natural environment, and even to humans, is unclear. Determining the risk to aquatic life is difficult because of the inherent qualities of toxicity tests. These tests are often purposefully simplified to represent acute dosage conditions and are applied to a single representative species (Daughton and Ternes, 1999). Extrapolating the risk to the community level of an entire aquatic ecosystem is complex and often loses practicality when describing the spectrum of possible effects.

Nevertheless, studies have been conducted that look at the toxicity risk of various PPCPs on bacteria, algae, crustaceans, amphibians, and fish (Schwartz et al., 2003; Franz et al., 2008;

Schnell et al., 2009). Antibiotic-resistant bacteria were observed in sewage and receiving waters, with their resistance likely proliferated via gene-carrying plasmids (Costanzo et al., 2005). Algae are compromised by antibiotics and antibacterial compounds (e.g., sulfamethoxazole, erythromycin, and triclosan); these compounds were reported to be as toxic to algae as to bacteria (Harada et al., 2008; Al Aukidy et al., 2012). The antibiotics affected generation time, cell membranes, and chloroplast function of the algal cells. For fish, the major PPCPs of concern are estrogens, musks, antidepressants, and the ultraviolet (UV) filters found in sunscreens (which are compounds that mimic estrogens). These compounds bind to hormone receptors, leading to possible developmental and reproductive effects (Coronado et al., 2008; Dévier et al., 2011). In each of these studies, the effects of individual PPCPs on a single organism were investigated; the results do little to explain the overall impact on an aquatic ecosystem.

Current research is concerned with the uncertainties in assessing the toxicity of PPCPs. Few studies have previously taken into account chronic versus acute exposure, the synergistic effects of multiple PPCPs, and the possibility of bioaccumulation (Sanderson, 2003). The impact to human health is also largely unknown. PPCPs, only partially removed by conventional wastewater treatment, are discharged into natural water supplies and eventually reach drinking water sources. PPCP removal at the wastewater stage would eliminate the major entry point of PPCPs into the environment and help prevent contamination of source waters.

2.1.3 Current Regulations

To date, neither the U.S. Congress nor the U.S. EPA has adopted rules specifically aimed at PPCPs in wastewater, drinking water, or land applications of municipal sludge (Eckstein, 2012; Harrison et al., 2006). Despite the ubiquitous presence and potential risks of PPCPs, enacting specific regulations is difficult. Legislators must ensure that regulations are focused on a broad list of PPCPs and that the list of PPCPs is prioritized based on toxicity data and widespread presence. To detect such priority PPCPs, analytical tools must improve to deliver information at ultra-trace concentrations while screening a wide variety of compounds with large differences in physicochemical properties (e.g., pKa, pKow, Kd, or functional groups; Dévier et al., 2011). Regulators must also be aware of the potential costs of monitoring and treating PPCPs. To minimize the expected costs of enacting a law, an effective treatment process must be identified that can remove the most ubiquitous and toxic compounds. Therefore, governing bodies have focused their efforts on supporting various studies of PPCPs, including fate and transport, human and ecological exposure, detection tools, and new and effective treatment technologies.

2.2 Proposed Treatment Technologies

The potential risk of PPCPs is now a recognized problem in the scientific community, and significant research has focused on removing PPCPs from wastewater before they enter the natural environment and drinking water treatment plants. Therefore, current research has studied PPCP removal by several existing and alternative treatment technologies. Some of that research is described briefly in the following. Each of these treatment technologies has its own impact on the environment and on the life cycles of PPCPs. It would be useful to compare these treatment options based on these factors, but that task is not considered herein.

2.2.1 Advanced Oxidation Processes

Advanced oxidation processes (AOPs) involve the generation of hydroxyl radicals and other strong oxidant species that are able to degrade recalcitrant chemical compounds (Andreozzi et al., 1999). AOPs investigated for PPCP removal include ozonation, photocatalysis, and combinations of these processes.

Oxidation can be a dark or light process. Substantial removal of several PPCPs was achieved using dark oxidation with ozone alone or with Fenton's reagent. Unfortunately, incomplete mineralization was often observed, and oxidation byproducts were produced. Determining the behavior and toxicity of ozonation byproducts from parent PPCPs is an expansive, time-consuming task that may be impossible for the broad spectrum of PPCPs that exist. In light oxidation, UV irradiation of titanium dioxide or of hydrogen peroxide produces hydroxyl radicals that attack PPCPs. These processes resulted in greater PPCP removal compared with dark oxidation but required large UV contact times, indicating substantial costs (Ternes et al., 2003; Esplugas et al., 2007). Also, competitive inhibition of the hydroxyl radical by dissolved bicarbonate and natural organic matter was observed in a study of TiO_2 photocatalysis of diatrizoate, an X-ray contrast compound (Sugihara et al., 2013). This result indicates that free radical scavenging is likely to impede the degradation of recalcitrant compounds.

2.2.2 Activated Carbon

Activated carbon is not common in wastewater treatment plants, but it is occasionally used as a polishing process to remove odors and residual dissolved organic carbon. Therefore, the use of both powdered and granular activated carbon has been studied for PPCP removal. Adsorption on activated carbon varies among PPCPs and depends on the individual compound's affinity for the carbon (Serrano et al., 2011). Treatment by activated carbon does not generate byproducts, but the carbon has to be disposed of or regenerated, generally off-site, which increases treatment costs (Reungoat et al., 2011). Moreover, in considering the effectiveness of activated carbon treatment, the regeneration waste of the carbon and the ultimate fate of the unoxidized PPCPs should be considered.

2.2.3 Constructed Wetlands

Constructed wetlands have recently been considered as a passive tertiary treatment step for many conventional wastewater discharge effluents. They are land-based treatment systems consisting of shallow ponds that contain floating or emergent rooted vegetation (Matamoros et al., 2008). Several studies have looked at the potential removal of PPCPs in existing constructed wetlands or pilot studies. Removal efficiencies were significant and were attributed to the coexistence of the various microenvironments of the constructed wetlands. Wetlands provide different physicochemical conditions that allow for degradation of PPCPs following several metabolic pathways (Hijosa-Valsero et al., 2010). However, major disadvantages of this passive treatment technology are the large land requirement and the inherent exposure that the contaminated wetlands provide to the surrounding wildlife (Haberl et al., 2003). The unknown ecotoxicological risks cannot be ignored, and further review should be considered.

Thus far, no proposed treatment is ideal to effectively remove PPCPs from wastewater. The proposed processes are hindered by incomplete PPCP removal, cost, the formation of unknown byproducts, and the lack of applicability to a broad range of treatment plants.

Consequently, research is ongoing, and a novel biochemical process using enzymes has recently been proposed.

2.3 Enzymatic Treatment

The use of enzymes to treat xenobiotics has been in place for several decades. Enzymes were first recognized for their potential to degrade pesticides in bioremediation processes (Scott et al., 2008), and recent efforts aim to study their application to wastewater. Enzymes act as biological catalysts by lowering the activation energy of reactions and increasing the reaction rates (Madigan et al., 2012). Enzymes facilitate reactions but are not consumed or transformed, allowing them to be used repeatedly. Enzymatic treatment offers distinct potential advantages including application to recalcitrant materials, operation at high and low contaminant concentrations and over wide pH and salinity ranges, and easy process control (Durán and Esposito, 2000).

White rot fungi and their extracellular lignin-modifying enzymes have recently been studied for PPCP degradation. The fungi secrete lignin-modifying enzymes that allow the organisms to mineralize extremely recalcitrant lignins in an aerobic oxidative process (Pointing, 2001). The lignin-modifying enzymes—lignin peroxidase, manganese peroxidase, and laccase—have unusually low specificity in substrate choice (Call and Mu, 1997), making these enzymes highly versatile in terms of treatment applications. The two most common classes of white rot fungi enzymes that are studied for PPCP degradation in wastewater are peroxidases and polyphenol oxidases.

Peroxidases catalyze the oxidation of aromatic lignin compounds by reducing hydrogen peroxide (Wesenberg et al., 2003). In a study investigating the oxidation of endocrine disruptors by lignin peroxidase, researchers found that the oxidation of 17β-estradiol produced colloidal dimers and trimers that could be filtered out of solution and showed that the estrogenic activity was effectively eliminated (Mao et al., 2010). Another study used fungal bioreactors in which manganese peroxidase was employed to oxidize anti-inflammatories (Rodarte-Morales et al., 2012). Significant degradation (65-99%) was observed, but the reactors required controlled oxygen pulses for optimal performance, which could be costly and difficult to manage if fully implemented.

Polyphenol oxidases catalyze the oxidation of phenolic compounds by reducing dissolved oxygen (DO). Laccase, a type of polyphenol oxidase, has been specifically studied in the treatment of PPCPs in wastewater. A major potential advantage of laccase over peroxidase is that laccase uses dissolved molecular oxygen for catalytic activity rather than using the hydrogen peroxide that is required in peroxidase systems (Auriol et al., 2007). Several treatment applications have been studied, with the studies focused on estrogens. Auriol et al. (2007) attained complete degradation of estrogens and estrogenic activity of the byproducts in synthetic wastewater, and Lloret et al. (2010) demonstrated the complete degradation of the same estrogens with only one-tenth as much laccase in acetate buffer. Estrogens contain phenolic groups and are therefore direct substrates of laccase. When treating PPCPs that do not contain readily oxidizable phenolic groups, the presence of a mediator compound can expand the oxidative range of laccase.

2.3.1 The Laccase-Mediator System for PPCP Degradation

Nonphenolic groups represent more than 70% of the total residues of lignin (Higuchi, 1997). Therefore, in addition to secreting lignin-modifying enzymes, the white rot fungi also

naturally secrete a mixture of low-molecular-weight compounds termed mediators. The small-sized mediators can access the active site of the laccase, whereas phenolic groups cannot (d'Acunzo et al., 2006). The oxidized mediator can then react with the nonphenolic compounds of the lignin to begin the degradation process. In essence, the use of mediators expands the spectrum of compounds that the enzymes are able to oxidize (Cabana et al., 2007).

The application of the laccase-mediator system to the treatment of PPCPs has vast potential. In a study testing the degradation of anti-inflammatories and estrogens in acetate buffer, natural and synthetic mediators improved not only the degradation capability of laccase but also the rate of degradation (Lloret et al., 2010). The researchers also noted that the use of a mediator substantially improved estrogen removal and that oxidation products exhibited lower or no estrogenic activity. Finally, it was shown in our laboratory that oxybenzone, a UV filter common in sunscreens, could be degraded by the laccase-mediator system in municipal wastewater (Garcia et al., 2011). Complete oxybenzone degradation was achieved using a synthetic mediator, and 95% removal was achieved with a natural mediator. The promising results suggest that the laccase-mediator system, with further investigation and development, might be a viable option for treatment of PPCPs.

2.3.2 A Mechanistic Overview

Laccase, a "blue copper" oxidase, contains four copper ions. In the simple laccase system, that is, without a mediator, laccase directly oxidizes phenolic substrates. In the presence of oxygen, four molecules of the reducing substrate are oxidized, coupled with the four-electron reduction of oxygen to water (d'Acunzo et al., 2006). The oxidation of the phenolic substrate proceeds through an outer-sphere electron-transfer process that generates a radical cation, which, after deprotonation, generates a phenoxyl radical (Crestini et al., 2003). The phenoxyl radicals then serve as substrates for further oxidative coupling and polymerization reactions (Garcia, 2011). A schematic of simple laccase catalysis is presented in Figure 2.1.

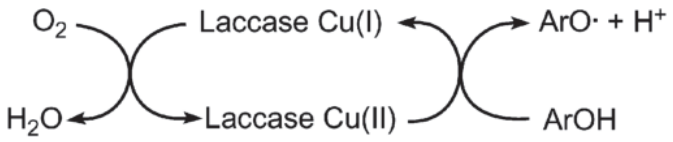

Figure 2.1. The biocatalytic redox cycle of laccase and an aromatic substrate in the presence of oxygen.

Note: AR=aromatic

Source: d'Acunzo et al., 2006

In the laccase-mediator system, there are two oxidative steps (Cabana et al., 2007). First, laccase oxidizes the primary substrate, the mediator, with the oxidized mediator acting as an electron-transferring compound. The oxidized mediator is a phenoxyl radical that can react in a variety of ways. The phenoxyl radical can react with other compounds via hydrogen abstraction, radical-radical coupling reactions, or electron transfer reactions (Crestini et al., 2003; Kunamneni et al., 2008). A schematic of a laccase-mediated hydrogen abstraction reaction is depicted in Figure 2.2.

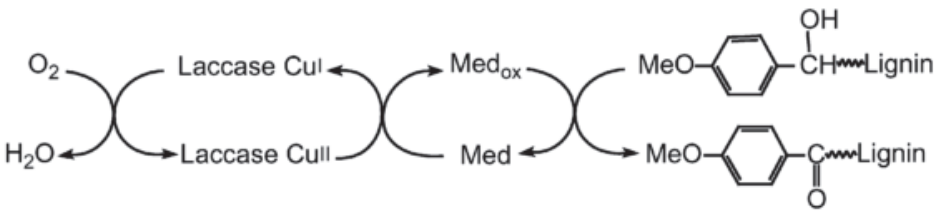

Figure 2.2. The biocatalytic redox cycle of the laccase-mediator system as applied to a methylated lignin compound.

Note: Me=methylated

Source: d'Acunzo et al., 2006

Another advantage of the laccase-mediator system is the stability of the phenoxyl free radicals. The phenoxyl radicals generated by the laccase-mediator system are long-lived, with reported half-life times of hours (Crestini et al., 2003; d'Acunzo et al., 2006). In comparison, the half-life of hydroxyl radicals is on the order of seconds. The stability of the phenoxyl radicals provides increased opportunities for the radicals to interact with the target PPCPs during enzymatic treatment, especially when considering the vast array of contaminants present in a wastewater matrix.

