Quantification and Distribution of Pharmaceuticals and their Human Metabolite Conjugates in a Municipal Wastewater Treatment Plant

By

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Thesis Abstract

Pharmaceuticals have the potential to persist environmentally through constant anthropogenic input via wastewaters. Toxic effects, both acute and chronic, can be elicited on non-target organisms within the aquatic environment depending on both species sensitivity and chemical class. Overall toxicity can be due to not only parent compounds but transformation products (TPs) as well. It was hypothesised that levels of pharmaceutical TP conjugates would rival those of the parent compounds within a major point source of pharmaceuticals (i.e. wastewater). This thesis successfully developed quantitative methods, for the first time, for four different classes of pharmaceuticals and three different types of conjugate TPs using weak anion exchange solid phase extraction in conjunction with liquid chromatography-tandem mass spectrometry for wastewaters and associated solids; and was validated using the North End Waste Pollution Control Centre located in Winnipeg, Canada. A three-month pilot experiment was conducted using these methods and highlighted the levels of acetaminophen, propranolol, sulfamethoxazole, and thyroxine, in addition to their associated conjugate TPs: acetaminophen sulfate, propranolol sulfate, *N*-acetyl sulfamethoxazole, sulfamethoxazole glucuronide, thyroxine glucuronide. Four different stages of wastewater processing were analysed (primary effluent, secondary effluent, mixed liquor, and final effluent), and levels in aqueous and solid phases assessed. Overall, acetaminophen was rapidly attenuated from primary to secondary effluent (>99%), propranolol and thyroxine persisted without any notable attenuation, and sulfamethoxazole were attenuated by approximately 67-78% from primary to secondary effluent; however the ratio of the three compounds remained consistent across treatments. Several batch bioreactor experiments using primary and secondary effluent, with or without aeration, were conducted to backstop what was seen environmentally. In addition, plausible mechanisms for

temporospatial variation (i.e. removal/ attenuation) were inferred. For the first time, bioreactor results showed concomitant effects on TP levels in the laboratory that were seen environmentally. In conclusion, levels of conjugates across all four classes of compounds, whether an acid, base, or zwitterion, did indeed rival those of the parent compounds within wastewaters. Sorption of ionisable pharmaceutical conjugates seems to be driven by hydrophobicity in the presence of substantial organic matter. Thus, dependent on pedoclimatic conditions, exposure levels and ostensibly the fate of these pharmaceutical TPs can vary. In light of the possibility for deconjugation to occur in surface waters via microbial enzymes, conjugates may provide an ancillary source for parent pharmaceuticals, and thus toxic effects to non-target organisms.

Acknowledgements

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A special thank you to The University of Winnipeg who has afforded my family and I every practical opportunity in providing state of the art analytical chemistry facilities, deferring to me first in choosing the sections of chemistry to teach that fit my family's schedule the best,

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Chapter 1

Introduction to Human Pharmaceutical Metabolite Conjugates

1. Thesis Hypothesis and Objectives

The overall objective of this PhD dissertation was to characterise the occurrence and distribution of pharmaceutical metabolite conjugates within wastewater using a municipal treatment plant as the model site. It was hypothesised that levels of these conjugates would rival those of the parent compounds. This dissertation has three main objectives: 1. Develop analytical techniques to extract and quantify pharmaceutical conjugates from environmental samples. 2. Perform a pilot study to take samples from the primary clarifier, the mixed liquor of the secondary treatment, the secondary clarifier, and the final effluent of North End WWTP using the developed methods to explore the current inventory of related conjugates. 3. Conduct a bench-top bioreactor experiment to corroborate larger scale wastewater treatment plant (WWTP) findings and infer mechanisms responsible for the findings of the resultant kinetics of various drug TPs *in vitro* in a natural/ engineered environment.

The following in this chapter introduces the impetus behind pursuing the objectives of the thesis contained herein. This includes understanding how pharmaceutical metabolite conjugates are made and how they persist environmentally. Given this persistence, it is important to highlight their potential for toxicity to non-target organisms and how to account for establishing subsequent risk to biota using a suite of assumptions via modelling software in the absence of concrete data. This current chapter also describes the source of pharmaceutical conjugates, and the justification for sampling specific locations within a WWTP to develop validated quanititative methods.

Integral to method development and monitoring regimes is the understanding of the solubility and stability of pharmaceutical conjugates in both standard solutions and the aquatic environment. Of important note, is also to highlight the sorptive tencencies of pharmaceuticals

and their respective conjugtes to suspended solids in addition to sludge within the WWTP. Given that pharmaceuticals are ionisable compounds, the complexity of mixed modes of sorption are also explored using a model compound to illustrate the many potential mechanisms at play that affect the distribution, transformation, and thus persistence within a WWTP environment.

Therefore, with these aforementioned rationales in mind, this thesis sought to quantify pharmaceutical TPs that were diverse in physicochemical parameters, and whose parent compound concentrations are quantified commonly within wastewaters, and proximal surface waters receiving wastewater input. It was also important to analyse for compounds that are "pseudo-persistent" in that they are continuously present within these waters due to human usage and excretion. North American prescription sales are one of the only guidelines available to estimate usage/ consumption due to privacy issues involving medical history of the population. Moreover, actual consumption of pharmaceuticals is variable due to patient compliance with medical guidance.

It was essential to analyse for TP conjugates that are stable within aqueous enviornments. This was not only to ensure stability during the method development process in terms of solubility, but also to increase the likelihood that these conjugates would survive wastewater treatment and be found environmentally. Therefore, the chemicals chosen were based on availability, solubility, aqueous stability, and whose parent compounds were found in sufficient quantities. These caveats notwithstanding, the potential toxicity to non-target organisms (e.g. fish, invertebrates) within the aquatic environment also becomes more relevant.

Therefore, during the initial method development, two distinct pharmaceuticals, propranolol (base) and sulfamethoxazole (acid), and their respective metabolite conjugates, 4-OH-propranolol sulfate and sulfamethoxazole-β-glucuronide, were explored. By the summation

of this thesis, the list of chemicals increased to acetaminophen (acid), acetaminophen sulfate, thyroxine (zwitterion), thyroxine glucuronide, and a third class of conjugate, *N*-acetylsulfamethoxazole.

1.1. Persistence of Pharmaceuticals Environmentally

Pharmaceuticals are designed to elicit strong biological responses at low doses in their recipient organism. Their presence in aquatic environments globally has led to concerns regarding the potential for adverse toxicological effects by these contaminants on non-target organisms (fish, invertebrates, microbial communities, plants, etc.) [1]. Human pharmaceutical conjugates typically have at least one polar moiety that enables the drug to be sufficiently soluble in the intestines or blood stream in order to be excreted. Upon human phase I biotransformation, parent compounds have the potential to be hydroxylated to a more polar compound, thus promoting their water solubility for either phase II conjugation or phase III excretion. As a result, humans excrete both parent and metabolised drug, which largely enter aquatic environments via wastewater effluent, and contribute to the pseudo-persistence of pharmaceuticals. WWTPs typically facilitate processes by which bacterial enzymes can either further biodegrade these parents and metabolites, or deconjugate metabolites back into the parent compound [2, 3]. In addition to biotransformation, abiotic processes (e.g. photolysis, hydrolysis) have the potential to transform parent compounds and metabolites into pharmacologically-active TPs that may pose a hazardous threat to aquatic biota found in surface waters receiving wastewater input.

1.2. Understanding Human Biotransformation Processes

Anthropogenic contaminants like pharmaceuticals can be described by toxicokinetics that are dependent on four main factors known as the acronym ADME. Those are the processes of entering the organism (absorption), how quickly/ where the chemical goes once in the organism (distribution), transformation of the chemical within the organism into products/ metabolites (metabolism), and the rate/ how the chemical gets removed (excretion). For these intents and purposes, the term metabolism refers to the transformation of natural substances necessary for life. Biotransformation is the more general term that refers to the "metabolism" of foreign chemical compounds. In general, there are three different phases of biotransformation, Phase I hydroxylation, Phase II conjugation, and Phase III excretion. As can be seen in Figure 1.1, the antidepressant fluoxetine (Prozac®) is shown to be affected by the two phases of biotransformation and ostensibly resulting in excretion from the human body[4]. Of important note in this case is the pharmaceutical design to take advantage of the purposeful demethylation into the pharmacologically-active structure.

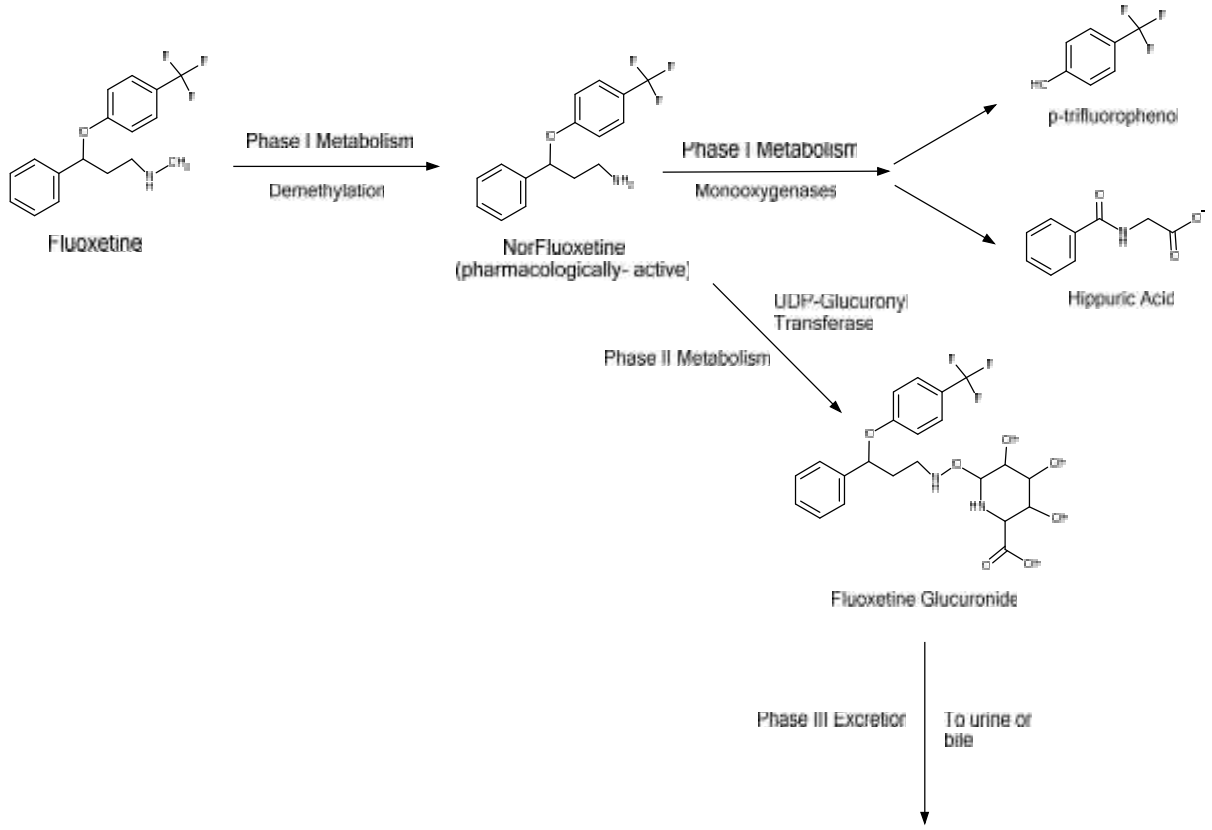

Figure 1.1 The antidepressant selective serotonin reuptake inhibitor, fluoxetine (Prozac), is shown to be transformed through Phases I and II biotransformation and ostensibly resulting in Phase III excretion from the human body. Of note is another mechanism of Phase I biotransformation, demethylation, which is very common in the detoxification process for many pharmaceuticals. Phase I products are from the pharmacological yields in DeVane (1999)[4].

These processes are generally found in the liver microsomes of vertebrates (e.g. humans). Lipophilic pharmaceuticals tend to migrate into hydrophobic membranes of the endoplasmic reticulum (ER) which is where most typical Phase I enzymes are found. The three main types of

Phase I reactions are oxidation, reduction, and hydrolysis, of which cytochrome P-450 enzymes are the most important enzymes. They are considered mixed function oxygenases or monooxygenases and are named due to their absorption peak of the reduced form at 450nm. The more polar product diffuses out of the hepatocyte ER membrane and into the cytosol where Phase II conjugation can take place.

Major types of conjugation that occur in humans: glucuronidation, sulfation, and glutathione. Glucuronidation is catalysed by the enzymes UDP-glucuronosyl transferases. These enzymes are ER membrane-bound; and there are many different forms with overlapping substrate specificities. –OH, -SH, and –NH group moieties on pharmaceuticals are the substrates that conjugate to anionic glucuronides through this reaction. Sulfate conjugation occurs in the cytosol of hepatocytes where 3-phosphoadenine-5-phosphosulfate (PAPS) is synthesised by a series of transferase steps. The sulfate group of PAPS is transferred to a wide range of xenobiotics that possess an –OH group (steroid alcohols, phenols, aliphatic-OH, aromatic amines. Glutathione (GSH) is a tripeptide cellular antioxidant comprised of glutamate, cysteine, and glycine. The thiol in the cysteine scavenges free radicals by donating two hydrogens. The result is reduced free radicals and a dimer of oxidised glutathiones. This dimer is reduced back to two GSHs via glutathione reductase with NADPH. The thiol group is also a good nucleophile that has a tendency to attack xenobiotic electrophilic compounds. Glutathione transferases exist primarily in hepatocyte cytosol and are responsible for binding GSH very tightly to substrates at hydrophobic binding sites. These three pathways are primarily responsible for creating the most favourable conditions in order for excretion to occur.

Phase III excretion in humans occurs via urine for molecular weights <300 Daltons and via the bile >600 Daltons. Conjugates and metabolites move across the hepatocyte membrane

and into bile canaliculi. Some conjugates pass completely through the gastrointestinal (GI) tract and into the feces. Very polar conjugates are not as readily reabsorbed by passive diffusion due to the hydrophobicity of the GI tract wall. These conjugates can become de-conjugated in the GI tract by glucuronidases and sulfatases to regenerate Phase I metabolites once again. These compounds are then reabsorbed thought the GI tract wall and return to the liver where they are hopefully re-conjugated once more. This process is known as enterohepatic recirculation, and provides the potential to facilitate toxic effects in organisms possessing these biochemical pathways. Given the environmental persistence of pharmaceuticals and their potential to recirculate within biota or humans, it is important to determine the toxicity of these compounds in order to infer risk.

1.3. Ecotoxicological significance of pharmaceutical conjugates

Measuring phase II biotransformation conjugates of personal care products and pharmaceuticals (PPCPs) will help distinguish their overall environmental attenuation from other processes such as phase I biotransformation or transfer to other phases (e.g. sorption to sediment/ soil, volatilisation to air, uptake by biota). Studies have reported the rates of removal of PPCPs under both nitrifying (aerobic) and denitrifying (anaerobic) conditions [5, 6]. The remaining sludge upon active digestion processes in WWTPs can be applied to agricultural fields; and the eventual fate of pharmaceutical conjugates becomes more complex. Groundwater chemistry and soil chemical reactions further convolute these processes. Sorption to particulates in soils and sediments[7], and especially in suspended organic matter within effluents and receiving waters can potentially account for levels of pharmaceuticals that could pose a threat to aquatic biota [8].

Pharmaceuticals can be found in the environment in either water or sediments depending on their polarity and hydrophobicity [7]. It is becoming more apparent that TPs, including metabolite conjugates, have the potential to comprise a large hidden component to the overall environmental load of current pharmaceuticals[9]. Reflective of this is the fact that levels of pharmaceuticals can actually be greater in WWTP effluents than influents through biotic (see figure 1.2) and abiotic deconjugation[10] (e.g. venlafaxine, sulfamethoxazole). Thus, substantial cumulative amounts of drugs are released into receiving waters. This further supports the necessity to create analytical techniques that can accurately account for the conjugate inventory in both aqueous and solid phases.

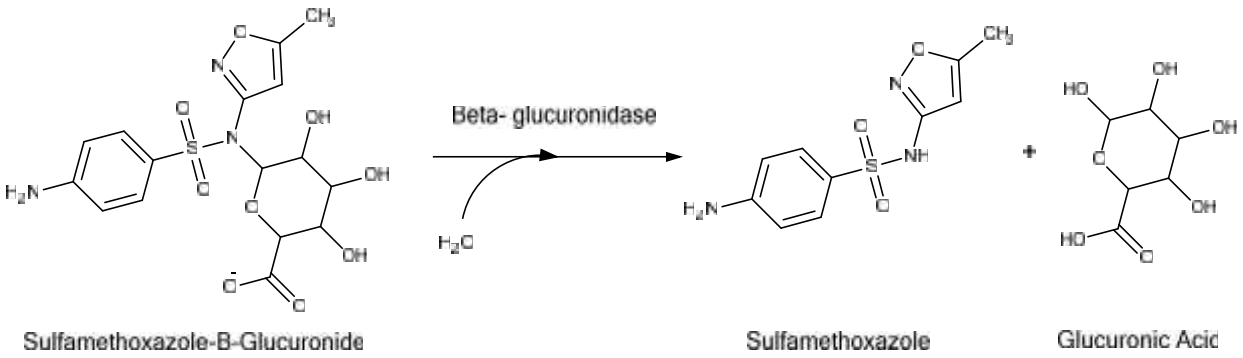

Figure 1.2 Beta-glucuronidase mechanism found in *E. coli* bacteria within the microbial consortia in wastewater and natural waters. Here the antibiotic sulfamethoxazole human conjugate is shown to undergo the enzyme-mediated hydrolysis at approximately pH 7. In an environmentally relevant pH range 6-9, the vast majority of sulfamethoxazole-β-glucuronide is of single anionic charge, as predicted by the ChemAxon Marvin Sketch™ pKa predictor application.

In addition, *if conjugates are found to be a significant source of parent compound, and they are determined to be toxic, then human health could be directly at risk*. For example, the risk of human exposure to pharmaceuticals in water is increasing due to the necessity of many countries with arid regions to provide an alternative source of potable water (e.g., Australia) through wastewater recycling, where levels of emerging contaminants like organic pollutants were previously measured by genotoxicity[11]. Currently additional ecotoxicological endpoints such as pharmaceutical estrogenicity, bacterial and algal toxicity, acetylcholinesterase inhibition and aryl hydrocarbon receptor activity have recently become more relevant health indicators for populations served by this technology[11]. Children and developing foetuses are of a particular concern in being exposed to any level of contaminant given their emerging immune, physiological, and neural[12] systems. Drugs approved to be administered to children are given in specific doses that correlate with body mass and age[13]. Many drugs not approved for children could be potentially hazardous to their development if they are chronically exposed to low levels that exist in the environment. Antibiotics like macrolides (e.g. clarithromycin) have been shown to be environmentally persistent[14], suggesting the danger of enabling waterborne resistant bacteria to come into contact with people through water usage or environmental exposure. Thus, it is essential to know all contributions of the pharmaceutical inventory that could directly impact infants' health (i.e. metabolites). While there is substantial literature identifying and quantifying a number of pharmaceuticals[15], there have been far fewer studies done on metabolites[16], and very few regarding human metabolite conjugates. Thus, subsequent to calculating/ estimating toxicity to various biota/ humans, it is essential to understand how to account for establishing risk using a suite of assumptions via modelling software in the absence of concrete data.

1.4. Practical application for risk assessment

Regulatory parameters for estimating risk of TP conjugates requires pre-existing knowledge of the environmental exposure levels and the associated toxicity values for each given compound with respect to biota of interest. In light of *lacking the analytical methods to quantify some of the highlighted TPs within this dissertation*, a probabilistic environmental risk assessment (PERA) of the potential TPs of selective serotonin reuptake inhibitors (SSRI) antidepressants and β-blockers (heart rhythm regulators) in fluvial systems was conducted using a suite of modeling software to estimate physicochemical parameters, exposure, and toxicity values [17]. A PERA estimates the probability of finding a certain concentration of chemical within an environment of interest, and then estimates the probability of eliciting a certain toxic effect to an organism of interest within that system using a hazard quotient.

$$H = \frac{E}{T} \quad \frac{C}{C}$$

Hazard quotients in this PERA were calculated using parent compound exposure data from North American and European surface waters as surrogates for TPs. Photolysis and pharmacokinetic literature, as well as the Swiss institute, Eawag's Biocatalysis/Biodegradation modeling software were used to estimate which TPs were environmentally plausible. This program uses 332 biotransformation descriptions for 249 biotransformation rules. This includes 46 descriptions for 25 "super rules", and 39 rules subsumed by the super rules. The comprehensive extent of descriptions for all rules can be found at http://eawag-bbd.ethz.ch (URL accessed Oct.9, 2018).

The Ecological Structure Activity Relationships (ECOSAR) Class Program is modeling software designed by the US Environmental Protection Agency for estimating untested organic

compound toxicities based on structure or simplified molecular-input line entry (SMILES) parameters. The program estimates a chemical's acute and chronic toxicity to aquatic organisms (fish, invertebrates, and aquatic plants) by using developed structure-activity relationships. ECOSAR is also packaged as a component of the Estimation Program Interface Suite (EPI Suite™), a physicochemical parameter estimation modeling software. EPI Suite™ uses information from >40000 chemicals to predict various properties including log octanol-water partitioning coefficient using atom/fragment contributions, and gas-phase reaction rate between the most prevalent atmospheric oxidant, hydroxyl radicals, and a particular chemical. The air-water partitioning (Henry's) coefficient is estimated using both group and bond contribution. Melting point, boiling point, and vapour pressure are estimated using a variety of techniques. Aerobic and anaerobic biodegradability of organic chemicals are estimated using 7 different models, including estimation of the biodegradation half-life of chemicals containing only hydrocarbons. Organic carbon normalised partitioning coefficient (K_{OC}) is estimated using both the Sabljic molecular connectivity model and the "traditional" method using log octanol-water partitioning coefficient.

The cumulative results of EPI Suite™, ECOSAR, the Eawag Biocatalysis/Biodegradation modeling software, in addition to experimental photolysis and degradation parameters from the peer-reviewed literature, can be seen in figure 1.3 using the SSRI fluoxetine (Prozac) is shown as an example.

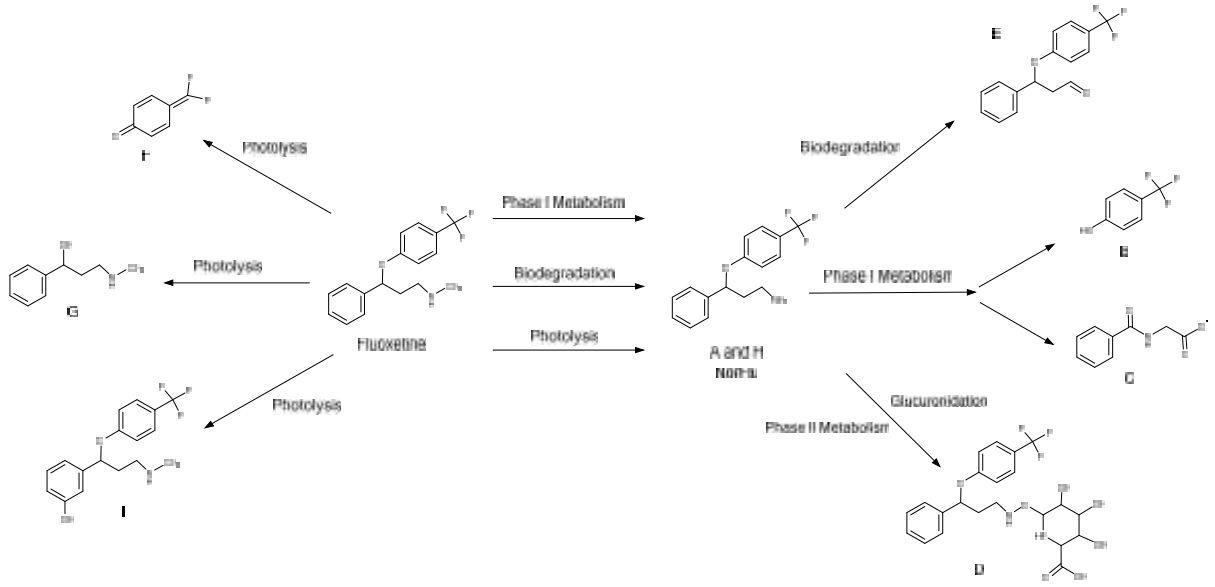

Figure 1.3 The antidepressant selective serotonin reuptake inhibitor, fluoxetine (Prozac), is shown to be transformed through Phases I and II biotransformation in the human body as was found in DeVane (1999)[4] . Photolysis products were representative of experimentally determined structures found in the peer-reviewed literature. Plausible biodegradation products were estimated using the Eawag Biocatalysis/Biodegradation modeling software.

The literature searched for in this PERA was typically representative of worst-case scenarios of exposure to these compounds (parent or TP). The majority of exposure data found was for WWTP or lagoon effluent, and the remainder was for proximal (within 1 km of release) receiving waters. If non-target toxic effects of TPs were to be seen in biota, it would be most likely in effluent-dominated systems where observed environmental concentrations are greatest. As shown in Figure 1.4, a 'worst-case' exposure data for parent compounds, and a 1:1 full conversion to TP was assumed. Based on pharmacological literature of SSRIs and β-blockers, this 1:1 conversion ratio is approximately a 10 fold overestimation of TP exposure, at a minimum. This is especially true considering our threshold of relevancy for human metabolites

was set at 5%. This overestimation helps establish a conservative first-tier risk assessment that does not necessitate the application of an uncertainty factor.

Salient to understanding toxicity and subsequent risk calculation, is that pharmaceuticals are metabolically-active ingredients that elicit therapeutic effects through specific modes of action (i.e. cellular pharmaceutical receptors) that are not accounted for by the estimation software employed in this thesis. Thus, the risk calculated using a hazard quotient approach as outlined above, should be considered a baseline, first-tier screening process. Toxicity, as estimated by ECOSAR, relys on knowledge regarding physical and stereochemical makeup (also chemical class) of a given contaminant. The results of which are dependent on a given chemical's "activity", or disruption of cell membrane processes leading to narcosis. These cellular disruptions are usually due to chemical diffusion and/or active transport. Moreover, chemcial mixtures have the potential to exascerbate the toxic potential of active pharmaceutical ingredients; thus further convoluting the accuracy of results in light of a lack of experimental data.

Therefore, in order to fully infer risk of these pharmaceuticals to biota and humans, it is essential to first understand where these compounds come from and how much is being released into the environment through WWTP effluent release.

