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### Publication Date

2016

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UNIVERSITY OF CALIFORNIA  
RIVERSIDE

Enantiomer Specific Fate and Toxicity of Chiral Pharmaceuticals in the Environment

A Dissertation submitted in partial satisfaction  
of the requirements for the degree of

Doctor of Philosophy

in

Environmental Toxicology

by

Edmond Sanganyado

June 2016

Dissertation Committee:

Dr. Jay Gan, Chairperson

Dr. Daniel Schlenk

Dr. Haizhou Liu

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The Dissertation of Edmond Sanganyado is approved:

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Committee Chairperson

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## ACKNOWLEDGEMENTS

I would like to offer my sincere gratitude to Dr. Jay Gan, my advisor, who gave me valuable assistance, support and guidance throughout the duration of my study as I pursued my research and career goals. I would like to thank Dr. Daniel Schlenk and Dr. Haizhou Liu for serving as my dissertation committee member, respectively. I am greatly indebted to Dr. Michael Anderson and Dr. Sharon Walker who served as my qualifying examination committee members. I will forever be grateful for their wise counsel and critic that helped the early development of my research project.

I would like to thank the United States Department of State, Bureau of Educational and Cultural Affairs who provided the funding for my studies through the Fulbright Program.

I would also like to thank *Journal of Chromatography A* and *Environmental Pollution* for publishing my research and permit me to use them in this dissertation. Chapter 2 is reproduced with permission from Sanganyado, E., Lu, Z., and Gan, J. (2014), Mechanistic insights on chaotropic interactions of liophilic ions with basic pharmaceuticals in polar ionic mode liquid chromatography, *Journal of Chromatography A* 1368: 82-88. Chapter 3 is reproduced with permission from Sanganyado, E., Fu, Q., and Gan, J. (2016), Enantiomeric selectivity in adsorption of chiral  $\beta$ -blockers on sludge, *Environmental Pollution* 214: 787-794. I would like to thank Environmental Toxicology and Chemistry for permission to reproduce figure 5-3 from Fabbri, E., and Franzellitti, S. (2016), Human

pharmaceuticals in the marine environment: Focus on exposure and biological effects in animal species, *Environmental Toxicology and Chemistry* 35: 799–812.

I had the honor of interacting with the most brilliant people daily as lab mates who helped me to become a better researcher. I would like to thank my previous and current lab members, particularly Dr. Fred Ernest, Dr. Weiying Jiang, Dr. Sherry Wu, Dr. Jeremy Conkle, Dr. Fang Jia, Dr. Zhijiang Lu, Dr. Laurel Dodgen, Jaben Richards, and Qiuguo Fu, for their vital assistance and companionships in the past five years.

Finally, I would like to thank my wife, Surprise and *my* mother, Wendy Bagley-Smithy for their unconditional support and care.

## ABSTRACT OF THE DISSERTATION

Enantiomer Specific Fate and Toxicity of Chiral Pharmaceuticals in the Environment

by

Edmond Sanganyado

Doctor of Philosophy, Graduate Program in Environmental Toxicology  
University of California, Riverside, June 2016  
Dr. Jay Gan, Chairperson

Pharmaceuticals are contaminants of emerging concern because they are designed to elicit biological responses at low doses. Antidepressants and  $\beta$ -blockers, which are largely chiral compounds, are the most frequently and abundantly detected pharmaceuticals in the environment. Enantiomers of chiral pharmaceuticals behave differently in biological systems. However, most studies on occurrence, fate and toxicity overlook chirality. There is an inadequate understanding of mechanisms of stereoselectivity in adsorption, biodegradation and environmental toxicity of chiral pharmaceuticals.

We determined the mechanism of chiral separation of atenolol and fluoxetine in a macrocyclic glycopeptide based column using different types of liophilic ions as mobile phase additives. Liophilic ions affected the retention factor, and the magnitude of the effect depended on their hydrophobicity:  $\text{CH}_3\text{COO}^- > \text{HCOO}^- > \text{NO}_3^-$ . The enantioresolution decreased when the concentration of liophilic ions was increased from 4 to 20 mM suggesting analyte retention was predominantly due to dynamic ion exchange.

We systematically studied the effect of chirality on the adsorption of  $\beta$ -blockers to wastewater sludge. The (*S*)-enantiomers of acebutolol and metoprolol had  $K_d$  values approximately twice that of the (*R*)-enantiomers, that is,  $109 \pm 11$  and  $57 \pm 8$  L/kg compared to  $52 \pm 13$  and  $22 \pm 8$  L/kg, respectively. The relatively more hydrophobic compounds, pindolol and propranolol did not exhibit stereoselectivity toward adsorption. Our results suggest that ionic interactions and hydrogen bonding contribute significantly to stereoselectivity in adsorption of the tested compounds.

We comprehensively investigated stereoselectivity in the biodegradation of  $\beta$ -blockers in wastewater treatment plants. The degradation of 5  $\beta$ -blockers in wastewater microcosms was also investigated. We observed *S*-enrichment in the degradation of metoprolol, pindolol and propranolol with the EF value changing from 0.5 to  $0.30 \pm 0.01$ ,  $0.37 \pm 0.0$  and  $0.32 \pm 0.05$ , respectively. The results from the wastewater microcosm parameterized the Rayleigh equation offering valuable quantitative assessment data that can be used to improve the accuracy of the environmental risk assessment of chiral pharmaceuticals.

The applicability of the read-across hypothesis in predicting stereoselective toxicity of 11 pharmaceuticals to aquatic organisms was performed using the Fish Plasma Model. We found metoprolol had high risk because its effect ratio, ER (ratio of human therapeutic plasma concentration to fish plasma concentration at steady state) was less than 1.0, whereas propranolol, salbutamol, fluoxetine and venlafaxine had medium risk ( $1.0 < ER < 30$ ). Stereoselectivity was observed in all compounds except atenolol and pindolol. We



showed that the read-across hypothesis is a useful tool for predicting stereoselective toxicity of chiral pharmaceuticals.

The results suggest stereoselectivity is prevalent in wastewater and the environment, and that pharmacological data can be used in predicting enantiomeric differences in the aquatic toxicity of chiral pharmaceuticals.

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## Chapter 1 Introduction

### 1.1 Pharmaceuticals in the Environment

Pharmaceuticals are frequently detected in the aquatic environment at concentrations ranging from ng/L to µg/L. Pharmaceuticals are biologically active compounds designed to cause a biological change at a low dose, and thus they pose a potential toxicological risk to non-target organisms after emission into the environment. Studies have shown that drugs such as propranolol, fluoxetine, and carbamazepine induce chronic toxicity in aquatic organisms at trace levels (Ankley et al., 2010; Brooks et al., 2003; Huggett et al., 2003). In a recent study, Fong et al. observed that the feet of snails (*L. americanum*) became detached following exposure to venlafaxine or fluoxetine (Fong et al., 2015). Therefore, a thorough understanding of factors influencing the fate, transport and non-target risks of pharmaceuticals in the environment is imperative.

Pharmaceuticals contain many diverse classes of compounds with a broad spectrum of physicochemical properties. Municipal wastewater treatment plants often serve as the initial collection point for these compounds, from which many of these compounds may be emitted into the natural environment via effluent discharges (Kümmerer, 2009). Although numerous studies have shown the ubiquitous occurrence of pharmaceuticals in wastewater treatment systems and in the impacted surface water, a largely overlooked aspect is that more than 50% of pharmaceuticals are chiral compounds with at least one stereogenic center (MacLeod and Wong, 2010). Accurate risk assessment of

pharmaceuticals in the environment therefore must consider the enantioselective fate and ecotoxicity of chiral pharmaceuticals.

A number of studies under field and laboratory conditions showed that degradation of chiral pharmaceuticals in wastewater and surface water was stereoselective (Caballo et al., 2015; Fono and Sedlak, 2005; Ribeiro et al., 2013). Enantiomers of chiral pharmaceuticals often exhibit different pharmacological and toxicological activities in biological systems (Kasprzyk-Hordern, 2010; Ribeiro et al., 2012; Wong, 2006). For example, in the late 1950s, the chirality of thalidomide, a drug for treating morning sickness, was ignored, resulting in the birth of over 10,000 children with birth defects (Ribeiro et al., 2012; Smith, 2009). Subsequent research uncovered that only R-thalidomide was therapeutic, while S-thalidomide was teratogenic. Therefore, influences of chirality should be carefully characterized in the environmental risk assessment of chiral pharmaceuticals. Although increasing in recent years, the published studies on chiral pharmaceuticals as environmental contaminants are rather scattered. The purpose of this review is to summarize progress in this area and identify knowledge gaps for future research.

### 1.1.1 Terminology

Chiral compounds are molecules with one or more stereogenic centers that have identical chemical structures, but different spatial arrangements of the atoms (Ribeiro et al., 2012). Enantiomers are non-superimposable stereoisomeric pairs of chiral compounds, while diastereomers are stereoisomers that are not mirror images (Figure 1-1). Enantiomers

are commonly identified using the R/S system where Cahn–Ingold–Prelog rules are used to prioritize substituent groups around the stereogenic center. Alternatively, enantiomers are identified according to the direction with which they rotate polarized light, with (+) rotating the polarized light clockwise and (–) anticlockwise. A racemate does not rotate polarized light, as it contains an equimolar mixture of the enantiomers.

### 1.1.2 Chirality in Biological Systems

Enantiomers usually engage in stereospecific interactions in chiral environments such as biological systems due to their three dimensional structures that result in stereoselective binding, catalysis, and stabilization (Wong and Warner, 2010; Wong, 2006). When an enantiomer has high affinity for the receptor, it is called an eutomer, while a distomer has less affinity (Ariens et al., 1988; Ariëns, 1984). Stoschitzky and coworkers (Stoschitzky et al., 1993) showed (*S*)-atenolol was the eutomer since it contributed most of the  $\beta$ -adrenergic antagonism whereas (*R*)-atenolol was the distomer. However, eutomer-distomer classification is not always straightforward. For example, *trans*-tramadol is an analgesic comprised a racemic mixture of (*R,R*)-(+)-tramadol and (*S,S*)-(–)-tramadol. The (+)-enantiomer inhibited serotonin reuptake due to a higher affinity for the  $\mu$ -receptor, but the (–)-enantiomer inhibited noradrenalin reuptake (Ardakani et al., 2008).

Enantiomers can have significantly different non-target toxicity in a chiral environment (Stanley et al., 2007). For example, in sublethal standardized and behavioral endpoints studies, (*S*)-fluoxetine was found to be more toxic than (*R*)-fluoxetine to fathead minnow (*P. promelas*) (Stanley et al., 2007). However, most published studies on

ecotoxicity of chiral pharmaceuticals ignored the enantiospecific nature of toxicity, with the underlying assumption that enantiomers have identical biological activity. The aquatic toxicity of chiral pharmaceuticals is often determined using the racemate. Therefore, there is an urgent need to obtain enantiospecific ecotoxicological data for chiral pharmaceuticals by considering their chirality.

### 1.1.3 Chiral Pharmaceuticals as a Regulatory Dilemma

The rapid development of chiral separation methods for chiral pharmaceuticals has established that enantiomers often have different biological properties. Ariëns et al. (1986) urged that “health authorities should be fully aware of the fact that acceptance of racemates implies acceptance of 50 % or more of possibly harmful pollutants for the milieu interne of patients and the environment in general”. The regulatory authorities in the United States, Europe, Japan and China recommended that pharmaceutical companies should try to separate enantiomers, evaluate the therapeutic activity of each enantiomer, and if possible, only market the eutomer (Agranat et al., 2002; Shindo and Caldwell, 1995; U.S. Food and Drug Administration, 1992). Consequently, there has been a decline in the number of racemic drugs approved by the U.S. Food and Drug Administration over the recent years (Figure 1-2). For instance, between 2000 and 2015, only 4 new drugs approved by FDA were racemic mixtures, as compared to 91 single enantiomers. However, despite the current trends in developing and marketing single enantiomers, there has not been the corresponding interest in elucidating the implications of chirality in environmental risk of pharmaceuticals. Daughton (2015) attributed the dilemma in regulating chiral pollutants in



the environment to challenges in stereoselective analysis. However, as discussed in the next sections, during the past three decades has seen significant advancement in development of chiral separation methods. Another potential reason is the fact that there are thousands of chiral pharmaceuticals, and it is infeasible to carry out a comprehensive risk assessment for this large number of compounds. Hence, there is a need for developing a tool for prioritizing chiral pharmaceuticals so that their environmental risks may be correctly evaluated.

## 1.2 Separation and Quantification of Chiral Pharmaceuticals

Enantioseparation and detection of chiral pharmaceuticals is problematic in an achiral environment since pairs of enantiomers have similar physicochemical properties (Evans and Kasprzyk-Hordern, 2014; Hashim and Khan, 2011; Pérez and Barceló, 2008). The goal of chiral separation and detection in environmental analysis is to 1) establish the chiral configuration of the enantiomers, 2) determine the enantiomeric composition of a compound, and 3) evaluate the concentration of the enantiomers in the environmental compartment under study. The separation, identification and quantification of enantiomers in environmental monitoring depend on the ability of the analytical technique to attain baseline separation consistently. Therefore, besides the limit of detection, limit of quantification, recovery, reproducibility, and method bias, enantioresolution ( $R_S$ ) (Equation 1-1) and enantioselectivity ( $\alpha$ ) (Equation 1-2) are the most critical parameters considered in the development and optimization of chiral analytical methods:

$$R_S = 2 \times \frac{t_2 - t_1}{w_1 + w_2} \quad (1-1)$$

$$\alpha = \frac{(t_2 - t_0)}{(t_2 - t_0)} \quad (2-2)$$

where  $t_1$  and  $t_2$  are the retention times of the first and second eluted enantiomers, respectively,  $t_0$  is the column void time found by recording the first baseline perturbation and  $w_1$  and  $w_2$  are the base peak widths of the first and second eluted enantiomers, respectively. In environmental monitoring, an  $R_s$  value greater than 1.5 is often preferred, since the enantiomers under such conditions have essentially baseline separation.

Enantiomeric fraction (EF) (Equation 3-3) is one of the most important parameters in chiral analysis as it determines the enantiomeric composition of the chiral compound in a sample:

$$EF = \frac{(R)}{(R)+(S)} \quad (3-3)$$

where (R) and (S) are the amount of R and S enantiomers, respectively. If the stereoconfiguration is not known, (+) and (-) or *E1* and *E2* may be used instead. The EF value of a racemate is 0.5 and for a single enantiomer, it is 1.0.

### 1.2.1 Polarity of Mobile Phase

In chiral selective liquid chromatography, enantioseparation can be achieved in both normal phase, where hydrophobic analytes are separated using a mobile phase comprised of a mixture of alkanes and alcohols such as hexane-methanol, hexane-ethanol, hexane-2-propanol and heptane-ethanol, and reversed phase, where analytes are separated using polar solvents such as water and methanol (Ali et al., 2009a; Tang, 1996; Younes et al., 2011).

Although enantioseparation of chiral pharmaceuticals in normal phase liquid chromatography offers high selectivity, sensitivity, and sample recovery in preparative studies, it is not compatible with Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) in liquid chromatography with a mass spectrometer (LC-MS) systems when the flow rate is high due to the risk of explosion (Nie et al., 2013). Unfortunately, attempts to avert the explosion hazard such as lowering the nebulizer temperature or increasing the fraction of polar organic solvent may often result in poor sensitivity (Nie et al., 2013). Besides a better compatibility to mass spectrometer detectors, reversed phase liquid chromatography has an additional advantage in that there are many commercially available chiral columns for reversed phase, as compared to normal phase analysis. Therefore, the reversed phase mode is generally preferred over the normal phase when analytes are separated and detected using LC-MS systems.

### 1.2.2 Direct and Indirect Separation Methods

Enantiomer pairs may be separated by indirect or direct methods. Indirect methods involve formation of diastereomers by derivatization using chiral reagents. The diastereomers are then separated on conventional columns. Furthermore, derivatization is a useful technique in gas chromatography when the target analytes are less volatile or in liquid chromatography when the functional groups close to the chiral center offer limited interactions with the stationary phase. Chiral analysis of profens, a group of non-steroidal anti-inflammatory drugs (NSAIDs) including ibuprofen, ketoprofen and naproxen by gas chromatography involved derivatization with chiral reagents such as R-1-

phenylethylamine (Hashim and Khan, 2011; Hashim et al., 2011; Khan et al., 2013). However, since derivatization is time-consuming, requires the enantiomer to possess a group that can be derivatized, and can cause racemization and formation of reactive by-products, direct methods are the preferred approach in chiral analysis of environmental samples (Cavazzini et al., 2011).

### 1.2.3 Chiral Columns

In direct enantioseparation, the chiral analytes undergo stereospecific interactions to form diastereoisomeric complexes with either the chiral stationary phase (CSP) or a chiral selector as a mobile phase additive (Hashim and Khan, 2011; Ribeiro et al., 2012). Since the selector and CSP are chiral, their interactions with the enantiomers are thus stereospecific. Chiral selectors are expensive and often not compatible with mass spectrometers; hence, CSPs are often preferred in the analysis of environmental samples for chiral contaminants. The mechanism of chiral recognition between the enantiomers and the chiral selector or CSP is often explained by the three-point model (Figure 1-3). Due to the stereoconfiguration of the CSP, three functional groups on only one enantiomer of the analyte undergo molecular interactions with three sites on the CSP to form a diastereomeric complex, resulting in the slower elution of that enantiomer due to a higher affinity for the CSP (Berthod, 2006; Davankov, 1997; Evans and Kasprzyk-Hordern, 2014). This model was developed when the main molecular interaction is attraction, but repulsion sometimes is also important in chiral recognition. The three point model is not applicable when chiral recognition is due to an analyte undergoing steric fitting in a cleft on the CSP or when the

analyte interacts with the CSP involving four configuration dependent points (Berthod, 2009, 2006; Berthod et al., 2010). However, the three-point model is normally adequate at explaining stereoselectivity in separation because of its simplicity. Since the development of a commercial CSP by Pirkle et al. in 1981, several materials have been employed as CSPs (Fernandes et al., 2013; Pérez and Barceló, 2007; Pirkle et al., 1981). The most commonly used CSPs in the environmental analysis of chiral pharmaceuticals are polysaccharides (Ali et al., 2009a; Bagnall et al., 2012; Barclay et al., 2012; Mskhiladze et al., 2013), proteins (Ardakani et al., 2008; Barclay et al., 2012, 2011), and macrocyclic antibiotics (Aboul-Enein and Ali, 2002; Bagnall et al., 2012; Barclay et al., 2012; MacLeod et al., 2007; Ribeiro et al., 2013).

Since the development of the first macrocyclic glycopeptide based CSP by Armstrong et al. (1994) over two decades ago, macrocyclic glycopeptide, particularly vancomycin and teicoplanin, have been exploited in the determination of occurrence and fate of chiral pharmaceuticals in the environment (Bagnall et al., 2012; MacLeod et al., 2007; Ribeiro et al., 2014; Barclay et al., 2012; Ribeiro et al., 2013, 2012). For example, Wong and coworkers employed reversed phase liquid chromatography coupled to a tandem mass spectrometer in their investigation of chiral pharmaceuticals in wastewater and surface water (MacLeod and Wong, 2010; MacLeod et al., 2007; Nikolai et al., 2006). The mobile phase was comprised of methanol, water and 0.1% triethylammonium acetate, with pH adjusted using acetic acid. However, since the majority of chiral pharmaceuticals are ionizable, enantioseparation is often achieved in the polar ionic mode (Sanganyado et al., 2014). Several researchers have employed the polar ionic mode in determination of

enantiomeric compositions in environmental matrices where the mobile phase consisted of an organic solvent and mobile phase additives only. For example, Bagnall et al. (2012, 2013) measured the enantiomeric compositions of illicit and prescription drugs in rivers using 4 mM ammonium acetate in methanol and 0.005% formic acid. However, the mechanism of chiral separation of pharmaceuticals in the polar ionic mode is not well understood. There is a need for a systematic study to establish the chiral separation mechanisms so that the benefits of different chiral stationary phases can be fully realized.

#### 1.2.4 Importance of Stereochemical Configuration for Risk Assessment

Stereochemical configurations are critical for understanding the enantioselective distribution, fate and toxicity of chiral pharmaceuticals in the environment. Most of the chiral pharmaceuticals studied to date are commercially available only as racemic mixtures. Therefore, it is important to characterize the stereoconfiguration of the eluted stereoisomers before reporting enantiomer-specific findings. Identifying enantiomers using their order of elution is sufficient in revealing the phenomenon of enantioselectivity in the environment, but inadequate for environmental risk assessment. Several researchers reported that enantiomers may undergo reversal in the order of elution during chromatographic analysis on polysaccharide or macrocyclic glycopeptide-based columns. For example, Gyllenhaal and Stefansson (2008) observed solvent-induced reversal of the enantiomer elution order following changing the organic modifier from 2-propanol to methanol/ethanol (2:1, v/v). The elution order of enantiomers is influenced by the types of stationary phases, temperature, pH, the amount of analyte injected onto the column, mobile

phase additives, and mobile phase modifiers (Okamoto, 2002; Xiang et al., 2011). Moreover, researchers have also used many different methods of chiral separation and different columns in the analysis of chiral pharmaceuticals. Without the knowledge of the absolute configuration, it is difficult to compare results from different studies. Therefore, identifying enantiomers using the order of elution is not sufficient for promoting further research, identifying trends through meta-analysis of available data, or applying the data for environmental risk assessment.

Since the announcement of the FDA recommendation on drug stereochemistry, the number of studies on absolute configuration of pharmaceuticals increased (He et al., 2011). For example, researchers established the absolute configuration of oxadiazol-3-one, a compound that had myocardial calcium entry channel blocking activity higher than diltiazem, using vibrational circular dichroism and optical rotation (Stephens et al., 2007). Specifically, the absolute configuration of oxadiazol-3-one was obtained by comparing vibrational and electronic circular dichroism spectra, and optical rotation obtained from density functional theory calculations to their experimental data (Stephens et al., 2007). Stephens et al (2007) found the enantiomers were (*S*)-(+)- oxadiazol-3-one and (*R*)-(-)- oxadiazol-3-one. Chiral molecules reflect, absorb, refract and scatter polarized light in different ways. Chiroptical techniques measure these variations to establish the absolute configuration of an enantiomer. Vibrational optical activity is the most widely used chiroptical technique in pharmaceutical analysis, and it involves spectroscopic determination of their differences in response to left and right circularly polarized light by vibrating molecules (He et al., 2011). In vibrational circular dichroism, the incident

polarized light is infrared and the enantiomer pairs produce spectra that are mirror images yet equal in intensity. The absolute configuration of the enantiomers is then determined by comparing the experimental and the predicted vibrational circular dichroism spectrum. Although sample preparation in vibrational circular dichroism is easy and does not require derivatization, it requires a relatively large sample between 5 to 10 mg and is only applicable for small and rigid samples (McConnell et al., 2014). However, X-ray crystallography only requires a single crystal and offers absolute configurations of higher confidence. In X-ray crystallography, the absolute configuration is established from the differences in resonant scattering of X-ray by a crystal formed by co-crystallization of the enantiomer and a reference chiral molecule (Flack and Bernardinelli, 2008). Nuclear magnetic resonance is sometimes used in establishing the absolute configuration of chiral pharmaceuticals. Determination of absolute configuration of an enantiomer in nuclear magnetic resonance involves formation of diastereomers using a chiral derivatization agent and establishing their differences in magnetic anisotropy (Allenmark and Gawronski, 2008).

### 1.3 Chiral Analysis of Pharmaceuticals in the Environment

The main sources of chiral pharmaceuticals in the environment are discharge of treated wastewater from wastewater treatment plants (WWTPs), manufacturing plants, and seepage from landfills and households (Kümmerer, 2010, 2009, 2008). Biotic processes such as microbial degradation may alter the enantiomeric composition of chiral pharmaceuticals in an environmental compartment, whereas abiotic processes should not



elicit any chiral selective effect (Baker and Kasprzyk-Hordern, 2013; Kasprzyk-Hordern and Baker, 2012; Kasprzyk-Hordern et al., 2010; MacLeod and Wong, 2010; MacLeod et al., 2007; Moreira et al., 2014; Nikolai et al., 2006; Ribeiro et al., 2012; Wong and MacLeod, 2009). Unfortunately, many studies on the fate and effects of chiral pharmaceuticals in the environment have ignored the role of chirality (Ariëns, 1984; Kasprzyk-Hordern, 2010). For instance, from 2002 to 2014, over 6,500 peer-reviewed articles on pharmaceuticals in the environment were published, yet less than 5% of these studies considered chirality (Figure 1-4). Neglecting chirality in environmental risk assessments introduces inaccuracies because distribution, fate and toxicity of chiral pharmaceuticals is usually stereoselective (Borges et al., 2011; Hashim and Khan, 2011; Kasprzyk-Hordern, 2010; Smith, 2009).

### 1.3.1 Occurrence of Chiral Pharmaceuticals

#### 1.3.1.1 Analgesics

Analgesics are the most frequently detected class of pharmaceuticals in the environment (Ali et al., 2009b; Buser et al., 1999; Hashim and Khan, 2011; Suzuki et al., 2014). Examples of the most common analgesics include ibuprofen, ketoprofen and naproxen. The enantiomeric composition of ibuprofen was the first to be determined in the environment among chiral pharmaceuticals (Buser et al., 1999). This finding led to a rapid surge in investigations considering stereoselective distribution of chiral pharmaceuticals (Kasprzyk-Hordern, 2010; MacLeod and Wong, 2010; Pérez and Barceló, 2008; Ribeiro et al., 2012). A number of studies demonstrated the enantiomeric occurrence of analgesics

in wastewater and the impacted surface water using gas chromatography tandem mass spectrometer (GC-MS/MS) (Khan et al., 2013) or liquid chromatography tandem mass spectrometer (LC-MS/MS) (Camacho-Muñoz and Kasprzyk-Hordern, 2015; Suzuki et al., 2014). The enantiomers of ibuprofen were found in surface water at concentrations from 0.02 µg/L to 1.02 µg/L (Camacho-Muñoz and Kasprzyk-Hordern, 2015; Khan et al., 2013). Both *R*- and *S*-enrichment was observed in studies conducted in Switzerland (Buser et al., 1999), Spain (Camacho-Muñoz and Kasprzyk-Hordern, 2015) and Australia (Khan et al., 2013) with EF values ranging from 0.41 to 0.77. The observed differences in EF values could be due to differences in compositions in sewage influent and effluent. For example, Buser et al. (1999) reported EF values in wastewater effluent ranging from 0.47 to 0.67 and 0.41 to 0.61 values in the impacted surface water. Additionally, the observed variations could be due to differences in operating conditions of the wastewater treatment plants, or differences in the microbial population and diversity in different river systems.

The EF values of chiral pharmaceuticals in wastewater influent are often influenced by the original enantiomeric composition of the prescribed drugs. For example, naproxen is sold as a single enantiomer, and hence wastewater influent samples had EF values of approximately 1.0 (Caballo et al., 2015; Camacho-Muñoz and Kasprzyk-Hordern, 2015; Khan et al., 2013). However, it was found that the EF value of naproxen decreased to 0.7–0.9 following the wastewater treatment process (Khan et al., 2013). This observation indicated that (*R*)-naproxen was formed during wastewater treatment likely due to chiral inversion. The enantiomeric composition may be also influenced by the stereoselectivity in human pharmacokinetics and pharmacodynamics of the pharmaceuticals. For example,

ketoprofen is prescribed as a racemic drug like ibuprofen. However, Caballo et al. (2015) found that the EF values of ketoprofen in the wastewater influent ranged from 0.54 to 0.68. In an earlier study, Grubb et al. (1999) observed that the elimination rate of (*R*)-ketoprofen was greater than its antipode after human consumption. Therefore, the deviation of EF values from 0.50 could be due to several processes spanning from human metabolism and elimination, and microbial transformations during wastewater treatment or in the natural environment.

#### 1.3.1.2 $\beta$ -blockers

The  $\beta$ -blockers are pharmaceuticals commonly used to treat cardiovascular diseases. They are one of the most prescribed therapeutic classes. Examples of  $\beta$ -blockers include atenolol, metoprolol, salbutamol, propranolol and sotalol. Fono and Sedlak (2015) detected enantiomers of propranolol in a wastewater effluent-dominated river in Southern California (Fono and Sedlak, 2005). Following a surface water and lake water incubation experiment, they concluded that stereoselectivity in the occurrence of propranolol was due to microbial degradation (Fono and Sedlak, 2005; Fono et al., 2006). Research groups from the United Kingdom, the United States, Spain, Portugal, and Canada investigated the enantiomeric composition of  $\beta$ -blockers in surface water, wastewater and sludge (Table 1-1). The environmental concentrations of  $\beta$ -blockers are often low compared to analgesics. Several studies showed the concentration of atenolol, metoprolol and propranolol in surface water to range from <1.0 ng/L to 0.02  $\mu$ g/L (Bagnall et al., 2012; Kunkel and Radke, 2012). The corresponding EF values were between 0.47 and 0.50.

The EF values of atenolol in different wastewater influent samples was close to 0.50 (Evans et al., 2015; López-Serna et al., 2013; Vazquez-Roig et al., 2014). However, the EF values of atenolol in wastewater influents exhibited significant inter-day variations (Vazquez-Roig et al., 2014). The  $\beta$ -adrenergic antagonism of atenolol is mainly associated with the S-enantiomer (Mehvar and Brocks, 2001). The elimination rate of the (R)-enantiomer is often greater than that of its antipode. Hence, at certain times R-enrichment in the domestic sewage occurs. There was little to no apparent change in EF values between the wastewater influent and effluent for atenolol (Table 1-1). However, S-enrichment wastewater effluent was reported for atenolol and metoprolol with EF values  $>0.54$  (Bagnall et al., 2012), whereas R-enrichment was observed for propranolol with EF values  $<0.45$  (Bagnall et al., 2012; Evans et al., 2015). Therefore, stereoselective distribution of chiral pharmaceuticals varied even within the same chemical group, as well as under different environmental settings.

#### 1.3.1.3 Antidepressants

Together with  $\beta$ -blockers, antidepressants make up more than 20% of all pharmaceuticals prescribed in Europe and the US. Citalopram, venlafaxine, sertraline and fluoxetine are the most commonly prescribed antidepressants that have at least one chiral center. Antidepressants and their metabolites have been found in wastewater (Table 1-1) at concentrations ranging from 0.008 to 0.70  $\mu\text{g/L}$ , and 20 to 107 ng/g in sludge (Table 1-2). Their EF values in wastewater influent ranged from 0.50 to 0.60. For example, fluoxetine and venlafaxine had an EF value of 0.50, whereas citalopram had an EF value

of 0.60 (Li et al., 2013; Liu et al., 2007; MacLeod et al., 2007), likely due to the fact that citalopram products are marketed as both a racemic mixture and S-citalopram. Therefore, the observed S-enrichment was mainly due to chiral switching at the stage of manufacturing (Agranat et al., 2002). The EF values for citalopram and fluoxetine were measured to be 0.70 for both compounds in wastewater effluent (Evans et al., 2015). However, studies from the United Kingdom and Portugal found EF values of venlafaxine to vary from 0.43 to 0.55 (Bagnall et al., 2012; Castrignanò et al., 2016; Evans et al., 2015; Ribeiro et al., 2014). Differences in physicochemical properties of the compounds, operating conditions of the wastewater treatment plants, and microbial population and diversity in the plants, all contributed to different EF values of chiral antidepressants.

### 1.3.2 Stereoselective Fate of Chiral Pharmaceuticals

Table 1-3 summarizes recent studies on biodegradation studies of chiral pharmaceuticals in the environment. Abiotic environmental processes such as volatilization, sorption and abiotic transformations are non-selective for enantiomers because of their identical physicochemical properties (Wong, 2006). However, biotic degradation is usually enantioselective due to the stereospecificity in the interactions of a chiral compound with enzymes in the microbes. Therefore, enantioselective analysis of enantiomers may serve as an indicator for microbially mediated degradation and may provide essential information on biochemical fate of chiral pharmaceuticals in the environment (Hashim et al., 2010; Kasprzyk-Hordern, 2010). Chiral pharmaceuticals such as ibuprofen, propranolol and venlafaxine have been used as chiral signatures to obtain an

understanding on biological attenuation processes and to distinguish between abiotic and biotic processes in surface water (Fono and Sedlak, 2005; Fono et al., 2006; Kasprzyk-Hordern and Baker, 2012; Li et al., 2013).

#### 1.3.2.1 Biodegradation in Wastewater Microcosms

Wastewater treatment plants are the main source of entry of chiral pharmaceuticals in both aquatic and terrestrial environments. Microbial degradation is the main removal pathway of chiral pharmaceuticals at a wastewater treatment plant (Khan et al., 2013; Martín et al., 2012; Subedi and Kannan, 2015). Several field and laboratory studies showed that microbial degradation of chiral pharmaceuticals in wastewater was stereoselective (Table 1-1). For example, the EF values of atenolol in different wastewater influent were close to 0.50 (Evans et al., 2015; López-Serna et al., 2013; Vazquez-Roig et al., 2014). However, S-enrichment was reported for atenolol and metoprolol in wastewater effluent, with EF values  $> 0.54$  (Bagnall et al., 2012), whereas R-enrichment was observed for propranolol with EF values  $< 0.45$  (Bagnall et al., 2012; Evans et al., 2015). Therefore, stereoselective removal of chiral pharmaceuticals varied even within the same therapeutic group. This was corroborated by Evans et al. (2015) who observed that the EF value of citalopram increased from 0.60 to 0.70, but there was no stereoselectivity in removal of venlafaxine and fluoxetine with EF remaining at 0.50 and 0.70, respectively (Evans et al., 2015). Therefore, a systematic understanding of the mechanism of removal of pharmaceuticals in wastewater treatment plants is critical in both exposure assessment and developing mitigation efforts.

To better understand stereoselectivity in wastewater treatment plants, researchers often use laboratory studies where control of various parameters is possible. The most commonly used approach is a wastewater microcosm where mixed liquor from an aeration basin is diluted to a total suspended solids concentration ranging from 1 and 5 g/L. Adding an additional source of nutrients such as acetate to the activated sludge inoculum was shown to have no effect on the stereoselectivity of degradation of alprenolol or propranolol (Ribeiro et al., 2013). Most studies on biodegradation of chiral pharmaceuticals did not introduce other sources of nutrients (Fono and Sedlak, 2005; Gasser et al., 2012). A slight *R*-enrichment was observed in microbial degradation of both alprenolol and propranolol. In a previous study, Ribeiro et al. (2012) also found that the degradation of (*S*)-metoprolol was more rapid. However, other studies on metoprolol and atenolol showed that *R*-enantiomers dissipated at a rate greater or similar to the antipode (Ribeiro et al., 2012). The differences in enantiomeric composition following biodegradation may be attributed to differences in microbial population and diversity and operating conditions of the wastewater treatment plants where the activated sludge were sampled. Some studies further showed diurnal variations in the EF value of chiral pharmaceuticals in wastewater effluent (Evans et al., 2015). There is a need for additional research to determine the effects of operating conditions on stereoselectivity during degradation of chiral pharmaceuticals.

