LAB-SCALE TREATABILITY OF PHARMACEUTICALS IN WASTEWATER

A Thesis

Presented in Partial Fulfillment of the Requirements for the

Degree of Master of Science

with a

Major in Civil Engineering

in the

College of Graduate Studies

University of Idaho

by

Ryan J. Rehder

May 2014

Major Professor: Erik R. Coats, Ph.D

AUTHORIZATION TO SUBMIT THESIS

This thesis of Ryan J. Rehder, submitted for the degree of Master of Science with a Major in Civil Engineering and titled “Lab-Scale Treatability of Pharmaceuticals in Wastewater,” has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____ Date: _____
Erik R. Coats, P.E., Ph.D.

Committee Members: _____ Date: _____
Richard Nielsen, P.E., Ph.D.

_____ Date: _____
Armando McDonald, Ph.D.

Department Administrator: _____ Date: _____
Richard Nielsen, P.E., Ph.D.

Discipline’s College Dean: _____ Date: _____
Larry Stauffer, Ph.D.

Final Approval and Acceptance

Dean of the College of Graduate Studies: _____ Date: _____
Jie Chen, Ph.D.

ABSTRACT

A primary source of pharmaceutically active compound (PhAC) introduction into the environment is water resource recovery facilities (WRRFs), a.k.a. wastewater treatment plants. Some attention has been given to evaluating existing full-scale WRRFs for their ability to remove PhACs. However, less research attention has been given to PhAC removal at the laboratory-scale, wherein more comprehensive investigations can be conducted.

This study evaluated, at the lab-scale, anaerobic fermentation, conventional activated sludge (aerobic), and anaerobic digestion for treatability of PhACs in wastewater. Additionally, because most of the analytical methods for PhACs are designed around large sample sizes available while evaluating full-scale plants, a modified sample processing and analysis method was developed, based on EPA Method 1694, but tailored for use during lab-scale studies involving small sample sizes.

ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my major professor and committee chair, Dr. Erik R. Coats, P.E. for many years of guidance and patience. Not only did Dr. Coats drive me to complete this work, he became my good friend in the process.

I would also like to thank my committee members, Dr. Richard Nielsen, P.E. and Dr. Armando McDonald, for their guidance throughout this process.

Most importantly, I would like to thank my wife, Courtney, for sacrificing years of her life in dedication to supporting the kids and me while I spent the long hours completing this work. Of all of the people I have to thank, she is the one that without her help, this would not have been possible.

DEDICATION

This work is dedicated to Courtney, Austin, Cassidy, and Lela.

TABLE OF CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

Standard Curves

Fermentation Treatability Results

Conventional Activated Sludge Treatability Results

Anaerobic Digestion Treatability Results

CHAPTER 1: BACKGROUND

1 INTRODUCTION

Modern wastewater treatment began in the early 20th Century. Initially, the focus of treatment was primarily on the removal of settle-able organic solids prior to effluent discharge into surface and ground waters. Such contaminants, if not removed, could excessively pollute water bodies through both the depletion of oxygen (eutrophication) and introduction of microbial pathogens. Around 1920 a novel process was introduced in the United States known as activated sludge (AS). AS relies on microbial populations to biologically remove both solid and dissolved contaminants from wastewater; the additional capability to remove soluble organic contaminants significantly improved effluent quality. As the field of microbiology progressed, and with the advancement of analytical equipment used to detect contaminants, additional potentially pollutive substances were identified and targeted for treatment and removal, or simply eliminated from industrial production. As an example of the latter, the emergence of gas chromatography (GC) in the 1970s exposed widespread polychlorinated biphenyl (PCB) pollution and was instrumental in the eventual elimination of PCB production and importation into the US.

Beginning in the late twentieth century, further advances in analytical capabilities induced the scrutiny of a broad class of compounds termed emerging contaminants (ECs). ECs are defined as any contaminant that causes a potential, perceived, or real threat to the safety of the public or environment and warrants further investigation (EPA, 2010). More specifically, ECs are chemicals most commonly of synthetic origin, being principally very large, complex organic molecules specifically designed to achieve some advanced function or purpose.

ECs can be any chemical or material such as pharmaceuticals, industrial wastes, or pesticides.

One sub-class of ECs is known as pharmaceutically active compounds (PhACs), which include human and veterinary pharmaceuticals, some active ingredients in human grooming and cleansing products, and disinfectants. Many water quality professionals consider PhACs to be of greater concern than other ECs because of the nature of the compounds, as they are produced with the intention of creating a biological effect at low concentrations (Halling-Sørensen, 1998).

The main sources for the introduction of PhACs into the water environment are improper disposal of unused medications and bodily excretion in the urine or feces following ingestion (Gros et al., 2010). Under either scenario, PhACs ultimately end up at water resource recovery facilities (WRRF). In addition to parent PhACs, many drugs are also excreted entirely as metabolites, adding to the already large number of individual compounds present in wastewater (Langford and Thomas, 2011). Recognizing this potential barrier (i.e., the WRRF) to ultimate release into the water environment, some research has been conducted on PhAC removal in WRRFs. For example, results have indicated that some PhACs are fully removed while others pass through with little change (Ternes et al., 2005; Yang et al., 2011). However, treatment effects are clearly mixed. As evidence of the potential limited treatment at WRRFs, parent PhACs or their metabolites have been detected in drinking water, groundwater, wastewater, sewage sludge, and biosolids land application site soils (Golet et al., 2003; Kolpin et al., 2002; Ternes et al., 2005; Ternes, 1998). Considering the broad number and array of PhACs (including metabolites), ultimately much remains to be examined.

In the broader examination into alternative means to control PhAC efflux into the environment, it is clear that the alternative of controlling pharmaceutical use is not an

option. Clearly, pharmaceuticals have contributed greatly to the heightened life expectancy and quality of life in the modern era (Schwab et al., 2005), and use will not end anytime soon. As evidence of the increasing challenge, there are in excess of 3,000 different pharmaceuticals in use today, and that number grows annually (Gros et al., 2010). There are between 20 and 30 new compound identities produced per year with an increase in usage volumes of 6% - 10% annually from 1999 through 2011 (Health, 2011). A main driver of increased usage has been a 15.1% rise in the number of individuals aged 65 and older between the years 2000 and 2010, a trend that is likely to continue (Census, 2010). Thus, as engineers we must develop appropriate solutions.

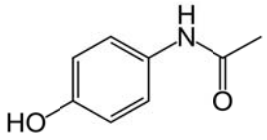
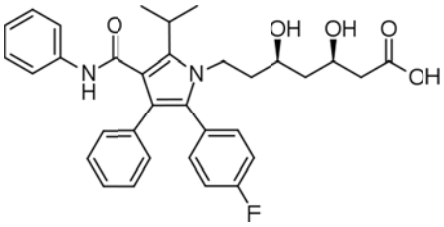
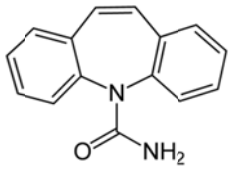
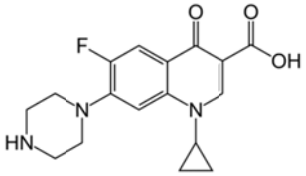
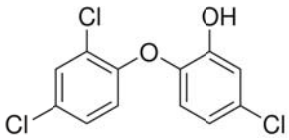
Recognizing the potential concern about PhACs in the water environment, and also considering that limited research has ultimately been performed in this regard, the purpose of this research project, and intrinsically this thesis, was to evaluate the potential to remove certain PhACs from wastewater. The research specifically focused on conventional biological treatment processes, based on the reality that, globally, trillions of dollars have been invested in such processes and ultimately little is known about the real potential for this existing infrastructure to positively effect PhACs in the water environment. In the course of executing these investigations, it was also determined that research was required to advance appropriate analytical methods for PhAC quantification in lab-scale systems. Through analytical optimization and the experimental targeting of functioning biological treatment systems, one can gain an understanding of the role of wastewater treatment in preventing or limiting environmental contamination of ECs.

2 BACKGROUND

2.1 PhAC Compounds Targeted for Research

Due to the extremely large number of PhACs presently prescribed medicinally in the U.S. (all of which can enter the sanitary sewer system and ultimately the WRRF) and further considering the very difficult analytical complexities in quantifying the many PhACs, to maintain an appropriate level of focus, in this study specific target compounds were selected to represent characteristics that could be correlated to other similar agents (i.e., "model" compounds). The suite of compounds chosen needed to represent broad classes of PhACs. To that end, compounds were chosen to represent both hydrophobic and hydrophilic behaviors along with those shown indicated by prior research to be biodegradable versus those that tend to be recalcitrant. The PhACs selected for this study were atorvastatin (Lipitor), acetaminophen (Tylenol), carbamazepine (Tegretol), ciprofloxacin (Cipro), and triclosan. As noted, another complication in PhAC research pertains to parent compounds vs. metabolites. The majority of published studies in wastewater treatment deal only with the parent compounds, while documentation of PhAC metabolites and transformation products is relatively rare (Kosjek et al., 2007). While parent compounds are certainly of concern in the water environment, the activity of PhAC metabolites can be equal to or exceed the activity associated with the parent chemical. Additionally, excreted inactive metabolites can even be transformed back into the original active drug during the wastewater treatment process (Carballa et al., 2004). Nevertheless, while metabolites are a concern, this study focused only on the parent compounds. Ultimately, the broad environmental effects of these PhACs are truly unknown. However, anecdotal evidence supporting environmental concerns is presented below for most of the analytes targeted in this study (Verscheren, 2008).

Table 1: Target Analytes for this Study

Common Name	Chemical Structure	MW	pKa	log K_{ow}
Acetaminophen		151.16	9.38	0.46
Atorvastatin		1155.34	4.46	5.70
Carbamazepine		236.27	7.00	2.45
Ciprofloxacin		331.34	5.90	0.28
Triclosan		289.54	7.90	5.25

2.1.1 Atorvastatin

Atorvastatin (AT), selected because of its widespread use, is the top selling prescription drug of all time (A.P., 2011). AT is a lipid regulating compound that is prescribed to reduce LDL cholesterol in patients with elevated levels. Remarkably, given its significant prescriptive use, the fate of AT in WRRFs is less studied than compounds in other PhAC classes. A survey by Metcalfe et al. indicated AT measured in WRRF influent and effluent at mean concentrations of 76 ng/L and 32 ng/L respectively (Metcalfe and Eddy, 2003). This survey was only for the parent compound. Additionally, several active metabolites have been identified in wastewater. Two main active metabolites of atorvastatin are *ortho*-

hydroxyatorvastatin and *para*-hydroxyatorvastatin; both oxidative products (Nirogi et al., 2006).

In regard to environmental concerns with AT, studies have indicated potential phytotoxicity or growth-related inhibitory effects on aquatic plants with sustained exposure to AT from plants grown in spiked growth media. Concentrations of 30 µg/L or less were documented to have significant impacts on *Lemna gibba,* a widespread aquatic plant, resulting in decreased concentrations of sterols in plant extracts. Sterols are critical components of plant membranes (Brain et al., 2006). However, typical environmental concentrations of <50 ng/L indicate little potential for impact of *Lemna* in real systems (Verscheren, 2008). It is likely, because of the mode of action of AT (inhibits cholesterol production), that other plant species or aquatic life could see negative impacts from elevated concentrations of AT in natural systems.

2.1.2 Acetaminophen

There are a number of PhACs that belong to the broad class of compounds known for their analgesic and antipyretic properties. Acetaminophen (AC) is one such compound. AC is often ingested alone or as a part of compounded medicines. The mode of action in the human body of AC is not fully understood. The primary reasons acetaminophen was chosen for this study is it widespread prevalence and its tendency to sorb to solids making it an excellent candidate (i.e., model compound) for studying PhACs in solids.

A number of studies have indicated concerns over AC in the environment (Joss et al., 2006; Ng et al., 2011; Yu et al., 2012). Most have concluded that this is because AC is the most widely used drug in the world and it has been shown to persist in the environment. Kim et al. suggest it is likely that local environmental concentrations of AC are in excess of their observed “predicted no-effect concentration (PNEC).” The PNEC was based on the studies observation of negative effects of AC on *Daphnia magna* (Kim et al., 2007).

2.1.3 Carbamazepine

Carbamazepine (CZ) belongs to a class of PhACs known as anticonvulsants. CZ is used to treat epilepsy and is also a specific analgesic for trigeminal neuralgia (severe acute pain typically in the face or neck). While the mechanism of action in the human body of CZ is not well known, it is believed to act by reducing polysynaptic responses and blocking post-tetanic potentiation (Novartis, 2014).

CZ is of particular interest to water professionals and was selected for this study because of its documented resistance to removal in WRRFs (Carballa et al., 2004; Joss et al., 2006; Ternes et al., 2005; Yang et al., 2011). This recalcitrant behavior is important because if methods to remove CZ effectively could be established, CZ could then potentially serve as an indicator that many other less-difficult-to-remove compounds were also reduced. Several metabolites of CZ have been identified, with the most common being carbamazepine-10,11-epoxide (Langford and Thomas, 2011). This metabolite maintains pharmacokinetic effects similar to those found with the parent compound (Novartis, 2014). Both have been found in WRRFs and natural waters (Langford and Thomas, 2011; Ternes et al., 2005). A 2010 study by Gros et al. identified CZ in all samples from a broad survey of WRRF and natural waters (Gros et al., 2010).

As an example of the environmental concerns with respect to CZ, a 2003 study by Cleuvers indicated that CZ acted by non-polar narcosis against *Daphnia*, a water flea, and that in the presence of some other PhACs, the combination effects on *Daphnia* were approximately additive in nature. This report also documented inhibited growth for *Lemna minor* (Duckweed) in the presence of CZ. However, EC_{50} concentrations were considerably higher than those in natural waters or WRRF influent and effluent (Cleuvers, 2003).

2.1.4 Ciprofloxacin

Antibiotic agents represent a large fraction of all prescribed medications. Overprescription of some antibiotics is believed to have contributed to the development of antibiotic resistant strains of certain pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA). Modes of action for differing antibiotic classes vary widely, allowing for the treatment of otherwise resistant strains of microorganisms. Ciprofloxacin (CP) is commonly employed when there is indication of resistance to other agents. However, it is also a "first-line" antibiotic for the treatment of some serious or hard-to-treat pathogens such as the Gram-positive *Bacillus anthracis*, the bacteria that causes anthrax. There is no known cross-resistance between CP and other antibiotic classes (Bayer, 2013).

CP acts by inhibiting DNA gyrase, the enzyme responsible for helping fold and unfold DNA prior to and following DNA replication (Drlica and Zhao, 1997). The inhibition of DNA "unfolding" prevents transcription which, in turn, prevents bacterial replication. CP is the most widely prescribed drug of a larger class of PhACs known as fluoroquinolones, all of which act in a similar manner (Golet et al., 2003).

CP was chosen for this study because of its unique mode of action and the high potential for harm from widespread bacterial resistance. CP is highly sorptive and thus is a good candidate compound for the study of PhACs in solid matrices.

2.1.5 Triclosan

Triclosan (TC) is an antibacterial agent present in many over-the-counter household and personal care products such as hand soaps and toothpaste. It can also be found in the plastic of some containers and in household cleaners. TC's primary function is the slowing or inhibition of microbial growth. It is effective against bacteria, fungi, and mildew (EPA, 2010).

TC characteristics include a very high solids-partitioning coefficient and moderate biodegradability (Karnjanapiboonwong, 2011). For this reason, TC was chosen as a

candidate for experiments focused on solid matrices. Also, there exists profound public perception that TC may be detrimental to health, especially in children and pregnant women (FDA, 2013).

TC, while maintaining general bactericidal effects has been largely vindicated of the once speculated severe ecological effects it may have in the environment. Several studies have evaluated TC in wastewater effluent and land-applied wastewater sludge with a study by Reiss et al. finding little evidence of inhibitory or negative effects on plants, earthworms, birds, fish, mammals, or soil microorganisms (Reiss et al., 2009). However, Kookana et al. implicated TC in disrupting the nitrogen cycle in sensitive soils, albeit at above environmentally relevant concentrations (Kookana, 2011).

2.2 Wastewater Treatment Technologies

Biological liquid-stream wastewater treatment processes can be grouped into two major classes; respirative (oxygen and/or nitrate available in bulk solution as a terminal electron acceptor) and non-respirative (effectively no exogenous terminal electron acceptor). Examples of respirative processes include conventional activated sludge (CAS) and trickling filters. Fermentation of primary sludge and upflow anaerobic sludge blanket (UASB) reactors are examples of non-respirative wastewater treatment processes that could be associated with liquid stream treatment. Beyond the influent wastewater stream, biological treatment processes generate sludge (biomass) that must also be treated, and both respirative and non-respirative processes similarly apply. In this regard, anaerobic digestion (AD, a non-respirative process) remains the most common method for biomass treatment; fermentation is also applied to partially digest sludge.

The potential need to treat wastewater for PhACs has been recognized. However, much of the PhAC research-to-date has focused either on a relatively small number of existing full-

scale WRRFs (with no consistent plan or trend as to the WRRF technologies evaluated) or on advanced wastewater treatment processes such as advanced oxidative processes (AOPs; which are not broadly applied at WRRFs) for their PhAC removal capabilities (Carabineiro et al., 2011; Jelic et al., 2011; Li et al., 2012; Martucci et al., 2012; Sirtori et al., 2009; Van Doorslaer et al., 2011; Yuan et al., 2013). Because of the significant investment (trillions of dollars) into wastewater treatment both in the U.S. and globally, there is a need to more thoroughly assess the capabilities of conventional processes as well as to evaluate which operational parameters could play a key role regarding pharmaceutical removal. To that end, this research has focused on the more conventional wastewater treatment processes detailed below. The below descriptions explain why and how each respective process is used within a WRRF; a brief overview of PhAC treatment capability follows.

2.2.1 Fermentation

Municipalities that discharge to eutrophically sensitive waters are experiencing increasingly stringent effluent contaminant limits, especially for phosphorus. Thus, phosphorus removal in WRRFs has received great attention over the last decade (with continued focus well into the 21st century). While there are two main process configuration options for removing phosphorus, research has indicated the biological phosphorus removal process should be selected as a first line of defense over chemical/physical processes (Coats et al., 2011). The process known as enhanced biological phosphorus removal (EBPR) relies on the volatile fatty acid (VFA) content (either inherent or generated) of wastewater to drive critical bacterial metabolisms to achieve effective phosphorus removal. A primary mechanism to produce VFAs is to separate and ferment organic solids present in the influent wastewater. This process is known as primary solids fermentation. Fermentation and VFA production can also be achieved utilizing waste sludge from a secondary treatment process.

Fermentation is an anaerobic process that relies on microorganisms to hydrolyze and then convert organic solids (proteins, complex carbohydrates, lipids) into VFAs in a process known as acidogenesis. These VFAs are then comingled back into the liquid wastewater stream where they become available for secondary suspended growth microbes to facilitate EBPR.

2.2.2 Conventional Activated Sludge

Aerobic biological suspended-growth wastewater treatment processes are collectively known as conventional activated sludge (CAS). CAS systems make up a large majority of all WRRFs in the U.S., and have been designed and constructed in a variety of configurations to achieve removal of organic carbon and ammonia-nitrogen (Metcalfe and Eddy, 2003).

CAS systems can be configured as sequenced bio-reactors (SBRs), semi-plug continuous flow racetrack type, or complete mixed continuous flow systems. The configuration and operation of CAS reactors are target-compound specific. However, the biological processes for all types are similar.

CAS systems not only produce reclaimed water, but also produce sludge. Because CAS is a biological process, microbial growth occurs. The biomass (sludge) generated must be removed from the system daily to maintain quasi steady state operations. The sludge that is removed must then be disposed of or treated. Typically composting, lime stabilization, or digestion (anaerobic or aerobic) is utilized to reduce pathogens and minimize odors. The stabilized sludge is then either sold as a soil supplement or land applied agronomically, depending on the extent of stabilization.

2.2.3 Anaerobic Digestion

Anaerobic digestion (AD) has been implemented for decades across the U.S. and globally as a means to reduce volumes of organic-rich wastes. AD finds its primary application in

treating waste sludge (biomass) produced from WRRFs; AD reduces both the mass of sludge and number of pathogens in the sludge so that it can be used as a soil supplement/fertilizer for agricultural or residential use. AD also generates methane-rich biogas that can be used to produce electricity to supplement grid power(Metcalfe and Eddy, 2003).

As its name implies, AD relies on anaerobic biological processes. Typical AD microbial consortia are capable of breaking down complex feedstock and converting the metabolites to methane and carbon dioxide. AD has experienced recent increased implementation because of increased pressure by regulatory agencies to reduce the carbon footprints of WRRFs.

2.3 Overview of PhAC Oxidation

There are many chemical and biological processes currently employed in conventional wastewater treatment that have the potential to reduce PhACs in wastewater. These processes principally include chemical oxidation, biological oxidation, photo-oxidation, and sorption. However, as discussed previously, PhACs are only removed incidentally (i.e., processes are not specifically designed with PhAC removal as a target) as current biological wastewater treatment systems principally target “conventional” wastewater contaminants (e.g., phosphorus, nitrogen, organic carbon).

Whether induced chemically or biologically or by another mechanism, oxidation is the process by which electrons are stripped from compounds such as PhACs and transferred to an electron acceptor (oxidizing agent). Although this process occurs naturally in the presence of oxygen (an oxidizing agent), the rate is slow. Most of the time, chemical oxidation is induced by the addition of oxidizing agents such as hydrogen peroxide or ozone. Other oxidizing agents commonly used in wastewater treatment include

permanganate, chloride dioxide, and chlorine (Metcalfe and Eddy, 2003). Biological oxidation is typically facilitated by a microorganism's need to either gain some energy value from the substrate (PhAC or other compound), or to eliminate a contaminant that is potentially inhibitive to the organism.

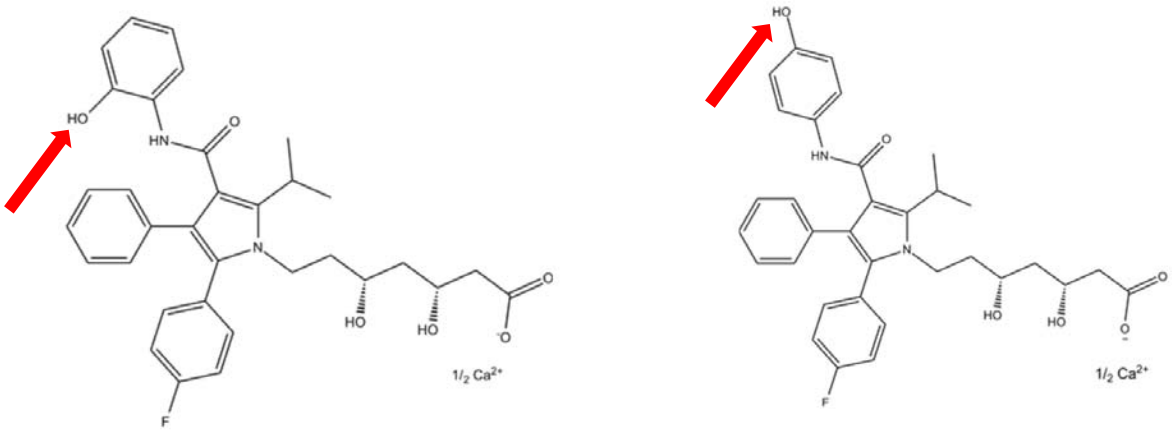
As simple examples of oxidation, reactions 1 through 4 below represent hydrogen peroxide or ozone induced oxidation of iron. These reactions are typically catalyzed by the presence of an acid represented in the equations as free H^+. The reactions are shown as two distinct steps known as half-reactions; however, these half reactions occur simultaneously as one reaction.

Eqn. No.			
1.	Hydrogen Peroxide:	$Fe \rightarrow Fe^{2+} + \mathbf{2e^-}$	(Fe loses 2 electrons)
2.		$H_2O_2 + 2H^+ + \mathbf{2e^-} \rightarrow 2H_2O$	(H_2O_2 gains 2 electrons)
3.	Ozone:	$Fe \rightarrow Fe^{2+} + \mathbf{2e^-}$	(Fe loses 2 electrons)
4.		$O_3 + 2H^+ + \mathbf{2e^-} \leftrightarrow O_2 + H_2O$	(O_3 gains 2 electrons)

It can be seen from the equations above that the electrons (e^-) are transferred from the iron to the oxidizing agent. Additionally, during this process, hydroxyl radicals are formed upon the decomposition of H_2O_2 which induce additional oxidative reactions. Within the context of chemical oxidation, these secondary reactions are primarily responsible for the oxidation of complex compounds such as PhACs. Similar chemical reactions can also be induced biologically, as many microbial species and, commonly, liver cytochromes can facilitate the transfer of electrons from an electron donor to an electron acceptor. Most PhACs for instance, undergo at least partial oxidation in the liver following ingestion in a process known as hydroxylation. Hydroxylation occurs when a hydrogen atom in a C-H bond is replaced with a hydroxyl group resulting in the formation of a C-OH^- bond. The hydroxyl comes as a hydroxyl radical, formed as discussed above. An example of this is presented with the identification of the primary metabolites of atorvastatin; *ortho*-hydroxyatorvastatin

and *para*-hydroxyatorvastatin (see Figure 1). This type of metabolization always renders the metabolites more water soluble than the parent compound (Halling-Sørensen, 1998).

Figure 1: Examples of hydroxylated PhACs, *ortho*-hydroxyatorvastatin (left) and *para*-hydroxyatorvastatin. Note the hydroxyl (OH) group attached to the upper left benzene ring



2.4 Fate of PhACs in WRRFs

Untreated PhACs are found in WRRFs partitioned into two fractions. Hydrophilic compounds tend to remain dissolved into the liquid fraction (mainly water) while the lipophilic or hydrophobic compounds will tend to partition onto/into the solids (mostly organic). While the liquid or aqueous fraction that is not oxidized will exit the WRRF with the effluent, most of the solid-bound PhACs will be maintained in the facility for a longer period of time.

The potential for PhACs to sorb and accumulate in, or be discharged from, biological WRRFs can be best understood by characterizing WRRF solids accumulation. The typical hydraulic retention time (HRT), and thus retention time for hydrophilic and untreated PhACs, in a biological secondary WRRF (e.g., CAS system) ranges from 8 to 24 hours. However, it is common for the solids (biomass) to be retained in the system for much longer

periods of time; a typical solids retention time (SRT) in a CAS system would range from four to 20 days. As the incoming wastewater contacts the internally recycled solids, those PhACs that have a high sorption coefficient (k_d; proportional to K_{OW}) are likely to sorb to particulate organic matter; a continuous buildup of solids partitioned PhACs is thus likely to occur.

Sorption to biomass and other particulate matter typically occurs due to one of two phenomena depending on the nature and chemistry of the PhAC. If the PhAC is highly polar, hydrogen bonding to the negatively charged bacterial cell membrane will occur. CP, like many antimicrobials, contains highly polar functional groups which can mediate their strong adsorption to soils and solids (Aristilde et al., 2010). For hydrophobic, lipophilic compounds, the solubilization of the PhAC into the lipid containing bacterial cell membrane is the likely mode of adsorption.

WRRF performance is typically driven by regulatory permits designed to target contaminants in the liquid effluent. Currently there are no established standards or treatment requirements for PhACs in the liquid stream effluent. In addition to liquid stream treatment requirements, there are regulatory requirements for sludge disposal (depending on the disposal method chosen). However, few regulations exist for PhACs in sludge and biosolids. As described, many PhACs readily sorb to biomass and primary sludge in biological WRRFs and to chemical flocs in chemically induced treatment processes. When the sludge is then disposed of, the sorbed PhACs (potentially still active) are disposed of as well. Biosolids are often applied agronomically wherein the ECs (PhACs) accompany the biosolids to the agricultural field, where they have been found to persist (Golet et al., 2003).

2.4.1 PhACs in Wastewater – Current State of Knowledge

As noted, much of the PhAC research to-date has been focused on evaluating existing full-scale WRRFs for their ability (or inability) to remove PhACs. Through this research,

treatment potential has been intrinsically assessed, albeit only within the context of the specific process configurations examined; the potential to modify or modulate operations of conventional treatment processes to achieve measureable removal has not been broadly evaluated. Table 2 summarizes a sample of studies specifically targeting AC, AT, CP, CZ, and TC removal in WRRFs.