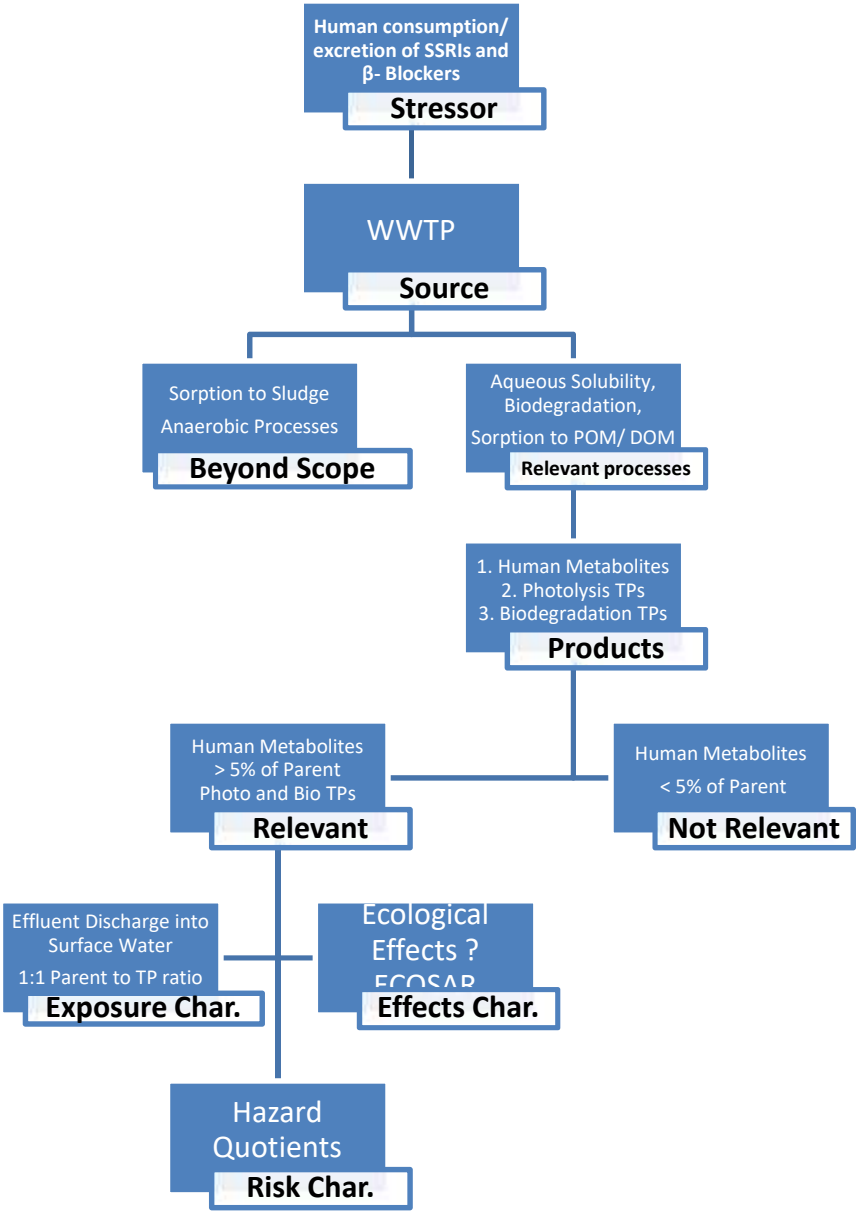

Figure 1.4 Conceptual model for a probabilistic environmental risk assessment of pharmaceutical transformation products (human metabolites, aerobic biodegradation products, and photodegradation products) in waste water effluent dominated systems.

1.5. Sources of Pharmaceuticals and Conjugates

The distribution of active pharmaceutical ingredients becomes more problematic when the disposal of wastewater products is considered (Figure 1.5). Upon release from WWTP, compounds are compartmentalised into the effluent and biosolids; 5.4 million metric dry tons of sludge is produced per year in the US where 22% is applied to agricultural land with the potential for run-off into surface waters. Alternatively, 34% is buried in landfills where the pharmaceuticals and TPs could potentially be found in the leachate[18].

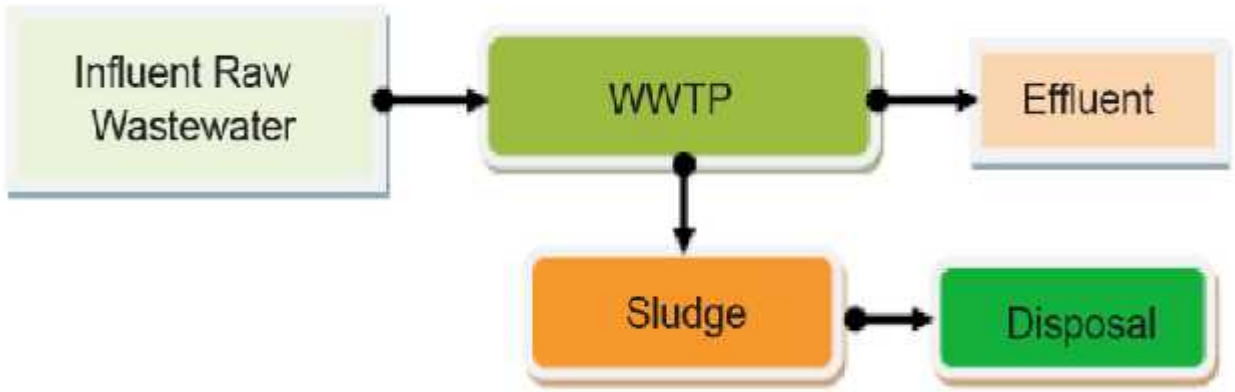

Figure 1.5 Schematic of overall processes involved in wastewater processing. Taken from www.in-pipe.com (Accessed Oct. 12, 2015)

Effluent compounds can be susceptible to abiotic degradation, and as such will affect their temporal and spatial distribution. Direct and indirect photolysis[19-21], air-water exchange, hydrolysis, sorption to dissolved organic matter (DOM), and particle settling can be dependent on physical characteristics of the surface waters, seasonality, as well as the specific physicochemical characteristics of the drug in question.

1.5.1. Wastewater Treatment Plant Sampling Site

The sampling site for this dissertation was from the North End Water Pollution Control Centre located in the City of Winnipeg, Manitoba, Canada (Figures 1.6, 1.7). All samples analysed within the context of this dissertation were obtained with permission from the City of Winnipeg Analytical Standards Division manager, Shaun Walker, and Operations Manager David Maxwell. All samples collected were 24 hour composite samples, and where applicable (i.e. mixed liquor, return activated sludge, and waste activated sludge) samples were composites of 3 channels that flowed through the plant. Thus, 1/3 of each sample was mixed from each channel, so as to reduce the temporospatial variability in sampling, and eliminate bias in choosing one channel over another at any given sampling time-point. All proprietary information regarding the wastewater treatment process is publicly available and was given by the operations manager, entitled, "*Wastewater Treatment Process Summary- North End Water Pollution Control Centre*" created by the Water and Waste Department.

Figure 1.6 Locations of the three wastewater treatment plants within Winnipeg, Manitoba. The North Main Water Pollution Control Centre (NEWPCC) is located in the north end of Winnipeg.

Figure 1.7 The Winnipeg North End Water Pollution Control centre. Image taken from Google Earth (2015).

The North Main WWTP was originally designed to serve 395,000 people, and a designed dry weather flow rate of 332 ML/day, and a total firm design capacity of the main pumps of 860 ML/day. Approximately 50% of the city currently uses the combined sewage system, which implies that wastewater generated by the served population is diluted by storm run-off to an extent prior to arriving at the WWTP. The overall mandate of the WWTP is to reduce the solids content of the wastewater by 95%. The general wastewater process at the WWTP is as follows: raw influent (2 hours), primary treatment (1.5-2 hours), aerobic reaction (1.4-2.16 hours), secondary treatment (1.5-4 hours), tertiary UV treatment (<10 seconds), then release to fluvial

receiving waters. Additionally, solids are processed through anaerobic digestion (10-24 days) with a subsequent dewatering process, with ultimate landfill disposal.

Upon arrival at the main interceptor pipe, wet wells are filled with incoming sewage in addition to municipal landfill groundwater termed, leachate, and also pump truck septage that is trucked to this location for processing. This combined raw influent is pumped into giant wells (total capacity 1925 m^3) in the grit house for screening/ removal of bulk garbage and pre-aeration (Figure 1.8). This bulk waste is fed onto conveyor belts and subsequently trucked away to the landfill. During this process, the waste activated sludge (WAS) is introduced prior to primary treatment in the next stage.

Figure 1.8 The grit house pre-aeration stage, including the holding tanks in the background, and the screeners and conveyor belt in the foreground.

Primary treatment is conducted by 5 large tanks (total capacity of 24,300 m^3) where several simultaneous processes occur. Mechanical skimmers remove greases and scum that are floated to the surface. Finer solid waste known as suspended solids starts to settle to the bottom where it becomes termed sludge. Mechanical scrapers move the sludge into hopper bins at the bottom of each tank where pumps remove the sludge for anaerobic digestion, dewatering, and ultimate disposal of dewatered "cake". The liquid left after primary treatment is termed settled sewage and is pumped towards the next stage of secondary treatment.

Secondary treatment is separated into two processes: oxygen reactor treatment and secondary settling. Oxygen reactors consist of 6 large tanks (total capacity 31,200 m^3) arranged into three trains (channels). Here the incoming settled sewage is vigorously mixed with high-purity oxygen (90-95%) oxygen generated by cryogenic air separators on site), and heavily bacteria laden return activated sludge (RAS) to initiate the biodegradation process of organic materials. Immediately upon oxygen reaction the sewage homogenate is termed the mixed liquor which typically contains 2000-3000 mg/L of bacteria-laden solids. The mixed liquor is flowed into the 26 final clarification tanks (41,275 m^3) for the settling of these solids/ bacteria (Figure 1.9).

Figure 1.9 One of the 26 final clarification tanks during the secondary treatment process. Water flows from the rear to the front of the tank moving over the combed concrete teeth prior to collection and movement to UV disinfection.

Settled sludge from this area is either returned to the oxygen reaction stage of treatment as aforementioned or diverted to waste (WAS) in the pre-aeration stage to mix with the primary sludge prior to digestion; this is known as co-thickening.

Sludge digestion consists of diverting co-thickened solids into holding tanks (total capacity 15,400 m^3) for 10-20 days where temperatures are held at 38^o C to digest the solids prior to release to reduce odors and destabilise organic matter through microbial consortia. Of note, is the biogas generated from this process which is approximately 65% methane; this is used to heat the WWTP during moderate temperatures.

Upon the final clarification process, the wastewater is flowed through an ultraviolet disinfection process to reduce the amounts of pathogens being released into the Red River receiving waters (Figure 1.10). This process consists of 6 large blocks of UV bulbs arranged into 7 removable banks of 24 bulbs, for a total of 1008 bulbs. Immediately after this process the final effluent is released to the Red River just south of the Chief Peguis Overpass east of Main St.

Figure 1.10 Close up view of each bank of UV bulbs used during the tertiary disinfection treatment process.

Now that the engineered technologies within a WWTP has been highlighted, it is possible to understand why specific sampling points were chosen for the the validation of the analytical methods (i.e. primary effluent, mixed liquor, secondary effluent, and final effluent). Moreover, it is important to note that biotic processes are separate from the abiotic processes that occur via these engineered technologies. Integral to these processes, is the importance of determining

analyte stability in solution in order to ascertain the specific removal/ augmentation of pharmaceutical conjugate levels within a WWTP. Moreover, this analyte stability is critical in determining transformation of the suite of analytes in bioreactor kinetics experiments.

1.6. Stability of Pharmaceutical Conjugates

The labile nature of human conjugates provided a unique challenge for the solubility and stability of authentic standards. It has been previously reported that there is a marked difference in the stability of aryl and acyl conjugates, both glucuronide and sulfate. For example, acyl glucuronides are potentially pharmacologically-active, and have much less stability in protic solvents such as methanol or water, primarily due to alkaline hydrolysis to the parent or aglycone in an environmentally relevant pH range (i.e. 6-9). To further convolute future method development using other compounds, there also exists the potential for pH-dependent intramolecular acyl migration as shown in Figure 1.11, thus limiting the amount of time available to work with freshly dissolved standards[22].

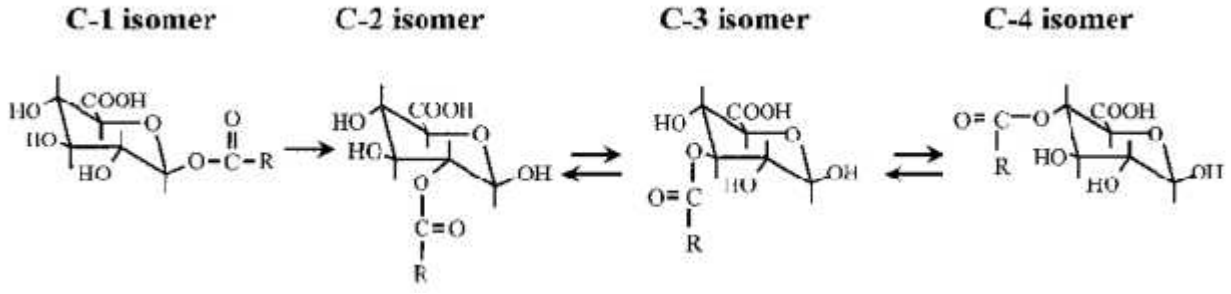

Figure 1.11 Rearrangement of acyl glucuronides by intramolecular transesterification. After formation of the C-1-O-acyl glucuronide, the acyl residue can “migrate” within the glucuronic acid molecule, forming positional C-2, C-3, and C-4 isomers. The rearrangement between the last three isomers is reversible. From Shipkova (2003)[22].

It has also been reported that transacylation and glycation reactions with other proteins (Figure 1.12), nucleic acids, and other nucleophilic components found in typical environmental matrices such as urine and wastewater also contributes to the chemical reactivity of these acyl conjugates[22].

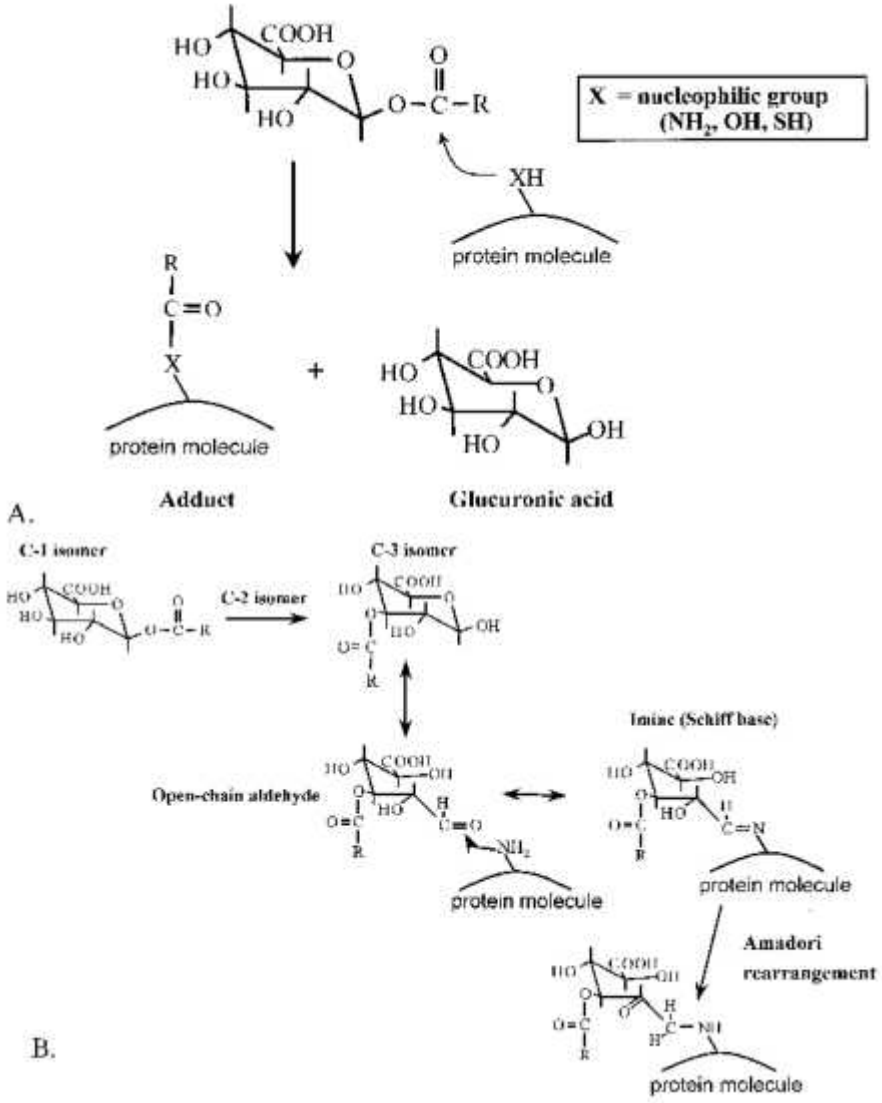

Figure 1.12 Mechanisms for covalent binding of acyl glucuronides to protein molecules. (A) Transacylation pathway, in which the aglycone moiety is directly bound to the protein. (B)

Glycation pathway, in which the aglycone moiety is bound to the protein via the glucuronic acid group. From Shipkova (2003)[22].

Carboxylic drugs that typically conjugate on the acyl chain have different intrinsic degradation half-lives. There appears to be several reasons for this. *In vitro* studies have shown that the extent of covalent binding is dependent on pH, exposure time, and acyl glucuronide concentration[23]. It is also possible that degree of substitution adjacent to the carboxylic group affects the magnitude of covalent binding (i.e. greater substitution less covalent binding)[22, 23].

To attempt to limit the aforementioned chemical reactions, two solutions wewre chosen: First, I used acetonitrile (an aprotic solvent) to dissolve my two initial conjugates. Second, a purposeful choice of 4-OH-propranolol sulfate and sulfamethoxazole glucuronide to represent not only two different classes of consistently prescribed pharmaceuticals for which there should be substantial environmental input, but also for their increased stability due to their aryl conjugation stereochemistry.

Once stability of the analytes was addressed, then the fate and distribution of these compounds between the aqueous and solid phases (e.g. water and suspended solids/ sludge) should be less problematic. Integral to understanding the fate of ionisable chemicals such as pharmaceutical conjugates in natural/engineered waters is the sorption mechanisms by which conjugates can be both removed from the aqueous phase, and protected from biodegradation.

1.7. Sorption of Ionisable Pharmaceuticals

Distribution of chemicals within the aquatic environment is not necessarily simple to model. Creating a mass balance is problematic due to the variations in simultaneous phases in

which chemicals can exist. In calculating the extent of sorption, many environmental conditions may need to be defined (e.g. pH, pE, humic/fulvic material content, etc). Understanding the capacity of an environmental system to sorb ionisable chemicals due to non-hydrophobic interactions is essential in estimating mass balance equations to determine the fate of anthropogenic compounds. This brief critical review illustrates the potential ionic or electrostatic interactions of the antibiotic class, sulfonamides, to a spectrum of solid surfaces. This understanding is important because hydrophobic partitioning has been typically used as a first (and often only) cut to estimate sorption for ionisable pharmaceuticals. However, cation exchange, cation bridging at clay surfaces, other surface complexations, and hydrogen bonding also play influential roles in the sorption of these compounds to soils and sediments [24].

Sorption specifically can affect the persistence of pharmaceuticals in porous media. The porous regions within soils and sediments, which could potentially account for up to 90% of the total mineral surface, can inhibit abiotic and biotic transformations [25]. Slow desorption due to hindered diffusion, and sorption in hydrophobic micropores of geological media can preserve anthropogenic organic contaminants in the subsurface and may therefore increase their persistence [25]. Therefore, deducing sorption mechanisms became much more important in governing transformation processes as well. Endocrine-disrupting compounds can be transformed in soils, but not mineralized. Thus, compounds such as estradiol can potentially be transformed into estrone and environmentally persist [26]. It is also possible that metabolites could compete with parent compounds for the same sorption sites, and potentially desorb the parent compounds back into the aqueous phase.

Depending on the mechanism of action, sorption can be described by Freundlich or Langmuir isotherms, either linear or non-linear, or combinations of both. In addition, the surface

complexation model [27] (described below) initially proposed in the early 1970s by Stumm and Morgan details the complexations of the inner (less than a water molecule distance) or outer (greater than one water molecule distance) spheres of solid surfaces affinities for metal ions. Ionisable chemicals such as pharmaceuticals could complex in similar fashion. Inner surface complexes can be involved in cation exchange, as well as ligand exchange (e.g. hydrous oxides) including ternary complexes. Outer surface complexes involve processes such as hydrogen bonding and ion pairs [27]. Numerous studies across multiple genres of aquatic chemistry and engineering have arisen in the past decade that attempted to define and potentially model pharmaeuticals due to their potentially toxic effects to aquatic or terrestrial organisms [28-39].

There exist several issues in establishing concrete predictive sorption models (for a thorough comparison of ionisable chemical sorption models, see the review by Webster [40]) due to the heterogeneity of natural organic matter (NOM) associated with either soils or sediments [41]. First, the main difficulty in extrapolating K_D values from one location to another was that batch experiments may not reflect the true nature of sorption in more complex environmental matrices. Second, the differences in pedoclimatic conditions, rainfall pattern, rainfall intensity and other temporal and spatial variability of soils including carbon content, mineralogical characteristics (such as clay and silica content) or soil pH [24] can have influential effects on sorptive tendencies. Third, the clay portions of solid surfaces have an additional challenge in modelling the sorption mechanisms of protons and metals. This is due to the dichotomy of possessing both a pH independent negative charge on basal planes due to isomorphic substitution and pH dependent charge on edge surfaces and defects due to surface protonation. Thus, there exist combinations of permanently charged cation exchange sites and variable surface complexation sites [42].

1.7.1. Limitations in Using K_{OW} to Estimate Ionic Partitioning

K_{OW} which is the octanol-water partitioning coefficient of a chemical, represents a chemical tendency to sorb to a hydrophobic environment. This is typically used to estimate other partitioning coefficients like the organic carbon normalised K_{OC} via:

$$\log K_{OC} = a * \log K_{OW} + b \quad (1)$$

where a and b are fitting parameters dependent on the compound class [43]. The hydrophobic partitioning ratio of ionic compounds is described by the term D_{OW}:

$$\log D_{OW} = \log K_{OW} + \log \frac{1}{1+10^{pH-pKa}} \quad (2)$$

This equation estimates the hydrophobic portioning for an acidic analyte; where pKa is the acid dissociation constant for a given chemical. Basic analytes use the exact same equation with the exception of reversing the pH and pKa values in the equation [44]. Therefore, at a given pH the magnitude of the ionic form of the acid or base can be calculated.

The compound-specific properties examined for correlation to sorption are log K and charge state. These two properties, however, are examined as one in the log Dow parameter, which assumes that any charged species is completely water soluble and only the neutral fraction of an acidic or basic chemical can partition to the solid phase [44]. The validity of this assumption breaks down when we consider that charged species can participate in electrostatic interactions; and so sorption of those analytes which carry a charge is likely a function of both the electrostatic properties of both sorbent and sorbate [44]. The proposed equations may fail if soil or sediment samples show an unusual ratio of organic carbon to clay (or other material that can adsorb (e.g. acridine and benzo[*f*]quinolone-two cations with delocalised charge) [45]. This probably was due to their special affinity to the negatively charged clay.

1.7.2. Sorption Types Beyond Non-polar Ionising Organic Acids and Bases

Highly polar and/or charged functional groups contribute significantly to a compound's physicochemical properties and potential environmental fate. A combination of specific electrostatic, ligand exchange, or covalent interactions and non-specific van der Waals forces may therefore govern the behavior of ionisable organics [44]. If the pH of an aqueous environment is at minimum 2 units above the pKa of the organic acid (i.e. >99% dissociated), then the neutral component was usually neglected in estimating sorption mechanisms [27, 40, 43]. Cation exchange, ligand exchange, oxide surface complexation, and hydrogen bonding became much more relevant in the overall sorption of ionisable pharmaceuticals [28-39].

It is important to note that hydrophobic associations through non-specific van der Waals interactions are weaker primarily due to the π-π interactions [46] in comparison to complexes formed through ionic interactions. Because of this difference, desorption of ionic pharmaceuticals varies as well. For example, naproxen and sulfamethoxazole are both organic anions in the environmental pH range; however, under increasing ionic strength, sulfamethoxazole was retained to natural sediment much stronger than the more aromatic naproxen (35 and 20%, respectively) due to ionic ligand exchange with the inorganic surfaces. Moreover, much less desorption was seen as ionic strength increased (4.9 and 31.2% for sulfamethoxazole and naproxen respectively)[46]. This will possibly affect the overall K_D estimation of an ionisable compound that exhibits more ionic interactions [30, 34, 37, 47].

1.7.3. Heterogeneity of Natural Organic Matter

Many studies have shown that both soils and sediments had varying components of "soft" amorphous, homogenous, gel-like humic matter; and also a "hard" carbon source like black carbon, soot, or biochar[36, 48, 49]. It has been shown that "soft" amorphous carbon can be mathematically accounted for by linear partitioning, whereas "hard" exhibited both absorption and adsorption. Depending on the relative combination of these two types of carbon, sorption ranged from linear to highly non-linear[41].

Humic and fulvic substances are a highly variable organic component in soils or sediments. They can range in size from a few hundred to several hundred thousand Daltons[48]. This size difference alone can potentially influence the ability for any sorbate, polar or non-polar, to absorb deeper into the carbonaceous matrix. What seemed to be a dominant restriction was the difference in amounts of functionally reactive groups and surface ionisable groups between humic matter and "hard" or "glassy" carbonaceous material[50]. In contrast, the mechanism of sorption to organic matter for non-polar compounds seemed to dominate over the mineral-based association due to the strong suppression by water of solute adsorption on polar mineral surfaces[51].

1.7.4. Cation Exchange for Sorption to Solid Surfaces

Ion-pair adsorption seems to occur to a larger extent through multivalent ions [52]. Moreover, ion-pairing K values for pairing increased as the number of valences increased [27]. However, it should be noted that this complexation is generally not the mechanisms of choice for dilute solutions of solute [27]. Ligand complexation such as cation exchange capacity (CEC) and

mineral complexation (e.g. cation bridge) may play a more descriptive role when dealing with pharmaceuticals that do not uniformly follow the D_{OW} prediction.

One significant drawback to isolating cation exchange as a dominating mechanism of sorption is when strong covalent interactions occur, resulting in a non-extractable fraction of sorbate. This is especially relevant in the interactions of ionisable organic compounds in the presence of heterogeneous humic acids [40]. For example, the antibiotic, sulfamethazine, can covalently bond to soil organic matter [32], further exemplifying the need for a thorough understanding of heterogeneity in not only the sorbent system, but also in modeling this variability.

It is important to note however, that when ionisable compounds are in the presence of polyelectrolytes (e.g. humic acids) at high pH or low ionic strength, layering potential decreases [27]. Moreover, if anionic humic acids are present they can align themselves in flat configurations. If polyelectrolyte and ionisable pharmaceutical are of opposite charge then the attraction can be coulombic (attractive) in nature. However, if both are of the same sign, then adsorption will only take place if non-electrostatic attractions outweigh the electrostatic repulsion [27]. What is especially important to note is that single sorbate- sorbent interactions could be described by isotherms, but multiple sorbate competitive adsorption became problematic.

1.7.5. Summary of Sorption Mechanisms Using the Model Pharmaceutical, Sulfamethoxazole

For the purposes of this dissertation, it is important to summarise the known potential sorption mechanisms and distribution of pharmaceuticals using the organic acid, sulfamethoxazole (pKa values of 1.7 and 5.6, and a low $\log K_{OW}$ of 0.89 [24]) as a model (Figure

1.13). Hydrophobic interactions can occur between aromatic or alkyl chains (typically longer than eight carbon chain) of the natural organic matter throughout the environmentally relevant pH range (4-10). Cation exchange can occur when the anilinic nitrogen is positively charged most likely at pH greater than pKa2. Cation bridges can be formed between the sulfonamide nitrogen and divalent cations most likely at pH greater than pKa1. It is possible that trivalent ions could form inner sphere complexes with sulfonamides; however, desorption becomes facilitated with the formation of bidentate ligand complexes between the solid surface and any of the three valence electrons (e.g. Al^{3+}, Fe^{3+}). This results in the previously available sites for cation bridges becoming preferentially associated with the trivalent ion over the pharmaceutical [48]. Moreover, the possibility of the formation of ternary complexes exists, thereby limiting the number of sorptive sites. The rare but strong negative charge assisted hydrogen bonding can occur when both the sulfamethoxazole and solid surface pKa's are approximately equal.

Values from one compound to another structurally-related, compound can be made under certain conditions. First, sorbate interactions with soils must not be via hydrophobic partitioning but via inner and outer sphere interactions such as cation exchange, cation bridging and/or surface complexation. An extensive body of literature has documented that, for sorption dominated by hydrophobic partitioning, small substituent changes can have a large effect on the broad application of K_D (e.g.OH^- replacement with Cl^-) [43]. Second, the base compound structure along with the arrangement of ionisable functional groups must be the same within the family. For example, insights from naproxen sorption will not be representative of sulfamethoxazole sorption trends; even though both compounds have been established to sorb via cation exchange, surface complexation, Furthermore, the same ionisable functional groups must be present for all compounds of a family.