#### 1.3.2.2 Degradation in Surface Water

Stereoselectivity in degradation of chiral pharmaceuticals in surface water has been reported in several field and laboratory studies. In a natural attenuation experiment lasting

92 d, temporal variation of the EF value of venlafaxine was observed (Li et al., 2013). When the flow in the river was low, a slight R-enrichment was observed, whereas an apparent S-enrichment occurred under normal or high flow conditions (Li et al., 2013). The temporal variation was probably caused by changes in microbial diversity due to changes in water chemistry over time. However, in a laboratory study, the S-enrichment was more pronounced with EF values increasing from 0.50 to 0.80. A lake water microcosm study showed that (*S*)-ibuprofen degraded faster than the R-enantiomer ( Buser et al., 1999). However, Winkler et al. ( 2001) found the *R*-form to degrade more rapidly in river water samples. Similarly, R-enrichment occurred when ibuprofen was incubated in a constructed wetland under aerobic conditions, whereas no stereoselectivity was observed under anaerobic conditions (Matamoros et al., 2009). Differences in bacterial communities in WWTPs, lakes, rivers or aquifers may explain the differences in enantioselective degradation observed in these matrices. Due to the complex nature of stereoselectivity, variations in microbial communities in different environments, and different chemical structures of chiral pharmaceuticals, the results highlighted above may not be extrapolated to other compounds or other environmental systems. The fact that inconsistent stereoselectivity occurred under different conditions points to the need for a more systematic approach in elucidating the effects of environmental conditions such as pH, co-pollutants, dissolved organic matter content, biological oxygen demand and temperature on chiral selective degradation.



### 1.3.2.3 Chiral Inversion in the Environment

Chiral inversion is a process whereby the three-dimensional structure of a compound is changed by enzymes through conversion of one enantiomer to its antipode without changing the molecule (Khan, 2014). Unlike chiral inversion, epimerization is not mediated by enzymes, but depends on the stability of the stereoconfiguration of the molecule and is sometimes affected by pH, types of solvent, and temperature (Wsól et al., 2004). When epimerization results in the formation of a racemic mixture, it is called racemization. Drug stability is critical in drug discovery and development because it affects dosage, shelf-life, and biological activity or toxicity of the compound (Wsól et al., 2004). Hence, pharmaceuticals usually display a high stability under normal conditions, hence epimerization and racemization of chiral pharmaceuticals has not been reported in environmental studies.

Previous studies on the fate of chiral pharmaceuticals in wastewater and the environment often overlooked chiral inversion and assumed that the observed differences in EF were caused by differences in the rate of degradation of the enantiomers. However, several pharmacological studies demonstrated that some pharmaceuticals were able to undergo chiral inversion (Suzuki et al., 2014). For example, in humans, ibuprofen underwent unidirectional chiral inversion whereby the *R*-enantiomer became (*S*)-ibuprofen but the *S*-enantiomer did not invert to the *R*-enantiomer (Hao et al., 2005). The phenomenon is common among the profens in general. For example, enzymes in the human body stereospecifically change (*R*)-fenoprofen and (*R*)-benoxaprofen to their respective

(*S*)-enantiomers (Khan, 2014). The degree and/or direction of chiral inversion may vary in different organisms. However, some drugs such as 2-phenylpropionic acid may undergo bidirectional chiral inversion (Khan, 2014). Chiral inversion may complicate the interpretation of changes in EF values as observed with chiral pharmaceuticals in the environment. For example, changes in EF values of ibuprofen may be caused by 1) chiral inversion of (*R*)-ibuprofen concurrent with non-stereoselective degradation, 2) enantioselective degradation, or 3) chiral inversion with concurrent enantioselective degradation (Khan, 2014). In a study on the occurrence of naproxen and ibuprofen in wastewater, Khan et al. (2014) did not detect (*R*)-naproxen in the influent, suggesting 1) and 2) were less likely to be the source of the (*R*)-enantiomer. By comparing the EF value of naproxen in the influent and effluent, Khan et al. observed that the EF value decreased from 1.0 to between 0.7 and 0.9. Thus, (*S*)-naproxen underwent chiral inversion to form (*R*)-naproxen in the WWTP. The degree of chiral inversion varied between individual WWTPs, possibly due to differences in microbial diversity and population or operating conditions. Suzuki et al. (2014) identified the role of chiral inversion in the fate of naproxen using single enantiomers, where single enantiomers were incubated in activated sludge and river water. It was further observed that there was a seasonal variation in the EF values due to enantioselective degradation and possibly chiral inversion. The occurrence of (*R*)-naproxen in the aquatic environment may be thus attributed to chiral inversion due to wastewater treatment. Therefore, whenever chiral inversion is suspected, a more accurate risk assessment should treat the individual enantiomers as single compounds. Moreover, the assumption that enantioselective degradation exclusively causes enantiomeric

enrichment may be invalid for some chiral pharmaceuticals, and the role of chiral inversion in the fate of chiral pharmaceuticals needs to be better understood through research.

### 1.3.3 Stereoselective Sorption in the Environment

Adsorption is one of the major removal pathways of chiral pharmaceuticals in wastewater and the environment. The concentration of  $\beta$ -blockers in sludge varied with the relative hydrophobicity of the compounds. Atenolol exhibited the lowest concentration, followed by alprenolol with values less than 0.02 ng/g (Table 1-2). However, the concentrations of sotalol and propranolol in sludge were 7 and 60 ng/g, respectively. A change in EF values was observed between the wastewater influent and sludge for some of these compounds. For example, EF values of propranolol and alprenolol increased from 0.40 to 0.50 and from 0.50 to 0.70, respectively, in sludge (Evans et al., 2015). The change in EF values could be attributed to enantioselective degradation or adsorption. Several studies have shown that microbially mediated biodegradation of chiral pharmaceuticals was enantioselective, however no studies demonstrated enantioselective sorption to sludge. Although organic matter contains numerous chiral centers, the adsorption of chiral pharmaceuticals to soil, sediment and sludge is often considered non-stereoselective (Kasprzyk-Hordern, 2010; Oravec et al., 2010). However, the sorptive behavior of enantiomers of trenbolone acetate, a synthetic growth hormone used in beef production, was shown to be stereoselective (Khan et al., 2009). Khan et al (2009) found that the adsorption of  $17\beta$ -trenbolone was two-fold greater than  $17\alpha$ -trenbolone. Stereoselectivity was recently observed in adsorption of human pharmaceuticals to sludge (Sanganyado et

al., 2016). Using batch experiments, stereoselectivity in the adsorption of enantiomers of acebutolol, atenolol, metoprolol, pindolol and propranolol, on sludge was shown to increase with a decrease in hydrophobicity of the adsorbate. The EF values of the amount of acebutolol, atenolol and metoprolol adsorbed on sludge were 0.27, 0.55 and 0.32, respectively, whereas for propranolol and pindolol it was about 0.50 (Sanganyado et al., 2016). These results suggested that polar interactions rather than hydrophobic interactions increased stereoselectivity in adsorption (Khan et al., 2009; Sanganyado et al., 2016).

#### 1.4 Conclusions

Since pharmaceuticals are used in large quantities, continuously emitted into the environment and designed to cause biological effects at low doses, the occurrence, fate and toxicity of pharmaceuticals in the environment merit close attention. However, because many pharmaceuticals are chiral, assuming that stereoisomers exhibit identical environmental behavior may be grossly inaccurate in terms of both their fate and ecotoxicity. Thus, determination of their enantiomeric composition in various environmental compartments is necessary. Additionally, microbial degradation of chiral pharmaceuticals in both the engineered (e.g., wastewater treatment plants) and natural environments can be stereoselective due to the stereospecific interactions of the micropollutant with the degrading microbes, yielding chiral or achiral metabolites at different rates and with different biological activity. Qualitative and quantitative biodegradation data are therefore important for improving the accuracy of environmental risk assessment of chiral pharmaceuticals. Laboratory studies are valuable for qualitative

investigation to understand the mechanisms of biodegradation of chiral pharmaceuticals under simulated conditions. However, laboratory experiments may be inadequate for a quantitative assessment (Gasser et al., 2012; Jammer et al., 2016, 2015; Souchier et al., 2016). Therefore, a quantitative tool for assessing the enantioselective degradation of chiral pharmaceuticals is needed and may be used to predict their behavior in the environment.

### 1.5 Research Objectives and Hypotheses

The primary goal of this project was to develop and employ chiral analysis methods to establish a systematic understanding of the mechanisms of adsorption and biodegradation, and identify a potential prioritization model, thus improving the accuracy of the risk assessment of enantiomers of antidepressants and  $\beta$ -blockers in the aquatic environment. The specific objectives were:

Objective 1: Develop a chiral method for analysis of enantiomers of fluoxetine and atenolol to understand the mechanism of chiral separation in vancomycin-based chiral columns.

Hypothesis: Previous studies showed that separation of basic pharmaceuticals by liquid chromatography can be improved by adding liophilic ions. Liophilic ions interact with both the analyte and the stationary phase and might affect enantioselectivity. It was hypothesized that adding different types and amounts of liophilic ions will affect chiral separation of fluoxetine and atenolol and at optimum conditions their enantiomers will be adequately separated.

Objective 2: Evaluate enantiomer specific adsorption of  $\beta$ -blockers to sludge during wastewater treatment and establish the sorption mechanism.

Hypothesis: Previous research demonstrated that the adsorption to soil of chiral pesticides and veterinary drugs was stereoselective. Adsorption is the second most important removal pathway of pharmaceuticals during wastewater treatment. Therefore, since sludge is primarily organic matter which in turn is chiral in nature, it may be hypothesized that adsorption of acebutolol, atenolol, metoprolol, pindolol and propranolol to sludge may exhibit enantiomeric specificity. Furthermore, previous studies showed that stereoselectivity was influenced by the hydrophobicity of the compounds. It was further hypothesized that the degree of stereoselectivity in sorption decreased when the  $\beta$ -blocker had a higher relative hydrophobicity.

Objective 3: Investigate the enantiomeric selective biodegradation of  $\beta$ -blockers in wastewater under laboratory conditions and establish the enantiomeric enrichment-degradation relationships.

Hypothesis 3: Previous research showed that the major removal pathway of pharmaceuticals in wastewater and the aquatic environment is biodegradation. Further studies demonstrated that the biodegradation of other pharmaceuticals, such as venlafaxine, ibuprofen and propranolol in surface water microcosms was stereoselective. Thus, it may be hypothesized that biodegradation of acebutolol, atenolol, metoprolol, pindolol and propranolol in wastewater may also be stereoselective. Previous research in PAHs showed enantiomeric enrichment-degradation relationships provide critical quantitative

assessment data essential for improving the accuracy of environmental risk assessment of chiral pharmaceuticals. It may be further anticipated that there is a linear correlation between change in enantiomeric composition and the degradation of  $\beta$ -blockers.

Objective 4: Estimate the environmental effect of chiral pharmaceuticals to non-target organisms using the read-across hypothesis.

Hypothesis 4: Previous studies showed that the read-across hypothesis was an adequate and reliable tool for predicting ecotoxicity and prioritization of pharmaceuticals in environmental risk assessment. Several studies showed that several primary drug targets in humans were evolutionarily conserved in fish. Therefore, it is hypothesized that by leveraging stereospecific pharmacological data, the potential risk of  $\beta$ -blockers and antidepressants on non-target aquatic organisms can be predicted. Furthermore, assuming there was conservation of the three dimensional structure of the primary drug targets, stereoselectivity in toxicity may also be predicted.

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Tables

Table 1-1 Occurrence and analysis of chiral pharmaceuticals in wastewater and surface water.

Drug	Country	Chiral analysis		Initial concentration			Final Concentration			Ref.
		Matrix	Detection	E1, µg/L	E2, µg/L	EF	E1, µg/L	E2, µg/L	EF <sup>c</sup>	
Ibuprofen	Switzerland	Wastewater	GC-MS		0.99-3.30	0.85-0.89		0.002-0.08 <sup>d</sup>	0.47-0.67	(Buser et al., 1999)
Ibuprofen	Switzerland	Surface water	GC-MS					n.d.- 0.01 <sup>d</sup>	0.41-0.60	(Buser et al., 1999)
Ibuprofen	Australia	Wastewater	GC-MS/MS				0.07	0.18	0.73	(Khan et al., 2013)
Ibuprofen	Australia	Creek	GC-MS/MS	0.05	0.12	0.71	0.03	0.09	0.77	(Khan et al., 2013)
Ibuprofen	Australia	Creek, downstream	GC-MS/MS				0.02	0.02	0.60	(Khan et al., 2013)
Ibuprofen	UK	Wastewater	LC-MS/MS				0.24	0.46±0.06	0.66	(Camacho-Muñoz and Kasprzyk-Hordern, 2015)
Ibuprofen	Spain	Wastewater	LC-MS/MS	0.25±0.01	0.96±0.01	0.79	0.04±0.0	0.07±0.0	0.62	(Caballo et al., 2015)
Ibuprofen	Spain	Wastewater	LC-MS/MS	0.25±0.0	1.54±0.05	0.86	0.10±0.0	0.21±0.01	0.68	(Caballo et al., 2015)

Drug	Country	Chiral analysis		Initial concentration			Final Concentration			Ref.
		Matrix	Detection	E1, µg/L	E2, µg/L	EF	E1, µg/L	E2, µg/L	EF <sup>c</sup>	
Ibuprofen	Spain	Surface water	LC-MS/MS	0.09	0.11	0.48	0.77	1.02	0.46	
Ketoprofen	Spain	Wastewater	LC-MS/MS	0.19±0.01	0.22±0.0	0.54	0.03±0.0	0.06±0.0	0.68	(Caballo et al., 2015)
Ketoprofen	Spain	Wastewater	LC-MS/MS	0.09±0.0	0.12±0.0	0.56	0.02±0.0	0.04±0.0	0.61	(Caballo et al., 2015)
Naproxen	Australia	Wastewater	GC-MS/MS				n.d.	0.02	1.0	(Khan et al., 2013)
Naproxen	Australia	Creek	GC-MS/MS	n.d.	0.03	1.0	n.d.	0.60	1.0	(Khan et al., 2013)
Naproxen	Australia	Creek, downstream	GC-MS/MS				n.d.	0.06	1.0	(Khan et al., 2013)
Naproxen	UK	Wastewater	LC-MS/MS				0.09±0.02	1.33±0.50	0.94	(Camacho-Muñoz and Kasprzyk-Hordern, 2015)
Naproxen	UK	Surface water	LC-MS/MS				n.d.	0.136	1	(Camacho-Muñoz and Kasprzyk-Hordern, 2015)
Naproxen	Japan	Surface water						0.08 <sup>d</sup>	0.84-0.98	(Suzuki et al., 2014)
Naproxen	Spain	Wastewater	LC-MS/MS	0.02±0.0	1.05±0.01	0.98	0.01±0.0	0.18±0.00	0.93	(Caballo et al., 2015)

Drug	Country	Chiral analysis		Initial concentration			Final Concentration			Ref.
		Matrix	Detection	E1, µg/L	E2, µg/L	EF	E1, µg/L	E2, µg/L	EF <sup>c</sup>	
Naproxen	Spain	Wastewater	LC-MS/MS	0.02±0.0	1.75±0.0	0.99	0.01±0.0	0.24±0.01	0.96	(Caballo et al., 2015)
Alprenolol	UK	Wastewater	LC-MS/MS		0.22±0.02 <sup>d</sup>	0.50		0.20±0.01 <sup>d</sup>	0.50	(Evans et al., 2015)
Atenolol	UK	Surface water	LC-MS/MS				0.01±0.0	0.02±0.0	0.47	(Bagnall et al., 2012)
Atenolol	Spain	Surface water	LC-MS/MS	0.002	0.002	0.50	<0.001	n.d.	-	(López-Serna et al., 2013)
Atenolol	UK	Wastewater	LC-MS/MS				0.59±0.02	0.50±0.01	0.55	(Bagnall et al., 2012)
Atenolol	Spain	Wastewater	LC-MS/MS	0.80-1.32	0.80-1.13	0.48-0.53	0.39-0.95	0.41-1.08	0.42-0.50	(Vazquez-Roig et al., 2014)
Atenolol	Spain	Wastewater	LC-MS/MS	0.54-0.95	0.91-1.23	0.34-0.44	0.30-0.67	0.31-0.79	0.46-0.51	(Vazquez-Roig et al., 2014)
Citalopram	UK	Wastewater	LC-MS/MS		0.65±0.05 <sup>d</sup>	0.60		0.05±0.004 <sup>d</sup>	0.70	(Evans et al., 2015)
DMC	UK	Wastewater	LC-MS/MS		0.002±0.0 <sup>d</sup>	1.0		n.d.	n.d.	(Evans et al., 2015)
Metoprolol	UK	Wastewater	LC-MS/MS				0.002±0.0	0.002±0.0	0.54	(Bagnall et al., 2012)

Drug	Country	Chiral analysis		Initial concentration			Final Concentration			Ref.
		Matrix	Detection	E1, µg/L	E2, µg/L	EF	E1, µg/L	E2, µg/L	EF <sup>c</sup>	
Venlafaxine	UK	Surface water	LC-MS/MS				0.008±0.0	0.01±0.0	0.58	(Bagnall et al., 2012)
Venlafaxine	UK	Wastewater	LC-MS/MS				0.06±0.0	0.04±0.0	0.43	(Bagnall et al., 2012)
Venlafaxine	Portugal	Wastewater	LC-MS/MS				0.11±0.01	0.13±0.01	0.54	(Ribeiro et al., 2014)
Venlafaxine	Portugal	Wastewater	LC-MS/MS				0.04±0.0	0.05±0.0	0.55	(Ribeiro et al., 2014)
Venlafaxine	UK	Wastewater	LC-MS/MS		0.35±0.03 <sup>d</sup>	0.50		0.22±0.02 <sup>d</sup>	0.50	(Evans et al., 2015)
Venlafaxine	UK	Wastewater	LC-MS/MS				0.07-0.11	0.09-0.12	0.44-0.49	(Castrignanò et al., 2016)
DMV	UK	Wastewater	LC-MS/MS				0.29-0.32	0.19-0.25	0.53-0.60	(Castrignanò et al., 2016)
DMV	UK	Wastewater	LC-MS/MS		0.70±0.02 <sup>d</sup>	0.50		0.29±0.01 <sup>d</sup>	0.50	(Evans et al., 2015)
Fluoxetine	UK	Wastewater	LC-MS/MS		0.05±0.004 <sup>d</sup>	0.70		0.03±0.0 <sup>d</sup>	0.70	(Evans et al., 2015)
Propranolol	UK	Surface water	LC-MS/MS				0.001±0.0	0.001±0.0	0.45	(Bagnall et al., 2012)
Propranolol	UK	Wastewater	LC-MS/MS				0.03±0.0	0.05±0.0	0.43	(Bagnall et al., 2012)

Drug	Country	Chiral analysis		Initial concentration			Final Concentration			Ref.
		Matrix	Detection	E1, µg/L	E2, µg/L	EF	E1, µg/L	E2, µg/L	EF <sup>c</sup>	
Propranolol	UK	Wastewater	LC-MS/MS		0.11±0.0 <sup>d</sup>	0.40		0.04±0.0 <sup>d</sup>	0.40	(Evans et al., 2015)
Salbutamol	UK	Wastewater	LC-MS/MS		0.28±0.03 <sup>d</sup>	0.50		0.46±0.01 <sup>d</sup>	0.50	(Evans et al., 2015)
Sotalol	UK	Wastewater	LC-MS/MS		0.25±0.02 <sup>d</sup>	0.50		0.15±0.009 <sup>d</sup>	0.50	(Evans et al., 2015)
Tramadol	UK	Wastewater	LC-MS/MS				0.69-0.80	0.60-0.68	0.51-0.57	(Castrignanò et al., 2016)

E1 and E2 first eluted or (R)-enantiomer, E2 second eluted or (S)-enantiomer

<sup>c</sup> Enantiomeric Fraction,  $EF = [S]/([S] + [R])$  or  $EF = [E2]/([E1] + [E2])$

<sup>d</sup> concentration of racemic mixture

DMV – Desmethylvenlafaxine

DMC - Desmethylcitalopram

n.d. not detected

Table 1-2 Occurrence and analysis of chiral pharmaceuticals in different types of sludge.

Drug	Country	Chiral analysis		Initial concentration			Final Concentration			Ref.
		Type of sludge	Detection	E1, ng/g	E2, ng/g	EF	E1, ng/g	E2, ng/g	EF	
Fluoxetine	UK	Digested	LC-MS/MS				90±10 <sup>a</sup>		0.70	(Evans et al., 2015)
Venlafaxine	UK	Digested	LC-MS/MS				80		0.50	(Evans et al., 2015)
DMC	UK	Digested	LC-MS/MS				107±30 <sup>a</sup>		0.60	(Evans et al., 2015)
DMV	UK	Digested	LC-MS/MS				20±6 <sup>a</sup>		0.50	(Evans et al., 2015)
Propranolol	UK	Digested	LC-MS/MS				60±10 <sup>a</sup>		0.50	(Evans et al., 2015)
Sotalol	UK	Digested	LC-MS/MS				7±3 <sup>a</sup>		0.50	(Evans et al., 2015)
Citalopram	UK	Digested	LC-MS/MS				120±60 <sup>a</sup>		0.60	(Evans et al., 2015)
Atenolol	UK	Digested	LC-MS/MS				0.002±0.0 <sup>a</sup>		0.40	(Evans et al., 2015)
Alprenolol	UK	Digested	LC-MS/MS				0.02±0.01 <sup>a</sup>		0.70	(Evans et al., 2015)

E1 and E2 are first eluted or (R)-enantiomer and second eluted or (S)-enantiomer respectively

second eluted or (S)-enantiomer

Enantiomeric Fraction,  $EF = [S]/([S] + [R])$  or  $EF = [E2]/([E1] + [E2])$

<sup>a</sup> concentration of racemic mixture

Table 1-3 Degradation of chiral pharmaceuticals in wastewater and the environment.

Compound	Incubation			Observation	Reference
	Matrix	Initial conc., µg/L	Period, d		
Alprenolol	Activated sludge inoculum	10,000 and 1000	15	Slight R-enrichment with half-lives of 4.95 and 5.70 for R-alprenolol and S-alprenolol, respectively	(Ribeiro et al., 2013)
Atenolol	Activated sludge inoculum	-	15	Degradation did not show enantioselectivity	(Ribeiro et al., 2012)
Metoprolol	River water	-	14	Degradation did not show enantioselectivity	(Fono et al., 2006)
Metoprolol	Activated sludge inoculum	-	15	Degradation of (S)-enantiomer was faster than the antipode	(Ribeiro et al., 2012)
Metoprolol	Effluent-sediment	10	65	S-enrichment was observed and a Rayleigh dependency was reported	(Souchier et al., 2016)
Propranolol	Activated sludge inoculum	10,000 and 1000	15	Slight R-enrichment with half-lives of 9.68 and 10.13 for R-propranolol and S- propranolol, respectively	(Ribeiro et al., 2013)



Compound	Incubation			Observation	Reference
	Matrix	Initial conc., µg/L	Period, d		
Propranolol	Wastewater microcosm	1.00 <sup>c</sup>	6	EF <sup>a</sup> decreased from 0.5 to 0.43 as the compound underwent biodegradation	(Fono and Sedlak, 2005)
Propranolol	River water	1.00 <sup>c</sup>	20	Degradation did not show enantioselectivity	(Fono and Sedlak, 2005)
Ibuprofen	Wastewater microcosm	0.99	0.33	No dissipation in first 2 h, then ER <sup>b</sup> changed from 5.7 to ER of 2.7 after 8 h.	(Buser et al., 1999)
Ibuprofen	Lake water	0.20	37	S-enantiomer degraded faster in the dark, thus R-enrichment was observed	(Buser et al., 1999)
Ibuprofen	Constructed wetlands	25	20	R-enrichment occurred under aerobic conditions, whereas no stereoselectivity was observed under anaerobic conditions	(Matamoros et al., 2009)
Ibuprofen	Membrane bioreactor	6–43	1	EF ranged from 0.88 to 0.94 in the influent and 0.38 to 0.40 in the effluent.	(Hashim et al., 2013)
Ibuprofen	Membrane bioreactor	2 <sup>d</sup>	1	R-enrichment occurred with an average decrease in EF from 0.52 to 0.39	(Hashim et al., 2011)

Compound	Incubation			Observation	Reference
	Matrix	Initial conc., µg/L	Period, d		
Naproxen	Membrane bioreactor	0.8–69	1	EF value in the influent decreased from 0.99 to between 0.86 to 0.94 in effluent.	(Hashim et al., 2013)
Naproxen	Membrane bioreactor	2 <sup>d</sup>	1	Chiral inversion was observed with EF decreasing from 0.99 to 0.65	(Hashim et al., 2011)
Naproxen	Constructed wetlands	25	20	EF decreased from between 0.88 and 0.90 to between 0.71 and 0.86.	(Matamoros et al., 2009)
Ketoprofen	Membrane bioreactor	0.1 to 27	1	Degradation did not show enantioselectivity	(Hashim et al., 2013)
Ketoprofen	Membrane bioreactor	2 <sup>d</sup>	1	S-enrichment occurred with an average increase in EF from 0.52 to 0.63	(Hashim et al., 2011)
Fluoxetine	Activated sludge inoculum	-	15	Degradation of did not show enantioselectivity	(Ribeiro et al., 2012)
Fluoxetine	<i>Labrys portucalensis F11</i>	618	30	(R)-enantiomer degraded faster than (S)-enantiomer.	(Moreira et al., 2014)

Compound	Incubation			Observation	Reference
	Matrix	Initial conc., µg/L	Period, d		
Venlafaxine	Wastewater microcosm	15	90	ER did not change significantly, but S to R enrichment under aerobic conditions but none under anaerobic was observed for O-desmethylvenlafaxine.	(Gasser et al., 2012)
Venlafaxine	River water	0.20	28	R-enantiomer degraded faster with EF values increasing from 0.5 to 0.8	(Li et al., 2013)
Venlafaxine	Natural attenuation, river	-	92	EF values were 0.62, 0.74 and 0.46 in rainfall, normal flow and low flow conditions, respectively	(Li et al., 2013)

<sup>a</sup>EF =  $E1/(E1 + E2)$  where E1 and E2 are concentration of first and second eluted enantiomers.

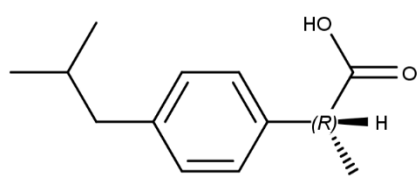
<sup>b</sup>ER =  $(\text{concentration of } R - \text{enantiomer}) / (\text{concentration of } S - \text{enantiomer})$ .

<sup>c</sup>Spiking concentration, initial concentration in microcosm not available.

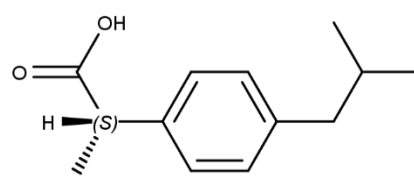
<sup>d</sup>Analyte was continuously introduced

Figures

Non-superimposable mirror images



(R)-(-)-ibuprofen



(S)-(+)-ibuprofen

Figure 1-1 R and S enantiomers of ibuprofen showing their chiral centers.

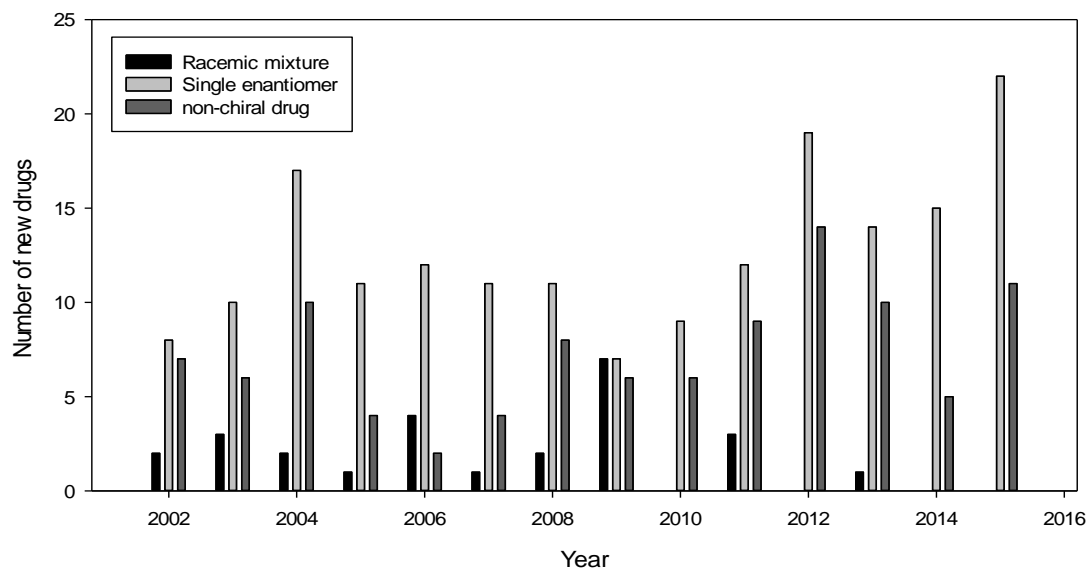


Figure 1-2 Number of new drugs approved by US Food and Drug Administration (FDA) for the period 2002 to 2014.

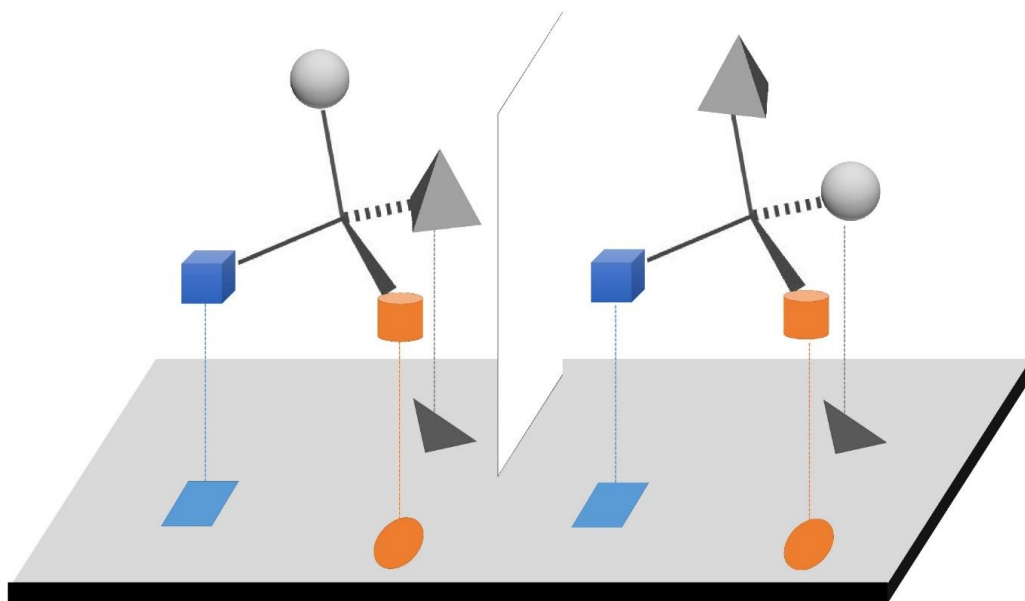


Figure 1-3 One enantiomer has stronger affinity for the reactive site than the other.

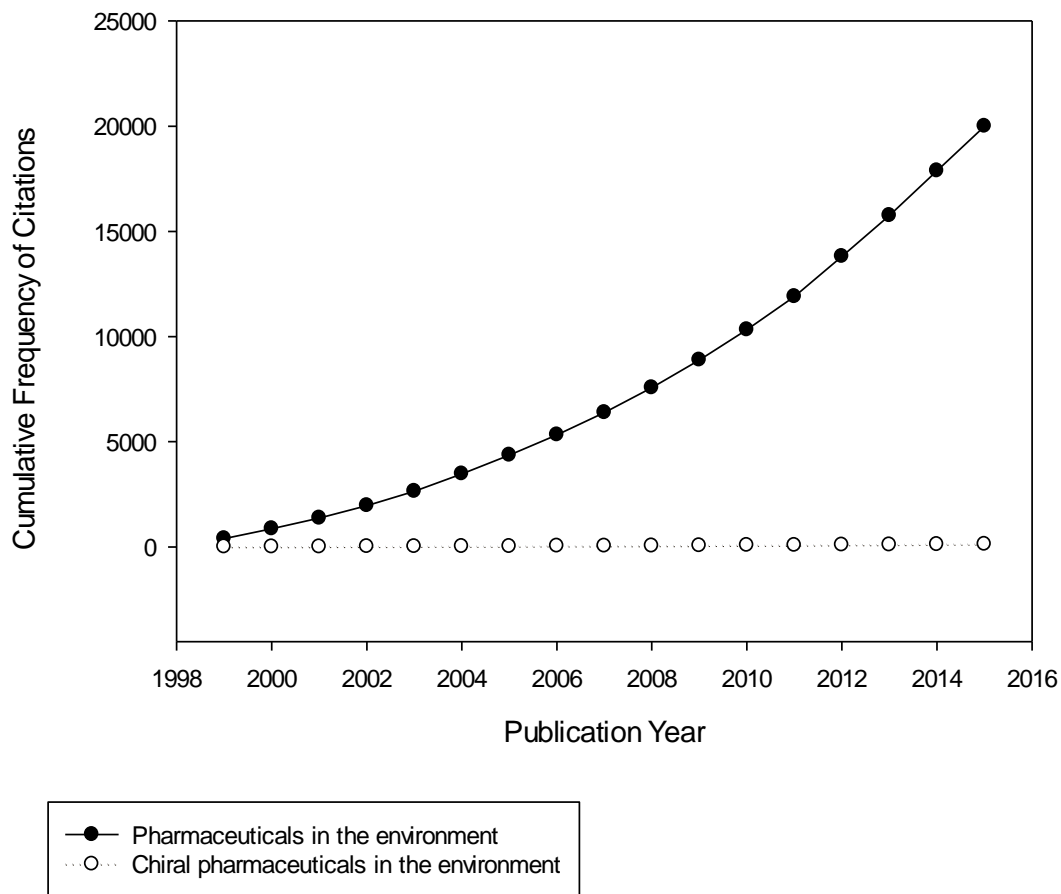


Figure 1-4 Comparison of increase in number of peer-reviewed publications related to pharmaceuticals in the environmental and chiral pharmaceuticals from 1998 to 2015. Citation information from SciFinder.

## **Chapter 2 Mechanistic Insights on Chaotropic Interactions of Liophilic Ions with Basic Pharmaceuticals in Polar Ionic Mode Liquid Chromatography**

### 2.1 Introduction

Over 50% of the current-use pharmaceuticals are chiral chemicals and about 70% are ionizable weak bases. Therefore, chiral analysis of stereoisomers is important in pharmacological studies and environmental risk evaluations since enantiomers typically have different therapeutic and toxicological activities due to stereoselectivity in biological interactions (Agranat et al., 2012, 2002). However, modulating mobile phase pH is challenging in chiral analysis since changes in pH alter retention and selectivity of the ionic enantiomers. However, disparities in mobile phase pH often result in changes in ionization of both the analyte and chiral stationary phase (CSP), causing variations in chromatographic performance. As a result, characterization of chromatographic behaviors of chiral ionogenic compounds, and understanding the effect of mobile phase additives, has been an important topic in chiral analysis (Kasprzyk-Hordern, 2010; Ward and Ward, 2010).