Table 2: Survey of Research on PhACs in WRRFs

Compound	WRRF Configuration	Medium	Removal efficiency (%)	% sorbed	Reference
AC	CAS	liquid	96-100		(Gros et al., 2010)
	CAS/CA+F	liquid/liquid	100		(Lubliner et al., 2010)
AT	CAS	liquid	40-80		(Gros et al., 2010)
	CAS	liquid/solids	30-90	10-60	(Jelic et al., 2011)
	Variable	liquid	66 (median)		(Lee et al., 2009)
CP	CAS	liquid	37-99		(Gros et al., 2010)
	EBPR	liquid/solids	63	85*	(Jia et al., 2012)
	CAS/CA+F	liquid/liquid	44-78/100		(Lubliner et al., 2010)
	CAS	solids		<10	(Karnjanapiboonwong, 2011)
CZ	CAS	liquid	n.d.		(Carballa et al., 2004)
	CAS/MBR	liquid,liquid	0,0		(Clara et al., 2005)
	CAS (lab-scale)	liquid	0		(Fernandez-Fontaina et al., 2012)
	CAS	liquid	0		(Gros et al., 2010)
	CAS	liquid, solids	0	1-5	(Jelic et al., 2011)
	CAS/MBR/FB	liquid/liquid/liquid	0-18/0-22/0-10		(Joss et al., 2005)
	CAS/MBR/FB	solids/solids/solids		0/0/0	(Joss et al., 2005)
	CAS/Primary	liquid/liquid	10/5		(Lajeunesse et al., 2012)
	CAS/CA+F	liquid/liquid	-41-51/-71-29		(Lubliner et al., 2010)
TC	CAS/CA+F	liquid/liquid	60-100/95-100		(Lubliner et al., 2010)
	CAS	solids		> 90	(Karnjanapiboonwong, 2011)

AC = acetaminophen, AT = atorvastatin, CP = ciprofloxacin, CZ = Carbamazepine, TC = triclosan
CAS = conventional activated sludge, CA+F = chemical addition followed by filtration, MBR = membrane bioreactor
EBPR = enhanced biological phosphorus removal, FB = fixed bed bioreactor, Primary = primary treatment only
n.d. = not detected
* Exceeds removal efficiency (greater than 100% of influent calculated using a system mass balance)

It can be seen in Table 2 that the removal potential for the target analytes in this study varies greatly. For CZ, as previously indicated, very little removal was observed in any WRRF configuration. AC and TC, on the other hand, were easily removed by activated sludge facilities. However, as in the case of TC, it is possible that the apparent "removal" of analyte from the aqueous fraction is simply due to its partitioning to solids rather than chemical or biological degradation. Likewise, CP and AT both indicated an affinity for WRRF solids. Ultimately, one must use caution when viewing the "removal" efficiency claimed in these studies. The removal observed from the aqueous phase may only be the result of the PhAC transitioning from the aqueous phase to the solid phase.

In this present study, both the aqueous fraction and solid bound fraction were investigated. In this manner, one can observe the potential for a particular process to remove the target PhACs, and can also draw conclusions about the potential fate of individual compounds studied, i.e. where the compounds that are not eliminated in the process may end up (aqueous or terrestrial environments).

2.5 PhAC Analytics

Complementing PhAC treatability and fate in WRRF is the intrinsic need to quantify PhACs in wastewater, solids, and reclaimed water. PhAC quantification methods can be divided into two distinct process steps, sample preparation and sample analysis. Sample preparation includes the sampling method and sample pretreatment. Sample analysis entails the use of precision laboratory analytical equipment to detect and quantify PhACs in prepared samples. Details on these specific procedures, based on current methods, are discussed below; **however, it must be noted that the following narrative is intended to be more general**. As this research project evolved, it became clear that this thesis would need to focus, in part, on the analytical methods as related to conducting lab-scale research. **Ultimately a major component of this thesis was to advance and refine the very complicated PhAC analytical methods, and these details will be addressed in a subsequent chapter.**

2.5.1 Sample Preparation

Typical sample preparation protocols for samples thought to contain PhACs include multiple steps. First, the type of sample is determined; WRRF and environmental samples can be either grab or composite in nature (in accordance with Standard Methods). Next, depending on the estimated PhAC concentrations within the sample, a sufficient sample size is determined. For example, if natural ground or surface water is being tested, larger samples would be required than would be the case with wastewater samples due to the

likely presence of lower concentrations of PhACs than that present in the wastewater. Another example would be testing for PhACs in biosolids and/or reclaimed water land application site soils or riverbank sediments versus testing WRRF sludge effluent. The likely existence of considerably higher PhAC concentrations in the raw wastewater sludge would dictate a smaller sample size requirement. PhACs have been found in both the aqueous phase and bound to solid particulates in both wastewater influent and effluent.

2.5.1.1 Solid Phase Extraction

Solid phase extraction (SPE) is employed to concentrate target analytes and to reduce the presence of potentially interfering compound in sample matrices. SPE is often a critical step in the preparation of environmental and WRRF PhAC samples because of the low concentration and complex matrices. SPE is a form of chromatography in the sense that compounds can be separated by their differing physical or chemical characteristics. SPE utilizes a sorptive media to selectively capture target analytes while minimizing the capture of other substances of no interest. While there are many different sorptive media used for SPE, some of the more common media are summarized in Table 3, organized by their mode of action (Supelco, 1998).

Table 3: SPE Sorbent Summary

Category	Liquid Phase	Solid Phase	Mechanism	Examples
Reversed Phase	Polar	Non-polar	Hydrophobic Interactions	C-18, C-8, C-4
Normal Phase	Non-polar	Polar	Hydrophilic Interactions	C-N, C-NH_2
Ion Exchange	Any	Any	Electrostatic Interactions	SAX, WAX
Adsorption	Any	Any	Adsorption	Proprietary Sorbents

Typical SPE cartridges are constructed of plastic or glass containing a specific mass of sorptive media (30 – 5000 mg). As the liquid sample is passed through the media, the target analytes are removed from solution and are thus bonded to the sorbent. Then, as an

elution solvent is passed through the cartridge, the affinity that the target analyte has for the solvent is greater than that for the media, resulting in the resolubilization of the analytes into the elution solvent. Following elution, the now concentrated analytes can be subject to further processing.

The first step in performing SPE on PhAC-containing matrices is to identify the target analytes. Once analytes are identified, an SPE cartridge can be selected that contains the applicable sorbent of an appropriate mass. The mass of sorbent is typically dependent on the sample size and the estimated analyte concentration. For a given sample matrix, larger sample sizes may require more sorbent. Of note, non-target analytes such as humic substances or fats can also compete for sorption sites, therefore sample matrices known to contain high concentrations of interfering compounds will indicate larger sorbent mass requirements. Interference in SPE will likely decrease the recovery of the target analyte due to competitive sorption by the interfering compound. Interfering compounds can be any material present in a sample that has the possibility of impacting the results of the PhAC analyses.

Steps involved in SPE include the following:

Conditioning: Involves pre-rinsing the sorbent media with solvent to eliminate interfering compounds that may be introduced by the sorbent itself when exposed to said solvent

Equilibration: Water rinse which reduces the solvent content from the conditioning step to eliminate the potential for analyte “pass-through” due to the presence of residual solvent

Application of mobile phase (sample): Applies the sample to the cartridge typically through vacuum or positive pressure manipulation

Drying: Similar to the equilibration step but involves reducing the mobile phase liquid such that it will not interfere with elution

Wash: Optional step wherein a low organic content aqueous solvent is applied to reduce the presence of unwanted/interfering compounds

Elution: The application of organic solvent to disrupt analyte-sorbent bonding, resolubilizing the compounds of interest

Once elution is complete, the eluted solvent containing the target analytes may be ready for analysis. It is common to evaporate the eluted solvent in order to further concentrate the analytes. Also, for some analytical methods or sample handling and storage requirements, a solvent exchange may be necessary. Solvent exchange typically involves drying the elution solvent to near dryness and then adding the desired solvent (typically water + low % formic acid) until a target final volume is achieved.

Variations of this process are summarized in Table 4 along with associated references.

Table 4: SPE Literature Review Summary

Relevant PhACs	Sample Size	Filtration	SPE Cartridge	Cartridge conditioning	Sample pH Adjustment	Sample application method	Application Flow rate	Cartridge Wash	Cartridge Drying	Eluant	Post processing	Analysis Method	Reference
carbamazepine	200 ml (acidic PhACs); 500 ml (neutral PhACs)	Whatmann GF 6 (0.45 µm)	Waters Oasis HLB, 60 mg (acidic PhACs); RP-C_{18ec}, 500 mg (neutral PhACs)	1 ml MeOH; 1 ml Milli-Q water	pH 2-3 (acidic PhACs); pH 7-7.5 (neutral PhACs)	-5 psi vacuum	20 ml min^{-1}	not specified	not specified	4 x 1 ml MeOH	Evap. to 1 ml under N_2	LC/MS	Salgado et al., 2010
acidic PhACs	500 ml	Yes, filter not specified	RP-C_{18}, 1 g	not specified	pH 2	not specified	not specified	not specified	not specified	1.5 ml MeOH	PFBBr derivatization	GC/MS	Kimura et al., 2005
atorvastatin, carbamazepine	not specified	Glass fiber	Waters Oasis MCX	6 ml MeOH; 4 ml Milli-Q water	pH2	not specified	not specified	6 ml Milli-Q water	under vacuum	4 ml MeOH; 4 ml MeOH + 5% formic acid	evap. to dry; resuspend in AcN + ammonium acetate	LC-MS/MS	Tarcomnicu et al., 2011
acetaminophen, triclosan	250 ml (raw WW); 500 ml (treated WW)	1.2 µm Millipore GF/C	Phenomenex Strata X, 200 mg	5 ml MeOH; 5 ml Milli-Q water pH 2	pH 2 with 2 M sulfuric acid	vacuum manifold	6 ml min^{-1}	10 ml 10% MeOH in Milli-Q water	not specified	6 ml AcN	PFBBr derivatization	GC/MS	Yu et al., 2012
carbamazepine	500 ml (raw WW); 1000 ml (treated WW)	Filter paper; 0.7 µm glass fiber	Waters Oasis HLB, 60 mg	10 ml MeOH; 10 ml ultra-distilled water	none	vacuum	10 ml min^{-1}	none	15 min under vacuum	25 ml MeOH	evap. to dry under N_2; resuspend in 1 ml 25% MeOH in ultra-distilled water	LC-ESI-MS/MS	Muz et al., 2012
ciprofloxacin	500 ml (surface water)	Yes, filter not specified	Two-stage: Waters Sep-pak C_{18}; Fisherbrand SAX (100mg)	Unspecified volumes: MeOH; 0.2% formic acid	none	not specified	1-1.5 ml min^{-1}	3 ml 0.1% formic acid	15-20 min under vacuum	3 ml 0.1% formic acid: MeOH (10:90)	evap. to 300 µl under N_2	LC-DAD/MS	Cardoza et al., 2005
acetaminophen, carbamazepine, ciprofloxacin, triclosan	200 ml	not specified	Waters Oasis HLB, 500 mg	4 ml MeOH; 6 ml ultra-pure water	not specified	not specified	not specified	none	air dry: 3 min	5 ml MeOH	evap. to 0.5 ml	LC-MS/MS	Ferrer et al., 2010
ciprofloxacin	10 ml (test water); 10 ml (NH_4OH)	0.45 µm Versapor	Waters Oasis MAX, 60 mg	1 ml MeOH; 1 ml 10% NH_4OH in distilled water	none	not specified	< 1 ml min^{-1}	none	air dry: 5 min	0.1 ml 0.2 M HCl; 1.5 ml 0.2 M HCl in MeOH; 0.4 ml 5 mM PO_4 buffer pH 3	evap. to 0.8 ml under N_2; brought up to 1 ml with PO_4 buffer	HPLC-FD	Belden et al., 2007
ciprofloxacin	500 ml (surface water)	0.7 µm baked glass fiber	Two-stage: Waters Oasis HLB, 60 mg; Waters Oasis MCX, 60 mg	Unspecified volumes: Ultra pure water; MeOH; 5% NH_4OH in MeOH	pH 3 with sulfuric acid	vacuum manifold	not specified	none	not specified	MeOH; MCX cartridge additional 5% NH_4OH in MeOH	Add 300 µl ammonium acetate	LC/MS-ESI	Kolpin et al., 2002
acetaminophen	1000 ml (surface water)	0.7 µm baked glass fiber	HLB (500 mg)	not specified	not specified	not specified	15 ml min^{-1}	not specified	not specified	Unspecified volumes: MeOH; trichloroacetic acidified MeOH	Both eluants dried separately under N_2 near dryness; combined, brought to 1 ml with 10% AcN in water	HPLC	Kolpin et al., 2002
atorvastatin + metabolites, carbamazepine + metabolites	2500 ml (WW effluent)	Whatmann GF/C	Phenomenex Strata X, 200 mg	not specified	not specified	not specified	not specified	10 ml ultra pure water	not specified	6 ml MeOH (1% NH_4OH); 6 ml MeOH (1% acetic acid)	Evap. to 1 ml under N_2	LC-MS/MS	Langford et al., 2011
atorvastatin	10 ml (plant growth media)	not specified	Waters Oasis HLB, 200 mg	3 ml MeOH; 3 ml HCl; 3 ml distilled water	none	vacuum manifold	1 ml min^{-1}	2 x 5 ml distilled water	5 min	7 ml MeOH	evap. to dryness; resuspended in 50:50 MeOH:water	HPLC-UV	Brain et al., 2006
Ciprofloxacin	200 ml	Fisher P5 filter paper	Waters Oasis HLB, 1000 mg	5 ml MeOH; 10 ml water pH 3	pH 3 with glacial acetic acid	not specified	10-15 ml min^{-1}	35 ml 6:1 water (pH 3.5):MeOH	not specified	10 ml 20:75:5 (v:v:v) MeOH:AcN:Formic acid	evap. to 500 µl under N_2; brought to 2 ml with AcN	HPLC-UV	Karnjanapi-boonwong et al., 2011
Triclosan	200 ml (WW); 500 ml (groundwater)	Fisher P5 filter paper	Honeywell Burdick & Jackson C_{18}	3 ml AcN; 3 ml Milli-Q water	none	not specified	<5 ml min^{-1}	none	not specified	3 x 1 ml AcN	not specified	HPLC-UV	Karnjanapi-boonwong et al., 2011

SPE - Aqueous Fraction – The aqueous fraction refers to that portion of a PhAC that is solubilized into the liquid portion of wastewater or environmental samples. Preparation of aqueous phase samples typically includes filtration followed by SPE. The filtration step can range from doing nothing to filtration through a 0.45 µm membrane filter. The chosen procedure is sample-specific and is typically dictated by SPE hydraulic requirements. Large impurities can blind the pore openings of the built-in cartridge prefilter or the sorbent bed itself resulting in hydraulic blockage.

SPE - Solid Fraction – As noted, hydrophobic PhACs will commonly sorb to solid matrices. Surface water sediment, agricultural field soil, and wastewater sludge/biosolids are all examples of solid matrices in which the analysis of PhACs is likely to be conducted. Generally, the preparation of solid samples consists of PhAC extraction (washing) from the solid matrices followed by filtration and SPE. There are several methods for extracting the target analytes from the solid matrices. Accelerated solvent extraction (ASE) uses common solvents (acetone, methanol, acetonitrile, etc.) at elevated temperatures and pressures (e.g. 100˚C/100 bars) (Ding et al., 2011). USE, or ultrasonic solvent extraction, is conducted by combining solvent washing and ultrasonication. While ASE is emerging as a much quicker alternative recovery method, USE is still the most common method employed (Belden et al., 2007; Brain et al., 2006; Karnjanapiboonwong, 2011; Ottmar et al., 2010; Salgado-Petinal et al., 2006). Common solvents for use in solid matrix extraction are acetone, acetonitrile, and methanol.

USE is conducted by adding a selected solvent to the solid pellet following centrifugation and drying (optional). The sample is then vortexed to break up and resuspend the pellet. The solvent-solids matrix is then subjected to ultrasonication in a sonicator bath for a designated period of time (typically 5 to 45 minutes). Once complete, the mixture is centrifuged and the centrate is collected for subsequent processing. It is common to repeat

the solvent addition/sonication/centrifugation procedure and to combine the centrate from each repetition (EPA, 2007; Salgado-Petinal et al., 2006).

Following PhAC recovery from the solids matrix, the centrate is subjected to SPE. Because SPE is sensitive to the content of organic solvents, it is typical to either evaporate the centrate and combine with water or simply dilute the entire volume of centrate with water such that the organic concentration is below the level required for effective SPE recovery. Additionally, prefiltration of diluted solids wash solvent can improve flow through SPE cartridges reducing sample preparation time.

The following list identifies parameters of interest in developing an USE method.

- *Solids separation*: the extent to which the liquid phase is removed from the solids during centrifugation or filtration. The drying of the remaining solids is optional.
- *Solvent selection*: the most common solvents for solid fraction analyte extraction are acetone, acetonitrile, and methanol. However, other solvents may be used depending on the PhAC targeted and its associated bond with the solids. pH adjustment has also been shown to improve USE (EPA, 2007).
- *Temperature*: many sonicator baths have adjustable temperature settings. Appropriate temperature settings can improve USE efficiencies.
- *Sonication time*: the length of time the samples experience sonication can have a profound impact on USE efficiency
- *Repetitions*: following the first sonication and subsequent centrifugation, additional repetitions of the procedure can be included which can involve differing solvents and sonication times

2.5.1.2 Sample Analysis

Quantitative measurement of PhACs is very difficult due principally to the extremely low concentrations present in WRRF and environmental samples (typically from the ng/L range

up to the low µg/L range). Accordingly, highly sensitive analytical techniques are required to detect and quantify the PhACs. Historically, the most common techniques used were gas chromatography coupled with mass spectrometry (GC-MS) and tandem mass spectrometry (GC-MS/MS) (Muz et al., 2013). However, gas chromatography (GC) can be difficult to apply in the analysis of some compounds because highly polar (non-volatile) compounds require time-consuming and complicated derivatization prior to analysis. Derivatization is the process of chemically modifying a compound to produce a new compound which has properties that are suitable for analysis using GC (Knapp, 1979). There are several other methods for addressing the analysis of PhACs, mainly using tandem mass spectrometry (Petrović et al., 2003; Ternes, 1998; Yang et al., 2011). In this sense, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) seems to be the currently preferred method for analysis of PhACs in environmental and wastewater samples. To this end, the Environmental Protection Agency (EPA) in 2007 published an analytical methodology in an attempt to standardize the analysis of PhAC containing samples, EPA Method 1694 (EPA, 2007).

2.5.2 EPA Method 1694

In 2007, the US Environmental Protection Agency (EPA) introduced Method 1694 in an attempt to standardize the multi-residue testing of PhACs (EPA, 2007). While the test procedures detailed in the EPA method are robust and applicable to most compounds, unfortunately there is limited applicability to lab-scale studies because of sample size limitations (i.e., large sample sizes are required in the EPA method). In addition to sample size concerns, others have concluded that Method 1694 is too complex to be applied uniformly and is in need of some modification (Ferrer et al., 2010).

While the EPA method provides a thorough baseline of the major steps in sample processing and analysis, its real benefit may be to provide a platform in which researchers

can begin to build and refine equipment, technique, and laboratory specific protocols that meet individual testing objectives. As noted, a major part of this thesis was the advancement of a PhAC analytical method for laboratory-scale research. To that end, Chapter 2 describes and details the research. As context, Table 5 provides the specific procedures from within EPA Method 1694 targeted for optimization in this study.

Table 5: EPA Method 1694 Modification Overview

		EPA 1694	This Study	Reason for Change	Reference
Sample Size	Aqueous	500 mL - 1 L	50 mL - 70 mL	Lab scale volumes	
	Solid	1 g dry	150 mg - 250 mg wet		
Filtration		Yes, if visible particles present - glass fiber	Yes, every sample - nylon syringe filter	Consistency, ease of filtration (glass fiber vs. nylon syringe)	
pH Adjustment		Yes	No	Minimal improvement in recoveries	(Muz et al., 2013)
Solid fraction extraction solvent		Acetonitrile	Acetonitrile/Methanol/ Acetone	More diverse solvent range allows for smaller solvent volumes	(Salgado-Petinal et al., 2006)
Solid fraction extraction solvent volume		70 mL	8 mL	Different solvents used (see above)	(Salgado-Petinal et al., 2006)
Drying and reconstituting solid extract		Rotary evap to 30 mL add 200 mL reagent water	Block heated nitrogen drier to <1 mL, add 25 mL reagent water	Smaller solvent volumes require less dry time and less aqueous dilution. Results in lower SPE processing times	
Final sample solvent		Methanol	Water (<5% methanol)	Analytical instrument requirement	
Analysis method		LC-MS/MS	SPE-MS/MS	Available equipment	

2.5.3 QA/QC

As noted, sample preparation and analysis methods vary greatly across research teams, due in part to the availability of analytical equipment. Some researchers have ready access to GC technology while others may have the latest LC systems at their disposal. Because chromatography equipment is very expensive, the analytical method chosen is largely

based on cost effectiveness and the availability of equipment. The use of differing analytical methods and equipment can make comparing data between studies difficult. In addition, because PhACs typically occur at trace concentrations, extreme care must be taken during sample collection, preparation, and analysis to maximize data quality.

One of the main challenges faced by researchers is their ability to account for the analytical interference and matrix effects of differing sample matrices. Not only are differing interfering substances present in samples from the various sources, but variations in the sample solvent itself can introduce variability into the analyses. Variations in sample solvents can include the water-to-organic solvent ratio, and varying organic solvents (methanol, acetonitrile, etc.). For example, an aqueous solvent with high organic percentages can lead to low analyte recovery during SPE. While there are several methods proposed to account for these phenomena, the most common practices include the method of standard addition and the addition of internal standards.

The principle of standard addition is invoked by splitting unknown samples into aliquots and adding a progressively increasing concentration of target analytes into each aliquot. The resulting data from analyses can then be plotted against the known concentration additions. When the data is plotted, the concentration in the unknown sample is determined to be the concentration at which this graph crosses the concentration axis. Essentially, this method calibrates each sample with its own calibration curve/line.

The internal standard method is the more commonly employed quality control method. Typically, a compound is selected that elutes chromatographically different than the target analytes but yet maintains similar chemical characteristics to the analyte. The following is a list of compounds from literature that have been used as internal standards (does not include deuterated standards):

- 2,3-dichlorophenoxy-acetic acid (Kimura et al., 2005)
- meclofenamic acid, mirex (Salgado-Petinal et al., 2006)
- meclofenamic acid, dihydrocarbamazepine (Carballa et al., 2004)
- β-estradiol-17acetate (Karnjanapiboonwong, 2011)
- flumequine, nalidixic acid (Cardoza et al., 2005)

Alternately, some PhAC analysis methods utilize deuterium (heavy hydrogen) or radio labeled compounds as internal standards ((Gros et al., 2010; Jelic et al., 2011; Ng et al., 2011; Tarcomnicu et al., 2011; Yang et al., 2011; Yu et al., 2012). Deuterium is a hydrogen isotope in which the hydrogen nucleus contains a proton and a neutron. This is contrasted with typical hydrogen (protium) in which only one proton makes up the nuclear structure. The deuterated compounds are either purchased or synthesized through D_2O reaction with the target analytes resulting in deuterium-hydrogen exchange (C-H bond replaced with C-D bond). Typically, the labeled standards are the deuterated form of each target analyte. This method is especially productive when using mass spectrometry (MS) for compound identification. The addition of deuterium, in this case, results in an additional atomic mass unit (AMU) for each hydrogen replaced with a deuterium (one for each neutron added). MS can detect differences in AMUs. In this manner, the addition of a known mass of deuterated standards to unknown samples can be used to identify and eliminate recovery, processing, and analytical errors that may occur during sample preparation and analysis.

3 RESEARCH OBJECTIVES

The focus of the research presented and discussed herein was to (i) develop a method for the determination of PhACs in small sample lab scale experiments and (ii) then apply that method to evaluate PhAC removal in conventional wastewater and sludge treatment processes. Specific goals in the method development phase were to (i) catalog sample preparation and analytical methods from peer-reviewed literature; (ii) select and evaluate candidate methods for their ease and robustness with respect to the selected PhACs; and (iii) build on the previous method evaluation to develop a reasonable and robust sample preparation and analytical method accommodative to the target PhACs, available equipment, and small sample sizes. For the evaluation of wastewater and sludge treatment processes, the primary goal was to conduct a preliminary evaluation of fermentation, CAS, and AD for their ability to remove the target PhACs.

The WRRF processes were selected for this research because they represent processes employed at many active WRRFs; however, ultimately the selection of processes was also influenced by uncontrolled dynamics on project funding. Fermentation was chosen for the first experiment because it is the most simple of these processes operationally. CAS followed due to the ease of converting the fermenters to CAS processes (add aeration and additional operational steps). While the analysis of these liquid-stream WRRF processes was originally intended to continue for the remainder of the project, financial support (from the INL) was unexpectedly terminated unexpectedly after one year (due to program re-prioritization at INL). New project funding was then secured from the Northwest Biosolids Management Association (NBMA) specifically for research associated with solid residuals from WRRFs. Accordingly, the research focus shifted to AD, a technology specifically developed for application to WRRF solids residuals.

CHAPTER 2: ESTABLISHING PHAC ANALYTICAL METHODS FOR LABORATORY-SCALE RESEARCH

1 INTRODUCTION

As noted previously, most PhAC research to date has focused on the analysis of samples collected from the water environment or from select full-scale WRRFs. Importantly, the nearly unlimited volumetric availability of these types of samples allows researchers to use large sample volumes in the preparation and analysis of PhACs, and thus to more strictly follow EPA guidelines and procedures. Considering the trace concentrations of PhACs present in these water samples, the larger sample volumes contain a larger mass of analyte for analysis and quantification. With the detection and quantification of target analytes being highly dependent on the mass of analyte present in the sample, a larger mass of analyte suspended in a small final sample volume will have a higher concentration that can be more easily detected and quantified.

In contrast to research conducted wherein large sample volumes can be readily collected, this research project focused on laboratory-scale bioreactors and PhAC treatability wherein only very small sample volumes (typically <70mL volumes) would be available. Thus, alternate sample processing and preparation methods were required to produce sufficient quantities of analyte for analysis and quantification. EPA Method 1694 was used as a platform from which to build sample preparation methods tailored to the research objectives and small sample sizes of this study. Additionally, opportunities to simplify the method, when present, were exploited to reduce sample processing times and costs. Table 5 provides a comparative overview of the EPA methods vs. those elements investigated in this study.

The goal for the analytics component in this project – which was ultimately a major focus of this research and thesis – was to evaluate and develop methods that would be useful in laboratory-scale research with small sample sizes and accordingly small analyte masses. This chapter presents and discusses the methods and results from this phase of the research project.

2 REAGENTS AND MATERIALS

All chemicals used throughout this study were of appropriate analytical grade. The methanol, acetonitrile, and cyclohexane were HPLC grade. For the compounds selected as analytes of interest, atorvastatin was purchased from ALLCHEM LLC (Fisher Scientific, Waltham, MA), triclosan from Crescent Chemical Co. (Ausburg, Germany), and ciprofloxacin from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Acetaminophen and carbamazepine were purchased from Acros Organics (New Jersey, USA). Oasis HLB (3 mL, 560 mg) SPE cartridges were purchase from Waters Corp. (Milford, MA, USA). Sterile 0.22 µm Millipore PVDF and unsterile 5.0 µm nylon syringe filters were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

3 PhAC ANALYTICAL METHODS

As discussed in Chapter 1, methods have been developed by others for the analysis of a matrix of PhACs in environmental samples using principally LC-MS/MS, GC-MS, or GC-MS/MS. Other methods have been published on quantification of single PhACs. However, because of the large number of potential PhACs present in WRRFs, broader and less specific methods have been the focus of recent research because of the ability to measure larger numbers of analytes across a larger number of drug classes. In this research project, two analytical methods were evaluated for target analyte quantification, SPE-MS/MS and GC-MS. GC-MS for this study was only evaluated preliminarily. Research herein principally focused on SPE-MS-MS, and all method development in this chapter and the experimental results in Chapter 3 were evaluated utilizing SPE-MS/MS.

3.1 SPE-MS/MS

Generally, SPE-MS/MS involves injecting a purified sample through an SPE cartridge and into a tandem MS. To conduct SPE-MS/MS for this project, immediately following sample processing and preparation, 100 µL of each sample was transferred to a 96-well assay plate. Sample preparation and processing was conducted at UI in the Environmental Engineering laboratory. The prepared plates were frozen and shipped to Agilent Technologies (Wakefield, MA) for analysis.

Once received by Agilent, plates were transferred onto a high-throughput RapidFire200 integrated autosampler/solid-phase extraction (SPE) system (Agilent Technologies, Wakefield, MA) coupled to an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, Ontario, Canada). Samples were aspirated under vacuum directly from assay plates for 250ms. The aliquot was then loaded onto a C4 SPE cartridge to remove buffer salts, using solvent A at a flow rate of 1.5 mL min^{-1} for 2.5s. The retained

analytes were eluted to the mass spectrometer by washing the cartridge with solvent B at 1.25 mL/min for 3s. The cartridge was then re-equilibrated with solvent A for 1.5s at 1.5 mL/min prior to injection of a subsequent sample. The entire sampling cycle was approximately 8s per well, enabling analysis of a 96-well plate in approximately 13min. Solvent A was water containing 0.01% (v/v) trifluouroacetic acid (TFA) and 0.09% (v/v) formic acid. Solvent B was acetonitrile/water (8:2, v/v) containing 0.01% (v/v) TFA and 0.09% (v/v) formic acid.

The mass spectrometer was operated in positive electrospray multiple reaction monitoring (MRM) mode, and transitions Q1 (quadrupole 1) and Q3 (quadrupole 3) for each analyte (which were optimized by Agilent) were continuously monitored. A dwell time of 100ms was used for all of the MRM transitions. The mass spectrometer was operated with a spray voltage of 4500 V and at a source temperature of 650°C. The peaks detected by mass spectrometry were approximately 1.5s wide at half-height, and they were integrated and processed using Agilent's RapidFire peak integration software.