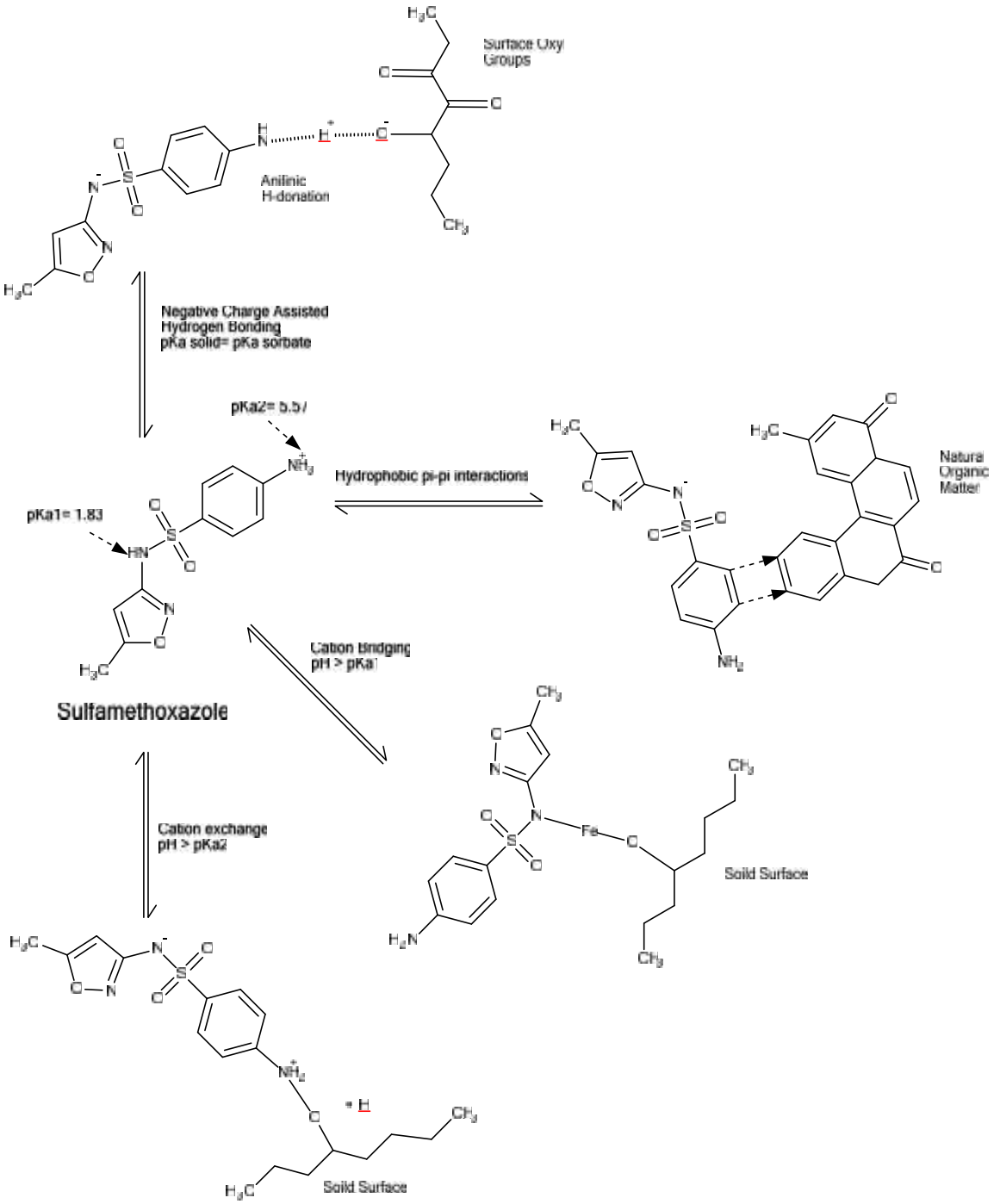

Figure 1.13 The probable mechanisms of sorption to various solid phases (mineral, natural organic matter, char, sediment, soil, clay) of the antibiotic sulfamethoxazole. Each mechanism notes at what pH range they are plausible. When no range is given it is assumed that the mechanism could occur throughout environmentally relevant pH ranges.

The size of minor substituents on the base compound structure must be small so that cation exchange, cation bridging and surface complexation interactions are not dominated by additional hydrophobic free energy contributions (e.g., alkyl chain length longer than eight carbons on a charged surfactant head group) [53].

Ionic strength and the type of ions present in the aquatic system help determine the magnitude of the various mechanisms that can occur. Divalent cations have been shown to assist in cation bridging, and trivalent cations have a propensity to form bidentate complexes and/or ternary structures [35, 38] which may or may not aid in the adsorption of sulfonamides. Moreover, it should be noted that these ions can form aqueous complexes with other anions like OH^-, thus limiting their availability to react. Sulfamethoxazole was strongly retained by the inorganic components of the aforementioned sandy loam sediment due to the fact that ligand exchange bonds were stronger than the interaction of non-hydrophobic compounds with NOM. The resulting strong sorption through these interactions would help explain why previously reported low levels of biodegradation have been seen in sediment and soil experiments [54]. The stronger the adsorption, the less bioavailable these ionisable compounds are to microbial consortia and other aquatic biota, in addition to potentially being shielded from abiotic processes (e.g. photolysis).

These results can help understand the potential for sulfonamides to persist and potentially elicit toxic effects to aquatic or terrestrial biota via physiological activation of endogenous receptors. Moreover, advances in engineered technologies could take advantage of the specific complexation reactions, whether electrostatic or covalent, to help prevent these types of chemicals from impacting sensitive environments. Through thorough examinations of the

concurrent mechanisms involved in sorption to solid surfaces, the ultimate intent would be to support or modify existing models for predictive and preventative purposes.

1.8. Outline of Dissertation Chapters

Chapter 2 discusses the current trends in environmental analysis of human metabolite conjugates of pharmaceuticals. These studies encompass environmental analytical chemistry, as well as biological chemistry, which can be extrapolated to environmental chemistry. Sample collection, qualitative and quantitative analysis, and types of mass analysis were highlighted. The author of this dissertation researched all topics, interpreted the peer-reviewed literature and wrote the manuscript.

Chapter 3 discusses the initial stages of this dissertation's analytical methods using propranolol, sulfamethoxazole, and their respective major conjugates 4-OH-propranolol sulfate, and sulfamethoxazole-β-glucuronide as model compounds. Discussed was the successful simultaneous extraction through weak anion exchange solid phase extraction cartridges from primary and secondary clarification wastewaters from the North End Winnipeg Water Pollution Treatment Plant in Winnipeg, Canada. Subsequent separation and quantification was achieved by reversed-phase C_{18} chromatography coupled to positive electrospray ionisation tandem mass spectrometry. The author of this dissertation designed the experiment along with along with his supervisor, performed the experiments, interpreted the data, and wrote the manuscript.

Chapter 4 discusses the expansion of this dissertation's analytical methods to include the zwitterionic compound thyroxine and its associated glucuronide conjugate. Of important note, was the addition of an extraction method for the conjugate from wastewater solids in addition to

the aqueous phase. Modification to the reversed-phase C_{18} chromatographic method was done to allow for better retention of the conjugate using a medium-density ligand tridentate C_{18} column from Waters® corporation, in addition to modification of the binary gradient using the same solvents as the previous chapter. This chromatographic method became the basis for all quantification during the subsequent studies (and chapters) contained within this dissertation, including the monitoring and bioreactor studies.

Chapter 5 discusses the occurrence and distribution of four different classes of pharmaceuticals and their metabolite conjugates in a wastewater treatment plant over four months. Aqueous and suspended solids fractions of primary, mixed liquor, secondary, and final effluent, along with return activated sludge, and waste activated sludge were quantified for acetaminophen, acetaminophen sulfate, propranolol, 4-OH-propranolol sulfate, sulfamethoxazole, *N*-acetyl sulfamethoxazole, sulfamethoxazole-β-glucuronide, thyroxine, and thyroxine-*O*-β-D-glucuronide. The author of this dissertation designed the experiment along with along with his supervisor, performed the experiments, interpreted the data, and wrote the manuscript.

Chapter 6 discusses the benchtop bioreactor studies conducted in order to corroborate or refute what was seen in the monitoring experiment contained herein chapter 6. Of note was the attempt to isolate mechanisms for removal, possible transformation and back-transformation to the parent compounds. Both primary and secondary effluent wastewaters were spiked with unlabelled analyte and analysed with or without the addition of forced air to elucidate these trends. The author of this dissertation designed the experiment along with along with his supervisor, performed the experiments, interpreted the data, and wrote the manuscript.

Chapter 7 discusses how a probabilistic ecological risk assessment was conducted for the TPs of three β-blockers (atenolol, metoprolol, and propranolol) and five selective serotonin re-uptake inhibitors (SSRIs; citalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline) to assess potential threats to aquatic organisms in effluent-dominated surface waters. To this end, the pharmacokinetic literature, the Swiss Institute Eawag's Biocatalysis/Biodegradation Database Pathway Prediction System aerobic microbial degradation software, and photolysis literature pertaining to β-blockers and SSRIs were used to determine their most likely TPs formed via human metabolism, aerobic biodegradation, and photolysis, respectively. Monitoring data from North American and European surface waters receiving human wastewater inputs were the basis of the exposure characterizations of the parent compounds and the TPs, where available. Monitoring data from North American and European surface waters receiving human wastewater inputs were the basis of the exposure characterizations of the parent compounds and the TPs, where available. The author of this dissertation wrote the manuscript in its entirety, collected all SSRI occurrence data, plausible transformation mechanisms and resultant TPs, estimated toxicity values for all compounds, and compiled all experimentally-verified toxicity values of which to compare our species sensitivity distribution. The author also researched the format of the PERA in conjunction with Mark Hanson of the University of Manitoba, Department of Environment and Geography. Jonathan Challis of the University of Manitoba, Department of Chemistry researched the majority of the occurrence data for the β-blockers and associated plausible transformation mechanisms and resultant TPs.

Chapter 8 summarises the major findings of all previous studies/ chapters, and postulates future directions for projects, extrapolating on the thesis findings.

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Chapter 2

Introduction to the Current Trends in Environmental Analysis of Human Metabolite Conjugates of Pharmaceuticals

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The author of this dissertation researched all topics, interpreted the peer-reviewed literature and wrote the manuscript.

2. Abstract

Analytical techniques are essential to the accurate identification and quantification of human pharmaceutical conjugates. Conjugates have the potential to comprise a significant hidden environmental load that could rival those of the parent compounds. This review reflects the current trends in the processing and analysis of human pharmaceutical conjugates. The primary focus was to outline trends in environmental analytical chemistry. However, it seemed valuable to include techniques involved in analysis of bile acid conjugates associated with biological fluids. These studies provided insight into steroid conjugate analysis that may prove potentially applicable to the environmental analysis of estrogen conjugates. Currently, sample collection is typically done by grab samples, and extraction from matrices is mainly achieved by hydrophilic-lipophilic balance cartridges. Reversed-phase liquid chromatography is by far the most common form of separation. The most common column choice was C18, with some inroads being made by the zwitterionic ion chromatography-hydrophilic interaction liquid chromatographic columns to take advantage of the polar moieties of conjugates for separation. The majority of studies used a binary gradient comprised of aqueous buffer and acetonitrile, which afforded good separation and preparation for mass analysis. Quadrupole-time-of-flight-MS was most commonly used for unknown conjugate identification. There is a noted increase in linear ion traps and high resolution mass spectrometers (e,g., Orbitrap™) for the identification and quantification of conjugates, and as such, some hybrid technologies are emerging. However, triple quadrupole instruments remain used for the greatest sensitivity and reproducibility for conjugate quantification. The multi-faceted combination of quadrupole-time-of-flight and triple quadrupole will be of great value.

2.1. Introduction

Pharmaceuticals are designed to elicit strong biological responses at low doses in their recipient organism. Their presence in aquatic environments globally has led to concerns regarding the potential for adverse toxicological effects by these contaminants on non-target organisms (fish, invertebrates, microbial communities, plants, etc.) [1]. Human pharmaceutical conjugates typically having at least one polar moiety that enables the drug to be sufficiently soluble in the intestines and/or blood stream in order to be excreted. Current pharmacological synthetic techniques are exploring the increase in bioavailability by conjugating hydrophobic drugs to a soluble moiety (ex. vitamin E). Upon human phase I detoxification these compounds have the potential to be hydroxylated to a more polar compound, thus promoting their water solubility further for either phase II conjugation or excretion. As a result, humans excrete both parent and metabolised drug, which largely enter aquatic environments via wastewater effluent, and contribute to the pseudo-persistence of pharmaceuticals. WWTPs typically facilitate processes by which bacterial enzymes can either further biodegrade these parents and metabolite compounds, or deconjugate metabolites back into the parent compound [2, 3]. In addition to biotransformation, abiotic processes (e.g. photolysis, hydrolysis) have the potential to transform parent compounds and metabolites into either metabolically active, or inactive TPs, either of which may or may not lead to complete mineralisation.

Many conjugates, especially estrogenic, have been found in various forms of WWTP: municipal, hospital, pharmaceutical manufacturer, and livestock [4, 5]. Moreover, livestock waste lagoon samples from different concentrated animal feeding lots in the US have shown estrogenic conjugates to comprise about one third of the total estrogen load. In fact, high levels of the rarely reported non-human EE2-α are most likely due to transformation processes within the lagoon,

given absence of other treatment [6]. Many different forms of pharmaceutical (i.e., various forms of parent compounds such as estrogens, as well as transformation products, and conjugates) have been seen at various stages of transport and wastewater treatment from toilets to holding tanks to WWTP influents to receiving waters [7].

Studies have reported the rates of removal efficiencies of personal care products and pharmaceuticals under both nitrifying (aerobic) and denitrifying (anaerobic) conditions [8 9]. Although mass balances can account for the "removal" of these compounds from the effluent, these by no means reflect actual degradation, but rather transfer to another phase (i.e. aqueous to sludge or gas phase). The remaining sludge upon active digestion processes in WWTPs can be applied to agricultural fields; and the eventual fate of pharmaceutical conjugates becomes more complex. Groundwater chemistry and soil chemical reactions further convolute these processes. Sorption to particulates in soils and sediments, and especially in suspended organic matter within effluents and receiving waters can potentially account for significantly unforeseen amounts of pharmaceuticals [10]. This further supports the necessity to create analytical techniques that can accurately account for the conjugate inventory in both aqueous and solid phases.

The environmental significance of metabolites and more specifically, conjugates is two-fold: potentially creating an unforeseen reservoir from which direct toxicological effects could be seen, or transformation via microbial deconjugation into an active form, and thereby eliciting toxic effects. For example, a comprehensive comparison of experimental data surrounding the toxicity of pharmaceuticals (i.e. β-blockers and SSRIs) is shown in section 7.4, Table 7.5. Thus demonstrating that environmentally-relevant levels of parent/deconjugated pharmaceuticals have the potential to elicit toxic effects in both acute and chronic ways. The exploitation of transforming some pharmaceuticals is evident by the purposeful metabolism of the inactive

forms to active (e.g. fluoxetine demethylation to norfluoxetine[11]). As a result, certain metabolites could actually be more toxic than the parent compound (e.g. acridine from carbamazepine [12]. Moreover, metabolism of pharmaceuticals can change the propensity of these chemicals to undergo abiotic or biotic degradation, thus potentially contributing to their persistence.

These above variables notwithstanding, pharmacokinetic literature is beneficial to give an estimate as to what proportions of pharmaceuticals should be excreted as parent or metabolite. However, levels of conjugates are more difficult to assess given the potential of hydroxylated moieties on metabolites to be conjugated in any number of proportions (i.e. single, double, mixed etc. conjugations). Therefore, robust procedures need to be developed that can identify and quantify potentially dynamic amounts of conjugates in the environment.

One of the primary limitations in identifying and quantifying conjugates is the need for quality standards. There are very few internal standards for quantification of metabolites, especially given the almost innumerable ways metabolites can be transformed. The synthesis of conjugates for analysis or subsequent standards using liver microsomes or supersomes™ [13] [14] is sometimes necessary, otherwise there is a high cost involved in getting an authentic, quality-controlled standard made.

The primary focus was to critique the analytical techniques within the context of environmental matrices and specifically human pharmaceutical conjugates, to address the needs of identification and quantification of these compounds. Sample preparation and extraction, separation, and detection are three main categories outlined in this review for comparative purposes. The aim was to elucidate common trends in the various techniques associated with these categories, in order to establish which are the most viable for future directions. Sample

preparation and extraction is vital for environmental samples to be reflective of their true conjugate levels (i.e. good recovery) prior to separation. Separation techniques that allow for baseline distinction of conjugates are essential for accurate identification and quantification. The choice of instrumentation for mass analysis is especially important for the differentiation of certain conjugates from their associated TPs that can arise through biotic/ abiotic mechanisms.

2.2. Methodology for review

This review was compiled and contrasted by searching the literature between June 28, 2014 and August 31, 2014 using Academic Search Premier EBSCO Host, Science Direct CRKN- Elsevier, ProQuest Research Library, Taylor and Francis Library CRKN, CRKN Wiley Online Library, and Web of Science database search engines, for all journal entries involving the analysis of pharmaceutical conjugates. It became apparent that the analytical techniques used in biological tissue and fluid analyses can potentially be extrapolated to the environmental domain given the similar instrumentation used in both. In total, forty-four papers were cited: 18 pharmaceutical, antimicrobial, and fungicidal papers; as well as a limit of five recent papers on bile acid conjugates, which were limited to the most recent publication years given the redundancy of the findings that echoed previous studies in the past 2 decades; four papers on the analysis of illicit drug and alcohol conjugates, in addition to one paper on phenol conjugate uptake by freshwater fish. Another sixteen papers found were on the topic of steroid analysis. Fourteen of which were centred on estrogenic compounds (endogenous and birth control conjugates); and the remaining two were focused on androgens and bisphenol A. Eleven of the forty-four were from environmental sources (river water, receiving water, lagoon, influent,

effluent, drinking water, sludge, soil, and reclaimed water. Eight papers were estrogenic conjugates, one antidepressant (venlafaxine), a cough expectorant (dextromethorphan), and one antibiotic (sulfamethoxazole). The remaining thirty-four papers were focused on biological tissues and fluids.

Table 2.1 Overall trends of experimental design for environmental analysis of various conjugates. Most frequent sample preparation, analytical separation, and mass analysis are highlighted, as well as all other alternative or complementary techniques.

	Most Common	Alternative or Complementary
Sample Preparation		
Sampling Type	Grab (environ)/ single (bio) [3, 6, 15-52]	Composite[7]
Matrix	Plasma[22, 31, 32, 37, 43, 53-56], Urine[3, 7, 21, 22, 30, 37, 38, 45, 52, 57-61], Stock solutions[27, 28, 31, 35, 36, 40, 42, 46, 56]	WWTP effluent[3, 7, 15, 17, 44, 47-51], receiving waters[20, 27, 39, 41, 47, 55], sludge[29, 41], raw sewage[6, 41, 44], drinking water[39], milks[16], soil[18], biota tissues (liver, heart, brain)[23, 24, 26, 34, 37, 62-64], horseradish[33], plants[65, 66]
Extraction	SPE cartridge or well plate (e.g. Oasis HLB) [3, 6, 7, 15, 17, 20, 22, 25-27, 29, 30, 32, 36, 37, 39, 41, 43, 47, 48, 51, 53, 56, 58-60, 64-66]	Liquid-liquid[33], microdialysis[55], microextraction[19], tandem amino SPE[16], precipitation[16, 32, 54], centrifugation[33, 34, 38, 40, 54], anion exchange chromatography[17], isolute SLE column[58], QuEChERS[23], mixed cellulose ester filtration[62]
Instrumental Analysis		
Separation	High performance liquid chromatography- C18 [6, 7, 15-20, 23, 24, 26, 29, 30, 32-34, 37, 39-42, 44, 46-57, 59-61, 64-66]	UHPLC[22, 35] GC[6, 18, 25], ZIC-HILIC[28, 45], immunoassay[21], mixed phase anionic exchange lipophilic[26], capillary liquid[24]
Sample Volume (µl)	10, 20, 5	1-50, 100, 150, 200
Mobile Phase	Water and acetonitrile with and without buffers [3, 6, 7, 15, 17-20, 23, 27, 30, 32-35, 39, 41, 42, 44, 52-55, 57, 59-62, 64]	Water and methanol with and without buffers [16, 28, 29, 31, 36-38, 47-51, 56, 65]
Detection		
Mass Analyser	QQQ [6, 7, 15-18, 23-29, 31, 32, 34-36, 41, 43-45, 48-54, 56-61, 64-67]	Q-TOF-MS[3, 17, 26, 30, 33, 35, 39, 52, 65, 66], UV-Vis[17, 28, 42, 44, 46, 55], Q-LIT-MS[19, 22, 27, 46, 47, 66], ^{13}C & ^{1}H NMR[46], Liquid scintillation[18, 57], Orbitrap^{TM}[20, 40], immunoassay[21], fluorescence[18], flame ionisation[18], FT-MS[37]

The various analytical procedures used for all compounds were broken down into three initial categories for discussion of the advantages and disadvantages of the current state of the art: sample preparation and extraction, separation, and detection (Table 2.1). Sample preparation was broken down into compound, sample type, sample matrix, extraction techniques. Separation was broken down into separation instrument, sample volume, and solvent gradient. Mass analysis was broken down into detector (mode if applicable), LOD (limit of quantification were stated if LODs were unavailable), recovery percentage of conjugate standard, analytical range, and linearity.

Since the original review was published in 2015, an update to the study was conducted that included 9 additonal scientific studies published from 2015-2018. Three of the studies were involved in monitoring regimes conducted on either wastewater or surface waters, three were conducted on biota, two were conducted on human fluids (i.e. plasma and urine), and one was conducted on membrane bioreactor performance. The additional suite of chemicals studied was: acetaminophen, estrogens, diclofenac, morphine, ibuprofen, trantinterol, and parabens. It is becoming increasingly more common to analyse for metabolites and conjugates during routine analyses; and it is becoming increasingly understood that these compounds have the potential to persist environmentally via conjugation/deconjugation reactions, thereby convoluting the true inventory these compounds. However, of these publications, no new technologies were employed in the analyses. It is of important note that the *in situ* knowledge on transformation/ back-transformation of conjugates and their respective parent compounds is still lacking.

2.3. Results and Discussion

2.3.1. Sample collection and extraction

The sampling and processing techniques used in the collection and extraction of both environmental and biological analytes are chosen based on the complexity of the matrix. Homogenisation and dissolution of biological samples in solvent with subsequent precipitation and/or centrifugation are typical steps for removing cellular components prior to instrumental analysis. Environmental matrices as well as biological have issues of effects that can arise due to co-extraction of impurities along with conjugates. These can be organic compounds or inorganic ions. Moreover, there is also the issue of levels of conjugates being too low to detect without pre-concentration. Environmental levels of conjugates are typically found at concentrations ranging from pg/L to ng/L, whereas the range in biological tissues is high ng/L to low mg/L, particularly given that human doses are generally on the order of mg. Therefore, filtration and SPE are almost always used for the pre-concentration of conjugates prior to chromatographic separation to address the issue of limits of detection.

In the analysis of pharmaceutical conjugates, grab samples (environmental) or single samples (biological) were used exclusively with the exception of one study involving 24 samples of 24-hour composites taken over a 1 month period [15]. Grab samples has the advantage of being simple. The main disadvantage is that they are not necessarily reflective of temporal or spatial trends, as effluents and receiving waters are dynamically changing in composition. Passive samplers for aquatic pharmaceutical sampling are gaining more attention, because they can provide continuous, time-weighted-average concentrations while deployed [68]. Because this averaging encompasses changes in concentrations and compositions of the water in which the passive samplers are deployed, they may be more reflective of conjugate levels that can be in

dynamic flux, especially around points of WWTP effluent discharge (i.e. due to seasonality of water flow). For example, the ratio of parent pharmaceuticals to metabolites can not only fluctuate on a daily basis, but also within a 24 hour period, by up to three orders of magnitude [69]. Thus, passive samplers can more accurately reflect a realistic exposure for biota over a given period of time, whereas grab samples can potentially give aberrantly high or low values, even if samples were obtained with sufficient replication to obtain error estimates on the precision of the measurement. The most interesting thing of note is the absence of any passive sampling for conjugate quantification in the existing literature to date. This could be due to the relative infancy of techniques available to analyse conjugates across multiple drug classes, or compound classes.

Of the studies reviewed, the most frequent objective for conjugate analysis was isolation and identification of the analyte of interest from the matrix, rather than quantification. In fact, the vast majority of these studies were concerned with structural determination and/or analytical method development to quantify pure conjugates in solution. The exceptions were Tso and Aga (2010)[16], who characterised and quantified estrogenic conjugates in commercial cow's and goats' milks using HPLC-QTOF-MS and subsequently Q-LIT-MS (see Figure 2.1); and Reddy *et al.* (2005)[17], who used UV-Vis and QTOF-MS to identify and QQQ to quantify estrogenic conjugates in wastewater influent and effluent. Both studies used grab samples in their analyses.

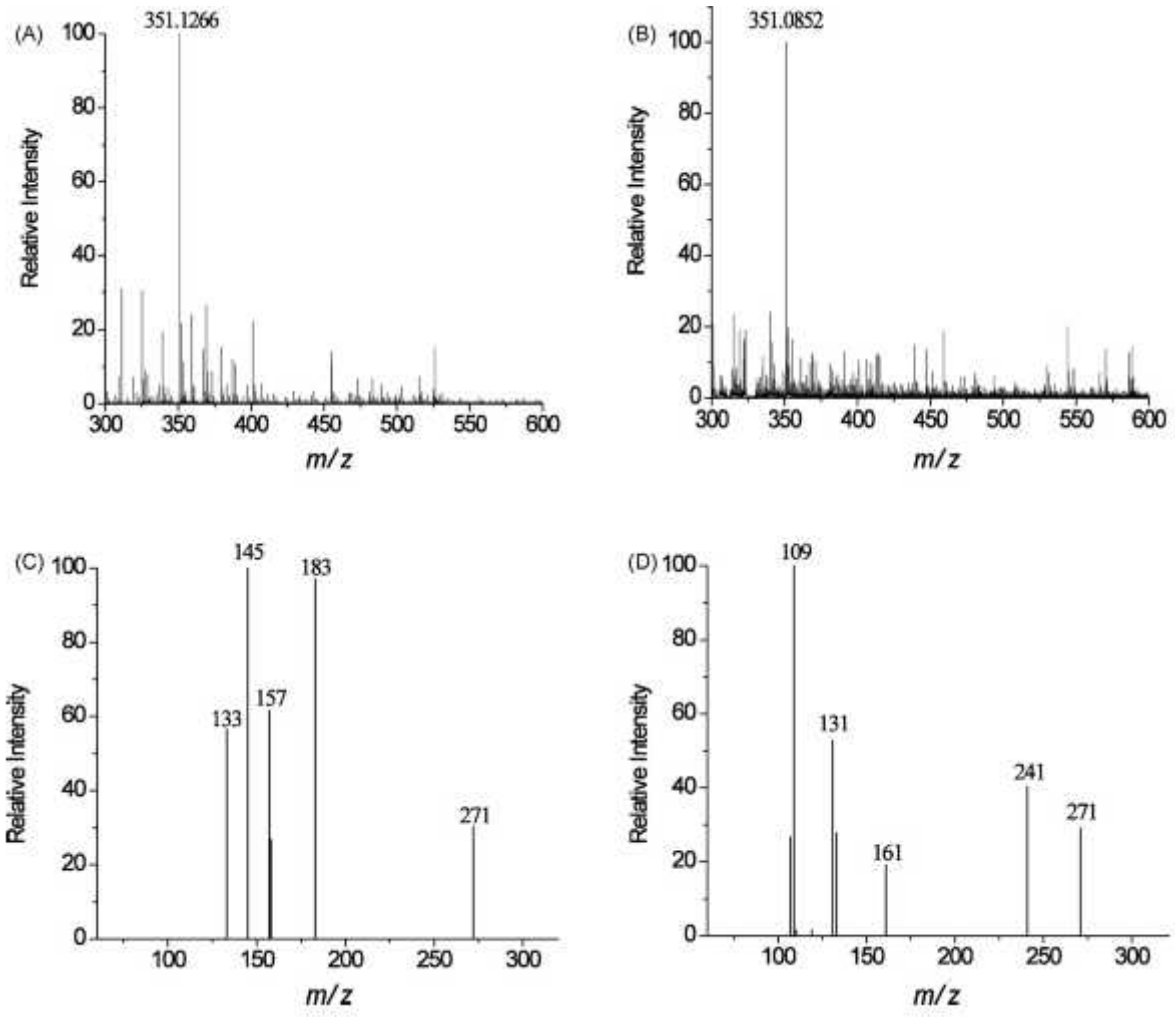

Figure 2.1 A direct contrast of complementary mass analyses, Q-TOF and Q-LIT, by accurate mass measurements of the molecular ion obtained by direct infusion of E2-3-Sulfate standard (A), and purified unknown peak at 11.0 min (B) using Q-TOF MS. Figure also shows LC–IT-MS^3 fragmentation pattern (product ion scan of 351 $\rightarrow$ 271) of E2-3-Sulfate standard (C) and unknown (D), indicating different product ions (145 and 183 vs. 109 and 131). Reproduced with permission from Tso and Aga, (2010) J Chromatogr A, 1217.