Macrocyclic glycopeptides are widely used as CSP because they can work in normal phase, reversed phase (RP-LC), polar ionic mode (PIM) or polar organic mode liquid chromatography. Vancomycin is a macrocyclic glycopeptide extensively used in chiral analysis of chiral polar organic compounds including  $\beta$ -blockers and antidepressants. With a molecular weight of 1449 and 18 stereogenic centers, vancomycin ionizes at different pH values due to its ionizable functional groups: one carboxylic, two amines and nine hydroxyl



groups (Xiao and Armstrong, 2004). The isoelectric point of vancomycin is 7.2 and its pKa values are 2.9, 7.2, 8.6, 9.6, 10.4, and 11.7 (Xiao and Armstrong, 2004). In PIM, enantioselectivity is mainly due to electrostatic forces, steric hindrance and hydrogen bonding whereas in RP-LC hydrophobic, electrostatic and steric interactions are more dominant. Since the introduction of macrocyclic glycopeptide two decades ago, research has primarily focused on their application in pharmaceutical, pharmacology and environmental studies (Berthod et al., 2004). Subsequent studies investigated their chiral recognition mechanism focusing primarily on RP-LC (Berthod, 2009, 2006; Berthod et al., 2010). In subsequent years, extensive studies by LoBrutto and co-workers (Kazakevich et al., 2005; LoBrutto et al., 2001b; Makarov et al., 2008; Pan et al., 2004) and Flieger and co-workers (Flieger and Czajkowska-Żelazko, 2011; Flieger, 2009, 2007, 2006; Flieger et al., 2013) showed that lipophilic ions mobile phase additives enhance chromatographic performance in achiral RP-LC. Previous studies by Thompson and coworkers using a crown ether column in chiral RP-LC with mobile phase pH 2.0 showed that retention factor ( $k$ ) and enantioselectivity ( $R_s$ ) of isomers of aminoindanol were affected by the type of lipophilic ions with  $k$  and  $R_s$  decreasing in the order  $\text{ClO}_4^- > \text{CF}_3\text{COO}^- > \text{NO}_3^- > \text{H}_2\text{PO}_4^-$ , following the Hofmeister series (Thompson et al., 1995). Since retention involves ion interactions, the separations using lipophilic ions retention are called ion interaction chromatography (Dai and Carr, 2005; LoBrutto et al., 2001a). The main models describing retention mechanisms in ion chromatography are partition model, where analyte and lipophilic ions form ion-pairs in either the mobile phase (ion-pair formation) or stationary phase (dynamic ion exchange) and electrostatic model, where retention is influenced by

the CSP's surface charge density and the ionic strength of the mobile phase (Smuts et al., 2014).

Understanding chiral recognition mechanisms of analytes in polar ionic mode liquid chromatography (PIM) is critical since it is one of the most widely used enantioseparation methods in pharmaceutical analysis of ionogenic analytes because it offers better chromatographic performance than reversed phase (RP-LC) or normal phase mode [22]. To our knowledge, there are no systematic studies on the effect of chaotropic interactions on retention and enantioselectivity in PIM on a macrocyclic glycopeptide-based CSP. In this study, we evaluate for the first time the mechanistic aspects of chaotropic interactions of liophilic ions and two structurally diverse basic chiral pharmaceuticals in PIM on a vancomycin based CSP. Atenolol and fluoxetine, two of the most widely used pharmaceuticals with ubiquitous environmental occurrence, were used as model analytes (MacLeod and Wong, 2010; Nikolai et al., 2006). The role of liophilic ions and their effects were discerned by altering the type and concentration of liophilic ions. Thermodynamic parameters of the enantioseparation were calculated for enantiomers of atenolol and fluoxetine using van't Hoff plots.

## 2.2 Materials and Methods

### 2.2.1 Chemicals

Racemic standards of atenolol and fluoxetine and analytical reagent grade triethylamine, formic acid and glacial acetic acid were purchased from Sigma Aldrich (St.

Louis, MO). HPLC grade methanol, ethanol and acetonitrile and the volatile ammonium salts (acetate, formate, chloride and nitrate) were purchased from Fisher Scientific (Fair Lawn, NJ). Stock solutions of individual racemic atenolol and fluoxetine at 1.0 mg/mL were prepared in methanol and stored in amber glass vials at -20 °C before use.

### 2.2.2 Instrument and Chromatographic Conditions

The racemic chiral drugs in methanol at 1.0 mg/ml was injected for analysis. The signals on an Agilent 1100 HPLC system (Agilent, Wilmington, DE, USA) with a multiple wavelength UV detector were recorded at 224 nm. In preliminary experiments, several mobile phases, including ethanol, methanol and acetonitrile, and various ratios and concentrations of triethylamine and acetic acid, were tested for improving analyte retention and resolution performance. Methanol, triethylamine and acetic acid (100/0.1/0.1, v/v/v) offered best enantioseparation. The mobile phase was degassed using an ultrasonic bath for 30 min followed by online degassing during elution. The flow rate was 1.0 ml/min, and the sample injection volume was 10  $\mu$ L. The elution was in the isocratic mode with separation carried out on a Chirobiotic V column (250 mm x 4.6 mm I.D., Sigma-Aldrich, St. Louis, MO, USA). The sign of optical rotation was confirmed using an in-line laser polarimeter detector (PDR-Chiral, Lake Park, FL).

The effect of type or amount of acid modifier was investigated using glacial acetic acid and formic acid by varying their fraction in the mobile phase (100 % methanol and 10 mM ammonium acetate) from 0.005 to 0.1 % (v/v). Furthermore, the effect of lipophilic ions on enantioseparation was determined using different volatile ammonium salts (10 mM) as

a buffer in 100% methanol. The pH was adjusted using 0.005% (v/v) formic acid. In order to characterize the effect of concentration, the amount of liophilic ions in the mobile phase was varied from 4, 10, 15 and 20 mM while pH was adjusted using formic acid. In all treatments, the column temperature was kept at 25 °C, except when the effect of temperature was evaluated. In the latter case, the temperature was varied from 13 to 40 °C. Data were treated and processed using Minitab 17 (State College, PA).

### 2.2.3 Thermodynamics

The thermodynamics in the chiral recognition of analytes may be evaluated through the determination of  $k$  and  $\alpha$  at different column temperatures using the van't Hoff equation 2-1 and 2-2:

$$\ln k = -\left(\frac{\Delta H^\circ}{RT}\right) + \frac{\Delta S^\circ}{R} + \ln \Phi \quad (2-1)$$

$$\ln \alpha = -\left(\frac{\Delta \Delta H^\circ}{RT}\right) + \frac{\Delta \Delta S^\circ}{R} \quad (2-2)$$

where  $k$  is the retention factor of the analyte,  $\alpha$  the enantioselectivity,  $R$  the ideal gas constant,  $T$  the absolute temperature, and  $\Phi$  the phase ratio (Tian et al., 2010).  $\Delta H^\circ$  and  $\Delta S^\circ$  are standard changes of enthalpy and entropy changes, respectively, between the analyte, mobile and stationary phases as determined using the van't Hoff plots ( $\ln k$  vs  $1/T$ ). However, the value of  $\Phi$  complicates the van't Hoff equations because  $\Phi$  can change with temperature and for most columns including Chirobiotic V column it is unknown.

Since, the terms  $\frac{\Delta H^\circ}{RT}$  and  $\frac{\Delta S^\circ}{R} + \ln \phi$  are the enthalpy and entropy contributions to  $\ln k$  at a given temperature,  $\Delta S^{0*}$  can be used to represent the entropy term.

## 2.3 Results and Discussion

### 2.3.1 Preliminary Examination

We compared  $R_s$  and  $\alpha$  of atenolol and fluoxetine enantiomers when an acid and a base or a volatile ammonium salt were used as mobile phase additives. Enantiomers of atenolol and fluoxetine were successfully separated using methanol/acetic acid/triethylamine (100/0.1/0.1, v/v/v). The values of  $\alpha$  and  $R_s$  were 1.01 and 1.12 for the enantiomers of atenolol, respectively, and 1.77 and 2.70 for the enantiomers of fluoxetine, respectively. However, triethylamine is known to cause degradation of Chirobiotic V column (Hashem et al., 2011). Alternatively, volatile ammonium salts, which are examples of liophilic ions, can be used to control changes in retention and selectivity due to pH changes through chaotropic interaction with positively charged analytes in the mobile phase or on the CSP (Makarov et al., 2008). When ammonium acetate was used, enantiomers of atenolol and fluoxetine were separated using methanol and 10 mM ammonium acetate with 0.005 % formic acid. The use of ammonium acetate offered better enantioseparation because the resolution and selectivity were above 2.20 and 1.08, respectively for both atenolol and fluoxetine (Table 2-1).

### 2.3.2 Effect of Type and Amount of Acid Modifiers

The mobile phase pH is a critical parameter significantly affect enantioselectivity and retention in PIM. Using 10 mM ammonium acetate, the effect of the nature and amount of acid modifier on  $R_s$ ,  $\alpha$  and  $k_1$  is shown in Table 2-1. When pH was adjusted to 6.61 using acetic acid, atenolol had  $R_s$ ,  $\alpha$  and  $k_1$  values of 2.54, 1.11 and 2.75, respectively. However, when pH was kept at 6.63 using formic acid, similar  $R_s$ ,  $\alpha$  and  $k_1$  values (2.50, 1.11 and 2.72, respectively) were obtained. Therefore, the nature of the acid additives did not appear to affect enantioselectivity or enantioresolution of the enantiomers of atenolol or fluoxetine. When pH was decreased by adding acetic acid or formic acid up to 0.10 % (v/v) resulted in a decrease of the retention factor. In RP-LC using polysaccharide based CSPs, Perrin et al. (Perrin et al., 2002) observed that increasing pH from 2.0 to 9.0 resulted in an increase in retention of basic  $\beta$ -blockers and attributed this to a decrease in ionization with increase in pH. In contrast, in the present study, decreasing pH increases the number of protonated species, thus increasing the electrostatic repulsion between the positively charged analyte and cationic CSP. This interaction lead to a decrease in the retention factor, as observed.

The degree to which the ionization changes may depend on the nature of the acid modifier. Decreasing pH resulted in a decrease in both  $k$  and  $R_s$ , but did not significantly affect the enantioselectivity of atenolol or fluoxetine, except when formic acid was used where the enantioselectivity of fluoxetine decreased with increasing pH. Formic acid, with pKa 3.77, is easily protonated as compared to acetic acid (pKa 4.76). The pH ranges of

formic acid and acetic acid were varied from 4.85 to 6.72 and 6.01 to 7.27, respectively, where vancomycin CSP is in cationic form. The mobile phase pH modified ionization of the functional groups close to the chiral center and thereby changed the mechanism of chiral recognition by altering the spatial arrangement of the molecule or electrostatic interactions (Stalcup, 2010).

### 2.3.3 Effect of Type of Liophilic Ions

Figure 2-2 shows the effect of liophilic ion additives in the mobile phase on the retention of atenolol and fluoxetine. When 20 mM  $\text{Cl}^-$ ,  $\text{NO}_3^-$ ,  $\text{HCOO}^-$  or  $\text{CH}_3\text{COO}^-$  was added to the mobile phase, the  $k_1$  obtained for atenolol was 0.11, 0.78, 1.34 or 2.07, respectively. The influence of the liophilic ion on the retention factor varied according to their hydrophobicity. A similar trend was observed when separating enantiomers of fluoxetine. Furthermore, the enantioresolution of both atenolol and fluoxetine increased with increased hydrophobicity of the liophilic ion. Ammonium acetate resulted in the highest resolution for enantiomers of both atenolol and fluoxetine with values of 2.23 and 1.81, respectively. There was little or no variation in enantioselectivity of atenolol when  $\text{NO}_3^-$ ,  $\text{HCOO}^-$  or  $\text{CH}_3\text{COO}^-$  was used and the respective  $\alpha$  values were 1.11, 1.10 and 1.10. However,  $\alpha$  of fluoxetine decreased with an increase in hydrophobicity of the liophilic ions with  $\text{CH}_3\text{COO}^-$  exhibiting the lowest selectivity (1.07). Statistical analysis was conducted on the  $\alpha$  values using one-way ANOVA. The P value was 1.0, error was 0.0001 and F was 0, thus there were no statistically significant changes in  $\alpha$  values.

An unusual result was obtained on the retention of enantiomers of atenolol and fluoxetine in the presence of liophilic ions. Unlike previous studies in achiral and chiral liquid chromatography, liophilic ions used in this investigation followed the Hofmeister series:  $\text{CH}_3\text{COO}^- > \text{HCOO}^- > \text{NO}_3^-$ . In contrast, Thompson and coworkers (Thompson et al., 1995) investigated the effect of liophilic anions on the chiral discrimination of protonated isomers of aminoindanol on a crown ether column in RP-LC at pH 2. The retention factor of the isomers increased in the order  $\text{ClO}_4^- > \text{CF}_3\text{COO}^- > \text{NO}_3^- > \text{H}_2\text{PO}_4^-$ . Furthermore, a study by Smuts et al. in PIM (reported as polar organic mode) on enantioseparation of 16 chiral sulfonic and phosphoric acids using a barium-doped cyclofructan as a chiral selector corroborated with this observation (Smuts et al., 2014). Smuts et al. postulated that anions with low chaotropicity like  $\text{CH}_3\text{COO}^-$  readily adsorb on the positively charged chiral selector. Since the analytes were negatively charged, adsorption of the counteranions would reduce the surface potential of the charged chiral selector, hence the decrease in retention. However, in the present study the analytes and CSP were positively charged, hence the inversed observation. Furthermore, the type of liophilic anion did not affect  $\alpha$  of the pairs of the stereoisomers suggesting probably the nature of the cation might influence  $\alpha$ . A study in RP-LC using bovine serum albumin as chiral selector by Tao and Gilpin corroborated this finding, as they observed  $\alpha$  values of D- and L-isomers of tryptophan to vary with type of cation of the phosphate buffer in the order  $\text{K}^+ > \text{NH}_4^+ > \text{Na}^+$  (Tao and Gilpin, 2001).



#### 2.3.4 Effect of Amount of Liophilic Ions

The relationship between retention of atenolol and fluoxetine, and liophilic ion concentration was investigated by incrementally changing the concentration from 4-20 mM for each of the ammonium salts (Figure 2-2). Increasing the concentration of  $\text{CH}_3\text{COO}^-$  and  $\text{NO}_3^-$  from 4 mM to 20 mM resulted in decrease in  $k$  and  $\alpha$  of the enantiomers of both atenolol and fluoxetine (Figure 2-2). Petterson and Schill found increasing concentration of chiral counterion, (+)-10-camphorsulfonate, in enantioseparation of amines in PIM on LiChrosob-DIOL column resulted in decrease in retention (Pettersson and Schill, 1981). Petterson and Schill speculated (+)-10-camphorsulfonate adsorbed on the column and acted as a CSP with retention and separation occurring through an ion-exchange mechanism. However, in RP-LC, Flieger (2009) and Courderot et al. (2002) found  $k$  and  $\alpha$  of acidic analytes on a macrocyclic glycopeptide-based CSP increased when the concentration of the chaotropic salts in the mobile phase was increased. They attributed the observation to competing dynamic ion exchange and ion pair formation. In ion pair formation, the ion-pair is formed in the mobile phase whereas in dynamic ion exchange it is formed at the CSP. Hence, it is difficult to distinguish the equilibria of the two partition models (Dai and Carr, 2005). Furthermore,  $R_s$  decreased with increase in concentration of liophilic ions because increasing concentration resulted in decrease in chiral interactions between the analyte and the CSP. However, there was no marked difference in  $\alpha$  with changes in concentration (Figure 2-3).

At pH 6.7, ionic interactions will be dominant since liophilic ions will be negatively charged, and the CSP and analytes will be positively charged. The pKa of atenolol and fluoxetine are 9.6 and 10.1, respectively. Smuts and coworkers found increasing concentration of chaotropic salts resulted in decrease in retention of 16 chiral phosphoric and sulfonic acids on a barium doped cyclofructan-6-based CSP and attributed this to electrostatic theory. In electrostatic theory, the liophilic ions alter the electrostatic surface potential of the CSP by creating an electrostatic double layer at the CSP-mobile phase interface (Bartha and Ståhlberg, 1994). Depending on the ionization of the CSP and the analyte, the change in surface potential can either promote electrostatic repulsion or attraction thus changing retention of the analytes and adsorption isotherms of the liophilic ions (Bartha and Ståhlberg, 1994; Ståhlberg, 1999). In the present study, the plots of retention factor versus concentration of liophilic ions were fitted to a Bartha-Ståhlberg equation 2-3,

$$\log k = -\frac{1}{2} \frac{z_A}{z_X} \log [X^-] + K \quad (2-3)$$

where  $k$  is the retention factor,  $[X^-]$  is concentration of the liophilic ion,  $K$  is a constant and  $z_A$  and  $z_X$  are charges of the analyte and the interacting ion, respectively. The slope of all plots were negative since retention decreased with increase in concentration of liophilic ions. The plots for  $\text{NO}_3^-$  and  $\text{CH}_3\text{COO}^-$  were linear with  $r^2$  values of 0.92 and 1.00, respectively, thus under the conditions studied, retention was probably due to ion interaction mechanisms. However, the plot when formate was used indicate retention first increased with pH, in typical ion-pair formation mechanism, but then dropped with

increase in concentration. A plot retention factor versus concentration of formate from 10 mM to 20 mM was linear. In a similar study in PIM separating enantiomers of acid analytes, Smuts et al. obtained a linear logarithmic relationship between counterion concentration and retention (Smuts et al., 2014). When the mechanism of chiral recognition is dominated by electrostatic interactions, the plots of  $\log k$  versus  $\log [X^-]$  is linear (Lämmerhofer, 2010). Negative slopes, such as found in this study, are often common in ion exchange chromatography (Smuts et al., 2014).

#### 2.3.5 Effect of Temperature

To elucidate the effect of temperature on the retention and enantioseparation behavior, the influences of temperature over the range 13 to 40 °C were evaluated (Figure 2-4). Increasing temperature resulted in a decrease in retention of the enantiomers. However, the increase in retention time in the polar ionic mode was very limited, at only about 3 s/°C. This was about one second greater than that reported by Berthod et al. (Berthod et al., 2004) who used acetic acid and triethylamine instead of volatile ammonium salts. Moreover, in the separation of atenolol, as the amount of ammonium acetate decreased from 20 to 15, 10 or 4 mM, the change in retention time with temperature increased from 2.85 to 2.97, 3.01 or 3.02 s/°C, respectively. Decreasing column temperature may result in an increase in viscosity of the mobile phase and this may account for the trend observed. Selectivity for both analytes using different liophilic ions in the mobile phase decreased when the temperature was increased. The influence of temperature on  $R_s$  was more complex. For all liophilic ions, there was little or no change in  $R_s$  of

atenolol; however,  $R_s$  decreased with increasing temperature when fluoxetine was separated in the presence of  $\text{CH}_3\text{COO}^-$ . When  $\text{NO}_3^-$  or  $\text{HCOO}^-$  was used,  $R_s$  increased when the temperature was increased from 13 to 20 °C and subsequently decreased as the temperature was further increased from 20 to 40 °C.

### 2.3.6 Thermodynamic Characterization

The mechanism of chiral recognition is often explored by varying different experimental parameters such as mobile phase composition, pH, polarity or ionic strength, and a chemical group of the analyte (Berthod, 2009, 2006). In this study, we varied the mobile phase composition and chemical groups and measured the effect on retention and selectivity factors. Both chiral and achiral interactions may influence  $k$  and the effect of achiral interactions should be the same for the enantiomers of the same chiral analyte. However,  $\alpha$  measures the difference in stereoselective retention between the enantiomers, and thus is more suitable in ascertaining the mechanism of chiral recognition. The retention factor of an analyte is associated with the change in partial molar free energy incurred when the analyte transfers from the mobile phase to the CSP (Thompson et al., 1995). Plots of  $\ln \alpha$  against  $1/T$  were generated for the enantiomers of atenolol and fluoxetine as a function of acetate concentration. The  $\text{pK}_a$  values of atenolol and fluoxetine are 9.6 and 10.1, respectively, but the operational pH of the Chirobiotic V column was between 3.5 and 7. The interactions of the analytes and chaotropic anion was not affected by the concentration within the pH range studied since the analytes were protonated whereas the acetate anion was deprotonated. Linear van't Hoff plots were obtained for the analytes in the polar ionic

mode over the temperature range of 13 to 40 °C (Table 2-2). At concentrations 4 to 20 mM of ammonium acetate,  $r^2$  values ranged from 0.989 to 0.999, which indicates that there was one predominant chiral recognition mechanism and that the mechanism of retention of the analytes did not change within the given temperature range.

As the concentration of the acetate ion in the mobile phase was increased, there was a corresponding increase in entropic term ( $\Delta S^\circ + \ln \phi$ ). According to the chaotropic theory, increasing concentration should result in more counterions disrupting the methanol solvation shield on the analyte. Therefore, increasing the acetate ion concentration resulted in decreased hydrophobic interactions between the analyte and the CSP, thus permitting fast elution and improved adsorption desorption kinetics (Berthod, 2009). Both  $\Delta\Delta H^\circ$  and  $\Delta\Delta S^\circ$  were found to be negative, suggesting that enantioseparation was enthalpically driven. The entropy of the first eluted enantiomer had less favorable entropy for separation as compared to the second eluting enantiomer, contributing to the chiral recognition.

## 2.4 Conclusions

Enantiomers of basic pharmaceuticals were successfully resolved on a vancomycin based column in polar ionic mode. The nature and amount of liophilic ions added to the mobile phase influenced the enantioresolution and retention of the analytes. The results obtained suggest enantiorecognition mechanism of basic pharmaceuticals involves chaotropic interaction between the liophilic ions and the analytes on the CSP. Increasing the amount of chaotropic salt in the mobile phase resulted in a decrease in the analyte retention. In accordance to the Hofmeister series, the more hydrophobic anion, acetate,

resulted in relatively stronger analyte retention. However, changing the amount of liophilic ions did not significantly affect the enantioselectivity of atenolol or fluoxetine. Therefore, the type of liophilic ion had no or minimal effect on enantioselectivity. The van't Hoff plots were linear in the temperature range of 13–40 °C, and the thermodynamic parameters  $\Delta H^\circ$ ,  $\Delta(\Delta H^\circ)$ ,  $\Delta S^{\circ*}$  and  $\Delta(\Delta S^\circ)$  depended on the amount and type of liophilic ions in the mobile phase and the structure of the compounds. The chiral recognition mechanism in the presence of inorganic salts appeared to be enthalpy driven. These findings are only limited to basic analytes separated on a vancomycin based CSP in PIM. Although, additional studies are required for understanding the enantioselective mechanism of acidic or neutral analytes, the present study provides mechanistic insights essential for predicting chiral separations critical for developing methods for pharmaceutical and environmental studies.

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Tables

Table 2-1 Effect of amount of liophilic ions on the retention factor and enantioresolution of atenolol and fluoxetine enantiomers.

Drug	Concentration	Acetic acid				Formic Acid			
		pH	$k_1'$	Rs	$\alpha$	pH	$k_1'$	Rs	$\alpha$
Atenolol	0	7.43	3.56	2.70	1.10	7.43	3.56	2.70	1.10
	0.005	7.27	3.15	2.63	1.11	6.72	2.83	2.51	1.11
	0.01	7.02	3.07	2.60	1.11	6.63	2.72	2.50	1.11
	0.04	6.61	2.75	2.54	1.11	5.37	1.29	1.67	1.11
	0.10	6.01	2.21	2.36	1.11	4.85	0.71	1.05	1.11
Fluoxetine	0	7.43	2.83	2.19	1.07	7.43	2.83	2.19	1.07
	0.005	7.27	2.78	2.20	1.08	6.72	2.55	2.25	1.08
	0.01	7.02	2.75	2.20	1.08	6.63	2.46	2.23	1.08
	0.04	6.61	2.46	2.21	1.08	5.37	1.15	2.25	1.11
	0.10	6.01	1.97	2.13	1.09	4.85	0.57	1.80	1.20

Table 2-2 Thermodynamic parameters and correlation coefficients of atenolol and fluoxetine enantiomers on a Chirobiotic V column.

Analyte	Liophilic ion	Conc. mM	$-\Delta H^\circ$ kJ/mol	$-\Delta S^{\circ*}$ J/mol·K	$R^2$	$-\Delta\Delta H^\circ$ kJ/mol	$-\Delta\Delta S^\circ$ J/mol·K	$-\Delta\Delta G^\circ_{298}$ kJ/mol
Atenolol	Acetate	4	1.16	-9.38	0.99	0.56	1.02	0.26
			1.72	-8.36	1.0			
		10	2.37	-1.26	1.0	0.62	1.20	0.26
			2.99	-0.06	1.0			
		15	3.12	3.12	1.0	0.60	1.17	0.25
			3.72	4.29	1.0			
	20	3.50	5.61	0.99	0.61	1.25	0.24	
		4.11	6.86	0.99				
	Formate	4	-0.47	-8.33	0.91	3.78	1.32	0.41
			3.32	2.98	0.92			
		10	0.74	-3.76	0.99	0.60	1.13	0.26
			1.34	-2.62	1.0			
		15	1.23	0.43	1.0	0.49	0.81	0.25
			1.72	1.23	0.99			
20	1.59	0.49	0.99	1.02	2.49	0.28		
	2.62	2.98	1.0					
Nitrate	10	0.63	2.00	0.68	0.47	0.72	0.26	
		1.11	2.72	0.88				

Analyte	Liophilic ion	Conc. mM	$-\Delta H^\circ$ kJ/mol	$-\Delta S^{\circ*}$ J/mol·K	$R^2$	$-\Delta\Delta H^\circ$ kJ/mol	$-\Delta\Delta S^\circ$ J/mol·K	$-\Delta\Delta G_{298}^\circ$ kJ/mol
Fluoxetine	Acetate	4	1.86	-6.82	1.0	0.96	2.51	0.21
			2.82	-4.31	1.0			
		10	3.09	2.00	1.0	0.93	2.45	0.21
			4.02	4.44	1.0			
		15	3.80	6.70	1.0	0.89	2.32	0.20
			4.68	9.03	1.0			
	20	4.05	9.34	1.0	0.94	2.49	0.20	
		4.99	11.82	1.0				
	Formate	4	0.05	-5.93	0.10	0.94	2.39	0.22
			0.99	-3.54	0.94			
		10	1.22	-0.81	0.99	1.43	3.96	0.25
			2.65	3.15	0.99			
		15	1.67	3.51	1.0	0.81	2.00	0.21
			2.47	5.51	1.0			
20	2.13	4.02	1.0	0.75	1.89	0.19		
	2.88	5.91	1.0					
Nitrate	10	1.21	6.52	0.81	1.24	3.16	0.30	
		2.45	9.73	0.94				

## Figures

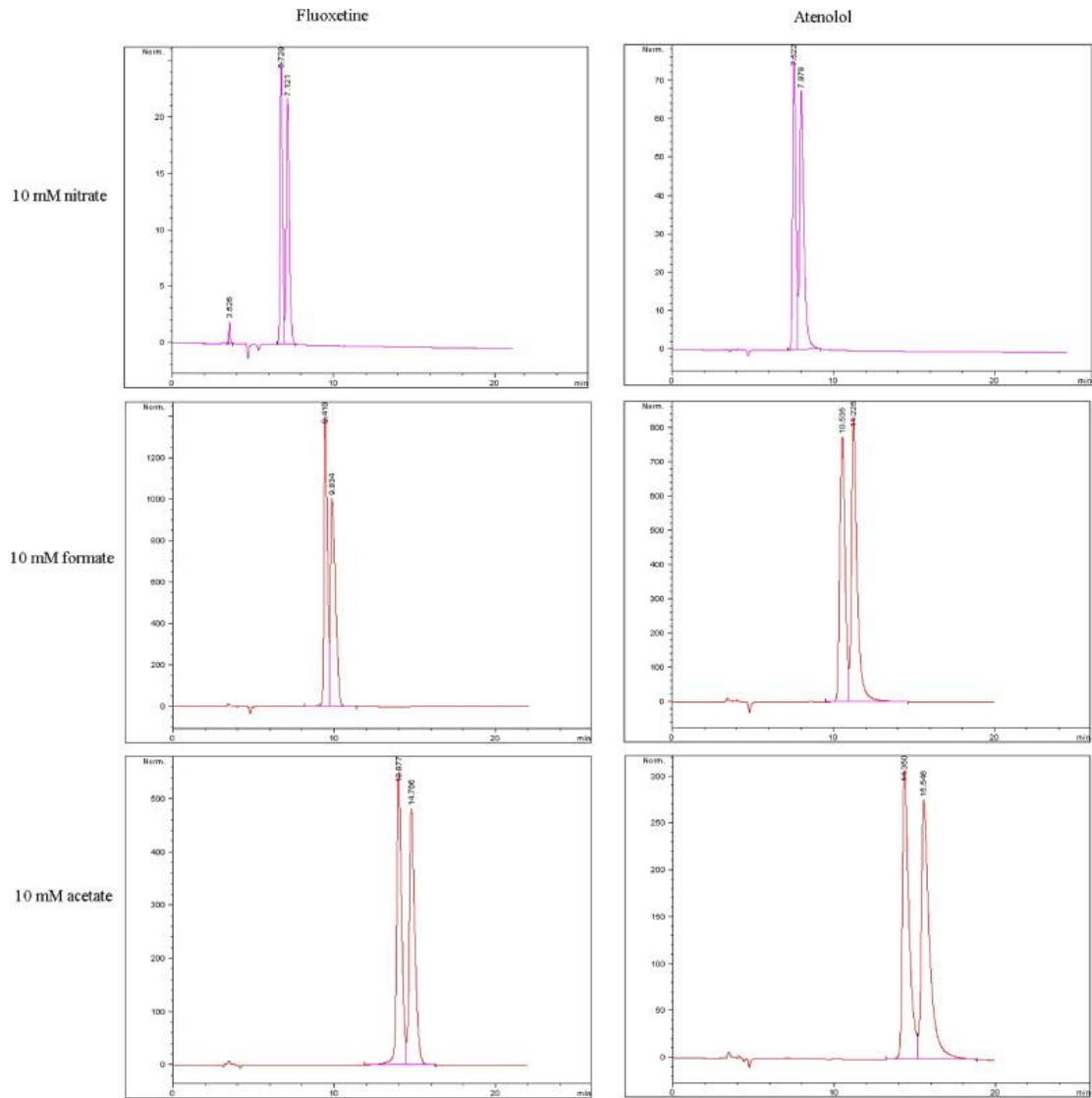


Figure 2-1 Chromatograms showing retention and separation of enantiomers of atenolol and fluoxetine on a Chirobiotic V column using 10 mM ammonium salts and 0.005% formic acid in methanol.

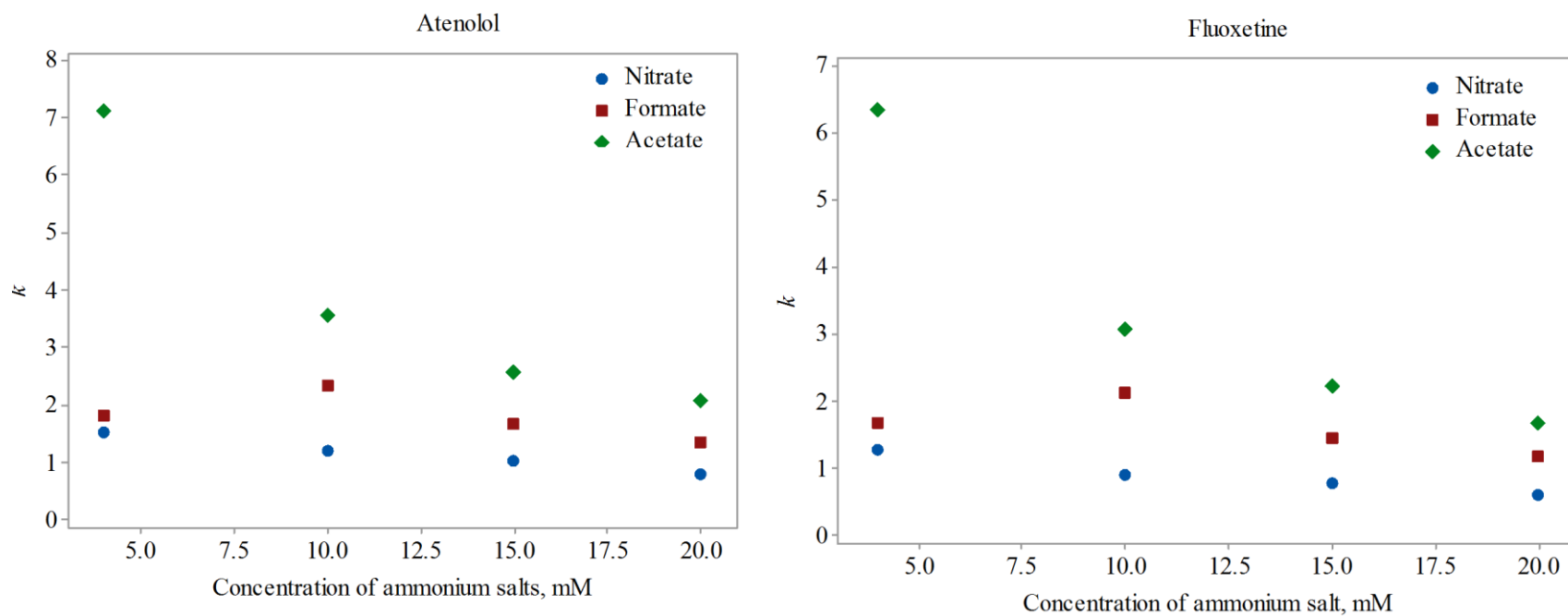


Figure 2-2 Effect of type and concentration of liophilic ions on retention factor of the first eluted enantiomer of atenolol and fluoxetine in PIM with pH kept at 6.7.