Analytes targeted with SPE-MS/MS must be separable from other analytes and interfering substances under the conditions listed above. For this study, all of the target PhACs could be quantified using SPE-MS/MS, with the exception of triclosan, which requires a negatively charged electrospray. While TC was originally targeted for this study, the Agilent Rapid-Fire method proved ineffective at measuring it. A GC-MS method was preliminarily evaluated to analyze for TC, but more work is required to further develop this method's usefulness. MS/MS mass spectra for AT, CP, and CZ are included in the Appendix.

3.2 GC-MS

A GC-MS method was developed through a modification to the method of Yu et al., 2012 (Yu et al., 2012). Derivatized samples were loaded onto a PolarisQ Iontrap GC–MS

instrument (Thermo Electron Corporation, Waltham, MA, USA) set up in positive electron impact mode. An injection volume of 1.0 µL was injected into the column in split mode (17:1) with an injector temperature of 290ºC. Separation was achieved on a ZB1 capillary column (30 m x 0.25 mm Ø, Phenomenex, Torrance, CA, USA) with helium as the carrier gas (1.2 mL $min^{-}1$) using a temperature program of 105ºC (1min) ramped to 285ºC at 8ºC min^{-1} and held for 10min. The transfer line to the MS was held at 290ºC. The purpose of evaluating the GC-MS method was to enable the research team to analyze for some of the target analytes in-house. AC and TC were the only two compounds that appeared to be applicable to this method at experimentally relevant concentrations. AT and CZ could be measured with GC-MS but at one to two orders of magnitude higher concentrations in pure samples.

4 PhAC SAMPLE PREPARATION METHODS

Analytical methods sufficiently sensitive to quantify PhACs at concentrations observed in this study (and at full-scale WRRFs) are highly susceptible to interference from undesired constituents present in wastewater and potentially present in prepared samples. Therefore, in order to analyze wastewater samples using the above-described methods, the samples must first be processed to yield a clean product. Beyond interference concerns, failure to provide adequate clean-up of samples prior to analysis could lead to instrument damage or chromatographic column fouling, resulting in unreliable results. A concentrated sample is also required for PhAC analysis, considering the very low concentrations observed in WRRFs and the environment. Finally, sample volume must be considered relative to the experimental setup.

This section describes the methods employed and evaluated in this research to fully prepare samples for analysis. In particular, this chapter will highlight components of EPA Method 1694 that were targeted for refinement in this study (see also Table 5). Recognizing that PhACs can both be present in bulk solution and sorbed to biomass solids, methods were separately developed and evaluated for both fractions. Section 5 discusses results associated with evaluating these methods.

4.1 Filtration

EPA Method 1694 only recommends filtration if “visible particles” are present in the pre-SPE samples and suggests a filter size and filtration apparatus. It was the opinion of the researchers in this study that filtration was a necessary component of sample preparation worth further evaluation.

Filtration of samples prior to SPE will improve subsequent processing steps and decrease total sample preparation time. Failure to filter samples prior to SPE can result in extremely

low flows through the SPE cartridge, because residual solids will plug the pre-filter that is part of the SPE cartridge. In the worst case, the SPE cartridge pre-filter (built into most cartridges) or the sorbtive media can become completely blocked, resulting in hydraulic lock leading to failure of passage of the entire sample through the media. This worst case scenario results in potential loss of the sample as well as significant expense, as the SPE cartridges are very expensive. This research evaluated a number of filtration options as follows.

- Gravity flow through Whatman filtration paper
- Vacuum driven filtration using Whatman glass fiber filters
- Syringe filtration using 0.22 µm PVDF filters
- Syringe filtration using 5.0 µm nylon membrane filters

4.1.1 Whatman Paper Filter

12.5 cm No. 42 circular Whatman (Kent, UK) paper filters were folded into a cone and placed into pre-washed HDPE funnels that were then placed into the opening of acid-washed (1N HCl) glass flasks. The filter papers and funnels were oversized enough to contain the entire sample. Nine samples at a time were applied to separate funnels/flasks, then covered to minimize light exposure (ciprofloxacin undergoes photodegradation) and allowed to gravity drain into the flasks (Belden et al., 2007).

4.1.2 Glass Fiber Filter

Sample processing through glass fiber filters was conducted as follows. Millipore No. APFC04700 glass fiber filters (1.2 µm particle retention, 47 mm Ø) were installed onto a vacuum manifold apparatus previously rinsed with a 50:50 MeOH:acetonitrile solution. The samples were then poured onto the surface of the filter and collected into an acid-washed (1N HCl) glass vacuum flask. Samples filtered in this manner were processed one at a time.

4.1.3 0.22 µm PVDF Syringe Filter

0.22 µm PVDF syringe filters (Millipore Corp., Billerica, Massachusetts) were assembled onto acid-washed (1N HCl) 60 mL HDPE syringe filters. Samples were then loaded into the syringe and manually pressurized through the filter one at a time into new 60 mL conical bottom HDPE test tubes. In the event that blinding of the filter cartridge occurred, additional filters were used until the entire sample had been filtered.

4.1.4 5.0 µm Nylon Syringe Filter

For filtration through 5.0 µm nylon syringe filters (Thermo-Fisher Scientific, Waltham, MA, USA), the same procedure was used as that for the 0.22 µm syringe filters described above.

4.2 Solid Phase Extraction

Concentration and cleanup of samples is necessary to enhance detection for trace compounds such as PhACs found in wastewater. For instance, assuming a particular analytical method has a detection limit of 1 µg L^{-1}, by concentrating a 100 mL sample into a volume of 2 mL, a 50 fold decrease in the detection limit (20 ng L^{-1}) is achieved. SPE also aids in sample cleanup by removing some interfering compounds through selective capture and recovery.

SPE was conducted utilizing a RapidTrace model 50000/16 automated SPE apparatus (Zymark/Biotage, Uppsala, Sweden), with some modifications. RapidTrace units are set up to sample from an eight mL test tube. However, target sample sizes for this study were 50 mL. For this reason, the SPE units were retrofitted with large sample capacities. RapidTrace units are factory set up to allow the use of up to eight different solvents. Because the current research only required three solvents, the remaining five ports were used to draw samples from larger containers such as those required by this research. Ultimately, four sampling ports were utilized on three RapidTrace apparatuses allowing the

automated manipulation of 12-50 mL samples at a time. Figure depicts the RapidTrace instruments used in this study including positioning of the sample bottles (glass bottles left of machines).

Figure 2: RapidTrace SPE System

To minimize the potential for sample cross-contamination during SPE due to the consecutive use of the RapidTrace syringe with each sample, a custom-made vacuum system was utilized to apply samples to the SPE cartridges for the last two data collection series. The vacuum system consisted of a vacuum manifold containing of a series of fish tank aeration manifolds and Tygon tubing was used to link the SPE cartridges to a KNF filtration pump (Trenton, NJ, USA). Samples were drawn through the sampling tubing and passed through the cartridges under vacuum. Vacuum was adjusted to achieve a target sample flow rate (≤ 1 mL min^{-1}). For this modified approach, the RapidTrace SPE units were only used for cartridge conditioning and cartridge elution.

4.2.1 SPE Cartridge selection

SPE cartridge selection is based on the chemistry of the target analyte and its potential reaction with a given SPE sorbent. A number of manufacturers produce a large number of different SPE cartridges. Many cartridges utilize a silica-based sorbent while others use activated carbon. While silica based cartridges are the most common for recovering single analytes, the variability of chemistry between compounds in trace PhAC analysis typically calls for proprietary polymeric sorbents. Based on an extensive review of the literature (Brain et al., 2006; Ferrer et al., 2010; Joss et al., 2005; Muz et al., 2013; Ng et al., 2011; Salgado-Petinal et al., 2006), ultimately the cartridge chosen for this study (based on its widespread use in PhAC research as well as its broad applicability) was the Waters Oasis HLB. HLB stands for "hydrophilic-lipophilic balance," meaning it is useful for recovering easily solubilized compounds as well as those that are likely to partition to solids.

4.2.2 SPE Protocol

Several method iterations were performed to develop a robust SPE method for use in this study. The relativeness of the method developed in this study, as compared with the EPA method, are discussed further below. Goals for SPE of PhACs included the following.

- High retention and subsequent concentration of target analytes
- Low retention of potentially interfering non-target compounds
- Ease of processing
- Speed of processing
- Repeatability of target analyte recoveries
- Applicability to broad variety of compound chemistries

The following sections detail the primary method iterations for SPE.

The initial, pH-specific SPE method utilized in this study followed EPA Method 1694 (EPA, 2007). This method utilizes split samples, one for acidic compounds (CP, AT, AC) and one for neutral/basic compounds (CZ, TC).

The acid fraction sample SPE cartridges were conditioned by serially adding 20 mL MeOH, 6 mL of reagent water, then 6 mL of reagent water at pH 2.0 +/-0.5 (pH adjusted with 6N HCl). Care was taken to ensure the cartridges did not dry out between conditioning and sample application. The acidified samples (pH 2.0 with 6N HCl) were then applied to the SPE utilizing the RapidTrace's integrated solvent lines at a flow rate of 5 mL min^{-1}. Following sample application, the cartridges were washed with 10 mL of reagent water followed by drying under air for 5 min. Elution was conducted with 8 mL of MeOH at a flow rate of 2 mL min^{-1}. The eluant was collected in 13mm x 100 mm borosilicate glass test tubes, transferred to a SpeedVac/freeze drier system, and evaporated to near dryness. The analytes were then re-suspended by the addition of 3 mL of reagent water and 1 mL of 0.1% formic acid content reagent water, and vortexed vigorously. Following re-suspension, samples appearing visually cloudy or containing suspended particles were filtered through a 0.22 µm PVDF syringe filter.

Neutral/basic fraction sample SPE cartridges were conditioned by serially adding 20 mL MeOH and 6 mL of reagent water. Care was taken to ensure the cartridges did not dry out between conditioning and sample application. The samples (pH 9.0 adjusted with NH_4OH) were then applied to the SPE utilizing the RapidTrace's integrated solvent lines at a flow rate of 5 mL min^{-1}. Following sample application, the cartridges were dried under air for 5 min. Elution was conducted by the serial addition of 4 mL of MeOH and 4 mL of 2% formic acid in MeOH at a flow rate of 2 mL min^{-1}. Sample eluents were then collected and processed the same as for the acid fraction samples detailed above.

4.2.3 pH Adjustment

pH of the analyte sample must be carefully considered when conducting SPE because changes in pH can cause changes in the electrostatic sorptive forces between substances. Because positively charged ions will tend to be attracted to negatively charged sorbents and vise-versa, changes in pH can affect the magnitude of the electrostatic forces by increasing or decreasing the net charge of analytes in solution. In this regard and depending on the sorbent used, optimal pH ranges are thought to exist for each compound of interest (EPA, 2007). Two methods were evaluated with respect to sample pH. One method is imbedded in the EPA Method 1694 while the other was discovered in relevant peer-reviewed literature as noted below.

EPA Method 1694 divides analytes into two groups: acidic and basic/neutral. For acidic analytes, decreasing the pH of the aqueous solvent by the addition of an acid will tend to re-associate protons to the analyte, resulting in the compound gaining an increase in net charge. For basic compounds, a decrease in pH will likely not result in a major change in charge because the chemical structure is already nearly saturated with protons. However, an increase in pH can protonate the analyte, creating hydroxyl groups thus giving the compound a lower overall charge. The EPA method requires acidic compounds to be in their more-positive form by artificially reducing the pH of their containing solution to approximately 2.0. Conversely, the pH of basic/neutral compound-containing solutions is increased to approximately 10.0, resulting in the charge of the analyte becoming more negative. The theory behind this is that it will increase the recovery efficiency for positively charged compounds using a negatively charged sorbent, with the opposite being true for the negatively charged analytes (Waters, 2010-2011). According to EPA Method 1694, selection of the proper recovery sorbent, aligned with the pH of the sample, is thus critical to achieve high recovery efficiencies.

Upon further review of literature it was determined that the EPA method could be simplified, requiring less sample preparation time and fewer chemicals. Specifically, a 2012 study evaluated the impact of pH on the recoveries of a broad suite of compounds (basic/neutral and acidic; including CZ) from solids and during SPE. Muz et al. determined that the optimal pH for the recovery of analytes from both classes (acidic, basic/neutral) was pH 7.0 with little variation between pH 6.5 and pH 9.0. At neutral pH, the recoveries observed were all in excess of 99 percent (Muz et al., 2013). This was likely due to the use of advanced sorbent polymers such as that contained in the Oasis HLB SPE cartridge (which was used in this study). This sorbent is effective at recovering compounds ranging from moderately acidic to moderately basic. Ultimately, based on the findings described above, no pH adjustment was applied to samples processed in this study. Rather, samples collected during this study were periodically checked for pH. The pH of all tested samples was between 6.5 and 7.5. More generally, for samples collected from wastewater matrices (regardless of the process configuration), the pH will almost certainly be near circumneutral; this is due to the fundamental biological operations required to treat wastewater that must function near pH 7.

4.2.4 Cartridge Conditioning, Sample Application, and Analyte Elution

The steps in EPA 1694 involved in preparing the SPE cartridge for use, applying the sample to the prepared cartridge, and drying and eluting the analytes from the cartridge were adjusted based on the expected sample makeup and size anticipated from the experiments in this study. Additionally, without the need for pH adjustment (as discussed in section 4.2.3), the pH specific steps could be simplified with both the acidic and basic/neutral fractions combined into one conditioning, application, and elution protocol.

Initially, samples were applied to the SPE cartridges using the RapidTrace units as described in Section 4.2. Latter experiments, specifically the last two conducted in this

study, a vacuum manifold was used to initiate the flow of the samples into the cartridge. The remainder of the sample was then allowed to flow under gravity. Cartridge conditioning and elution were still conducted according to Section 4.2. The cartridges were conditioned, immediately transferred to the vacuum manifold taking care to ensure that the sorbent did not dry out, and the samples were applied. Vacuum was reapplied and the cartridges were allowed to dry for 5 minutes. The cartridges containing the analyte were then transferred back to the RapidTrace units for subsequent elution.

Cartridge conditioning consisted of the following:

- 10 mL MeOH applied at 30 mL min^{-1}
- 10 mL DDI water applied at 30 mL min^{-1}

Elution was accomplished with

- mL MeOH:acetonitrile (1:4) applied at 5 mL min^{-1}

4.3 Solid Fraction Extraction

PhAC concentrations in solids cannot be measured directly, but rather must be recovered from the solids matrix for analysis. The solid fraction extraction protocol requires “washing” the concentrated solids with solvent to extract the analyte bound to the solid matrix in the sample.

The following protocol was used to process samples from the initial fermentation experiments to extract the fraction of analyte sorbed to solid matrices (i.e., biomass). The methods in Sections 4.4.1 and 4.4.2 follow EPA Method 1694, which were then modified for the subsequent CAS experiments as discussed in Section 5.2 and summarized in Table 5: EPA Method 1694 Modification Overview. Bioreactor samples (containing liquid and biomass solids) were centrifuged at 10,000 rpm for 5 min. The centrate was then either sampled directly, or subsequently processed through SPE. By sampling before and after SPE, the

analyte in the remaining small fraction of liquid contained in the solid sample could then be subtracted from analysis results. Solid fraction sample sizes ranged from 150 mg wet to 250 mg wet. The solid fraction aliquots were then placed in clean (soap/1N HCl acid washed) 50 mL centrifuge tubes labeled with the appropriate analyte name(s). Based on the target analyte the following procedures were used as a baseline for the remaining solid fraction extractions.

4.3.1 Acidic Compounds (atorvastatin and ciprofloxacin)

The following protocol was used to complete the solid fraction extraction of acidic analytes. 15 mL of pH 2.0 phosphate buffer was added to the centrifuge tube containing the sample and vortexed vigorously for five minutes. The pH of the suspension was then adjusted to 2.0 +/- 0.5 with phosphate buffer. The remaining steps are detailed as follows.

- Add 20 ml acetonitrile, sonicate for 30 min., centrifuge (3000 rpm for 5 min)
- Remove supernatant and place in 250 ml bottle/flask
- Add 15 ml phosphate buffer to remaining solids and adjust pH to 2.0 with HCl
- Add 20 ml acetonitrile, sonicate for 30 min., centrifuge (3000 rpm for 5 min)
- Remove supernatant and place in 250 ml bottle/flask from before
- Add 15 ml acetonitrile, sonicate for 30 min., centrifuge (3000 rpm for 5 min)
- Remove supernatant and place in 250 ml bottle/flask from before
- Process through the SPE method designed for acidic samples

4.3.2 Basic Compounds (i.e., carbamazepine)

The following protocol was used to complete the solid fraction extraction of basic/neutral analytes. 15 mL of reagent water was added to the sample and vortexed vigorously for five minutes. The pH was then adjusted to 10 +/- 0.5 with ammonium hydroxide. The remaining steps are detailed as follows.

- Add 20 ml acetonitrile, sonicate for 30 min., centrifuge (3000 rpm for 5 min)
- Remove supernatant and place in 250 ml bottle/flask
- Add 15 ml reagent water to remaining solids and adjust pH to 10.0 dropwise with NH_4-OH
- Add 20 ml acetonitrile, sonicate for 30 min., centrifuge (3000 rpm for 5 min)
- Remove supernatant and place in 250 ml bottle/flask from before
- Add 15 ml acetonitrile, sonicate for 30 min., centrifuge (3000 rpm for 5 min)
- Remove supernatant and place in 250 ml bottle/flask from before
- Process through the SPE method designed for basic/neutral samples

4.4 Sample Drying

Two methods were evaluated for drying pre-SPE and post-SPE samples, in lieu of rotary evaporation as prescribed in EPA Method 1694 (rotary evaporation equipment was not readily available). Pre-SPE samples were dried to reduce the solid extract volume (i.e., solvent), thus resulting in lower organic solvent fraction and overall reduced sample volume for SPE as detailed above. The post-SPE samples were dried for the purpose of solvent exchange (with water) necessary for shipping and downstream analytics. By exchanging the organic solvent for water (< 5% organic content, v/v) the samples could be frozen for shipping to Agilent. Additionally, lower organic concentrations are required for analysis by SPE-MS/MS because, as noted, high solvent concentrations will prevent capture on the in-line SPE cartridge resulting in lost analyte. The two drying methods evaluated were SpeedVac rotary evaporation and block heated drying under nitrogen gas.

The SpeedVac unit was connected in series to a vacuum pump via a freeze-dryer. The freeze-dryer was used to condense the methanol-based solvent to prevent it from entering

the vacuum pump. The rotary evaporator (SpeedVac) uses heat and vacuum to evaporate solvents and centrifugal force to prevent boiling.

To achieve block heated drying, a nitrogen gas manifold was constructed using a baseplate and glass Pasteur pipettes formatted to fit a Hach digestion block. Tygon tubing was then used to connect the Pasteur pipettes to a low-flow rotameter used to control nitrogen flow. Nitrogen was supplied to the rotameter from a 20 psi regulated bulk nitrogen gas cylinder. A photo of the nitrogen drying apparatus is included in Figure .

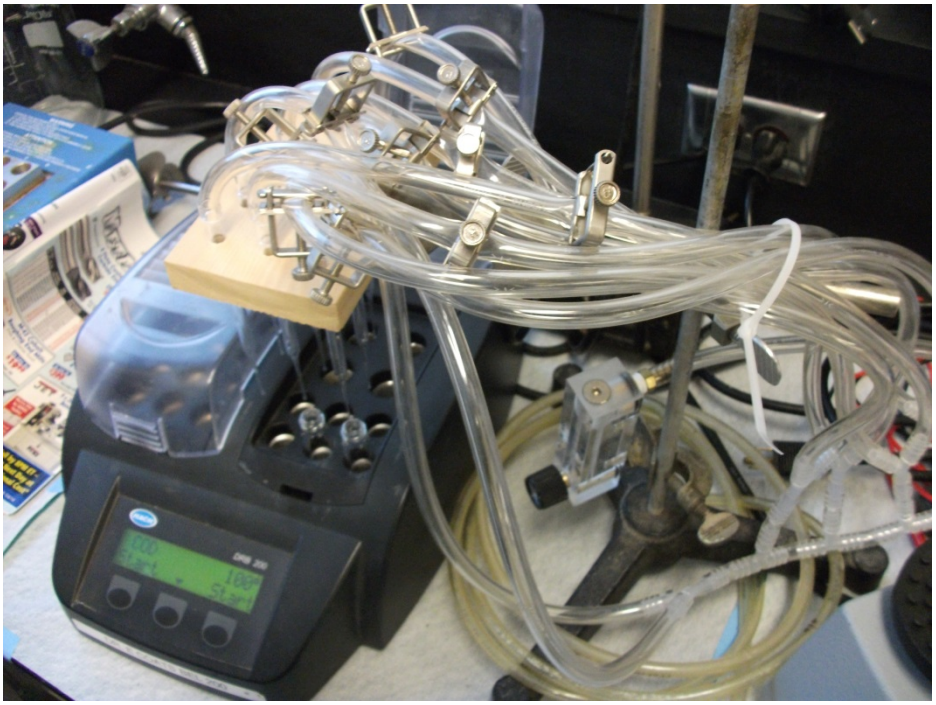


Figure 3: Nitrogen Drying Manifold and Block Heater

4.5 Sample Plate Preparation and Shipping

100 µL samples were assembled into Bio-Rad 96-well skirted analytical plates (Hercules, CA, USA). The plates were sealed with adhesive aluminum sealing tape. The sealed plates were then placed into a -20ºC freezer. The samples remained in the freezer for a minimum of 2 hours in order to allow the samples to freeze completely. The sample plates

were then placed into a Zip-Lock type bag and into a Bio-Rad insulated closed-cell foam cooler packed with ice packs. The cooler lid was taped shut and the unit was immediately transported to FedEx.

Each shipment was received by FedEx at approximately 3:00 PM PST and were shipped Priority Overnight to Agilent Technologies in Massachusetts. The shipment arrived and was accepted at Agilent at approximately 10:00 AM EST, the next morning, where they were immediately placed into refrigeration.

It was observed that if excess organic solvent (methanol) remained in the samples, they would either not freeze completely initially, or they would thaw by the time they reached Agilent. Care was taken to ensure that the methanol content of the finished samples was low enough to allow for transportation without sample thawing. A final methanol content of ≤5% was targeted for this purpose.

5 EXPERIMENTAL OPTIMIZATION OF METHODS

The sample preparation methods in Section 4.0 generally followed EPA 1694, with modifications and improvements as described to account for smaller sample sizes (see also Table 5). Specifically, the challenges associated with processing small sample sizes in this study required further modification of the solid fraction recovery and SPE for all samples (aqueous and solid). Additionally, sample processing had to be tailored to available lab equipment and operator knowledge. Of the methodological elements discussed in Section 4.0, filtration, solid fraction extraction, and sample drying procedures required refinement and were thus studied in more detail in this research. Described and discussed below (Sections 5.1-5.3) are the results from the associated procedures investigations. Results from these investigations are discussed qualitatively using two criteria as follows.

1. Impact on result quality
2. Ease of implementation and reproducibility

Section 5.4 presents and discusses quantitative results relative to standard curve development.

5.1 Filtration

All of the filtration methods evaluated were sufficient to achieve the primary objective: prevent complete hydraulic blockage of the SPE cartridges and subsequent failure and error reporting of the RapidTrace SPE apparatuses. However, the ease of use, time required per sample, and the potential for cross-contamination varied with the method chosen. Table 6 summarizes the conclusions drawn from these trials. Nylon syringe filters were ultimately chosen as the filter of choice for this study. Due to the very low concentrations encountered while studying PhACs, a consistent filtration step will help minimize systematic errors associated with subsequent sample processing steps.

Table 6: Filtration Method Evaluation Summary

	Advantages	Disadvantages
Whatmann filter paper	Multiple samples at a time Inexpensive Ease of use	Sample loss from absorption to the filter Funnel reuse allows opportunity for cross-contamination Long processing time may allow opportunity for photodegradation
Glass fiber filters	High rate of sample application Low sample volume retained	Cross-contamination potential in hard-to-clean vacuum manifold Potential of cross-contamination in vacuum tubing One sample processed at a time
0.22 µm PVDF syringe filters	High rate of sample application High post-filtration sample quality Low sample volume retained Ease of use	Higher pre-filtration centrifugation standard Small pore size - may require multiple cartridges if blinding occurs High per-sample cost compared to glass fiber and paper filters
5.0 µm Nylon syringe filters	High rate of sample application High post-filtration sample quality Low sample volume retained Ease of use	High per-sample cost compared to glass fiber and paper filters Reduced solids removal as compared with the other methods (due to larger pore openings)

5.2 Solid Fraction Extraction

Modifications to the EPA Method's solid fraction extraction protocol, as used in this study, were as follows.

5.2.1 Solid Fraction Extraction Solvent Volume

To reduce the sample processing time required for extracting PhACs from the solid fraction, and the prevalence of problems that may arise thereof, smaller extraction solvent volumes can be used. Alternately, larger volumes can be used, and the extraction solvent can be evaporated to reduce the volume prior to dilution with water. While smaller extraction solvent volumes can reduce sample processing time, larger solvent volumes will likely result in more analyte mass recovery, which is an important consideration in the pursuit to evaluate PhAC treatability in WRRFs.

The recovery of PhACs from solids was evaluated by utilizing peat moss as a surrogate for the sample solid matrix. A known concentration of analyte was added in aqueous solution to clean centrifuge tubes containing one gram of autoclaved, rinsed peat moss each. The

centrifuge tubes were then placed on a shaker table and shaken slowly for 24 hours. The surrogate samples were centrifuged at 3,000 rpm for 5 minutes and the centrate was decanted and discarded. Two of the solid fraction surrogate samples were processed using the solid fraction extraction protocol dictated by EPA Method 1694 and two were subjected to the modified extraction method of Salgado et al. (Salgado-Petinal et al., 2006). The latter, simplified method uses a smaller volume of extraction solvent (8 mL total vs. 70 mL for Method 1694). The smaller solvent volumes were made up of 4 mL methanol/acetonitrile (1:1) and 4 mL acetone, as compared to only acetonitrile per Method 1694. This comparative analysis was conducted independently for each compound (AT, CP, CZ). The analysis results indicate nearly identical concentrations for AT and CZ extracts using the Salgado method as compared to the EPA method. Slightly higher recoveries were observed for the modified method for CP (9% greater arithmetic mean).

In this research, a balance was established between having enough solvent to achieve high recoveries while minimizing the solvent used to minimize sample processing times. Sufficient analyte recoveries, for these experiments were observed at much smaller wash solvent volumes (approximately 8 mL per gram of solids) than those recommended in EPA 1694 (approximately 70 mL per gram). It appears as though the EPA method uses an overly conservative approach toward solvent wash volume with approximately 50% of the solvent recovering less than 1% of the analytes. In part, what allowed the solvent volumes to be reduced was the use of varying solvent types as discussed in Section 5.2.2.

5.2.2 Solid Fraction Extraction Solvent

Methods published for solid fraction recovery require a varying range of solvent types and volumes. EPA Method 1694, for instance, requires 70 mL of solvent (acetonitrile). By contrast, Belden et al. used 10 mL of extraction solvent (NH_4OH) (Belden et al., 2007). The difference in solvent volumes is likely the result of the latter's refinement of the EPA

method. Decreased solvent volumes, in this regard, will always lead to shorter overall processing times. In addition to differing volumes, different solvents have been utilized for their varying nature of action on sorbed compounds. Methanol, for instance, is of special importance for extracting analytes from solid matrices because it is classified as “protic.” This means that it can/will disrupt hydrogen bonding between polar analyte molecules and the solid matrix. While ethyl acetate and acetonitrile are stronger solvents, with respect to solids extraction, a protic solvent such as methanol should always be included in the extraction series (Waters, 2010-2011).

Following extraction from the solids, the solvent concentration in the eluent must be reduced or diluted with water to ensure capture via SPE. Specifically, if the solvent concentration is too high, the analytes will pass through the SPE cartridge without sorbing (because the same solvent is used to ultimately recover sorbed analyte from the SPE). The final concentration of organic solvent immediately prior to SPE should be ≤ 5% (EPA, 2007). Thus, for proper dilution, 950 mL of water would be required to dilute the 50 mL of solvent required by the EPA method. Here again, managing large samples presents a real challenge. Specifically, the larger sample size would require very long SPE processing times. At the typical hydraulic loading rate of 5 mL min^{-1}, a 1000 mL sample would take 200 minutes to process. Longer processing times may increase the potential for photodegradation or ambient contamination of samples. To reduce solid fraction extraction sample processing time and the prevalence of problems that may arise thereof, smaller extraction solvent volumes can be used. Alternately, the extraction solvent can be dried down to reduce the volume prior to dilution (the method employed in this study and detailed below).

Solvents used in this study included methanol, acetonitrile, and acetone. Three step extractions were conducted. The first two washes used 1:1 methanol:acetonitrile while the

last used acetone. The centrifuged supernatant was collected and combined for further dry-down, dilution, and SPE.

5.3 Sample Drying

While the SpeedVac sample drying apparatus allowed the dry-down of more samples at-a-time, this sample drying method did not perform consistently between samples. The rate of dry-down often varied, leaving some samples evaporated completely while others still contained several milliliters of solvent. Additionally, the large volume of methanol evaporated from the high number of samples and re-condensed in the freeze-drier proved difficult to manage, often leading to ice buildup and loss of vacuum. The nitrogen drying system proved to be much more reliable, consistent, and controllable. While the capacity of the nitrogen drying system was only 10 samples at a time, those 10 samples could be completely evaporated in approximately one fourth the time required to dry the same 10 samples in the SpeedVac.