Extraction is particularly useful if the conjugates of interest are found within any matrix aside from pure solutions. In fact, with the exceptions of estrogen glucuronide analysis in soil [18],

resveratrol conjugates isolated from heart, liver, and brain tissues [19]; [20], and alcohol glucuronides assayed from urine by serial dilution for immunoassay [21], all other studies used some form of SPE or micro-elution well plate. Both methods used HLB stationary phase, to isolate and concentration conjugates. Conjugate extraction provided the desired retention and pre-concentration of highly polar moieties like glucuronides and separation of them from the surrounding matrix. This step was critical in minimising ion suppression or enhancement common in electrospray ionisation. An alternative was use of SPE micro-extraction plates [22] with MCX sorbent which supported an affinity for bases and is stable in organic solvents. The main advantage compared to SPE cartridges (e.g., Oasis HLB) is the small amount of eluent necessary for conditioning of the plates (200 µL) and elution of the samples (400 µL). However, the optimal pKa should be reflective of the analytes of interest, limiting broader use if the pKa varies greatly.

Tandem SPE, using an amino phase in conjunction with an HLB phase, was used by Tso and Aga (2010)[16] for elimination of carbohydrates, proteins, and fats from milk samples prior to chromatography. This procedure provided similar separation of conjugates from biological matrices as the centrifugation approach. The benefits were the cost effectiveness of using several cartridges over the high capital cost of centrifugation instrumentation, as well as the means for cleanup to be done without centrifugation. Internal standard recoveries (62-112% and 89.7-105.5, respectively), and linearity (0.996-0.999 and 0.93-0.99, respectively) were similar between the two methods. However, much better sensitivity was found for tandem SPE (LOD values of 2 for E1-3-sulfate, E2-3-sulfate, and 7 ng/L for E1-3-glucuronide and EE2-3-glucuronide), compared to the 39000 ng/L value for the same compounds using centrifugation [18]. The trade-off for the cost effectiveness of cartridges was the laborious steps involved in

extraction and purification. Namely, precipitation was followed by primary extraction using HLB SPE with three steps which included a loading wash, subsequent acid and base washes, secondary SPE with HLB, SPE with amino phase, elution to collection vials, evaporation to dryness, and reconstitution in methanol.

One other interesting method of SPE was the use of dispersive SPE after extraction by QuEChERS [23]. Dispersive SPE was useful in that acetonitrile was mixed with anhydrous $MgSO_4$ and primary secondary amine sorbents in one step, thereby reducing extraction time. It was successful in yielding good recoveries for parent estrogens and other estrogenic parent analytes such as atrazine and bisphenol A (86-110%), but was poor for estrogen glucuronides and sulfate conjugates (20-61%). Moreover, RSD values were upwards of 47% during inter-day comparisons, in spite of the optimisation of acetonitrile to water ratios for extraction of parent compounds such as atrazine, E1, and E2. In fact, when this method was applied to analytes in rat testes, no estrogenic conjugates (E1S, E2S, E1G, and E2G-28) were detectable in 7 samples. Therefore, this procedure's validation for conjugates is in question.

2.3.2. Instrumental Analysis

The dominant form of separation was HPLC of various forms, including capillary LC and turbulent flow LC), as well as supercritical fluid chromatography. Other non-LC techniques like immunoassay, NMR, liquid scintillation, and GC were uncommon. The advantages and disadvantages are outlined below.

2.3.2.1. Gas Chromatography

In studies of drug conjugates, GC was rarely used because of one major disadvantage. Conjugates are most likely not volatile, and thus derivatisation is necessary to convert these compounds into a volatile form to achieve good separation and quantification [70]. However, some researchers have suggested that certain steroid sulfates, upon hydrolysis and derivatisation, could actually be other conjugates, such as steroid glucuronides or lipid conjugates, thereby resulting in skewed results [24]. In biological samples, autoxidation of cholesterol during insufficient sample preparation and hydrolysis has also been presented as a possible source of erroneous steroid sulfates [24].

This review found only three instances of GC analysis. First, bisphenol A- glucuronide was measured using GC-QQQ after β-glucuronidase hydrolysis of the spiked and labelled glucuronide, followed by derivatisation [25]. Second, headspace analysis of volatile metabolites derived from soluble radiolabeled E2-glucuronide was done by splitting the GC column effluent, reacting with Cu(II)O, and trapping the resulting CO_2 with Carbosorb E sorbent and, diluting with Permafluor, and then counting using liquid scintillation[18]. Finally, unconjugated E1, E2, and E3 were measured by GC-QQQ to compare to conjugates, analysed by LC-QQQ using SPE, derivatisation, from animal feeding lagoon [6]. The advantages of LC compared to GC are shorter analysis time, complete automation, much less derivatisation, low instrument clean up and maintenance, medium to high sample throughput, compound thermal stability and no real limitation by molecular mass or polarity [71].

2.3.2.2. Immunoassay and Other Techniques

Immunoassay [21], ^{13}C and ^{1}H nuclear magnetic resonance (NMR), liquid scintillation, UV-Vis, fluorescence coupled to HPLC, and flame ionisation (coupled to GC) were found within the literature for conjugate analysis. The major disadvantages of immunoassay are long preparation times, lack of dynamic range (3-5 orders of magnitude, depending on detector), and the inherent cross-reactivity of various forms of class-common conjugates to the antibody in question. In fact, Arndt *et al.* (2014) demonstrated through LC-MS/MS that during an ethyl alcohol glucuronide assay that the common disinfectant, isopropanol, showed 69-84% cross- reactivity, a considerably high non-specificity for an analyte.

Liquid scintillation has a benefit of only "counting" radiolabeled analytes, and is this quite specific and sensitive. However, the major downfalls in environmental analysis are the long preparation and extraction times, and the corroboration of the quantification with HPLC-fluorescence, which in itself has limitations as noted below. Moreover, the issue of disposing of radioactive materials becomes necessary. Of course, radiolabeled materials must be used, which are expensive to synthesize and typically used only only for bench-scale studies of specific fate processes (e.g., production and degradation of conjugates).

Fluorescence requires that analytes have moieties that will fluoresce (e.g. aromatic moieties), which is not the case across all classes of conjugates. In fact, only one study [18] used fluorescence for qualitative identification prior to LSC. This detector was used in a suite of instruments (HPLC-QQQ, GC-LSC, and GC-flame ionisation). Moreover, Permafluor™ aqueous mounting medium was necessary to reduce fade and increase identification. Thus, the only ostensible role for fluorescence in the future would be for enzyme kinetic analysis using

more complex fluorescent cocktails. NMR has been used for decades for structural determination. However, NMR lacks the sensitivity needed for environmental applications, as exemplified by the single LOD value reported by Gagne (2008)[26] in analysing 17-ethylnylestradiol-3-glucuronide and 6 gingerol-4-glucuronide by NMR of 150,000 ng/L (150 µg/L).

2.3.2.3. Liquid Chromatography

HPLC, including UHPLC, was by far the most dominant form of LC used in isolating pharmaceutical, antimicrobial, and estrogenic conjugates. This also included other HPLC techniques such as capillary liquid chromatography [24], mixed phase anionic exchange lipophilic stationary phase liquid chromatography [26], ZIC-HILIC [27, 28]. Of these, HILIC seems to be the most promising for future pharmaceutical analysis.

Capillary LC coupled to QQQ has definite advantages in that the LODs for glucuronide and sulfate conjugates of estrogen, androsterone, and pregnalone isolated from brain tissue were 0.006-0.08 ng/L and 0.013-0.032 ng/L, respectively [24]. As mentioned above, LODs for analysis of environmental samples will most likely need to be in the pg to ng/L range in order to be effective and give an accurate inventory. Moreover, the recovery was 94.9% for glucuronides, and the range was 0.01-1000 nmol/L. The main advantage was that hydrolysis of the conjugates to measure parent compounds was not necessary.

Gorga *et al.* (2014)[29] used online turbulent–flow LC for separating estrogenic conjugates using Turboflow™ columns followed by Hypersil C18 analytical columns and methanol and water mobile phases for negative mode and the additions of 20mM ammonium formate and 0.1%

acetic acid for positive mode. LODs of 0.0083-1.6 ng/g for sediment samples and 0.10- 125ng/g for sludge (i.e. ppb to ppt range), recoveries were 65-92% for sediment and 40-83% for sludge, analytical ranges of 2.5-3000ng/L, and showed a linearity of 0.992-0.999. The LODs and analytical range speak to this procedure's potential for accuracy and relevance at environmentally levels when compared to those of Badoud *et al.*[30] who conducted HPLC on estrogenic conjugates from urine. Their LODs were in the range of 1-500 μg/L, which reflects the reduced necessity in having very low LODs in biological sample analysis.Taguchi *et al.* (2013)[31] analysed for bile acid conjugates in rat serum using supercritical CO_2 fluid LC coupled to a choice of four different C18 columns: an ethylene-bridged hybrid (BEH), a fluorophenyl, a BEH amide, and a HSS cyano. The main advantage was the cost effectiveness of CO_2 over liquid solvents for mobile phase. The drawback is the high pressures necessary for efficient separation using supercritical CO_2. Upon analysis by QQQ, the LOD were 400-1500 ng/L, with a range of 500- $2x10^6$ ng/L, which is not particularly relevant for environmental analyses in the low ng/L range.

2.3.3. LC Stationary Phases

There are a number of different types of HPLC stationary phases used for separating conjugates. Regardless of type of detector used, the C-18 reversed-phase stationary phase was the most common one used for conjugate analysis. Major column vendors such as Waters, Agilent, and Phenomenex all produce C18 columns (e.g. Xbridge, Alltech, Zorbax, Luna) of various pore sizes, packing materials (e.g. BEH) and end-capping technologies. These commercially available columns all have stable bonded phases, and can withstand high back

pressures of up to approximately 400 bar (HPLC) and above (for UHPLC). The major purpose of end-capping is to prolong the life of the column by protecting the silica from dissolution by preventing hydrolytic agents from accessing the siloxane backbone. Thus reducing undue retention of conjugates to un-capped silanol groups, yielding peak tailing; this leads to the trends being seen in polar conjugate separation.

There have been developments on multiply-endcapped silica C18 columns to reduce the rate of cleavage of the siloxane bond, and thus increase the chance of odd peak shapes, changed retention times, and poor separations [72]. Four of the most recent studies on conjugates have used the proprietary Waters Acquity HSS T3 column for separating very hydrophilic polar compounds. Zhao [32] (rat plasma) and Macherius [33] (horseradish extract) analysed various antimicrobials including triclosan, Lou [34] analysed resveratrol, and Xie[35] (standards) analysed 52 glutathione conjugates of various pharmaceuticals. The glutathione conjugates were prepared by microsomal incubation of the parent compounds from stock solutions. The main advantage of the HSS T3 column was to maximise separation due to the stationary phase's resistance to degradation through extensive end-capping. Moreover, the small particle (e.g. 1.7 μm diameter) increases the efficiency in UHPLC by decreasing the amount of time the analytes are sorbed (Rivera *et al.*, 2012).

HILIC exploits the polar functional groups of conjugates. This was achieved by creating a hydrophilic layer around the stationary phase due to the use of an aprotic solvent such as acetonitrile. Although the retention in HILIC increases with increasing polarity or hydrophilicity, opposite to the trend observed in reversed phase, HILIC is not a variation of normal phase LC. The HILIC technique employs water-miscible solvents compatible with mass spectrometry[73]. Qin *et al.* (2008)[27] analysed river water samples for estrogen sulfates and glucuronides using

high organic content mobile phase and achieved LOD values ranging from 0.17-6.78 ng/L with 68-105% recovery and a calibration range of 0.2-2000 ng/mL. Vikingsson *et al.* (2008)[28] also used HILIC to analyse various morphine conjugate and achieved a LOD of 1 ng/L.

2.3.4. LC Mobile Phase Composition

All LC studies in this review used a binary gradient, with the majority consisting of acetonitrile and water, with or without buffers. The six exceptions were not only studies primarily prior to 2010, but also the majority were bile acid conjugate studies [31, 36, 37], with two estrogen conjugate studies [16, 29], and one methamphetamine glucuronide study [38]

The above study using HILIC seems to take advantage of the potential for efficient electrospray ionisation due to high organic content in the binary mobile phase (e.g., 95/5 (v/v) acetonitrile/aqueous 5 mM ammonium acetate (pH 6.80) and 75/25 acetonitrile/aqueous 5 mM ammonium acetate (pH 6.80) [27]. In comparison, high aqueous contents of greater than 90% [74] necessary to elute highly polar compounds in reversed-phase chromatography, have the potential to de-wet HILIC columns, resulting in retention loss between runs.. In light of this, the three studies that used water and organic phases, with no buffers, started the elution with at least 10% organic composition. This fact notwithstanding, it has been shown that steroid conjugates yielded a two- to three-fold increase in ion intensity when using acetonitrile/ water over methanol/water. This may be due to a lower viscosity in the former mixture [17], resulting in more rapid creation of proper sized droplets necessary for ion formation in electrospray ionisation.

2.3.5. Mass Analysis

At this point in time for conjugate analysis, the objective is the identification of naturally occurring conjugates *in vitro*, followed by optimisation of sensitivity and of peak isolation and shape. Quantification is currently limited to pure chemicals, whether commercial or synthesised in pure matrices, with bile acids and estrogenic/androgenic conjugates predominating in the environmental/ biological samples. The "holy grail" of identification and quantification would be to use an elegant form of extraction, along with a single solvent system with a single series of LC columns. Eluents would be characterised by a single mass analyser capable of structural determination, and quantification of conjugates at environmentally relevant levels. Issues in mass analysis are compounded and influenced by all previous steps combined; and the uncertainties involved are also multi-faceted.

The current standard for quantification is QQQ, with inroads being made by Q-LIT and Thermo's Orbitrap™. The current trend in qualitative identification is by various TOF mass spectrometers (e.g., Q-TOF) to provide high mass resolution.

2.3.5.1. Quadrupole-Time-of-Flight MS

The majority of the eight studies employing Q-TOF, Xie[35] (pharmaceuticals); Macherius[33] (triclosan); Gagne[26] (estrogenic); Ferrer and Thurman[39] (lamotrigine); Thurman and Ferrer[3] (dextromethorphan) used Q-TOF to analyse qualitatively for unknown metabolites of the compounds studied. Reddy (2005-estrogenic), Tso and Aga[16] (estrogenic), and Badoud[30] (steroid conjugates) all quantified conjugates using Q-TOF.

The complexities in analysing conjugates by TOF was exemplified in Xie *et al.* (2013)[35] studying 52 synthesized pharmaceutical glutathione conjugates. They used a T_3 HSS polar column combined with HPLC and a binary gradient consisting of acetonitrile and water with added buffer, and corroborated their findings with QQQ in both negative and positive modes. Disulfide and thioester conjugates produced qualifier ions in negative and positive mode, respectively. However, the aliphatic and benzylic conjugates major routes of fragmentation at the glutathione and cysteinyl C-S bonds potentially yielded more parent compound, making conjugate identification and quantification difficult. Additionally, the aromatic conjugates were resistant to fragmentation. Thus accurate measurement of conjugates by diagnostic ions or neutral loss became troublesome.

Table 2.2 Ranges of instrumental properties using various mass analysers for conjugates. Top and bottom rows show the range of results possible for MS/MS, Q-TOF, and Q-LIT factoring acceptable LOD, recovery, linear range, and linearity combined.

Mass Analyser	Conjugate	Sample Type	Extraction	LOD (ng/L)	Recovery (%)	Range (ng/L)	Linearity
MS/MS	[a]Estrogen-glcuronide, sulfate, acetate	Wastewater 24h composite	HLB SPE	10-100	23-78	30-1500	NA
	[b]dihydroartemisinin - glc	Grab- urine	HLB SPE	1840 (LOQ)	90-115	1840-4.6×10^6	NA
Q-TOF-MS	[c]Lamotrigine-2N-glc	Grab- receiving waters, wastewater effluent.	Offline syringe pump	NA	99	10-5000	0.99
	[d]Estrogen-sulf, Testosterone-sulf	Grab-urine	HLB SPE	NA	74-120	1000-5×10^5, 5×10^4-8×10^6	NA
UV-Vis	[e]Estrogen- glc, sulf	Grab- wastewater, milliQ	HLB SPE and anion exchange	0.14-0.16	62.5-81.2	NA	NA
	[f]Paclitaxil-PEG-GSH	Grab-Pure compounds	None	NA	98.8-100.8	2.5×10^6-6×10^7	0.9994
Q-LIT-MS	[g](nor)buprenorphine-glc	Grab- plasma,urine	MCS µ-extraction well plate	1-10	92-98, 68-84	20- 1×10^5	NA
	[h]Oxazepam-glc	Grab-blood	µ-elution well plate	250	36-51	500-1×10^6	NA
Orbitrap	[i]Sulfamethoxazole-glc	Grab- reclaimed water	HLB SPE	67	NA (only parent compound info)		
FT-MS	[j]Bile acid- sulfates	Grab- urine, plasma,liver	HLB SPE	10	74-140	5000-5×10^5	NA

[a]Pedrouzo (2011); [b]Geditz (2014); [c]Ferrer and Thurman (2010); [d]Badoud (2011); [e]Reddy (2005); [f]Sutariya (2012); [g]Regina (2013); [h]Wang (2013); [i]Wang (2014); [j]Bobeldijk (2008).

As shown in Table 2.2, quantitative analysis using QTOF for which there are conjugates in existing mass spectral databases were helpful for Ferrer and Thurman (2010)[39] who studied the antidepressant, lamotrigine-2-glucuronide, in 118 drinking water, ground water, river water,

and WWTP effluent samples. Using an offline SPE syringe pump coupled to a Zorbax Eclipse C18 column in HPLC and acetonitrile and water with 0.1% formic acid as mobile phase, they found 99% recovery of the glucuronide using QTOF in positive mode using an internal standard with a wide linear dynamic range of 10-5000 ng/L. In their subsequent study on the cough syrup expectorant in 2012, dextrorphan glucuronides were analysed by Q-TOF and accounted for 92% of the phase II conjugates [3]. The high mass resolution provided a number of benefits, including the ability to identify specific isomers based on the presence of a single hydrogen atom, and good signal-to-noise ratios because fewer isobaric interferences existed.

The paradigm of Q-TOF identification and QQQ quantification was demonstrated by Masia *et al.* (2013)[75] in their analyses of 43 pesticides in WWTP influent, effluent, and proximal surface waters. After analyte identification by Q-TOF, which included 13 pharmaceuticals and two drugs of abuse not originally targeted, QQQ was able to achieve LOD values of 0.04-2ng/L through multiple reaction monitoring. Thus, the combination of these two instruments is an effective tool. However, the drawback of expense involved in purchasing and maintaining both instruments can overshadow the potential ubiquity of this method.

2.3.5.2. Quadrupole-Linear Ion Trap

Q-LIT-MS operates as a Paul ion trap detector, with the added advantage of allowing tandem MS in time as well as in space. LIT detectors are usually the third quadrupole in a QQQ, so parent ion selection is already achieved in the first quadrupole. LIT also provides the ability to do MS^3, MS^4, etc. through fragmentation and ejection of grand-daughter (etc.) ions, with a

greater *m/z* range than conventional Paul ion traps due to the larger size of the LIT resulting in fewer space-charge effects.

It is apparent from the studies analysed that Q-LIT has good quantification potential. Regina *et al.* (2013)[22] quantified morphine metabolite glucuronides with environmentally-applicable results using MCX micro-extraction well plates, HPLC using a Kinetix core-shell column eluting with a gradient of water with 0.1% formic acid and acetonitrile. As shown in Table 2.2, they reported LODs of 1-10 ng/L, 68-98% recoveries, and an environmentally-relevant linear dynamic range of 20-1×10^5 ng/L.

For a qualitative comparison to Regina *et al.*[22] *above*, Wang *et al.* (2013)[19] analysed various oxazepam and temazepam glucuronides extracted from whole blood using HLB micro-extraction well plates. They also separated their conjugates by HPLC using a C18 column eluting with water and acetonitrile with 2mM ammonium formate buffer and 0.1% formic acid. This resulted in a LOD of 250 ng/L, low recoveries of 36-51%, and a high range of 500- 1x10^6ng/L compared to Regina *et al.*[22] The major difference between binary gradients was Regina *et al.*[22] ramped linearly for a total of 8 minutes, and Wang ramped and held organic contents over seven intervals for a total of 23 minutes. Although these are different compounds, qualitatively it is possible that the difference in gradient profiles could affect the linear separation of the analytes for analysis by Q-LIT due to the conjugates that eluted at longer retention times presenting potential peak broadening and tailing by the retention on the column for longer periods of time. Moreover, choosing a more broad-range sorbent like HLB for extraction of many forms of analyte as opposed to MCX polar technology for the retention of conjugates could affect the recoveries of conjugates. Regina *et al.*[22] showed recoveries of buprenorphine glucuronide of 92-98%, and the metabolite norbuprenorphine glucuronide of 68-84%. In comparison, Wang *et al.*[19]

showed recoveries of only 36-51% for oxazepam and temazepam glucuronides. However, Wang *et al.*[19]was in fact quantifying both parents and conjugates. Thus, the trade-off in increased recovery and quantification for conjugates could also limit the efficiency of analysing the parent compounds. Also, as mentioned above, it is possible that the core-shell technology of the Kinetix column used by Regina *et al.*[22] could result in shorter plate heights and shorter diffusion distances due to the smaller pore size on the surface of the sorbent particles, as opposed to fully porous particles. This could allow more uniform elution and better resolution due to less peak broadening, or tailing.

Figure 2.1 is a good example of how using both Q-TOF and Q-LIT can be complementary in elucidating unknown metabolites. Tso and Aga (2010)[16] used Q-TOF to profile commercial cow and goat milks for estrogen conjugates, both endogenous and anthropogenic. Mass spectra A and B in Figure 2.1 show a very close possible identification of an unknown peak at 11 min, but upon Q-LIT-MS analysis, the ions generated did not correspond to the anticipated E2-3-sulfate. Initially, Q-TOF identified an unknown ion with similar *m/z* 351to a sulfated E2 with similar retention times of 11.2 and 11.3 minutes. Upon subsequent isolation of the unknown peak (*m/z* 351) in an ion trap, a product ion (*m/*z 271) was further fragmented (MS^3). Also, as seen in the mass spectra C and D an E2-3-sulfate standard was analysed under the same conditions for comparison. The standard yielded product ions of *m/z* 145 and 183, and the unknown product ions were *m/z* 109 and 131. The conclusion was that the unknown was not the suspected E2-3-sulfate. Therefore, this comparison not only shows the power of Q-TOF to elucidate traces of unknown metabolites, but also Q-LIT's ability to be corroborative and quantitative when searching for specific accurate mass fragments.

2.3.5.3. High-Resolution Accurate Mass Spectrometry

Orbitrap™ is the trademark name of Thermo Corporation for their mass analyser for HRAMS. Fragmented ions orbit around a cathode within a casing that serves as the anode. Thus, depending on the *m/z* ratio, the ions maintain a specific speed and orbit, and that information is then interpreted by software.

As with TOF, HRAMS can detect a difference in mass defect. For instance, the loss of one ^{1}H, one ^{16}O, or one ^{35}Cl yields specific differences in molecular mass of an unknown compound in question, thus, structure can be inferred. Both TOF and Orbitrap™ have the capability of identifying < 5ppm. Thus, these instruments have a great advantage over QQQ of measuring single atom changes to any given ion fragment, and are thus very useful for characterising novel metabolites (conjugates). Moreover, the flight path of the ions is orthogonal, and is thus very consistent in the reproducibility and differentiations between very small mass defects. Q-TOF however, has the potential to co-ionise impurities, much like QQQ, making new conjugate screening tedious and necessitates the purification of analytes from complex environmental matrix [76]. Moreover, difficulties associated with using TOF instruments are the limited dynamic range of 4-5 orders of magnitude (compared to 5-8 orders of magnitude for QQQ), and the dependence on lock mass or internal calibration to maintain high mass accuracy [20].

Current models of Orbitrap™ HRAMS hybridise a linear ion-trap and Orbitrap™ mass spectrometer together which combines the high resolution in the transient signal mode with a large intrascan dynamic range and sub-ppm mass accuracy under both MS and MS^{n} modes. It allowed a direct coupling of mono or two-dimensional chromatography and lead to a rapid

increase in the number of metabolites identified [76]. As can be seen in Table 2.2, Orbitraps™ have the ability to analyse qualitatively for novel metabolites but have difficulties in quantifying analytes with certainty with longer dwell times. The two studies found in this review used HRAMS for identification of troglitazone-GSH [40] and sulfamethoxazole-glucuronide metabolites[20]. Interestingly, Wang determined that upon HRAMS identification of phase II metabolites, acetylsulfamethoxazole and sulfamethoxazole glucuronide that the concentrations were 2000±1400 ng/L, and 2900±1500 ng/L, respectively, collectively accounting for 54% of the total sulfamethoxazole content of the reclaimed water. This was apparently the first instance of this conjugate surviving WWTPs, and appearing in receiving waters. This capability demonstrates the necessity of mass spectrometry to have not only good resolving power, but sensitivity, and reproducibility in environmental analytical chemistry.

2.3.5.4. Triple Quadrupole-Tandem Mass Spectrometry

Although QQQ has the capability of high sensitivity and specificity through single or multiple reaction monitoring, it can have difficulty in the quantification of an extended number of metabolites due to the necessity of decreased dwell times. This would significantly reduce sensitivity and reproducibility of measurements [77]. Thus, in the present state of conjugate analysis the question becomes is identification or quantification the goal of the experiment.

LC-QQQ, and to a lesser extent, ion trap mass spectrometers, is undoubtedly the most used approach in such targeted analyses. With the high sensitivity and selectivity that tandem mass spectrometry offers when encountering complex matrices, most parent drugs can be detected and quantified at the low ng/L level [20].Therefore, within the scope of this review,

HPLC-QQQ has been by far the dominant technology in quantification of *known* human metabolite conjugates (Table 2.1). That is to say, the standards have been commercially or individually created and standardised to exhibit known ion fragments that QQQ can select for or against.