2.3.3 Natural Mediators

Synthetic mediators, which are used in laccase-mediated delignification in the paper pulp industry, are raising concerns because of their high cost and possible toxicity. Camarero et al. (2007) found that the use of a natural mediator, acetosyringone or syringaldehyde, resulted in comparable delignification (up to 25%) to that of synthetic mediators such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 1-hydroxybenzotriazole. These two synthetic mediators have also been used in PPCP treatment using the laccase-mediator system, and similar toxicity and economic concerns should be considered. Acetosyringone, a natural mediator, was shown to effectively degrade oxybenzone in municipal wastewater (Garcia et al., 2011). However, because it is manufactured in a chemical laboratory, acetosyringone is still prohibitively expensive when considering full-scale implementation of enzymatic treatment. Thus, easily accessible natural phenolic substrates should be considered in future work.

2.4 Summary

The presence of PPCPs in our natural environment is indisputable. These compounds have been detected in a majority of streams receiving treated wastewater effluent, indicating that conventional treatment processes are not an effective means of treatment. Given that human consumption of PPCPs is unlikely to drastically decrease and given the relative engineered stability of these compounds, PPCPs are considered "pseudo-persistent." The ecotoxicological effects of thousands of compounds are unknown, making regulation decisions difficult. Several treatment technologies have been proposed to remove PPCPs from wastewater, but few are ideal in terms of economics, nontoxic oxidation byproducts, and potential implementation.

However, enzymatic treatment of PPCPs has offered promising results. Laccase, produced by the white rot fungi, is a polyphenol oxidase enzyme capable of degrading lignin. Laccase is capable of degrading phenolic-like PPCPs such as estrogens but cannot degrade the vast majority of PPCPs. However, studies show that in the presence of a mediator compound, the

oxidative range of laccase is expanded, and it can degrade nonphenolic compounds. The potential of the laccase-mediator system has not been fully explored.

The objective of this study is to enhance the laccase-mediated treatment of PPCPs by investigating critical treatment parameters that influence removal efficiency. Parameters such as pH, mediator and enzyme concentrations, treatment time, wastewater background matrices, and reactor configuration are all considered. Alternative, low-cost, natural mediators are also investigated. These parameters are important in the design and optimization of a full-scale treatment process that can be implemented at an existing wastewater treatment plant.

Chapter 3

Methods and Materials

Successful removal of PPCPs from municipal wastewater will improve public acceptance of water reuse and reduce the adverse impacts of wastewater discharge on aquatic life. In this research, experiments were devised to determine if the laccase-catalyzed oxidation of PPCPs is an effective treatment process that can be implemented at existing wastewater treatment plants.

3.1 Research Design

Several series of experiments were designed to achieve the main objectives of this work and were delineated as follows:

1. Evaluation of enzymatic treatment configuration: Experiments were conducted to enhance PPCP removal with two reactor configurations using oxybenzone as the target PPCP. The selected configuration was used in further experimentation.

2. Investigation of inexpensive mediator sources: Alternative mediator sources that were high in phenolic compounds were considered. The selected alternative mediator was tested in PPCP oxidation experiments in wastewater, and the removal was evaluated.

3. Assessment of simultaneous treatment of two PPCPs: Once the reactor configuration was selected, enzymatic treatment of sulfamethoxazole was tested using the treatment parameters that had been optimized for oxybenzone degradation. The degradation of oxybenzone and sulfamethoxazole when treated simultaneously was then evaluated.

4. Bench-scale simulation of enzymatic treatment in a continuous flow regime: The treatment configuration and experimental conditions that yielded the best PPCP removal were used to simultaneously treat oxybenzone and sulfamethoxazole in a continuous flow reactor. The success of these experiments may indicate the viability of enzymatic treatment for PPCP removal from municipal wastewater.

The performance of enzymatic treatment in all phases was evaluated in terms of PPCP removal, treatment time, and amount of enzyme and mediator required.

3.2 Chemicals and Materials

The laccase enzyme was obtained from Novozymes A/S (1000 U/mL, where U is the unit of enzyme activity, defined in the following). Oxybenzone (CAS 131-57-7), sulfamethoxazole (CAS 723-46-6), gallic acid (CAS 149-91-7), ABTS diammonium salt (CAS 30931-67-0), hydrochloric acid (CAS 7647-01-0), and Folin-Ciocaltaeu's phenol reagent (MDL MFCD00132625) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Acetosyringone (CAS 2478-38-8) was purchased from Indofine Chemical Company (Hillsborough, NJ, United States). Sodium phosphate monobasic (CAS 10049-21-5) was purchased from Fisher Scientific (Pittsburgh, PA, United States). Ultra pure water was produced by filtering distilled water through a water purification system (Barnstead Nanopure, Thermo Scientific, Asheville, NC, United States). Liquid chromatography-mass

spectrometry (LC/MS)-grade methanol and water were purchased from JT Baker (Phillipsburg, NJ, United States). HLB extraction cartridges (Oasis, Product Number 186001880) were purchased from Waters Corporation (Milford, MA, United States).

Two sources of wastewater from the Austin, TX area were used to simulate PPCP oxidation in wastewater with two different levels of biological oxygen demand (BOD) and Total Suspended Solids (TSS). The two sources were Walnut Creek (WC) and South Austin Regional (SAR) wastewater treatment plants. The BOD and TSS of each wastewater are presented in Table 3.1. WC is a weaker wastewater than SAR: It has a lower BOD and less TSS. Secondary effluent wastewater samples were also obtained from each of these sources for use in the free radical generator experiments.

Table 3.1. Ten-year Average BOD and TSS of Two Wastewater Sources—WC and SAR Wastewater Treatment Plants

Treatment Plant	BOD (mg/L)	TSS (mg/L)
WC	101	66
SAR	139	117

3.3 Representative PPCPs

Two representative PPCPs, oxybenzone and sulfamethoxazole, were chosen for the initial work of testing the efficacy of enzymatic treatment. Oxybenzone is commonly used in personal care products, specifically cosmetics and sunscreens, as a UV filter. It has been detected in treated wastewater effluent, rivers, and even drinking water at concentrations between several ng/L and μg/L (Yang and Ying, 2013). Oxybenzone could also pose significant risks to aquatic life. It has been shown to mimic estrogen compounds in fish, leading to possible disruptions in their endocrine and reproductive systems (Coronado et al., 2008). Perhaps most importantly, however, oxybenzone represents a class of PPCPs that is not directly oxidizable by laccase (Garcia et al., 2011). If the laccase-mediator system can effectively remove oxybenzone from wastewater, it will not only decrease the risks to aquatic life, but perhaps more importantly, it might indicate that the system is able to degrade a broader range of PPCPs in one treatment step.

The second target PPCP, sulfamethoxazole, was chosen because it represents an important class of sulfonamide antibiotics. Sulfonamides are widely used as human and veterinary pharmaceuticals. Sulfamethoxazole has been almost universally detected in treated wastewater discharges and even at significant distances (~100 m) downstream of wastewater discharge points (Batt et al., 2006; García-Galán et al., 2012; Kolpin et al., 2002). The presence of antibiotics, and specifically sulfamethoxazole, has been shown to have toxic effects on phytoplankton, the base of the food chain in aquatic environments (Waiser et al., 2011). Given the persistence and toxicity of sulfamethoxazole, it is a critical PPCP to investigate for removal by enzymatic treatment. In addition, successful degradation of sulfamethoxazole will prove that other PPCPs might be effectively treated by the laccase-mediator system.

3.4 Experimental Series and Procedures

All experiments to test the efficacy of the laccase-mediator system were conducted in real municipal wastewater and followed the general experimental format discussed in the following. Enzymatic oxidation experiments were performed in amber glass batch reactors that were placed in a constant-temperature orbital shaking water bath at 23 °C. The reaction mixture consisted of the laccase enzyme, a specific mediator compound, and the target PPCP in unfiltered primary effluent. The commercially produced laccase was used in all experiments. Samples were taken at time zero (i.e., before laccase addition) and at 0.5 and 2 h after laccase addition to measure the PPCP concentration. The samples were analyzed using liquid chromatography-tandem mass spectrometry (LC/MS/MS). Samples were also collected at 0 and 2 h to measure enzyme activity. Further details of each experimental series and the procedures of various measurement techniques are explained in the following sections.

3.5 Evaluation of Enzymatic Treatment Configuration

Enzymatic treatment is likely to be most effective when performed after primary sedimentation and before biological treatment. The decrease in BOD and TSS through primary treatment will provide a cleaner background matrix, when compared with raw wastewater, in which the reaction mechanism can occur. Perhaps more importantly, however, the placement of enzymatic treatment before biological treatment will provide an opportunity for further degradation of partially oxidized PPCPs and of oxidation byproducts in the activated sludge. Therefore, in both of the reactor configurations considered and shown schematically in Figure 3.1, enzymatic treatment is placed between the primary and secondary treatments.

In the direct addition (DA) configuration (Figure 3.1(a)), the laccase and mediator are added directly to the oxidation reactor, where they react to create free radicals that can then oxidize PPCPs. In the free radical generator (FRG) configuration (Figure 3.1(b)), the enzyme and mediator react in a separate, smaller reactor to generate free radicals that are then introduced into the oxidation reactor to oxidize PPCPs. Because of the small size of the FRG reactor, the conditions (e.g., pH) can be more carefully controlled to maximize free radical generation. An additional benefit of the FRG configuration may be that secondary effluent can be recycled and used as the FRG background matrix. As compared with primary effluent, secondary effluent has a lower BOD and total solids concentration and therefore might enhance free radical production.

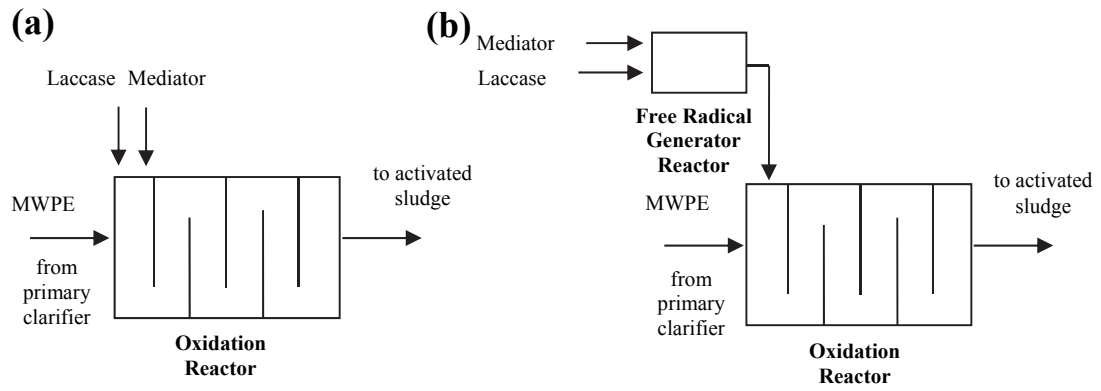

Figure 3.1. Enzymatic treatment of PPCPs in primary effluent performed in an oxidation reactor before the activated sludge process.

Notes: (a) The enzyme and mediator are added directly to the oxidation reactor. (b) The enzyme and mediator are added to a separate reactor, where they produce free radicals, which are then introduced to the oxidation reactor that is fed with primary effluent.
MWPE = Municipal Wastewater Primary Effluent

3.5.1 Direct Addition Experiments

To optimize the PPCP treatment efficiency in the DA configuration, oxybenzone was used as the target PPCP. A series of optimization experiments was conducted by varying one of four treatment parameters at a time. The treatment parameters were the initial pH of the reaction mixture (adjusted with hydrochloric acid), initial enzyme activity, initial mediator concentration, and treatment time. The reaction mixture consisted of unfiltered primary effluent, mediator, and an initial oxybenzone concentration of 43.8 nM (10 µg/L).

For each treatment parameter, a set of experiments was performed to isolate the effects of that parameter on oxybenzone removal. A summary of the DA experiments is listed in Table 3.2. The experimental parameters of each experiment were determined via a step-wise process: If oxybenzone removal was greater than 90%, the experimental treatment parameter that resulted in the target PPCP removal was used for the next set of experimental conditions. In cases where equivalent removal occurred for more than one treatment condition, the most economical condition was chosen.

Table 3.2. Experimental Conditions for the DA Configuration Experiments Using Oxybenzone as the Target PPCP (10 µg/L) and Two Sources of Wastewater—WC and SAR Wastewater Treatment Plants

Experiment	Wastewater Source	Initial pH	Initial Mediator Concentration (µM)	Initial Enzyme Activity (U/mL)	Oxidation Reactor Size (L)
DA-1	WC	5, 6, 7	87.6	1.0	0.1
DA-2	WC	6, 6.5, 7	87.6	1.0	1.0
DA-3	aerated WC, WC	6.5	87.6	1.0	1.0
DA-4	WC	7	26.3, 87.3	0.1	1.0
DA-5	WC	7	26.3, 87.3	1.0	1.0
DA-6	WC	7	87.6	0.3, 1.0	1.0
DA-7	SAR	7	87.6, 105	1.0	0.1
DA-8	SAR	7, Unadjusted	105	1.0	0.1
DA-9	SAR	Unadjusted	105	0.5	1.0

3.5.2 Free Radical Generator Experiments

To optimize PPCP treatment using the FRG configuration, the conditions of the FRG reactor were optimized instead of those of the oxidation reactor. The parameters investigated were the initial pH of the FRG reactor, initial enzyme activity, initial mediator concentration, and detention time of the FRG reactor. The first FRG experiments were based on the successful results of the DA configuration. At the start of the optimization process, a pH and laccase-to-mediator ratio that were similar to those that were successful in the DA configuration were used. The remaining condition, the FRG detention time, was estimated based on preliminary experiments investigating the stability of the free radicals.