5.4 Optimization of Standard Curve Development

Standard curves are a critical and necessary tool used in the quantification of experimental unknowns. By analytically processing and then plotting known concentrations of an analyte against instrument responses, one can determine unknown sample concentrations using a linear or curvilinear model based on the known values. Preparation and investigation of standard curves is also useful in developing and validating new methods for analytes.

There are a number of factors to consider when developing and using a standard curve. Each factor holds a certain level of significance with respect to accuracy of the model (standard curve). The following questions target important factors for developing standard curves.

- Do the expected values of the unknowns fall within the range of quantification of the standard curve?
    - What are the upper and lower limits of quantification for the method and instrument?
    - What is the limit of detection for each analyte and the instrument used?
- Do the standard curves exhibit good fit?
- How does the standard curve compare across iterations?
    - How often does the standard curve need to be prepared?
    - What is the magnitude of the difference in standard curves between iterations?
- Do the standards used for developing the standard curves accurately represent the experimental unknowns?
    - Are the standards and unknowns prepared in the same solvent? Is this necessary?
    - Are there potentially interfering constituents in the unknowns that are not present in the standards?
    - Are the standards prepared at the same time as the unknowns? Is this necessary?

Over the course of conducting this research, and in particular in advancing the methods for preparing/processing small volume laboratory samples, each of the above questions was addressed. Results from these investigations are detailed below, with a focus on the optimization process specifically relating to standard curve development within the context of these factors.

The sample preparation and analytical methods investigated in this study, and detailed in this chapter, were developed concurrently with investigations of alternate wastewater

treatment technologies for the removal of PhACs (treatability results are separately presented and discussed in Chapter 3). In this regard, the following discussion centers on important and relevant observations within the context of the standard curves and the impact of those observations on the advancement of the sample preparation and analytical protocols.

Seven independent, discrete sets of standard curves were developed during the course of this research, with each set developed to advance the PhAC analytical method. Graphically presented in 5.4-A through Figure 5.4-L are the standard curves from each iteration along with a discussion relating to the factors listed above.

5.4.1 Experiment I

The reagent grade analytes used for PhAC standards were first prepared at high stock concentrations (100 mg/L) in methanol due to their low solubilities in water. The stock solutions were then batched into DDI water to achieve concentrations of 0.1, 0.5, 1.0, 5.0, 10, and 20 µg/L (thus retaining relatively high concentrations of methanol). Aliquots of each standard were pipetted into individual wells of a 96-well plate and subjected to SPE-MS/MS analysis.

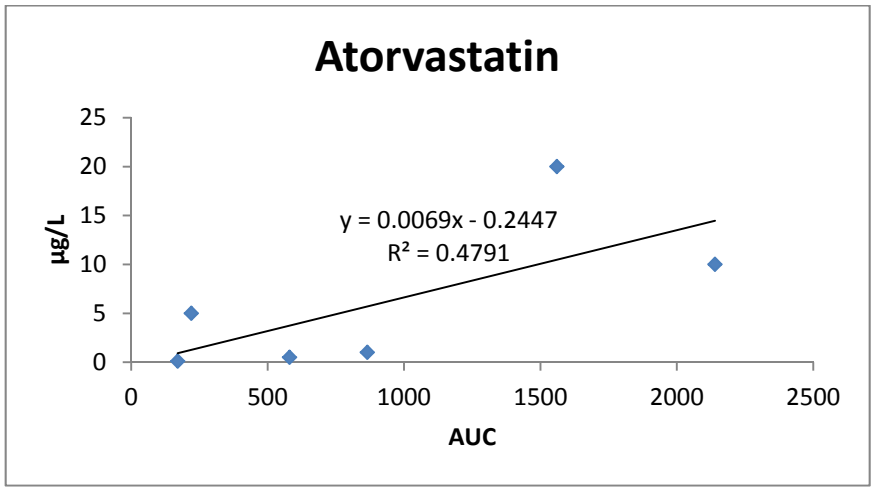


Figure 5.4-A

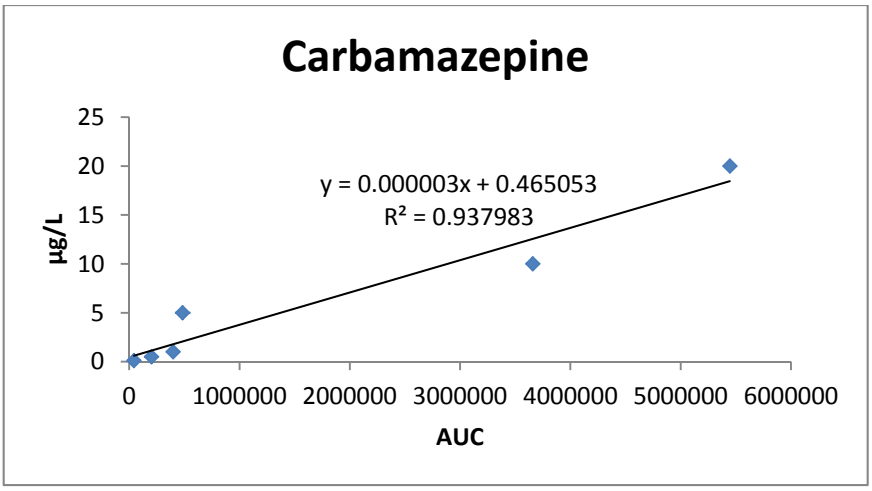


Figure 5.4-B

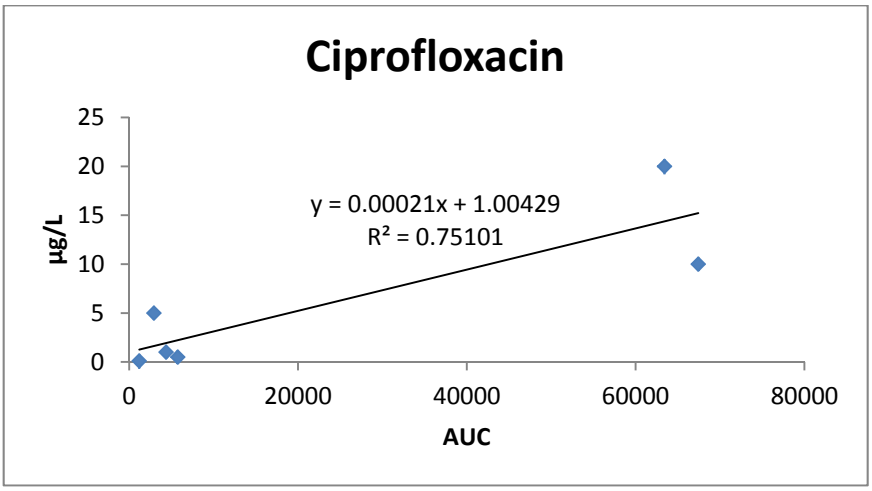


Figure 5.4-C

The standard curves depicted in Figures 5.4.1 A-C represent the analytical response - area under the curve (AUC) – versus the concentration of analyte in the batched standards, as processed through SPE-MS/MS. As can be seen, the data are not particularly linear for any of the analytes. Applying a linear regression provides additional clarity to the data. Specifically, the R^2 value, included on each graph, is a measure of how well the linear model fits the data points. Values of R^2 closer to 1.0 indicate a better model fit. In other words, the closer the R^2 is to 1.0, the better the model (linear equation on each graph) will predict the unknown values. For most parameters, R^2 values above about 0.9 indicate a

reasonably well fit model. The linear models depicted in Figures 5.4.1-A and 5.4.1-C (for AT and CP, respectively) indicate relatively low predictability, whereas the model in Figure 5.4.1-B (for CZ) appears to be more useful in predicting unknown values.

For this set of data representing the first round of analyses, it was assumed that the non-linearity observed is likely due to the presence of excess methanol in the standard solutions. In the SPE-MS/MS process, the samples are first subjected to SPE in order to remove potentially interfering compounds. As noted, if the organic solvent (methanol) content is too high, the analyte will be washed through the SPE cartridge instead of collecting on the sorbent. The analytical instrument appears to be highly sensitive with respect to carbamazepine, likely dampening the deviation of the data points from the linear model due to excess methanol.

5.4.2 Experiment II

Building on the first round of results, new standards were prepared for a second experiment, this time batched in replicate but in different solutions. One set of standards was dissolved in DDI water while the other set was diluted into raw wastewater filtered through a 1.2 µm glass fiber filter. The concentrations of analyte for these standards ranged from 0 to 50 µg/L. Care was taken to ensure that the methanol content was less than 1% by volume to eliminate SPE pass-through.

Because the substrate provided to the experimental reactors was real wastewater, it likely contained background levels of some or all of the analytes. By using wastewater as a matrix for standards, background levels of PhACs could be accounted for intrinsically in the development of the standard curve.

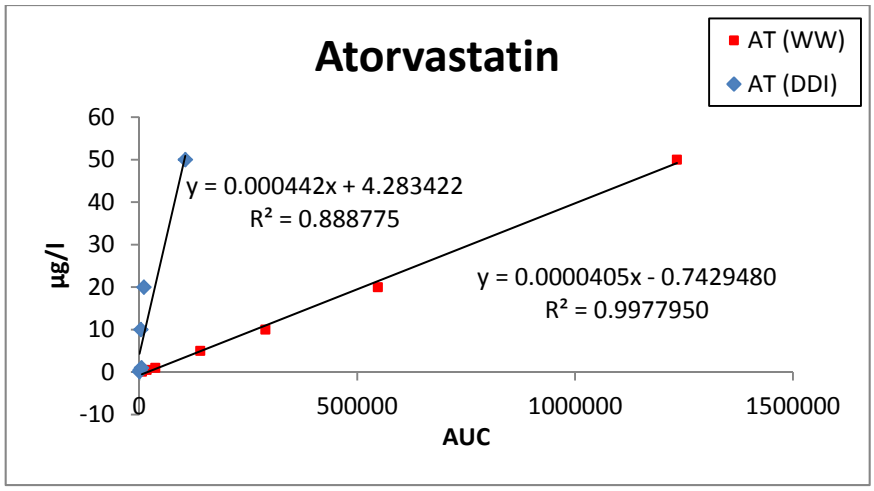


Figure 5.4-D

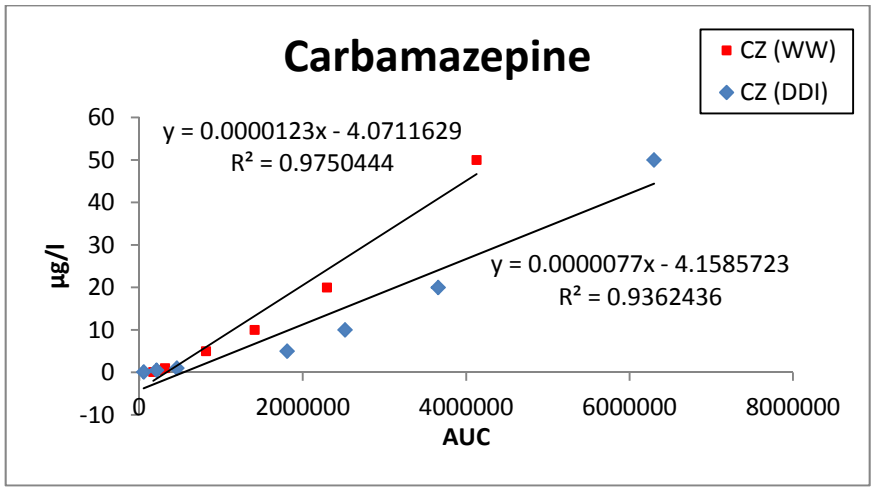


Figure 5.4-E

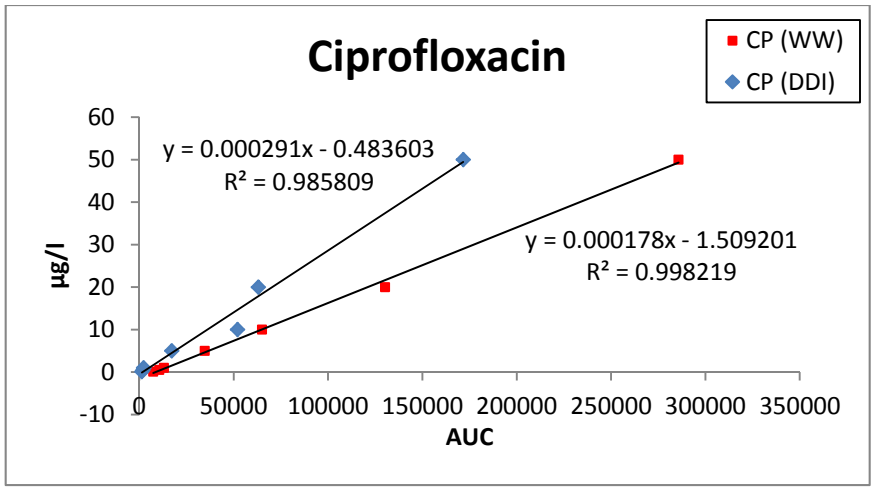


Figure 5.4-F

Results from this second iteration yielded significantly improved, and different, results as compared to the first round of study. The main improvement noted was much higher R^2 values compared to those from Experiment I.

While model fit improved, some interesting anomalies were observed related to the different solvents used. The standard curves in Figure 5.4.2-A for atorvastatin indicate nearly an order of magnitude difference in sensitivity for the standards batched in filtered wastewater compared to those in DDI water. In other words, the instrument response (AUC) for the same concentration of analyte was nearly ten times higher when batched in wastewater than when diluted in DDI water. A similar response was observed for CP (Figure 5.4.2-C). Interestingly, this phenomenon is reversed for CZ, in which the AUC for the analyte in DDI water is larger than the same concentration batched in wastewater. Additionally, it is apparent that some curvature exists for the CZ standards assembled in both mediums. While a polynomial may be a better fit, in this case, the linear model indicated on the plot in Figure 5.4.2-B maintains an R^2 of over 0.97 for standards batched in wastewater, considered in this research to be sufficiently representative.

The difference between the analyte responses noted above (related to solution in which the standards were prepared) is most likely the result of matrix effects. A matrix effect occurs when particulate or dissolved constituents present in the finished samples cause systematic errors in the instrument response. These errors can result from either signal suppression in which the other constituents “shadow” the analyte signal causing it to be artificially low, or signal amplification in which the signal response from the interfering compound is incidentally included in the analyte AUC. The latter typically results from the failure of the analytical method to adequately separate the analyte from the interfering material, i.e. insufficient chromatographic separation. Tarcomnicu et al. observed similar matrix effects for PhACs with variable signal interference depending on the media in which the standards

are batched (Tarcomnicu et al., 2011). In their study, both signal suppression and signal amplification were noted and tended to be highly variable between analytes.

The standards assembled in filtered wastewater in this experiment resulted in excellent linearity for all three analytes. Due to the apparent matrix effects, it was determined that the remainder of the experimental results be based on standards prepared in filtered substrate in which unknowns would be present and targeted for quantification. In this manner, potential interferences by unknown contaminants could be accounted for, with the same matrix effects expected in the unknown samples and the standards. While it is improbable to exactly match the medium in which the unknown samples exists, the more similar the standard and unknown matrices are, the more that matrix effects can be minimized. Other researchers have also used this technique known as “matrix-matched calibration” (Malysheva et al., 2013). While it is understood that additional background PhAC compounds could be present in the wastewater and would thus potentially underestimate actual PhAC concentrations, the importance of capturing matrix effects was deemed more important.

An alternative to using DDI water in lieu of real wastewater would be to employ internal standards. Other research using standards batched in reagent purity solvents (water, methanol, acetonitrile, etc.) employed internal standards as a method to eliminate matrix effects (Golet et al., 2003; Karnjanapiboonwong, 2011; Salgado-Petinal et al., 2006; Yang et al., 2011). However, internal standard compounds may be insufficient to account for the error-producing effects of differing solvents and differing responses to interfering substances present in real samples. For example, CZ would not work as an internal standard for AT because the matrix effects appear to act oppositely on AT as compared to their action on CZ. Additionally, with the great number of PhACs in use today, there are not always standards available (deuterated or otherwise) for each compound.

5.4.3 Experiments III, IV

Based on the work in Experiment II, as noted the remainder of the experiments in this study utilized standards prepared in matrices similar to that expected in the unknown finished samples. Experiments III and IV once again utilized filtered wastewater as the standard medium. The variability inherent in the analytical method and instrumentation can be assessed by comparing the standard curves from these experiments.

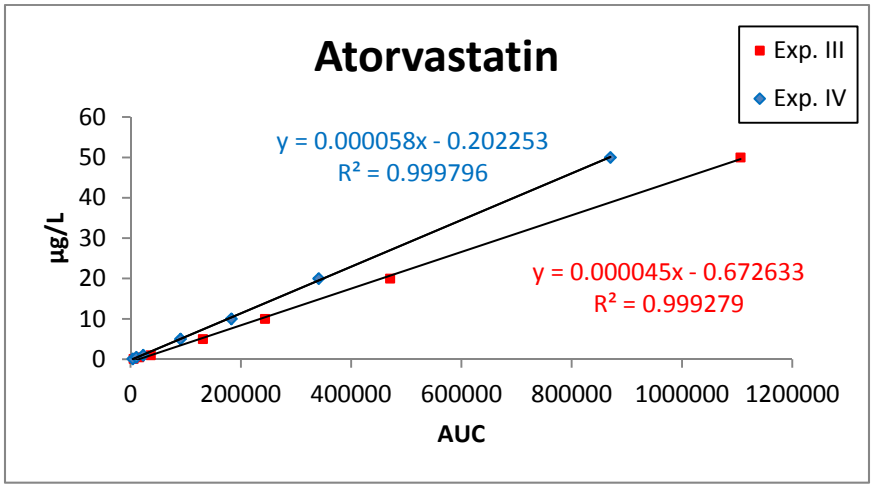


Figure 5.4-G

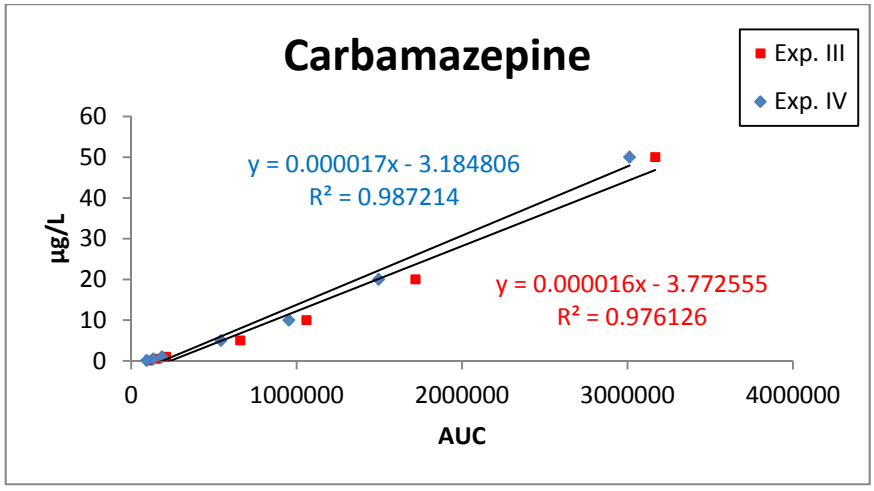


Figure 5.4-H

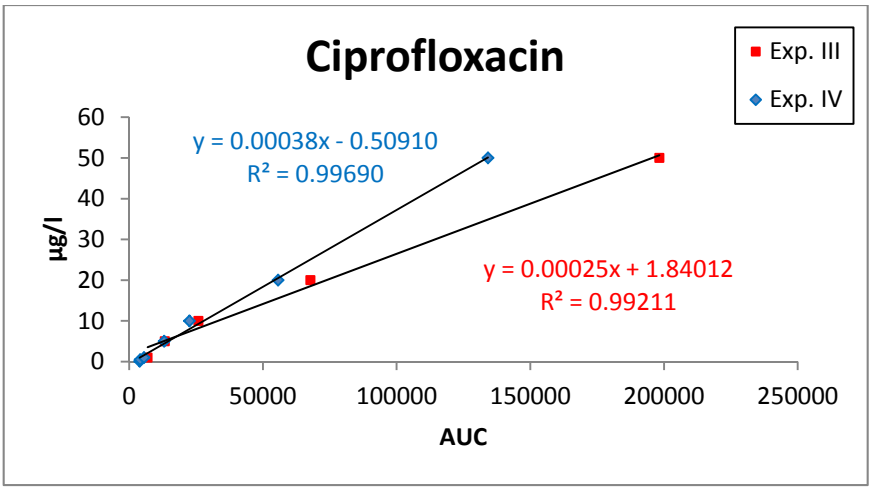


Figure 5.4-I

Figures 5.4.3-A through 5.4.3-C represent the standard curves for each analyte from both experiments. These standards were submitted for analysis exactly 12 days apart. It is apparent by comparing the slope coefficient of the standard curve for each analyte that the instrument response does indeed vary over time.

In addition to the slope of the standard curve, one can note the variation in the model x-axis intercept. All curves except Experiment III in Figure 5.4.3-C intersect the x-axis at a positive AUC value. It also appears that a linear model for the lower concentrations (0-10 µg/L) from Experiment III in Figure 5.4.3-C would also cross the x-axis at a positive AUC, even though the full linear regression intercepts at a negative AUC value. The AUC values at which the model line crosses the x-axis represents the background response of the instrument for a given sample matrix. Some background signal will likely always be present with highly sensitive instruments even when evaluating DDI water.

5.4.4 Experiments V, VI

Building on Experiments I-IV, which focused on fermentation and CAS WRRF processes, experiments V and VI evaluated anaerobic digestion for its impact on PhACs. One unique characteristic of these experiments compared to the previous experiments was that the substrate source for the assessed reactor was a laboratory-scale conventional activated

sludge SBR supplied entirely by synthetic feed. These PhAC treatability investigations also added acetaminophen, while discontinuing carbamazepine. A complete discussion on the experimental setup for these sampling events can be found in Chapter 3.

For these evaluations, matrix-match calibration was again attempted. Standard curves were developed using filtered supernatant from the synthetic wastewater fed activated sludge reactor as the standard medium. By using this medium, the possibility of encountering background levels of the target analytes in the standards was eliminated, since the synthetic medium contained no PhACs. This contrasts with the standards from the previous experiments that utilized filtered wastewater as the substrate source and standard medium, which likely contained trace concentrations of some or all of the analytes of interest (Golet et al., 2003; Karnjanapiboonwong, 2011; Ternes et al., 2005; Yang et al., 2011).

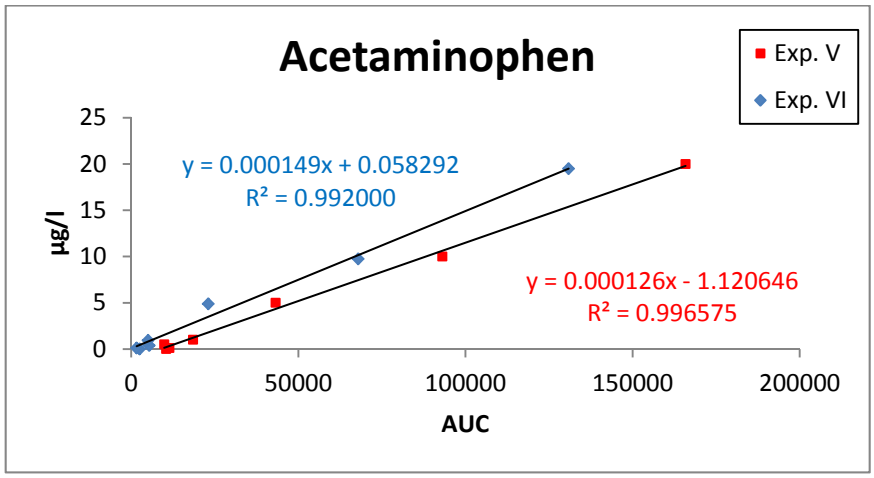


Figure 5.4-J

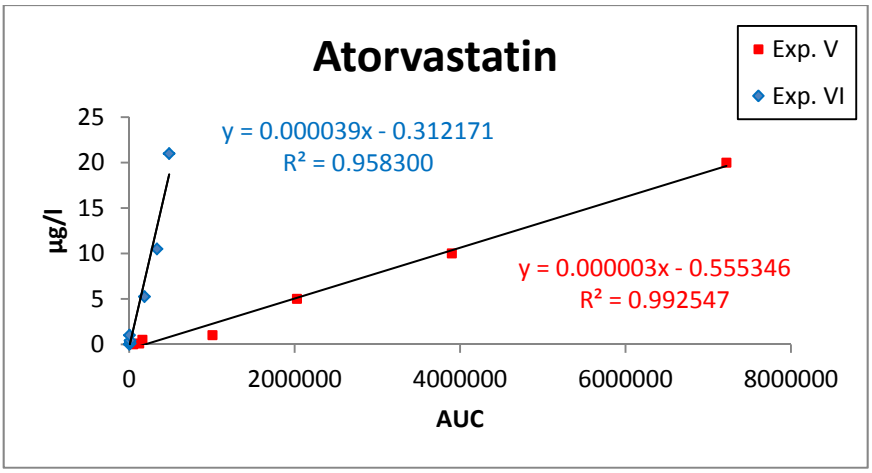


Figure 5.4-K

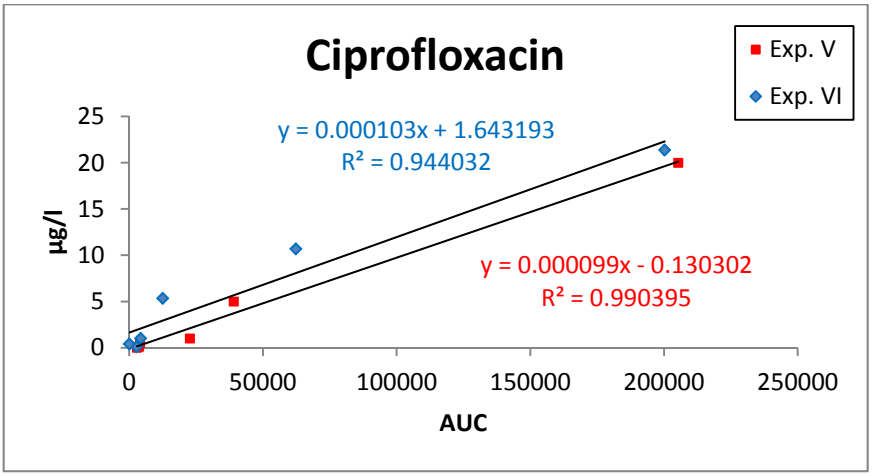


Figure 5.4-L

By comparing the standard curves in each figure (5.4.4-A through 5.4.4-C), there appears to be two phenomenon worth discussing. First, the reproducibility of standard curves seems to be compound specific. For instance, the standard curve slope coefficient for AC and CP are very similar between the experiments with a variation of 18% and 4% respectively. However, the slope of the standard curves for AT differed greatly with a 13-fold increase in slope between experiments V and VI. Second, the x-intercept for each set of standard curves varied quite significantly even while the R^2 values remained well over 90%.

Table 7: Standard Curve Slope Coefficients

	Exp	AT	CZ	CP	AC
DDI	I	0.00690	0.000003	0.00021	
	II	0.00042	0.000008	0.00029	
Filtered WW	II	0.000041	0.000012	0.00018	
	II	0.000058	0.000017	0.00038	
	IV	0.000045	0.000016	0.00025	
CAS Supernatant	V	0.000039		0.00010	0.000149
	VI	0.000003		0.00010	0.000126

Summarized in Table 5-1 are the linear slope coefficients of the standard curves from each experiment discussed in this chapter. For a given medium (DDI, WW, CAS) the slope coefficient for each analyte appear to be relatively consistent. However, by comparing the linear slopes between media it is obvious that the medium in which the standards are prepared affects the analytical response. This is most likely the result of matrix effects as discussed above. The exception to this is the linear models for CP that appears to be less impacted by differing matrices.

5.4.5 Experiment VII

It was theorized from previous experiments that the amount of organic solvent in the finished samples might be an important variable in the analytical outcome. During SPE, the analytes are eluted from the SPE cartridges using organic solvents. While these solvents may be acceptable for analysis using GC-MS, Agilent's RapidFire SPE-MS/MS method may be susceptible to analyte loss if the concentration of organic solvents is too high in the injected sample (similar to that discussed in Section 5.4.1).

In order to evaluate the effect of organic solvent content on SPE-MS/MS, standards were batched in filtered wastewater containing 5% methanol. The standards containing the methanol returned AUCs 2% to 30% lower than the AUCs observed for the standards batched in natural matrices as detailed in Experiments III through VI. In most cases, the

results of the methanol-containing samples indicated analyte concentrations less than the background levels observed by analyzing filtered wastewater containing no added PhACs. Importantly, these observations contrast with EPA Method 1694 in which a methanol concentration up to 5% is considered acceptable. However, according to Agilent Technologies, Inc., RapidFire SPE-MS/MS method used in this study is likely much more susceptible to losses associated with excess organic solvent than the LC-MS/MS method recommended in EPA 1694.