The most common conjugates that have been extensively profiled and quantified are estrogenic, both endogenous chemicals and synthetic ones for birth control. For instance, Reddy *et al.* (2005)[17] and Kumar *et al.* (2012)[41] reported LOD values in the ng/L range, but did not report range, linearity, or recoveries. On the other hand, Pedrouzo *et al.* (2011)[15], demonstrated sulfate LODs of 15, 10 ng/L (influent, effluent respectively), and glucuronide LODs of 50, 25 ng/L (influent, effluent respectively- Table 2.2), with moderate recoveries of 23-78%, and an environmentally relevant range of 30-1500 ng/L. Gorga *et al.* (2014)[29] showed that coupling turbulent flow to HPLC-QQQ can yield very low LOD values of E3-3-sulfate, E2-17-glucuronide, E1-3-glucuronide, and E3-16glucuronide of 0.0083-1.6 ng/g and 0.10-125 ng/g for receiving water sediment and wastewater sludge, respectively. Moreover, recoveries of 40-92% (higher for sediment), and an analytical range of 2.5-3000ng/L, with a linearity of 0.992-0.999 was seen. This shows the power of current QQQ technology when coupled to effective SPE, specialty columns (Kromasil 100, Cyclone MCX), and binary gradient elution. Having standards available to gauge matrix suppression or enhancement properly, and to accurately predict ion fragments, is paramount for precision and reproducibility.

2.3.5.5. Comparing HRAMS to QQQ

Of interest is the study conducted by Herrero *et al.* (2014)[78] who studied the direct comparison of veterinary drugs in WWTP influent and effluent, between QQQ and HRAMS by Orbitrap (Figure 2.2).

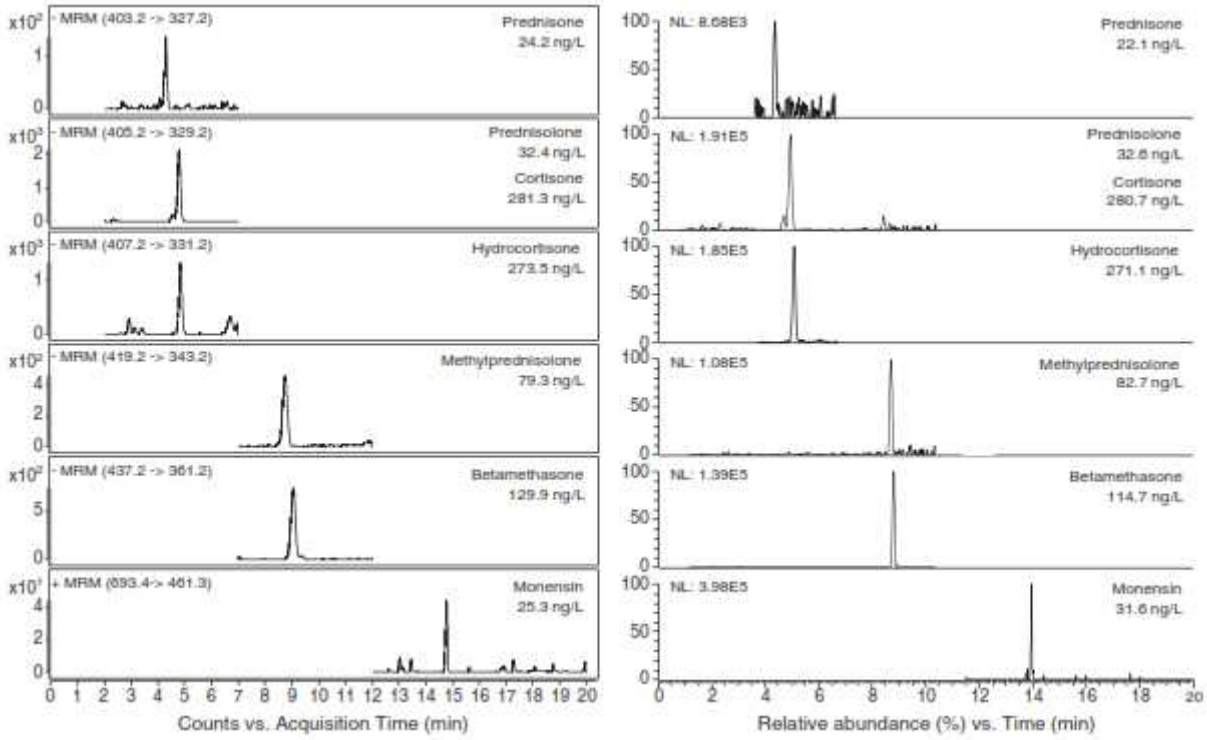

Figure 2.2 A direct comparison of a selected reaction monitoring chromatogram (left) from a UHPLC-MS/MS and UHPLC- high-resolution mass spectrometry chromatogram (right) of an influent sewage sample from a WWTP. This shows the similarities in quantification between the two mass analysers for these specific pharmaceuticals. Both mass analysers were coupled to the same column under the same SPE, column, and elution conditions. The extrapolation to conjugates can be inferred from this data. Reproduced with permission from Herrero *et al.* (2014) J Mass Spectrom, 49, 585.

Although they did not specifically analyse the conjugates, the study notes that for 9 glucocorticoids and 5 polyether ionophores, for both mass analysers the LOD and LOQ values ranged from 2.5-5 ng/L and 2.5-20ng/L respectively, with replicability all under 10% RSD. A correlation plot to compare the same samples analysed by both methods had linearity of 0.996. These results indicate that both QQQ and HRAMS mass analysers were almost in direct quantitative comparison with one another for these parent compounds.

It was also noted that the use of HRAMS can help to prevent false results because data is acquired in full-scan mode, so interferences coming from the matrix can be identified and evaluated. However, it should be mentioned that Orbitrap™ mass analysers do have a drawback in that a minimum of at least one ion ratio must be measured. For this reason, a large number of transitions must be monitored simultaneously (in the same time window) in multiresidue analytical methods. This fact compromises the number of points across the peak without losses in sensitivity because of the very low dwell time required, especially when ultrahigh performance liquid chromatography (UHPLC) is used, because of the narrow peaks achieved (Herrero *et al.*, 2014)[78]. This work notwithstanding, there are other studies comparing the two technologies in a variety of matrices (e.g. milk, tissues, plasma, honey), but there is very few conducted using environmental matrices.

2.4 Conclusions

Knowledge and data on human conjugate analysis in environmental matrices is sparse. By far, estrogen compounds are the most studied primarily due to the redundancy in multiple analytical disciplines. For instance pharmacological, environmental and food chemistries all have

different motivational interests for studying the same steroid conjugates. Through biotransformation and excretion by the human body, conjugates have the potential to persist based on their stereochemistry, and the biotic and abiotic processes impacting the conjugate during processing through wastewater treatment and release into receiving waters. Therefore, techniques and databases need to be made that can isolate for and quantify various classes of compounds. The major considerations for environmental analysis are that the conjugates likely exist at the pg to ng level, *and* that a non-trivial proportion of transformation products of either the conjugate or parent drug are not yet identified. Many studies cumulatively show that previously unidentified metabolites and conjugates have the potential to make up a non-trivial component of the total pharmaceutical inventory in an aquatic ecosystem.

There is no single component to conjugate analysis that is trivial in comparison to another. Extraction, pre-concentration, non-destructive separation, and mass analysis are all interconnected in their effectiveness. However, technologies that take advantage of the polar nature of the human conjugate, whether through SPE or liquid chromatography, have the greatest chance of isolating conjugates from complex matrices. Sensitive and selective mass analysers like the Q-LIT, Q-ToF, Orbitrap™, or QqQ-MS will be of great benefit to isolating and quantifying said conjugates. The forefront of these technologies appears not to be in direct competition with one another, but rather complementary. Thus, hybrid technologies such as the Q-LIT-Orbitrap™ for robust conjugate identification though multiple fragmentations via MS^n capabilities provide power in identifying unknown TPs especially at trace environmental levels. However, QqQ-MS remains the staple in sensitive, reproducible, quantitative analysis of human conjugates.

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Chapter 3

Simultaneous Quantification of Propranolol and Sulfamethoxazole and Major Human Metabolite Conjugates 4-Hydroxy-Propranolol Sulfate and Sulfamethoxazole-β-Glucuronide in Municipal Wastewater- A Framework for Multiple Classes of Drug and Conjugate

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The author of this dissertation designed the experiment along with his supervisor, performed the experiments, interpreted the data, and wrote the manuscript.

3. Abstract

Recent data suggests there are non-trivial amounts of human pharmaceutical conjugates potentially entering environmental surface waters. These compounds could contribute to eliciting toxic effects on aquatic biota either directly or indirectly, via de-conjugation. The need for developing a single method for quantifying both parents and conjugates is necessary. Propranolol (PRO), sulfamethoxazole (SMX), and their respective major conjugates 4-OH-propranolol sulfate (PRO-Sul) and sulfamethoxazole-β-glucuronide (SMX-Glc) were successfully simultaneously extracted through weak anion exchange solid phase extraction cartridges from primary and secondary clarification wastewaters from the North End Winnipeg Water Pollution Treatment Plant in Winnipeg, Canada. Subsequent separation and quantification was achieved by reversed-phase C_{18} chromatography coupled to positive electrospray ionisation tandem mass spectrometry. Linearity for all compounds throughout the 7-point calibration range was > 0.99. Recovery RSD ranges across all matrices for PRO, SMX, PRO-Sul, and SMX-Glc were 2.1-13.2%, 2.3-10.2%, 9.8-19.2%, and 2.0-10.3% respectively. Primary and secondary filtrates respectively showed a significant increase of PRO from 0.039 to 0.045 µg/L; a significant decrease for SMX from 1.56 to 0.58 µg/L; significant decrease of PRO-Sul from 0.050 to 0.020 µg/L; and a significant decrease of SMC-Glc from 0.41 to 0.019 µg/L. These observations indicate that there was removal of all compounds from the aqueous phase occurring at some point between the stages of treatment. To our knowledge, this is first study that simultaneously separated and quantified two different classes of parent compounds and two different kinds of human metabolite conjugates (glucuronide and sulfate) from a major urban wastewater treatment plant.

3.1. Introduction

Pharmaceuticals can be found in the aquatic environment in either water or sediments depending on their polarity and propensity to sorb to particulates [1, 2]. It is becoming more apparent that transformation products, including metabolite conjugates, have the potential to comprise a large hidden component to the overall environmental load of current pharmaceuticals [3]. Reflective of this is the fact that concentrations of pharmaceuticals in wastewater treatment plant (WWTP) effluents could exceed that in influents through biotic and abiotic deconjugation [4] (e.g. venlafaxine, sulfamethoxazole). For example, phase II metabolites of sulfamethoxazole can be abiotically back-transformed to the parent compound, indicating that these metabolites may be an additional environmental source of the drug [5]. Therefore, it is increasingly important to include phase II transformation products when assessing the occurrence, fate and transport of pharmaceuticals in the environment [6], given that substantial cumulative amounts of drugs are released into receiving waters from human and veterinary use. Accordingly, it is necessary to create analytical techniques that can accurately account for the conjugate inventory in the aquatic environment.

Two drugs commonly found in environmental waters in the ng/L to µg/L range [6-9] are propranolol (PRO), an adrenergic receptor antagonist typically used to prevent cardiac arrhythmias [7], and sulfamethoxazole (SM), a commonly used antibiotic [10]. Based on their known pharmacokinetics, 90% of administered PRO was recovered in urine with approximately 17% undergoing glucuronidation, 41% side-chain oxidation, and 42% ring oxidation [11]. One of the primary transformation products is 4-OH-propranolol, which has an apparent equal potency to PRO, but a shorter human biological half-life [12] through naphthalene ring hydroxylation by cytochrome P-450 2D6. Upon administration of SMX, 45-70% is excreted via urine and 43% is

primarily metabolised to acetyl-sulfamethoxazole, and approximately 15% as glucuronides [1]. The most likely site for glucuronidation is via the central –NH group, and thus yielding sulfamethoxazole-β-glucuronide as a primary human conjugate potentially present in wastewaters. However, levels of conjugates can be difficult to predict given the potential mixture of multiply-conjugated moieties in any number of proportions (i.e. glucuronide/sulfate, single, double, mixed etc. conjugations). Therefore, robust procedures need to be developed that can identify and quantify potentially dynamic amounts of conjugates in the environment.

One of the primary limitations in identifying and quantifying environmentally-relevant conjugates is the need for quality standards. There are limited commercially-available standards for analysis of conjugates across many drug classes. The synthesis of conjugates standards using liver microsomes or Supersomes™ [13-15] is sometimes necessary in order to accurately account for ion suppression or enhancement through the LC-MS/MS analytical procedures. High-resolution instruments such as Orbitrap™ provide insight as to elucidating transformation products for which quality standards are unavailable [6]. However, the superior selectivity, sensitivity, and linear quantification range of liquid chromatography-triple quadrupole mass spectrometry is of great benefit in quantifying compounds in complex matrices (e.g. wastewater) [16]. While many studies have been conducted on the quantification of estrogen conjugates in environmental waters [17-20], this study provides a rapid and consistent LC-MS/MS method for simultaneous analysis of two completely different classes of pharmaceuticals, PRO and SMX, and their respective major human transformation product conjugates 4-OH-propranolol sulfate (PRO-Sul) and sulfamethoxazole-β-glucuronide (SMX-Glc). These conjugates were selected to limit alkaline hydrolysis and acyl migration [21] that could transform other metabolites of these drugs (e.g., acyl conjugates). Our method takes advantage of a single mode of electrospray ionisation

and a single binary phase of solvents for both parent compounds and two different types of conjugates, both sulfate and glucuronide, for the first time to the best of our knowledge.

3.2. Materials and Methods

3.2.1. Chemicals and consumables

HPLC-grade methanol, formic acid, ammonium hydroxide (28.9%), and isopropanol (for sterilization) were obtained from Fisher Scientific (CITY, NJ, USA), while acetonitrile was purchased from Fisher and EMD Millipore). Ultrapure Milli-Q (18 MΩ-cm) was produced from a Synergy™ Milli-Q purification system from Millipore (Billerica, MA). Nitrocellulose filter paper (0.45 µm) was obtained from Merck (Ireland), and 13 mm, 0.22 µm white PTFE luer lock inlet syringe filters was purchased from Restek (Bellefonte, PA, USA). Syringe filters were attached to an Agilent 1.0 mL glass syringe (Australia). All solid phase extraction cartridges were Oasis 3 cc, 60 mg from Waters Corporation (Milford, MA), including hydrophilic-lipophilic balance (HLB), mixed anion exchange (MAX), and weak anion exchange (WAX). Nalgene® 250 mL white HDPE bottles were purchased from Thermo Fisher, Rockwood, Tennessee, USA. Centrifuge bottles (50 mL) were purchased from VWR, Mississauga, Ontario, Canada. Glassware was pre-cleaned by ashing at 450°C for 1 hr to destroy organic materials unless otherwise indicated. PEEK tubing (Fisher Scientific, Toronto, ON) was used in the syphoning of environmental matrices through SPE cartridges.

3.2.2. Chemical Standards

Unlabelled standards of propranolol, sulfamethoxazole (Sigma Aldrich, Oakville, ON), 4-OH-propranolol sulfate and sulfamethoxazole-glucuronide (Toronto Research Chemicals, Toronto, ON); and matching isotopically-labeled standards propranolol-d_7 (C/D/N Isotopes Inc, Pointe- Claire, QC), sulfamethoxazole-d_4 (ICN Biomedicals, Irvine, CA), 4-OH-propranolol-d_7 sulfate and sulfamethoxazole-d_4-glucuronide (Toronto Research Chemicals) were obtained as neat powders (Table 3.1, Figure 3.1).

Table 3.1 Physicochemical parameters of propranolol, sulfamethoxazole, 4-OH-propranolol sulfate, and sulfamethoxazole-glucuronide.

Compound	Molar mass (g/mol)	Aqueous Solubility (mg/L)	pK_a	$LogK_{ow}$
Propranolol	295.80[a]	62.0[b]	9.24[c] 9.67, 14.09[a]	3.48[c]
Sulfamethoxazole	253.28[d]	600[d]	1.83, 5.57[d] 1.97, 6.16[e]	0.89[d]
4-OH Propranolol Sulfate	355.41[f]	120900[f]	-1.92, 9.67, 14.09[e]	-0.369[f]
Sulfamethoxazole-glucuronide	430.41[f]	1000000[f]	1.95, 3.32, 12.45, 13.39, 14.8[e]	-2.892[f]

[a]PHYSPROP Database – www.srcinc.com. [b]EPI Suite™ via ECOSAR. [c]Escher *et al.* (2006). [d]Srinivasan *et al.* (2014). [e]Marvin Sketch pKa prediction. [f]Solubility and $LogK_{OW}$ predicted by EPISuite via ECOSAR

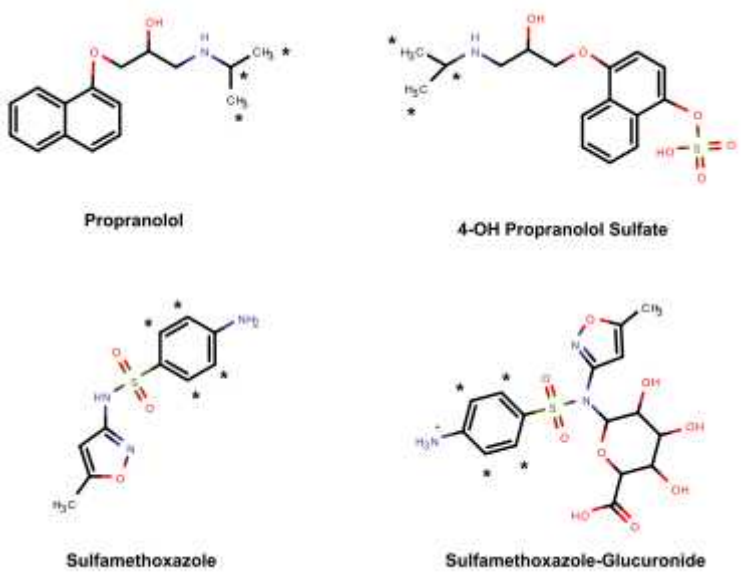

Figure 3.1 The chemical structures of both parent compounds PRO and SMX, and conjugates PRO-Sul and SMX-Glc, all in fully protonated state. Note that at environmentally relevant pH conditions, including laboratory analysis, SMX, and the conjugates are in anionic form, whereas PRO is predominantly cationic. All isotopically-labelled standards are deuterated, with asterisks indicating labeled deuterium atoms in the analogous compound.

Master stock solutions ranging from 36-44 mg/L were prepared in 5/20 (v/v) acetonitrile: methanol and stored at -20^{0}C. Typically, acetonitrile is the solvent of choice for stock solutions and standards given its aprotic properties, thus limiting hydrolysis. Moreover, acetonitrile is generally beneficial to conduct hydrophilic liquid-interaction (HILIC) chromatography. However, the lack of complete miscibility of acetonitrile with PRO and PRO-Sul necessitated the use of methanol as the stock solvent, and is the reason for our use of 5/25 (v/v) acetonitrile: methanol. Subsequent 1000 µg/L qualitative optimiser solutions were prepared in methanol and stored at -20^{0}C. Unlabelled (50 µg/L and 1000 µg/L) and isotopically-labelled (1000 µg/L) stock

solutions for making calibration curves and spiking various matrices were prepared in methanol. To verify the spiking technique in the single and dual elution experiments in the aforementioned matrices, 2000 µg/L of propranolol was used instead of 1000 µg/L in the unlabelled mixture. Unlabelled and labelled mixed working standard solutions for chromatography parameter qualitative assessments (1, 10, and 100 µg/L) were prepared from these previous stock solutions and stored at 4°C. Calibration curve standard solutions (0.1, 0.5, 1, 5, 10, 50, and 100 µg/L) for quantitative assessments were prepared from the master stock solutions in 50/50 (v/v) Milli-Q water: methanol and stored at 4°C.

3.2.3. UHPLC-MS/MS Methods

Chromatography was performed with a Agilent 1200 UHPLC, with separation using an Agilent Eclipse Plus C_{18} column (2.1 mm × 50 mm, 1.8 µm dp), coupled to a Phenomenex SecurityGuard Ultra C_{18} guard column (2.1 mm × 5 mm) at 42^0C at 0.4 mL/min. Injection volumes during optimisation were 5 µL loaded through a 2 µL sample loop, and during analysis 15 µL was loaded through a 10 µL sample loop. Mobile phase A1 was 0.05% formic acid (FA) in Milli-Q water, B1 was acetonitrile with 0.05% formic acid, A2 was 95/5 (v/v) Milli-Q water: methanol, and B2 was 100% methanol. Gradient elution was performed as follows: 0-2.00 min linear ramp from 10% B1 to 60% B1, 2.01-5.5 min re-equilibration at 10% B1. Upon completion of all analytical runs the columns were flushed with 20 min of 10% B2, then 25 min of 95% B2 to eliminate formic acid residues for column storage.

Qualitative assessment and quantification was performed through multiple reaction monitoring (MRM) on an Agilent 6410 triple quadrupole mass spectrometer (MS/MS) in

positive electrospray ionisation mode (ESI+), a capillary voltage of 4000 V, and a source temperature of 300°C. Nitrogen was used for desolvation and drying gas at 11 L/min, and for nebulization at 15 psi. Ultrapure nitrogen was used as collision gas at a flow of 16.8 L/min. The MS1 and MS2 heaters were set at 100°C. Compound-specific mass spectrometric parameters and ion fragments are found in Table 3.2.

Table 3.2 Compound-specific mass spectrometric parameters determined during optimisation such as retention time, fragmentation voltage (frag), collision energy (CE), and collision voltage (CV) (all in V) and mass transitions of precursor to product ions.

Compound	Retention Time (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Dwell (ms)	Frag	CV	CE (eV)
Propranolol	2.00	260.2	183	200	110	7	16
Propranolol-d7		267.2	116.1	200	110	7	16
Sulfamethoxazole	1.84	254.1	156	200	107	7	12
Sulfamethoxazole-d4		258.1	96.1	200	101	7	28
4-OH Propranolol Sulfate	1.39	356.1	276.1	200	138	7	16
4-OH-d7-Propranolol Sulfate		362.2	282.2	200	122	7	16
Sulfamethoxazole-glucuronide	1.39	430.1	254	200	100	7	0
Sulfamethoxazole-d4-glucuronide		434.12	112	200	104	7	44

Criteria for positive identification and quantification was on the most abundant $[M+H]^+$ product ion fragment (quantifier) without the use of a qualifier given that human conjugates are simply the parent compounds coupled to an anionic moiety (whether glucuronide or sulfate) that could readily de-conjugate during fragmentation. Thus, identical transitions of *m/z* lesser than the parent compound could potentially represent fragments of either the parent or conjugate (Table 3.2), although these fragments are most likely sufficiently separated by retention time.

3.2.4. Extraction and Pre-concentration of Samples

3.2.4.1. Sample Matrices and Collection

Spike and recovery tests were performed first using Milli-Q water, then river water, and primary and secondary clarifier wastewater to assess matrix effects in a typical receiving water. A large 40 L grab sample of Elm River water was obtained from Oakville, Manitoba, Canada and stored at 4°C for use during method development. The Elm River water initial pH was approximately 7.7, with a specific conductivity of 311.45 µS-cm, dissolved oxygen was 81.54%. The Elm river had a flow rate of 0.1728 m^3/s, a catchment area of 105.32 km^2 with 0.6529% cropland, contributing sewage of 1.225 persons/km^2, and livestock ratio of 10.0446 N/km^2 [22]. Primary and secondary clarifier 24 h composite samples were obtained from the North Main Waste Water Treatment plant of Winnipeg, Canada, servicing approximately 70% of Winnipeg's population of approximately 700,000 people. The facility treats combined sewage of residential, commercial, industrial, and hospital waste, in combination with municipal street run-off, suggesting that its wastewaters would likely contain measurable quantities of pharmaceutical

conjugates. As the treatment process flow rate is greatly dependent on precipitation, samples were obtained using 24-h composite active samplers, which collected into 20L Nalgene® HDPE bottles.

Wastewater was collected into 2 L amber solvent bottles (pre-conditioned with methanol and Milli-Q water) for initial spike and recovery experiments. For subsequent quantitative analysis, 1 L bottles were filled at the WWTP by the staff, with no headspace and refrigerated at 4°C overnight. Upon wastewater retrieval, the samples were filtered immediately upon arrival in the university laboratory through 0.45 µm nitrocellulose filters into ashed and pre-conditioned amber bottles to reduce the likelihood of microbial transformation or degradation. Coliforms that contain a great deal of glucuronidases and sulfatases are typically between 0.6-1.2 µm in diameter and 2-3 µm in length [23] and should be largely isolated from the matrix through this method.

3.2.4.2. Nalgene® HDPE Sorption Test

The possibility of pharmaceutical conjugates adsorbing to HDPE Nalgene® bottles was considered. Quadruplicates of Milli-Q water and river water (200 mL each) were spiked with analytes as outlined below. The only exception to these methods was that upon spiking with unlabelled and labelled standards, the bottles were capped and stirred for approximately one minute each to homogenise (ensuring no splashing into the cap), then the bottles sat undisturbed in the dark for 24 hours prior to extraction through solid phase extraction (SPE).

3.2.4.3. Off-line Solid Phase Extraction

Aliquots of desired matrices were put in ashed amber jars and spiked with unlabelled and internal standard mixture solutions, to extract and recover an environmentally relevant concentration of 0.1 or 0.2 µg/L in 200 mL and 100 mL aliquots respectively, and reconstitute them in 1 mL, for a concentrated total of 20 µg/L. SPE cartridges were placed on a vacuum manifold , and PEEK "sippers" (tubing) were attached to pass water through the cartridges at 5 mL/min. Cartridges were then eluted as outlined in 2.4.4 and the eluent evaporated to dryness under nitrogen at 42°C in a water bath. Each sample was reconstituted in 1 mL 50/50 (v/v) Milli-Q: methanol and vortexed. Samples were transferred using ashed Pasteur pipettes to glass syringes for syringe filtering into ashed amber LC vials. All samples were refrigerated at 4°C until instrumental analysis. Specific SPE conditions are outlined in 2.4.4.

3.2.4.4. SPE Cartridge Treatments

All SPE cartridges were preconditioned as per the Waters Oasis™ generic 2×4 method, using 3 mL aliquots of each appropriate solvent. The 2×4 method is used as a generic approach to identify which sorbent will extract, retain, and elute the conjugates of interest, and is outlined for our procedure below. MAX was conditioned with a series of 0.2% FA in Milli-Q to reduce basic interferences, then methanol, then 0.2% NH_4OH in Milli-Q to reduce acidic interferences, then Milli-Q water. Samples were loaded to MAX as in 2.4.3, washed with 0.2% NH_4OH, then eluted with 0.2% FA in methanol for single elutions, or in 2 stages of methanol then 0.2% FA in methanol for dual elutions. WAX was conditioned with a series of 0.2% NH_4OH in Milli-Q to reduce acidic interferences, then methanol, 0.2% FA in Milli-Q to reduce basic interferences,

then Milli-Q water. Samples were loaded to WAX, washed with 0.2% FA, then eluted with 0.2% NH_4OH in methanol for single elutions, or in 2 stages of methanol then 0.2% NH_4OH in methanol for dual elutions. In comparison, HLB was preconditioned with methanol then Milli-Q water, and then eluted in single 3 mL methanol aliquots.

3.2.4.5. Spike and Recoveries for SPE

An unlabelled standard mixture (20 ng each analyte) and deuterium-labelled internal standard mixture (50 ng each) were used for spiking to quantify losses at each stage of the extraction processes. The unlabelled mix was spiked at the beginning of extraction in the amber jars containing 200 mL of the matrix of interest. Internal standard mix spikes were done at four different extraction stages: first, in the initial jar; second, after SPE but before N_2 evaporation; third, after N_2 evaporation at reconstitution in 50/50 (v/v) Milli-Q: methanol; and fourth, in the LC vial after syringe filtering. IS spiking in the jar accounts for losses throughout the entire SPE process. Mathematical differences in unlabelled recoveries represent the differences in losses of unlabelled analyte at each stage due to the by-passing of IS spike from the previous stage(s).