In the preliminary FRG experiments, the stability of free radicals was investigated by monitoring the absorbance spectra of the laccase-mediator system over time. Previous work (Garcia, 2011) suggested that the stability of free radicals is a function of enzyme activity but that the background matrix could play a role in scavenging the free radicals. Therefore, the absorbance spectra of several reaction mixtures with different enzyme activities and background matrices were measured using a UV-visible spectrophotometer (Agilent Technologies, Waldbronn, Germany). Two background matrices were studied, a clean 0.1 M sodium phosphate buffer and WC secondary effluent, at two enzyme activity concentrations. All experiments were conducted with 1.7 mM acetosyringone at 23 °C and adjusted to an initial pH of 6.5. The reaction mixture of each experiment was diluted in clean Millipore water to ensure that the measured absorbance was less than 1.0 absorbance units. The specific conditions of each experiment are listed in Table 3.3.

Table 3.3. Preliminary FRG Experimental Conditions to Investigate Free Radical Stability

Experiment	Enzyme Activity (U/mL)	Background Matrix	Duration (min)	Dilution (Reaction Mixture: Millipore Water)
SPEC-1	3	0.1 M HPO_4^{2-}	30	1:40
SPEC-2	10	0.1 M HPO_4^{2-}	30	1:30
SPEC-3	10	WC 2° Effluent	180	1:20

Notes: Experiments were performed at 1.7 mM acetosyringone and adjusted to an initial pH of 6.5

In the FRG preliminary experiments (discussed in depth in Chapter 4), it was confirmed that the stability of the free radicals is a function of enzyme activity. Thus, during the optimization of enzymatic treatment in the FRG configuration, the FRG reactor detention time and enzyme activity were investigated simultaneously. The oxidation reactor contained unfiltered primary effluent with unadjusted pH and an initial oxybenzone concentration of 43.8 nM (10 µg/L). In each experiment, the volume of the FRG reaction mixture that was delivered to the oxidation reactor remained a constant 5% of the oxidation reactor volume. Therefore, for the 100 mL and 1 L oxidation reactors used during FRG experimentation, 5 and 50 mL of the FRG reaction mixture were delivered to the oxidation reactor, respectively.

A summary of the FRG experimental conditions is listed in Table 3.4. Treatment conditions were first optimized using 0.1 M sodium phosphate buffer in the FRG reactor and then using secondary effluent from both wastewater sources. As in the optimization of the DA configuration, the experimental parameters of each FRG experiment were determined via a step-wise process: If oxybenzone removal was greater than 90%, the experimental treatment parameter that resulted in sufficient removal was used for the next set of experimental conditions. In cases where equivalent removal occurred for multiple treatment parameters, the most economical condition was chosen.

Table 3.4. Experimental Conditions for the FRG Configuration Experiments Using Oxybenzone as the Target PPCP (10 µg/L) in 0.1 M Na_2HPO_4 Buffer or in Secondary Effluent Wastewater from WC or SAR Wastewater Treatment Plants

Experiment	Background Matrix	Detention Time (min)	Initial pH	Initial Mediator Concentration (mM)	Initial Enzyme Activity (U/mL)	Oxidation Reactor Size (L)
FRG-1	Buffer	2.5	6	0.7, 1.1, 1.4, 1.8	10.0	0.1
FRG-2	Buffer	0.5, 2.0, 3.5, 5.0	6	1.4	2.5	0.1
FRG-3	Buffer	0.5, 2.0, 3.5, 5.0	6	1.4	5.0	0.1
FRG-4	Buffer	0.5, 1.5, 2.5, 3.5	6	1.4	10.0	0.1
FRG-5	Buffer	2.0	5, 6, 7	1.4	5.0	0.1
FRG-6	Buffer	2.0	6	1.4	5.0	0.1, 1.0
FRG-7	WC 2°	2.0	6	1.4	5.0	0.1
FRG-8	WC 2°	0.5, 2.0, 3.5, 5.0	6	2.1	10.0	0.1
FRG-9	SAR 2°	2.0	6	2.1	10.0	1.0

Notes: Secondary (2°)

3.6 Study of Alternative Mediators

Identifying an alternative mediator source could significantly reduce the cost of enzymatic treatment. Potential alternative mediator sources include food-processing wastes that have high phenolic content. Winery wastewater was initially proposed as an alternative mediator source because its average polyphenol concentration has been reported to be 140 mg/L (Bustamante et al., 2005) and because several wineries are in the vicinity of Austin, TX. The phenol content of the alternative mediator source is important because laccase shows specificity for aromatic-containing compounds (Zhao et al., 2012).

For initial experiments, red wine (instead of winery wastewater) was used as a surrogate alternative mediator source. The total phenol content of red wine can be up to ten times that of white wine because of extended contact time with grape stems, seeds, and skins during the red wine vinification process (Jaitz et al., 2010). A merlot, which has been found to have a higher phenolic content than other red wines (Atanacković et al., 2012), was used.

The total phenol content of the diluted red wine and several standards of acetosyringone, the laboratory-grade mediator, was measured using the Folin-Ciocalteau method, which is described in a subsequent section. In this method, gallic acid is used as a standard measurement of phenols, and the measured concentration is expressed in gallic acid equivalents (GAE). Based on the acetosyringone standard curve, there is 0.32 mg GAE per mg acetosyringone. To test red wine as an alternative mediator, diluted wine was used as an alternative mediator in a DA configuration experiment. The amount of red wine delivered to the oxidation reactor had a phenolic content equivalent to that of the acetosyringone used in previous experiments.

3.7 Removal of Two PPCPs Simultaneously

The removal efficiency of sulfamethoxazole, an antibiotic, was also tested in the laccase-mediator system. Experimental parameters resulting in the greatest oxybenzone removal were applied to primary effluent spiked with 39.5 nM (10 μg/L) sulfamethoxazole. The experimental parameters tested were the reactor configuration, initial pH, mediator and enzyme concentrations, and finally, treatment time. Additional experiments investigated an increased mediator concentration and an increased treatment time. The conditions of the individual sulfamethoxazole treatment experiments are listed in Table 3.5.

Table 3.5. Experimental Conditions for Testing Enzymatic Treatment of Sulfamethoxazole

Experiment	Initial Mediator Concentration (μM)	Oxidation Reactor Size (L)	Treatment Time (h)
SULFA-1	105	1.0	2
SULFA-2	123	0.1	2
SULFA-3	105	1.0	3

Notes: All experiments were conducted in the DA configuration using SAR primary effluent with unadjusted pH, 0.5 U/mL laccase activity, and an initial sulfamethoxazole concentration of 10 μg/L

The simultaneous treatment of both sulfamethoxazole and oxybenzone at initial concentrations of 10 μg/L each was also investigated. Experiments were conducted using the DA configuration in 1 L oxidation reactors containing SAR primary effluent with unadjusted pH. The initial laccase activity and acetosyringone concentration were 0.5 U/mL and 105 μM, respectively.

3.8 Control Experiments

Control experiments were conducted for both oxybenzone and sulfamethoxazole in the DA configuration by omitting the laccase, acetosyringone, or both. Samples from all of the control reactors were taken at 0 and 6 h and measured for relevant constituents (oxybenzone or sulfamethoxazole content and laccase activity). Oxybenzone control experiments were performed using 87.6 μM acetosyringone or 1.0 U/mL laccase activity in WC wastewater, and sulfamethoxazole control experiments were performed using 105 μM acetosyringone or 0.5 U/mL laccase activity in SAR wastewater. In the sulfamethoxazole control experiment that omitted the mediator, 30% of the PPCP was degraded after 2 h. This indicates that sulfamethoxazole is directly oxidizable by laccase to a certain extent.

3.9 Continuous Flow Experiments

3.9.1 Tracer Test

To test the effectiveness of enzymatic treatment under more realistic conditions, 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 feeds distilled water from a large reservoir into a small reservoir, which overflows back into the large reservoir. This small reservoir provides a constant head for a second peristaltic pump, which pumps from the small reservoir to the

reactor. The flow enters the reactor via tubing that is secured to the wall of the reactor with the opening pointed downward at a depth of half the water depth. Just before the tubing enters the reactor, there is a T-connection with a septum, which allows 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 2 h (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 min intervals for the first 90 min, at 5 min intervals from 90 to 180 min, at 10 min intervals from 180 to 240 min, and at 15 min intervals from 240 to 375 min, 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 a UV-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.

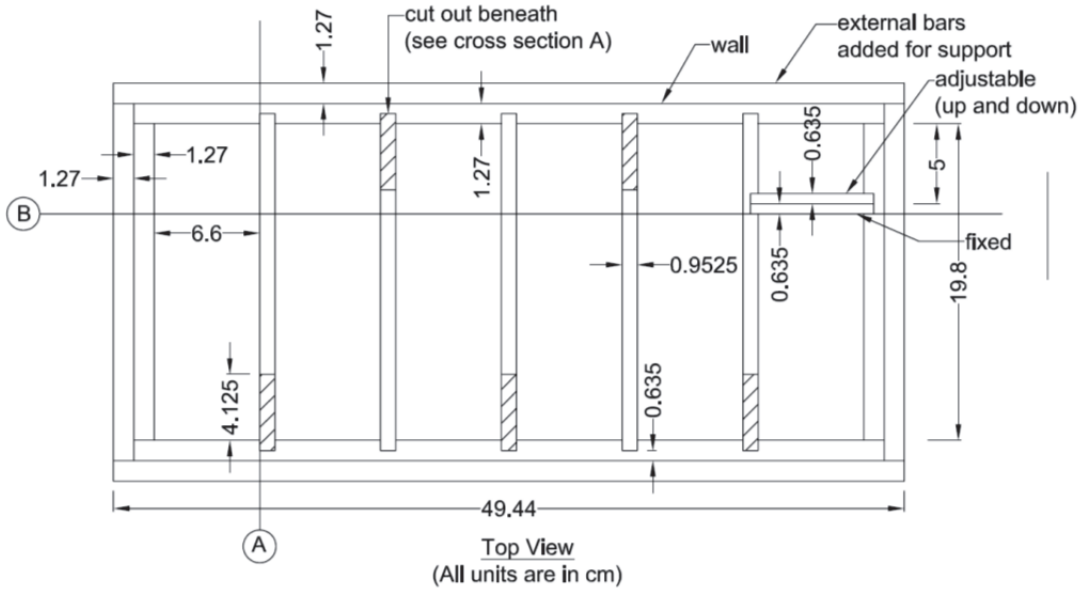

Figure 3.2. Plan view of the reactor.

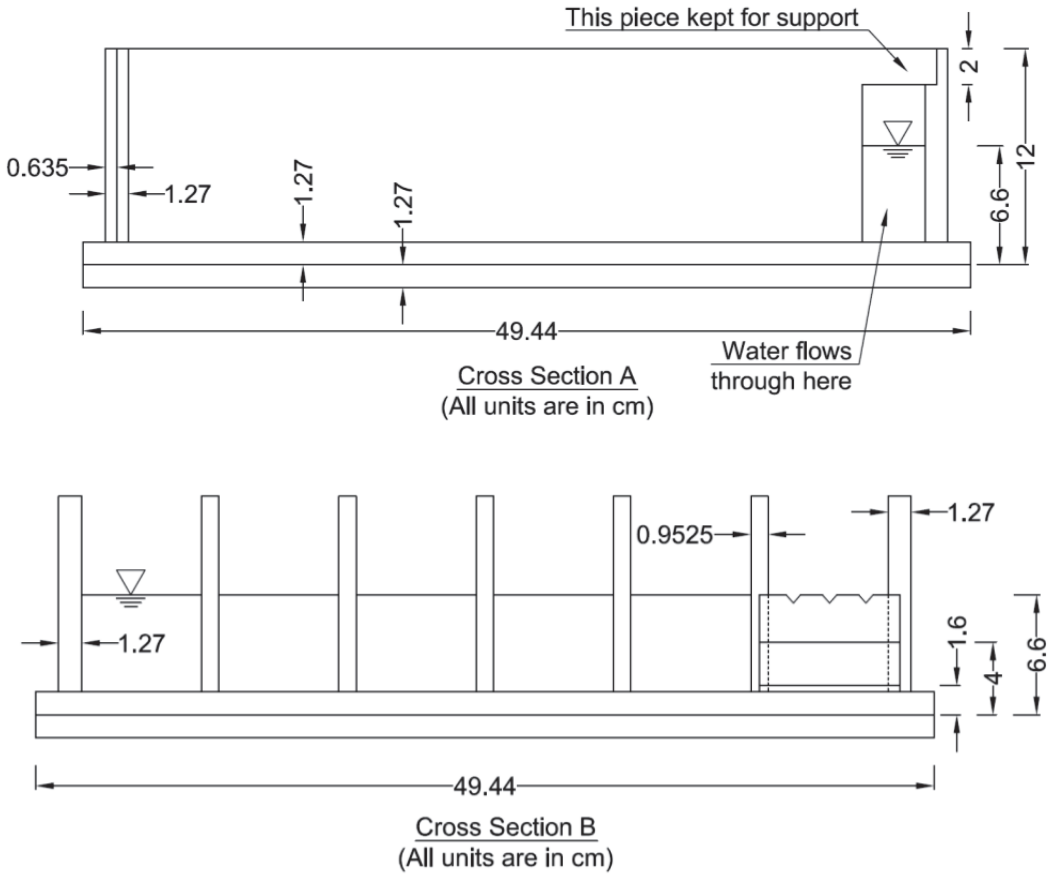

Figure 3.3. Vertical cross sections of the reactor.