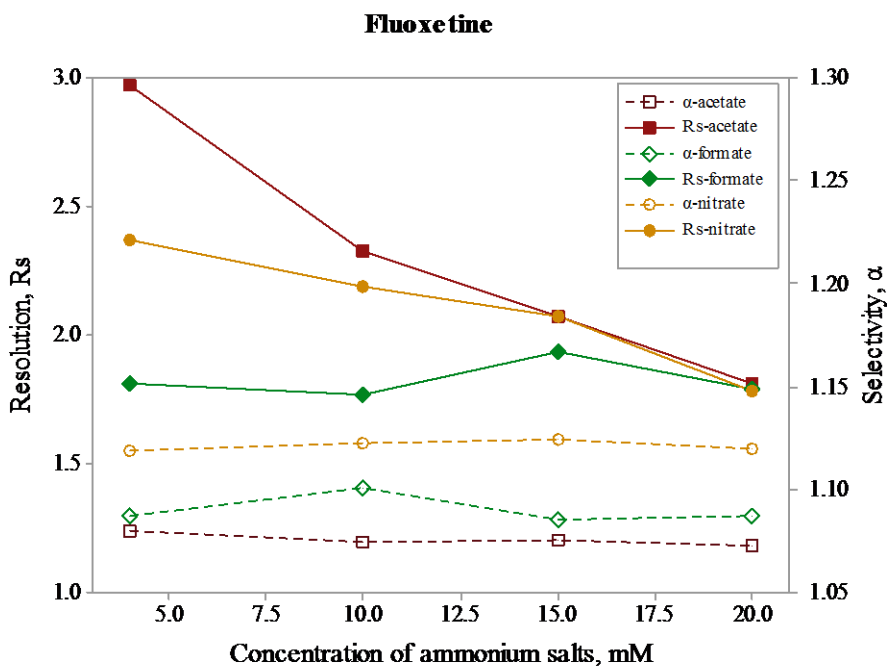
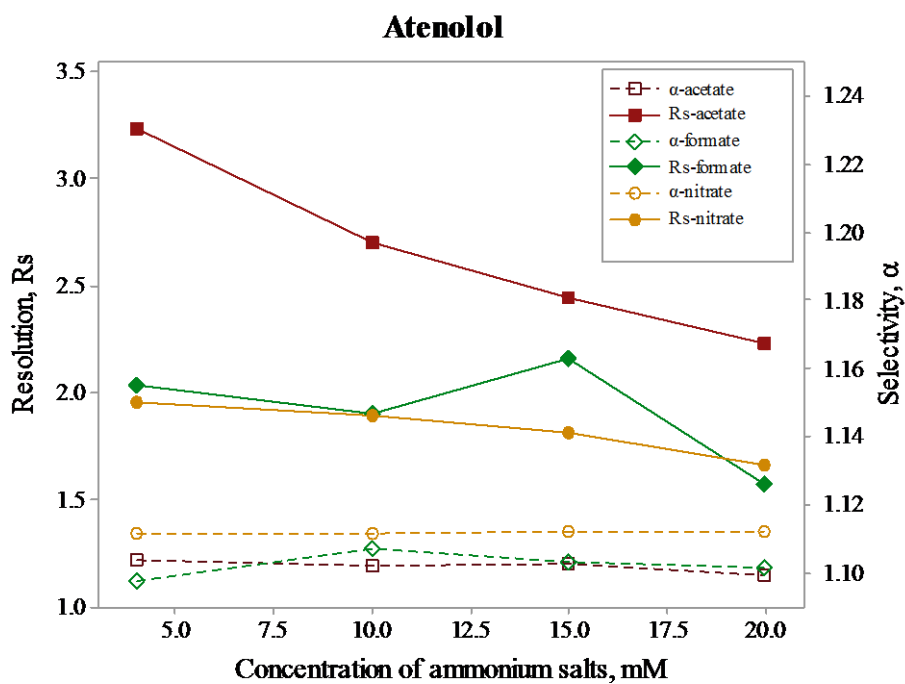


Figure 2-3 Effect liophilic ions on Rs and  $\alpha$  of the enantiomers of atenolol and fluoxetine.

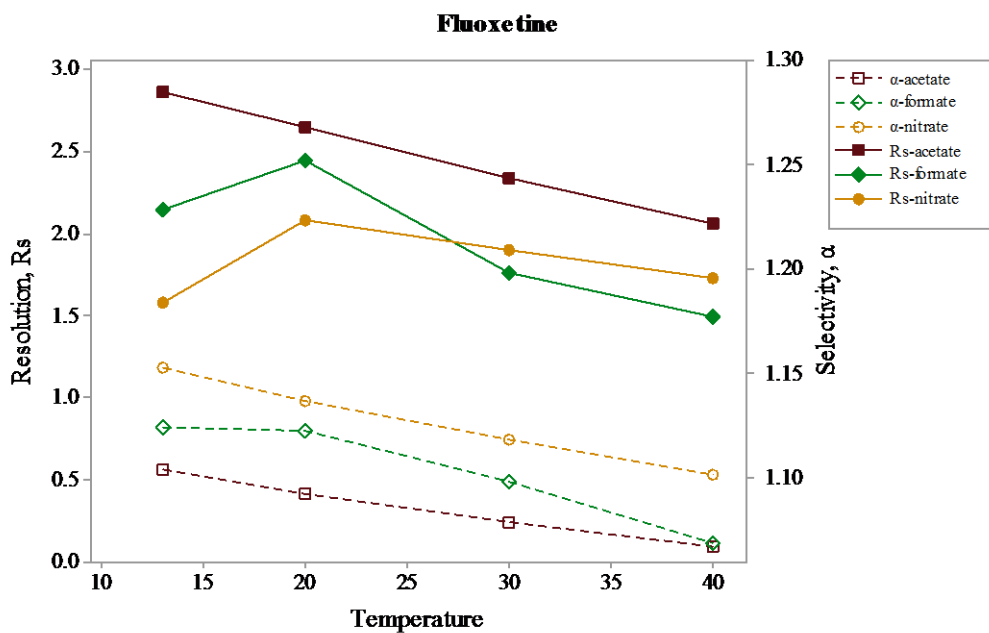
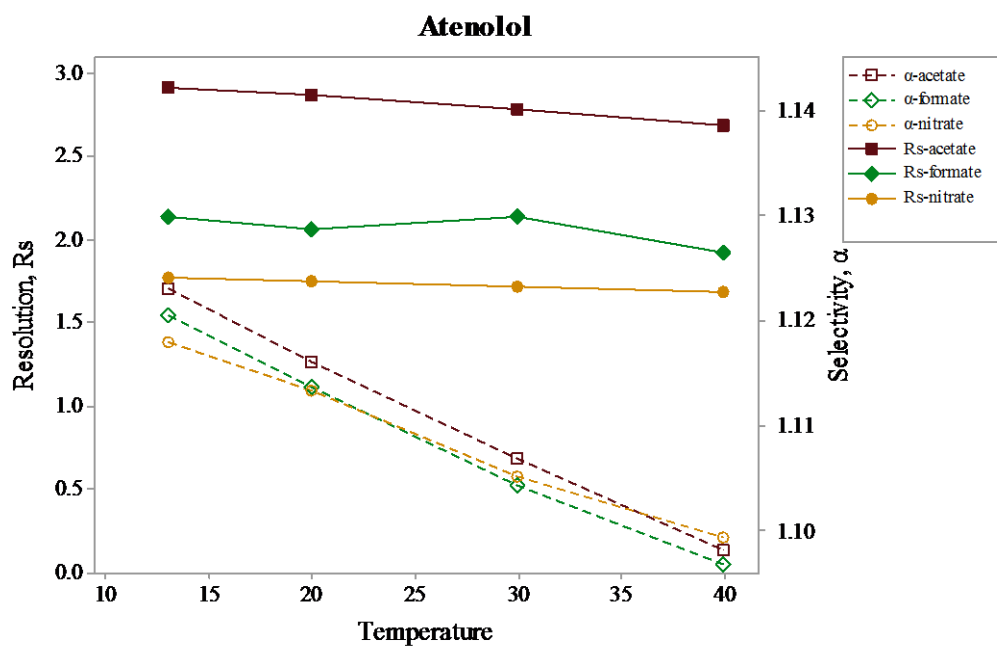


Figure 2-4 Effect of temperature on resolution and selectivity of atenolol and fluoxetine using a mobile phase comprising of methanol and 10 mM ammonium salts.



## Chapter 3 Enantiomeric Selectivity in Adsorption of Chiral $\beta$ -Blockers on Sludge

### 3.1 Introduction

There is a growing interest on the environmental fate, transport and risks of pharmaceuticals since they are designed to effect a biological response at low doses (Brooks et al., 2003; Flaherty and Dodson, 2005; Huerta et al., 2012; Stamm et al., 2008). A wide range of human pharmaceuticals, such as antidepressants (Metcalf et al., 2010; Schultz and Furlong, 2008; Schultz et al., 2010), analgesics (Gracia-Lor et al., 2010; Kosjek et al., 2005; Ziylan and Ince, 2011), antibiotics (Hernández et al., 2007; O'Connor and Aga, 2007),  $\beta$ -blockers (MacLeod et al., 2007; Ribeiro et al., 2014), and steroidal hormones (Kolpin et al., 2002), have been detected in the environment at concentrations ranging from ng/L to  $\mu$ g/L. Pharmaceuticals are pseudo-persistent and may cause sublethal or chronic toxicity to nontarget organisms (Crane et al., 2006; Niemi et al., 2013). To better predict the environmental fate of pharmaceuticals and assess their risks, it is essential to obtain accurate parameters such as phase distribution coefficients ( $K_d$ ) that govern their behaviors in both engineered and natural systems.

The determination of fundamental parameters such as  $K_d$  is complicated by the fact that over 50% of current-use pharmaceuticals are chiral and may display stereoselectivity in environmental processes (Evans and Kasprzyk-Hordern, 2014). Pharmaceuticals enter the environment via wastewater plants (WWTPs) where they are partially removed via abiotic and biotic processes such as adsorption and biodegradation. A number of studies show that biodegradation of chiral pharmaceuticals are often stereoselective, resulting in

different concentration profiles of enantiomers for the same compounds in WWTP effluents (Barclay et al., 2012; Fono and Sedlak, 2005; Moreira et al., 2014; Ribeiro et al., 2012). To date, however, stereoselectivity in phase distribution processes has been largely overlooked. It is known that humic substances, which are abundant in sludge at WWTPs, contain chiral centers (Oravec et al., 2010). It may be hypothesized that surface interactions of chiral pharmaceuticals with sludge are likely stereoselective. Moreover, WWTPs often receive surfactants in their influents, and many surfactants are also chiral compounds. It may be further expected that the co-existence of surfactants may modify the phase distribution of chiral pharmaceuticals between sludge and water.

In this study we used  $\beta$ -blockers as model chiral pharmaceuticals to evaluate the occurrence of stereoselectivity in their adsorption to sludge materials and explore the influence of surfactants on this interaction.  $\beta$ -Blockers, such as atenolol, acebutolol, metoprolol, pindolol and propranolol, are among the most widely used chiral pharmaceuticals. Monitoring studies have shown ubiquitous occurrence of  $\beta$ -blockers in wastewater effluents (Caballo et al., 2015; MacLeod and Wong, 2010; Vazquez-Roig et al., 2014), and in some cases, different concentration profiles of enantiomers in sludge (Evans et al., 2015). In addition to biodegradation, studies also demonstrated adsorption as an important removal pathway for  $\beta$ -blockers at WWTPs (Baker and Kasprzyk-Hordern, 2011; Wick et al., 2009).  $\beta$ -Blockers are highly polar weak bases (Stevens-Garmon et al., 2011), and their partition into sludge may be attributed mainly to electrostatic interactions, hydrogen bonding and van der Waals forces (Berthod et al., 2014). However, it is unknown if selectivity in adsorption to sludge would contribute to enantiomer-specific behaviors of

these compounds at WWTPs. Furthermore, surfactants are common chemical constituents in WWTP influents and co-existence of certain surfactants was previously shown to influence the adsorption of pharmaceuticals to solid matrices (Hari et al., 2005). However, no research to date has considered the potential effect of various surfactants on the stereoselectivity of chiral pharmaceuticals in their phase distribution among WWTP compartments. Results from this study are expected to help ascertain stereoselectivity of chiral pharmaceuticals during wastewater treatment processes, and improve our understanding of mechanisms underlying adsorption of basic chiral pharmaceuticals such as  $\beta$ -blockers to sludge.

## 3.2 Materials and Methods

### 3.2.1 Materials

A set of commonly used and environmentally relevant  $\beta$ -blockers was considered in this study. As shown in Table 3-1, the selected  $\beta$ -blockers, acebutolol, atenolol, metoprolol, pindolol, propranolol and salbutamol, have different  $\log K_{ow}$  and  $pK_a$  values. Standards of the  $\beta$ -blockers were purchased as racemic mixtures from Sigma-Aldrich (St. Louis, MO) with minimum purity of 99%. Analytical grade methanol and ammonium trifluoroacetate with purity >99% were obtained from Sigma-Aldrich.

Different types of surfactants were used for this study to evaluate their effect on the adsorption of  $\beta$ -blockers to sludge (Table 3-2). These included the nonionic surfactant Triton X 100, anionic surfactant sodium dodecyl sulfate (SDS), cationic surfactant

cetyltrimethylammonium bromide (CTAB), and chiral cationic surfactant (1R,2S)-(-)-N-dodecyl-N-methylephedrinium bromide (DMEB). All surfactants were purchased from Fisher Scientific (Fair Lawn, NJ).

### 3.2.2 Sludge

Samples of secondary sludge were collected from the aeration basins at the Riverside Water Quality Control Plant (Riverside, CA) and Eastern Municipality Water District (Moreno Valley, CA) in southern California that treated municipal waste at 151,000 m<sup>3</sup>/d and 60,500 m<sup>3</sup>/d, respectively. The sludge was collected by grab sampling and stored at 4 °C until use. The concentration of the total suspended solids in the sludge was determined gravimetrically. The sludge sample was centrifuged and then freeze-dried for 2 d to obtain the dry material. The freeze-dried sludge was further sieved, homogenized and sterilized by autoclaving for 20 min at 121 °C. Although autoclaving may potentially alter the physicochemical properties of sludge, it is a commonly used method for sterilization and was used in this study to inhibit biodegradation during adsorption measurements. The sterilized sludge was stored at -20 °C before use.

### 3.2.3 Adsorption Experiments

Adsorption of the selected  $\beta$ -blockers to sludge before and after surfactant modification was determined using the batch-equilibration method. To mimic the solid-water interactions in WWTPs, the sterilized sludge was diluted to approximately 2,000 mg/L using deionized water. The suspension was autoclaved and kept in a fume hood

overnight to ensure hydration of the sludge. The autoclaved sludge from Riverside Water Quality Plant was used to determine the adsorption isotherms of the  $\beta$ -blockers. The isotherms were constructed using five adsorbate concentrations (10, 20, 50, 100 and 200 ng/mL). To evaluate the effect of co-existence of surfactants, the sludge suspension was amended with a surfactant at 10 mg/L, and the selected concentration was below the critical micelle concentration (CMC) of the selected surfactants (Table 3-2) to avoid the formation of micelles during the adsorption measurement. The effect of surfactants was investigated using a single  $\beta$ -blocker concentration of 100 ng/mL. For all adsorption measurements, 8 mL of sludge suspension was placed in a 10 mL-glass tube with Teflon lined-screw cap and mixed on a mechanical shaker at 150 rpm for 48 h at ambient temperature. Previous studies on both soils and sludge showed that  $\beta$ -blockers would reach phase equilibrium within 48 h of equilibration (Barron et al., 2009; Hari et al., 2005). The initial pH of the samples was approximately 7.4. After equilibration, the slurry was centrifuged at 4,000 rpm for 30 min, and approximately 1.0 ml supernatant was withdrawn and filtered through a 0.22- $\mu$ m polytetrafluoroethylene (PTFE) filter membrane (Millipore, Carrigtwohill, Cork, Ireland) before instrumental analysis.

To ensure the rigorousness of adsorption measurement, triplicate samples were used for each chemical concentration step. A control with only the sludge suspension (2,000 mg/L) but no analytes was included and similarly analyzed. The background concentrations of the selected  $\beta$ -blockers were below the detection limits. Furthermore, adsorbent-free controls were included using deionized water spiked with five concentrations (10, 20, 50, 100 and 200 ng/mL) of the  $\beta$ -blockers. No apparent chemical

loss due to photodegradation, hydrolysis, adsorption to the glass wall, or chiral inversion was observed.

The adsorption isotherms were fitted to the Freundlich adsorption model using SigmaPlot (version 13, San Jose, CA) as described below:

$$C_s = K_f C_w^n \quad (3-1)$$

where  $C_s$  represents the chemical concentration on the sludge at equilibrium (ng/kg),  $C_w$  is the chemical concentration in the aqueous phase (ng/L), the dimensionless value  $n$  indicates the degree of linearity and  $K_f$  is the Freundlich adsorption coefficient ( $\text{ng}^{1-n} \text{L}^n/\text{kg}$ ). The adsorption isotherms were assumed to be linear when  $n$  equals 1 at the 95% confidence interval. For linear adsorption isotherms, the data were subsequently fitted to:

$$C_s = K_d C_w \quad (3-2)$$

where  $K_d$  is the linear adsorption coefficient.

### 3.2.4 Chiral Separation and Analysis

Chiral separation of the selected  $\beta$ -blockers was performed on an Agilent 1100 high performance liquid chromatograph (HPLC) (Agilent, Wilmington, DE, USA) interfaced with an electrospray ionization mass spectrometer (Thermo Finnigan LCQ Deca XP Plus San Jose, CA) (ESI LC-MS/MS). After passing through the 0.22- $\mu\text{m}$  membrane, the filtrate was injected directly into the system for quantitative analysis. Samples (10  $\mu\text{L}$ ) were injected by an autosampler onto a Chirobiotic V column (4.6 mm  $\times$  250 mm, particle size

5  $\mu\text{m}$ ) with a guard column (10 mm  $\times$  20 mm, particle size = 5  $\mu\text{m}$ ), all purchased from Sigma Aldrich (St. Louis, MO). A mobile phase comprising methanol and ammonium trifluoroacetate in methanol (0.1% w/v) at 40/60 (v/v) was used to elute the enantiomers in the isocratic mode at a flow rate of 0.5 mL/min. The run time was 35 min and the retention times for individual enantiomers are shown in Table S3-1. Mass spectra were acquired using ESI in the positive ion mode. The ionization voltage was +4.5 kV, collision energy 25 eV, auxiliary gas temperature 350  $^{\circ}\text{C}$ , and the nebulizing and auxiliary gas settings were 60 and 20 arbs, respectively. Table S3-2 shows the monitored precursor and product ions and instrument parameters for each  $\beta$ -blocker considered in this study.

Quantitation was achieved using matrix-matched external standards to compensate for signal suppression or enhancement caused by the matrix. The standards were prepared in sterilized sludge (2,000 mg/L) to a final concentrations ranging from 2 to 200 ng/ml. The sign of optical rotation for each enantiomer was confirmed using an in-line laser polarimeter detector (PDR-Chiral, Lake Park, FL). The limit of detection was calculated as three times the standard deviation of the lowest detected concentration.

### 3.2.5 Quality Assurance and Data Analysis

The linearity, repeatability and enantiomer resolution were established for the analytical method (Figure S3-1, Figure S3-2 and Table S3-1). Deming regression analysis was used to test matrix effects (Figure S3-3 and Table S3-1). All data are presented as mean and standard deviation of triplicates. A one-way ANOVA test was conducted at

$\alpha=0.05$  to evaluate the significance of differences among the treatments. Statistical analysis was performed using SigmaPlot.

### 3.3 Results and Discussion

#### 3.3.1 Adsorption and Stereoconfiguration Data Quality

The enantiomers were completely resolved under the used analytical conditions (Figure S3-1 and Table S3-1), with  $R_s > 1.0$ . The optical activity for each enantiomer was used to establish its exact stereoconfiguration (Table S3-1). The first eluted peak of each compound was identified as the (-)-enantiomer and the second the (+)-enantiomer. Furthermore, (-) and (+)-enantiomers of the five  $\beta$ -blockers corresponded to the (*S*)- and (*R*)-enantiomers, respectively. A Deming regression analysis comparing matrix matched standards and calibration standards in deionized water gave a slope greater than 0.80. Hence, at the 95% confidence level, matrix matched external calibration curves compensated for matrix effects.

Fitting  $C_s$  values obtained through the aqueous phase loss method and  $C_w$  at phase equilibrium to the Freundlich equation consistently generated a good fit (Table S3-3). Calculation for  $C_s$  was based on the assumption that a decrease in the mass of  $\beta$ -blockers in the aqueous phase corresponded to the amount adsorbed to the sludge solid phase. The  $K_d$  values for surfactant-modified sludge were further determined using a single point concentration (100 ng/mL) (Table 3-3) The maximum standard deviation in all adsorption experiments, with or without surfactant modification, was approximately 30 L/kg for (*S*)-



acebutolol following amendment with DMEB. The standard errors in the determination of  $K_f$  values were less than 11.4.

### 3.3.2 Adsorption in Sludge in Absence of Surfactants

The effect of chirality on adsorption of  $\beta$ -blockers to sludge was investigated using five  $\beta$ -blockers in triplicate to determine the reproducibility of derived EF values and  $K_d$ . The degree of stereoselectivity in adsorption was determined using the enantiomeric fraction ( $EF = (R)/(S + R)$ ) of the  $\beta$ -blockers adsorbed on the sludge. Stereoselectivity in adsorptive behavior was observed when  $EF \neq 0.5$ , that is, when  $EF < 0.5$ , the (*S*)-enantiomer preferentially adsorbs to the sludge, whereas when  $EF > 0.5$  the antipode would be preferred. For each  $\beta$ -blocker, the average EF value was measured in 18 samples, comprising 15 samples from the Riverside Water Quality Control sludge (comprising analytes spiked from 10 to 200 ng/mL) and 3 samples from the Eastern Municipality Water District (Figure 3-1). Atenolol and acebutolol demonstrated overall stereoselectivity, but pindolol and propranolol had average EF values of approximately 0.5. All further investigations were subsequently conducted using the Riverside sludge. The mean EF values for acebutolol, atenolol and metoprolol on the Riverside sludge were 0.27, 0.55 and 0.32, respectively (Figure 3-2 and Figure S3-4). Evans et al. (2015) reported that the EF values of atenolol, metoprolol and propranolol in digested sludge from a wastewater plant in the U.K. were  $0.4 \pm 0.0$ ,  $0.3 \pm 0.3$  and  $0.5 \pm 0.1$ , which were comparable to our finding. Our observations suggest that the stereoselectivity observed in the Evans et al. study could be due to selective adsorption of enantiomers.

The Freundlich adsorption isotherms showed a non-linearity index close to unity, suggesting that the relationship between  $C_s$  and  $C_w$  was generally linear. The adsorption data were subsequently fitted to a linear relationship to estimate  $K_d$ . The fit was good with  $r^2$  values greater than 0.94, except for metoprolol (Table S3-3). The linearized isotherms of  $\beta$ -blockers on the sludge sample are depicted in Figure S3-5. The  $K_d$  values on sludge and surfactant-modified sludge, the charge and number of neutral species of the  $\beta$ -blockers at pH 7.4 are listed in Table 3-3. Statistically significant differences between  $K_d$  values of the enantiomers were observed for acebutolol, atenolol and metoprolol ( $p < 0.006$ ). The  $K_d$  values for (*R*)-enantiomers were twice that of the corresponding (*S*)-enantiomers of acebutolol and metoprolol. For example, (*S*)-acebutolol had a  $K_d$  value of  $109 \pm 11$  L/kg, as compared to  $52 \pm 13$  L/kg for (*R*)-acebutolol. However, stereoselectivity was not observed in the adsorption of enantiomers of pindolol or propranolol ( $\alpha = 0.05$ ,  $p = 0.834$  and  $0.836$ , respectively) in the sludge. The  $K_d$  values of propranolol and pindolol (between 304 and 375 L/kg) were also substantially larger than that for atenolol, acebutolol or metoprolol (ranging from 22 to 134 L/kg). Pindolol and propranolol are moderately hydrophobic compounds with  $\log K_{ow}$  of 1.84 and 2.76, respectively. The relatively high hydrophobicity of pindolol and propranolol may have contributed to their stronger adsorption on the solids. Previous studies showed a similar trend reporting low  $K_d$  values for acebutolol, atenolol and metoprolol in sludge, sediment or soil ( $<150$  L/kg), but moderate to high values for pindolol and propranolol ( $>150$  L/kg), although stereoselectivity was not examined in these studies (Maurer et al., 2007; Radjenović et al., 2009; Ramil et al., 2010; Yamamoto et al., 2009).

### 3.3.3 Adsorption in Sludge Amended with Surfactants

Surfactants are present in WWTP as co-sorbates and their effect on adsorption of  $\beta$ -blockers was evaluated by amending sludge with different types of surfactants (Figure 3-2 and Figure S3-4). When the sludge was modified with an anionic surfactant, SDS, the  $K_d$  values of acebutolol and metoprolol increased. For example, the  $K_d$  of (*R*)- and (*S*)-metoprolol increased from  $22 \pm 8$  to  $58 \pm 6$  L/kg and  $57 \pm 8$  to  $96 \pm 15$  L/kg, respectively. However, for atenolol,  $K_d$  of (*R*)-atenolol decreased from  $134 \pm 3$  to  $112 \pm 4$  L/kg, and while that of (*S*)-atenolol increased from  $123 \pm 2$  to  $155 \pm 11$  L/kg. Pindolol and propranolol only exhibited a small change in  $K_d$  and no discernable change in EF values. At pH 7.4, the  $\beta$ -blockers were positively charged and they may have formed ion pairs with the anionic surfactant that had higher apparent hydrophobicity, resulting in a higher affinity for the sludge surfaces (Sütterlin et al., 2008). In a similar study without consideration of chirality, adsorption of negatively charged nalidixic acid on natural aquifer materials increased by approximately 400% in the presence of a cationic surfactant cetylpyridinium chloride (Hari et al., 2005). However, in the same study, there was no apparent change in adsorption of a similarly charged norfloxacin. Assuming ion pair formation is the dominant mechanism governing adsorption of ionogenic compounds in the presence of charged surfactants, then a similar observation may be expected for the effect of cationic surfactants on negatively charged compounds (Kibbey et al., 2007).

When the sludge was amended with a cationic chiral (CTAB) or achiral (DMEB) surfactant, there was no apparent change in the amount of  $\beta$ -blockers sorbed except for

metoprolol (Figure 3-2). This suggests that there was probably little, if any, sorbate-sorbate interaction between the positively charged surfactant and the positively charged  $\beta$ -blockers. The lack of sorbate-sorbate interaction could be due to the low concentration of the surfactant in the system or the weakness of the repulsive forces in influencing adsorption. Again, adding CTAB or DMEB did not alter the adsorption of the more hydrophobic  $\beta$ -blockers pindolol and propranolol. However, the amount of (*R*)- and (*S*)-metoprolol sorbed to sludge increased by approximately 100 and 47%, respectively, on the CTAB-modified sludge.

Amending sludge with a nonionic surfactant, Triton X 100 decreased the stereoselectivity in the adsorption of metoprolol and acebutolol, but switched the enantiomeric preference for atenolol. Adsorption of (*R*)-atenolol was preferred without the surfactant, but addition of Triton X 100 resulted in (*S*)-atenolol being preferentially adsorbed to the sludge. However, the EF values for metoprolol and acebutolol approached 0.5, from  $0.27 \pm 0.05$  to  $0.42 \pm 0.07$  and  $0.32 \pm 0.07$  to  $0.43 \pm 0.02$ , respectively, following amendment of Triton X 100. Furthermore, Triton X 100 increased adsorption of (*S*)-atenolol, acebutolol and metoprolol, but decreased that of (*R*)-atenolol. However, there was no apparent change in adsorption of propranolol in the presence of Triton X 100. This suggests that the co-occurrence of neutral surfactants may affect the stereoselective distribution of ionizable chiral micropollutants in the environment.

### 3.3.4 Effect of Hydrophobicity in Stereoselective Adsorption

This study demonstrated for the first time that adsorptive behavior of less hydrophobic ionizable micropollutants in sludge was stereoselective. Figure 3-3 shows the effect of hydrophobicity on stereoselectivity in the adsorption of the selected  $\beta$ -blockers on sludge. The EF values of  $C_s$  were determined at five environmentally relevant concentrations ranging from 10 to 200 ng/ml using secondary sludge collected from the Riverside Water Quality Control Plant (Figure 3-2 and Figure S3-4). The extent of enantiomeric discrimination in adsorption varied with the hydrophobicity of the  $\beta$ -blockers, with less hydrophobic compounds (atenolol, acebutolol and metoprolol) showing a greater degree of stereoselectivity. Atenolol was the least hydrophobic compound and its *R*-enantiomer was preferentially adsorbed to the sludge solids while the level of the *S*-enantiomer was elevated in the aqueous phase. In contrast, the (*S*)-enantiomer was preferred over the antipode for metoprolol and acebutolol which are more hydrophobic than atenolol, but less than pindolol or propranolol. However, the more hydrophobic  $\beta$ -blockers, propranolol and pindolol, had EF values close to 0.50 ( $0.50 \pm 0.02$  and  $0.48 \pm 0.02$ , respectively). These observations together suggest that there was likely a dependence between stereoselectivity in adsorption and a compound's hydrophobicity.

The relationship between hydrophobicity and stereoselectivity in adsorption was further assessed using a 3D surface plot constructed using  $\log D_{\text{pH}=7.4}$  and the relative hydrophobicity values (measured in equivalent methylene units,  $N(\text{CH}_2)$ ) at pH 7.4 (Gulyaeva et al., 2002) to predict EF (Figure 3-3). At high  $\log D$  and  $N(\text{CH}_2)$ , there was

limited to no stereoselectivity, and inverse enrichment of (*S*)-enantiomers in aqueous phase was predicted. However, at high  $\log D$  and low  $N(\text{CH}_2)$ , enrichment of (*R*)-enantiomers in the aqueous phase was predicted. Enantiomeric enrichment occurred below 12  $N(\text{CH}_2)$  units and remained constant above 12 units. Relative hydrophobicity is known as a critical parameter in pharmacodynamics and pharmacokinetics. For instance, Gulyaeva et al. demonstrated that  $\log D$  and  $N(\text{CH}_2)$  were better chemical descriptors for understanding distribution and adsorption of  $\beta$ -blockers in biological systems using quantitative structure–activity relationship analysis (Gulyaeva et al., 2002). At high  $\log D$  and  $N(\text{CH}_2)$ , adsorption on sludge is mainly caused by hydrophobic interactions. In the present study, at pH 7.4, the fraction of species in neutral form was less than 0.70 % (predicted using MarvinSketch version 15.11, Cambridge, MA). Thus, ionic interactions are expected to drive adsorption onto the sludge, especially for relatively less hydrophobic compounds like atenolol and metoprolol. However, Kibbey et al. observed that the type of species abundant in a system at a given pH does not necessarily govern the adsorption mechanism if there are other competing species that exhibit a stronger affinity for the sorbent (Kibbey et al., 2007). For example, the adsorption behavior of pindolol and propranolol was potentially dominated by hydrophobic interactions even though less than 0.60% species were in the neutral form. Furthermore, adsorption of pindolol and propranolol was mainly non-stereoselective potentially due to the dominance of hydrophobic interactions. Hence, the stereoselective sorptive behavior observed for acebutolol, atenolol and metoprolol may be attributed to the stereoselective ionic and/or hydrogen bonding interactions. In a previous study on the stereoselectivity in adsorption of  $17\alpha$  and  $17\beta$ -trenbolone, Khan et al. (2009)

noted that the ionic and hydrogen bonds were stereoselective, thus promoting enantiomeric adsorption, whereas hydrophobic interactions were not selective.

### 3.4 Environmental Implications

Enantiomer selectivity in adsorption of chiral compounds in wastewater and the environment has been generally ignored. This study offered evidence that adsorption of ionizable chiral compounds to environmental surfaces may be stereoselective. Thus, enantiomeric enrichment may occur during wastewater treatment due to selective adsorption of enantiomers to sludge as a removal process. For example, the EF value for atenolol was  $0.55 \pm 0.03$ , indicating that there was a higher concentration of (*R*)-atenolol sorbed to the sludge than (*S*)-atenolol. Hence, a higher level of (*S*)-atenolol may be expected in the effluent if adsorption is the dominant removal pathway. Several studies reported relatively higher concentrations of (*S*)-atenolol during wastewater treatments (MacLeod et al., 2007; Nikolai et al., 2006). Therefore, chirality is an important parameter regulating phase distribution of enantiomers of chiral pharmaceuticals in both treated effluents and biosolids.

The results obtained in the present study showed that phase distribution of ionogenic micropollutants such as  $\beta$ -blockers in WWTPs may be influenced by their chirality as well as the co-occurrence of other chemical constituents (e.g., surfactants). Anionic surfactants increased the adsorption of relatively less hydrophobic (acebutolol and metoprolol)  $\beta$ -blockers as compared to neutral or cationic surfactants. This observation suggests formation of ion pairs between the  $\beta$ -blockers and surfactant. The resultant ion pair

complex likely increased hydrophobicity and thus decreased stereoselectivity. Therefore, enantiomer specific partitioning in the sludge may diminish in the presence of surfactants. Additionally, the effect of chiral surfactants on adsorption may provide more insights on the adsorption of ionizable compounds on sludge. Some surfactants prevalent in the environment, especially biosurfactants, are chiral compounds, and may form diastereomeric ion pairs with chiral compounds, contributing to enhanced stereoselectivity in adsorption. Furthermore, surfactants are ubiquitously found in the environment, but at varying concentrations. An important question for further research is the effect of levels of surfactants on adsorption of pharmaceuticals on sludge.

Stereoselectivity in adsorption can potentially be predicted from chemical descriptors, such as hydrophobicity and  $\log D$ . Our results indicate that stereoselectivity in adsorption increased with decreases in hydrophobicity of the sorbate. For example, atenolol and metoprolol used in this study exhibited stereoselective adsorption on sludge. The EF values reported by other researchers were in general agreement with our findings, even though the earlier studies did not consider the exact stereoconfiguration of the enantiomers of the  $\beta$ -blockers (Evans et al., 2015). Thus, it may be expected that pindolol and propranolol are present in biosolids or digested sludge as a racemic mixture whereas less hydrophobic compounds such as atenolol, acebutolol and metoprolol (to a lesser extent) may show enantiomeric enrichment.

This study only considered the adsorption of basic pharmaceuticals, and there is a need to evaluate stereoselectivity in adsorption of neutral and acidic pharmaceuticals, such



as ibuprofen, ketoprofen, naproxen, among others. Stereoselectivity is enhanced by polar interactions and diminished by non-specific hydrophobic interactions. It is valuable to quantitatively establish the contribution of each mechanism on selective adsorption of chiral compounds. In addition, given the role of polar interactions, it may be also important to understand the effect of ionic strength, pH, surface charges of the sorbent and charge density of the sorbate.

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Tables

Table 3-1 Physicochemical properties of  $\beta$ -blockers.

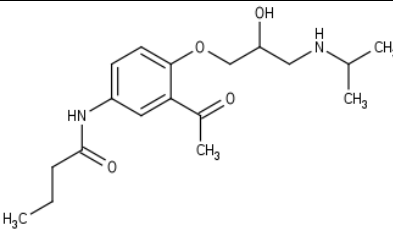
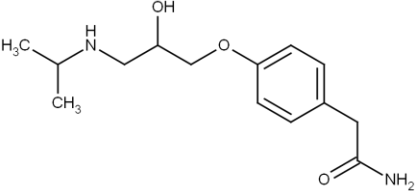
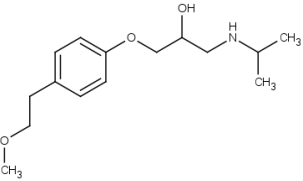
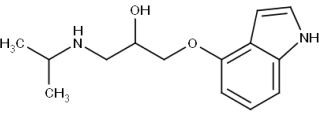
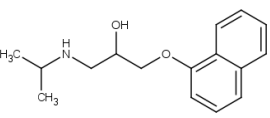
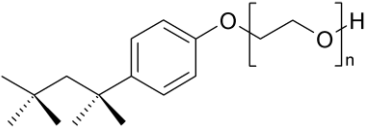
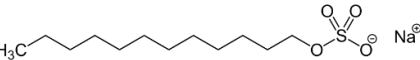
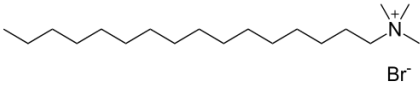
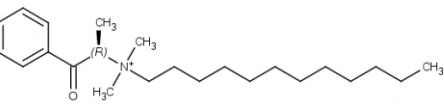
$\beta$ -blocker	Structure	MW, g/mol	Water solubility, mg/L	$\log K_{ow}$	$pK_a$
Acebutolol		336.20	259	1.71	9.2
Atenolol		266.16	13,300	0.16	9.6
Metoprolol		267.18	16,900	1.69	9.7
Pindolol		248.15	7,880	1.84	9.67
Propranolol		259.16	61.7	2.76	9.67

Table 3-2 Physicochemical properties of surfactants.