3.2.4.6. Method Evaluation

One sample each of the primary and secondary wastewater filtrates along with triplicates of fresh 24 h composite aliquots of primary and secondary wastewater filtrates from the North Main treatment plant were processed as described in 2.4.1-2.4.5. However, there was no spiking of unlabelled parent and conjugate compounds so as to quantify these compounds in the

wastewater. One positive control of Milli-Q water was spiked with unlabelled standard mix to ensure that the compounds of interest were being retained and eluting properly; and a working lab blank was analysed to assess for laboratory contamination. In addition, a single sample each of primary and secondary filtrates stored at 4°C for a month prior to processing as noted, were analysed to evaluate analyte stability under storage. Moreover, a single Milli-Q positive control spiked with the same unlabelled and IS standards as above in addition to a Milli-Q field blank, were subjected to the same filtration, extraction, pre-concentration, and LC-MS/MS protocols as outlined in 2.4.1, 2.4.3, and 2.4.4.

3.2.4.7. Linearity, Precision, MLD, and MLQ

Calibration curves were constructed using unlabelled standard samples, as noted in 2.2, spanning the predicted environmentally relevant range and spiked with internal standard for quantitative assessments. Curves were best fitted using a least squares linear regression model, weighted by the inverse of the analyte concentration. Linearity ranges were determined from regression of the 7 point calibration curves' unlabelled standard responses in relation to the analogous internal standard over 10 separate river and wastewater extraction runs on different days. The method limit of detection (MLD) and limit of quantification (MLQ) were defined as the mean concentrations of 7 replicates of standard mixtures of 0.1, 0.5 and 1 µg/L that yielded *S/N* ratios greater than 3 and 10, respectively. Intraday and interday precision was calculated by spiking and extracting 3 separate aliquots of primary wastewater filtrate and analysing each (total of 3) over a 24 h period, and over 3 separate days, respectively. Calibration curve accuracy was calculated by quantifying the mass of unlabelled parent and conjugate standard mixture spiked in

50/50 (v/v) Milli-Q water: methanol compared to the internal standard mixture response ratio over 4 separate days.

3.2.4.8. Statistical Analysis

GraphPad Prism v.5 (GraphPad Software, La Jolla, CA, USA) was used to conduct all analyses during method development. One-way ANOVA was conducted for all components of method development, and Tukey's post-hoc test was used for comparison of multiple means between stages of SPE. Dunnett's post-hoc test was conducted for comparing the target expected value of IS spikes to actual recoveries. For method validation, unpaired two-tailed t-tests ($\alpha = 0.05$) were conducted to determine if quantities of endogenous analyte differed significantly from primary to secondary clarification within the wastewater filtrates.

3.3. Results and Discussion

3.3.1. Liquid Chromatography-Tandem Mass Spectrometry

While it is possible to analyse some of these analytes under ESI negative mode, positive mode was chosen for several reasons. First, both PRO-Sul and SMX-Glc product ion fragments were found to be approximately 5 to 10 times greater in positive mode compared to the abundances in negative mode, while SMX product ion fragments were approximately double in positive mode, and PRO was virtually non-ionisable in negative mode. Second, we wanted to have a method that required only one method with one binary solvent, for which positive mode was necessary.

The conjugates analysed were baseline-separated from their parent compounds (Figure 3.2). The most abundant single product ion fragments were used for quantification. The other product ions (Table A1.1) isolated for the conjugates through optimisation were identical to the product ions isolated for the parent compounds. Thus, these were not chosen because of the potential for mischaracterisation of the analyte of interest if the retention times of parent and conjugate species are relatively similar during LC-MS/MS.

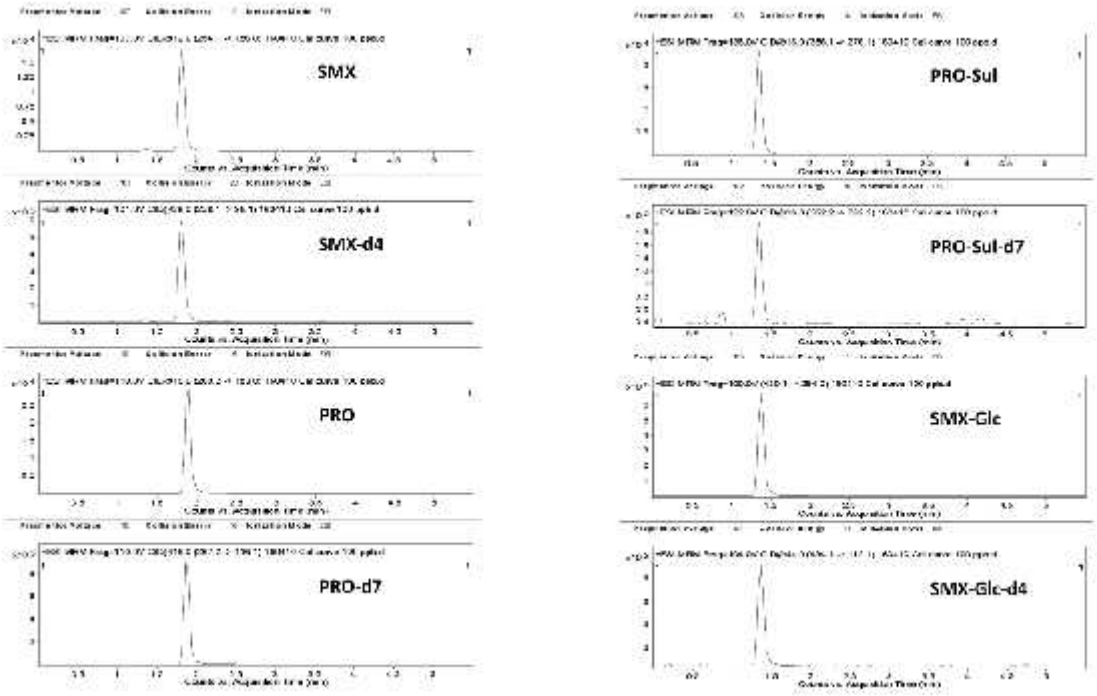

Figure 3.2 Extraction of the multiple reaction monitoring acquisition of a 100 ng/mL standard mixture of unlabelled and analogous deuterated compounds of PRO, SMX, PRO-Sul, and SMX-Glc.

It was important to note that due to the storage of the C_{18} column in negative mode solvents such as 95/5 (v/v) Milli-Q: methanol, a minimum of 45 to 60 mins of flushing was necessary. This was done with positive mode solvents A1 and B1 listed in 2.3, at 0.4 mL/min with the column heated at 42^0C, the column outlet PEEK tubing diverted directly to waste, and not to the MS. Several single injections of a greater concentration calibration curve standard (i.e. 50 or 100 μg/L) were usually necessary to get the retention times back down to the linear ramping phase of the timetable (i.e. < 2 mins) to prevent peak broadening.

In addition, a sufficiently clean guard column was necessary to achieve consistent counts in the chromatograms. Moreover, back-flushing of the guard column was sometimes necessary to achieve consistent retention times and increase analyte counts of PRO-Sul and SMX-Glc. During subsequent flushing or blank injections, no conjugate analyte breakthrough occurred, indicating entrapment within the contaminants in the guard column. In contrast, PRO and SMX counts appeared unaffected by the presence of a slightly contaminated guard column. This suggests that the conjugates could potentially have been sorbed or complexed to the foreign matter contained in the guard column through non-hydrophobic interactions. This fact affirmed the overall necessity for filtration of wastewater matrix prior to SPE, and for dual elution protocol to eliminate neutral interferences by initially eluting with 100% methanol prior to instrumental analysis.

We note that HILIC was also tried with limited success in retaining the conjugates, and with no success at retaining the more hydrophobic parent compounds, which was to be expected. HILIC is most likely to be successful by column-switching subsequent to C_{18} separation [24]. Moreover, changing the reconstitution solvent to 80/20 (v/v) Milli-Q: methanol and to 20/80

(v/v) Milli-Q: methanol did not sufficiently change retention of analytes, promotion of baseline separation, or sharpen peak shapes to justify deviation from 50/50 (v/v) Milli-Q: methanol.

3.3.2. Method limits of detection, method limits of quantification, precision, and accuracy

Precision for PRO, SMX, PRO-Sul, and SMX-Glc (Table A1.2) was (intraday and interday): 2.2 and 3.7, 2.7 and 1.7, 18.2 and 15.6, and 4.2 and 5.3% respectively (Table 3.3). PRO-Sul seemed to have a greater RSD even though he *S/N* ratio was always in the 40 to 70 range for all analyses of this conjugate. The precision of all compounds were less than approximately 5% aside from this analyte. It is possible that the C_{18} column was becoming more equilibrated as the analyses continued for their respective time frames. Linearity for PRO, SMX, PRO-Sul, and SMX-Glc, was 0.9964-0.9989, 0.9965-0.9997, 0.9884-0.9999, and 0.9969-0.9999 respectively (Table 3.3). Calibration curve accuracy (Table A1.3) was calculated by quantifying the mass of unlabelled parent and conjugate standard mixture spiked in 50/50 (v/v) Milli-Q water: methanol compared to the internal standard mixture response ratio over 4 separate days. The accuracy of 0.1, 0.5, 1, 5, 10, 50, and 100 µg/L standard mixtures were 76.2% to 172.5%, 68.3% to 128.5%, 68.1% to 142.4%, 88.5% to 111.2%, 89.9% to 116.5%, 74% to 102.6%, and 98.3% to 101.4%, respectively. Thus, not only are these conjugates stable over the short-term, but also that the analytical method provides recoveries of concentrations that are representative of environmentally relevant concentrations.

Table 3.3 Method limits of detection (MLD), method limits of quantification (MLQ), intraday precision, and interday precision.

Compound	MLD (µg/L)	MLQ (µg/L)	Linearity (R^2)	Interday Precision (%)	Intraday Precision (%)
PRO	0.1	0.5	0.9964- 0.9989	2.2	3.7
SMX	0.1	0.5	0.9965- 0.9997	2.7	1.7
PRO-Sul	0.1	0.5	0.9884- 0.9999	18.2	15.6
SMX-Glc	0.1	0.5	0.9969- 0.9999	4.2	5.3

3.3.3. Solid Phase extraction

Offline solid phase extraction using Waters WAX cartridges was the most effective of our SPE options for isolating our analytes from the surrounding environmental matrices. Various mechanisms beyond hydrophobicity can be used to separate ionic compounds from matrices [25-27]. WAX sorbent is essentially a mixed mode, hydrophilic-lipophilic balance macroporous copolymer poly(divinylbenzene-co-*N*-vinylpyrrolidone) backbone with an acid-base nitrogenous group (pK_a~ 6). This additional functional group is sufficient for sorbing acids such as sulfates and glucuronides. SPE is beneficial for pre-concentration of analytes from water samples, given that concentrations will be very low (high parts-per-trillion and below) [25-27]. Thus, large volumes of matrix can be passed through the cartridges. Despite pre-filtration (2.4.1), wastewater typically contains a non-trivial amount of dissolved or suspended organic matter. For example, yellow-green extractions resulted from any elution of environmental matrix with 0.2% ammonium hydroxide in methanol, whether using single or dual elutions. This observation suggests that the coloured compounds in these matrices could be anionic in character. Indeed,

certain ion exchange SPE sorbents show generally low LC–MS/MS recoveries given strong matrix effects in the first fraction due to hydrophobic interaction [28]. This is most likely because all four types of mixed ion and weak ion exchange sorbents have the same non-ionic poly(divinylbenzene-co-*N*-vinylpyrrolidone) backbone. The strong ion-exchanger such as MAX has been to shown to have lower recoveries and thus stronger suppression of extracts than WAX [28]. This observation indicates that during the elution of both weak cationic and anionic ion exchange compounds in the second fraction, fewer matrix compounds are eluted than in the second fraction of the strong ion exchanger sorbents. In comparison, urine mainly contains weak acids and bases and these are only poorly retained on the weak exchange resins [28].This may be analogous to what is seen in the retention and elution of analytes from wastewater as a whole, as they most likely originate from human urine and feces. Thus, similar interference may be due to retention of said weak acids and bases on MAX, and the limitation of interference using WAX.

MAX sorbent can isolate anionic glucuronide and sulfate conjugates from parent active pharmaceutical ingredients [15]. However, if quantification of parent compounds is also desirable then depending on the charge of the parent analyte, then different sorbents (e.g. HLB or WAX) and conditions for conditioning and washing are necessary. It is important to note that PRO, SMX, and PRO-Sul all eluted in the initial methanol fraction during the dual elution process. Thus, this methanol elution step cannot be simply considered a “washing” step as suggested by the Oasis 2×4 method. These fractions must be analysed. This observation indicated that hydrophobic sorption was the dominant mechanism in SPE of these compounds in both deionised water and complex matrices regardless of pKa or charge. SMX-Glc had very little affinity for both HLB and MAX cartridges in any matrix, necessitating the search for another all-encompassing extraction sorbent (WAX).

As reported in Brown and Wong (2015)[16] the vast majority of conjugate isolation for both qualitative and quantitative studies of pharmaceuticals and estrogenic compounds used either SPE cartridges or micro-elution well plates, both impregnated with HLB as stationary phase [29, 30]. As for the current compounds of interest, both PRO and SMX have been extracted from environmental matrices countless times using C18 and HLB sorbents, with good recoveries overall 60.4- 116%, the lower recoveries deriving from urine [8, 9, 31, 32]. Specifically, with respect to SMX-Glc, there are several studies that address the SPE of environmental waters. Bonvin (2013) [5] refers to SPE protocol cited in a 2012 document unavailable online through Google Scholar, Researchgate, or the Web of Science. Radke (2009)[1] reported linear calibration curves (>0.98), but no studies were conducted to report the LOD or LOQ. They reported that the analytical method was less reproducible with an average recovery rate of 168 (+/- 58% (n= 42)) due to matrix effects during the HPLC- MS/MS analysis. However, when spiked with the labeled surrogate standard D3-SMX-Glc results were generally reproducible at 94% (n= 2). Wang and Gardinali (2014) [6] used tandem SPE cartridges comprised of HLB and Sep-Pak tC18 Plus. For SMX they reported an MDL of 67ng/L (N=7), and a recovery of 94% (N=3), but there was no mention of the recovery of the SMX-Glc using high resolution mass spectrometry.

Partani (2009)[12] isolated both total parent PRO, and total PRO-Sul from human plasma with HLB SPE cartridges with a resulting LOQ of 100 µg/L for both compounds, and linearity of 1-500 µg/L and 1-360 µg/L respectively. However, the recoveries were variable with 200 µg/L PRO recovered at 95.2%, and 500 µg/L of PRO-Sul recovered at 62.5%. Comparison to our current study showed WAX SPE cartridges produced an LOQ of 0.5 µg/L for all compounds, linearity from 0.1- 100 µg/L, and recoveries of 122 and 101% for PRO and SMX respectively, and 91 and 99% for PRO-Sul and SMX-Glc respectively from environmental waters. The keys to

these recoveries were in the necessity of sequential dual elutions with 3 mL of methanol and 3 mL of ammonium hydroxide in methanol in order to reduce matrix effects, and taking advantage of the anion exchange mechanism in conjunction with hydrophobic interactions of the weak anion exchange sorbent.

3.3.4. HLB/MAX/WAX in Milli-Q and Elm River Waters Using Dual Elutions

All three sorbent types studied were moderately sufficient at extracting, retaining, and eluting all analytes except for SMX-Glc using the initial Milli-Q water matrix (Table A1.4). Herein, “mean differences” indicate the loss of quantified analyte from the expected target concentration of 20 µg/L. Specifically, PRO showed significant losses using WAX at the SPE stage (mean difference = 14.7 µg/L; P=0.0019). SMX showed significant losses using MAX after syringe filtering (mean difference = 9.43 µg/L; P=0.0220). 4-OH-propranolol sulfate showed no losses. SMX-Glc showed significant losses at all stages of SPE using HLB (mean differences = 18.8, 18.9, 18.6 µg/L; P<0.0001) and MAX (mean differences = 10.0, 14.0, 14.5 µg/L; P<0.0001), but no significant losses using WAX cartridges.

When river water was used (Figure 3.3) overall only WAX sorbent showed sufficient retention and elution. PRO using HLB showed significant losses at the syringe filter (mean difference= 7.75 µg/L; P=0.045). SMX using all sorbents showed significant losses at nitrogen evaporation and syringe filtering (mean differences = 4.85 to 9.77 µg/L; P<0.0001). PRO-Sul all stages showed significant losses across all sorbents except for spiking in the jar using WAX (mean differences=7.17 to 9.70 µg/L for HLB, 18.7 to 19.3 µg/L for MAX, and 4.68 to 4.69 µg/L for WAX; P<0.0001). SMX-Glc only HLB and WAX showed no significant losses in jar, all

other stages across all sorbents showed significant losses (mean differences=14.7 to 16.9 µg/L for HLB, 10.4 to 17.5 µg/L for MAX, and 9.1 to 11.8 µg/L for WAX; $P<0.0001$). Thus, it was determined that WAX sorbent cartridges were sufficient at retaining parent compounds through hydrophobic interactions, likely on the macroporous copolymer poly(divinylbenzene-co-*N*-vinylpyrrolidone) backbone, and the anionic conjugates, likely through associations via the ionisable nitrogenous moieties.

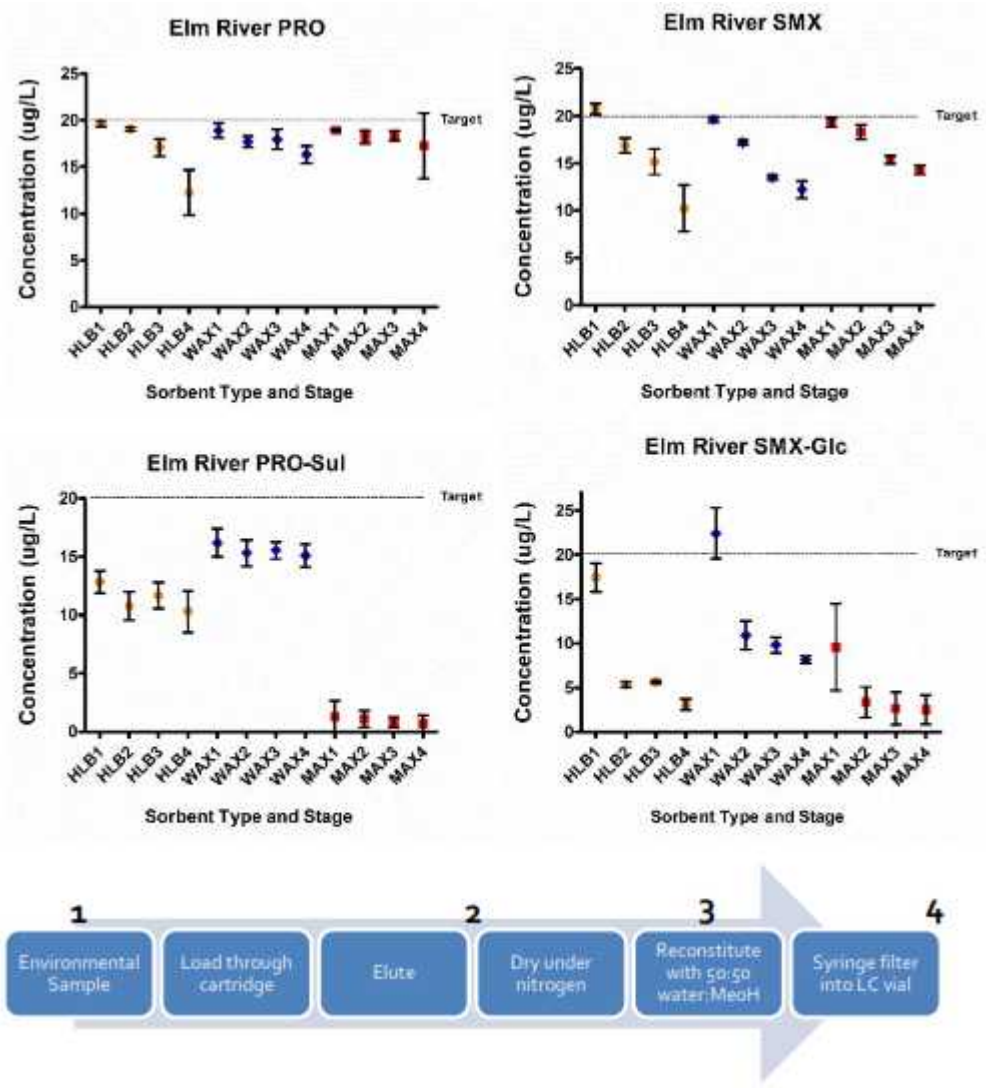

Figure 3.3 Spikes and recoveries of PRO, SMX, PRO-Sul, and SMX-Glc in Elm River matrix. The suffixes 1-4 represent an internal standard mixture spike at the various stages of extraction and reconstitution: 1) in original jar; 2) after solid phase extraction; 3) at reconstitution; 4) after syringe filtering in the LC vial. This analysis allows for elucidation of which stage(s) incurred the greatest losses of unlabelled analyte. The dots represent the mean value of triplicate trials; the error bars represent the standard error of the mean.

3.3.5. Spike and Recoveries Using River, Primary, and Secondary Wastewater Filtrates

Once the sorbent type was isolated as WAX, it became necessary to determine if unlabelled compound spikes could be quantified in the various matrices, which were primarily comprised of aqueous fractions of primary and secondary clarifier wastewater filtrates (Table 3.4).

Table 3.4 Recoveries of 20 µg/L native spike mixtures of parents and conjugates upon weak anion exchange solid phase extraction (3 cc, 60 mg) in Elm River water, primary wastewater filtrate, secondary wastewater filtrate, and an additional experiment testing potential sorption with Milli-Q deionised water and Elm River water in Nalgene HDPE bottles using dual elutions. Values shown are the recoveries in % (n=3) and relative standard deviation in % in parentheses, except the Nalgene values which are mean (n=4) and relative standard deviation in % in parentheses.

Compound	Mass of Recovery (%)							
	River		Primary Wastewater		Secondary Wastewater		Nalgene (Dual)	
	Single	Dual	Single	Dual	Single	Dual	Milli-Q	River
PRO	111 (2.9)	122 (13.2)	148 (4.5)	134 (3.4)	148 (7.4)	145 (4.3)	114 (4.0)	112 (2.1)
SMX	109 (9.9)	101 (10.2)	1325 (13.6)	1145 (2.3)	482 (5.0)	335 (3.2)	109 (7.4)	123 (6.9)
PRO-Sul	43 (28.2)	91 (10.5)	11 (15.0)	76 (19.2)	19 (11.6)	60 (16.0)	100 (15.0)	90 (9.8)
SMX-Glc	93 (12.2)	99 (5.8)	203 (14.6)	180 (5.5)	84 (3.3)	102 (2.0)	92 (8.1)	96 (10.3)

All analytes showed no significant difference in recovered quantities in river matrix with the exception of PRO-Sul (mean difference= 11.5 µg/L; $P<0.0001$ using single elution). This observation is consistent with our previous observation that WAX was appropriate for extraction, retention, and elution of all compounds, but that a dual elution was potentially helpful in removing interferences from the samples. Indeed, a dual elution of 3 mL methanol then 3 mL 0.2% ammonium hydroxide in methanol showed no significant difference for any analyte in river

water (Table 3.4), suggesting good retention and elution. Mean differences in recoveries for primary and secondary filtrates becomes less important, but rather quantities greater than the initially spiked amounts (and the precision) suggesting the presence of these conjugates. Reflective of this fact was the negative differences in mean recoveries for all compounds. PRO showed significant mean differences of -13.6 to -19.1 µg/L for both single and dual elutions across both wastewaters ($P< 0.0001$). SMX showed significant mean differences of -210 to -245 µg/L for primary filtrate, and -46.9 to -76.6 µg/L ($P< 0.0001$) for secondary filtrate for single and dual elutions, respectively. PRO-Sul showed significant mean differences of -17.7 and -4.91 µg/L for primary filtrate, and -16.1 and -7.98 µg/L ($P< 0.0001$) for single and dual elutions respectively, suggesting the persistence of both parent compounds and PRO-Sul through these two stages of water treatment. SMX-Glc showed significant mean differences of -20.6 and -15.9 µg/L ($P<0.0001$) in primary filtrate but no significant difference in secondary filtrate, suggesting the presence of this conjugate in primary and subsequent transformation or removal by the secondary clarification stage.

3.3.6. Potential Sorption of Analytes to Nalgene® HDPE Bottles

Some neutral or cationic pharmaceuticals (i.e. loratadine and sertraline, respectively) can potentially sorb to the surface of HDPE bottles [33], thus contributing to the underreporting of these compounds once removed from these containers. Hence, we needed to determine if our analytes would be retained by the 20 L HDPE bottles used by the North Main Treatment Plant for collection of the 24 h composite samples. The use of HDPE bottles is of logistical benefit when collecting environmental waters (i.e. no breakage, spillage, and reduction of headspace). We found statistically different means for Milli-Q and Elm River respectively of -5.55 and -4.76

µg/L (P= 0.0002) for PRO. Elm River showed a statistically different mean of -4.47 µg/L (P= 0.0035) for SMX (Table 3.4, Figure A1.1). However, both conjugates showed no statistical difference in the theoretical target of 20 µg/L to what was recovered. Thus, it could be concluded that all four compounds show no potential for sorption to HDPE bottles within a 24 h time frame.

3.3.7. Method Evaluation

Based on the previous recoveries outlined above, we expected in North End wastewater treatment plant waters to see a persistence of PRO, SMX, and PRO-Sul, but an attenuation of SMX-Glc due to wastewater treatment processes. Positive controls all had recoveries consistent with the ranges from the previous recovery experiments, and all field blanks contained no amounts of parents or conjugates, consistent with the lack of volatility of these compounds. What was found was promising, the results of which can be found in Figure 3.4.

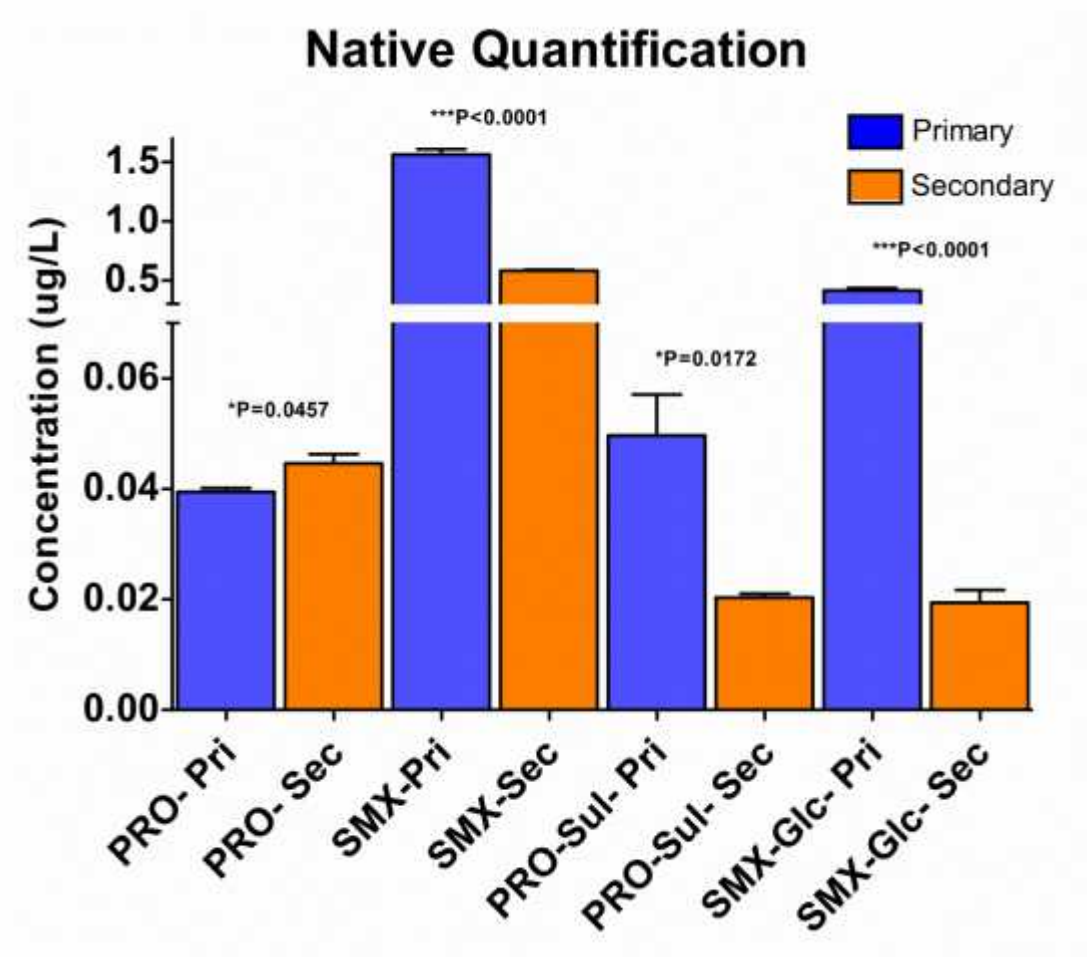

Figure 3.4 Method evaluation quantifying unlabelled endogenous levels of given parent compounds and conjugates in fresh primary wastewater and secondary wastewater filtrates (n=3; solid colours) with relative standard deviation in (%) in parentheses. The column bars represent the mean value of triplicate trials; the error bars represent the standard error of the mean.