6 CONCLUSIONS

The method development process presented and discussed in this chapter highlighted many challenges associated with lab-scale investigations targeting PhACs in complex matrices. Analytical methods sufficiently sensitive to quantify PhACs at concentrations observed in this study appear to be susceptible to interference from undesired constituents in prepared samples. Beyond interference concerns, failure to optimize the organic solvent content of finished samples can lead to reduced analytical sensitivity using SPE-MS/MS, up to and including complete loss of analytical response. In addition to organic solvent content, the data supports that the matrix in which the finished samples are contained must be matched to the matrix in which analytical standards are prepared. Further experimentation should be conducted to determine the extent the differing matrices has on results and what measures can be taken to minimize the error introduced therefrom.

6.1 Questions Answered

Addressing the questions posed in the introductory paragraph of Section 5.4 with the perspective gained from the results presented in Sections 5.4.1 through 5.4.4 proceeds as follows.

- Do the expected values of the unknowns fall within the range of quantification of the standard curve?

Yes. The expected values for experiments in this study ranged from zero (Limit of Detection) to approximately 20 µg/L, which would be well within the linear range for each standard curve.

    - What are the upper and lower limits of quantification for the method and instrument?
    - What is the limit of detection for each analyte and the instrument used?

Limits of detection and quantification are generally associated with the variability of standard curve data around the linear standard curve model. Qualitatively, the variability appears to be quite low. This would suggest a low potential limit of detection and low limit of quantification. However, numerical values for these parameters would only be speculative, as single standards prepared for each concentration does not allow for a sufficient statistical analysis of variability. As for upper limit of quantification, no instrument saturation was observed. Thus, for the standard concentrations used, the upper limit of quantification was not achieved.

- Do the standard curves exhibit good fit?

The data points for each compound included in the development of the standard curve all exhibit good fit to a linear model, with high R^2 values for all but Experiment I. However, one could argue that all of the standard curves for CZ could have possibly been more appropriately represented by a quadratic or polynomial rather than linear model due to the observed slight curvature of the data points over the concentration range.

- How do the standard curves compare across iterations?

As indicated in the discussion for each set of experiments, overall, the slope and intercept of the standard curve vary significantly between matrices. Additionally, the curves statistically varied over time as different standard curves were prepared on different dates. Most of the models maintained a coefficient of determination (R^2) of greater than 0.99. This means that these models accurately predict 99+% of the variability in the data. As noted in Section 5.4.4, the slopes of the linear models varied between analysis dates by a minimum of 4%, with most of the variations between iterations falling in the 15% to 30% range.

- How often does the standard curve need to be prepared?

Because of the variability between analysis events noted previously, and the corresponding failure of a given linear model to predict previous or subsequent events, a new set of standards and a new standard curve must be established each time an experiment is conducted. While some of the iterations appear to indicate ample repeatability, the extreme variation observed in Figure 5.4.4-B reaffirms why standards must be assembled each time samples are processed and analyzed.

- What is the magnitude of the difference in standard curves between iterations?

The magnitude of the variation in standard curves over time and in varying solvents are summarized in Table 5-1.

- Do the standards used for developing the standard curves accurately represent the experimental unknowns?

While it is not possible to say that the matrix in which the standards are prepared absolutely models the matrix in which the unknown samples are collected, it can be inferred or postulated that the closer the matrices are in makeup (background compounds present, etc.) the more likely the standard curve represents the unknowns. More importantly, quality standard curves were obtained using standards prepared in the various unknown matrices, which adds confidence to the interpretation of concentrations in unknown samples.

- Are the standards and unknowns prepared in the same solvent? Is this necessary?

As discussed in Section 5.4.2, other researchers have concluded that in lieu of precision internal standards, the only way to account for matrix effects in sample

analysis is to match the matrix in which the standards are prepared to the matrix of the unknown samples (matrix-match calibration). Based on the results presented herein, use of the unknown sample matrix is advised.

- Are there potentially interfering constituents in the unknowns that are not present in the standards?

Experiments on biological systems are unique in that the makeup of the system matrix is ever changing. As biological communities grow and adapt, and as they metabolize substrate, the potentially interfering substances in experimental samples constantly change. To that end, it is not possible to exactly match the standard matrix with the unknown sample matrix. However, by using the unknown sample matrix, potentially interfering substances are most likely sufficiently addressed.

- Are the standards prepared at the same time as the unknowns? Is this necessary?

As noted previously, new standards must be included each time unknown samples are prepared for analysis.

7 FINAL METHOD SUMMARY

The final sample preparation and analysis method developed according to the descriptions in Sections 3 through 6 is as follows:

- Sample size: 70 mL
- Centrifuge at 5,000 rpm for 10 min.
- Collect centrate in clean centrifuge tube
    - Filter through 5.0 µm nylon syringe filter
    - Collect 50 mL for processing
    - Spot verify pH approx. 7.0 +/- 1.0, adjust if necessary
    - Process through SPE according to Section 4.2
    - Bring eluent containing analyte to near dryness using nitrogen drying apparatus as described in Section 5.3 (do not let the sample go dry)
    - Bring up to 2 mL using DDI water.
    - Pipette into 96-well plate and transfer to analytical laboratory using the guidance in Section 4.5.
- Extract the solid fraction according to Section 5.2: Solid Fraction Extraction
    - Evaporate the extract using the nitrogen drying apparatus to less than 1 mL.
    - Dilute the extract with 50 mL of DDI water in a clean centrifuge tube
    - Spot verify pH approx. 7.0 +/- 1.0, adjust if necessary
    - Process through SPE according to Section 4.2
    - Bring eluent containing analyte to near dryness using nitrogen drying apparatus as described in Section 5.3 (do not let the sample go dry)
    - Bring up to 2 mL using DDI water.
    - Pipette into 96-well plate and transfer to analytical laboratory using the guidance in Section 4.5.

CHAPTER 3: PHAC TREATABILITY STUDY

1 INTRODUCTION

Reducing and/or eliminating PhAC emission from WRRFs is a tremendous challenge for environmental engineers and WRRF operational personnel. While ozone and activated carbon treatment have been shown to substantially reduce the concentrations of certain PhACs present in wastewater, as discussed in Ch. 1, more common WRRF treatment processes such as activated sludge (AS) have been only anecdotally evaluated for their abilities to remove these compounds (Carabineiro et al., 2011; Sirtori et al., 2009). However, considering the genesis and operational focus of these conventional WRRF configurations (i.e., on nutrient removal), it should be no surprise that PhAC removal in these systems is purely incidental and the elimination of PhACs is variable (Heberer, 2002). Ultimately, more information is required to assist WRRF operators in optimizing conventional liquid and solids stream biological treatment plant performance to remove PhACs.

Considering PhAC treatability within the context of existing WRRFs, PhACs present in wastewater can be removed (either complete or partial degradation), can pass through the system in solution untreated, or can sorb to the biomass. Untreated PhACs remaining in solution will end up in the watershed (either through direct discharge or through effluent irrigation). When the WRRF-generated sludge/biomass is treated (through AD or other means) and disposed of, the accompanying residual PhACs are also disposed of. Of significant concern is the land application of high PhAC-containing biosolids. Once land applied, the PhACs can potentially become mobile concomitant with erosion, or they can

persist in resident soils wherein there is potential for them to pose an environmental risk to soil microbes or plant life (Brain et al., 2006; Karnjanapiboonwong, 2011).

The research presented and discussed herein aimed at evaluating conventional biological WRRF processes - anaerobic suspended growth (fermentation), aerated suspended growth (conventional activated sludge, CAS), and anaerobic sludge stabilization (anaerobic digestion, AD) - for the ability to reduce or eliminate PhACs. This research was conducted while simultaneously developing and evaluating the analytical methodology detailed in the previous chapter. The data presented in this chapter should be viewed as foundational for subsequent investigations.

A key point in analyzing and interpreting the results from this study is to observe the trend in concentration and not the actual concentrations themselves (i.e., potential treatment, not treatment for removal). Additionally, a transition of the analyte from the aqueous to solid phase can be identified by observing a decreasing aqueous phase concentration while simultaneously observing an increasing solid phase concentration. However, the total mass of analyte observed in both phases must be compared between samples to evaluate whether the observed apparent transition between phases was authentic or was the result of degradation of the analyte itself.

2 FERMENTATION

2.1 Experimental Setup

Four discrete one liter fermenters were operated and analyzed. Each one-liter glass beaker fermenter was mixed using magnetic stir plates (Thermo Scientific Cimarec), and was inoculated with biomass from an operational four-liter municipal primary solids fermenter located in the same laboratory. The fermenters were operated as sequenced bioreactors (SBRs) with a hydraulic retention time (HRT) of six hours and a solids retention time (SRT) of approximately three days. To achieve these parameters, the reactors were fed unfiltered raw wastewater eight times daily at three-hour intervals; substrate was fed to each fermenter using Watson Marlow model 323 peristaltic pumps (Watson Marlow Bredel, Wilmington, Massachusetts). Raw wastewater (substrate) was collected regularly from the Moscow, Idaho WRRF, and stored at 4˚C until used. To achieve settling and decanting/wasting cycles, stir plates and separate peristaltic pumps were added. The feed and decant pumps were controlled automatically by a Guho™ 5000 series programmable logic controller (PLC).

To evaluate PhAC treatability via fermentation, each fermenter was spiked with a single PhAC, one each for AT, CP, and CZ. The fourth reactor was dosed with a combination of the three compounds. Sampling was conducted just prior to spiking and at times 0, 30, 90 and 180 minutes following the addition of PhACs.

70 mL samples were collected so that at least 50 mL would remain following solids separation and filtration through 1.2µm glass/fiber filters (Millipore Corp., Billerica, Massachusetts). The aqueous samples were immediately processed via SPE following the pH adjustment dictated by EPA 1694. The solid fractions were also processed using the complete procedure described in EPA Method 1694. Three separate treatability

assessments were performed, represented as Experiment I, II, and III, respectively. Samples from Experiment I were processed according to the unmodified EPA Method (1694). Samples from fermenter Experiments II and III were processed according to the methods in Chapter 2: Experiments I and II.

2.2 Results and Discussion

Potential treatment performance in the fermentation reactors is presented in Figures 3.2.1-A through 3.2.2-C for each analyte from the time of analyte addition to t = 3 hours (i.e., over an operational cycle). Note that the aqueous phase is indicated in µg/L while the solid fractions are in µg/kg on a dry solids basis.

2.2.1 Fermentation Experiment I

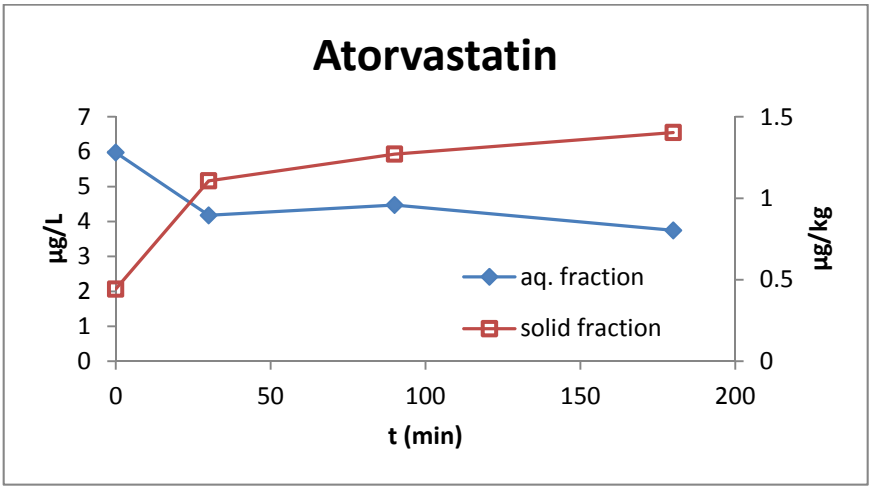


Figure 2.2-A

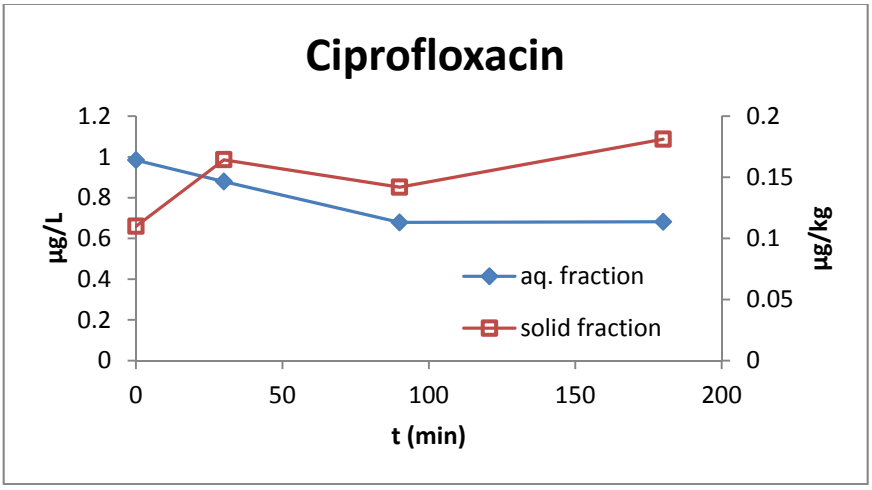


Figure 2.2-B

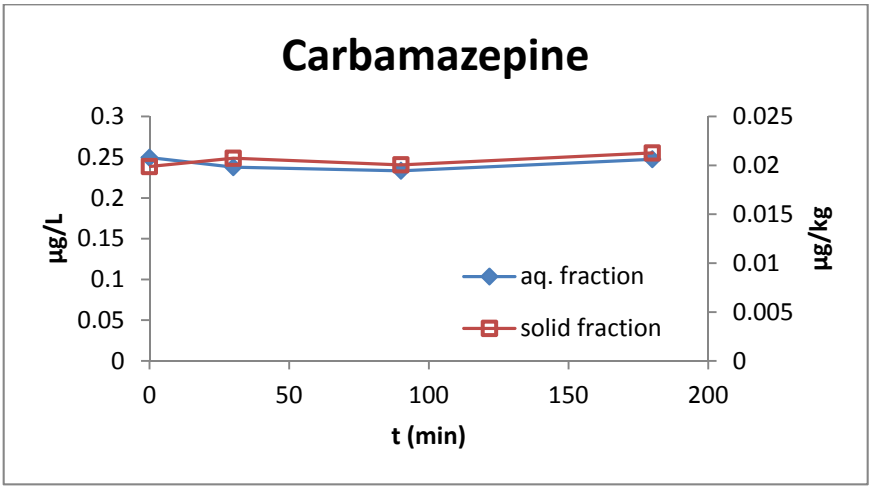


Figure 2.2-C

2.2.2 Fermentation Experiment II

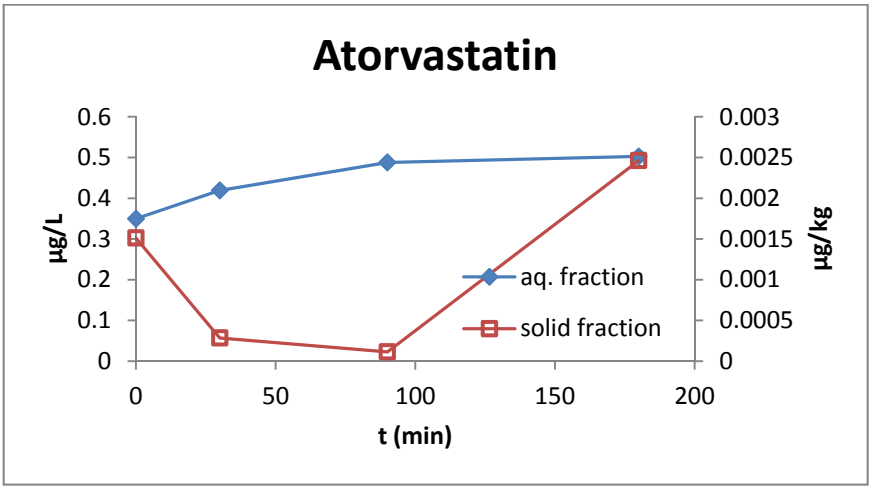


Figure 2.2-D

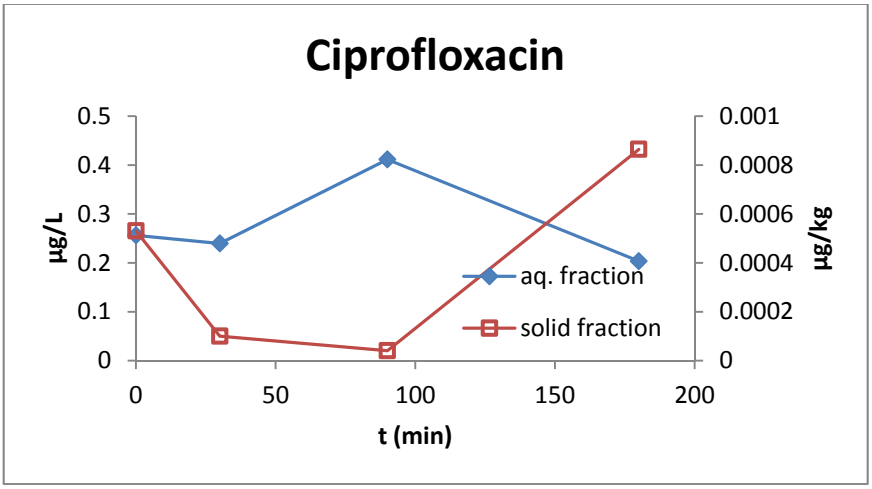


Figure 2.2-E

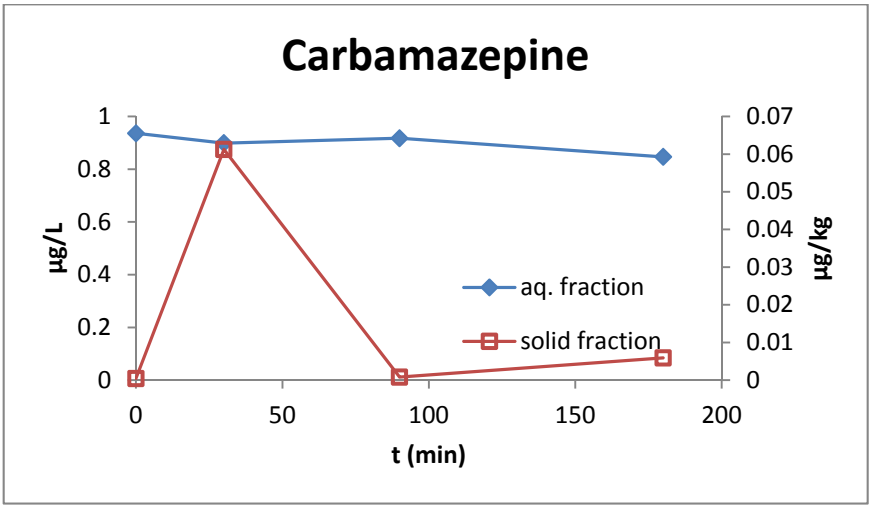


Figure 2.2-F

2.2.3 Fermentation Experiment III

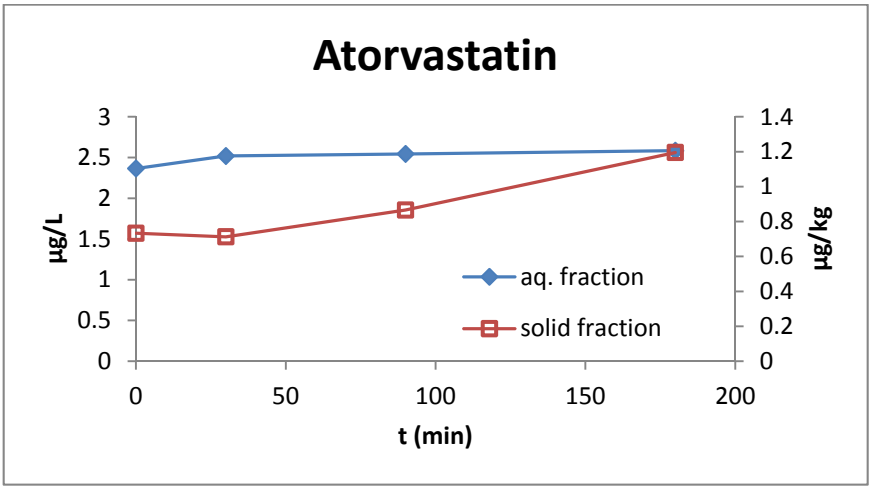


Figure 2.2-G

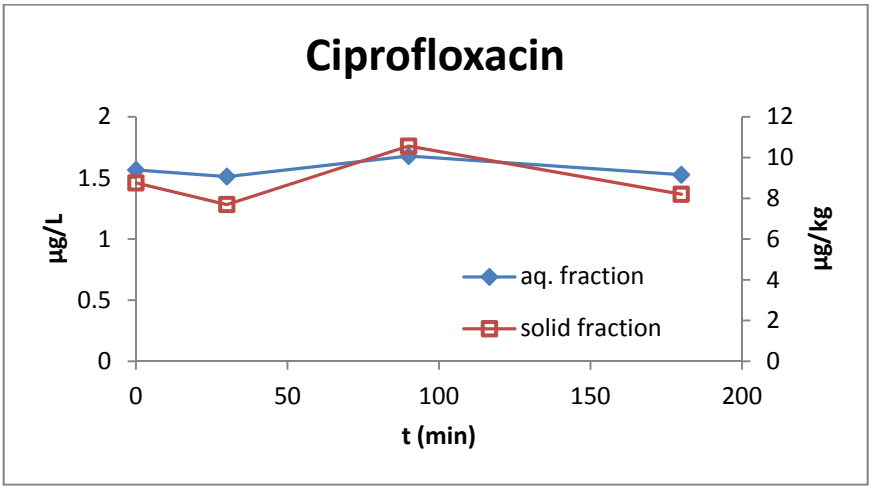


Figure 2.2-H

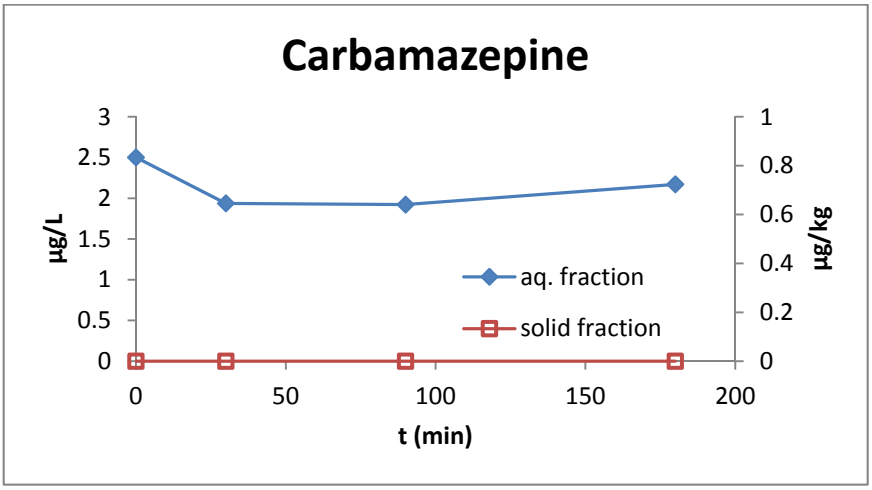


Figure 2.2-I

For each sampling event, collected samples were immediately centrifuged and split into aqueous and solid fractions. The aqueous and solid samples were then processed according to the methods discussed in Chapter 2, and then analyzed. By plotting both fractions onto the same graph, one can make qualitative inferences about the fate of the analyte of interest. For instance, the results from Experiment I (Fig. 3.2-A) would tend to indicate that atorvastatin will transition from the aqueous phase to the solid phase via sorption to biosolids. However, the observed values for the solid phase fraction were much lower than expected for each analyte in Experiments I. As indicated in Chapter 2, this is likely the result of inadequate dilution of solid extraction solvents prior to processing through solid phase extraction (SPE). An excessive solvent concentration will prevent adhering of the analyte on the SPE sorbent resulting in abnormally low recoveries. The reduced capture efficiency is likely what led to the falsely low solid fraction results. Because of the unpredictable results consequential of these analyte losses, the mass balances for Experiment I is not included in this discussion.

As discussed in Ch. 2, the analytical methods established for Experiments II and III exhibited sufficient confidence to warrant a more comprehensive treatment assessment. As such, mass balance analyses were completed for Experiments II and III. Results from these mass balance analyses for each analyte are presented in Table 8.

Table 8: Mass Balances for Fermentation Reactors

Mass Changes in Fermentation Reactor (µg)										
		Atorvastatin			**Ciprofloxacin**			**Carbamazepine**		
		t=0	*t=3 hr*	*Change*	*t=0*	*t=3 hr*	*Change*	*t=0*	*t=3 hr*	*Change*
Experiment II	Aqueous	0.350	0.503	0.153	0.256	0.203	-0.053	0.936	0.847	-0.089
	Solid	0.001	0.001	0.000	0.005	0.009	0.004	0.000	0.003	0.002
	Total	0.350	0.503	**0.153**	0.261	0.212	**-0.049**	0.936	0.850	**-0.087**
Experiment III	Aqueous	2.365	2.583	0.218	1.565	1.526	-0.039	2.501	2.171	-0.331
	Solid	0.043	0.070	0.027	0.456	0.427	-0.029	0.000	0.000	0.000
	Total	2.408	2.653	**0.245**	2.021	1.953	**-0.068**	2.501	2.171	**-0.331**

Included in Table 3.2-A is the total mass of analyte in each fraction (aqueous, solid) at the beginning and end of each experiment. The experimental period coincided with the reactor operational cycle length, three hours for the fermentation SBR. For AT, the results suggest no removal via fermentation. In fact, it appears that the influent substrate contained residual AT, as indicated by an increase in total mass of AT over an operational cycle for both experiments. Ultimately, though, the increase in aqueous fraction AT (without a commensurate decrease in solid fraction AT) cannot be explained.

Considering CP, measureable treatment was observed over the fermenter operational cycle; for Experiment II, the quantity of CP introduced into the fermenter was reduced by 18.8%. For Experiment III, much higher influent concentrations of CP were observed; while the total quantity of CP removed was comparable to that observed in Experiment II, the fraction removed was only 3.4%. Unexpectedly, the results indicated little potential for CP to sorb to the solids. This contrasts with literature wherein over 50% of the CP was observed to sorb to sludge within the treatment train (Belden et al., 2007; Golet et al., 2003).

Finally, considering CZ, a similar treatability potential was observed as with CP. Essentially all of the influent CZ was present in the aqueous phase, and overall removal ranged from 9.3% (exp. II) to 13.2% (Exp. III). These behaviors for CZ are reinforced by consensus with previous studies that indicate minimal ability for any conventional treatment processes to remove CZ, including by sorption (see Table 2: Survey of Research on PhACs in WRRFs).

3 CONVENTIONAL ACTIVATED SLUDGE

3.1 Experimental Setup

For the activated sludge experiments, porous stone air diffusers were added to convert the fermentation reactors to conventional activated sludge (CAS) reactors. All other structural components remained the same as for the fermentation experiments. Operationally, the SRT was increased to five days (typical for CAS systems focused principally on the removal of biochemical oxygen demand (Metcalfe and Eddy, 2003) while the HRT remained at six hours. Aeration was controlled (on/off) by the PLC, with compressed air provided via a Redhead electrically actuated diaphragm valve. Air flow was checked periodically and manually adjusted to ensure that aeration was sufficient to maintain a dissolved oxygen (DO) of ≥ 2 mg/L. The CAS reactors were aerated for 5 hrs and 19 minutes, with unfiltered influent raw wastewater supplied for 3.5 minutes at the beginning of each cycle. Settling was allowed to occur for 30 minutes at the end of each operational cycle, followed by a decant phase of 11 minutes. Raw wastewater (feed) was obtained from the Moscow, Idaho WRRF. This feed likely contained background levels of PhACs.

Periodic checks were conducted of influent and effluent soluble COD to ensure that the microbial consortia were performing typically with respect to COD removal compared to literature values (Metcalfe and Eddy, 2003). On a more frequent basis, DO was monitored continuously immediately following the feeding cycle. Microbial activity was assumed adequate if the DO decreased considerably immediately following feeding. This response to rapid organic loading is similar to that response observed during cell respiration tests.

3.2 Results and Discussion

Two independent PhAC spiking/sampling events were conducted on the CAS reactors. The figures included in Sections 4.2.1 and 4.2.3 illustrate the observed impact on PhAC

concentrations during these sampling events. Considering the analytical concerns expressed in Ch. 2, interrogation of results from these CAS investigations focused on relative changes in PhACs over any operational cycle. While there may be some uncertainty as to the actual value of the PhACs, there is much higher confidence in the relativeness over a tested operational cycle, and thus treatability can be reasonably assessed. Aqueous results are presented as μg/L, while the solid fraction results are presented as μg/kg (dry weight basis).