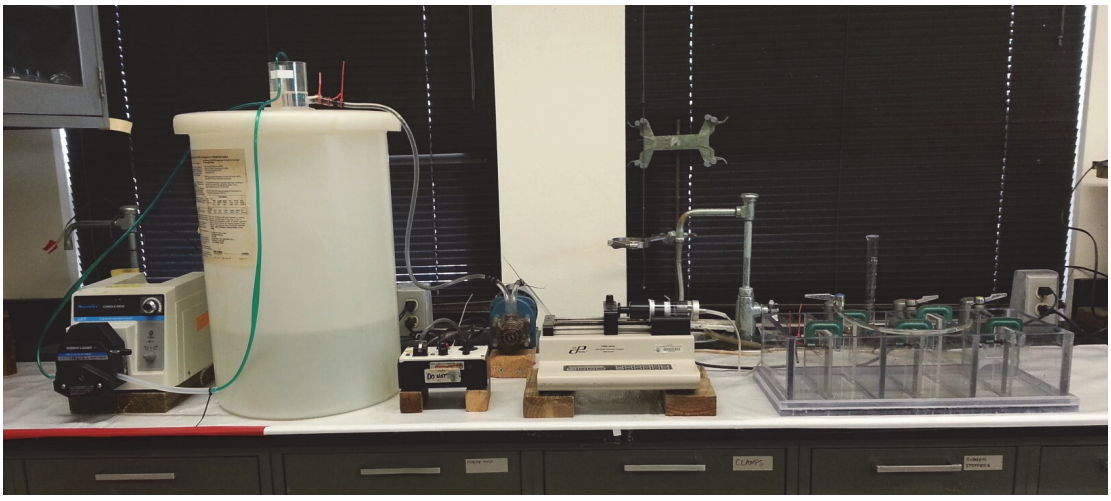

Figure 3.4. Continuous flow experimental setup.

3.9.2 Enzymatic Treatment

Continuous flow experiments for the enzymatic treatment of primary effluent containing PPCPs 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 in the reactor of 0.5 U/mL. Prior to time zero, primary effluent was flowing through the reactor at approximately 40 mL/min for two hours (approximately one detention time) to establish a steady 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 min intervals. At each sampling time, the flow rate and pH were measured, and samples were collected for the analysis of PPCP concentrations and enzyme activity.

3.10 Enzyme Activity Assay

Laccase activity was determined following the methods described by Garcia et al. (2011), in which the enzyme activity is measured by determining the oxidation rate of a substrate (ABTS) to its final product ($ABTS^+$). The colorimetric assay determines the formation of $ABTS^+$ in an assay mixture by measuring its absorbance every 1.5 min for 15 min at 37 °C in a microplate reader (Biotek, Winooski, VT, United States). $ABTS^+$ absorbs light at 420 nm with an extinction coefficient of 3.6×10^4 /(M cm). The absorbance of $ABTS^+$ is related to concentration via the Beer-Lambert law. Samples taken for enzyme activity assays were diluted so that assay mixtures had enzyme activities of approximately 1.0×10^{-3} U/mL. Assay mixtures consisted of 25 µL of diluted sample, 35 µL of 5.0 mM ABTS, and 190 µL of 0.1 M sodium acetate buffer at pH 5. All enzyme activity samples were measured in triplicate to provide a measure of repeatability.

One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of ABTS per minute at 37 °C. This rate was found by determining the slope of the $ABTS^+$ concentration measured over time, as shown in Figure 3.5. In Figure 3.5, the slope, 2.367×10^{-4} µmol/min formed in 250 µL reaction volume and accounting for a 1:50 sample dilution, corresponds to 0.47 µmol/(min mL), or 0.47 U/mL, enzyme activity.

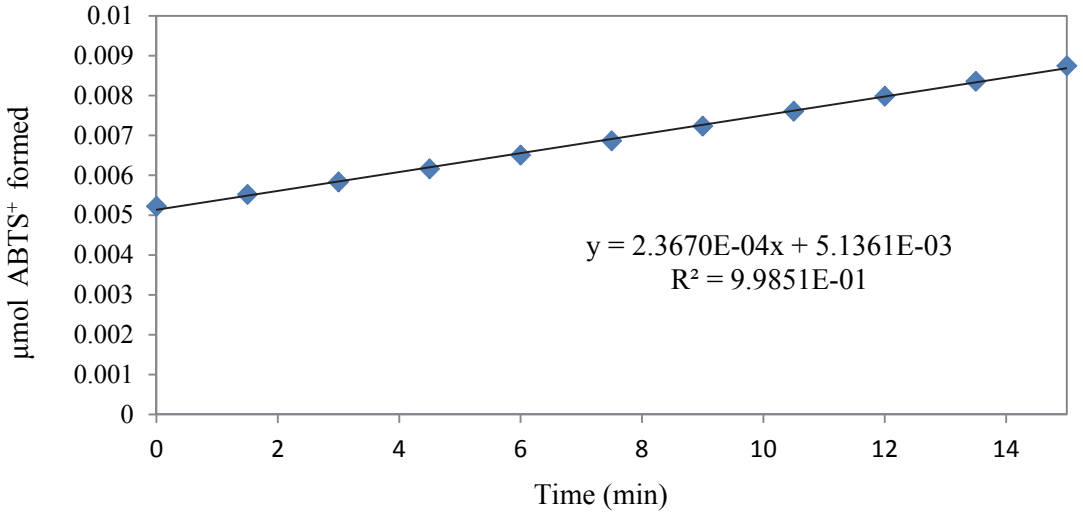

Figure 3.5. The U of laccase enzyme activity is determined by the rate at which $ABTS^+$ is produced over time at 37 °C, given by the slope.

3.11 Solid Phase Extraction

The oxidation reactor samples (20 mL) were first placed in 50 mL amber vials and acidified (with 5 N hydrochloric acid) to a final pH of approximately 1.5 to inactivate the enzyme. The samples were then concentrated using solid phase extraction cartridges (Oasis HLB extraction cartridges, Waters Corporation). Cartridges were primed with 3 mL LC/MS-grade methanol (JT Baker), rinsed with 9 mL LC/MS-grade water (JT Baker) in three equal increments, loaded with 20 mL samples, and finally, rinsed again with 9 mL water in three equal increments. The analyte, oxybenzone or sulfamethoxazole, was then eluted from the cartridges with 2.5 mL of LC/MS-grade methanol in three incremental volumes (800, 800, and 900 µL).

3.12 Liquid Chromatography and Mass Spectrometry Analysis

Quantitative analyses of the target PPCPs in all experiments were performed using LC/MS/MS. The analytes were isolated using a Shimadzu 150×4.6 mm C18 column with a particle size of 5 µm and a binary gradient of methanol and water. A Finnigan Surveyor autosampler, a Finnigan Surveyor mass spectrometer pump, and a TSQuantum mass spectrometer (Thermo Electron Corporation, Waltham, MA, United States) were used. Electrospray ionization in the positive mode was the ionization source. The quantitative LC/MS/MS method details for both compounds are summarized in Table 3.6. A second oxybenzone method, listed as Oxybenzone II in Table 3.6, was developed to improve the method detection limit.

Table 3.6. LC/MS/MS Analytical Parameters for Oxybenzone and Sulfamethoxazole

	Oxybenzone I	Oxybenzone II	Sulfamethoxazole
Liquid Chromatography			
Injection Volume (µL)	10	15	10
Flow Rate (µL/min)	700	400	400
Gradient: *Oxybenzone I*, *Oxybenzone II, and Sulfamethoxazole*: 5% methanol held constant for 3 min, increased linearly to reach 80% at 9 min, held constant at 80% for 2 min, stepped up to 100% and held constant for 10 min. At the end of each run, the methanol was stepped down to 5% and held constant for 2 min.			
Mass Spectrometry			
Collision Energy (eV)	29	21	15
Sheath Gas Pressure (arbitrary units)	50	60	30
Auxiliary Gas Pressure (arbitrary units)	10	40	10
Collision Gas Pressure (mTorr)	1.0	1.5	1.5
Ion Spray Voltage (eV)	4000	4000	4000
Capillary Temp. (°C)	400	350	350
Precursor-Product Ion Mass/Charge (m/z) Ratio	229–151	229–151	254–156

3.13 Total Phenol Content Assay

The total phenol content of the laboratory grade mediator, acetosyringone, and of several alternative mediator sources was measured using the Folin-Ciocalteau method, as described by Waterhouse (2003). In brief, 20 µL of sample was mixed with 1.58 mL of water and 100 µL of the Folin-Ciocalteau reagent. After 30 s to 8 min, 300 µL of sodium carbonate (1.88 M) was added. After 2 h at 20 °C, the absorbance of each solution was measured at 765 nm using a UV-visible spectrophotometer (Agilent Technologies, Waldbronn, Germany). Gallic acid was used as a standard for measurement of phenols, and the measured concentration was expressed in GAE. The gallic acid standard curve is shown Figure 3.6.

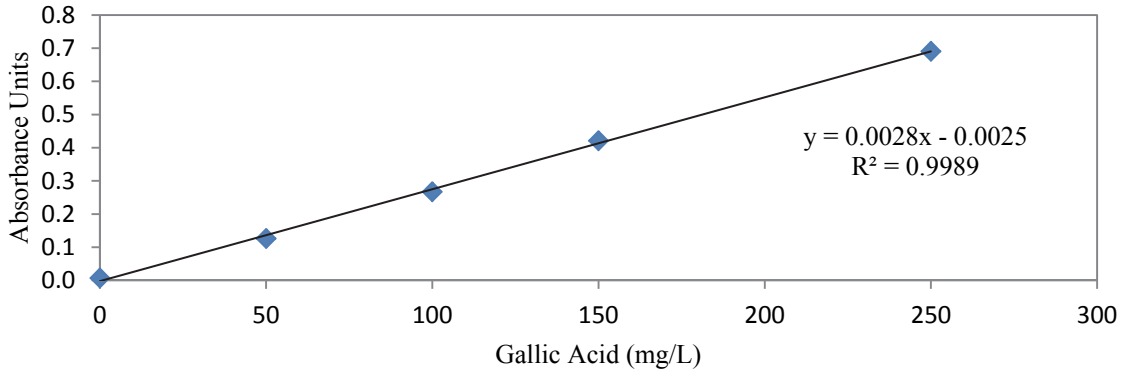

Figure 3.6. Gallic acid standard curve; absorbance measured at 765 nm.

Chapter 4

Experimental Results

As discussed in the previous chapter, several experimental series were designed to investigate enzymatic treatment. The first experimental series determined the most efficient treatment configuration. The selected configuration was then used for further study in the second and third experimental series, which investigated alternative mediator sources and the simultaneous treatment of multiple PPCPs. In each experiment, the performance of the enzymatic treatment was evaluated in terms of PPCP removal, treatment time, and amount of enzyme and mediator required.

4.1 Evaluation of Enzymatic Treatment Configurations

The objective of all of the sets of experiments was to determine the most efficient reactor configuration for enzymatic treatment. Series of experiments were conducted for two reactor configurations, shown in Figure 3.1. The experimental series were designed to vary a single treatment parameter at a time and to investigate its effect on PPCP removal. Treatment parameters were the initial pH of the reaction mixture, initial enzyme activity, initial mediator concentration, and treatment time. Oxybenzone was used as the target PPCP throughout this part of the experimentation. As discussed in Chapter 3, three types of data were obtained from each enzymatic treatment experiment: PPCP concentration, pH, and enzyme activity. The effects of the treatment parameters were first investigated in WC wastewater, which was the lower strength (or lower BOD) wastewater, and then in SAR wastewater, which was the higher strength wastewater.

4.1.1 Direct Addition Experiments

In the DA configuration, laccase and acetosyringone were added directly to the oxidation reactor. The conditions of each experiment are listed in Table 3.2. The pH was the first parameter investigated because it was expected to have a significant impact on removal. Previous work had shown that the optimal pH of laccase ranges from slightly acidic to neutral conditions (Auriol et al., 2007; Garcia et al., 2011). Therefore, to obtain the greatest PPCP removal while maintaining a practical pH for primary effluent, a pH range of 5–7 was investigated in Experiment DA-1 with WC wastewater.

All three reactors exhibited good to excellent removal and exhibited a substantial pH rise, as shown in Figure 4.1. The pH rise, or the consumption of protons, coincides with the reduction of oxygen to water during the laccase-catalyzed oxidation of aromatic compounds (Wesenberg et al., 2003). Therefore, the change in pH (or, more properly, alkalinity) might indicate the extent of the laccase reaction. The experiment conducted at pH 6 showed the greatest change in pH and also the most substantial oxybenzone removal (91%). However, significant removal (86%) was also achieved in the reactor that had an initially neutral pH. This result indicates that enzymatic treatment could possibly be implemented at full scale without pH adjustment, depending on the alkalinity and pH of the wastewater.

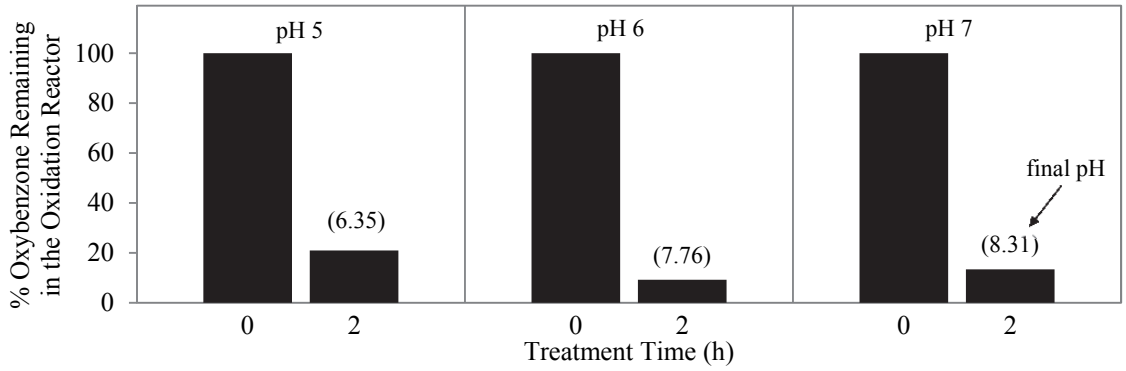

Figure 4.1. Percentage of oxybenzone remaining for Experiment DA-1.

Notes: Oxidation reactors were 0.1 L in size and contained WC wastewater with 87.6 μM acetosyringone concentration and 1.0 U/mL enzyme activity with a 2 h treatment time and varied initial pH (indicated at the top of each graph).