Surfactant	Type	Structure	MW (g/mol)	CMC (mM)	HL B
Triton X 100	Nonionic		647	189	13.4
SDS	Anionic		288	7.1	40
CTAB	Cationic		364.45	0.9	10
DMEB	Chiral cationic		428.49	4.0	-

MW molecular weight

CMC critical micelle concentration

HLB hydrophile-lipophile balance

Table 3-3 The K<sub>d</sub> values (L/kg) of five β-blockers estimated using single point calculations on surfactant-modified sludge.

Compound		No surfactant	Triton X 100	SDS	CTAB	DMEB
Atenolol	S	123 ± 2 <sup>a</sup>	147 ± 11 <sup>a</sup>	155 ± 11 <sup>a</sup>	122 ± 5 <sup>a</sup>	203 ± 22 <sup>a</sup>
	R	134 ± 3	117 ± 5	112 ± 4	100 ± 6	157 ± 13
Acebutolol	S	109 ± 11 <sup>a</sup>	151 ± 11 <sup>a</sup>	131 ± 8 <sup>a</sup>	122 ± 18	92 ± 30
	R	52 ± 13	113 ± 13	94 ± 2	84 ± 22	51 ± 9
Metoprolol	S	57 ± 8 <sup>a</sup>	86 ± 16	96 ± 15 <sup>a</sup>	84 ± 10 <sup>a</sup>	71 ± 6 <sup>a</sup>
	R	22 ± 8	65 ± 27	58 ± 5	46 ± 3	52 ± 7
Pindolol	S	374 ± 8	330 ± 8	320 ± 21	343 ± 22	299 ± 2
	R	372 ± 11	329 ± 14	323 ± 21	344 ± 25	303 ± 10
Propranolol	S	307 ± 17	308 ± 1	310 ± 4	284 ± 19	272 ± 4
	R	304 ± 16	302 ± 4	302 ± 4	286 ± 13	262 ± 7

<sup>a</sup>At  $\alpha = 0.05$ ,  $p < 0.050$  thus there is a significant difference between the mean of (R) and (S)-enantiomers.

SDS sodium dodecyl sulfate

CTAB cetyltrimethylammonium bromide

DMEB (1R,2S)-(-)-N-dodecyl-N-methylephedrinium bromide



Figures

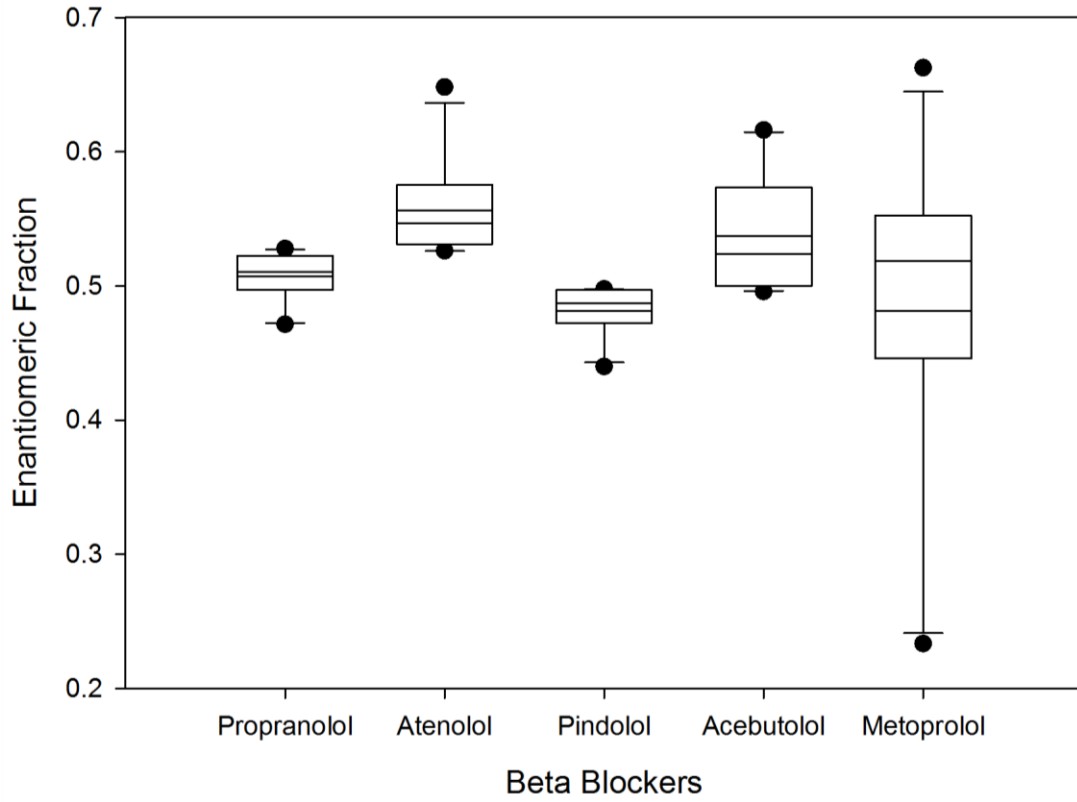


Figure 3-1 Differences in the enantiomeric fraction of  $\beta$ -blockers at different concentrations sorbed on two types of sludge.

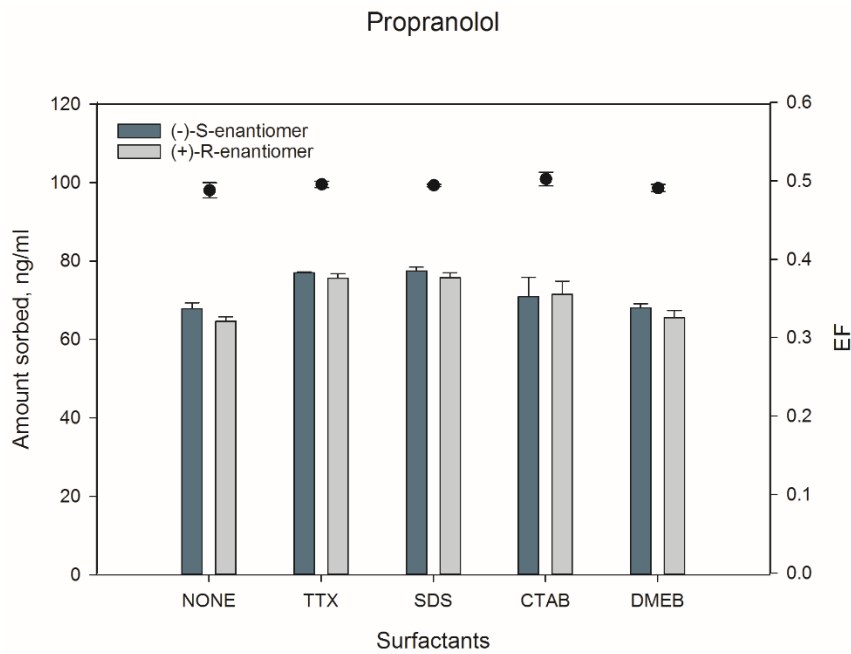
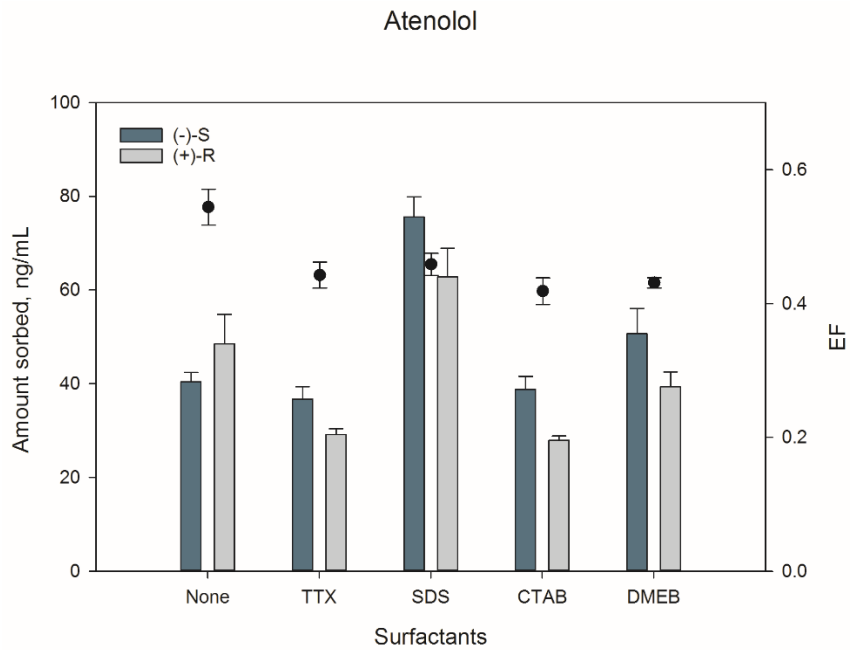


Figure 3-2 Effect of amending sludge with surfactants on the amount of atenolol and propranolol sorbed and the stereoselectivity in adsorption.

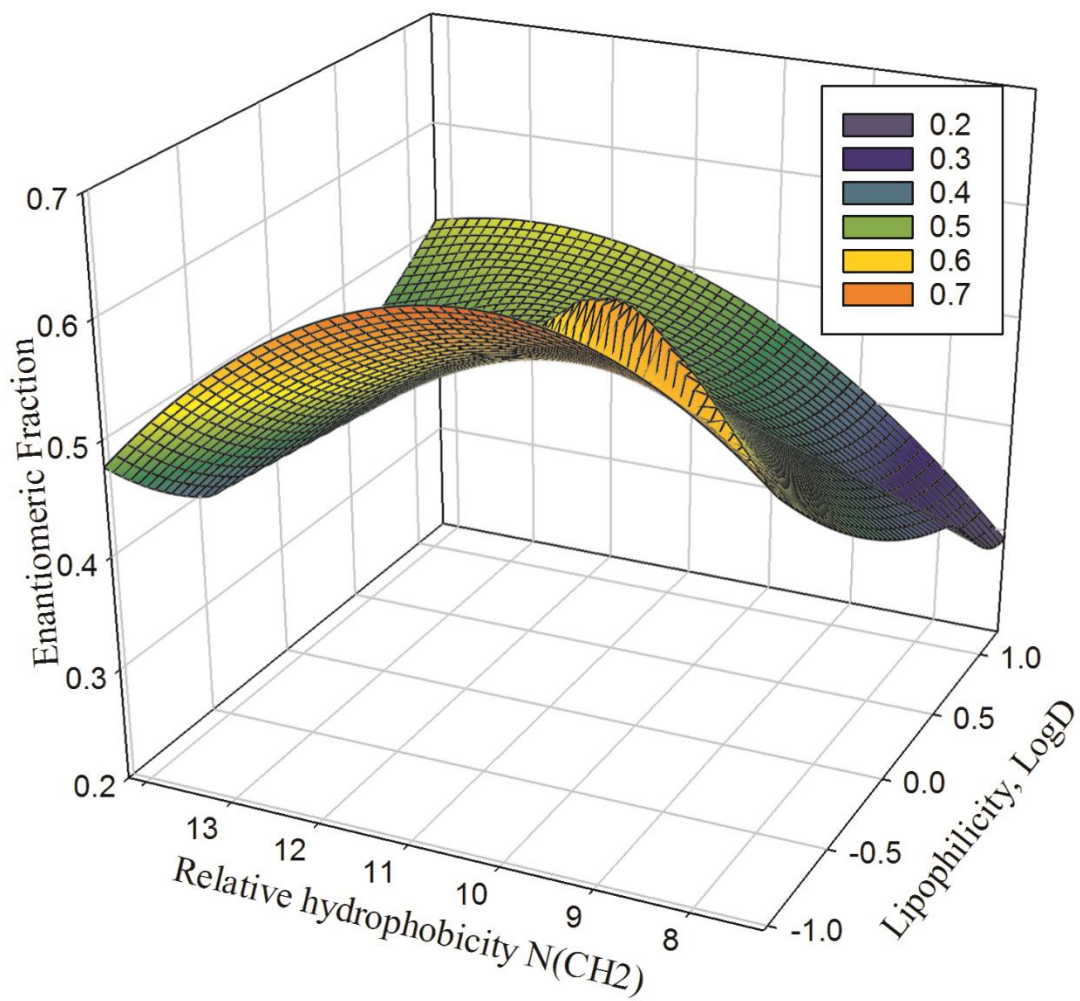


Figure 3-3 A correlation between hydrophobicity of the compounds and stereoselectivity.

## Supporting Information

We establish the identity of the  $\beta$ -blockers in this study using the retention time and MRM transitions, and optical rotation following separation and detection with LC-MS/MS and polarimeter. Enantioresolution ( $R_s$ ) of the  $\beta$ -blockers was calculated from equation S3-1.

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2} \quad (\text{S3-1})$$

Where  $t_1$  and  $t_2$  and  $w_1$  and  $w_2$  are the retention times and base width of the first and second eluting enantiomer, respectively. A baseline separation was achieved when  $R_s \geq 1.5$ . Quantification was considered adequate when  $R_s \geq 0.9$ .

To compensate for matrix effects matrix match external calibration standards were used. Approximately 2 g<sub>ss</sub>/L sludge was prepared and allowed to hydrate while mixed gently on a mechanical shaker. The suspension was autoclaved and then filtered using a 0.2-micron syringe filter. Each compound was dissolved in the filtrate to prepare the matrix matched solution (ranging from 5.0 to 100 ng/mL) and were injected in triplicate. Repeatability was determined by injecting the matrix matched standard 15 times. The limit of detection was determined from the standard deviation of the response of the lowest detected concentration. Deming regression analysis was used to establish the matrix effect. This involved comparing the responses obtained following spiking the compounds in the matrix and deionized water. When the slope of the curve is further from 1.0, quantification

will be challenging. External standard calibration curves were used for quantification and matrix effects were compensated by using matrix matching.

Table S3-1 Method validation of chiral analysis of  $\beta$ -blockers in wastewater using LC-MS/MS. Matrix effects were determined using Deming regression analysis.

Compound		Resolution		Repeatability %		Linearity (n=15)			Matrix Effects			LOD
		$t_r$	$R_s$	$t_r$	Peak Area	Calibration model	$R^2$	Error	Slope	$R^2$	Recovery, %	
Propranolol	S	14.6	1.0	3.4	3.6	$y = -4.374 + 0.000000973x$	0.99	2.6	0.96	0.98	$0.89 \pm 0.09$	4.2
	R	15.9		3.8	2.7	$y = -1.488 + 0.000000778x$	0.99	2.2	0.95	0.98	$0.94 \pm 0.01$	3.2
Pindolol	S	13.5	1.1	3.4	3.9	$y = -4.387 + 0.000000633x$	0.99	2.3	0.92	1.0	$0.94 \pm 0.04$	1.1
	R	14.5		3.6	4.3	$y = -3.302 + 0.000000565x$	0.99	3.2	0.83	1.0	$0.91 \pm 0.03$	1.2
Metoprolol	S	14.2	1.0	3.4	4.2	$y = -3.588 + 0.000000325x$	0.99	3.1	0.98	1.0	$0.93 \pm 0.05$	0.3
	R	15.3		3.6	3.4	$y = -3.261 + 0.00000028x$	0.99	1.5	0.95	1.0	$0.97 \pm 0.03$	0.2
Atenolol	S	19.5	0.97	4.0	5.8	$y = -5.877 + 0.000000522x$	0.99	2.7	0.92	1.0	$0.88 \pm 0.03$	1.9
	R	21.4		4.4	4.5	$y = -5.692 + 0.000000448x$	0.99	3.0	0.89	1.0	$0.89 \pm 0.04$	1.6
Acebutolol	S	17.6	1.2	4.1	3.4	$y = -0.808 + 0.00000177x$	0.99	3.2	0.84	1.0	$0.89 \pm 0.05$	4.2
	R	19.1		4.3	4.0	$y = 2.558 + 0.00000153x$	0.99	2.8	0.91	1.0	$0.89 \pm 0.02$	5.4

Table S3-2 Ionization parameters of five beta blockers using ESI LC-MS/MS in positive mode.

Compound	Precursor ions	Dwell time (ms)	Collision energy (eV)
Propranolol	266	25	38
Pindolol	249	18	36
Atenolol	267	25	36
Acebutolol	337	25	36
Metoprolol	268	20	38

Table S3-3 Isotherm data for five beta blockers on sludge and retention times and percentage of neutral species that participate in hydrophobic interaction at pH 7.4.

Compound	<i>r</i> <sup>2</sup>	<i>K<sub>f</sub></i> ( <i>n</i> = 1)			% neutral species at pH 7.4	Number of samples	
		Value	CV%	Std. error			
Propranolol	R	0.996	302	1.08	3.27	0.54	12
	S	0.997	298	1	3		12
Pindolol	R	0.998	359	0.72	2.6	0.54	12
	S	0.998	358	0.8	2.88		12
Atenolol	R	0.943	154	4.86	7.5	0.54	15
	S	0.944	159	4.88	7.77		15
Acebutolol	R	0.938	147	4.97	7.29	0.68	15
	S	0.96	155	3.95	6.14		15
Metoprolol	R	0.853	124	9.14	11.4	0.54	15
	S	0.861	129	8.62	11.1		15



Table S3-4 Stereoselective differences in Kd values with  $\alpha = 0.005$ .

Compound	RWQP	TTX	SDS	CTAB	DMEB
Atenolol	0.006	0.011	0.003	0.008	0.034
Acebutolol	0.004	0.018	0.001	0.081	0.091
Metoprolol	0.005	0.314	0.014	0.004	0.026
Pindolol	0.834	0.938	0.869	0.943	0.559
Propranolol	0.836	0.099	0.118	0.872	0.113

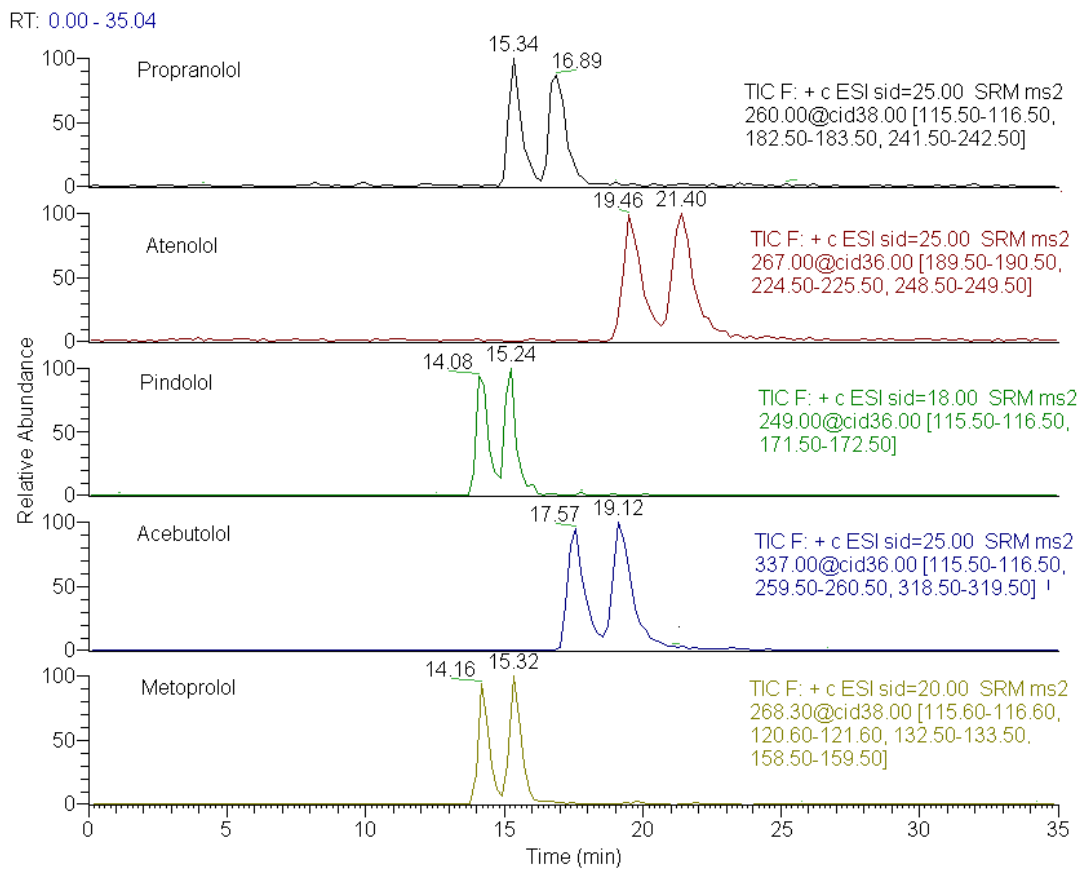


Figure S3-1 Chiral separation of five beta blockers using Chirobiotic V column.

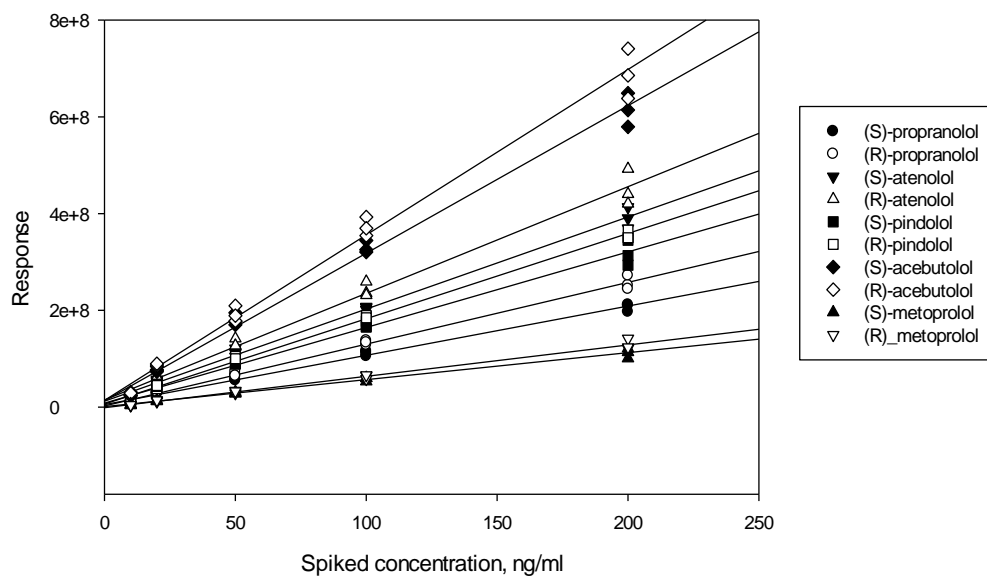


Figure S3-2 Matrix matched calibration curve for 5  $\beta$ -blockers.

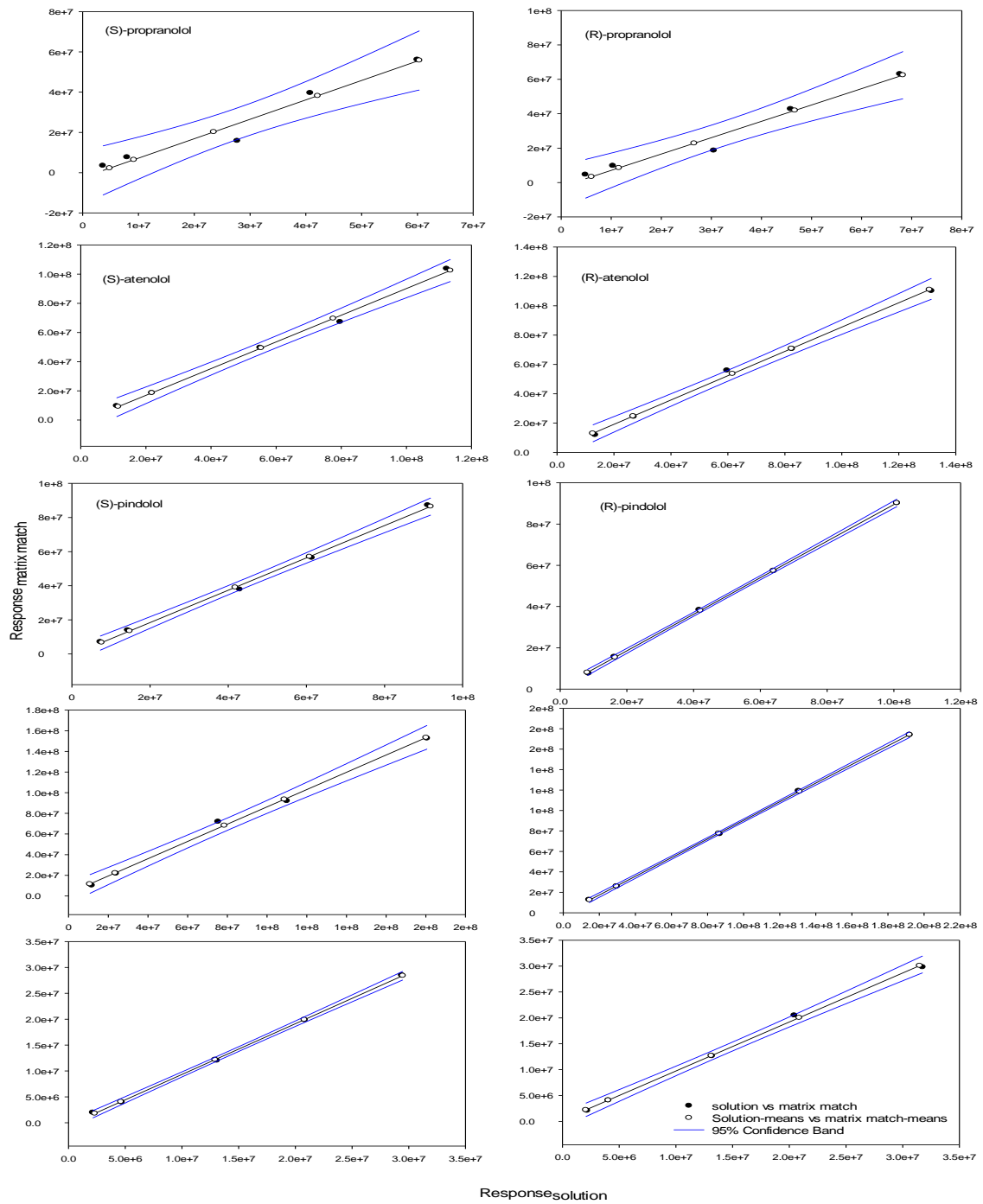
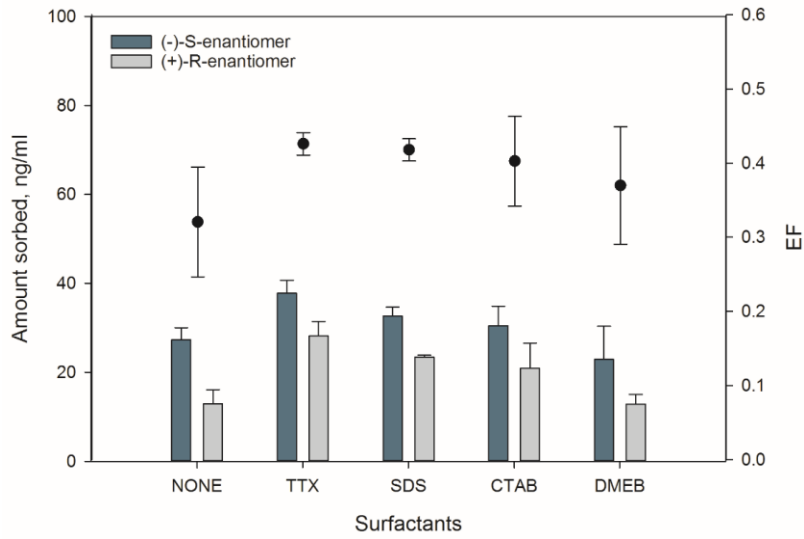
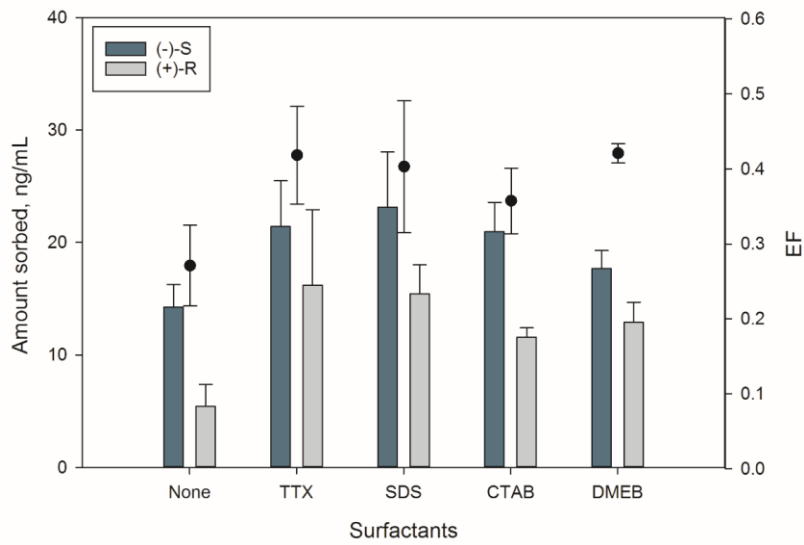


Figure S3-3 Deming regression analysis comparing matrix-matched and solution calibration curves obtained for each compound during the matrix effect procedure.

### Acebutolol



### Metoprolol



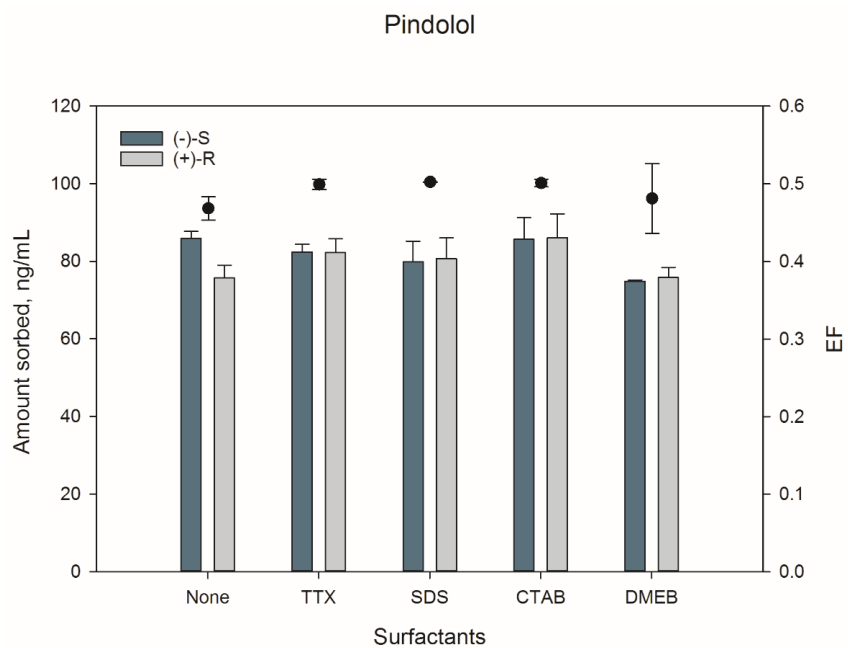


Figure S3-4 Effect of amending sludge with surfactants on the amount of beta blockers sorbed and the stereoselectivity in sorption.

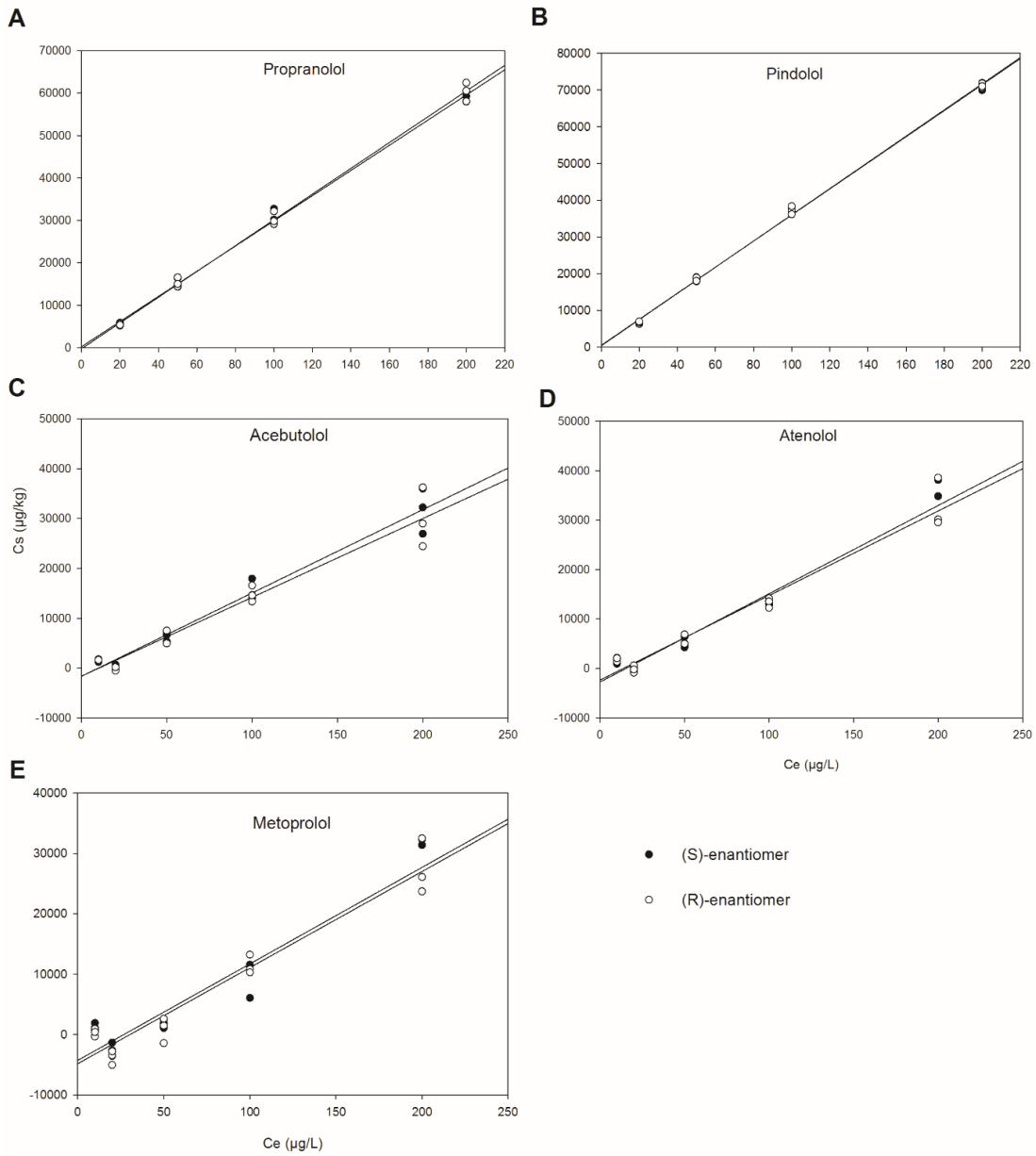


Figure S3-5 Sorption isotherm for five beta blockers in activated sludge. Five concentration samples were used in triplicate to fit  $C_s$  and  $C_e$  into a linear isotherm model for each compound except pindolol and propranolol where 4 samples were used.