3.2.1 CAS Experiment I

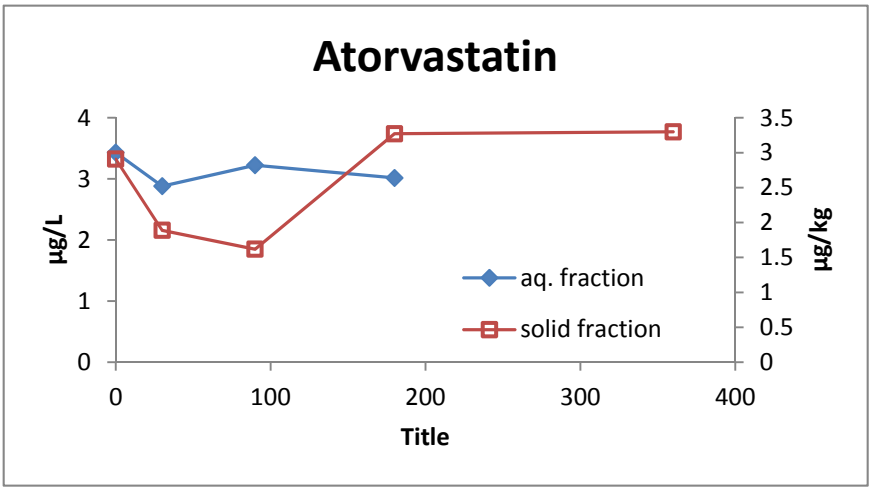


Figure 3.2-A

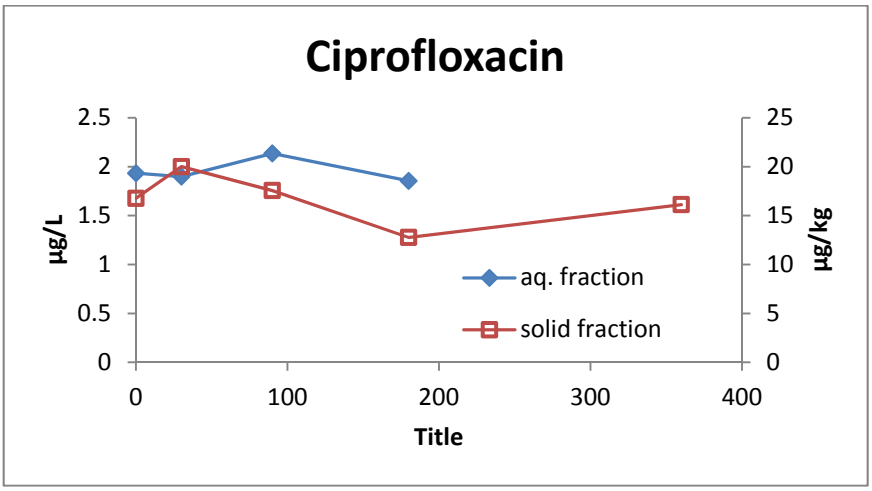


Figure 3.2-B

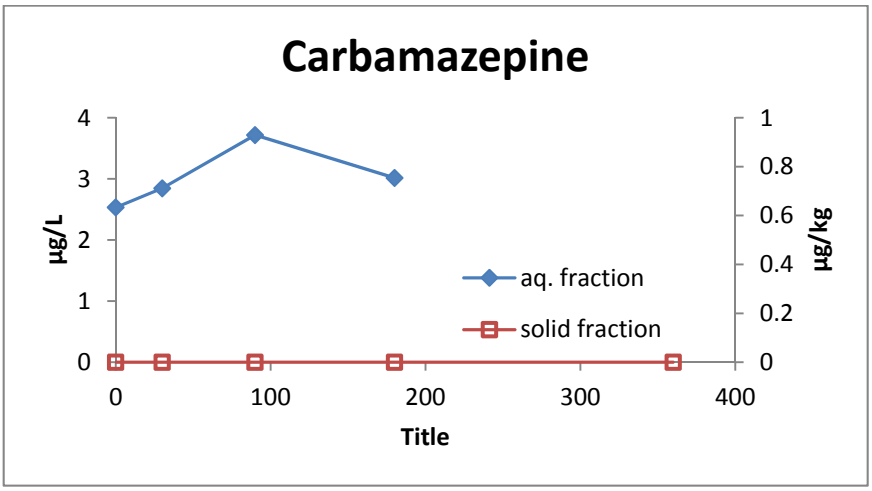


Figure 3.2-C

3.2.2 CAS Experiment II

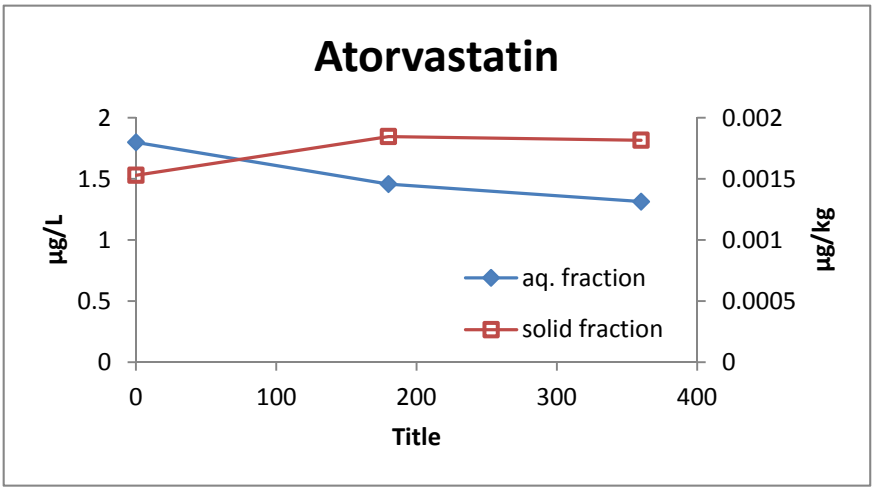


Figure 3.2-D

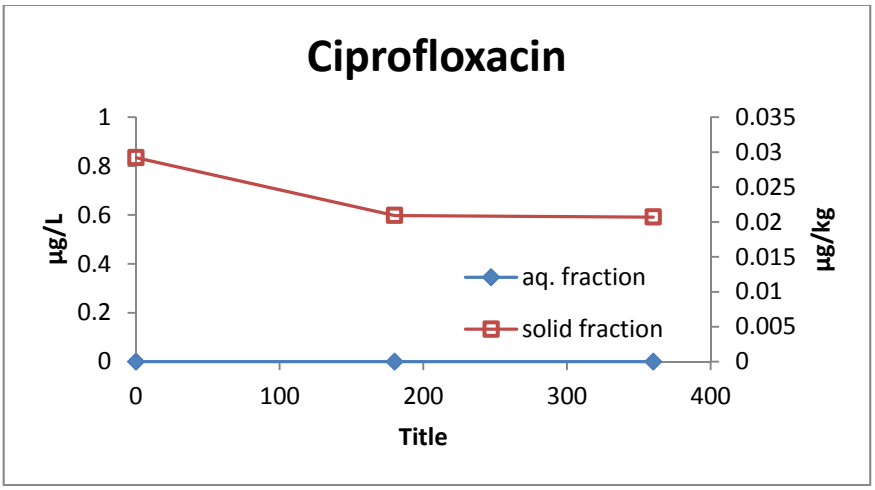


Figure 3.2-E

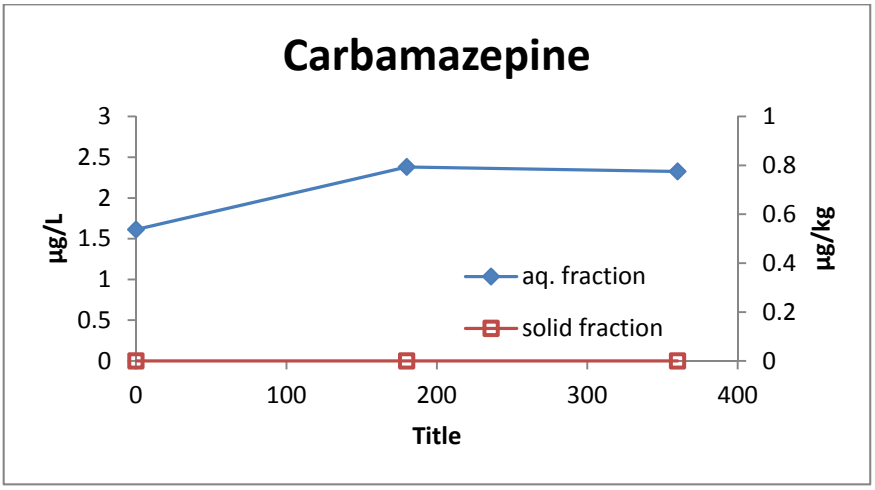


Figure 3.2-F

The experiments conducted under this operating scheme were designed to evaluate the treatability of PhACs by aerobic microbial consortia. To that end, one could conclude by examining all three series of results that, collectively, the aerobic consortia exhibited little potential to degrade PhACs. Observations over all three data series indicated a slight reduction in total ciprofloxacin although it is unlikely that much of this reduction can be attributed to biochemical reactions alone. It is more likely that the reduction was due, in part, to a number of phenomena including photo-degradation, reactions with hydroxyl radicals in the reactor aeration air, and some amount of biochemical degradation.

Some results indicated an increasing trend for total analyte mass over the observation period. For instance, the total mass of atorvastatin appears to increase moderately in each data series. While it is unlikely that the mass of AT is actually increasing, it is more likely that the observed increase is due to analytical variability and the previously discussed nature of interfering substances present in PhAC samples. One could infer from the discussions on the impact of sample solvents from Chapter 2, and the known evolution of constituents present in the aqueous phase of an SBR over the course of a cycle, that analytical results could indicate trends in PhAC concentrations that are actually trends in "other" non-PhAC contaminants. As an example, assume that humic substances present in the finished sample cause the analytical response for atorvastatin to be artificially low due to an unknown phenomenon. As the SBR progresses further into its aerobic cycle, the humic substances should biodegrade, leading to their reduced presence in the finished sample. The signal suppression seen early in the SBR sequence will be lessened, resulting in the appearance that atorvastatin is increasing when, in fact, the observed behavior is actually the reduction in humic substances.

It is important to point out that some results trended toward literature observations. For instance, carbamazepine indicated very little propensity to sorb to biosolids and no overall

reduction was observed over the three sampling events (Clara et al., 2005; Gros et al., 2010; Jelic et al., 2011; Joss et al., 2005). This is contrasted with the atorvastatin results that may indicate a slight tendency of the compound to transition into the solid fraction. Mass balances for each of the above experiments can be found in Table 9.

Table 9: Mass Balances for CAS Reactors

Mass Changes in CAS Reactor (µg)										
		Atorvastatin			**Ciprofloxacin**			**Carbamazepine**		
Aerobic SBR		*t=0*	*t=6 hr*	*Change*	*t=0*	*t=6 hr*	*Change*	*t=0*	*t=6 hr*	*Change*
Experiment I	Aqueous	3.431	3.015	-0.415	1.934	1.855	-0.079	2.533	3.016	0.482
	Solid	0.170	0.191	0.021	0.874	0.665	-0.208	0.000	0.000	0.000
	Total	3.601	3.207	**-0.394**	2.807	2.521	**-0.287**	2.533	3.016	**0.482**
Experiment II	Aqueous	1.799	1.314	-0.485	0.000	0.000	0.000	1.613	2.325	0.712
	Solid	0.140	0.127	-0.012	2.968	1.517	-1.451	0.000	0.000	0.000
	Total	1.939	1.441	**-0.497**	2.968	1.517	**-1.451**	1.613	2.325	**0.712**

Included in Table 9 is the total mass of analyte in each fraction (aqueous, solid) at the beginning (t = 0) and end (t=6 hr) of each CAS operational cycle for each experiment.

Based on the improved sample processing techniques gleaned from the first two sampling events, the observed in-reactor concentrations for Fermentation Experiment III and CAS Experiments I and II were very near the expected concentration of 2 µg/L. With the analytical method not changing, the convergence of the measured results with the expected values was likely due to improved sample processing techniques and standard solvent matching.

The results observed, in terms of overall mass reduction, were mixed for AT and CZ. The data indicate that AT appears to be susceptible to degradation in an aerobic environment; this observation is in direct contrast with the anaerobic-fermentation results (Table 8). Observed AT removal for the two aerobic experiments was 10.9% and 25.6%, respectively. This is somewhat less than the observed removals summarized in Table 8. CZ treatability was opposite that of AT, with no removal achieved. Similar to AT treatability, results

contrasted with those observed for the anaerobic-fermentation experiments; as discussed, CZ degradation was observed via fermentation. Results for CP indicate a propensity of the analyte to be removed under both aerobic (Table 9) and anaerobic-fermentative (Table 8) environments. For the aerobic experiments, the rate of CP removal observed ranged from 10.2% to 48.9%.

4 ANAEROBIC DIGESTION

4.1 Experimental Setup

To conduct the PhAC AD research, a four-liter AD was constructed from polycarbonate Plexiglas. The system incorporated a square plan section and a sloped bottom where the slurry inlet/outlet was located (see Figure 4). Mixing was accomplished with a mechanical shaft-drive mixer (Oriental Motor). As shown, the AD was heated using an integral (external) water bath, with building hot water supply controlled to the water bath via a Redhead solenoid valve and a MYPIN model TA4 PID controller (Mypin Electric Co. Ltd., Model TA4, Guangdong, China). The temperature was controlled at 37ºC (+/- 0.5ºC). This chemostat reactor was designed and operated at an SRT/HRT of 20 days. Feeding and wasting was conducted by pumping into and out of the vessel via the bottom fitting.

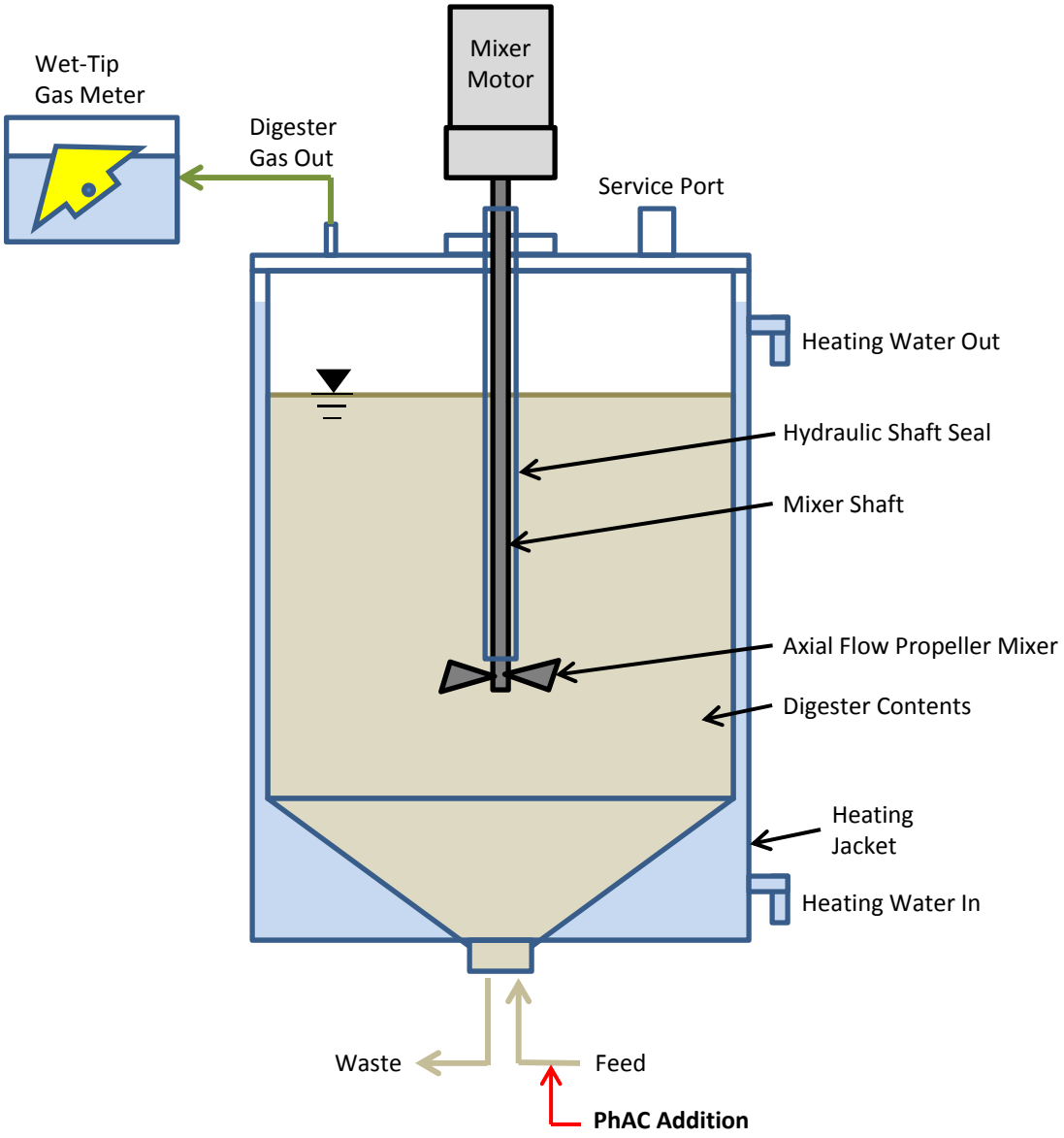


Figure 4: AD Reactor Diagram

Initially, substrate for the AD was provided as waste activated sludge (WAS) from the still operating CAS system fed real wastewater. Specifically, the four CAS reactors from the previous study were combined into a single four-liter vessel, from which the WAS was derived. Inocula from an operating dairy manure digester was added after one week of operation to help accelerate the AD toward process stability.

Because of the trace nature of the target compounds, it was decided that background levels of these PhACs present in real wastewater and the resultant waste sludge could be significant enough to impact results. Therefore, 60 days prior to the executed AD PhAC removal experiments, the substrate for the CAS reactor was changed from raw wastewater to synthetic feed. The synthetic feed used for this experiment was Syntho 2.0 (Nopens et al., 2001) (Table 10: Syntho 2.0 Consitituents).

Table 10: Syntho 2.0 Consitituents

Syntho 2.0	
Constituent	**mg/L**
Urea	91.7
NH_4Cl	12.8
Na-Acet•$3H_20$	131.6
Peptone	17.4
$MgHPO_4$•$3H_20$	29.0
KH_2PO_4	23.4
$FeSO_4$•$7H_2O$	5.8
Starch	122.0
Milk powder	116.2
Yeast	52.2
Soy oil	29.0
$Cr(NO_3)_3$•$9H_2O$	0.770
$CuCl_2$•$2H_2O$	0.536
$MnSO_4$•H_2O	0.108
$NiSO_4$•$6H_2O$	0.336
$PbCl_2$	0.100
$ZnCl_2$	0.208

Synthetic feed was batched at 100X concentration and autoclaved to achieve sterilization. The concentrated substrate was then metered into the CAS reactor via a Watson Marlow 323U peristaltic pump (Watson Marlow Bredel, Wilmington, Massachusetts). Distilled water was also metered into the CAS system using similar pumps simultaneous with the concentrated feed, such that the feed was diluted to the concentrations listed Table 10. Flow rates were also controlled to maintain the target SRT/HRT.

While the goal of the experiments conducted on the AD system was to evaluate the sludge treatment process for its impact on PhAC removal, conventional parameters were monitored to ensure that the digester was operating under typical operating conditions and that the PhACs did not adversely affect AD performance. Specifically, the following parameters were monitored twice weekly:

- Total and volatile solids (feed and waste sludge)
- pH
- Alkalinity
- Biogas production
- Temperature

All operational parameters were in line with those observed in a typical functioning AD. The operational pH of the AD system ranged from 7.3 to 7.6.

In order to evaluate the impact on PhACs by AD and vise-versa, two separate experiments were conducted: a single pulse experiment followed by a continuous feed experiment. The single pulse experiment was designed to evaluate the AD consortium's ability to withstand a one-time spike of a mixture of the PhACs of interest: acetaminophen (AC), atorvastatin (AT), ciprofloxacin (CP), and triclosan (TC). The target in-reactor concentration of each PhAC was 2.0 µg/L. Spiking was accomplished manually through the feed tubing during the feed cycle.

The continuous feed experiment consisted of a continuous addition of PhACs, for a period of seven days, into the influent sludge during AD feeding such that a substrate concentration of 2.0 µg/L (for each PhAC) was achieved. The continuous feed experiment commenced 6 hrs after the pulse addition of the PhACs, without further interruption of AD operations. A Watson Marlow peristaltic pump (Watson Marlow Bredel, Wilmington, Massachusetts) was used to inject PhAC concentrate directly into the sludge during

feeding. The goal of this second experiment was to evaluate the AD system for its ability to adapt (or fail) after extended exposure to the target PhACs. Conventional parameters (as listed above) were monitored during both experiments in order to assess the impact on the AD consortia of these compounds.

4.2 Results and Discussion

Figures 5.2-A, B, and C illustrate the aqueous and solid fraction of each analyte in the AD system over the six hour period immediately following the pulse addition of the PhAC mixture. Figures 5.2-D through 5.2-F show the analyte concentration profiles from the end of the pulse feed experiment (at t = 6 hours) through the end of the seventh day of AD operations.

4.2.1 Anaerobic Digestion: t = 0 to t = 6 hours

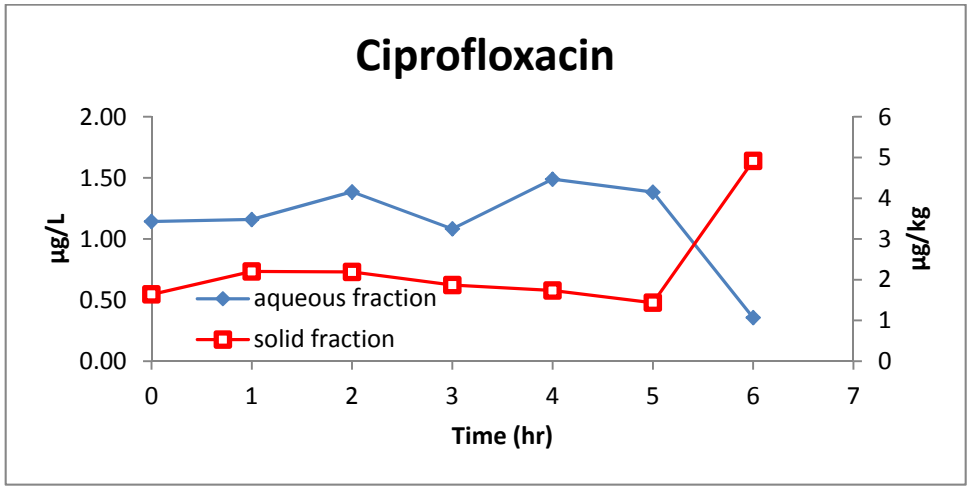


Figure 4.2-A

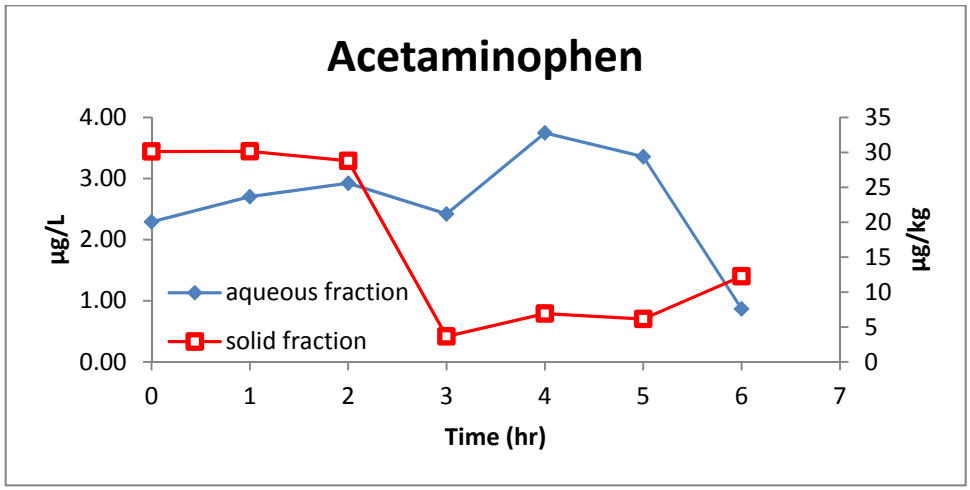


Figure 4.2-B

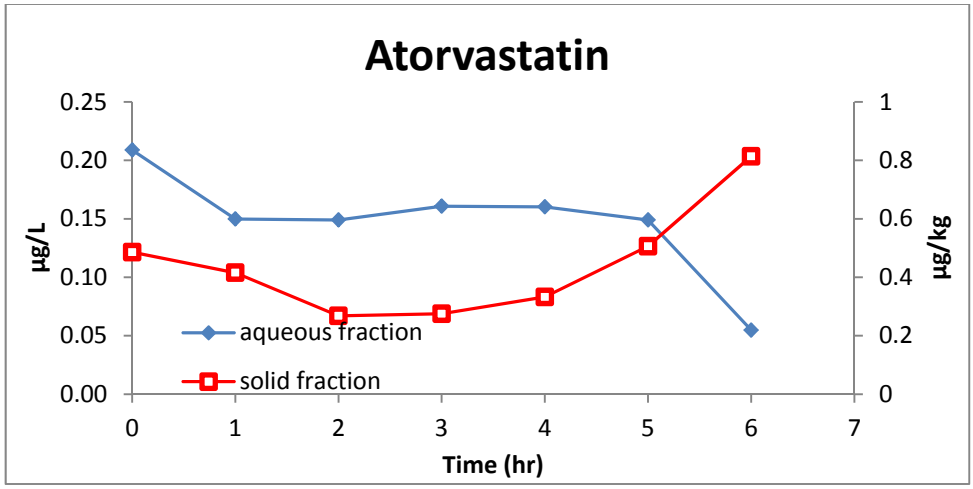


Figure 4.2-C

4.2.2 Anaerobic Digestion: t = 6 hours to t = 7 days

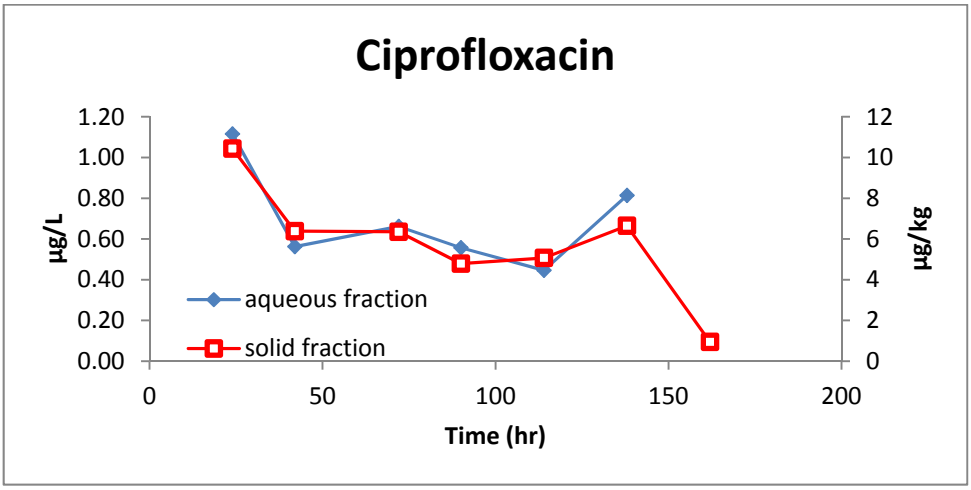


Figure 4.2-D

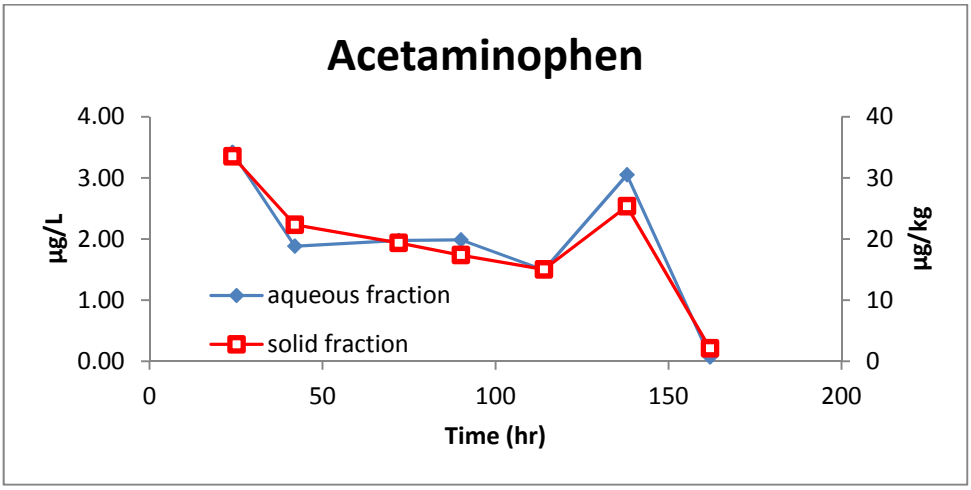


Figure 4.2-E

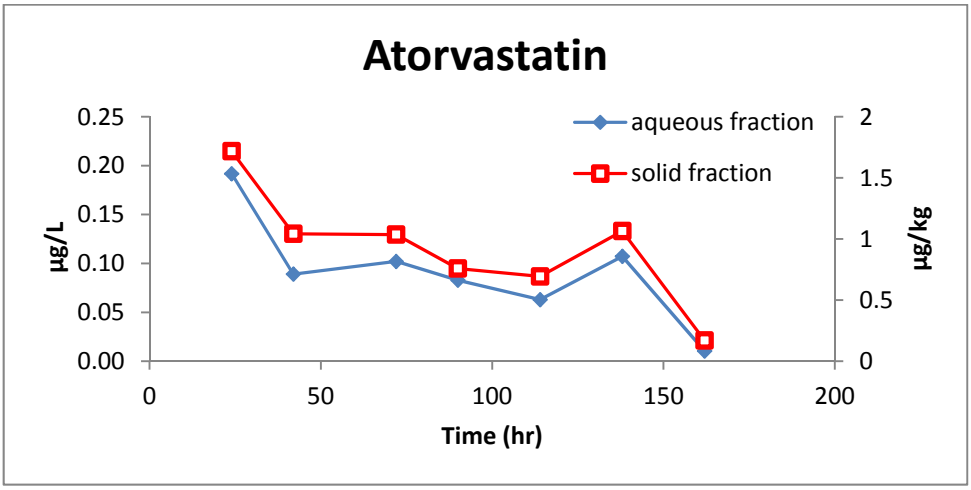


Figure 4.2-F

4.2.3 Pulse Feed

During the “pulse” fed six-hour cycle represented in Figure 4.2-A to Figure 4.2-C, the CP in each phase were relatively constant over the first five hours. However, the sample collected at t = 6 hours appears to indicate a transition of the CP in the aqueous phase to the solid phase.