To fully present all of the data that was collected during an oxidation experiment, the measured enzyme activity, which was the final data type collected, of Experiment DA-1 is presented in Table 4.1. The target enzyme activity of this experiment was 1.0 U/mL for all reactors. Although the measured initial enzyme activity was the highest in the pH 7 reactor, the loss in enzyme activity was greater in the pH 5 and pH 6 reactors. Similar effects have been reported, specifically for the laccase-catalyzed oxidation of other xenobiotics (e.g., estrogen, bisphenol A) and indicate that the enzyme is susceptible to significant inactivation at optimal pH (e.g., pH 4 and 5) (Kim and Nicell, 2006; Lloret et al., 2012). At acidic pH, the reaction is likely faster than at neutral pH, but almost equivalent PPCP degradation occurred.

Table 4.1. Measured Enzyme Activity for Experiment DA-1

Experimental Reactors	pH 5	pH 6	pH 7
Treatment Time (h)	**Enzyme Activity (U/mL)**		
0	1.01	1.00	1.21
2	0.43	0.48	0.74
% Remaining after 2 h	42.7	47.8	60.9

The pH resulting in the greatest removal during Experiment DA-1 (i.e., pH 6) was used in Experiments DA-2 and DA-3. Experiment DA-2 was designed to confirm the results of Experiment DA-1 in a scaled-up reaction volume (1 L). Comparable oxybenzone removals of 96%, 89%, and 95% were achieved in the larger reactor at pH 6, 6.5, and 7, respectively. Experiment DA-3 investigated the effects of aeration by aerating an oxidation reactor, initially adjusted to pH 6.5, throughout the 2 h treatment time. Aeration, or more specifically, the concentration of dissolved oxygen (DO), did not prove to be limiting in the laccase oxidation of oxybenzone as 92% removal was achieved. These results were promising for full-scale enzymatic treatment after primary treatment. To simulate the most economically viable conditions, further experiments were not aerated and were adjusted to an initial pH of 7.

Experiments DA-1 through DA-3 were conducted at 1.0 U/mL laccase activity and 87.6 µM acetosyringone concentration. These values were based on previous work conducted in our laboratory (Garcia et al., 2011), but to further pursue economically viable conditions, reduced enzyme and mediator concentrations were investigated in Experiments DA-4 and DA-5. The results, shown in Figure 4.2, indicate that the effect of decreased enzyme activity is more pronounced than the effect of decreased acetosyringone (mediator) concentration.

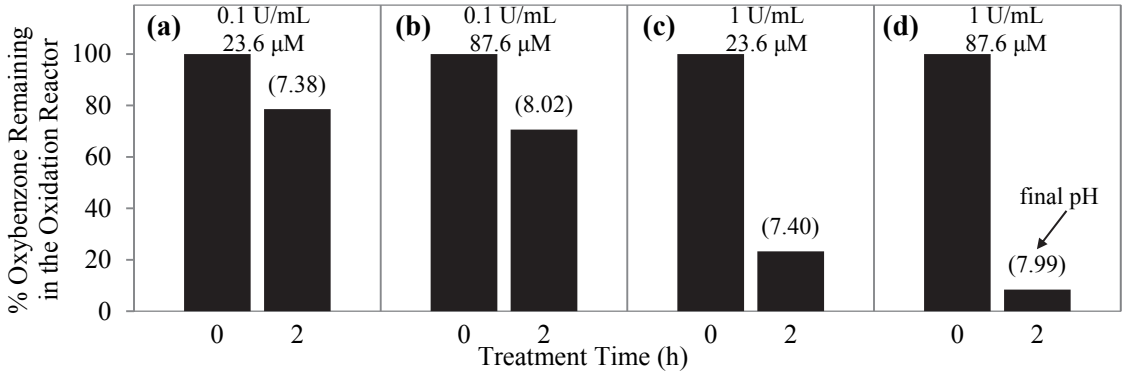

Figure 4.2. Percentage of oxybenzone remaining in Experiments DA-4 and DA-5.

Notes: Oxidation reactors were 1 L in size and contained WC wastewater adjusted to pH 7 with 0.1 U/mL enzyme activity and (a) 23.6 µM or (b) 87.6 µM acetosyringone or with 1.0 U/mL enzyme activity and (c) 23.6 µM or (d) 87.6 µM acetosyringone.

The final pH of each reaction mixture, also shown in Figure 4.2, might indicate that a similar extent of reaction, or a similar production of free radicals, occurred at each level of acetosyringone concentration that was tested. Fewer free radicals were produced at lower enzyme activities, and less oxybenzone removal was achieved, which suggests that the interaction between the laccase and the mediator was the limiting step of the reaction. It is hypothesized that the free radical mediator molecule can react with other compounds by hydrogen abstraction and regenerate the original mediator molecule (Crestini et al., 2003); perhaps there was not a sufficient concentration of laccase to interact with the available acetosyringone molecules in the reaction mixture. If this is true, the laccase-mediator reaction in wastewater is enzyme limited, not mediator limited, at the conditions in our experiments.

Treatment time was the final treatment parameter investigated in the DA experiments using WC wastewater. Treatment time is an important variable because it will determine the size, and therefore the cost, of the reactor that is required for enzymatic treatment. In Experiment DA-6, two levels of enzyme activity, 1.0 and 0.3 U/mL, were tested while PPCP concentration, pH, enzyme activity, and DO concentration were measured at 0, 0.25, 0.5, 1.0 and 2.0 h. Figure 4.3(a) and (b) show that substantial oxybenzone removal was achieved for both activity levels but that the full 2 h of treatment was required. The concomitant reduction of oxygen to water in the laccase-catalyzed reaction is confirmed in Figure 4.3(c), which documents the DO decrease and the pH increase. Moreover, it can be seen that the greatest change in pH occurs within the first 15 min of treatment and corresponds to the greatest change in oxybenzone concentration in both reactors: 43% and 37% in the 0.3 and 1.0 U/mL reactors, respectively. These results suggest that, if only partial PPCP degradation was desired, a significantly smaller oxidation reactor would be required.

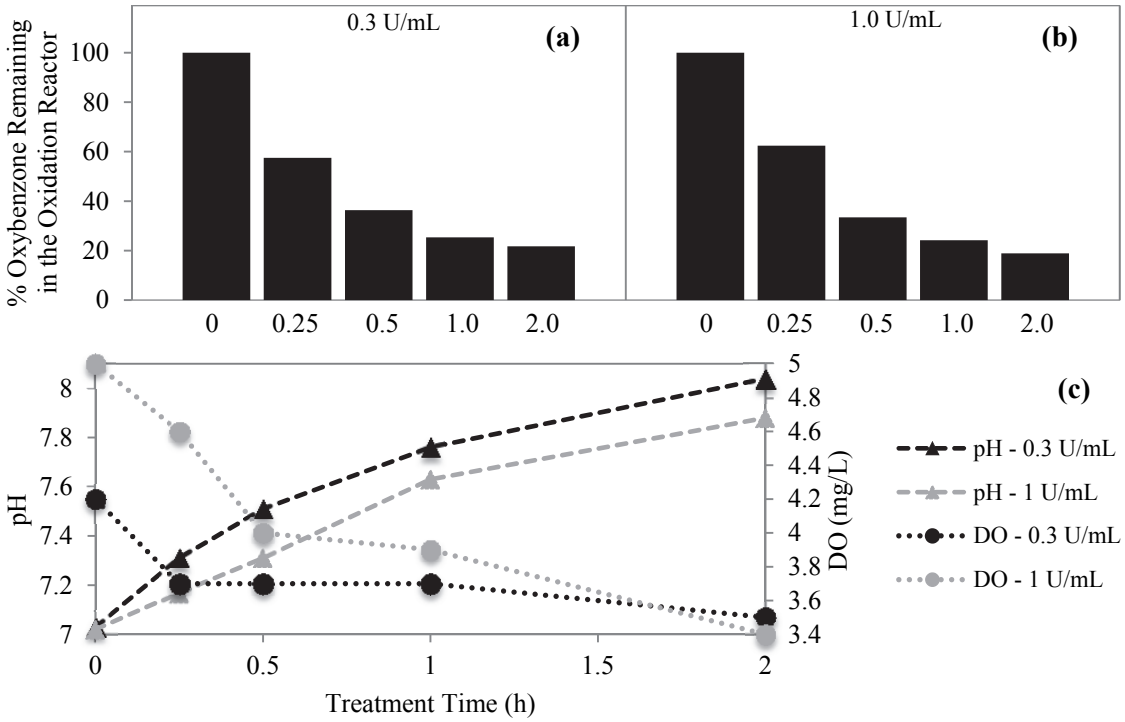

Figure 4.3. Percentage of oxybenzone remaining, DO, and pH for Experiment DA-6.

Notes: Oxidation reactors were 1 L in size and contained WC wastewater adjusted to pH 7 with 87.6 µM acetosyringone and (a) 0.3 U/mL or (b) 1.0 U/mL enzyme activity; (c) the pH and DO (mg/L) over the course of the experiment in both oxidation reactors.

The most economically feasible conditions that yielded significant oxybenzone removal in WC wastewater were applied to SAR wastewater in Experiments DA-7 and DA-8. The results of Experiment DA-8 indicate that a higher acetosyringone concentration of 105 µM was needed to achieve at least 80% oxybenzone removal, as compared with only 66% removal with 87.6 µM. A higher mediator concentration is likely required because of the effects of increased BOD and TSS of the SAR wastewater. Experiment DA-8 considered the effect of unadjusted pH compared with starting treatment at pH 7. The difference in oxybenzone removal was insignificant, with 80% removal at pH 7 and 76% removal at unadjusted pH.

4.1.2 Summary of the Direct Addition Experimental Series

The results, in terms of oxybenzone removal, of each DA experiment are presented in Table 4.2 along with each experiment's conditions. In this format, the pseudo-optimization process can be more easily seen.

Table 4.2. Experimental Conditions and Resulting Percentage of Oxybenzone Removed for the DA Configuration Experimental Series

Experiment	Wastewater Source	Initial pH	Initial Mediator Concentration (μM)	Initial Enzyme Activity (U/mL)	Oxidation Reactor Size (L)	Oxybenzone Removed (%)
DA-1	WC	5, 6, 7	87.6	1.0	0.1	79, 91, 87
DA-2	WC	6, 6.5, 7	87.6	1.0	1.0	90, 80, 85
DA-3	aerated WC, WC	6.5	87.6	1.0	1.0	76, 85
DA-4	WC	7	26.3, 87.3	0.1	1.0	21, 77
DA-5	WC	7	26.3, 87.3	1.0	1.0	29, 92
DA-6	WC	7	87.6	0.3, 1.0	1.0	78, 81
DA-7	SAR	7	87.6, 105	1.0	0.1	66, 80
DA-8	SAR	7, unadjusted	105	1.0	0.1	80, 76
DA-9	SAR	unadjusted	105	0.5	1.0	62

4.1.3 Free Radical Stability Experiments

As previously discussed, the laccase-catalyzed oxidation of aromatic compounds generates phenoxyl free radicals. In consideration of the free radical generator (FRG) configuration, depicted in Figure 3.1, the stability of the free radicals is imperative in ensuring that there is sufficient time for the free radicals to be generated in a separate, smaller reactor and then delivered to the oxidation reactor containing the target PPCP. By understanding the stability of the acetosyringone free radicals, it is possible to estimate a FRG detention time.

To investigate the stability of the free radicals, the absorbance spectra of three different reaction mixtures were measured over time. The conditions of each experiment are listed in Table 3.3, and the spectra are presented in Figure 4.4. Acetosyringone absorbs UV radiation with an absorption maximum at a wavelength of approximately 290 nm. For each reaction mixture, the initial spectrum (before laccase addition) peaks at this approximate wavelength. Immediately following laccase addition, the peak increases and shifts slightly to the left (i.e., to a lower wavelength). For several minutes, the peak is greater than the initial acetosyringone peak, and then the absorbance begins to diminish over time. It is hypothesized that the increase in absorbance corresponds to the production of a free radical mediator

compound and that the decrease corresponds to the free radical reacting with another compound.

Experiments SPEC-1 and SPEC-2 were conducted in a clean sodium phosphate buffer at pH 6.5. At the lower laccase activity, 3 U/mL (Figure 4.4(a)), the peak of each spectrum slowly climbs to its maximum absorbance, observed after 2 min 40 s of reaction time. At the higher laccase activity, 10 U/mL (Figure 4.4(b)), the maximum absorbance of the free radicals occurs at 15 s, and the peak remains close to this maximum for approximately 1 min 40 s. Given the clean background matrix, the free radicals are likely reacting in radical-radical coupling reactions (Crestini et al., 2003), and therefore the absorbance decreases over time.

Experiment SPEC-3 was conducted at 10 U/mL laccase activity in WC secondary effluent to simulate the proposed conditions, in which recycled secondary effluent would be the FRG background matrix. The stability of the free radicals was very similar to that observed in Experiment SPEC-2, in which the absorbance rapidly increases and then is stable for 3 min. The decrease in radical concentration in secondary effluent could be due to any of the three reaction pathways proposed by Crestini et al. (2003).

These results confirm that the rate of free radical generation is a function of enzyme activity. Therefore, during the analysis of FRG treatment parameters, the FRG detention time and enzyme activity were investigated simultaneously. The results also confirm that the radicals are stable for several minutes, indicating that FRG detention times should be studied in the range of 0.5 to 5 min.