## Chapter 4 Quantitative Assessment of Biodegradation of $\beta$ -blockers in Wastewater using Chiral Analysis

### 4.1 Introduction

$\beta$ -blockers are the most widely used class of pharmaceuticals and are frequently detected in wastewater and surface water at ng/L to  $\mu$ g/L concentrations (Scheurer et al., 2010; Wick et al., 2009). They are commonly prescribed for the treatment of cardiovascular diseases such as congestive heart failure and cardiac arrhythmia. Several environmental monitoring studies by researchers from Canada (MacLeod and Wong, 2010; MacLeod et al., 2007; Nikolai et al., 2006), Spain (Vazquez-Roig et al., 2014), the UK (Baker and Kasprzyk-Hordern, 2013), the USA (Fono and Sedlak, 2005) and Portugal (Ana Rita Ribeiro et al., 2014) reported a widespread distribution of  $\beta$ -blockers in sludge, wastewater, lakes, and rivers.  $\beta$ -blockers enter the aquatic environment following incomplete removal in wastewater treatment plants. The presence of  $\beta$ -blockers in the environment may pose a risk to non-target organisms since these pharmaceuticals are designed to elicit biological effects at low concentrations (Stanley et al., 2006).  $\beta$ -blockers contain at least one chiral center in their structure and are often sold as a racemic mixture. However, stereoisomers exhibit different behaviors in chiral environments such as biological systems or chiral surfaces (Kasprzyk-Hordern, 2010). For example, (*S*)- $\beta$ -blockers preferentially bind to  $\beta$ -adrenergic receptors to effect a therapeutic change, except for sotalol where the (*R*)-enantiomer is preferred (Mehvar and Brocks, 2001). Furthermore, in an aquatic toxicity study, Stanley et al. (2006) found (*S*)-propranolol was more chronically toxic to



*Pimephales promelas* than its antipode. Therefore, it is imperative to determine the enantiomeric composition and environmental behavior of chiral pharmaceuticals in order to perform more accurate ecological risk assessments. Consequently, there is a growing interest on the implications of their chirality on their distribution, fate, and toxicity in wastewater and the environment (Evans and Kasprzyk-Hordern, 2014; Petrie et al., 2014; Ribeiro et al., 2012).

Wastewater and environmental removal of chiral pharmaceuticals is mediated by microbial degradation, adsorption to sludge or sediment, photolysis, and chemical degradation (Kibbey et al., 2007; Prasse et al., 2015; Stevens-Garmon et al., 2011; Wick et al., 2009). However, several studies showed that chiral pharmaceuticals are primarily removed via microbial degradation, which is a stereoselective process. In the past 15 years, the stereoselective degradation and enantiomeric composition of chiral drugs in wastewater and surface water has gained considerable attention. Microbial degradation of chiral pharmaceuticals such as fluoxetine (Moreira et al., 2014; Ribeiro et al., 2012), ibuprofen (Hashim et al., 2011; Khan et al., 2013), propranolol (Ribeiro et al., 2014), metoprolol (Fono et al., 2006), and venlafaxine (Li et al., 2013) has been shown to be stereoselective. Several researchers have proposed that the difference in enantiomeric composition of the pollutants in wastewater and the environment can be used to establish their mechanisms of degradation and also identify their sources. Fono and Sedlak (2006) demonstrated that changes in the enantiomeric fraction ( $EF = \frac{E1}{E1+E2}$ , where E1 and E2 are the first eluted and second eluted enantiomers or (*R*)-enantiomer and (*S*)-enantiomer, respectively) can be used

for source tracking. Souchier et al. (2016) recently investigated the enantioselective degradation of metoprolol in a wastewater microcosm and found that it degraded into metoprololic acid with the (*R*)-enantiomer undergoing more rapid degradation. However, Ribeiro et al. (2013) found that the (*S*)-enantiomers of propranolol and alprenolol had higher biodegradation rates than their antipodes following incubation in an activated sludge inoculum. Therefore, determination of enantioselective degradation is essential for conducting more accurate ecological risk assessments of  $\beta$ -blockers.

Laboratory microcosm experiments are critical for understanding the mechanism of microbial degradation and the persistence of the micropollutants in wastewater and the environment. However, controlled laboratory experiments only offer qualitative data and not quantitative data, the latter of which is a crucial aspect of environmental risk assessments (Gasser et al., 2012; Jammer et al., 2016, 2015; Souchier et al., 2016). Thus, there is a need for a quantitative tool for evaluating enantioselective degradation with the potential for predicting chiral behavior of micropollutants at environmental concentrations. Recently, Jammer et al. (2015) investigated the structural basis of the enantioselective hydrolysis of 16 derivatives of 2-(phenoxy)propionate during enzymatic degradation by lipase. They found that the Rayleigh equation sufficiently described the relationship between the change in enantiomeric composition ( $\ln \left( \frac{[ER_t]}{[ER_0]} \right)$ ) and concentration ( $\ln \frac{[C_t]}{[C_0]}$ ) under environmentally relevant conditions. The constant of proportionality in the Rayleigh equation is called the enantiomeric enrichment factor,  $\epsilon_{ER}$ . Souchier et al. (2016) recently demonstrated that the biodegradation of metoprolol exhibited Rayleigh dependency and

concluded that *in situ* biodegradation can be quantified using enantiomeric fractionation. Hence, the relationship between changes in enantiomeric composition can be used as a tool for evaluating the biodegradation of chiral pharmaceuticals quantitatively.

Since  $\beta$ -blockers are ionogenic compounds their biodegradation can be influenced by changes in pH. During wastewater treatment, a change in pH of approximately 2.0 has been reported. In a soil degradation experiment, (*R*)-metalaxyl underwent more rapid degradation than its antipode at pH > 5, at the same rate when pH was between 4 and 5, and slower at pH < 4 (Buerge et al., 2003). However, little is known regarding the effect of pH on the stereoselective degradation of  $\beta$ -blockers in wastewater. Furthermore, to identify the role of physicochemical properties and structural features in stereoselective degradation in wastewater, five  $\beta$ -blockers (acebutolol, atenolol, metoprolol, pindolol, and propranolol) were analyzed in a wastewater microcosm experiment to measure changes in their enantiomeric composition over time. To the best of our knowledge, this is the first report on Rayleigh dependency and the effect of pH on stereoselective degradation of acebutolol, atenolol, metoprolol, pindolol, and propranolol in a wastewater microcosm.

## 4.2 Materials and Methods

### 4.2.1 Sampling Site

Mixed liquor activated sludge was collected from the Colton Wastewater Treatment Plant (CWWTP) in Colton, CA. At this facility, wastewater influent is treated by passing through screening and grit channels and primary clarifiers. The primary effluent undergoes biological oxidation using conventional activated sludge in aeration basins followed by secondary clarification. The CWWTP serves an estimated population of 65,900 people, treating  $24,000 \text{ m}^3 \text{ day}^{-1}$  with an average flow of approximately  $21,000 \text{ m}^3 \text{ day}^{-1}$ .

### 4.2.2 Materials

A set of commonly used and environmentally relevant  $\beta$ -blockers was considered in this study. As shown in Table 4-1, the selected  $\beta$ -blockers—acebutolol, atenolol, metoprolol, pindolol, propranolol, and salbutamol—have different  $\log K_{ow}$  and  $pK_a$  values. Standards of the  $\beta$ -blockers were purchased as racemic mixtures from Sigma-Aldrich (St. Louis, MO) with minimum purities of 99%. Other chemicals used included analytical grade methanol and ammonium trifluoroacetate with purities  $>99\%$  (Sigma-Aldrich).

### 4.2.3 Biodegradation Study

We adopted the experimental set-up of the biodegradation experiment from Gulde et al (2014). Briefly, we sampled mixed liquor activated sludge from an aeration basin at a municipal WWTP (Colton, California, USA). The activated sludge was diluted with deionized water to produce a solution with a concentration of total suspended solid of  $\approx$

2.0 g<sub>ss</sub>/L (where g<sub>ss</sub> is gram suspended solids). We conducted the experiments at a pH of 6.0, 7.19 and 8.26 in triplicate. The initial pH of the batch reactors was adjusted using the inorganic base sodium hydroxide. We assumed that the change in pH during the incubation period was not sufficient to confound the results since activated sludge has high organic content that provides a buffering capacity.

Amber bottles (100 mL) were filled with diluted activated sludge (50 mL). The batch reactors were incubated for 6 h under stirring conditions at 120 rpm. The batch reactors were spiked with a  $\beta$ -blocker mix solution (50 ppm) to obtain a final concentration of 20  $\mu\text{g/L}$  for each enantiomer of the  $\beta$ -blocker. To determine whether spiking using a mix solution or a single racemic mixture affects degradation, additional batch reactors were spiked each with pindolol and acebutolol separately. The samples (2 mL) were withdrawn from the batch reactors in triplicate at approximately 5 min, 0.5 d, 1 d, 3 d, and 5 d using a 10 mL glass syringe. The samples were added to a centrifuge tube and centrifuged at 12,000 rpm for 15 minutes. Following filtration with a 0.2-micron glass filter, the supernatant (1.0 mL) was transferred to analytical vials which were stored at 4°C for no more than 7 days before analysis while covered with aluminum foil. External calibration curves at different pHs were prepared using the diluted activated sludge and spiked with the standard solutions to yield concentrations ranging from 2.0 to 200  $\mu\text{g/L}$ . The loss of  $\beta$ -blockers due to sorption, volatilization and other abiotic processes was examined using a filtrate of autoclaved activated sludge. The diluted activated sludge was autoclaved at 121 °C and 103 kPa for 20 min. Triplicate samples were collected at 5 min, 0.5 d, 1 d, 3 d, and 5 d. External calibration curves at different pHs were prepared using the autoclaved activated

sludge and its filtrate spiked with the standard solutions to yield concentrations ranging from 5.0 to 200 µg/L.

#### 4.2.4 Chiral Analysis

##### 4.2.4.1 Instrumentation

The chiral  $\beta$ -blockers were separated and detected by an Agilent 1100 high performance liquid chromatograph (HPLC) (Agilent, Wilmington, DE, USA) coupled to an electrospray ionization mass spectrometer (Thermo Finnigan LCQ Deca XP Plus San Jose, CA) (ESI LC-MS/MS). After passing through the 0.22- $\mu$ m membrane, the filtrate was injected directly into the system for quantitative analysis. Samples (10  $\mu$ L) were injected by an autosampler onto a Chirobiotic V column (4.6 mm x 250 mm, particle size 5  $\mu$ m) with a guard column (10 mm  $\times$  20 mm, particle size = 5  $\mu$ m), all purchased from Sigma Aldrich (St. Louis, MO). A mobile phase comprised of methanol and ammonium trifluoroacetate in methanol (0.1% w/v) at 40/60 (v/v) was used to elute the enantiomers in the isocratic mode at a flow rate of 0.5 mL/min. The run time was 35 min and the retention times for individual enantiomers are shown in Table S4-2. Mass spectra were acquired using ESI in the positive ion mode. The ionization voltage was +4.5 kV, collision energy 25 eV, auxiliary gas temperature 350 °C, and the nebulizing and auxiliary gas settings were 60 and 20 arbs, respectively.

#### 4.2.4.2 Quantitation standards, chiral detection and control samples

A range of concentrations of  $\beta$ -blockers were spiked to autoclaved activated mixed liquor (2 g<sub>ss</sub>/L) to create a matrix-matched calibration curve. Matrix-matched external standards were used to compensate for ion suppression/enhancement in ESI. The matrix-matched standards were filtered and analyzed using the same procedure as the microcosm samples. The sign of optical rotation for each enantiomer was confirmed using an in-line laser polarimeter detector (PDR-Chiral, Lake Park, FL). The limit of quantitation was taken as the lowest concentration on the calibration curve, or 5 ng/mL.

#### 4.2.5 Quality Assurance and Data Analysis

All data are presented as mean and standard deviation of triplicates. A one-way ANOVA test was conducted at  $\alpha=0.05$  to evaluate the significance of differences among the treatments. Statistical analysis was performed using SigmaPlot.

### 4.3 Results and Discussion

#### 4.3.1 Chiral analysis of $\beta$ -blockers in Wastewater Microcosms

The enantiomers of five  $\beta$ -blockers were separated on a vancomycin-based chiral column yielding  $R_s$  values greater than 1.0 ( $R_s = 2 \frac{(t_2 - t_1)}{(w_2 + w_1)}$ , where  $t_1$  and  $t_2$ , and  $w_1$  and  $w_2$ , are the retention time and peak width of the first and second eluted enantiomers, respectively), which is sufficient for quantification (Table 4-1). Figure S4-1 shows the chromatograms for the separated enantiomers. Identification of the enantiomers was

achieved using retention time, mass spectrometry, and optical rotation. Table 4-1 shows the exact stereoconfiguration of the analytes and analytical performance of the direct injection chiral LC-MS/MS method employed in this study. The Deming regression analysis at 95 % confidence shows that the matrix effects were compensated for using a matrix-matched external calibration curve (Table 4-1 and Figure S4-2). Ion suppression and/or ion enhancement during mass spectrometry has been shown to introduce artifacts in chiral analysis using ESI-LC-MS/MS. In addition, loss of analytes due to abiotic processes was low (Table S4-2). The inter- and intra-day variations in retention time and peak area for each enantiomer were less than 5.0 %. The detection limits for the  $\beta$ -blockers were calculated from the calibration curve using three times the standard deviation of the lowest detected concentration. In activated sludge, the detection limit ranged from 0.2 to 5.4  $\mu\text{g/L}$  (Table S4-1). Therefore, the analytical technique was fit for chiral analysis of  $\beta$ -blockers in wastewater microcosm samples.

#### 4.3.2 Degradation of $\beta$ -blockers in Wastewater Microcosms

Laboratory experiments were conducted using activated sludge to simulate biotransformation in municipal wastewater treatment plants. Figure 4-2 shows the degradation patterns of four  $\beta$ -blockers. Atenolol degraded rapidly and was not detected in any of the samples after 12 hours agreeing with a previous study that reported a half-life in activated sludge of 6.0 hours (Maurer et al., 2007). No pharmaceutical was detected in the batch reactors after 5 days of incubation. All additional experiments focused on four  $\beta$ -blockers namely, propranolol, pindolol, metoprolol, and acebutolol. Acebutolol and



pindolol were degraded rapidly in 12 h, whereas the degradation of propranolol was relatively slower. However, there was a lag in the degradation of metoprolol in the first 12 hours as no apparent change in initial concentration was observed. Since the  $\beta$ -blockers were added to the microcosm as a mixture, apparently the microorganisms in the activated sludge had a preference for other micropollutants besides metoprolol and propranolol. The validity of this supposition was explored in a further experiment whose results are described later.

Enantioselectivity in the degradation of the  $\beta$ -blockers in activated sludge was determined by monitoring the changes in EF values with incubation time. After accounting for recoveries and initial spiking concentration for each enantiomer (20  $\mu\text{g/L}$ ), the initial EF value of each compound was normalized at 0.5. Degradation of the (*R*)-enantiomer of all of the selected  $\beta$ -blockers was faster than that of the antipode, except for acebutolol. As a result, the EF values for metoprolol, pindolol, and propranolol decreases from 0.5 to  $0.30 \pm 0.01$ ,  $0.37 \pm 0.0$  and  $0.32 \pm 0.05$ , respectively. However, there was enantiomeric enrichment of (*R*)-acebutolol in the batch reactors since (*S*)-acebutolol degraded more rapidly with a final EF value of  $0.63 \pm 0.6$ . The differences between degradation of acebutolol and the other  $\beta$ -blockers indicate that stereoselectivity may differ even between compounds in the same chemical group (Table 4-1). Acebutolol possesses a phenyl group that contains an ethyl aldehyde group and a propyl group linked through a peptide bond. Hence, acebutolol is more extensively branched and has a three dimensional configuration slightly different from the much more linear propranolol, pindolol, and metoprolol. Thus, stereoselectivity is expected to be similar between propranolol, pindolol, and metoprolol,

but not necessarily with acebutolol. Since loss due to adsorption and volatilization was negligible, the microorganisms responsible for removal of organic pollutants in wastewater treatments plants demonstrate a degree of stereoselectivity. Hence, removal of chiral micropollutants in wastewater may be stereoselective. Souchier et al. (2016) found no change in enantiomeric composition in the removal of metoprolol in wastewater plants by comparing EF values in influent and effluent. However, following an incubation experiment, they found that there was *S*-enrichment since (*R*)-metoprolol degraded faster. Fono and Sedlak (2005) observed that the EF value of propranolol in wastewater plants changed from 0.5 in the influent to 0.42 following secondary treatment. In contrast, Ribeiro et al. (2013) found slightly higher biodegradation rates for the (*S*)-enantiomers of alprenolol and propranolol. However, these studies were only qualitative as they only confirmed stereoselectivity in degradation.

#### 4.3.3 Effect of pH on Degradation of $\beta$ -blockers

Changing pH affects the stereoselectivity and degradation rates of  $\beta$ -blockers in activated sludge batch reactors (Figure 4-3). The  $\beta$ -blockers in the wastewater microcosms degraded stereoselectively with enantiomeric enrichment of either the (*R*)- or (*S*)-enantiomer. The EF values during degradation of acebutolol were above 0.5, indicating enrichment of the (*R*)-enantiomer. The highest EF value ( $0.75 \pm 0.05$ ) was observed in degradation of acebutolol at pH 8.26 and the lowest EF value ( $0.05 \pm 0.04$ ) was obtained during degradation of propranolol. Importantly, changing the pH accentuated the stereoselectivity. For example, in the degradation of acebutolol, increasing the pH from

7.19 to 8.26 resulted in a 19 % increase in the final EF. Decreasing the pH to 6.0 resulted in no apparent change in the degree of stereoselectivity, with initial and final EF values being  $0.63 \pm 0.05$  and  $0.64 \pm 0.4$ , respectively. In contrast, incubation of propranolol, pindolol, and metoprolol with an increase in pH to 8.26 resulted in the EF values decreasing by 84, 34 and 40 %, respectively. When the pH was decreased to 6.0 from 7.19, there was a relatively small decrease in EF. The EF of propranolol and pindolol decreased by 50 and 32 %, while the EF of metoprolol increased by 31 %. Plotting the EF values against the pH gave a poor correlation ( $R^2 = 0.32$ ,  $n = 14$ ), but excluding pH 6.0 increased the correlation to 0.64. The results suggested pH-dependence in the degree of stereoselectivity during degradation of  $\beta$ -blockers in activated sludge.

Adjusting the pH in the batch reactors to 6.0, 7.19 and 8.26 affected the degradation of  $\beta$ -blockers since they contain ionizable functional groups with  $pK_a$  values ranging from 9.2 to 9.7 (Caron et al., 1999). A single point matrix-matched and pH specific external standard was used to compensate for artifacts in detection and quantification caused by the change in ionization of the analytes. When the initial pH of the activated sludge was increased to 8.26, degradation of the less hydrophobic compounds (acebutolol and metoprolol) was relatively faster. For example, when the pH was changed from 7.19 to 8.26, the amount of acebutolol remaining in the batch reactor after three days decreased from  $1.29 \pm 0.8$  and  $2.20 \pm 0.9$  to  $0.27 \pm 0.2$  and  $0.80 \pm 0.4$   $\mu\text{g/L}$  for the (*S*)- and (*R*)-enantiomers of acebutolol, respectively. Degradation of ionizable compounds in wastewater has been shown to be dependent on the number of neutral species in the system (Gulde et al., 2014). When the pH was increased, the total neutral species of acebutolol

increased from 0.43 to 6.38 %. It is often assumed that only the neutral species are taken up and degraded by organisms. Metoprolol, pindolol, and propranolol had the same number of neutral species under basic conditions, but the effect of increasing pH was different. There was no apparent change and/or an increase in the rates of degradation for pindolol and propranolol (relatively more hydrophobic compounds), respectively. Specifically, (*S*)-enrichment occurred for propranolol when the pH was increased with amount remaining changing from  $1.64 \pm 0.3$  to  $6.88 \pm 2.7$   $\mu\text{g/L}$  (Figure 4-3). However, no change in amount of (*R*)-propranolol was observed. Decreasing the pH in the batch reactors to 6.0 decreased the rate of degradation of the  $\beta$ -blockers, except for metoprolol where no apparent change was observed. For example, the amount of (*S*) and (*R*)-enantiomers of propranolol and acebutolol remaining in a batch reactor at pH 7.19 and 6.0 changed from  $1.64 \pm 0.2$  and  $0.46 \pm 0.1$   $\mu\text{g/L}$  to  $10.2 \pm 1.1$  and  $2.3 \pm 2.1$   $\mu\text{g/L}$ , and  $1.29 \pm 0.8$  and  $2.20 \pm 0.9$   $\mu\text{g/L}$  to  $3.02$  and  $5.57$   $\mu\text{g/L}$ , respectively (Figure 4-3). At pH 6.0, the number of neutral species decreased to at most 0.03 %, thus decreasing the number of species available for uptake (Table S4-1). However, there was no apparent change in the rate of degradation of metoprolol when pH was decreased in the reactor. Furthermore, there is a general consensus that when pH in the wastewater aeration basins decreases, there is a corresponding reduction in nitrification rates (Cho et al., 2014). At pH 6.0, the growth rate of ammonia-oxidizing bacteria normally decreases, but the nitrite-oxidizing bacteria remain in the system (Fumasoli et al., 2015). In a study on pH-dependent biotransformation of pharmaceuticals in activated sludge, Gulde et al. (2014) found there was a bias toward lower values of  $k_{\text{bio}}$  due to a decrease in oxygen uptake during incubation. At low pH, the

growth rate of bacteria decreases, thus there will be a reduction in microbial population and diversity (Cho et al., 2014; Fumasoli et al., 2015; Gulde et al., 2014). Therefore, pH 6.0 introduced artifacts that potentially affected the correlation between pH and stereoselectivity. This suggests that the decrease in rate of degradation observed in this investigation could be due to a decrease in microbial activity caused by the acidic conditions. Therefore, a simple model of neutral speciation does not adequately explain the uptake and degradation of basic compounds such as  $\beta$ -blockers, and ionic speciation could be a possible mechanism of degradation (Gulde et al., 2014).

#### 4.3.4 Rayleigh Dependence in Wastewater Degradation

Data obtained from the microcosm experiment and data reported in the literature, whenever sufficient information was provided, was parameterized the Rayleigh equation. The goal of this experiment was to establish, for the first time, the relationship between enantiomeric enrichment and biodegradation of  $\beta$ -blockers in wastewater. At pH 7.12, a linear fit was obtained for the biodegradation of propranolol, pindolol, and metoprolol (Figure 4-4 and Table S4-1). The  $R^2$  value of the Rayleigh equation ranged from 0.94 to 0.99. However, the  $\epsilon_{ER}$  values of all of the  $\beta$ -blockers were low ( $< 10.0$ ). The enantiomeric enrichment factor,  $\epsilon_{ER}$ , for propranolol, pindolol, and metoprolol were -1.70, -.21 and -8.76, respectively. The  $\epsilon_{ER}$  value for acebutolol was -0.46, but there was substantial variability with an  $R^2$  of 0.62. Changes in Rayleigh dependence were observed when the pH of the wastewater microcosm was changed. At pH 6.0, a linear fit was obtained between the enantiomeric composition and the degradation of propranolol, acebutolol, and

metoprolol with an  $R^2$  of 0.95, 0.98 and 0.95, respectively. However, at pH 8.26, Rayleigh dependence was only observed for propranolol with pindolol, acebutolol, and metoprolol giving an  $R^2 < 0.55$ . Degradation of micropollutants in wastewater and the environment is largely mediated by enzymatic reactions (Jammer et al., 2014). Furthermore, pH changes affect the ionization state of the  $\beta$ -blockers, changing the number of neutral species in the environmental compartment. Hence, these results suggest that Rayleigh dependency is influenced by the pH of the environmental compartment. In a laboratory and field study on the biodegradation of metoprolol, Souchier et al. (2016) obtained a linear fit to the Rayleigh equation under both dark and light conditions with  $R^2 > 0.99$ . The  $\epsilon_{ER}$  value for metoprolol obtained by Souchier et al. (2016) was approximately 6 % of the one reported in this study because their investigation was performed in sediment and not activated sludge.

Besides the wastewater microcosm experiment, we parameterized the Rayleigh equation to reported studies on the biodegradation of  $\beta$ -blockers in wastewater treatment plants. For this investigation, the total concentration of each compound in the influent and the effluent was required. When not available, the sum of the concentration of each enantiomer was calculated. Since most studies use EF value rather than enantiomeric ratio, ER (Equation 4-1), we calculated the ER value as follows:

$$ER = \frac{E_1}{E_2} \quad (4-1)$$

$$ER = \frac{EF}{(1-EF)} \quad (4-2)$$

The initial and final conversion as well as enantiomeric enrichment were obtained from data from the influent and effluent, respectively. Data sufficient for reconstructing enantiomeric enrichment and degradation plots was obtained from studies on 7 wastewater treatment plants for the degradation of atenolol, metoprolol, salbutamol, propranolol, and sotalol (Figure S4-2) (Evans et al., 2015; MacLeod et al., 2007; Nikolai et al., 2006). Rayleigh dependency was observed for the removal of atenolol and salbutamol in wastewater since a good fit ( $R^2 > 0.80$ ) was obtained in the plots. Propranolol and sotalol had a poor fit with an  $R^2$  value of 0.09 and 0.14, respectively. However, the  $R^2$  value for metoprolol was 0.45, which is almost half of the value reported in a previous study (Souchier et al., 2016). Souchier et al (2016) studied the removal of metoprolol in 5 wastewater treatment plants and obtained a good linear fit for the Rayleigh equation with  $R^2 > 0.98$ . Enantiomers may not follow the same conversion mechanism since they form diastereomers in a chiral environment and their target sites and degradation rates may differ (Gasser et al., 2012). For example, the enantiomeric enrichment factor obtained by Souchier et al. in both the laboratory and field study differed from those we obtained in the microcosm study and determined from the reconstruction data (Souchier et al., 2016). Furthermore, changes in pH have been shown to result in changing the enantiomer enriched during soil degradation of metalaxyl (Buerge et al., 2003). However, since the results obtained in this study demonstrated a Rayleigh dependency, this suggests that the enantiomeric enrichment-degradation relationship can be a valuable tool for evaluating quantitatively the biodegradation of  $\beta$ -blockers in wastewater and potentially in the environment.

#### 4.4 Environmental Implications

There are few studies on the enantioselective degradation of chiral pharmaceuticals in wastewater and the environment. No previous studies have investigated the enantioselective degradation of acebutolol and pindolol even though they are one of the most widely prescribed  $\beta$ -blockers. To examine the effect of chemical structure on the stereoselectivity of degradation, atenolol, propranolol, and metoprolol were also included in the study. This study demonstrated for the first time that pindolol and acebutolol degraded stereoselectively in wastewater microcosms. The results show a level of structural dependency regarding which enantiomer is enriched with *S*-enrichment for propranolol, pindolol, and metoprolol, and *R*-enrichment for acebutolol. However, a study on enantioselective toxicity of propranolol to *Pimephales promelas* showed that (*S*)-propranolol was more toxic than its antipode (Stanley et al., 2007). Thus, the *S*-enrichment of propranolol in wastewater might pose an environmental risk to aquatic organisms.

The results obtained in the present study showed that changes in pH of wastewater microcosms affects the degree of stereoselectivity and the degradation rates of ionizable micropollutants. The removal rate of the  $\beta$ -blockers was low following the adjustment of pH to 6.0. However, a marked increase in stereoselectivity (become more negative or more positive) was observed after changing the pH from 7.19 to 8.26. The observation suggests that changing the percentage of neutral species in a system affects molecular docking of the chiral components. There was not sufficient data to suggest changing pH may also result in a change in target site. However, since pH changes of 2.0 are common in



wastewater treatment plants, intra-day, inter-day, and seasonal changes in enantiomeric composition of the effluents reported. For example, a previous study on wastewater effluent have shown inter-day variations in the EF values of ibuprofen, ketoprofen, and naproxen (Hashim and Khan, 2011). Therefore, pH is an important parameter to consider when investigating the removal of ionic micropollutants that have a chiral center. Adsorption to sewage sludge is another possible mechanism leading to removal of chiral pharmaceuticals.

Quantitative assessment of enantioselective degradation is essential for improving the accuracy of ecological risk assessments of chiral pharmaceuticals. Previous studies on the fate of  $\beta$ -blockers were primarily qualitative and thus inadequate for a robust risk assessment (Nikolai et al., 2006; Ribeiro et al., 2012). This study demonstrated for the first time that the enantiomeric enrichment of  $\beta$ -blockers in a wastewater microcosm had a linear relationship with the conversion rate (Rayleigh dependency). We obtained a linear fit for the Rayleigh equation for propranolol, pindolol, and metoprolol. Rayleigh dependency was also observed for atenolol and salbutamol through analysis of previous studies. However, there is a need to investigate Rayleigh dependency in other frequently detected micropollutants and to include different environmental compartments. The present study demonstrates that in addition to source tracking, enantiomeric enrichment can be utilized for quantitatively evaluating the biodegradation of  $\beta$ -blockers in environmental systems.

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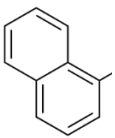
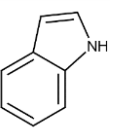
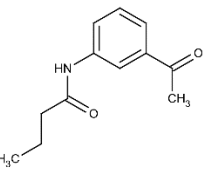
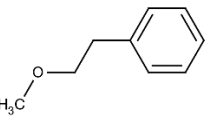
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Tables

Table 4-1 Chemical structure and Rayleigh enrichment factors for the dissipation of  $\beta$ -blockers in wastewater microcosm.

Drug	Substitute group <sup>a</sup>				Rayleigh enrichment factor <sup>b</sup>						
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	$\epsilon_{ER}$	R <sup>2</sup>	$\epsilon_{ER}$	R <sup>2</sup>	$\epsilon_{ER}$	R <sup>2</sup>	
					pH=7.19	pH=8.26		pH=6.0			
Propranolol	H	H	H		-1.70	0.94	-1.76	0.85	-	0.95	4.96
Pindolol	CH <sub>3</sub>	CH <sub>3</sub>	H		-0.24	0.95	-1.60	0.43	-	0.27	2.40
Acebutolol	CH <sub>3</sub>	CH <sub>3</sub>	H		-0.46	0.62	-0.37	0.52	-	0.98	0.71
Metoprolol	CH <sub>3</sub>	CH <sub>3</sub>	H		-8.76	0.99	-4.53	0.23	-	0.95	6.40

Figures

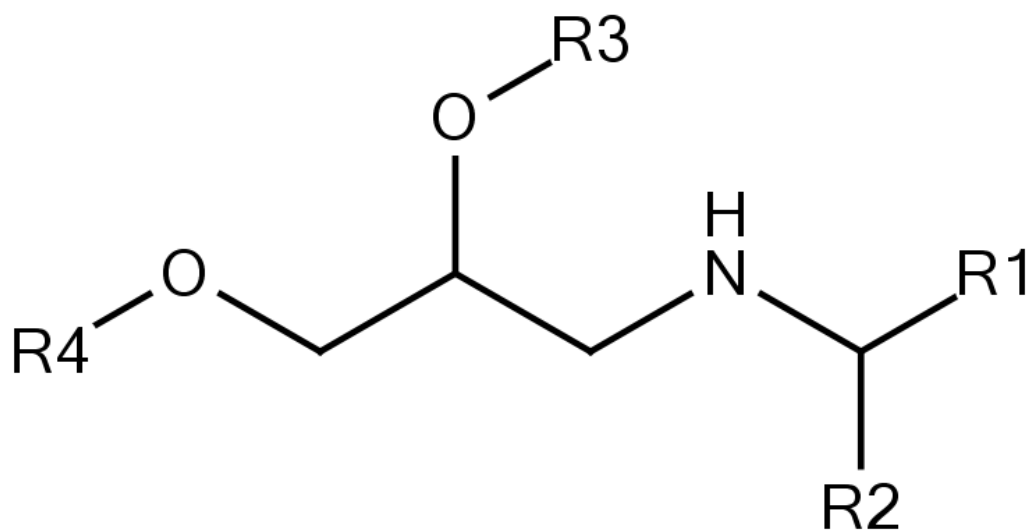


Figure 4-1 Structure of investigated  $\beta$ -blockers. R1, R2, R3 and R4 represent different substitute groups on  $\beta$ -blockers as shown in Table 4-1.