Like CP, AC concentrations were initially constant. In contrast to CP, the AC exhibited an earlier transition to the solid phase, and then appears to partially desorb into the aqueous phase during the final hour of the experiment. AT followed a pattern fairly similar to that observed for CP including the apparent sorption in the last hour of the test period.

4.2.4 Continuous Feed

While the pulse feed experimental results exhibited variability regarding the fate of the respective analytes, the fate of the PhACs during the continuously-fed experiment were nearly identical. Without regard to the differences in the vertical scale in Figure 4.2-D through Figure 4.2-F, CP, AC, and AT concentrations (aqueous and solid fractions) all were initially elevated at t = 6 hours and consistently decreased over the next seven days. An unexpected phenomenon was observed in the samples collected at t = 138 hours (between five and six days); the aqueous and solid phase concentrations of all three analytes are approximately 50% higher in that sample as compared to the previous sample.

As shown, it is apparent that the overall mass of each analyte decreased over the seven-day experimental period, confirming PhAC removal by anaerobic digestion. While the final sample for the aqueous phase CP was unfortunately contaminated and thus discarded, it is likely that it would have indicated a result near zero, similar to both AT and AC.

4.2.5 Mass Balance

Figure 4.2-G through Figure 4.2-I present the total mass of analyte in the AD over the experimental period (note that the x-axis scale is compressed after t=8 hrs). The first six

hours represent the “pulse” feed portion of the experiment. The remaining data represents the “continuous” feed period in which a 2 µg/L in-reactor concentration was targeted.

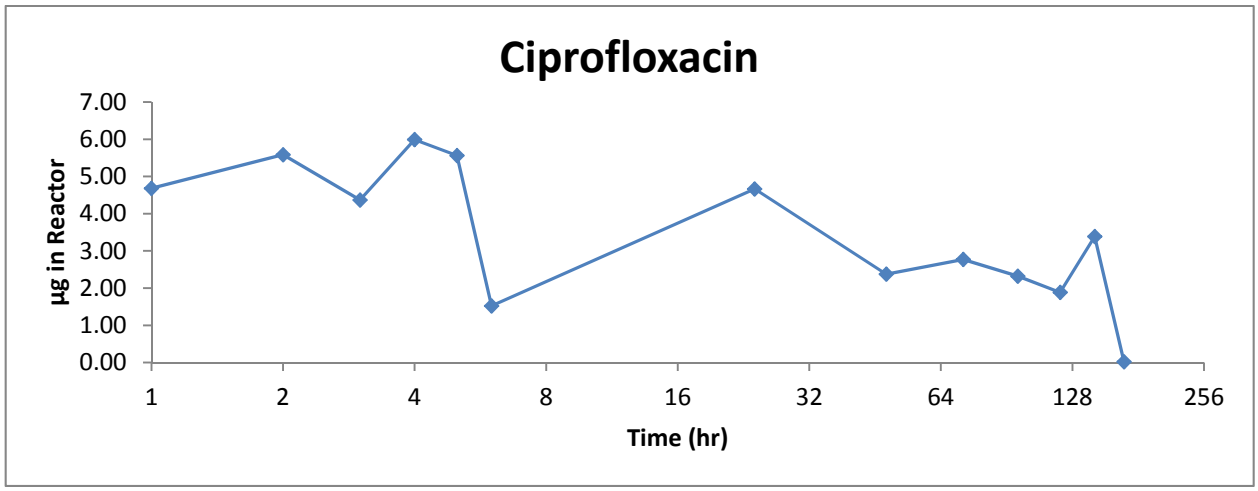


Figure 4.2-G

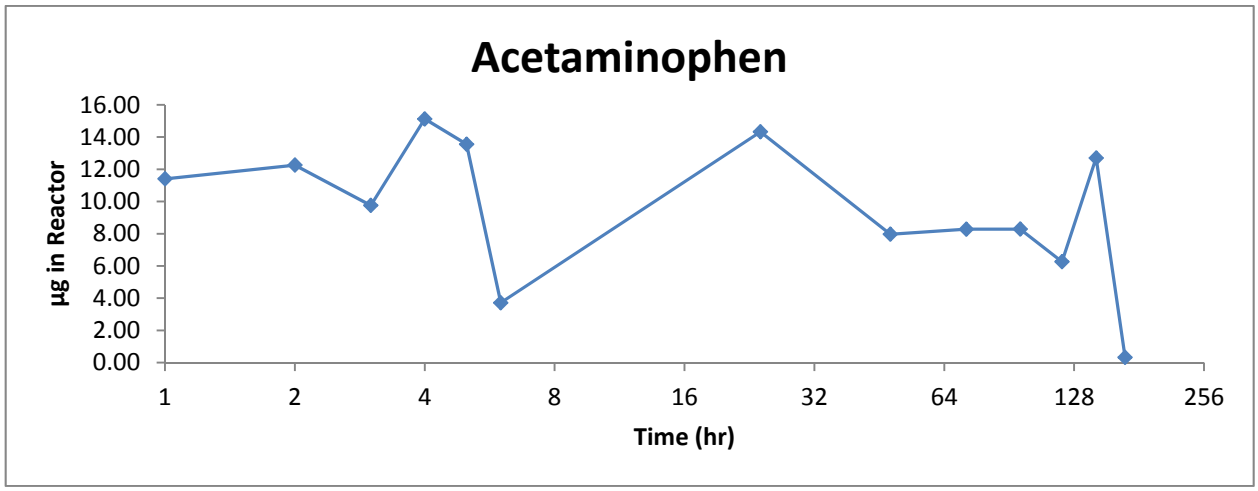


Figure 4.2-H

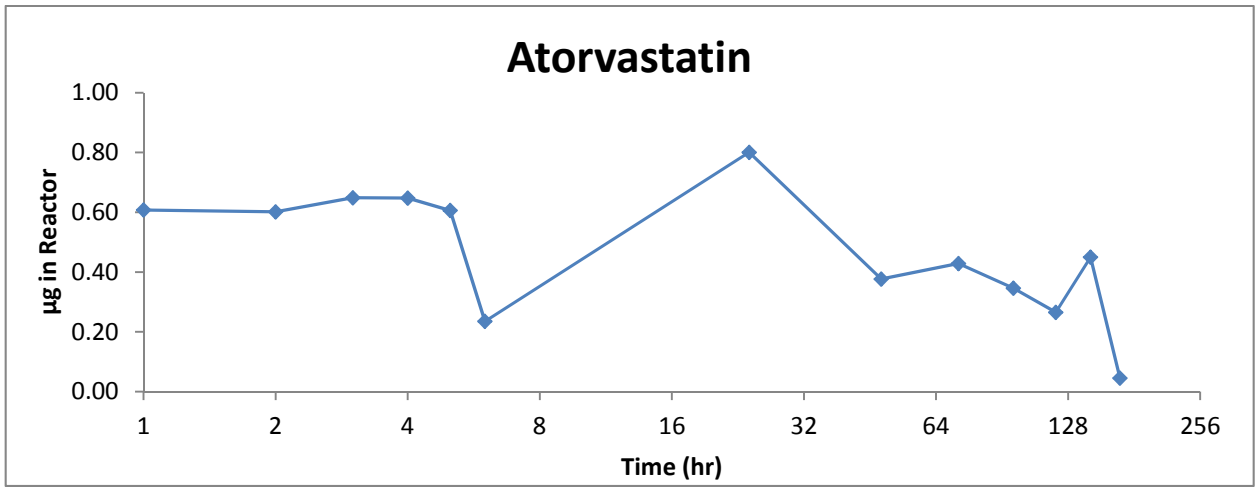


Figure 4.2-I

The mass balances indicate little ability for anaerobic digestion microbial consortia that are not acclimated to complex chemicals such as the PhACs to remove “slugs” of PhACs. This can be seen as a constant analyte mass during the first six-hour period in the figures above. However, the observed slow response could have been a metabolic lag or acclimation period allowing the microbial community to adapt to the new substrate. Some inhibition of the microbial community is also likely due to the addition of a known antimicrobial agent, ciprofloxacin. Over time, following the addition of PhACs, some compounds transition between the aqeous phase and solid phase or vice versa. Partitioning to the solid fraction appears to make the compounds less readily biodegradable. However, considering that the “solids” essentially were microbial aggregates, or flocs, after extended sorption period, the analytes either rehydrolyzed into solution, allowing the consortia to metabolize them, or were metabolized within the floc. The AD system does seem to have some ability to remove all of the analytes targeted in this study when fed constant doses over longer periods of time. This anecdotally appears to contradict Golet et al. in that they observed no significant removal of CP in AD systems (Golet et al., 2003). Other research conducted on AD observed 0% removal of CZ in a pilot-scale study (Carballa, 2007).

4.3 Analysis of the AD Microbial Consortium

Successful AD requires synergism between bacteria (that hydrolyze and ferment substrate to hydrogen, carbon dioxide, and acetate, all methane precursors) and methane-producing archaea. Recognizing the absolute importance of archaea in the successful AD of organic substrate, it was of interest to understand if the addition of PhACs had a potential effect on the consortium. In this regard, research focused principally on the most sensitive population in the AD – the methanogenic archaea. Specifically, quantitative polymerase chain reaction (qPCR) was applied to enumerate the methanogenic population in the AD.

Genomic DNA was extracted from biomass obtained from the AD using the MO BIO PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA USA). Biomass samples were collected weekly over a three-month period beginning approximately two months before the addition of PhACs. qPCR was applied using 16S rDNA-based oligonucleotide primers to estimate the relative abundance of the respective archael populations present in the AD. Specifically, oligonucleotide primers were used to quantify the three principal orders of hydrogenotrophic methanogens (*Methanococcales, Methanobacteriales, Methanomicrobiales)*, and the two most predominant families within *Methanosarcinales* (*Methanosarcinaceae,* and *Methanosaetaceae*), acetoclastic methanogens. Oligonucleotide forward and reverse primers were designed in accordance with (Yu et al., 2005). qPCR was conducted on a StepOne Plus™ Real-Time PCR system (Applied Biosystems, Foster City, CA) using iTaq™ SYBR® Green Supermix w/ROX (Bio-Rad Laboratories Inc., Hercules, CA, USA) with a total reaction volume of 25 µL. qPCR conditions were as follows: 3 min at 95 ºC, 45 cycles of 30 s at 95 ºC, 45 s annealing, and 30 s at 72 ºC. All unknown samples were assessed in triplicate with 5 ng of total genomic DNA and 500 nM final concentration of each primer per reaction. Relative microbial abundance (Figure 5) was estimated using the mean amplification efficiencies for each primer set, the Cq values for the individual samples, and the 16S rDNA copy numbers. The relative quantity of the respective families/orders was determined according to the ΔΔCq method as described by (Pfaffl, 2001). For quantification, the 16S rDNA gene copy number for archaea was set at 1.8, while the gene copy number for bacteria was set at 4.1. 16S rDNA gene copy numbers for the different archaeal orders/families were determined using the Ribosomal RNA Operon Database (rrnDB, http://www.rrndb.mmg.msu.edu/).

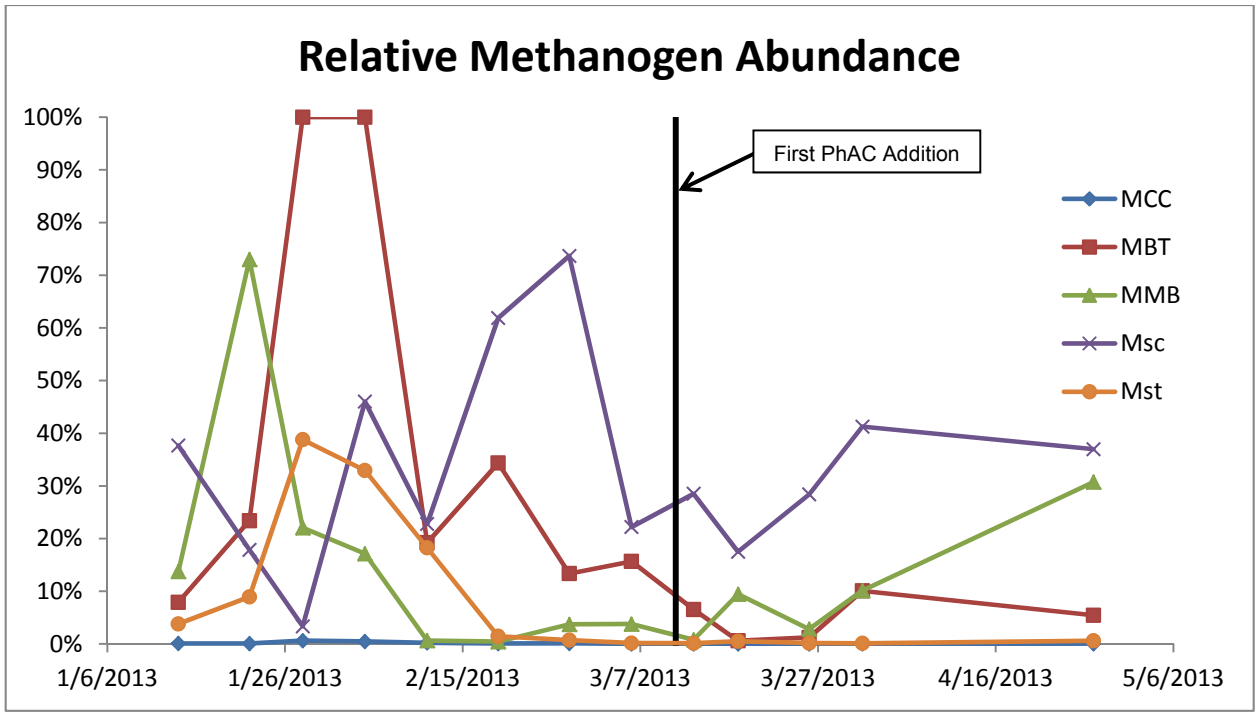


Figure 5: Methanogenic Populations Using qPCR

AD is employed to biodegrade organic-rich biomass and to produce methane-rich biogas for use as a fuel in power generation or digester heating. Methane (CH_4) is synthesized in AD by two distinct orders of methanogens: acetoclastic and hydrogenotrophic. Methanogens of the hydrogenotrophic orders (i.e., *Methanococcales* (MCC), *Methanobacteriales* (MBT), and *Methanomicrobiales* (MMB)) use H_2 and CO_2 to produce methane. Alternatively, acetoclastic methanogens use acetate to produce methane. Acetoclastic methanogens are combined into a single order known as *Methanosarcinales*, which can be subdivided into two principle families (*Methanosarcinaceae* (Msc) and *Methanosaetaceae* (Mst) (Khanal, 2008).

As depicted in Figure 5, initially it appears that the acetoclastic population (Msc) represents the majority of the archaea. Previous research on the lab-scale dairy manure AD from which the inocula was obtained revealed that the Msc dominated therein (Coats et al., 2012); thus, predominance of Msc near the beginning of AD operation was not necessarily

a surprise. Then a shift in the relative archaeal population seems to occur, and the hydrogenotrophs gain dominance. The population shift was likely the result of the maturation of the microbial population with respect to the new and markedly different substrate (WAS, which is living biomass, as contrasted with the inert organic matter present in dairy manure). Regarding the acetoclastic populations, Msc exhibits enhanced reproductive capability as compared to Mst (Speece, 2008). After approximately one month of operation, the Mst population is essentially non-existent where Msc becomes the most prevalent archaea for the remainder of the experiment.

4.4 Anaerobic Digester Performance

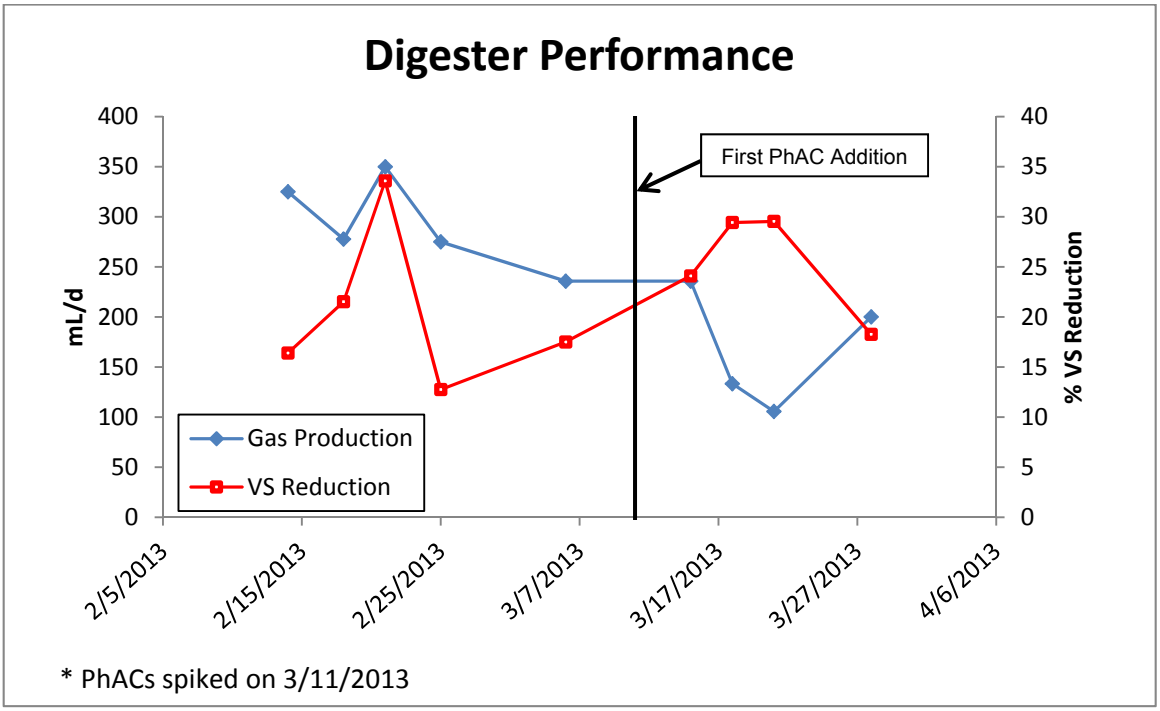


Figure 6: Anaerobic Digester Performance

AD system performance can be evaluated by observing the biogas production and volatile solids (VS) reduction. From a carbon flux perspective, the gas produced is a function of VS reduction. In other words, the more VS removed, the more biogas the digester should produce. For a constant AD feed source, the gas production should also increase as the percent VS reduction increases. Figure XX shows the percent VS reduction and gas

produced over time during the experimental period. It appears that for much of the period, the gas production is inverse to the VS reduction. However, the percent VS reduction would lag behind gas production over time. This is because the VS reduction is a bulk solution parameter that changes slowly whereas gas production is a “real time” parameter with greater sensitivity.

By comparing the gas production from Figure 6 and the methanogenic abundance from Figure 5, it is apparent that the addition of PhACs on March 11 has a significant impact on both. The decreased gas production is likely the direct result of the inhibition of the methanogens from the PhACs. One could speculate that the antimicrobial, ciprofloxacin, was a likely culprit of this observed phenomenon. Researchers have previously observed similar occurrences. One study established a genotoxicity threshold of 0.2-0.4 µg/L for *E.coli* PQ37 (Kümmerer et al., 2000).

5 CONCLUSIONS

Based on the results of the PhAC treatability experiments presented and discussed herein, the following conclusions can be drawn for each of the different processes evaluated.

- **Fermentation (anaerobic, short SRT)**

  The fermentation experiments indicated no ability to remove AT and minimal removal of CP. This contrasts with the results observed for CZ. Based on the mass balance for CZ, reductions in mass were noted for both fermentation experiments. As noted in Chapter 1, this contrasts with most literature that implicates CZ as a recalcitrant compound with typical conventional processes indicating little propensity to remove it. Because there are few anaerobic wastewater treatment plants remaining in service, little attention has been given to evaluating these processes for removal of PhACs. Further research into anaerobic treatment for PhACs deserves more focus.

- **Conventional Activated Sludge (aerobic SBR)**

  As can be seen in Table XX in Chapter 1, considerable research focus has been placed on CAS, particularly at full scale. CAS has been suggested as the primary option to target PhACs for most facilities (Gros et al., 2010; Joss et al., 2005). This research supports that theory, at least with respect to AT and CP. However, increased removal of CZ was observed in anaerobic fermentation reactors as compared to CAS. However, because of the widespread, predominant use of CAS facilities, previous research attention on process and operational optimization of CAS is well focused.

- **Anaerobic Digestion (anaerobic, long SRT)**

The literature review conducted for this research failed to uncover any previous studies focused on AT, CP, or AC treatment in AD. While the results presented for AD in this chapter should be considered preliminary, the observed reduction in mass for all of the analytes suggests it may be a good option for PhAC remediation. With the relatively high concentrations evaluated in this study, AD may also be a good option for the treatment of wastewater containing higher concentrations of PhACs. Sources of higher strength wastewater that could potentially be candidates for AD treatment include pharmaceutical production wastes, hospital wastewater, and membrane filtration centrate. Further research should be conducted focused on these waste streams.

It appears based on the results presented in this chapter and the discussions above regarding real-world PhAC treatment implications that a combination of treatment technologies may provide the greatest effectiveness in mitigating PhAC pollution. While late research has focused on advanced treatment technologies, more focus should be placed on conventional treatment technologies, even those that consider antiquated or out-of-date processes such as anaerobic wastewater treatment.

REFERENCES

A.P. (2011) Lipitor becomes world's top-selling drug, http://www.crainsnewyork.com/article/20111228/HEALTH_CARE/111229902.

Bayer (2013) Product Monograph: Cipro XL.

Belden, J.B., Maul, J.D. and Lydy, M.J. (2007) Partitioning and photodegradation of ciprofloxacin in aqueous systems in the presence of organic matter. Chemosphere 66(8), 1390-1395.

Brain, R.A., Reitsma, T.S., Lissemore, L.I., Bestari, K., Sibley, P.K. and Solomon, K.R. (2006) Herbicidal Effects of Statin Pharmaceuticals in Lemna gibba. Environmental Science & Technology 40(16), 5116-5123.

Carabineiro, S.A.C., Thavorn-Amornsri, T., Pereira, M.F.R. and Figueiredo, J.L. (2011) Adsorption of ciprofloxacin on surface-modified carbon materials. Water Research 45(15), 4583-4591.

Carballa, M., Omil, F., Lema, J.M., Llompart, M.a., García-Jares, C., Rodríguez, I., Gómez, M. and Ternes, T. (2004) Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Research 38(12), 2918-2926.

Carballa, M.O., F.; Lema, J.M.; Ternes, T. (2007) Fate of Pharmaceuticals and Personal Care Products (PPCPs) During Anaerobic Digestion of Sewage Sludge. Water Research 41(May), 2139-2150.

Cardoza, L.A., Knapp, C.W., Larive, C.K., Belden, J.B., Lydy, M. and Graham, D.W. (2005) Factors affecting the fate of ciprofloxacin in aquatic field systems. Water Air and Soil Pollution 161(1-4), 383-398.

Census, U.S.B. (2010) United States Census 2010.

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N. and Kroiss, H. (2005) Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. Water Research 39(19), 4797-4807.

Cleuvers, M. (2003) Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicology Letters 142(3), 185.

Coats, E.R., Watkins, D.L. and Kranenburg, D. (2011) A Comparative Environmental Life-Cycle Analysis for Removing Phosphorus from Wastewater: Biological versus Physical/ Chemical Processes. Water Environment Research (10614303) 83(8), 750-760.

Ding, Y., Zhang, W., Gu, C., Xagoraraki, I. and Li, H. (2011) Determination of pharmaceuticals in biosolids using accelerated solvent extraction and liquid chromatography/tandem mass spectrometry. Journal of Chromatography A 1218(1), 10-16.

Drlica, K. and Zhao, X. (1997) DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiology & Molecular Biology Reviews 61(3), 377.

EPA, U.S. (2007) Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS.

EPA, U.S. (2010) Triclosan Facts, http://www.epa.gov/oppsrrd1/REDs/factsheets/triclosan_fs.htm#summary.

FDA, U.S. (2013) Triclosan: What Consumers Should Know, http://www.fda.gov/forconsumers/consumerupdates/ucm205999.htm.

Fernandez-Fontaina, E., Omil, F., Lema, J.M. and Carballa, M. (2012) Influence of nitrifying conditions on the biodegradation and sorption of emerging micropollutants. Water Research 46(16), 5434-5444.

Ferrer, I., Zweigenbaum, J.A. and Thurman, E.M. (2010) Analysis of 70 Environmental Protection Agency priority pharmaceuticals in water by EPA Method 1694. Journal of Chromatography A 1217(36), 5674-5686.

Golet, E.M., Xifra, I., Siegrist, H., Alder, A.C. and Giger, W. (2003) Environmental Exposure Assessment of Fluoroquinolone Antibacterial Agents from Sewage to Soil. Environmental Science & Technology 37(15), 3243-3249.

Gros, M., Petrović, M., Ginebreda, A. and Barceló, D. (2010) Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environment International 36(1), 15-26.

Halling-Sørensen, B.N.N., S.; Lanzky, P. F.; Ingerslev, F.; Holten Lützhøft, H. C.; Jørgensen, S. E. (1998) Occurrence, fate and effects of pharmaceutical substances in the environment- A review. Chemosphere 36(2), 357-393.

Health, I. (2011) US Pharmaceutical Sales - Q4 2011.

Heberer, T. (2002) Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology Letters 131(1/2), 5.

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M. and Barcelo, D. (2011) Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. Water Research 45(3), 1165-1176.

Joss, A., Keller, E., Alder, A.C., Göbel, A., McArdell, C.S., Ternes, T. and Siegrist, H. (2005) Removal of pharmaceuticals and fragrances in biological wastewater treatment. Water Research 39(14), 3139-3152.

Joss, A., Zabczynski, S., Göbel, A., Hoffmann, B., Löffler, D., McArdell, C.S., Ternes, T.A., Thomsen, A. and Siegrist, H. (2006) Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. Water Research 40(8), 1686-1696.

Karnjanapiboonwong, A., et al. (2011) Occurrence of PPCPs at a Wastewater Treatment Plant and in Soil and Groundwater at a Land Application Site. Water Air Soil Pollution 216, 257-273.

Khanal, S.K. (2008) Anaerobic biotechnology for bioenergy production. Iowa: Wiley-Blackwell. p 179.

Kim, Y., Choi, K., Jung, J., Park, S., Kim, P.-G. and Park, J. (2007) Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea. Environment International 33(3), 370-375.

Kimura, K., Hara, H. and Watanabe, Y. (2005) Removal of pharmaceutical compounds by submerged membrane bioreactors (MBRs). Desalination 178(1–3), 135-140.

Knapp, D.R. (1979) Handbook of Analytical Derivatization Reactions, John Wiley and Sons, New York, NY.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B. and Buxton, H.T. (2002) Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999–2000: A National Reconnaissance. Environmental Science & Technology 36(6), 1202.

Kookana, R.S., G.-G. Ying and N.J. Waller (2011) Triclosan: It's Occurrence, Fate, and Effects in the Australian Environment. Water Science & Technology 63.4, 598-604.

Kosjek, T., Heath, E., Petrović, M. and Barceló, D. (2007) Mass spectrometry for identifying pharmaceutical biotransformation products in the environment. Trends in Analytical Chemistry: TRAC 26(11), 1076-1085.

Kümmerer, K., Al-Ahmad, A. and Mersch-Sundermann, V. (2000) Biodegradability of some antibiotics, elimination of the genotoxicity and affection of wastewater bacteria in a simple test. Chemosphere 40(7), 701-710.

Lajeunesse, A., Smyth, S.A., Barclay, K., Sauvé, S. and Gagnon, C. (2012) Distribution of antidepressant residues in wastewater and biosolids following different treatment processes by municipal wastewater treatment plants in Canada. Water Research 46(17), 5600-5612.

Langford, K. and Thomas, K.V. (2011) Input of selected human pharmaceutical metabolites into the Norwegian aquatic environment. Journal of Environmental Monitoring 13, 416-421.

Lee, H.-B., Peart, T.E., Lewina Svoboda, M. and Backus, S. (2009) Occurrence and fate of rosuvastatin, rosuvastatin lactone, and atorvastatin in Canadian sewage and surface water samples. Chemosphere 77(10), 1285-1291.