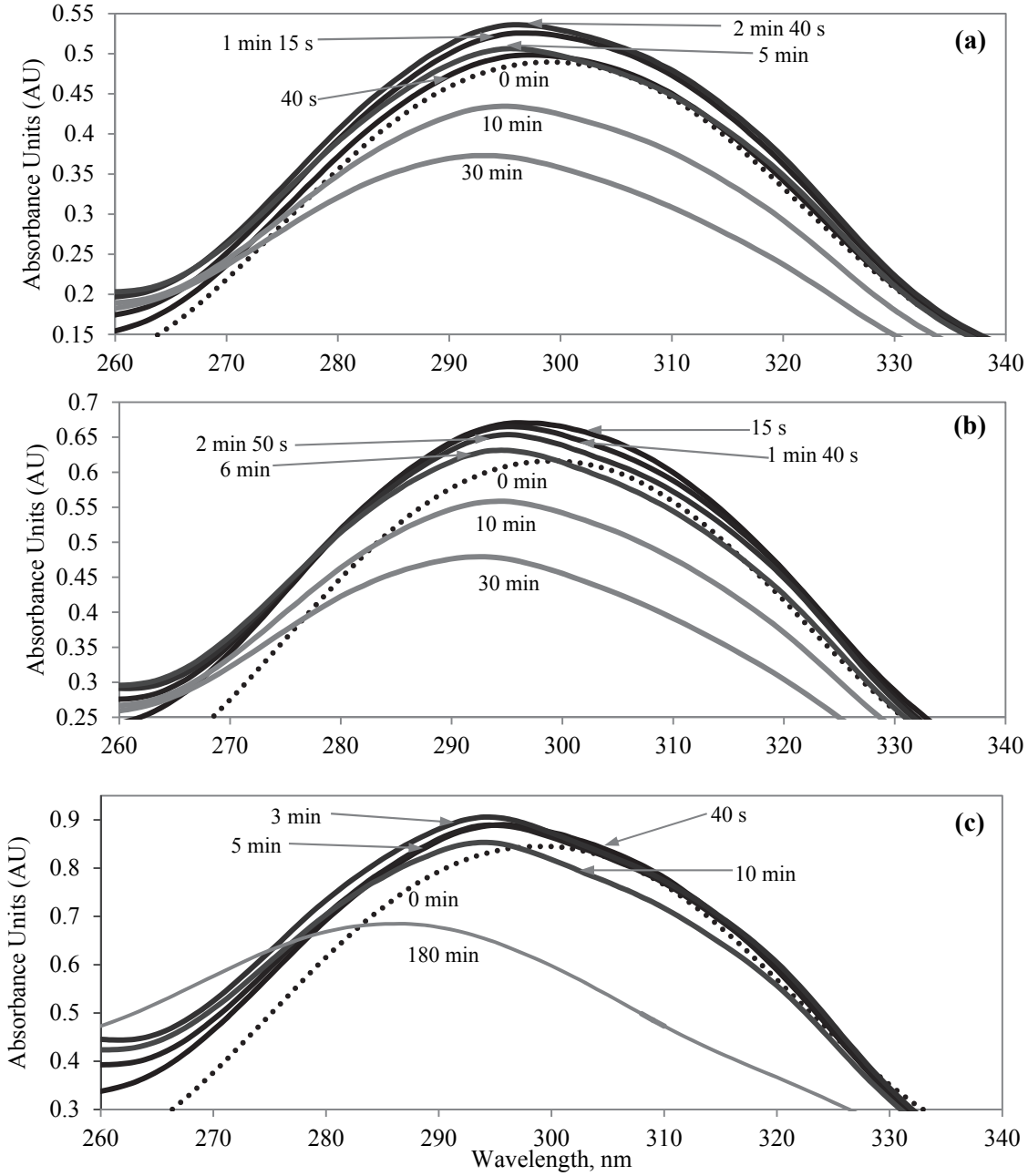

Figure 4.4. UV absorbance spectra of the laccase-catalyzed oxidation of acetosyringone over time for Experiments SPEC-1 through SPEC-3.

Notes: Reaction mixtures consisted of sodium phosphate buffer with 1.7 mM acetosyringone and (a) 3 or (b) 10 U/mL enzyme activity and of (c) WC secondary effluent with 10 U/mL enzyme activity.

4.1.4 Free Radical Generator Experiments

In the FRG configuration, the laccase and acetosyringone are added to a separate, smaller reactor where free radicals are generated under controlled conditions. The free radicals are then introduced to the oxidation reactor, which contains primary effluent wastewater and the target PPCP. The motivation of using the FRG configuration is that conditions, for example,

pH, can be more carefully controlled in the smaller reactor, which might provide an economic advantage over the DA configuration. The conditions of each experiment are listed in Table 3.4. Experiments were first conducted using a 0.1 M sodium phosphate buffer as the FRG background solution. Buffer was used before secondary effluent to confirm that enzymatic treatment was possible with the FRG reactor configuration.

The mediator concentration, a potentially significant expense in enzymatic treatment, was varied in Experiment FRG-1 of the FRG experimental series. In this experiment, the FRG detention time was 2.5 min, based on the results of the free radical stability experiments previously discussed. As shown in Figure 4.5, after 2 h of treatment, FRG mediator concentrations of 1.8 and 1.4 mM acetosyringone both provide sufficient PPCP removal (almost 90%). To minimize chemical usage, a 1.4 mM concentration was chosen for subsequent FRG experiments conducted in buffer.

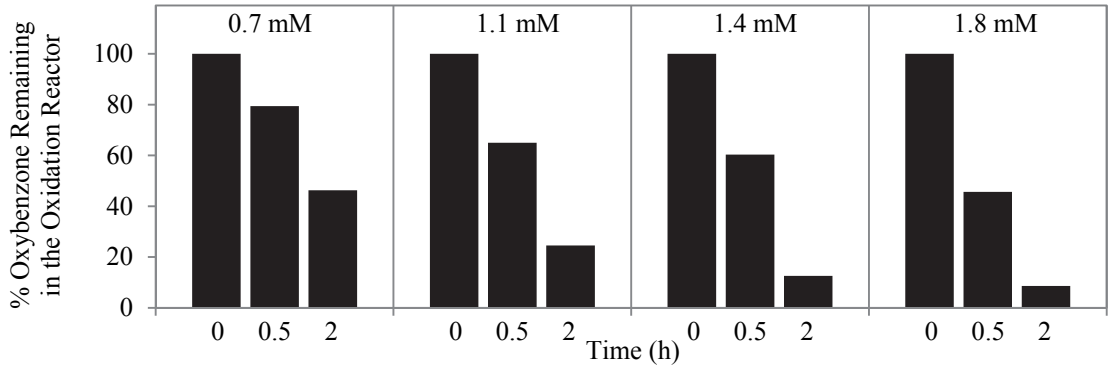

Figure 4.5. Percentage of oxybenzone remaining in Experiment FRG-1.

Notes: Oxidation reactors were 0.1 L in size and contained WC wastewater with pH unadjusted. The FRG reactor had a 2.5 min detention time and contained buffer adjusted to pH 6, 10 U/mL enzyme activity, and varying acetosyringone concentrations (indicated at the top of each graph).

Experiments FRG-2 through FRG-4 were designed to explore the effects of enzyme activity and FRG detention time. Each experiment was completed at a different enzyme activity with variable detention times. The results of these experiments, displayed in Figure 4.6, indicate that as enzyme activity increases, oxybenzone removal increases, but only slightly; for example, at a detention time of 3.5 min, oxybenzone removal after 2 h was 82%, 89%, and 91% for 3 U/mL, 5 U/mL and 10 U/mL, respectively. The variation in oxybenzone removal at a given enzyme activity and varied FRG detention times is minimal, denoting that the effect of FRG detention time was not prominent at the enzyme activities studied. Significant oxybenzone removal occurred in every experiment.

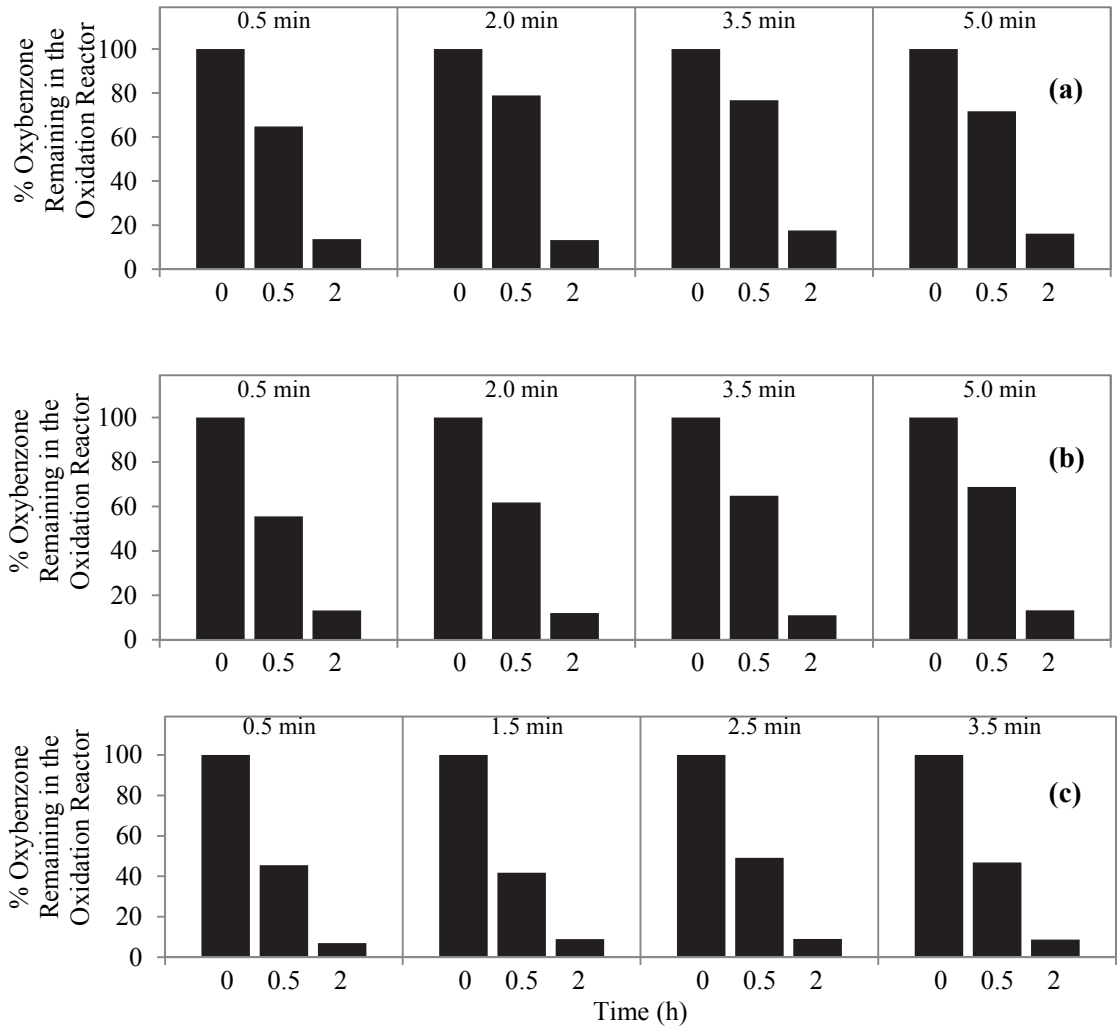

Figure 4.6. Percentage of oxybenzone remaining for Experiments FRG-2 through FRG-4.

Notes: Oxidation reactors were 1 L in size and contained WC wastewater with unadjusted pH. The FRG reactors contained 1.4 mM acetosyringone and (a) 2.5, (b) 5, or (c) 10 U/mL enzyme activity with varying FRG detention times for each (indicated at the top of each graph).

The effect of the pH in the FRG reactor was considered in Experiment FRG-5. In this experiment, the mediator concentration, enzyme activity, and detention time were chosen based on achieving an economically viable FRG reactor configuration (1.4 mM acetosyringone, 5 U/mL, and 2 min detention time) and applied to experiments ranging from pH 5 to 7. After 2 h of treatment, 86%, 86%, and 83% oxybenzone removal was achieved for FRG reactors that had been initially adjusted to pH 5, 6, and 7, respectively. The differences in removal are quite minor, but experiments performed at pH 5 and 6 were more effective than those at pH 7.

The purpose of Experiment FRG-6 was to confirm oxybenzone removal in a scaled-up reactor (1 L) using the best treatment parameters found for the FRG configuration with buffer: 1.4 mM acetosyringone, 5 U/mL enzyme activity, pH 6, and a 2 min detention time in the FRG reactor. Substantial removal of 90% was achieved after 2 h of treatment. These conditions are for an FRG reactor containing sodium phosphate buffer and an oxidation reactor containing WC primary effluent.

Experiments FRG-7 and FRG-8 of the FRG experimental series investigated the effects of using WC secondary effluent as the background matrix of the FRG configuration. The success of the FRG configuration depends on successful oxybenzone removal occurring with secondary effluent in the FRG reactor; otherwise, the expense of providing clean water for free radical generation would make treatment impractical. When the optimized conditions found using buffer, that is, the conditions of Experiment FRG-6, were applied in Experiment FRG-7 using secondary effluent, only 68% oxybenzone removal was achieved. The diminished removal indicates that the contaminants present in the secondary effluent inhibited free radical production or consumed free radicals before they could be introduced to the oxidation reactor containing the primary effluent and oxybenzone. Therefore, in Experiment FRG-8, both the enzyme activity and acetosyringone concentration were increased to observe the effect on oxybenzone removal. Given that the stability of the free radicals and hence, the optimum detention time of the FRG reactor, are related to enzyme activity, the FRG detention time was also varied in Experiment FRG-8. The differences in removal between the reactors with 0.5, 2.0, and 3.5 min detention times were insignificant; all reactors achieved 93–96% oxybenzone removal. The 5 min detention time resulted in 90% removal.

4.1.5 Summary of the FRG Experimental Series

The results, in terms of oxybenzone removal, of each FRG experiment are presented in Table 4.3 along with each experiment's conditions. In this format, the pseudo-optimization process can be more easily seen.