PRO showed a statistically significant increase from 0.039 to 0.045 µg/L (P= 0.0457) from primary and secondary filtrates respectively (Figure 3.4). These levels are consistent with other findings [12, 34] suggesting that PRO has the potential to not only survive this portion of the treatment process but could potentially increase. SMX was shown to exist at levels consistent

with other findings [6, 35], a significant decrease from 1.56 to 0.58 µg/L ($P < 0.0001$) in primary and secondary treatment respectively. PRO-Sul showed a significant decrease of 0.050 to 0.020 µg/L ($P = 0.0172$) from primary to secondary respectively. These findings suggest that levels of PRO-Sul could be greater than the levels of PRO entering the wastewater treatment process, which is consistent with pharmacokinetic human metabolism data that outline 4-OH-propranolol sulfate as one of the dominant transformation products excreted from the human body [11]. Moreover, this data suggests PRO-Sul could be de-conjugated in secondary treatment, where a significant amount of heterotrophic bacteria exist, resulting in the mild inventory increase in PRO observed.

SMX-Glc showed a significant decrease from 0.41 to 0.019 µg/L ($P < 0.0001$) in the primary and secondary filtrates, respectively. This indicates that there is transformation or degradation occurring at some point between these stages of treatment. Because these 24 h composite samples were collected simultaneously (i.e. the same time frame) they are not necessarily indicative of a quantifiable reduction between samples; these samples represent two different batches of wastewater which are processed at time frames proportional to flow rates. Further study would need to be conducted to identify what proportion of 24 h composite samples represents given batches of wastewater; in fact this will be a component of the following monitoring study subsequent to this manuscript.

Of particular note were the samples of previous primary and secondary filtrates filtered one month prior to the fresh samples. These filtrates should theoretically have had very little coliforms or other bacteria present that could potentially de-conjugate the conjugates while in storage at 4°C in the dark. These coliforms typically have diameters in the 0.6-1.2 µm range when provided with adequate nutrients for growth [36], and these wastewaters were filtered through 0.45 µm nitrocellulose filters prior to storage. The stored primary and secondary filtrates

contained these amounts of PRO, SMX, PRO-Sul, and SMX-Glc respectively: 0.080 and 0.11, 3.88 and 0.92, 0.076 and 0.073, and 0.44 and 0.046 μg/L. (Figure 3.4). This observation suggests that under conditions that are removed from elevated amounts of microorganisms (e.g. oligotrophic waters) these compounds are relatively stable and persistent in the short term (i.e. less than one month). These latter concentrations occurred when the flow rate of the WWTP was quite low (i.e. 125 ML/day) in comparison to the former (triplicate) concentrations which occurred during a greater flow rate of 178 ML/day (i.e. during the spring melt of the municipal street run-off. Thus, it is possible that dilution is a major factor in the levels of pharmaceuticals analysed; however, excretion from the population would be dynamic as well. Further characterisation of the potential correlation of drug levels to flow rate would prove useful in this system.

It is likely that both conjugates studied here are present primarily in the aqueous phase. Given that PRO is predominantly positively charged in the pH range of most environmental waters (6 to 9) due to its pKa of 9.5 [9] it should be attracted to the negatively charged sites in the NOM or to clay minerals with negative charges due to isomorphic substitution in the mineral structure. The sulfonamide nitrogen in SMX could potentially also undergo other complexations with negatively charged matter via cation bridging using positively-charged multivalent ions as the intermediary in natural environments [36-38]. Thus, depending on the characteristics of the NOM, dissolved or suspended solids, sorption of the parent compounds can vary. PRO-Sul and SMX-Glc, however, should be singly anionic in the environmentally relevant pH range (i.e. 6-9). Due to their highly polar nature, unless deconjugation or some other transformation occurs, these compounds would predominantly exist in the aqueous phase [39] given their very great solubilities and very low $\log K_{OW}$ values (Table 3.1).

3.4 Conclusions

Propranolol, sulfamethoxazole, and their respective major conjugates 4-OH-propranolol sulfate and sulfamethoxazole-β-glucuronide were simultaneously extracted through HLB, MAX, and WAX cartridges from waters. WAX was determined as the sorbent of choice for extracting primary and secondary clarification wastewaters. Primary and secondary filtrates from the North End wastewater treatment plant of Winnipeg respectively showed a significant increase of PRO, decrease of PRO-Sul, increase of SMX, and decrease of SMX-Glc concentrations, indicating that there was some sort of removal from the aqueous phase occurring at some point between these stages of treatment that affected SMX and both conjugates, but that PRO demonstrated persistence. To the best of our knowledge, this is the first study that simultaneously separated and quantified two different classes of parent compounds and two different kinds of human transformation product conjugates (glucuronide and sulfate) from a major urban wastewater treatment plant.

The innovative potential of this study is that multiple classes of pharmaceutical (as represented by PRO and SMX) and multiple types of conjugate (as represented by PRO-Sul and SMX-Glc) can be simultaneously collected, extracted, eluted, separated, and quantified using one chromatographic column, with one set of binary solvents on a low resolution mass spectrometer. The ubiquity of these instruments notwithstanding, the convenience of a low resolution instrument being able to quantify these compounds simultaneously allows high resolution instruments to be available for metabolite/ unknown compound investigation.
The strengths of this study are the isolation of loss of each conjugate at each sub-stage of the SPE process, and identifying the variation in recovery of these analytes across a spectrum of

matrices (i.e. deionised water to wastewater). We are confident that this study can be used as a framework to extract and quantify both glucuronide and sulfate conjugates of both acidic and basic pharmaceuticals in the aquatic environment as represented by SMX-Glc and PRO-Sul, and potentially be extrapolated to biological matrices as well (e.g. plasma, urine).

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Chapter 4

Measurement of Thyroxine and Its Glucuronide in Municipal Wastewater and Solids Using Weak Anion Exchange Solid Phase Extraction and Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry

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The author of this dissertation designed the experiment along with his supervisor, performed the experiments, interpreted the data, and wrote the manuscript.

4. Abstract

A solids extraction method, using sonication in combination with weak anion exchange solid phase extraction, was created to extract thyroxine (T4) and thyroxine-*O*-β-D-glucuronide (T4-Glc) simultaneously from wastewaters and sludges, and to quantify these compounds via reversed-phase ultra-high performance liquid chromatography-tandem mass spectrometry. The method limits of quantification were all in the low ng/g (dry weight solids) range for both T4 and T4-Glc: 2.13 and 2.63 ng/g respectively in primary wastewater, 4.3 and 28.3 ng/g for primary suspended solids, for 1.1 and 3.7 ng/g for return activated sludge. Precision for measurements of T4 and T4-Glc were 2.6 and 6.5 % (intraday) and 9.6 and 5.7% (interday) respectively, while linearity was 0.9967 and 0.9943 respectively. Overall recoveries for T4 and T4-Glc in primary suspended solids were 94% and 95%, and 86 and 101% in primary wastewater, respectively. Extraction efficiency tests using primary sludge determined that one methanol aliquot was sufficient during the extraction process as opposed to 2 or 3 aliquots. Mass loadings at the North Main Wastewater Treatment Plant in Winnipeg, Canada showed 316%, 714%, and 714% greater T4-Glc than T4 associated with the suspended solids of the primary, secondary, and final effluent respectively, yet 765% more T4 than T4-Glc associated with the solids of the mixed liquor. Moreover, 26% of T4 and 49% of T4-Glc were associated with the suspended solids during the treatment process. This method demonstrates the need to assess accurately both metabolite conjugates of contaminants of emerging concern, as well as the sorbed levels of particle-reactive analytes such as T4 in the aquatic environment.

4.1. Introduction

Thyroxine ((*S*)-2-amino-3-[4-(4-hydroxy-3,5diiodophenoxy)-3,5-diiodophenyl]propanoic acid); T4) is an essential hormonal regulator of several vertebrate metabolic processes. The follicular cells of the thyroid gland produce the total exclusive amount of T4 and approximately 20% of triiodothyronine (T3) in the human body, with the remainder of T3 generated by peripheral tissues (e.g. brain, placenta, muscle, pituitary) [1, 2]. T4 is converted into T3 most likely through a mono-deiodination process via deiodinases [3-5]. In normal euthyroid individuals, approximately 8 and 90 µg/ day respectively of T3 and T4 are secreted [6, 7], with approximately 42% of secreted T4 converted to T3, suggesting that mono-deiodination is an obligatory step in peripheral metabolism of T4 [3].

In developed countries, approximately 10-15% of the total population has clinical or sub-clinical hypothyroidism [1]. The aggregate sum of the various brands of thyroxine are the third most prescribed pharmaceutical in Canada, with the vast majority of this sum to women aged 6-79 between 2007-2011 at 1.08 million prescriptions. This represented 9.8% of the total scripts during that time frame [8]; moreover, more than 400 different formulations are distributed worldwide [2]. Thus, not only is endogenous T4 metabolised and secreted, but also non-trivial amounts of peripheral T4 derived from prescription medicines such as Synthroid® and Levothyroxine® [2] contributing to T4 in the environment due to incomplete removal in wastewater treatment [9].Thyroxine-binding globulin (TBG) is a high-affinity, low concentration binding protein that binds approximately 80% of T3 and 75% of T4; the remainder are associated with other lower-affinity proteins that circulate in the human body [1]. Only 0.02% T4 and 0.04% T3 are considered bioavailable in circulation [1]. Thus, it is likely that T4 would be excreted in the sorbed form to proteins found in the feces.

T4-Glc is a conjugate of T4 and glucuronic acid, and is known to be a major metabolite to inactivate and clear T4 from the body [10, 11]. It is important to note that bacteria in the lower intestine (jejunum and ileum) possess glucuronidases, which results in very little T4-Glc being excreted via the feces [12]. In addition to the intestine, the kidney is also a major site of T4-Glc deconjugation in rats [12]. Thus, T4-Glc potentially found in a wastewater environment would most likely be due to the sorption of T4-Glc to suspended particulate matter from the aqueous phase; and an analytical method needs to be developed that can account for this sorbed fraction of analyte.

The potential environmental concern for thyroid hormones being released into receiving waters is two-fold. First, as aforementioned T4 and the associated metabolites have the potential to elicit physiological effects on different tissues, and elicit biological effects involved in cell signaling [13, 14]. Second, given that T4 and T4-Glc bind to proteins within the human body for transport and storage, these compounds have the potential to be recalcitrant to hydrolysis, photolysis [13], or biotic transformation due to the lack of availability in the sorbed state. Levels of T4-Glc are also important to gauge, as biota (e.g. mammals and teleosts) can deconjugate and de-iodinate T4-Glc to active forms and elicit these aforementioned effects [15]. If much of the environmental load of T4 or T4-Glc is associated with particulate matter, then this proportion of the total inventory has the potential to persist environmentally, especially farther downstream from the WWTP where there is lesser potential for degradation or deconjugation via coliforms typically found throughout the wastewater treatment process [16].

While T4 and its metabolites have been analysed numerous times from biological tissues and standards [18,22-25], there is only one published report of T4 in wastewater [17]. As well, the only studies found that quantified T4-Glc was via analysing various radioisotopes [11, 12]. To date, no

studies have measured T4-Glc either in environmental waters or wastewater. Both provide analytical challenges, given likely low concentrations (ng/L and ng/g solids range) and potential matrix effects from the presence of copious amounts of organic matter.

This study highlights the importance of extracting both the parent hormone T4 and its metabolite conjugate T4-Glc using a single solid phase extraction (SPE) sorbent, and quantifying in a single chromatographic run, using identical mass spectrometric parameters. This is valuable because a single sorbent and single column using identical solvents reduces preparatory and analytical time. Additionally, this reduces costs on consumable materials for additional procedures, thus making this procedure universally cost-effective and yields the potential for higher throughput of quantifying both the parent and conjugate simultaneously. It was important to build upon the framework we previously developed [18] that utilised the weak anion exchange (WAX) SPE protocol for extracting parent and conjugate pharmaceuticals. It was originally postulated that the anionic characteristics of transformation products such as conjugates (e.g. glucuronides, sulfates) would have predisposed these compounds to be found in greater proportions in the aqueous phase. However, compounds that associate with proteinaceous materials (i.e. T4 and T4-Glc) for biological storage and transport were hypothesised to exist at non-trivial levels in the particulate phases within the wastewater treatment regimen due to moderate $\log K_{OW}$ values. Thus, a solids extraction process that was compatible with the previous framework was essential to develop before comprehensive quantification of T4 and T4-Glc occurred.

4.2. Materials and Methods

4.2.1. Chemicals and consumables

Methanol, formic acid, ammonium hydroxide (28.9% v/v), and isopropanol (for sterilization) were obtained from Fisher Scientific (Fair Lawn, NJ, USA), while acetonitrile was purchased from Fisher and EMD Millipore; all organic solvents were HPLC-grade. Ultrapure Milli-Q (18 MΩ-cm) water was produced from a Synergy™ Milli-Q purification system from Millipore (Billerica, MA, USA). Nitrocellulose filter paper (0.45 µm) was obtained from Merck (Ireland), and 13 mm, 0.22 µm white PTFE luer lock inlet syringe filters was purchased from Restek (Bellefonte, PA, USA). Syringe filters were attached to an Agilent 1.0 mL glass syringe (Australia). All WAX solid phase extraction cartridges were Oasis 3 cc, 60 mg from Waters Corporation (Milford, MA, USA), Nalgene® 250 mL white HDPE bottles were purchased from Thermo Fisher (Rockwood, Tennessee, USA). Centrifuge bottles (50 mL) were purchased from VWR (Mississauga, ON, Canada). Glassware was pre-cleaned by ashing at 450°C for 1 hr to destroy organic materials unless otherwise indicated. PEEK tubing (Fisher Scientific, Toronto, ON, Canada) was used in the syphoning of environmental matrices through SPE cartridges.

4.2.2. Chemical Standards

Standards of T4 (chemical purity 94.16%) and T4-O-β-D-Glc (chemical purity 98%); and matching isotopically-labeled standards T4-$^{13}C_6$ (chemical purity 98%, isotope purity 98.8%) and T4-$^{13}C_6$-O-β-D-Glc (chemical purity 96%, isotope purity 98.6%) (Toronto Research Chemicals, Toronto, ON) were obtained as neat powders (Table A2.1, Figure 4.1). Methanolic stock solutions (40 mg/L) were made and stored at -20^{0}C. Calibration curve standard solutions (0.1, 0.5, 1, 5, 10, 50, 100, 500, 750 µg/L) for quantitative assessments were prepared from stock solutions in 50/50 (v/v) Milli-Q water:methanol and also stored at -20^{0}C.

4.2.3. Ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) methods

Chromatography was performed with a Agilent 1200 UHPLC, with separation using a Waters Acquity HSS T3 C_{18} column (2.1 mm × 50 mm, 1.8 μm dp), coupled to a Waters Acquity HSS T3 C_{18} guard column (2.1 mm × 5 mm) at 42^0C at 0.4 mL/min. Injection volumes were 2 μL during optimisation and 10 μL during analysis. Precursor and product ion fragments, collision energy, abundance, and fragmentation energy for both parent and conjugate compounds were determined using Agilent's Mass Hunter Optimizer software. Optimizer results were derived from single injections of isolated analyte standards measured in positive mode ESI. Mobile phase A1 was 0.05% formic acid (FA) in Milli-Q water, B1 was acetonitrile with 0.05% formic acid, A2 was 95/5 (v/v) Milli-Q water:methanol, and B2 was 100% acetonitrile. A binary gradient elution using both channels A1 and B1 was performed as follows: 0- 3.00 min linear ramp from 5% B1 to 70% B1, 3.01- 5.00 hold at 70% B1, then re-equilibrated from 5.01- 12.00 min at 5% B1. Upon completion of all analytical runs the columns were flushed with a binary combination of solvents of A2 and B2 for 20 min, which consisted of 10% B2, then 25 min of 95% B2 to eliminate formic acid for column storage.

Table 4.1 Compound-specific mass spectrometric parameters used for MS/MS analysis: retention time, fragmentation voltage (frag), collision energy (CE), and collision voltage (CV) (all in V) and mass transitions of precursor to product ions. Dwell time was 200 ms for all optimisation; Q is the quantifier and q is the qualifier. For thyroxine-^{13}C, only one transition was produced.

Compound	Retention Time (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Q or q	Frag (V)	CE (eV)
Thyroxine	2.8	777.7	731.7	Q	147	24
			604.8	q	147	44
Thyroxine-^{13}C		783.7	737.7	Q	138	28
Thyroxine-*O*-B-D-glucuronide	2.2	953.7	777.7	Q	141	16
			731.7	q	141	40
Thyroxine-^{13}C-*O*-B-D-glucuronide		959.8	783.7	Q	144	16
			85.0	q	144	60

Qualitative assessment and quantification was performed through multiple reaction monitoring (MRM) on an Agilent 6410 triple quadrupole mass spectrometer in positive electrospray ionisation mode (ESI+), a capillary voltage of 4000 V, and a source temperature of 300°C. Nitrogen was used for desolvation and drying gas at 11 L/min, and for nebulization at 15 psi. Ultrapure nitrogen was used as collision gas at a flow of 16.8 L/min. The MS1 and MS2 heaters were set at 100°C. Compound-specific mass spectrometric parameters and ion fragments used are found in Table 4.1. Criteria for positive identification and quantification was on the most abundant $[M+H]^+$ product ion fragment (quantifier) and confirmation of analyte using the second most abundant $[M+H]^+$ ion fragment (qualifier) (Table 4.1).

4.2.4. Extraction and Pre-concentration of Samples

4.2.4.1. Sample Collection

Wastewater samples were obtained from the North Main Waste Water Treatment plant of Winnipeg, Canada. The facility treats sewage of residential, commercial, industrial, and hospital waste, in combination with municipal street run-off, suggesting that its wastewaters would contain measurable quantities of T4 and T4-Glc [17]. As the treatment process flow rate is greatly dependent on precipitation, all samples were obtained using 24-h composite active samplers. Primary sludge, return activated sludge (RAS), and waste activated sludge (WAS) were collected via 24 h composite samplers located in each channel of WWTP piping, and stored in 20L Nalgene® HDPE bottles until retrieval. All mixed liquor and waste activated sludge samples were composite samples of three channels within the WWTP to reduce in-pipe variation of the sample matrix, and subsampled for our purposes. Ten mL of shaken homogenous aliquots of these samples were then collected in one 50 mL centrifuge tube each. Tubes were capped and refrigerated upright until sample retrieval within one hour.

Suspended solids were sampled by collecting 250 mL of each wastewater type (primary, secondary, mixed liquor, and final effluent) into 250 mL Nalgene® HDPE bottles. Upon retrieval, 200 mL of each such sample was divided into 6 VWR centrifuge tubes (approximately 34 mL each) and centrifuged at 2200*g* for 10 min. The pellets were gradually combined using several 2 mL aliquots of identical wastewater into one tube, centrifuged, and decanted to waste. All samples were transported in ice packs in the dark to the laboratory, generally within 30 min, to minimise the potential for photolytic or other transformation.

4.2.4.2. Sludge Extraction

Upon sample retrieval, sludges were centrifuged into pellets as described in 4.2.4.1. A 3 mL aliquot of methanol was added, followed by vortexing for 30 sec, sonication at 37^{0}C for 10 mins, pulsing for 1 sec on the vortex, centrifuging again at 2200*g* for 10 min, and decanting. This procedure was done in triplicate. Each set of 3 centrifuged methanol supernatants (i.e. 3×3 ml) were collectively decanted into 250 mL ashed, pre-conditioned (with deionised water) amber bottles containing 200 mL of deionised water. Thus, the maximum amount of methanol per bottle was approximately 5% (v/v). Once compounds were extracted, they were filtered through 0.45 µm nitrocellulose filters into ashed and pre-conditioned amber bottles to reduce the likelihood of microbial transformation or degradation during SPE, in addition to preventing the SPE cartridges from clogging. As reported previously [18], coliforms that contain a great deal of glucuronidases are typically between 0.6-1.2 µm in diameter and 2-3 µm in length [19], and should be largely isolated from the matrix through this method. Subsequent to extraction, all samples were subjected to WAX SPE protocols outlined in 4.2.4.3.

4.2.4.3. Off-line Solid Phase Extraction

As per a previous SPE procedure [18], 200 mL aliquots of desired matrices were put in ashed amber jars and spiked with unlabelled (where applicable) and internal standard mixture solutions, to extract and recover an environmentally relevant concentration of 0.1 µg/L. All WAX SPE cartridges were preconditioned with a series of 3 mL aliquots of 0.2% NH_4OH in deionised water to reduce acidic interferences, then methanol, 0.2% FA in Milli-Q to reduce basic interferences, then deionised water. Samples were loaded through the WAX cartridge,

washed with 0.2% FA, eluted with methanol (3 mL) then 0.2% NH_4OH in methanol (3 mL), nitrogen evaporated at 42^0C, reconstituted in 1 mL 50/50 (v/v) Milli-Q: methanol, vortexed then syringe filtered, for a concentrated total of 20 µg/L. All samples were refrigerated at 4°C until instrumental analysis.

4.2.4.4. Solvent Extraction Efficiency Assessment

The extraction protocol above was applied to determine how many aliquots of methanol were necessary to extract endogenous T4 and T4-Glc effectively from primary sludge. For this experiment, sludge from February 23, 2017 was collected and treated as in 4.2.4.1-4.2.4.3 above, and nine ashed 250 mL amber jars were used to test 1, 2, or 3- 3 mL aliquots of methanol, in triplicate each. An additional separate experiment was conducted to determine the effect of using 0.2% ammonium hydroxide in methanol in extracting the hormones. This was to see if there was a benefit to exceeding the pKa of T4 and T4-Glc and reducing the proportion of the zwitterion positive charge, and increasing the proportion of the anion and double anion, respectively, thereby maximising the WAX mechanism during extraction.

4.2.4.5. Accuracy Assessment: Spike and Recoveries for SPE

An unlabelled standard mixture (20 µL of 1000 µg/L per analyte) and $^{13}C_6$-labelled internal standard mixture (50 µL of 1000 µg/L per analyte) were used for spiking to quantify recoveries, and to assess the matrix effects on the quantification of the analytes. The unlabelled mix was spiked at the beginning of extractions in the amber jars containing 100 mL of: first, deionised water; second, primary suspended solids, then lastly, primary wastewater. This would

then yield a back-calculated unlabelled concentration of 0.2 µg/L in aqueous samples. The target reconstituted concentration in 1 mL solvent in LC vial was therefore 20 µg/L. Another experiment was conducted using the same procedure by spiking suspended solids centrifuged from primary wastewaters (in triplicate) on December 13th, 2016 to ascertain systematically the recovery of T4 and T4-Glc from solids. Both experiments were subjected to the analytical procedure outlined in 4.2.4.1- 4.2.4.3.

4.2.4.6. Linearity, Precision, MLD, and MLQ

Calibration curves were constructed using unlabelled standard samples, as noted in 4.2.2, spanning the predicted environmentally relevant range and spiked with internal standard for quantitative assessments. Curves were best fitted using a least squares linear regression model, weighted by the inverse of the analyte concentration (1/x). Linearity (mean) was determined from regression of the nine- point calibration curves' unlabelled standard responses in relation to the analogous internal standard over 5 separate wastewater extraction runs on different days. Calibration curve accuracy (mean) was calculated by quantifying the mass of unlabelled parent and conjugate standard mixture spiked at each concentration level in 50/50 (v/v) Milli-Q water: methanol compared to the internal standard mixture response ratio over 4 separate days. The method limit of detection (MLD) and limit of quantification (MLQ) were defined as the concentration of the lowest quantified extracted T4 and T4-Glc from primary wastewater, primary suspended solids, and return activated sludge where known concentrations (<2 ng/ml) and *S/N* ratios were back-calculated as having *S/N* ratios of 3 and 10 respectively with the associated endogenous concentrations. Intraday and interday precision was calculated by spiking and extracting 3 separate (100 mL) aliquots of primary wastewater filtrate (as in 4.2.4.5) and

analysing each (total of 3) over a 24 h period (0, 12, 24 h), and over 3 separate days (0, 24, 48 h), respectively.

4.2.4.7. Method Application

Using the above methodology, unlabelled levels T4 and T4-Glc were extracted and quantified from primary, secondary, mixed liquor, and final effluent wastewaters, as well as suspended solids separated from these stages from the North Main WWTP on March 13th and March 15th, 2017. Aqueous concentrations were calculated by dividing concentrations in-vial by 200, solids concentrations were calculated by dividing the mass of analyte in-vial (ng) by the mass of solids (mg solids dry weight) from which the analyte originated.

4.2.4.8. Statistical Analysis

GraphPad Prism v.5 (GraphPad Software, La Jolla, CA, USA) was used to conduct all analyses during method development. One-way ANOVA was conducted for all components of method development, and Tukey's post-hoc test was used for comparison of multiple means among stages of SPE. Dunnett's post-hoc test was conducted for comparing the target expected value of IS spikes to actual recoveries.

4.3. Results and Discussion

4.3.1. Liquid Chromatography-Tandem Mass Spectrometry

Product ion fragments of T4 and T4-Glc were non-ionisable in negative mode. However, we wanted to have a method that required only one binary solvent compatible with other pharmaceuticals and their conjugates [18], for which positive mode was desirable. Dynamic MRM used in this study quantified analytes only within a short (ca. 1 min) time frame, thereby increasing signal-to-noise ratios and reducing matrix interferences. The reason for using dynamic MRM was to be able to adapt this working method for simultaneously analysing T4 and T4-Glc in conjunction with numerous other pharmaceutical residues. T4 was baseline-separated from T4-Glc (Figure 4.1) with retention times of 2.2 and 2.8 min respectively (Figure 4.1). The elution gradient used for T4 and T4-Glc necessitated modification of our previous framework [18] due to the greater log K_{OW} of thyroxine compounds than our previously analysed compounds (sulfamethoxazole and propranolol). It was also apparent that a longer re-equilibration time (7 to 12 mins, up from 3 to 5.5 min) [18] at 5% B1) was necessary to ensure that more polar and earlier-eluting matrix interferences were washed off the analytical column prior to the next injection. For multi-residue analysis, these steps can be readily incorporated for measurement of other analytes.