Li, W., Nanaboina, V., Zhou, Q. and Korshin, G.V. (2012) Effects of Fenton treatment on the properties of effluent organic matter and their relationships with the degradation of pharmaceuticals and personal care products. Water Research 46(2), 403-412.

Malysheva, S., Diana Di Mavungu, J., Goryacheva, I. and Saeger, S. (2013) A systematic assessment of the variability of matrix effects in LC-MS/MS analysis of ergot alkaloids in cereals and evaluation of method robustness. Analytical & Bioanalytical Chemistry 405(16), 5595-5604.

Martucci, A., Pasti, L., Marchetti, N., Cavazzini, A., Dondi, F. and Alberti, A. (2012) Adsorption of pharmaceuticals from aqueous solutions on synthetic zeolites. Microporous & Mesoporous Materials 148(1), 174-183.

Metcalfe and Eddy (2003) Wastewater Engineering Treatment and Reuse.

Muz, M., Ak, M.S., Komesli, O.T. and Gökçay, C.F. (2013) An ozone assisted process for treatment of EDC's in biological sludge. Chemical Engineering Journal 217, 273-280.

Ng, K., Lin, A., Yu, T. and Lin, C. (2011) Tertiary Treatment of Pharmaceuticals and Personal Care Products by Pretreatment and Membrane Processes. Sustain. Environ. Res. 21(3), 173-180.

Nirogi, R.V.S., Kandikere, V.N., Shukla, M., Mudigonda, K., Maurya, S., Boosi, R. and Anjaneyulu, Y. (2006) Simultaneous quantification of atorvastatin and active metabolites in human plasma by liquid chromatography–tandem mass spectrometry using rosuvastatin as internal standard. Biomedical Chromatography 20(9), 924-936.

Nopens, I., Capalozza, C. and Vanrolleghem, P.A. (2001) Stability analysis of a synthetic municipal wastewater, Universiteit Gent, Department of Applied Mathematics, Biometrics and Process Control.

Novartis (2014) Tegretol Consumer Information.

Ottmar, K., Colosi, L. and Smith, J. (2010) Development and Application of a Model to Estimate Wastewater Treatment Plant Prescription Pharmaceutical Influent Loadings and Concentrations. Bulletin of Environmental Contamination and Toxicology 84(5), 507-512.

Petrović, M., Gonzalez, S. and Barceló, D. (2003) Analysis and removal of emerging contaminants in wastewater and drinking water. TrAC Trends in Analytical Chemistry 22(10), 685-696.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9), e45.

Reiss, R., Lewis, G. and Griffin, J. (2009) AN ECOLOGICAL RISK ASSESSMENT FOR TRICLOSAN IN THE TERRESTRIAL ENVIRONMENT. Environmental Toxicology & Chemistry 28(7), 1546-1556.

Salgado-Petinal, C., Llompart, M., García-Jares, C., García-Chao, M. and Cela, R. (2006) Simple approach for the determination of brominated flame retardants in environmental solid samples based on solvent extraction and solid-phase microextraction followed by gas chromatography–tandem mass spectrometry. Journal of Chromatography A 1124(1/2), 139-147.

Schwab, B.W., Hayes, E.P., Fiori, J.M., Mastrocco, F.J., Roden, N.M., Cragin, D., Meyerhoff, R.D., D'Aco, V.J. and Anderson, P.D. (2005) Human pharmaceuticals in US surface waters: A human health risk assessment. Regulatory Toxicology and Pharmacology 42(3), 296-312.

Sirtori, C., Zapata, A., Oller, I., Gernjak, W., Agüera, A. and Malato, S. (2009) Decontamination industrial pharmaceutical wastewater by combining solar photo-Fenton and biological treatment. Water Research 43(3), 661-668.

Speece, R.E. (2008) Anaerobic biotechnology and odor/corrosion control for municipalities and industries, Archae Press, Nashville, Tenn.

Supelco (1998) Guide to Solid Phase Extraction, Sigma-Aldrich.

Tarcomnicu, I., van Nuijs, A.L.N., Simons, W., Bervoets, L., Blust, R., Jorens, P.G., Neels, H. and Covaci, A. (2011) Simultaneous determination of 15 top-prescribed pharmaceuticals and their metabolites in influent wastewater by reversed-phase liquid chromatography coupled to tandem mass spectrometry. Talanta 83(3), 795-803.

Ternes, T., Joss, A., Kreuzinger, N., Miksch, K., Lema, J., von Gunten, U., McArdell, C. and Siegrist, H. (2005) Removal of pharmaceuticals and personal care products: Results of the Poseidon Project, pp. 227-243, Washington, DC.

Ternes, T.A. (1998) Occurrence of drugs in German sewage treatment plants and rivers. Water Research 32(11), 3245-3260.

Van Doorslaer, X., Demeestere, K., Heynderickx, P.M., Van Langenhove, H. and Dewulf, J. (2011) UV-A and UV-C induced photolytic and photocatalytic degradation of aqueous

ciprofloxacin and moxifloxacin: Reaction kinetics and role of adsorption. Applied Catalysis B: Environmental 101(3/4), 540-547.

Verscheren, K. (2008) Handbook of Environmental Data on Organic Chemicals, John Wiley and Sons, New York, NY.

Waters, C. (2010-2011) Waters Quality Parts, Chromatography Columns, and Supplies Catalog. Corporation, W. (ed), Canada.

Yang, X., Flowers, R.C., Weinberg, H.S. and Singer, P.C. (2011) Occurrence and removal of pharmaceuticals and personal care products (PPCPs) in an advanced wastewater reclamation plant. Water Research 45(16), 5218-5228.

Yu, J., Bisceglia, K., Bouwer, E., Roberts, A. and Coelhan, M. (2012) Determination of pharmaceuticals and antiseptics in water by solid-phase extraction and gas chromatography/mass spectrometry: analysis via pentafluorobenzylation and stable isotope dilution. Analytical & Bioanalytical Chemistry 403(2), 583-591.

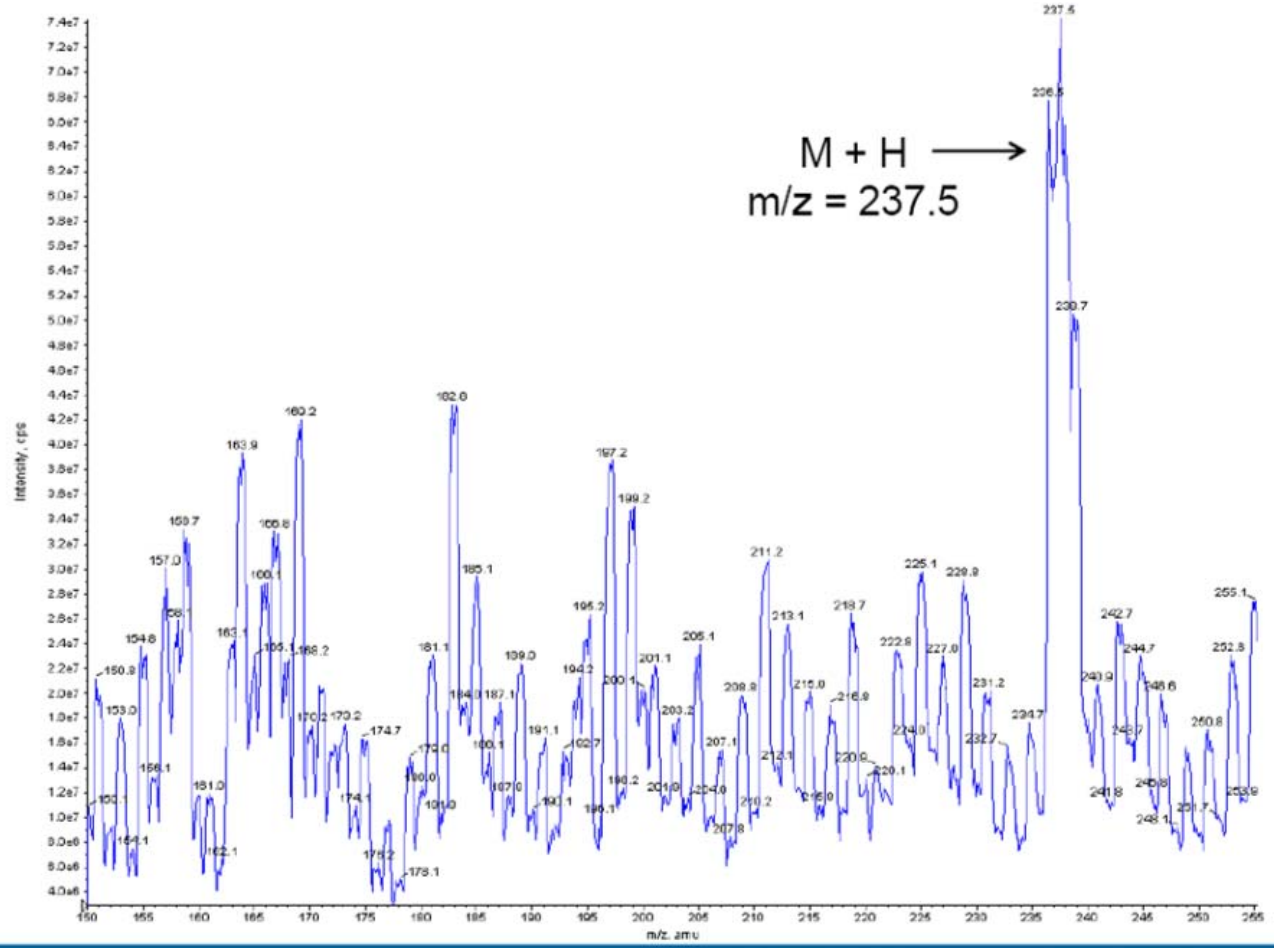
Yu, Y., Lee, C., Kim, J. and Hwang, S. (2005) Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnology and Bioengineering 89(6), 670-679.

Yuan, S., Jiang, X., Xia, X., Zhang, H. and Zheng, S. (2013) Detection, occurrence and fate of 22 psychiatric pharmaceuticals in psychiatric hospital and municipal wastewater treatment plants in Beijing, China. Chemosphere 90(10), 2520-2525.

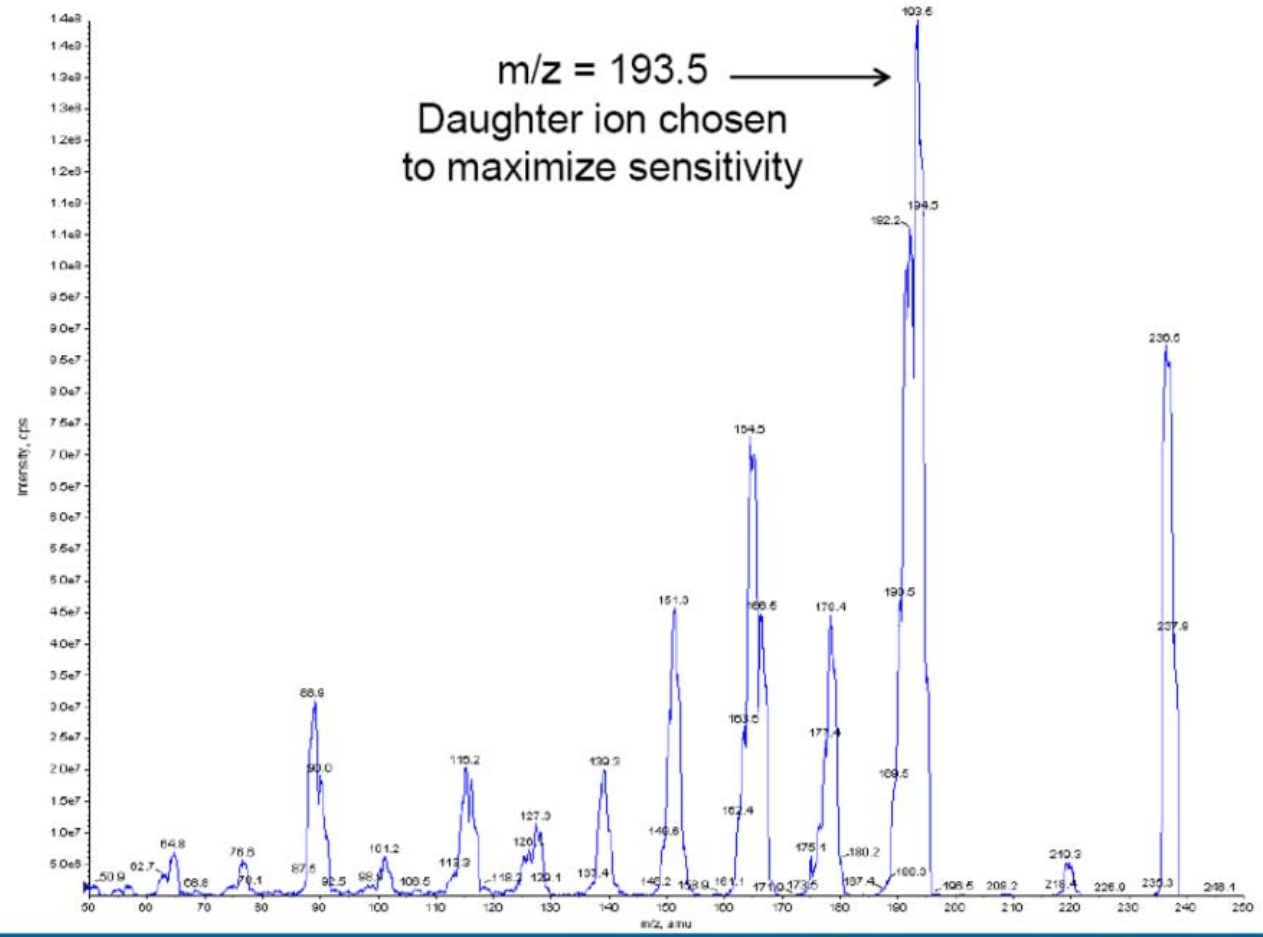
APPENDIX

SPE-MS/MS Spectra

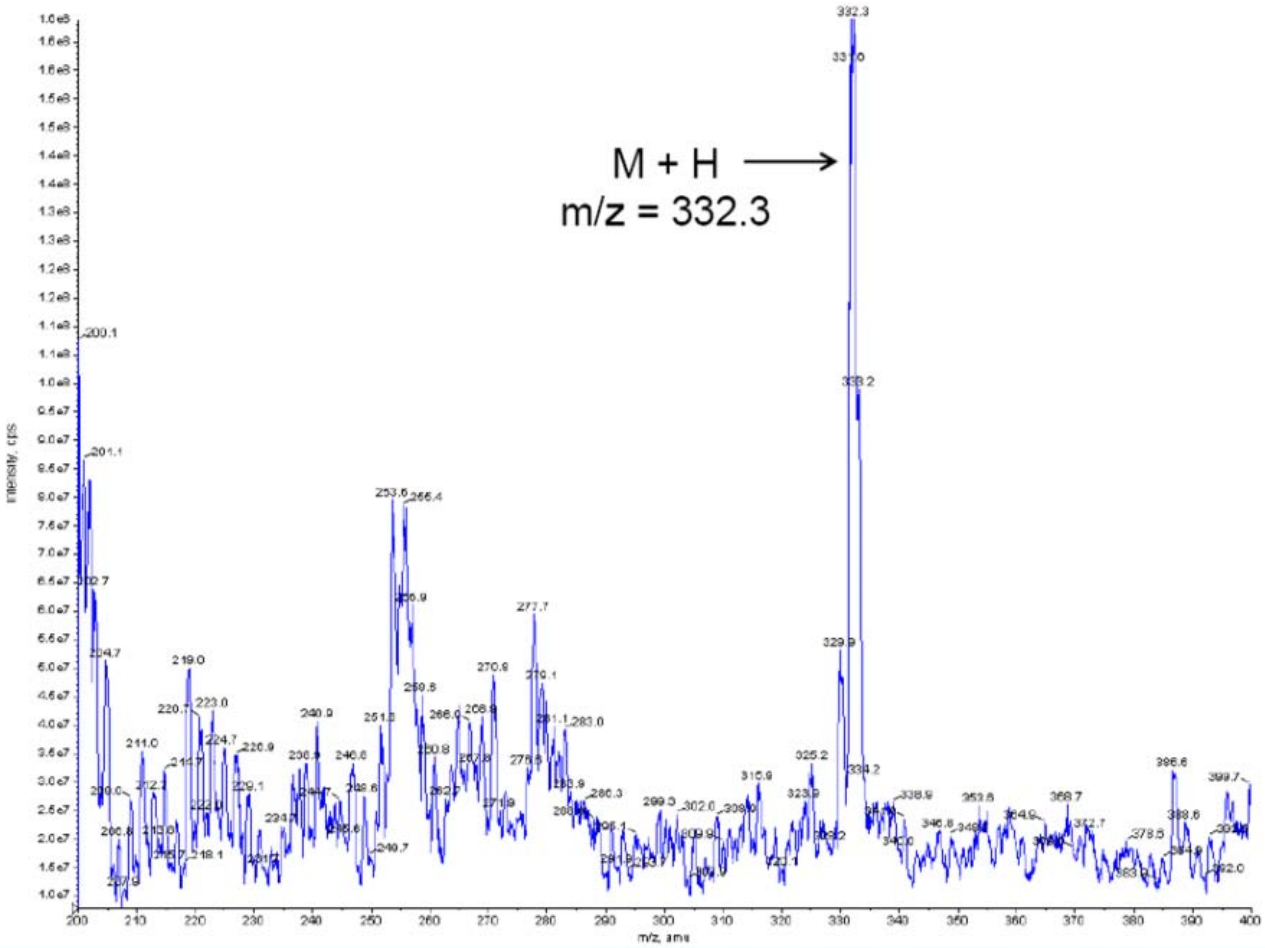
Carbamazepine Parent Ion (Q1) Scan



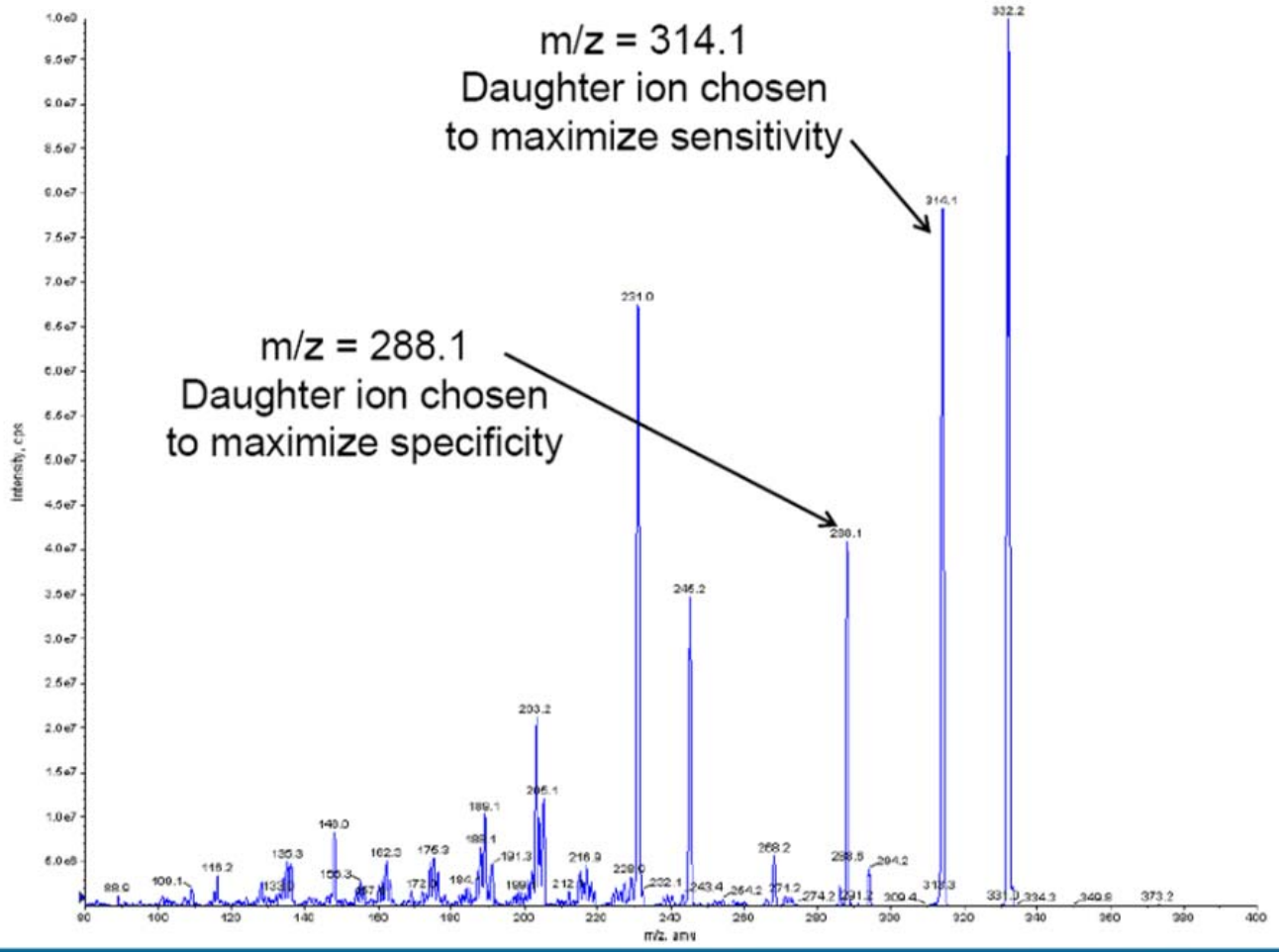
Carbamazepine Daughter Ion (Q3) Scan



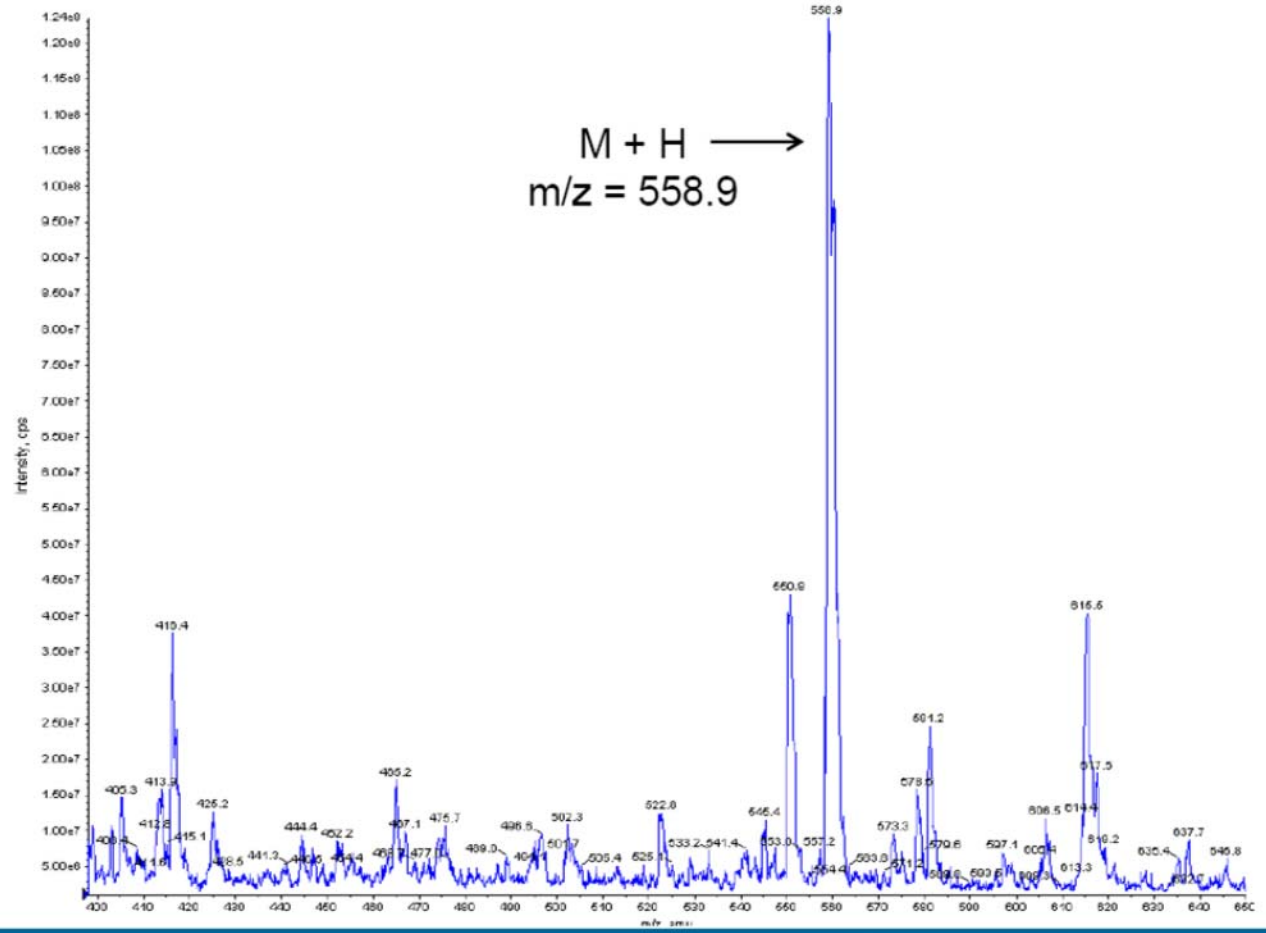
Ciprofloxacin Parent Ion (Q1) Scan



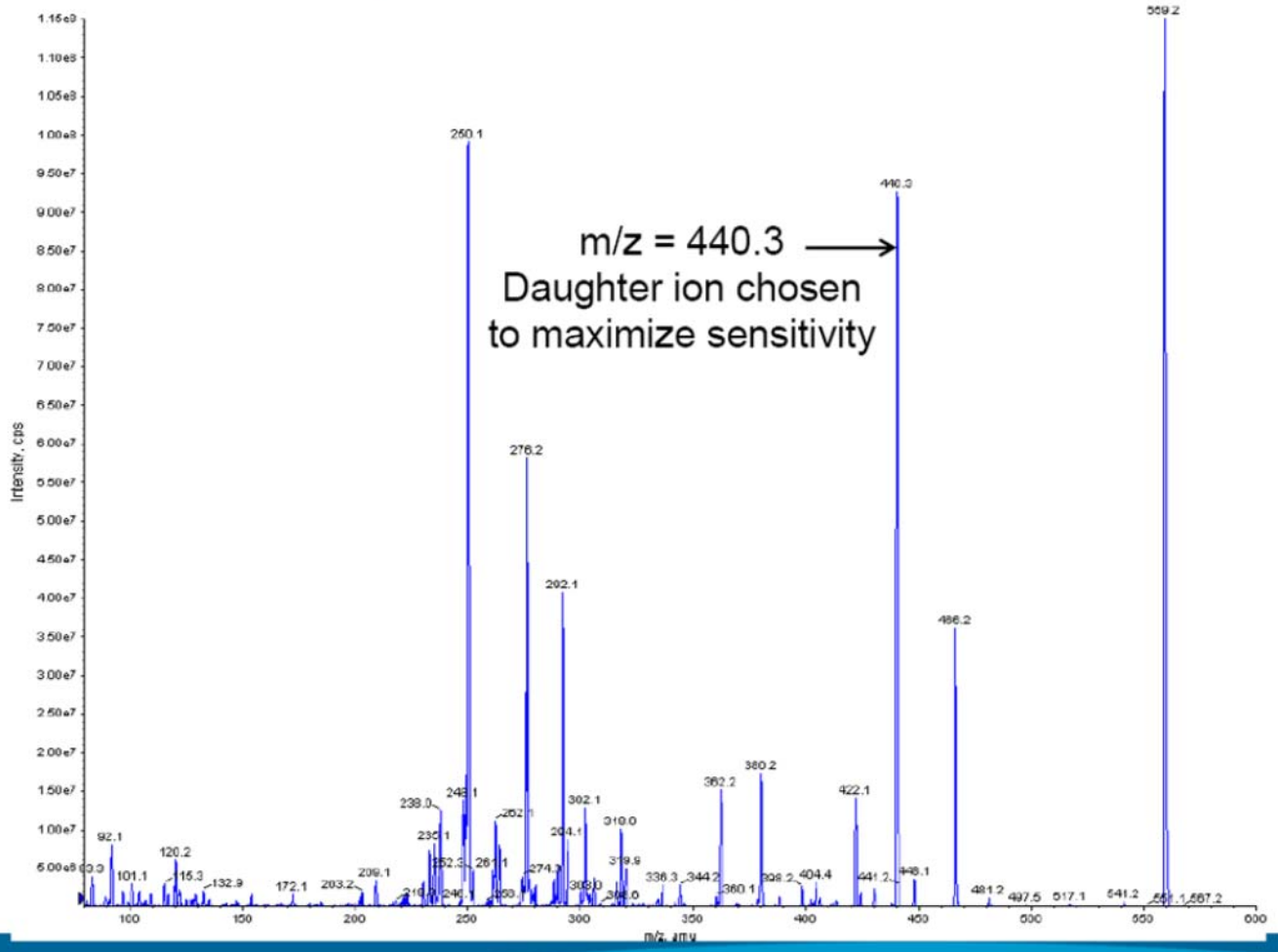
Ciprofloxacin Daughter Ion (Q3) Scan



Atorvastatin Parent Ion (Q1) Scan



Atorvastatin Daughter Ion (Q3) Scan



Agilent Technologies