Table 4.3. Experimental Conditions and Resulting Percentage of Oxybenzone Removed for the FRG Experimental Series

Experiment	Back-ground Matrix	Detention Time (min)	Initial pH	Initial Mediator Concentration (mM)	Initial Enzyme Activity (U/mL)	Oxidation Reactor Size (L)	Oxybenzone Removed (%)
FRG-1	Buffer	2.5	6	0.7, 1.1, 1.4, 1.8	10	0.1	54, 76, 87, 91
FRG-2	Buffer	0.5, 2.0, 3.5, 5.0	6	1.4	2.5	0.1	86, 87, 82, 84
FRG-3	Buffer	0.5, 2.0, 3.5, 5.0	6	1.4	5	0.1	87, 88, 89, 87
FRG-4	Buffer	0.5, 1.5, 2.5, 3.5	6	1.4	10	0.1	93, 91, 91, 91
FRG-5	Buffer	2.0	5, 6, 7	1.4	5	0.1	86, 86, 83
FRG-6	Buffer	2.0	6	1.4	5	0.1, 1.0	86, 91
FRG-7	WC 2°	2.0	6	1.4	5	0.1	68
FRG-8	WC 2°	0.5, 2.0, 3.5, 5.0	6	2.1	10	0.1	95, 94, 94, 90
FRG-9	SAR 2°	2.0	6	2.1	10	1.0	63

4.1.6 Determination of Optimal Configuration

Experiment DA-9 of the DA experimental series (see Table 3.2) and Experiment FRG-9 of the FRG experimental series (see Table 3.4) were designed to compare the treatment efficacy of the two proposed configurations given identical chemical usage in the higher strength wastewater, SAR. The use of SAR wastewater represents the most economically conservative conditions in terms of mediator and enzyme amounts. As shown in Figure 4.7, both configurations achieved 65% oxybenzone removal. The removal for the DA configuration is less than that in previous experiments, and this difference is ascribed to the attributes of the primary effluent sampled. The primary effluent was unusually dark in color and strong in odor, likely indicating greater BOD and TSS concentrations than the average BOD and TSS of SAR, 138 mg/L and 117 mg/L, respectively. Nevertheless, the fact that the same percentage of removal was achieved in both configurations means that the added expense and difficulty of operation of the FRG configuration would not be viable in full-scale enzymatic treatment, and the DA configuration is therefore considered the best reactor configuration.

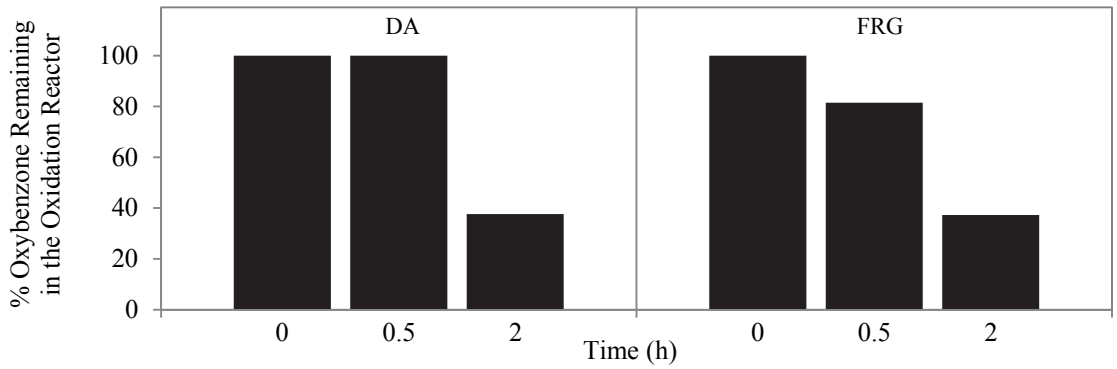

Figure 4.7. Percentage of oxybenzone remaining for Experiments DA-9 and FRG-9.

Notes: Both experiments were conducted using SAR wastewater in 1 L reaction mixtures. (a) The DA experimental conditions were unadjusted pH, 105 μM acetosyringone, and 0.5 U/mL enzyme activity. (b) The FRG experimental conditions were pH 6, 2.1 mM acetosyringone, 10 U/mL enzyme activity, and an FRG detention time of 2 min; 50 mL of the FRG reaction mixture was delivered to the 1 L solution in the oxidation reactor, resulting in the same solution conditions as in the DA reactor.

4.2 Alternative Mediator Experiments

Red wine was tested as a surrogate alternative mediator. In a set of experiments testing the use of red wine as an alternative mediator, a predetermined total phenolic content was delivered to two oxidation reactors, one with oxybenzone as the target PPCP and the other with sulfamethoxazole. The total phenol content, expressed as GAE, was determined by the Folin-Ciocalteau method for the acetosyringone concentration that had yielded successful enzymatic treatment (i.e., 105 μM). Unfortunately, no removal of oxybenzone or sulfamethoxazole occurred with wine as the mediator.

Wine might be an ineffective mediator because of the variety of phenolic compounds present in wine. The phenolic compounds can be simple phenolic acids or complex polyphenols including benzoic acids, coumaric acid, resveratrol, flavonoids, and tannins, to name a few (Jaitz et al., 2010). The form of the phenolate that is capable of reacting with laccase and its

ability to form relatively stable oxidized intermediates might determine the efficiency of a phenol as a mediator (Camarero et al., 2007). Given the variety of phenolic compounds in wine and the results of the enzymatic treatment experiments, winery wastewater is not a promising alternative mediator source. However, other food processing wastes, such as olive oil mill wastewater, have yet to be explored and could prove effective.

4.3 Removal of Multiple PPCPs Simultaneously

Enzymatic treatment was also investigated with sulfamethoxazole as the target PPCP. The experimental conditions of the sulfamethoxazole experiments are listed in Table 3.5. The conditions of Experiment SULFA-1 mimicked the conditions in which the greatest oxybenzone removal had been achieved in SAR wastewater. Enzymatic treatment resulted in approximately 65% removal of sulfamethoxazole (Figure 4.8(a)). In a control experiment that omitted the mediator, acetosyringone, only 30% sulfamethoxazole removal was obtained. Although sulfamethoxazole removal was not as substantial as oxybenzone removal (~90%), this result indicates that the laccase-mediator system is capable of removing other PPCPs from primary effluent.

Additional testing, Experiments SULFA-2 and SULFA-3, was done to determine if sulfamethoxazole removal could be improved. Experiment SULFA-2 was conducted with an increased mediator concentration of 123 µM acetosyringone, and Experiment SULFA-3 was conducted with an increased treatment time of 3 h. Both experiments (Figure 4.8(b) and (c)) resulted in approximately 70% removal, a minimal improvement. Optimization of the sulfamethoxazole treatment was not pursued because further degradation is expected to occur in the activated sludge phase of conventional wastewater treatment.

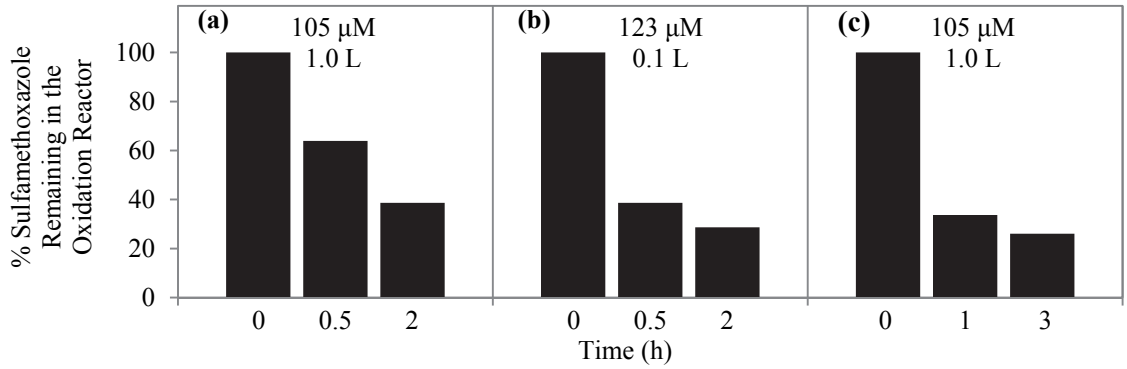

Figure 4.8. Percentage of sulfamethoxazole remaining in Experiments SULFA-1 through SULFA-3.

Notes: All experiments were conducted in the DA configuration with SAR primary effluent, unadjusted pH, and 0.5 U/mL enzyme activity, with varying acetosyringone concentrations and reactor sizes (as indicated at the top of each graph).

The simultaneous treatment of both sulfamethoxazole and oxybenzone at initial concentrations of 10 µg/L each was also investigated. The result of this experiment is shown in Figure 4.9. The data indicate that when the two PPCPs are treated together, the removal achieved during individual treatment is maintained: approximately 65% for sulfamethoxazole and 90% for oxybenzone. These results are extremely encouraging with respect to full-scale enzymatic treatment of a variety of PPCPs.

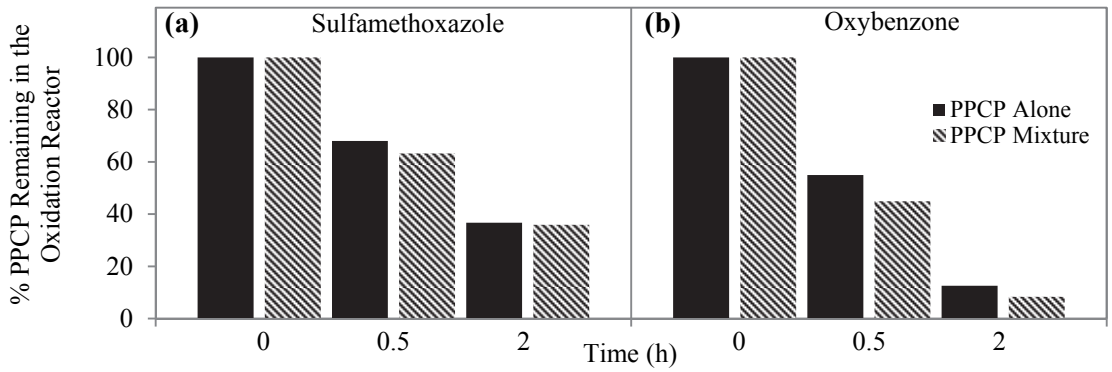

Figure 4.9. Percentage of PPCP remaining when (a) sulfamethoxazole is treated as the single PPCP and in a PPCP mixture with oxybenzone and when (b) oxybenzone is treated as the single PPCP and in a PPCP mixture with sulfamethoxazole.

Notes: All experiments were conducted in the DA configuration with unadjusted pH, 105 µM acetosyringone, and 0.5 U/mL enzyme activity.

4.4 Continuous Flow Experiments

4.4.1 Tracer Test

To investigate the effectiveness of enzymatic treatment under more realistic conditions, a bench-scale continuous flow reactor was constructed. The hydraulic flow through the reactor was characterized by a tracer test, which yielded the exit age distribution shown in Figure 4.10 and the cumulative age distribution shown in Figure 4.11; in both cases, the experimental data are shown as the points on the figures. The fact that the exit age distribution has two peaks indicates that the hydraulic flow through the reactor is made up of two separate flow patterns.

To model this phenomenon, two conceptual reactors within the actual reactor can be imagined that are operating in parallel with each other, with each representing one of the flow patterns. The first conceptual reactor is modeled as a series of equal-sized continuous flow stirred-tank reactors (CFSTRs). The second conceptual reactor is modeled as two reactors in series: a plug flow reactor (PFG) followed by a series of CFSTRs. The sum of Flow 1 Model and Flow 2 Model is the exit age distribution model. The mathematical model equations are shown in the following (Lawler, 2010; Benjamin and Lawler, 2013).

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

$$E_2(t) = (1-f_1) \frac{1}{\bar{t}_2} \frac{{N_2}^{N_2}}{(N_2-1)!} \left(\frac{t-t_{pfr}}{\bar{t}_2}\right)^{N_2-1} \exp\left(-\frac{N_2(t-t_{pfr})}{\bar{t}_2}\right) \qquad \text{(Flow 2 Model)}$$

$$E_{Tot}(t) = E_1(t) + E_2(t) \qquad \text{(Total Flow Model)}$$

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 equal-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 t_{pfr}, and the second is modeled as N_2 equal-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, Reactor 2 is responsible for the second peak, and the offset between them is caused by a combination of the PFR 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 min is accounted for in the model by the plug flow portion of Reactor 2. Hence, the model has six parameters: the two values of $\bar{t}$, the two values of *N*, the value of t_{pfr}, and the value of f_1. 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 is useful to be cognizant of a few characteristics of the model of *N*-CFSTRs in series (Lawler, 2010; Benjamin and Lawler, 2013). The spread of the model curve is dependent on the value of *N*, with a narrower curve being the result of a higher value of *N*. Also, the peak value in the exit age distribution occurs at a value of $t = \big((N-1)t\big)/\bar{t}$. And, 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 that shoulder in the experimental results shown in Figure 4.11 could be used to estimate t_{pfr}. With these ideas, the first trials for the parameter values were reasonably constrained, and the further efforts to fit the model to the experimental results were straightforward.

The chosen values for all of the model parameters are shown in Table 4.4, and the model results are shown along with the experimental results in Figure 4.10 for the exit age distribution and in Figure 4.11 for the cumulative age distribution. For both distributions, the model fits the experimental data quite well, with some deviation especially at the longer times.