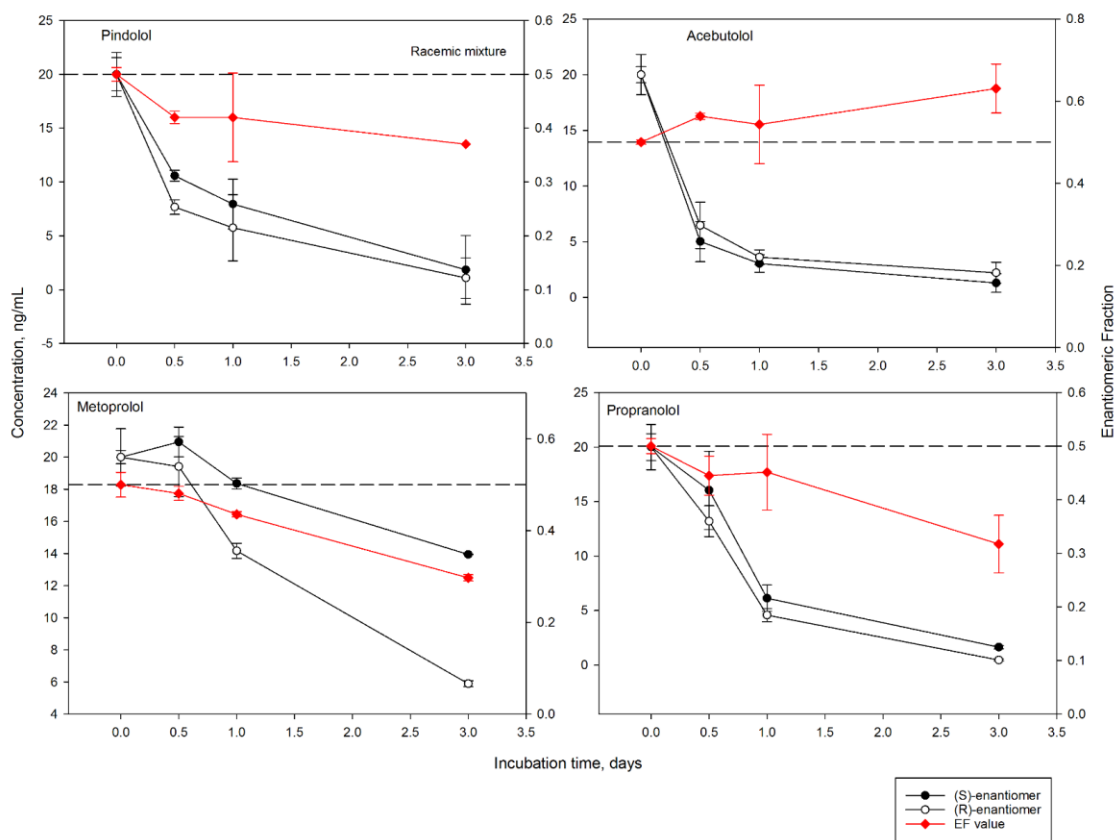
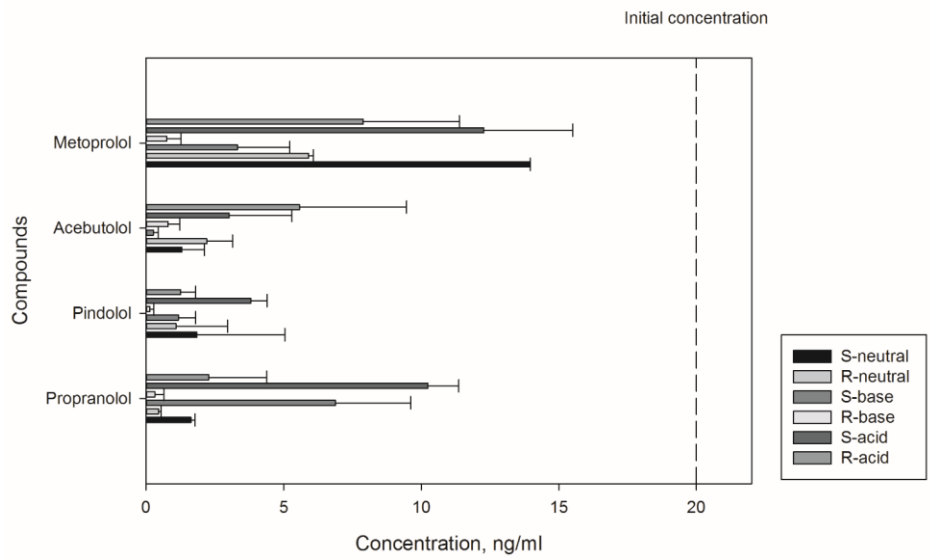
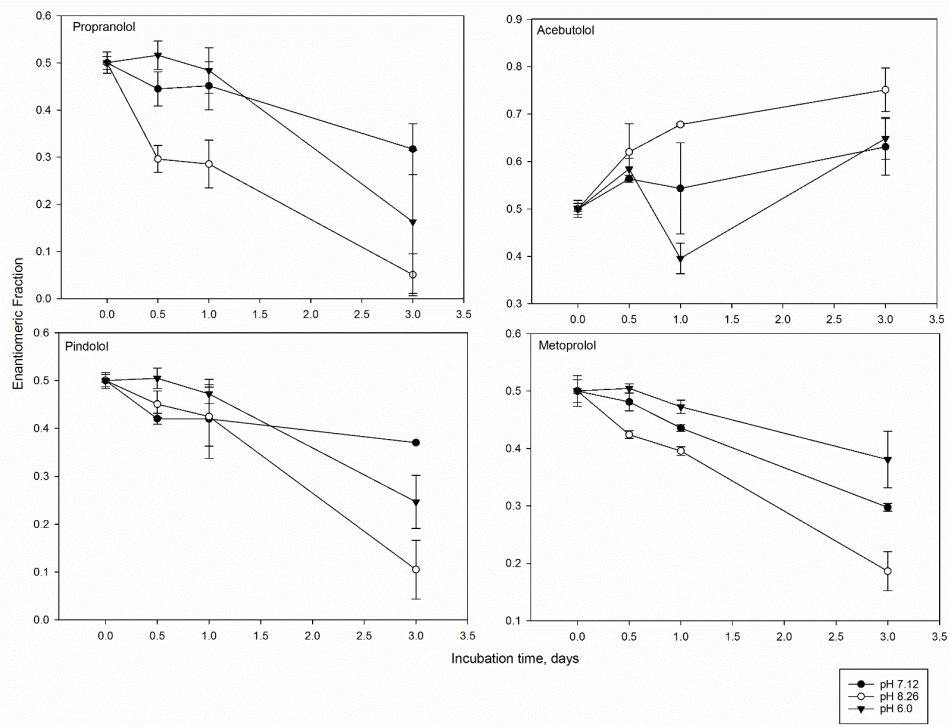


Figure 4-2 Stereoselective fate of  $\beta$ -blockers in wastewater microcosms at pH 7.12.





(a)



(b)

Figure 4-3 Effect of pH on stereoselective degradation (amount remaining in reactor (a) and change in EF values with time (b)) of  $\beta$ -blockers in wastewater.

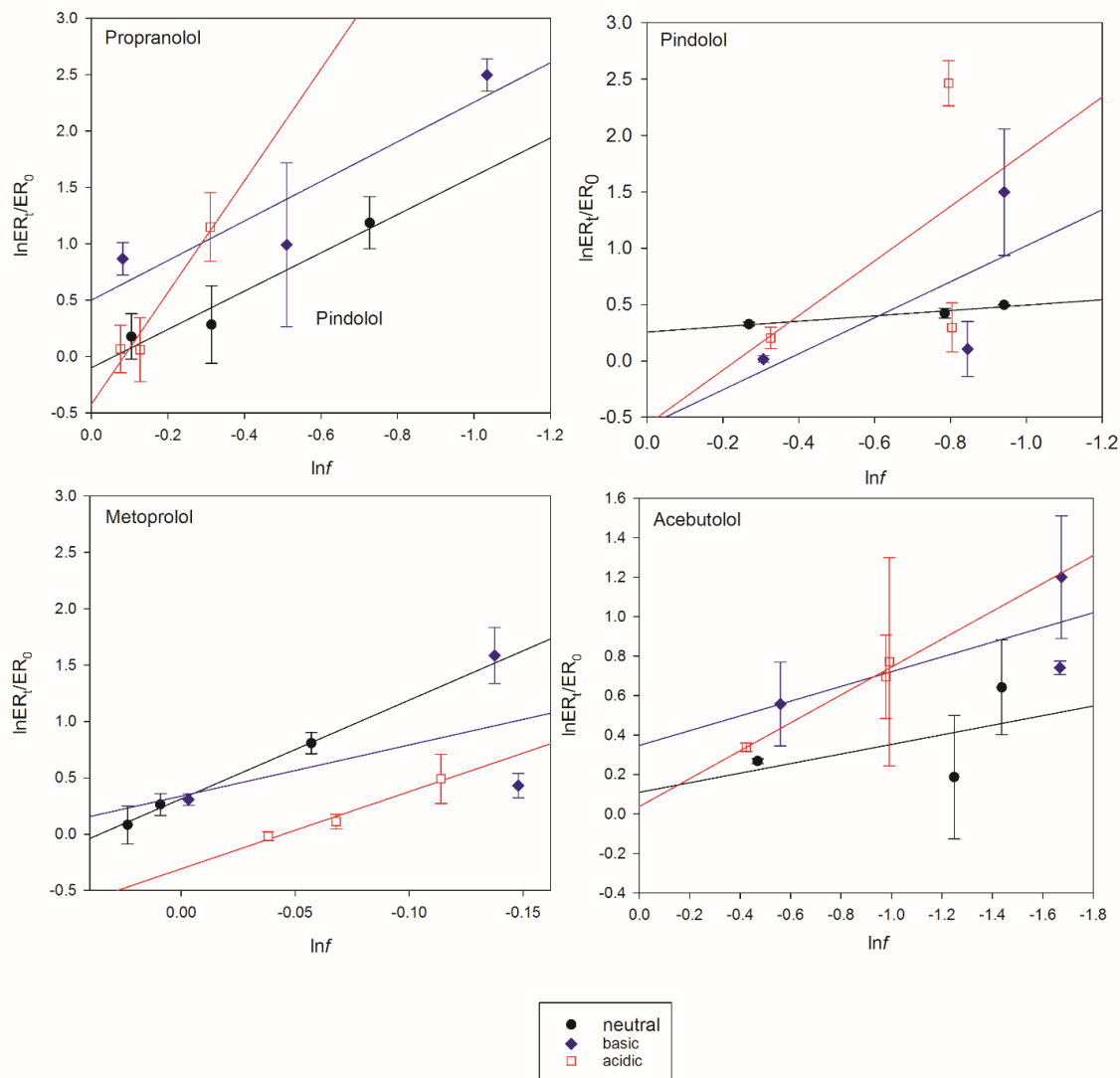


Figure 4-4 Rayleigh representation of the enantiomeric enrichment of fate of  $\beta$ -blockers in a wastewater microcosm in the dark.

## Supporting Information

Table S4-1 Physicochemical properties of selected  $\beta$ -blockers.

$\beta$ -blocker	MW, g/mol	Water solubility, mg/L	$\log K_{ow}$	$pK_a$	Ionization, % neutral species <sup>a</sup>		
					6.0	7.2	8.4
Acebutolol	336.20	259	1.71	9.2	0.03	0.43	6.38
Atenolol	266.16	13,300	0.16	9.6	0.02	0.34	5.13
Metoprolol	267.18	16,900	1.69	9.7	0.02	0.34	5.13
Pindolol	248.15	7,880	1.84	9.67	0.02	0.34	5.13
Propranolol	259.16	61.7	2.76	9.67	0.02	0.34	5.13

<sup>a</sup> % neutral species estimated using MarvinSketch 15.11.2.0 by ChemAxon.

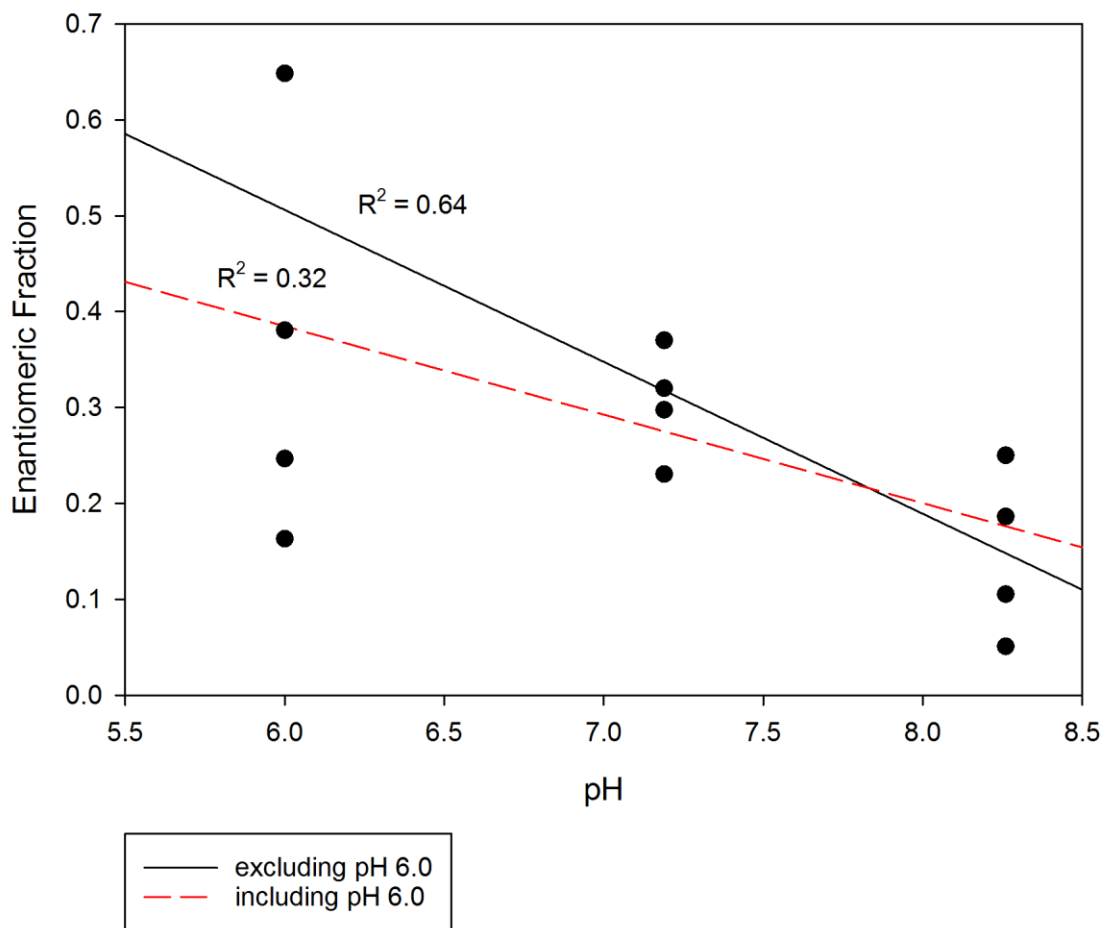


Figure S4-1 Effect of change in pH on stereoselectivity in degradation.

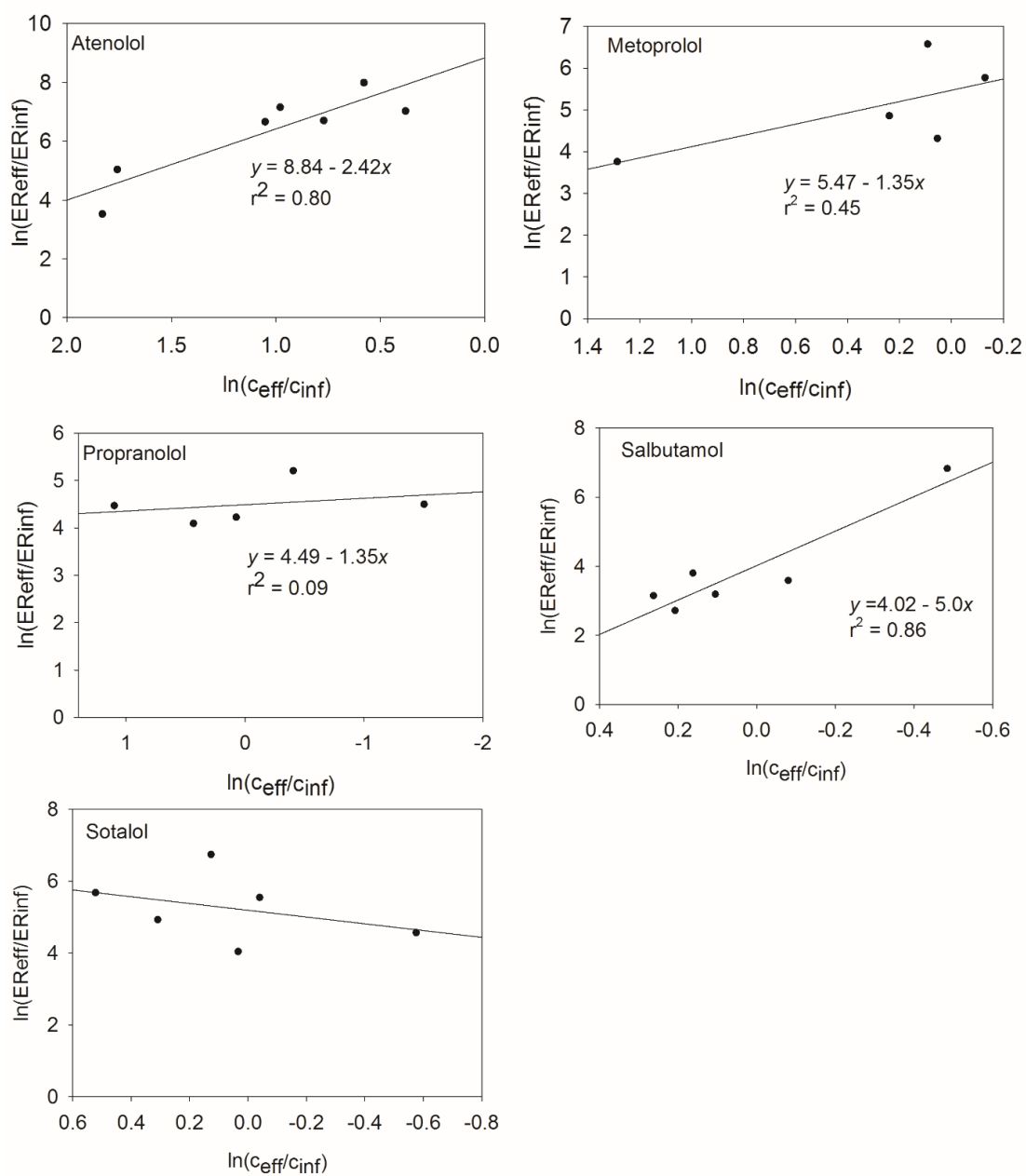


Figure S4-2 Rayleigh dependency in enantioselective degradation of 5  $\beta$ -blockers in 7 wastewater treatment plants.

## **Chapter 5 Leveraging Pharmacological Data for Prioritization of the Ecological Risks of Chiral Pharmaceuticals**

### 5.1 Introduction

Pharmaceuticals are frequently detected in wastewater and the environment at concentrations ranging from ng/L to  $\mu\text{g/L}$  (Kolpin et al., 2002; Subedi and Kannan, 2015). The most commonly detected therapeutic classes of pharmaceuticals are analgesics (Caballo et al., 2015; Kosjek et al., 2005), antidepressants (Metcalf et al., 2010; Niemi et al., 2013; Schultz and Furlong, 2008; Schultz et al., 2010), antibiotics (Hernández et al., 2007; O'Connor and Aga, 2007) and beta blockers (Parrilla Vázquez et al., 2012). Pharmaceuticals enter the environment through different point sources, such as discharge from manufacturing plants, landfills or wastewater treatment plants and from household waste (Baker and Kasprzyk-Hordern, 2013; Petrie et al., 2014). However, the major route of entry for pharmaceuticals is via wastewater effluent. It is estimated that the loading of pharmaceuticals into the environment will increase in the next decade with increasing life expectancies and general improvement in standards of living leading to higher rates of prescription drug use (Kümmerer, 2010). The occurrence of pharmaceuticals in the environment is an issue of major concern because they are biologically active compounds that can cause adverse effect at a low dose (Kümmerer, 2010).

Understanding the chirality of the pharmaceuticals and integrating exposure and effects based approaches through leveraging pharmacological stereoselective data is essential for accurate environmental risk assessment and prioritization. Approximately

2,500 pharmaceuticals have at least one chiral center and they are sold as racemic mixtures or single enantiomers (Kasprzyk-Hordern, 2010). Enantiomers have different physicochemical properties in chiral environments and it has been proposed that they should be treated using the mixture toxicity paradigm, that is to treat each enantiomer as a different compound (Stanley and Brooks, 2009). Therefore, it is implausible to carry out whole organism toxicity studies of all pharmaceuticals and their enantiomers. However, there is a wealth of knowledge available from drug discovery and development research that can be leveraged for predicting potential environmental exposure and effects of chiral pharmaceuticals (Rand-Weaver et al., 2013). Most of the data is available on stereoselective behavior of chiral pharmaceuticals in mammals because in 1992 the United States Food and Drug Agency issued a recommendation that pharmaceutical companies investigate the impact of chirality on pharmacology and toxicology (Mehvar and Brocks, 2001; U.S. Food and Drug Administration, 1992). The read-across hypothesis suggests that a pharmaceutical will have an effect on a non-target organism only if there is an evolutionary conservation of the primary drug targets such as molecular receptors and enzymes (Ford and Fong, 2015). It is possible to read-across stereospecificity in interaction of pharmaceuticals with the primary drug targets assuming the three dimensional structure of the drug targets is conserved. Hence, it is conceivable to leverage available pharmacological data to predict stereoselectivity in toxicity and establish a list of pharmaceuticals that need to be prioritized for further environmental monitoring and toxicity testing (Berninger et al., 2016).

With a view to aiding the environmental risk assessment of pharmaceuticals, we examined the factors that may affect the applicability of the read-across hypothesis in predicting stereoselective ecotoxicity in fish by investigating the peculiarities of pharmaceuticals in the environment and pharmaceuticals in biological systems. We investigated the validity of the assumption that there is evolutionary conservation of the primary drug targets, their major function and three dimensional structures so that we could establish whether extrapolating mammalian pharmacological data to fish toxicity was viable. This work focuses on stereoselectivity in environmental exposure, bioaccumulation and biological responsiveness of non-target organisms, specifically in aquatic environment.

## 5.2 Limitations of Traditional PBT Models

### 5.2.1 Exposure Assessment

Environmental risk assessment involves hazard assessment, exposure assessment and risk assessment and management. The goal of environmental risk assessment is protecting terrestrial and aquatic organism exposed to pollutants such as chiral pharmaceuticals. Since there are more than 5,000 pharmaceuticals on the market, evaluating the environmental risk of all of them is very ambitious and improbable. However, following the Stockholm protocol for persistent organic pollutants (POPs), environmental risk of pharmaceuticals evaluated using persistence, bioaccumulation potential and toxicity (PBT) model (Figure 5-1) (Christen et al., 2010). There are two integrated approaches used in the evaluation of environmental risk of pollutants, namely



establishing the exposure and the hazard factors. The hazard based approach focuses on hazard identification using the intrinsic properties of the pollutant, such as volatility, octanol-water partition coefficients, half-life, sorption, polarity and ionization. In contrast, exposure based approaches identify risk using extrinsic behavior of the pollutants in the environmental compartment. The exposure factor is mainly dependent on the magnitude, frequency and duration of exposure, thus influencing the occurrence, transport and partitioning of the pollutants in the environment. However, pharmaceuticals have different physicochemical and biological properties than POPs, and the PBT might require modifications in order to address the particular intrinsic and extrinsic properties of pharmaceuticals that make them a potential environmental risk (Tables

Table 5-1). Furthermore, the PBT model does not address the impacts of chirality on biological interactions between pharmaceuticals and non-target organisms.

The bioaccumulation potential of pharmaceuticals in non-target organisms is overlooked since pharmaceuticals are polar ionic compounds. However, most streams in the US are effluent-dominated, implying aquatic organism endure prolonged and frequent exposure to pharmaceuticals and this is termed pseudo-persistence. Pseudo-persistence may increase the bioaccumulation potential of the pharmaceuticals (Du et al., 2014). Additionally, pharmaceuticals are designed for optimal absorption and distribution into the human body through hydrogen bonding, ionic interaction and, at times, high lipophilicity (Du et al., 2014). Hence, in the past decade, several studies reported the bioaccumulation of pharmaceuticals such as  $\beta$ -blockers and antidepressants in fish (Garcia et al., 2012;

Valdés et al., 2014), algae and crustaceans (Vernouillet et al., 2010). However, the effect of enantiomeric composition on exposure risk can be incorporated in exposure assessment if a read-across of human pharmacological data is considered. For example, Williams et al. demonstrated a correlation between the  $K_d$  and  $V_D$  values for 13 pharmaceuticals with  $r^2 = 0.62\text{--}0.72$  in sediment (Williams et al., 2006) and soil systems (Williams et al., 2009). However, several mammalian pharmacological studies observed stereoselectivity in the  $V_D$  values of chiral pharmaceuticals (Brocks, 2006). Stereoselective in sorption of organic pollutants has previously reported in soil and sludge. Although no pharmaceutical studies on stereoselective bioaccumulation and plant uptake have been conducted this phenomenon has been observed in lizards (Wang et al., 2014), *Tubifex tubifex* (Liu et al., 2014), and *Brassica campestris* (H. Wang et al., 2013) exposed to racemic pesticides. Thus, bioaccumulation and plant uptake of chiral pharmaceuticals may be stereoselective.

### 5.2.2 Toxicity Assessment

Although pharmaceuticals have short half-lives ranging from hours to months, they are considered pseudopersistent because they are continually discharged into the environment via wastewater effluent (Niemi et al., 2013). Thus, non-target organisms are chronically exposed to chiral pharmaceuticals. Several studies reported beta blockers, antidepressants and other pharmaceuticals exhibited developmental (Airhart et al., 2007; David and Pancharatna, 2009), reproductive (Gust et al., 2009; Huggett et al., 2002), behavioral (Winder et al., 2009) and acute toxicity (Ferrari et al., 2004; Overturf et al., 2012) to various aquatic organisms. However, unlike pesticides, pharmaceuticals are

designed for therapeutic purposes and not for their toxicity. Consequently, there is a tendency to view them less as pollutants of major environmental concern. Numerous studies have shown that aquatic organisms are exposed to chiral pharmaceuticals in surface water, marine water and sediment (Kümmerer, 2010).

Since several studies have found chiral pharmaceuticals do not usually exist as racemic mixtures or single enantiomers in the aquatic environment, aquatic species are exposed to different doses of each enantiomer. In addition to the enantiomer specificity in exposure, numerous studies have demonstrated the adsorption, disposition, metabolism, and toxicity of chiral pharmaceuticals is stereoselective (Ardakani et al., 2008). Enantiomers usually engage in stereospecific interactions in chiral environments such as biological systems due to their three dimensional structure that results in stereoselective binding, catalysis, and stabilization of the chiral pharmaceuticals (Wong and Warner, 2010; Wong, 2006). When an enantiomer has high affinity for the receptor it is called a eutomer and a distomer has less affinity (Ariens et al., 1988; Ariëns, 1984). Stoschitzky and coworkers (Stoschitzky et al., 1993) showed (*S*)-atenolol was the eutomer since it contributed most of the  $\beta$ -adrenergic antagonism and (*R*)-atenolol was the distomer (Table 5-1). However, eutomer-distomer classification may be more complex. For example, tramadol is an analgesic comprising of a racemic mixture of (*R,R*)-(+)-tramadol and (*S,S*)-(-)-tramadol. The (+)-enantiomer inhibits serotonin reuptake due to a higher affinity for the  $\mu$ -receptor, but the (-)-enantiomer inhibits noradrenalin reuptake instead (Ardakani et al., 2008). However, most studies on ecotoxicity focus on acute toxicity ignoring chronic environmental exposure of non-target organisms. Therefore, understanding the

stereoselective toxicity of chiral pharmaceuticals to non-target organisms is essential for a more accurate risk assessment.

There are a few studies that have demonstrated stereoselectivity in the toxicity of pharmaceuticals to non-target aquatic organisms. Previous studies on environmental risk of fluoxetine demonstrated that there is a need for incorporating chirality, exposure and effects assessments for accurate pharmaceutical prioritization (Table 5-1) (Brooks et al., 2003; Scott et al., 2015; Stanley et al., 2007). The EC<sub>50</sub> of growth inhibition in *Daphnia magna* by atenolol and fluoxetine ranged from 6.9 to 1450 mg/L (De Andrés et al., 2009). In this study, De Andres et al. (2009) observed stereoselectivity in the adverse effects of atenolol with the (*S*)-enantiomer being more potent than the (*R*)-enantiomer, but not between enantiomers of fluoxetine. Even though environmental concentrations are below acute toxicity levels, pharmaceuticals are designed to effect physiological change at very low concentrations. Thus, pharmaceuticals may cause long-term effects, rather than short-term. After conducting a battery of traditional effects assessment, Brooks et al. (2003) found the environmental risk of fluoxetine was low, but they postulated that it could elicit adverse effects at non-traditional endpoints. However, fluoxetine was classified as a low priority pharmaceutical following a predicted exposure assessment using a corrected predicted environmental concentration (PEC)/predicted no effect concentration (PNEC) ratio (Oakes et al., 2010). A subsequent chronic toxicity study demonstrated that fluoxetine elicited sublethal and behavioral effects stereoselectively on *Pimephales promelas* (Stanley et al., 2007). These results agreed with the postulate made by Brooks et al. (2003) thus

showing knowledge of the evolutionary conservation of the primary target of the pharmaceuticals could help in their prioritization (Rand-Weaver et al., 2013).

### 5.2.3 Advantages of the Read-Across Hypothesis

Since there are more than 5,000 pharmaceuticals in current use, long-term toxicity studies cannot be conducted for all of them, hence the need for prioritization. The biological responses are very specific because the pharmaceuticals compounds are designed to target specific molecular receptors, enzymes, metabolic pathways, cell signal pathways, and/or plasma proteins (Fabbri and Franzellitti, 2016; Fabbri and Moon, 2015). However, the primary drug targets are often conserved across species, thus occurrence of pharmaceuticals in the environment could potentially elicit biological effects in non-target organisms (LaLone et al., 2013). The read-across hypothesis is often described as the ability for a pharmaceutical to elicit a physiological or behavioral response on a non-target organism due to evolutionary conservation of the primary drug target (Berninger et al., 2016; Lalone et al., 2014; Rand-Weaver et al., 2013). Since several studies reported that some primary targets of pharmaceuticals are conserved in aquatic organisms such as zebra fish, mussels and fathead minnow, the read-across hypothesis can be used for predicting ecotoxicity of pharmaceuticals and prioritization of pharmaceuticals in environmental risk assessments, through leveraging available mammalian pharmacological data (Berninger et al., 2016; Fabbri and Franzellitti, 2016; Lalone et al., 2014). In this systematic review we investigate the applicability of the read-across hypothesis in estimating stereoselectivity in environmental risk of chiral pharmaceuticals.

## 5.3 Approach

### 5.3.1 Selection of Compounds

To demonstrate the application of the read-across hypothesis to predicting stereoselective toxicity we selected 11 chiral pharmaceuticals for this review (Table 5-2). We chose compounds that represented the most widely used therapeutic class, that is analgesics, antidepressants and  $\beta$ -blockers. The compounds had pKa values ranging from 4.85 to 9.80 and predicted logKow between 0.04 and 4.20. Therefore, at environmental pH ranges the compounds exist as ionic and neutral species. We only investigated 11 pharmaceuticals because of the scarcity of studies on occurrence and toxicity of chiral pharmaceuticals in the environment. We only selected compounds that were detected in the environment following chiral analysis.

### 5.3.2 Determination of Predicted Environmental Concentration

We estimated the environmental concentration of the pharmaceuticals in surface water using usage patterns. The goal of exposure assessment of chiral pharmaceuticals is to provide data on their steady-state concentrations in a specific environmental compartment. The predicted environmental concentration (PEC) of pharmaceuticals is determined by estimating the total amount consumed by humans in a specific area, and the percentage that is excreted as parent compound factoring in the dilution effect of discharge (Arnold et al., 2013). In this review, we determined the PEC in surface water using the highest recommended daily dose ( $TD_{max}$ ), the market penetration percentage (M) (default

value is 0.01), the volume of sewage per person per day ( $V$ ) (default value is 200 L/person/day), and the rate of dilution of the WWTP effluent by surface water ( $D$ ) (default value is 10) (equation 5-1).

$$PEC_{surface\ water} = \frac{TD_{max} \times M}{V \times D} \quad (5-1)$$

However, as equation 1 shows, the factors considered in determination of PEC do not take chirality into account. However, occurrence of chiral pharmaceuticals has been shown to be mostly stereoselective in various environmental compartments (Ali et al., 2009; Ribeiro et al., 2012; Evans and Kasprzyk-Hordern, 2014; Kasprzyk-Hordern, 2010; Hashim et al., 2010). We sought to illustrate the limitations of PEC by calculating enantiomeric ratios (ER) using measured environmental concentrations. We selected previous studies that clearly identified the exact configurations of the enantiomers where possible. In all cases, the maximum detected concentration was selected because it is the worst case scenario, which is an important consideration when assessing environmental risk. It is important to note that spatial and temporal variations in enantiomeric composition of pharmaceuticals are often common in environmental monitoring. For example, the concentration and EF values of atenolol in wastewater effluent and influent was found to vary with the time of day due to drug consumption patterns or diurnal variations in wastewater operating conditions (Vazquez-Roig et al., 2014).

### 5.3.3 Application of the Read-Across Hypothesis

The effect of each enantiomer on non-target organisms was estimated using the Fish Plasma Model. The Fish Plasma Model states a biological effect is observed in non-target organisms when their plasma concentration is equal to or higher than the human plasma concentration that elicits the effects. Huggett et al (2003) proposed that the potential environmental effect in non-target organisms of a pharmaceutical can be predicted using the read-across model from the therapeutic dose. The important mammalian pharmacological data often used in read-across studies are the volume of distribution, human plasma concentration, clearance rate and half-life of elimination. Pharmaceuticals are partitioned from the plasma to the primary drug target and when the concentration reaches a certain threshold a biological response occurs (Giltrow et al., 2009). Therefore, the steady-state plasma volume of the distribution of a compound in an aquatic organism such as, a fish plasma steady-state concentrations ( $FPC_{ss}$ ), is compared to the human therapeutic plasma concentration ( $HPC_T$ ) to obtain the effect ratio (ER) (Figure 5-2). An adverse effect may occur when the  $ER > 1$ . The  $FPC_{ss}$  can be obtained experimentally or estimated using the Fish Steady State Plasma Model based on the following equations:

$$\text{Log}P_{\text{Blood:Water}} = 0.73 \times \text{log}D_{7.4} - 0.88 \quad (5-2)$$

$$FPC_{SS} = PEC \times P_{\text{Blood:Water}} \text{ or } FPC_{SS} = MEC \times P_{\text{Blood:Water}} \quad (5-3)$$

We obtained pharmacodynamic and pharmacokinetic data that was specific for each enantiomer from the literature and pharmaceutical databases. The daily dose and  $HPC_T$  for each enantiomer was obtained from previous studies that investigated the role of



stereochemistry in pharmacokinetics and pharmacodynamics. We obtained the primary drug targets for the 11 pharmaceuticals from DrugBank ([www.drugbank.ca](http://www.drugbank.ca)). We estimated the  $FPC_{SS}$  using equation 5-2 and equation 5-3.

#### 5.4 Results

The primary drug target for all the  $\beta$ -blockers we investigated was the  $\beta$ -1 adrenergic receptor, except for salbutamol which targeted the  $\beta$ -2 adrenergic receptor (Huggett et al., 2002). Antidepressants, namely fluoxetine, norfluoxetine and venlafaxine inhibited serotonin re-uptake by targeting the sodium-dependent serotonin transporter (Wacker et al., 2013). However, these pharmaceuticals also target other receptors, for example venlafaxine interacts with the noradrenergic re-uptake and propranolol also targets the 5-hydroxytryptamine 1A receptor (Schreiber et al., 2011). Table 5-3 shows that interaction of chiral pharmaceuticals with the primary drug targets is highly stereoselective. Except for sotalol and salbutamol, beta adrenergic receptors are effectively blocked by the (*S*)-enantiomers. As a result, Stanley et al. (2006) hypothesized that since (*S*)-propranolol was a more potent  $\beta$ -adrenergic receptor antagonist than its antipode, it should be more chronically toxic to fathead minnow. Their chronic toxicity study confirmed the hypothesis and thus suggesting the importance of evolutionary conservation of primary drug targets in the applicability of the read-across hypothesis. However, such enantiomer specific preference may not translate across different species which do not have the  $\beta$ -adrenergic system. For example, (*S*)-atenolol is more toxic than its antipode to a microalga, but less toxic than (*R*)-atenolol to a protozoan. This could be due to interspecies variations in

adsorption, metabolism, distribution and elimination of atenolol or existence of beta adrenergic receptor subtypes. Therefore, extrapolating stereoselectivity in pharmacology to environmental toxicity is challenging. However, data on chronic toxicity of chiral pharmaceuticals remains scarce, therefore there's need for more studies in this area.