Table 4.4. Flow Model Parameters

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

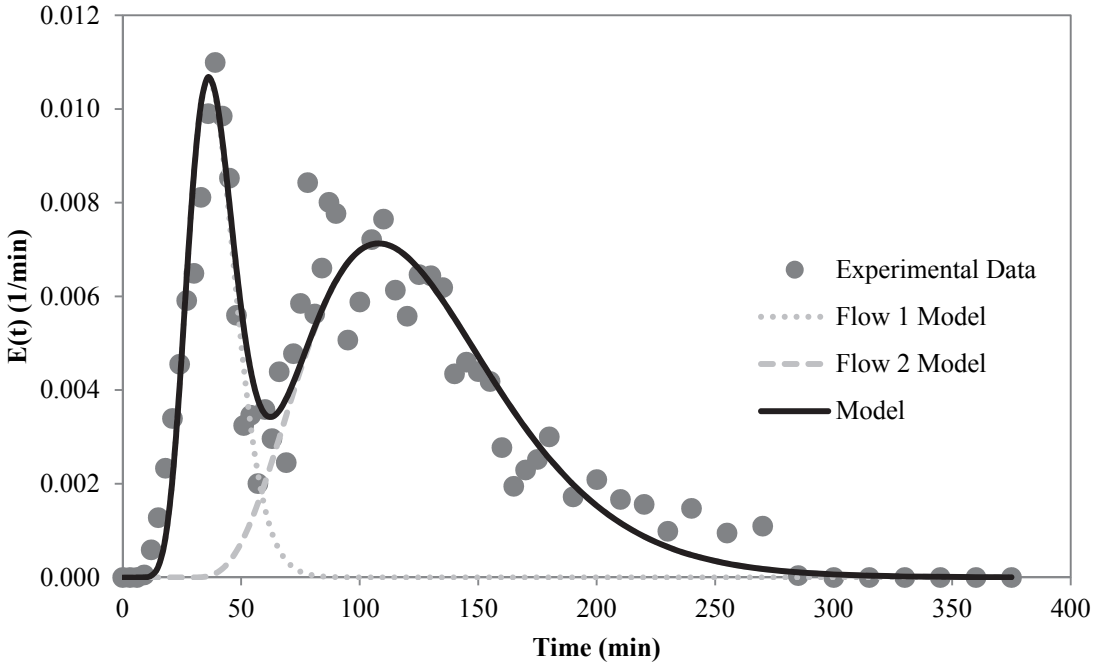

Figure 4.10. Exit age distribution, as determined by the tracer test.

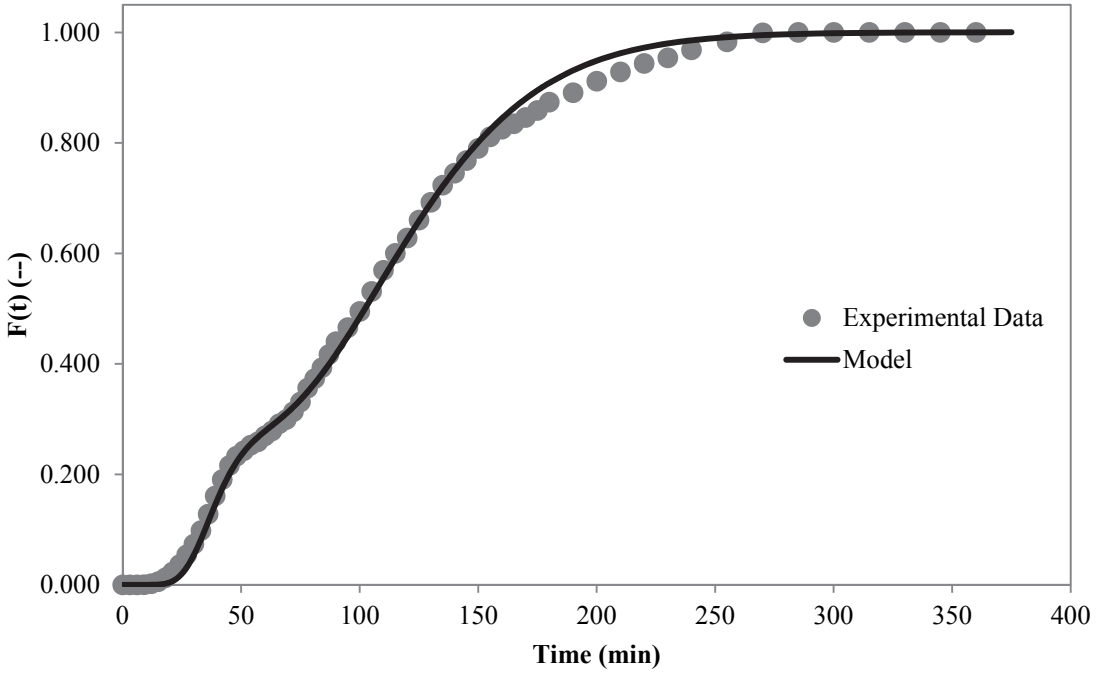

Figure 4.11. Cumulative age distribution, as determined by the tracer test.

4.4.2 Enzymatic Treatment

A continuous flow enzymatic treatment experiment with both oxybenzone and sulfamethoxazole was performed using the same experimental setup as the tracer test.

Effluent PPCP concentrations and enzyme activity were measured every 50 minutes, and these results are shown in Figure 4.12. 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. The results show that a steady state was not reached during the 400 minutes that the experiment ran, but several observations can be made about these results.

This first enzymatic treatment experiment in a continuous flow setting yielded much lower PPCP removals than those measured in batch experiments. This result can be partly, or wholly, explained by the hydraulic characteristics of the continuous flow reactor. The batch experiments previously conducted emulate an ideal PFR with a detention time of two hours, and as the tracer test results show, the continuous flow reactor does not behave like an ideal PFR. The cumulative age distribution (Figure 4.11) shows that 63% of the flow through the reactor had a detention time of less than 2 h and that 25% had a detention time of less than 1 h. In that 63% of the flow, there was less time for the reaction to occur than in the batch experiments, in which 100% of the reaction mixture had 2 h to react. In addition, batch reactors were mixed throughout the 2 h 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 min.

For a first-order reaction in a continuous flow reactor, the steady-state effluent concentration (or fraction remaining) can be predicted from the exit age distribution as follows (Lawler, 2010; Benjamin and Lawler, 2013):

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

The expected steady-state oxybenzone and sulfamethoxazole removal efficiencies under the conditions of this experiment were calculated using the exit age distribution model from the tracer test and the first-order rate constants of oxybenzone and sulfamethoxazole. The PPCP concentration data from the batch experiment shown in Figure 4.9, in which oxybenzone and sulfamethoxazole were treated simultaneously with an enzyme activity similar to that in this experiment, were used to calculate the first-order rate constants. The first-order rate constant (k_1) of oxybenzone is 0.02022 /min, and $k_{1,\text{sulfamethoxazole}}$ is 0.00801 /min. Combining these rate constants with the exit age distribution model yields predicted steady-state removal efficiencies of 80% and 52% for oxybenzone and sulfamethoxazole, respectively. The maximum removal efficiencies achieved in this experiment were 56% and 37% for oxybenzone and sulfamethoxazole, respectively. Because a 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 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, whereas loss of enzyme activity was observed in batch experiments.

Further continuous flow enzymatic treatment experiments need to be conducted. Future experiments will be run for a longer period of time to ensure that they reach a steady state.

Experiments with increased detention times and increased enzyme activity will be performed as well.

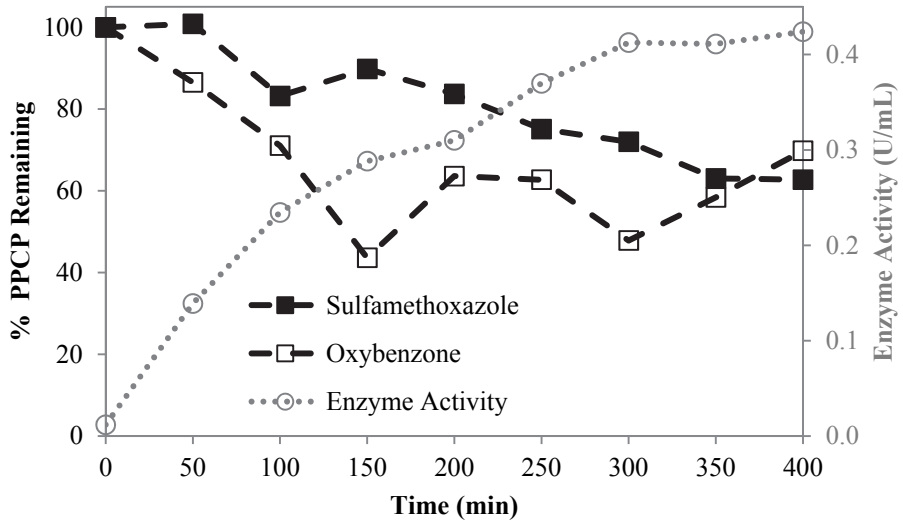

Figure 4.12. Left axis: Percentage of PPCP remaining when oxybenzone and sulfamethoxazole are treated in the continuous flow reactor. Right axis: Enzyme activity over the course of the experiment.

Note: This experiment was conducted with unadjusted pH, 20.63 mg/L acetosyringone, and 0.5 U/mL enzyme activity.

Chapter 5

Summary and Conclusions

Surveys and studies have shown that the public generally supports water reuse up to the point of personal usage (Rock et al., 2012). The public's fear that treatment plants are not doing enough to treat micropollutants is substantiated by the presence of PPCPs downstream of wastewater effluent discharges. Potable water reuse, either direct or indirect, is unlikely to achieve either public or regulatory acceptance if the specter of PPCPs in the water is not substantially removed. Implementing enzymatic treatment as a supplementary process at existing wastewater treatment facilities might be a viable solution for PPCP degradation. The main objective of this research was to exploit the natural laccase-mediator system to treat PPCPs in municipal wastewater in efforts to make direct water reuse safe.

In this work, experiments were devised to determine if the laccase-catalyzed oxidation of PPCPs is an effective process that can be implemented at existing wastewater treatment plants. Enzymatic treatment is likely to be most effective after primary sedimentation and before biological treatment. Studies have shown that biological treatment is accountable for the majority of PPCP degradation that occurs at existing conventional wastewater treatment plants. Enzymatic treatment before this step might enhance the removal of PPCPs by partial oxidation that would allow additional degradation during the subsequent biological treatment.

All oxidation experiments were conducted in batch reactors using locally collected municipal primary effluent wastewater. The first objective of this work was to investigate PPCP removal in two reactor configurations using oxybenzone as the target PPCP. Enzymatic treatment was deemed successful if approximately 90% of the target PPCP was removed. Removal was enhanced via a pseudo-optimization process that varied pH, mediator concentration, enzyme concentration, and treatment time in a step-wise process. The most efficient reactor configuration was chosen based on economics and on its potential for full-scale enzymatic treatment.

Because the mediator is likely to represent a significant cost to enzymatic treatment, the second objective of this work was to identify an alternative mediator source. Laccase can directly oxidize phenolic compounds; thus, waste streams with high phenolic content were sought to study as inexpensive alternative mediator sources. Winery wastewater has been shown to have very high phenolic concentrations; red wine was used as a surrogate in laboratory testing.

The final objective of this research was to investigate the efficacy of the laccase-mediator system for the treatment of other PPCPs as well as the simultaneous treatment of multiple PPCPs. Sulfamethoxazole was used as an additional target PPCP. Oxybenzone and sulfamethoxazole were treated simultaneously using the treatment conditions that had resulted in successful oxybenzone removal.

5.1 Conclusions

The major results and conclusions of this research are as follows:

1. Successful (75–91%) oxybenzone removal was achieved in municipal primary effluent at neutral or unadjusted pH and without supplemental aeration.

2. Wastewater with a greater BOD required a greater mediator concentration (105 vs 88 μM) to achieve successful (>75%) oxybenzone removal.

3. A comparison of the DA and the FRG treatment configurations yielded similar oxybenzone removals. Although both configurations could achieve nearly identical removal efficiencies, the DA configuration was favored because of its ease of operation and economic viability in full-scale enzymatic treatment.

4. Wine is an ineffective alternative mediator source most likely because of the type of phenolic compounds present.

5. Enzymatic treatment of sulfamethoxazole was less efficient than that of oxybenzone, but substantial removal (65%) still occurred, indicating that the laccase-mediator system is capable of removing other PPCPs from primary effluent.

6. The simultaneous treatment of sulfamethoxazole and oxybenzone is as effective as the individual treatment of each PPCP.

7. Simultaneous removal of oxybenzone and sulfamethoxazole was achieved in a continuous flow setting, but improvement of the continuous flow treatment conditions will be necessary.

The results of this work are remarkable in suggesting a biochemically-based treatment for PPCPs that can be easily applied to existing wastewater treatment plants. Because there is no need to adjust pH or provide supplemental aeration, the operational costs of enzymatic treatment are limited. Moreover, using the DA configuration would minimize capital costs. Perhaps most significantly, multiple PPCPs can apparently be treated with one process. Enzymatic treatment might provide a solution to PPCP degradation that several alternatively proposed technologies cannot. The feasibility of enzymatic treatment is thus far extremely promising.

5.2 Future Work

On the basis of this work, the most challenging limitation of enzymatic treatment might be the relatively high concentration of mediator required. Further investigation of alternative mediator sources is necessary to ensure that enzymatic treatment is a viable process. Other food processing wastes, such as olive oil mill wastewater, could be suitable alternative mediators, but additional research is required. Further study of the laccase-mediator system is also needed to identify the oxidation byproducts of enzymatic treatment. It is important that the oxidation byproducts pose less risk than the parent PPCP. Although enzymatic treatment of primary effluent provides an opportunity for further degradation in the subsequent biological treatment process, work should be done to characterize and track the byproducts throughout the activated sludge process to determine the fate of the parent PPCPs and the

byproducts of the enzymatic treatment. Finally, the efficacy of the laccase-mediator system should be evaluated with additional compounds to fully understand if enzymatic treatment can be broadly applied to the vast array of PPCPs present in our water.

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WATEREUSE RESEARCH

1199 North Fairfax Street, Suite 410
Alexandria, VA 22314 USA
(703) 548-0880
Fax (703) 548-5085
E-mail: Foundation@WateReuse.org
www.WateReuse.org/Foundation