#### 5.4.1 Determination of PECs and Plasma Concentrations

The environmental concentration of chiral pharmaceuticals can be predicted based on usage patterns. We estimated the PECs for 11 commonly used chiral pharmaceuticals using Equation 1 and the concentrations ranged from 1.0 to 1000 ng/ml (Table 5-3). Ibuprofen had the highest estimated PEC, this finding is supported with field studies (Ali et al., 2009). However, the major drawback of PECs is that they ignore the chirality of the compounds. Biotic processes have been shown to change the enantiomeric composition of chiral pharmaceuticals in the environment (Gasser et al., 2012; Moreira et al., 2014). The differences in  $HPC_T$  values between enantiomers corroborate this fact (Table 5-3). For example, (*R*)- and (*S*)-fluoxetine had  $HPC_T$  values of 22 and 130 ng/ml, respectively. However, the  $HPC_T$  values of betaxolol and sotalol were relatively less stereoselective with their differences ranging from 2.3 and 3.7 %. Since the investigation focused on stereoselectivity in effect ratio the  $FPC_{SS}$  values for each enantiomer were the same.

#### 5.4.2 Estimation of Effect Ratio

Table 5-1 and Table 5-3 show the experimental toxicity of the eleven chiral pharmaceuticals and their predicted effect ratios, respectively. Only two studies considered

the role of chirality in the ecotoxicity of pharmaceuticals. Data on stereoselective toxicity of chiral pharmaceuticals was only available for atenolol, propranolol and fluoxetine. Furthermore, the stereoselectivity studies did not use the same study organisms. Thus comparing estimated effect ratios to experimental toxicity results from different organisms helped to determine the applicability of read-across hypothesis in diverse organisms in which the primary drug targets were evolutionary conserved.

#### 5.4.2.1 $\beta$ -blockers

When the  $ER \leq 1.0$  the compound is considered to be high risk,  $1.0 < ER < 30$  medium risk but when  $ER > 30$  then it is a low risk pollutant. Of the seven  $\beta$ -blockers investigated, only metoprolol was found to be high risk. Betaxolol, propranolol, and salbutamol had medium risk whereas atenolol, pindolol and sotalol were predicted to be low risk. However, (*R*)-metoprolol was 3-fold lower than its antipode. Interestingly, stereoselectivity in effect ratio was found to be affected by the route of entry in humans. When salbutamol was administered by ingestion, the effect ratio for (*R*)- and (*S*)-enantiomers were 60 and 8.33, respectively. However, when (*R*)- and (*S*)-salbutamol were administered intravenously the effect ratios were 5.83 and 3.33, respectively. There is a need to establish which route of entry in humans can offer a better estimate of the effect ratio. However, although stereoselectivity was predicted in this investigation, Sun et al. (2013) found there was no stereoselectivity in the effect of (*R*)- and (*S*)-metoprolol on heart rate, hatching rate or mortality of zebrafish. Furthermore, Stanley et al. (2006) found (*S*)-

propranolol was more chronically toxic to *P. Promelas* than its antipode, which was the inverse of the predicted effect.

#### 5.4.2.2 Antidepressants

There were three antidepressants investigated in this systematic review, fluoxetine, norfluoxetine, and venlafaxine (Table 5-1 and Table 5-3). A degree of stereoselectivity was observed in the predicted risk of fluoxetine and venlafaxine. The effect ratio of (*R*)- and (*S*)-fluoxetine were 12 and 69, respectively. Venlafaxine and (*R*)-fluoxetine had an effect ratio between 1.0 and 10. However, compared to (*R*)-fluoxetine, venlafaxine had an effect ratio 4 times more potent. Norfluoxetine and (*S*)-fluoxetine were estimated to be low risk since their effect ratios were above 60. De Andrés et al. (2009) found that (*R*)-fluoxetine was more toxic to *P. subcapita* than (*S*)-fluoxetine agreeing with the prediction. However, there are no studies on the stereoselective ecotoxicity of norfluoxetine or venlafaxine.

#### 5.4.2.3 Analgesics

The ecotoxicity and environmental risk of ibuprofen was investigated (Table 5-1 and Table 5-3). Ibuprofen is one of the most widely used pharmaceuticals and it had a high PEC hence the estimated effect ratio was very low at 0.09 and 0.10 for (*R*)-ibuprofen and (*S*)-ibuprofen, respectively. However, the effect of ibuprofen on non-target organisms was predicted to be slightly stereoselective. There are no studies on the stereoselective ecotoxicity of ibuprofen or other analgesics.

## 5.5 Discussion

The conserved primary drug targets are chiral macromolecules, which potentially exhibit stereoselectivity when interacting with chiral pharmaceuticals. For example, propranolol is a chiral compound whose therapeutic properties are based on its  $\beta$ -adrenergic antagonist properties. It has also been shown to be an antagonist for the serotonin (5-hydroxytryptamine [5-HT]) receptor, which modulates physiology of invertebrates (Figure 5-3) (Fabbri, 2015). However, early human pharmacological studies demonstrated that interaction of 5-HT receptors in rat brain membranes with enantiomers of propranolol was stereoselective (Middlemiss, 1984). The enantiomer, (*S*)-(-)-propranolol was more active than the antipode. Stanley et al. (2006) found (*S*)-propranolol was more chronically potent to *P. promelas* in which  $\beta$ -adrenergic receptors are more conserved than (*R*)-propranolol with regard to chronic toxicity. However, *D. magna* does not possess  $\beta$ -adrenergic receptors and hence no apparent toxicity was observed (Stanley et al., 2006). These observations suggest that the evolutionary conservation of the primary drug target is a critical parameter for accurately predicting potential environmental effects of pharmaceuticals.

Evolutionary conservation of primary drug target approach can be a powerful tool for predicting environmental toxicity of chiral pharmaceuticals. Furuhaugen et al. (2014) demonstrated that evolutionary conservation of drug targets in the cladoceran, *Daphnia magna*, was positively correlated to the aquatic toxicity of levonorgestrel, miconazole and promethazine (Figure 5-4). They found that the primary targets of miconazole and

promethazine are conserved in *D. magna*. These conserved primary targets proteins are called orthologs. Levonorgestrel did not elicit any adverse effects because its primary target was not conserved in *D. magna*. However, in a more extensive study, orthologs for 1318 primary drug targets were predicted in 7 species that are frequently used in ecotoxicity studies (Gunnarsson et al., 2008). They found 86%, 61% and 35 % evolutionary conservation of the orthologs in *Danio rerio*, *D. magna* and *Chlamydomonas reinhardtii*, respectively. Importantly, Gunnarsson et al. (2008) found that orthologs of pharmaceuticals that effect change through enzyme mediation were well conserved across species suggesting pharmaceuticals targeting enzymes could cause adverse effects in a number of species (Gunnarsson et al., 2008; Rand-Weaver et al., 2013). These results and those reported by other researchers, showed that there is a need for incorporating the evolutionary conservation of molecular targets of the pharmaceuticals in establishing the endpoints for an ecotoxicity assessment (Berninger et al., 2016; Lalone et al., 2014; LaLone et al., 2013).

#### 5.5.1 The Challenges of Receptor Subtypes

Application of read-across method in environmental risk assessment can be challenging since evolutionary conserved primary drug targets may exist as different subtypes across species (Fabbri and Moon, 2015; Rand-Weaver et al., 2013). The subtypes of molecular receptors often have different ligand binding patterns and this may cause variations in the resultant biological response elicited by the pharmaceutical compounds (Wacker et al., 2013). For example, the ligand-receptor interaction of ergotamine with 5-

HT receptor was shown to vary between the two subtypes, 5-HT<sub>2B</sub> and 5-HT<sub>1B</sub> (Wacker et al., 2013). Wacker et al. (2013) found 5-HT<sub>2B</sub> demonstrated functional selectivity for the signaling of  $\beta$ -arrestin was very strong but the 5-HT<sub>1B</sub> receptor was non-selective. Although the residues on the active domain of 5-HT receptor family are well conserved, Wang et al. (2013) found that the ligand binding of ergotamine differed between 5-HT<sub>2B</sub> and 5-HT<sub>1B</sub>. Specifically, they found that, unlike 5-HT<sub>2B</sub>, the 5-HT<sub>1B</sub> receptor had an extended pocket that made the serotonin receptor subtype bind the ligand strongly hence the subtype functional selectivity.

#### 5.5.2 Challenges of Interspecies Variations

Brown et al (2014) found that there was evolutionary conservation of between 65 and 86 % human primary drug targets in 12 diverse species. They found that the ligand binding domain on nuclear steroid hormone receptors responsible for therapeutic properties of 45 estrogenic pharmaceuticals was highly conserved, but varied across species. Furthermore, there was interspecies variation in the activation of estrogen receptors and target-ligand binding. Brown et al (2014) suggested the interspecies differences were caused by variations in promoter sequences and amino acid residues in other domains besides the ligand-binding domain, such as the DNA binding domain. Therefore, although there is strong evolutionary conservation of primary drug targets in fish, interspecies variations or existence of subtypes may lead to diversions in drug receptor activation, physiological and behavioral responsiveness that complicates predicting population level effects (Brown et al., 2014). As a result, variations in stereoselectivity in biological

responses to chiral pharmaceuticals between species could be attributed to evolutionary divergence in primary drug targets.

### 5.5.3 Challenges of Chirality and Receptor Subtypes

The  $\beta$ -adrenergic receptors are one of the most studied molecular receptors in mammals. They mediate several biological processes and have been shown to be well conserved across species. Wang et al (2009) found five  $\beta$ -AR genes in adult zebrafish, which they named *adrb1*, *adrb2a*, *adrb2b*, *adrb3a*, and *adrb3b*. Sun et al (2013) investigated the effect of enantiomers of propranolol and metoprolol on the transcription of these  $\beta$ -AR genes in zebrafish. They observed enantioselectivity in the transcription of *adrb3b* with (*R*)-propranolol yielding a higher transcription level than treatment with (*S*)-propranolol. However, the expression of other genes was not altered following exposure to racemic, (*R*)- or (*S*)-propranolol (Sun et al., 2013). Enantioselectivity was more pronounced following exposure to metoprolol, with (*S*)-metoprolol downregulating transcription of *adrb1*, *adrb2b*, *adrb3a*, and *adrb3b*, whereas (*R*)-metoprolol upregulated *adrb3a*. Therefore, for better assessment of the environmental risk of pharmaceuticals there is a need for understanding the implications of chirality on ligand-receptor interactions. Stereoselective toxicity is influenced by the subtype and the chirality of the pharmaceuticals. However, as these results show, enantiomers might target different subtypes. There is a need for a better understanding of the structural basis of ligand-receptor interaction and biological functions of the different primary drug target subtypes.



## 5.6 Limitations of the Read-Across Method

Recent studies have shown that exposing fish to environmentally relevant concentrations resulted in fish plasma concentrations that exceeded human therapeutic concentrations (Ford and Fong, 2015; Giltrow et al., 2009; Scott et al., 2015). Giltrow et al. (2009) found that following exposure to 0.1 and 1.0 mg/L propranolol, the fish plasma concentration in fathead minnows exceeded the therapeutic human plasma concentration. Furthermore, they found that the fish plasma concentration increased with an increase in concentration of propranolol in water (Giltrow et al., 2009). Thus, the fish plasma model could be used to estimate the effect ratio of propranolol in fish. To that effect, Giltrow et al. (2009) found that the correlation between their experimental and predicted fish plasma concentration was within 1 order of magnitude; demonstrating the applicability of the read-across hypothesis. However, these results had three major drawbacks 1) the concentrations of propranolol in water used were above environmentally relevant concentrations, 2) propranolol is an ionic compound and its bioaccumulation in fish can be affected by the physicochemical properties of the surface water, such as pH, and 3) the chirality of propranolol was overlooked despite the fact that stereoselective mammalian pharmacological data is available. Scott et al. (2015) addressed the first two concerns by determining the plasma concentration of fish obtained from wastewater-dominated rivers. The fish plasma concentration of diltiazem consistently exceeded the therapeutic human plasma concentration over the study period (Scott et al., 2015). Similarly, the experimental fish plasma concentrations for diltiazem and diphenhydramine exceeded the predicted values (Scott et al., 2015).

## 5.7 Conclusion

Stereoselective distribution and fate has been the focus in evaluating environmental risk of chiral pharmaceuticals. However, such studies only cover exposure assessments, overlooking the effects assessments. In risk assessment paradigms, both the exposure and effect factors are critical and need to be determined experimentally or predicted using models. The enantiomeric composition of the pharmaceutical in the environment may contribute to stereoselective bioaccumulation of the compounds. A read-across model incorporating stereoselectivity in mammalian pharmacological effect can be used to predict environmental risk of chiral pharmaceuticals. Metoprolol was shown to be a high risk compound and its enantiomers showed a stereoselectivity. Venlafaxine, fluoxetine and propranolol were estimated to be medium risk pollutants. Prediction of the effect ratio will help in the prioritization of chiral pharmaceuticals in environmental risk assessments. However, there is need for additional studies on stereoselective chronic ecotoxicity of chiral pharmaceuticals.

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Tables

Table 5-1 Physicochemical properties of select chiral pharmaceuticals.

Drug	pK <sub>a</sub>		logK <sub>ow</sub>	K <sub>blood:water</sub>
	1	2		
Atenolol	9.4	13.9	0.33	0.059
Betaxolol	9.67	14.09	2.54	0.261
Ibuprofen	4.85		3.84	0.353
Fluoxetine	9.80		4.17	0.374
Metoprolol	9.67	14.09	1.76	0.199
Norfluoxetine	9.77		3.74	0.345
Propranolol	9.67	14.09	2.58	0.263
Pindolol	9.67	14.09	1.69	0.193
Salbutamol	9.40	14.18	0.88	0.120
Sotalol	9.43	14.43	0.05	0.015
Venlafaxine	8.91	14.42	2.74	0.275

pK<sub>a</sub> and LogK<sub>ow</sub> estimated from ACD/LogD Suite

K<sub>blood:water</sub> calculated value from Equation 1

Table 5-2 Pharmacological and ecotoxicological behavior of 11 chiral pharmaceuticals and their respective primary targets obtained from DrugBank (www.drugbank.ca).

Drug	Pharmacology		Ecotoxicity	Primary drug target	References
	Class	Stereoselectivity			
Atenolol	$\beta$ -blocker	activity due to ( <i>S</i> )-atenolol	( <i>S</i> )-atenolol was more toxic than the antipode to a microalga, but less toxic than ( <i>R</i> )-atenolol to a protozoan	$\beta$ -1 adrenergic receptor	(De Andrés et al., 2009)
Betaxolol	$\beta$ -blocker	activity due to ( <i>S</i> )-betaxolol	Toxicity was estimated to be low using ECOSAR	$\beta$ -1 adrenergic receptor	(Sanderson et al., 2003)
Ibuprofen	Antidepressant	activity due to ( <i>S</i> )-ibuprofen	Ibuprofen retarded development of zebrafish embryo at >10 $\mu$ g/L	Prostaglandin G/H synthase 2	(David and Pancharatna, 2009)
Fluoxetine	Antidepressant	activity due to ( <i>S</i> )-fluoxetine	( <i>R</i> )-fluoxetine was more toxic to <i>P. subcapita</i> than the antipode	Sodium-dependent serotonin transporter	(De Andrés et al., 2009)
Metoprolol	$\beta$ -blocker	activity due to ( <i>S</i> )-metoprolol	No stereoselectivity in observed changes in heart rate, hatching rate or mortality to zebrafish	$\beta$ -1 adrenergic receptor	(Sun et al., 2013)
Norfluoxetine	Antidepressant	-	-	Sodium-dependent	

Drug	Pharmacology		Ecotoxicity	Primary drug target	References
	Class	Stereoselectivity			
				serotonin transporter	
Propranolol	$\beta$ -blocker	activity due to ( <i>S</i> )-propranolol	( <i>S</i> )-propranolol was more chronically toxic to <i>P. promelas</i> than ( <i>R</i> )-propranolol	$\beta$ -1 adrenergic receptor	(Stanley et al., 2006)
Pindolol	$\beta$ -blocker	activity due to ( <i>S</i> )-pindolol	-	$\beta$ -1 adrenergic receptor	(Mehvar and Brocks, 2001)
Salbutamol	$\beta$ -blocker	activity due to ( <i>R</i> )-salbutamol	-	$\beta$ -2 adrenergic receptor	(Kasprzyk-Hordern, 2010)
Sotalol	$\beta$ -blocker	activity due to ( <i>R</i> )-sotalol	Increased number of embryos in New Zealand mudsnail following exposure	$\beta$ -1 adrenergic receptor	(Feiner et al., 2014)
Venlafaxine	Antidepressant		Increased mortality to fathead minnows exposed to 305 ng/L venlafaxine	Serotonin re-uptake and noradrenergic re-uptake	(Schultz et al., 2011)

Table 5-3 Estimation of the effect ratio for each enantiomer using predicted environmental concentrations to establish the FPCss values.

Drug	Dose	HPC <sub>T</sub> , ng/ml		PEC, ng/ml		FPC <sub>ss</sub> , ng/ml		Effect Ratio		Reference
		R	S	R	S	R	S	R	S	
Atenolol	50	332	323	13.0	13.0	0.77	0.77	435	423	(Egginger et al., 1993)
Betaxolol	50	41	42	13.0	13.0	3.38	3.38	12	12	(Stagni et al., 1991)
*Ibuprofen	400	30.9	34.2	1000	1000	352	352	0.09	0.10	(Canaparo et al., 2000)
Fluoxetine	20	22	130	5.0	5.0	1.87	1.87	12	69	(Silva et al., 2009)
Metoprolol	50	0.9	3.0	13	13	2.59	2.59	0.35	1.19	(Hermansson, 1982)
Norfluoxetine	20	108	161	5.0	5.0	1.73	1.73	62	93	(Silva et al., 2009)
Propranolol	80	34.5	48.7	20	20	5.27	5.27	6.56	9.25	(Pham-Huy et al., 1995)
Pindolol	15	36	33	4.0	4.0	0.77	0.77	46	42	(Somogyi et al., 1992)
Salbutamol- oral	4	7.2	1.0	1.0	1.0	0.12	0.12	60	8.33	(Schmekel et al., 1999)
Salbutamol - IV	0.2	0.7	0.4	1.0	1.0	0.12	0.12	5.83	3.33	(Schmekel et al., 1999)
Salbutamol - inhalation	0.8	2.0	1.2	1.0	1.0	0.12	0.12	16	10	(Schmekel et al., 1999)
Sotalol	80	270	260	20	20	0.30	0.30	912	878	(Badaloni et al., 2003)
Venlafaxine <sup>a</sup>	200	38.7	45.8	50	50	13.8	13.8	2.81	3.33	(Liu et al., 2007)
	75	17.9	21.7	19	19	5.23	5.23	3.42	4.15	(Liu et al., 2007)

Figures

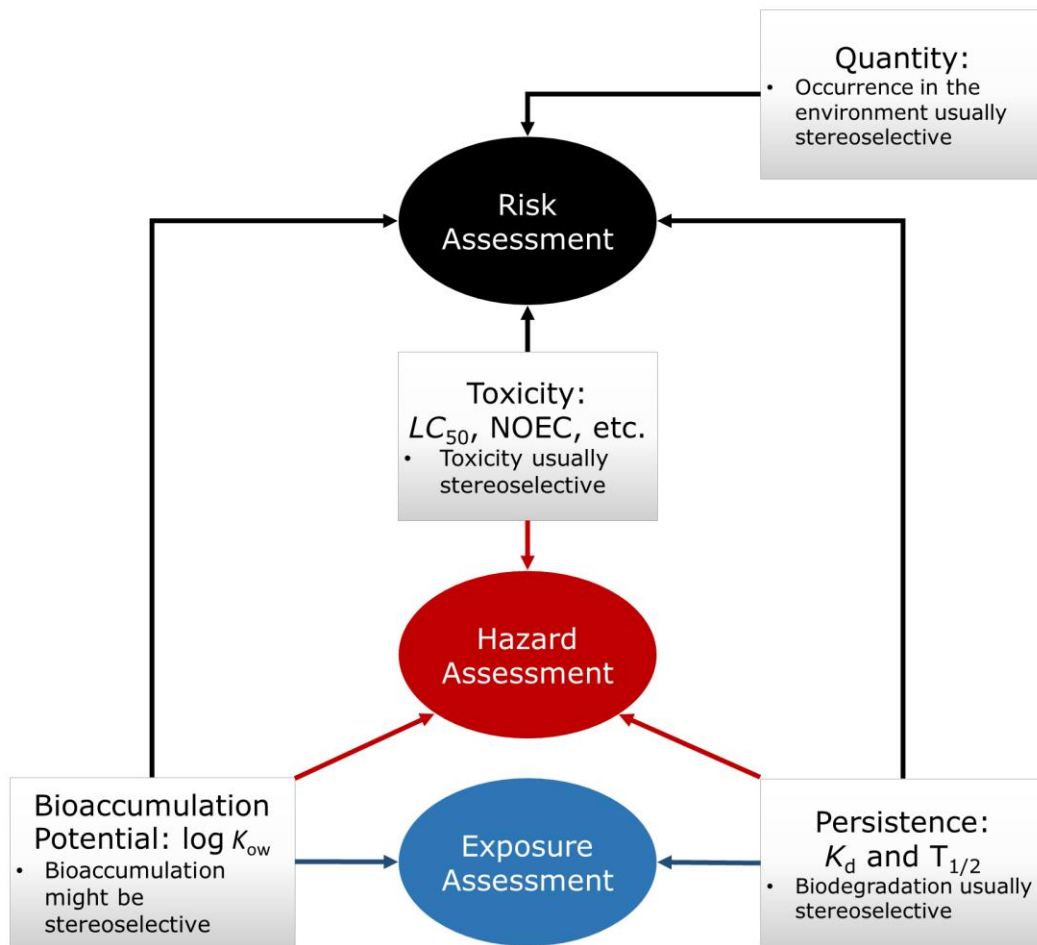


Figure 5-1 Limitations of persistence, bioaccumulation and toxicity on establishing the risk of chiral pharmaceuticals in the environment.

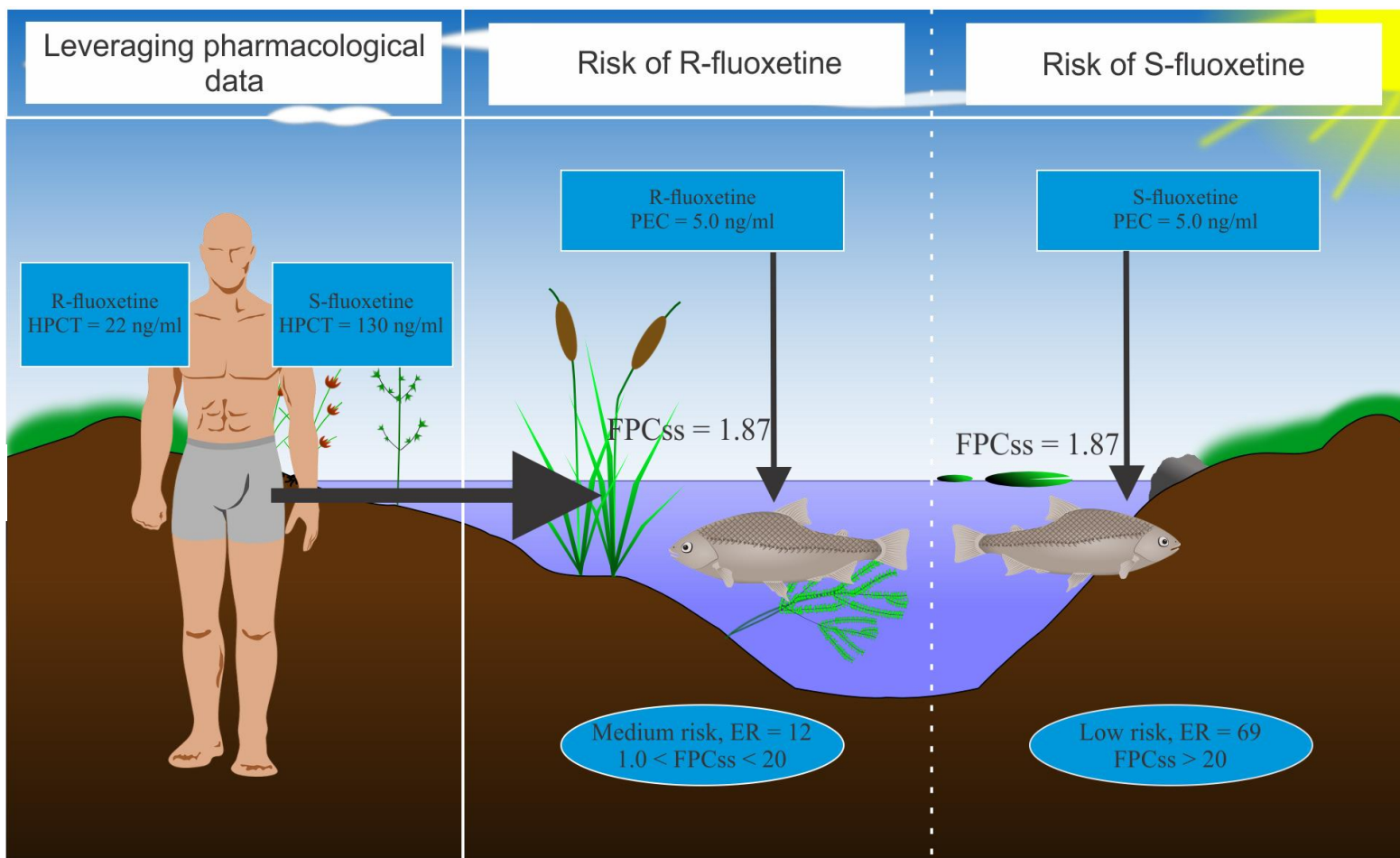


Figure 5-2 Estimating stereoselectivity in the environmental risk of enantiomers of fluoxetine through leveraging human steady state plasma concentration of each enantiomer.



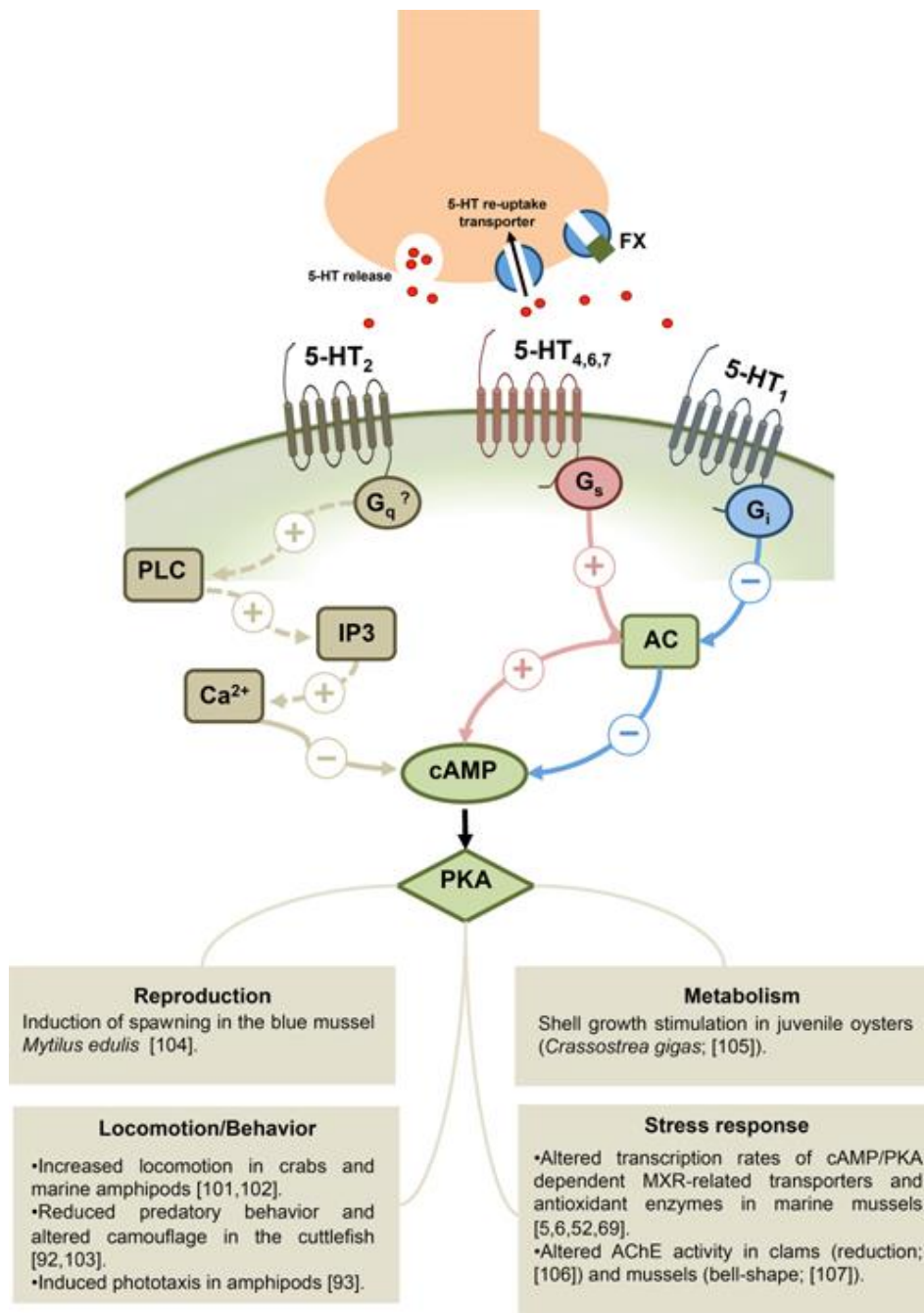


Figure 5-3 The mode of action of fluoxetine (FX): Serotonin reuptake inhibition (Fabbri and Franzellitti, 2016).

## Chapter 6 General Conclusions and Future Research Needs

### 6.1 General Conclusions

A great number of pharmaceuticals are chiral compounds. The occurrence, fate and toxicity of chiral pharmaceuticals in the environment is often stereoselective with one enantiomer becoming relatively more abundant than the antipode from enantioselective fate behaviors. In this dissertation, a chiral analysis method was developed and employed in evaluating stereoselectivity of a wide range of chiral pharmaceuticals in their adsorption to wastewater sludge and biodegradation during wastewater treatment. Wastewater treatment plants are the initial collection point for such pharmaceuticals and incomplete removal contributes to their occurrence in the open environment. The stereoselectivity in the effect of chiral pharmaceuticals on non-target aquatic organisms was further estimated using the read-across hypothesis by leveraging pharmacological data. Incorporating chirality in environmental fate and ecotoxicological studies significantly improves the accuracy of environmental risk assessment of chiral pharmaceuticals.

We first developed a chiral analysis method for the separation and quantitative determination of atenolol and fluoxetine using liquid chromatography. Decreasing the concentration of liophilic ions in the mobile phase resulted in an increase in enantioresolution and retention factor. The effect of the type of liophilic ions on the retention factor followed the Hofmeister series:  $\text{CH}_3\text{COO}^- > \text{HCOO}^- > \text{NO}_3^-$ . The results suggest that the enantiorecognition mechanism of basic pharmaceuticals involved chaotropic interactions between the liophilic ions, analytes and chiral stationary phase.

Using the chiral analysis method, adsorption of five  $\beta$ -blockers on wastewater sludge was then characterized using batch experiments. The sorption of acebutolol, atenolol and metoprolol was stereoselective with EF values 0.27, 0.55 and 0.32, respectively, but pindolol and propranolol were sorbed non-selectively. Thus, pharmaceuticals with relatively low hydrophobicity exhibited the highest stereoselectivity in adsorption, suggesting that stereoselectivity in adsorption was primarily governed by polar, instead of hydrophobic, bond interactions.

In a subsequent study, we investigated the enantioselective degradation of 5  $\beta$ -blockers in wastewater microcosms at different pH conditions. We observed S-enrichment in the degradation of metoprolol, pindolol, and propranolol with the enantiomeric fraction changing from 0.5 to  $0.30 \pm 0.01$ ,  $0.37 \pm 0.0$  and  $0.32 \pm 0.05$ , respectively. A Comparison of change in enantiomeric composition to degradation gave a good linear fit for propranolol, acebutolol, and metoprolol ( $R^2 > 0.94$ ), thus indicating Rayleigh dependence. Such enantiomeric enrichment-degradation relationships may be used to understand the role of biodegradation, and to improve the accuracy of environmental risk assessment of chiral pharmaceuticals by offering valuable quantitative assessment data.

In the last study, we leveraged pharmacological data to predict stereoselectivity in the aquatic toxicity of 11 chiral pharmaceuticals. Particularly, (*R*)-metoprolol was found to be more potent than (*S*)-metoprolol by a factor of 3 and (*R*)-fluoxetine was 5 times more potent than its antipode. We estimated that (*S*)- and (*R*)- ibuprofen, and (*S*)-metoprolol were high-risk pollutants since their effect ratio was less than 1.0, while betaxolol,

propranolol, venlafaxine and (*R*)-fluoxetine had medium risk with effect ratio between 1.0 and 10. Therefore, the read-across hypothesis may be used as a tool for predicting the certain environmental risk of chiral pharmaceuticals.

## 6.2 Future Research Needs

We developed a method for analyzing basic pharmaceuticals in environmental samples and employed it to study their environmental fate. Several studies determined the enantiomeric composition of chiral pharmaceuticals in wastewater, surface water and sludge. Thus, it is evident that aquatic organisms are frequently exposed to different concentrations of chiral pharmaceuticals. However, there is need to investigate stereoselectivity in uptake of chiral pharmaceuticals by aquatic organisms and the method we developed may be extended to the analysis of biological samples with modifications.

Some studies showed that sorption was an important removal pathway of acidic pharmaceuticals in wastewater treatment plants. We showed that adsorption of basic chiral pharmaceuticals was stereoselective, especially for chiral compounds with relatively low hydrophobicity. However, approximately 25% of currently available drugs are single acids or diacids, and hence there is a need to determine stereoselectivity in adsorption of acidic chiral pharmaceuticals on sludge. Furthermore, additional studies are needed to discern stereoselectivity in the adsorption of chiral pharmaceuticals to soil and aquatic sediments, as organic matter in these matrices may contain chiral surfaces and enable selective interactions.

We demonstrated that enantiomeric fraction can be used as a tool for determining quantitatively the biodegradation of chiral pharmaceuticals in wastewater. The enantiomeric enrichment-conversion relationship yields a single constant that can potentially be predicted using quantitative structure–activity modeling. There is need for developing such models for other scenarios because they may help in source tracking of the metabolites of chiral pharmaceuticals as environmental contaminants and for predicting environmental risks without extensive experimentation.

Furthermore, we showed that the read-across hypothesis could be used to predict stereoselective effects of chiral pharmaceuticals to non-target organisms. However, the applicability and validity of this hypothesis remains limited due to the lack of comprehensive chronic toxicity studies. There is a need for investigation on the chronic toxicity of chiral pharmaceuticals to non-target organisms and the possibility of employing the read-across hypothesis as a tool for prioritization should be further explored.