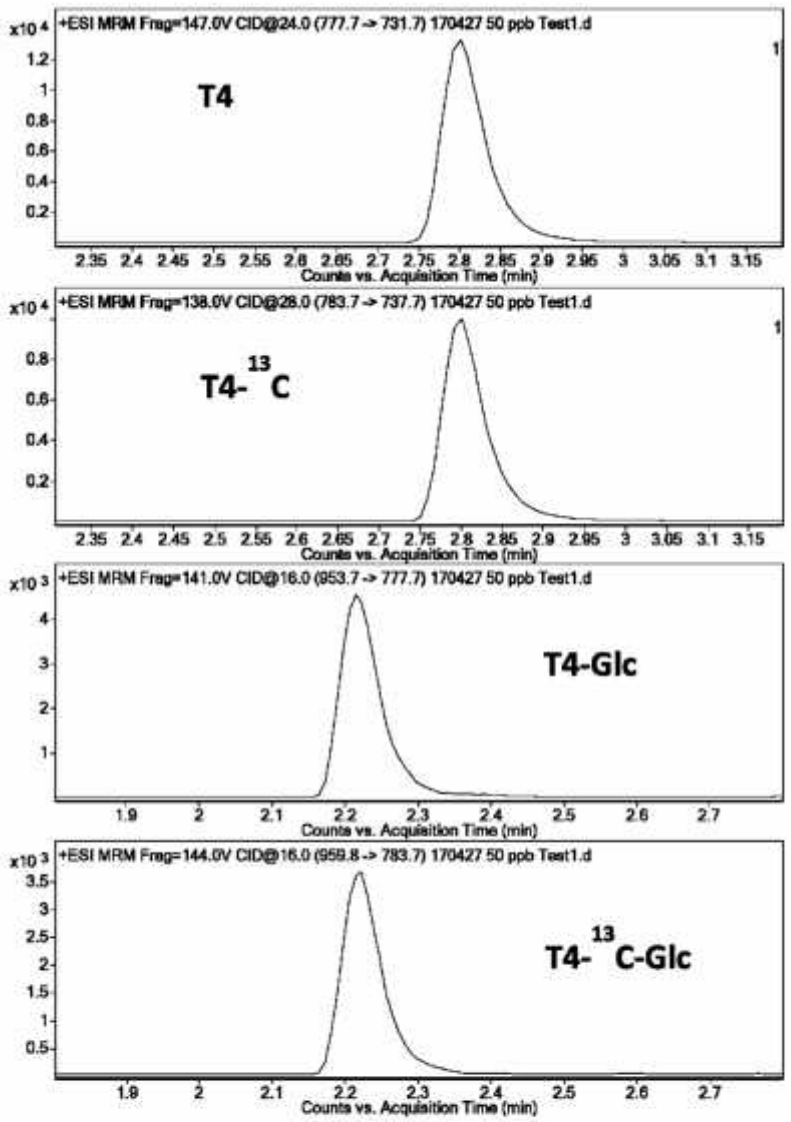

Figure 4.1 Chromatograms, extracted from dynamic multiple-reaction monitoring, of a 50 μg/L standard mixture of unlabelled and analogous $^{13}C_6$ isotopic compounds of T4 and T4-Glc. The parent compound T4, and conjugate T4-Glc are zwitterions under environmentally relevant pH conditions and during laboratory analysis.

4.3.2. Method Limits of Detection, Method Limits of Quantification, Precision, and Cal Curve Accuracy

The MLQ/ MLD were all calculated in in the low ng/L and ng/g solids dry weight range for both compounds in all three matrices (Table 4.2). The chemical purity of the isotopically-labeled standards (96%) did not seem to affect the MLD and MLQ due to the relative accuracy of the spike and recovery studies (>95%) and linearity of calibration curves (>0.99) listed below. Precision for T4 and T4-Glc (Table 4.2) was 2.6 and 6.5% (intraday) and 9.6 and 5.7% (interday). Linearity for T4 and T4-Glc was 0.9967 and 0.9943 respectively across 4.75 orders of magnitude (0.1- 750 µg/L) (Table 4.2). Calibration curve mean accuracy of both analytes in the 0.1, 0.5, 1, 5, 10, 50, 100, 500, 750 µg/L calibration curve standard mixtures were 139.1, 89.6, 85.2, 63.7, 80.0, 88.3, 81.1, 100.1, and 132.3 % respectively. The greater variation in the higher magnitude calibration curve value is due to the 1/x weighting of the linear regression, given that T4 and T4-Glc were determined to be found at approximately the 1 µg/L level (in LC vial). Medium-term stability and freeze-thaw stability of THY-Glc was determined by monitoring the peak areas of both unlabelled and internal standard quantifier ions in the 100 ng/mL calibration curve mixture which was stored at -20^{0}C when not in use. Over the duration of 5 weeks including 8 freeze-thaw cycles, the unlabelled and internal standard areas varied by only 17% and 6% respectively, with no trend of progressive loss. Thus, over the duration of this study the glucuronide can be considered to be stable.

Table 4.2 Method limits of quantification (MLQ), method limits of detection (MLD) for primary wastewater filtrate (WW) (ng/L), primary suspended solids (SS) (ng/g solids dry weight), return activated sludge (RAS) (ng/g solids dry weight), and linearity (mean). Intraday precision and interday precision (% relative standard deviation- RSD) were calculated using primary wastewater.

Compound	MLQ/ MLD			Intraday Precision (%RSD)	Interday Precision (%RSD)	Linearity (R^2)
	WW	SS	RAS			
T4	2.13/ 0.64	4.3/ 1.3	1.1/ 0.35	2.6	9.6	0.9967
T4-Glc	2.63/ 0.79	28.3/ 8.5	3.7/ 1.1	6.5	5.7	0.9943

4.3.3. Solid Phase Extraction

Offline solid phase extraction using Waters™ WAX cartridges was the most effective SPE option for isolating our analytes from the surrounding environmental matrices [18]. Various mechanisms beyond hydrophobicity can be used to separate ionic compounds from matrices [20-22]. WAX sorbent is a mixed mode, hydrophilic-lipophilic balance macroporous copolymer poly(divinylbenzene-co-*N*-vinylpyrrolidone) backbone with an acid-base nitrogenous group (pK_a~ 6). This additional functional group is sufficient for sorbing acids such as sulfates and glucuronides, typically produced by the human body to make these chemicals more water-soluble for excretion. In step with our previous framework, a sequential dual elution of T4 and T4-Glc was necessary once they were loaded onto the WAX sorbent to reduce matrix suppression. This step involved separate elutions using 3 mL each of methanol, then 0.2% ammonium hydroxide in methanol, to permit anion exchange with nitrogenous moieties of the WAX sorbent. Because aqueous 0.2% ammonium hydroxide has a pH of approximately 10.0

and the WAX has a pKa of approximately 6, the base will promote the release of the anionic moieties of the T4 and T4-Glc and their dissolution in the eluent. Wastewater contains urine, mainly comprised of weak acids and bases that are only poorly retained on the weak exchange resins and would thus be separated from our analytes by WAX. Moreover, T4 and T4-Glc have moderate log K_{OW} values of 4.12 and 2.65 respectively (ECOSAR via EPISuite V1.1) implying that hydrophobic sorption is most likely a dominant mechanism of extraction and retention, especially the parent compound [18]. In fact, T4 was always eluted within the methanol fraction, whereas T4-Glc was always eluted in the ammonium hydroxide fraction. This observation is similar to what we found previously with respect to sulfamethoxazole and its associated glucuronide [18].

4.3.4. T4 and T4-Glc Spike and Recoveries (Accuracy) in Deionised Water, Wastewater Filtrate and Suspended Solids

Using deionised water, the overall WAX sorbent showed a significant difference in recovery from the reconstituted target of 20 µg/L in solvent (Table 4.3) of T4 (114%; $P<0.001$) but not T4-Glc (90%; ns). Recoveries of T4 in primary wastewater filtrate (Table 4.3) showed a significant difference from the target concentration of 20 µg/L (86%; $P<0.001$) and no significant difference of T4-Glc (101%; ns). Overall, the primary suspended solids (Table 4.3) showed adequate recoveries with no significant difference ($P=0.7097$) of 94% and 95% for both T4 and T4-Glc respectively. As can be seen by using isotope dilution throughout quantification, regardless of the difference in signal (abundance area) when analysing various matrices, spiked internal standard and unlabelled analyte were proportionally augmented. Thus, quantification yielded good *S/N* ratios and values greater than the MLQ regardless of matrix effects.

Table 4.3 In-vial recoveries (accuracy) of thyroxine (T4) and thyroxine-*O*-*β*-*D*-glucuronide (T4-Glc) in various matrices (n=3): deionised water (DW), primary suspended solids (SS), and primary wastewater filtrate (WW). All spikes were 20 ng/mL, and recoveries are mean values with % relative standard deviation (%RSD) in parentheses, and percent recovery (%).

Compound	Recovery (ng/ml)					
	DW		SS		WW	
	Absolute (ng/mL)	Percent	Absolute (ng/mL)	Percent	Absolute (ng/mL)	Percent
T4	22.8 (3.0)	114	18.9 (3.0)	94	17.1 (3.0)	86
T4-Glc	18.0 (0.1)	90	19.0 (12)	95	20.2 (8.0)	101

The primary objective was to determine if the suspended solids would sorb our analytes too strongly and prevent an acceptable level of recovery (Table 4.3). There have been several studies conducted on various other organic pollutants where soils had amounts of analyte dripped onto the solids and allowed to dry [23, 24]. Given that many pharmaceuticals are ionisable contaminants, typical K_{OC}-type sorption to natural organic matter cannot automatically be assumed to be the dominant mechanism. It is possible that there could be limited sorption sites on the solids that could be saturated by the spiked standard compound mixture, especially with respect to electrostatic interactions. Thus, it is difficult to assign an appropriate mass of T4 or T4-Glc that could be considered realistic in being sorbed to the collected suspended solids or sludge, thereby overestimating the recoveries, a phenomenon observed elsewhere [24]. Nevertheless, recoveries for T4 and T4-Glc provided sufficient mass of analyte for quantification, and were corrected through isotope dilution. Thus, it was determined that WAX sorbent cartridges were sufficient at retaining T4 through hydrophobic interactions, likely on the

macroporous copolymer poly(divinylbenzene-co-*N*-vinylpyrrolidone) backbone, as well as the anionic conjugate T4-Glc, likely through associations via the ionisable nitrogenous moieties in combination with the non-polar tendencies.

4.3.5. Solvent Extraction Efficiency

Initially we hypothesised that we could extract the sludge/solids using 0.2% ammonium hydroxide without deleteriously affecting the subsequent SPE process, given both the anionic nature of T4 and T4-Glc and that the total amount of methanol in 200 mL of deionised water was less than 5%. If ionic complexation played a dominant role in sorption of T4 and T4-Glc to organic matter in wastewater, then facilitating a more alkaline environment where the sorption sites within the organic matter could become anionic would lend itself to ion repulsion, and thus desorption of the analytes. Thus, the same procedure outlined in 4.2.4.2 for methanol aliquots was used except with alkaline methanol. Virtually no analyte retention at all was observed. In short, all individual extractions of primary sludge in this manner resulted in either no analytes detected qualitatively, or a few quantitative values that were considered below the MLD. This was most likely due to the resulting aqueous 0.2% ammonium hydroxide extract solution promoting the de-protonation of the WAX SPE nitrogenous ring moieties, thereby making anionic exchange very unlikely.

The primary sludge tested was 3.47% total solids (v/v), expressed in wet weight due to the recalcitrance of primary sludge to dry completely during solids analysis. It was concluded in 3.4 that there was no statistical difference in recoveries from the spiked target (20 ng) concentration in suspended solids which used identical SPE protocol as our previous study; therefore the method extraction efficiency was good. However, for completion sake, endogenous

masses of T4 and T4-Glc were extracted, as outlined in 2.4, to determine the number of methanol washes necessary. Thus, of the homogenous 10 mL sludge aliquots of identical origin (9 in total), 0.347 mL/each were total solids wet weight. The mean extracted concentrations for each triplicate sub-sampling (Figure A2.1) showed no statistical difference at 8.9, 9.3, and 9.7 µg/L (sludge solids wet weight) for T4 (P=0.5990); and 1.5, 1.8, and 0.6 µg/L (sludge solids wet weight) for T4-Glc (P=0.3441), demonstrating good inter-subsample precision. Moreover, because there were no differences observed in extracted concentrations of either compound with increasing aliquots of methanol (i.e. 1, 2, or 3), we concluded that it only one aliquot is necessary to extract available endogenous T4 and T4-Glc from sludge or suspended solids.

4.3.6. Method Application

The developed method was applied to a complete suite of wastewater and solids collected on March 13th and March 15th, 2017. Flow was 158 ML/day on March 13th, and the solids data can be found in Table A2.2. For T4, the primary, secondary, mixed liquor, and final effluent yielded concentrations of 0.0051, 0.0050, 0.0038 and 0.0050 µg/L in water respectively (Table 4.4); and these values are in agreement with other literature findings within an order of magnitude for both primary (0.064 µg/L) and final effluent (0.022 µg/L)[17].

Table 4.4 Method application and mass loadings

Treatment Stage	Concentration				Daily Mass Loading (g/Day)			
	Aqueous Phase (µg/L)		Suspended Solids (µg/g solids dry weight)		Aqueous Phase[1]		Suspended Solids[2]	
	T4	T4-Glc	T4	T4-Glc	T4	T4-Glc	T4	T4-Glc
Primary	0.0051	0.0070	0.021	0.054	0.81	0.86	0.38	1.2
Secondary	0.0050	0.021	0.076	0.29	0.79	3.3	0.14	1.0
Mixed Liquor	0.0038	0.021	0.055	0.0071	0.60	3.2	20.8	2.7
Final Effluent	0.0050	0.013	0.11	0.81	0.80	2.1	0.28	2.0

[1] Concentration × Flow Rate

[2] (Concentration × Flow Rate × Concentration of Solids)/ 1000

The differences could be attributed to the different SPE sorbents used (i.e. MAX and HLB vs WAX), the difference in wastewater processing (i.e. absence of sand filtration in the current study's WWTP), the differences in WWTP population served, and/or comparing their raw influent values to the current primary clarifier samples. These concentrations correspond to daily mass loadings of 0.81, 0.79, 0.60, and 0.80 g/day. For the respective suspended solids, there were 0.021, 0.076, 0.055, and 0.11 µg/g solids (dry weight) respectively, which corresponded to mass loadings of 0.38, 0.14, 20.8, and 0.28 g/day. For the primary sludge there was a concentration of 5.31 µg/L (total solids wet weight) based on 3.31 %solids. For the RAS and WAS concentrations of 0.0113 and 0.0471 µg/g solids (dry weight) respectively were measured.

For T4-Glc, the secondary and final effluent yielded concentrations of 0.021 and 0.013 µg/L in water respectively, which corresponded to daily mass loadings of 3.3 and 2.1 g/day. For the mixed liquor and final effluent suspended solids, there was 0.0071 and 0.81 µg/g solid (dry weight) respectively, which corresponded to mass loadings of 2.7 and 2.0 g/day. For the RAS a

concentration of 0.076 µg/g solids (dry weight) was calculated. T4-Glc concentrations were below the MLQ in the primary and secondary on March 13th, likely due to matrix effects. Thus, on March 15th these extractions were conducted once more and resulted in T4-Glc concentrations in the primary and mixed liquor of 0.007 and 0.021 µg/L in water respectively, resulting in mass loadings of 0.86 and 3.2 g/day. In the primary and secondary suspended solids there were 0.054 and 0.29 µg/g (dry weight), resulting in mass loadings of 1.2 and 1.0 g/day, respectively.

There appeared to be 411% and 260% more T4-Glc than T4 in the secondary and final effluent respectively (Table 4.4), which is in keeping with the notion that glucuronidation occurs primarily to make chemicals more water soluble. Moreover, there was 765% more T4 associated with the solids of the mixed liquor (20.8 g/day) than T4-Glc (2.7 g/day) indicating that a non-trivial amount of both T4 and T4-Glc is potentially removed by the treatment plant (and cycled within the plant via the mixed liquor) through settling of the secondary sludge. This appeared to be an important mechanism of removal for T4, the more hydrophobic of the two compounds. However, when the mass loadings of the four stages were compared (Table 4.4) there was 316%, 714, and 714% more T4-Glc than T4 associated with the suspended solids of the primary, secondary, and final effluent respectively. These results support the idea that T4 is excreted as T4-Glc through the urine. Once present in a WWTP, the affinity of T4-Glc for the significant amounts of organic matter in wastewater results in greater levels of T4-Glc in the solid phase rather than the aqueous. Moreover, this observation suggests that there was consistently more T4-Glc found throughout the WWTP and that a significant mass of both compounds are released into proximal receiving waters in both aqueous and particulate phases.

To further compound the total inventory of biologically-interactive thyroid hormones, there are also numerous other metabolites of T4 that are only recently becoming more

understood in terms of their mechanisms of action: de-aminated T4 and T3 yield tetrac and triac respectively, as well as diiodothyronines, decarboxylated thyronamines, and 3-iodothyroacetic acid, which all have a broad suite of seemingly shorter biological actions [7, 25]. Thus, T4 is a parent hormone by which a diverse cascade of physiological actions and cell signaling can result upon transformation biologically and potentially environmentally. Thus, characterising the amounts of T4 and T4-Glc is a good initial step in deciphering transformative potential. This paradigm is much like the one seen in animal feedlot operations where estrogenic hormones and their biologically-inactive conjugates are found distributed throughout the aqueous and solid phases [26]. Moreover, it has been demonstrated that conjugated forms of estrogens were more water soluble, and thus available for aerobic digestion within a WWTP. However, upon hydrolysis or microbial enzyme biotransformation, the free-form of the estrogens become available once again and can potentially elicit biological and physiological effects [27] much like thyroid hormones can [11, 12, 28]. The cumulative amounts of T4 and T4-Glc serve as a minimum starting point for total thyroidal inventory, given the numerous other metabolites potentially present within a wastewater system (e.g. T3, tetrac, etc.). However, the scope of this study was to simultaneously extract and quantify the metabolite conjugate in conjunction with the parent T4 to roughly represent the range of hydrophobicity for all thyroid compounds (i.e. T4 more hydrophobic to T4-Glc more hydrophilic).

4.4 Conclusions

In conclusion, an effective solids extraction method using sonication in combination with weak anion exchange solid phase extraction was created to quantify T4 and T4-Glc simultaneously from wastewater, suspended solids, and sludge from a major municipal

wastewater treatment plant. This method demonstrates the need to assess accurately the total inventory of particle-reactive anthropogenic contaminants within a wastewater system. Not only is there a necessity to quantify both the parent and metabolite conjugate of thyroxine, but also to quantify the masses of these compounds entering the ecosystem in both the aqueous and particulate phases.

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Chapter 5

Distribution and Fate of Pharmaceuticals and their Metabolite Conjugates in a Municipal Wastewater Treatment Plant

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The author of this dissertation designed these experiments along with his supervisor, performed the experiments, interpreted the data, and wrote the manuscript.

5. Abstract

Some pharmaceutical conjugates can be excreted into wastewaters at levels rivalling those of the parent compounds; however, little is known about this potential reservoir of pharmaceuticals to aquatic systems. We evaluated the occurrence and distribution of four different classes of pharmaceuticals and their metabolite conjugates in a wastewater treatment plant over four months. Aqueous and suspended solids fractions of primary, mixed liquor, secondary, and final effluent, along with return activated sludge, and waste activated sludge were assessed. The only conjugate not found in the final effluent was acetaminophen sulfate. Moreover, thyroxine and thyroxine glucuronide were the only compounds quantified in the suspended solids in the final effluent. Propranolol, propranolol sulfate, thyroxine, and thyroxine glucuronide all had no significant decreases in concentration going through the wastewater treatment process, from primary to final effluent. However, there were significant decreases observed for acetaminophen (99.8%), sulfamethoxazole (71%), *N*-acetyl sulfamethoxazole (59%), and sulfamethoxazole glucuronide (79%). The mean (±SEM) mass loadings in the aqueous fraction of the final effluent for each compound ranged from 0.84±0.2 g/d for thyroxine to 45.3±4.2 g/d for acetaminophen. At least as much conjugate was released into receiving waters, if not more: 1.6±0.2 g/d for thyroxine glucuronide to 18.5±4.5 g/d for sulfamethoxazole glucuronide, and 61.2±9.6 g/d for *N*-acetyl sulfamethoxazole. Additionally, the mean loading of thyroxine was 0.29±0.025 g/day and thyroxine glucuronide 1.8±0.59 g/day in the suspended solids. This equates to 26% of total thyroxine and 53% of total thyroxine glucuronide associated with suspended particulate matter that reaches receiving waters. This study reflects the importance of including phase II conjugates in assessing overall compound load of

pharmaceutical discharge from wastewaters, and also that substantial amounts of such contaminants are associated with wastewater solids when drugs are in the pg/L to µg/L range.

5.1. Introduction

Various transformation processes can occur within the body that change ingested pharmaceuticals into a more water-soluble form in preparation for excretion. Pharmaceuticals can be biotransformed, depending on pharmacokinetics and various epigenetic factors of individuals, by phase I endogenous monooxygenases and mixed function cytochrome P-450 enzymes into more hydrophilic excretory products. Subsequent to this, various enzymes (e.g. UDP-glucuronosyl transferase [1] and sulfotransferase [2]) can attach hydrophilic moieties such as glucuronide, sulfate, and acetyl groups [3-5] to phase I products to create phase II conjugates. Excreted drugs, both unchanged and biotransformed, enter wastewater treatment facilities, where the effects of various treatment processes could attenuate or augment their respective concentrations as well [6-10].

Until recently, environmental quantitation of drugs was typically concerned with their unchanged state (i.e., parent compound) and some various phase I metabolites. However, simultaneous quantitation of a more thorough picture of both parent and conjugated forms across multiple classes of drugs has generally not been conducted, with the noted exception of estrogens [11-14], androgens [15, 16], and recent work on lamotrigine and its glucuronide [17, 18]. It is possible that significant amounts of pharmaceutical conjugates, rivalling or even exceeding the parent compounds, are surviving wastewater treatment in both the aqueous and suspended solid phases and directly entering receiving waters [17-21]. Sorption to solids, both suspended particulate [22] and dissolved organic matter [21, 23, 24], could contribute to the environmental persistence of metabolite

conjugates. Moreover, chemical sorption to solids has been considered to be a major factor in the protection from various biotic and abiotic transformation mechanisms (e.g. photolysis, hydrolysis) [25, 26]. Although conjugates are presumed to be quite hydrophilic based on lesser $\log K_{OW}$ values, complexations and/or hydrophobic interactions can occur that necessitate quantitation of conjugates in the solid phase. Thus, it is important to quantify drugs and conjugates in the solid phase that could potentially desorb in the environment and elicit toxic effects on non-target organisms.

Toxicological effects on non-target organisms could arise either directly by exposure to the conjugate [27] or indirectly via deconjugation back into the parent drug by microbial enzymes (e.g. glucuronidases, sulfatases) derived from the microflora resident in the human gut and by those found within the wastewater treatment process [9, 10, 17]. The labile nature of these conjugates is mainly governed by the stereochemical positioning of the conjugated moiety in relation to the parent compound (e.g. aryl vs acyl) [28]. The persistence of parent drugs through the wastewater treatment process is compound-specific [25, 29-33]; and it is plausible that the persistence of certain conjugates will be as well [17, 19]. Thus, there is sufficient evidence that significant masses of labile, potentially toxic conjugates are surviving wastewater treatment and are not being considered in monitoring or risk assessment analyses; however, data is lacking.

The aim of this study was to use developed frameworks, aqueous [19] and solids [34], for the extraction and quantitation of drugs and their respective major conjugates, and determine their distribution within a major municipal WWTP. We hypothesise that significant compound-specific levels of conjugates would be present in the primary clarifier, and that microbial activity during wastewater treatment would change the concentrations [34] and relative proportions of both parent and conjugate compounds. We also provide an assessment of the mass loadings within

the final effluent, to estimate the amount reaching major surface waters receiving wastewater input (i.e., the Red River, Manitoba, Canada).

5.2. Materials and Methods

5.2.1. Sampling

The North Main WWTP in Winnipeg (Figure A3.1), Canada services approximately 500,000 people and treats combined wastewater from residential, commercial, industrial facilities, as well as the combined street runoff from approximately 2/3 of the municipality. Samples were taken from the primary clarifier, mixed liquor (between aerobic reactor and secondary clarification), secondary clarifier, and final effluent (after the tertiary UV exposure treatment). Both aqueous and solid samples were collected as 24-hour composite samples via active autosampler, providing a representation of what goes through the WWTP daily (Table A3.1). Aliquots were collected weekly by WWTP staff during daily protocols, stored in 250 mL Nalgene® bottles with no headspace, capped and refrigerated until collection (typically within an hour), and processed to completion immediately that day. Chromatographic analysis was done immediately after processing, so as to minimise any potential in-vial transformation.

5.2.2. Materials

Methanol, formic acid, ammonium hydroxide (28.9% v/v), and isopropanol (for sterilization) were obtained from Fisher Scientific (CITY, NJ, USA), while acetonitrile was purchased from Fisher and EMD Millipore). All organic solvents were HPLC-grade. Ultrapure Milli-Q (18 MΩ-cm) water was produced from a Synergy™ Milli-Q purification system from Millipore (Billerica, MA). Nitrocellulose filter paper (0.45 μm) was obtained from Merck

(Ireland), and 13 mm, 0.22 µm white PTFE luer lock inlet syringe filters was purchased from Restek (Bellefonte, PA, USA). Syringe filters were attached to an Agilent 1.0 mL glass syringe (Australia). All WAX solid phase extraction cartridges were Oasis 3 cc, 60 mg from Waters Corporation (Milford, MA), Nalgene® 250 mL white HDPE bottles were purchased from Thermo Fisher, Rockwood, Tennessee, USA. Centrifuge bottles (50 mL) were purchased from VWR, Mississauga, Ontario, Canada. Glassware was pre-cleaned by ashing at 450°C for 1 hr to destroy organic materials unless otherwise indicated. PEEK tubing (Fisher Scientific, Toronto, ON) was used in the syphoning of environmental matrices through SPE cartridges.

Standards of acetaminophen CAS# 103-90-2 (chemical purity 94.16%), acetaminophen sulfate CAS# 32113-41-0, propranolol CAS# 525-66-6, 4-OH-propranolol sulfate (propranolol sulfate) CAS# 57075-33-0, sulfamethoxazole CAS# 723-46-6, *N*4-acetylsulfamethoxazole (*N*-acetyl-sulfamethoxazole) CAS# 21312-10-7, sulfamethoxazole-β-glucuronide (sulfamethoxazole glucuronide) CAS# 14365-52-7 , thyroxine CAS# 51-48-9, and thyroxine-*O*-β-D-glucuronide (thyroxine glucuronide) CAS# 21462-56-6 (chemical purities all >98%); and matching isotopically-labeled standards acetaminophen-d_4 CAS# 64315-36-2, acetaminophen-d_3 sulfate CAS# 1188263-45-7, propranolol-d_7 CAS# 344298-99-3, 4-OH-propranolol-d_7 sulfate CAS# NA, sulfamethoxazole-d_4 CAS# 1020719-86-1, *N*4-acetylsulfamethoxazole-d_4 CAS# 1215530-54-3, sulfamethoxazole-d_4-β-glucuronide CAS# NA, thyroxine-$^{13}C_6$ CAS# 1217780-14-7, and thyroxine-$^{13}C_6$-*O*-β-D-glucuronide CAS# NA (all chemical purities >96%, and isotope purity >98%) (Toronto Research Chemicals, Toronto, ON) were obtained as neat powders, the structures and properties can be seen in Figure A3.1. Methanolic stock solutions were made and stored at -20^{0}C. Calibration curve standard solutions (0.1, 0.5, 1, 5, 10, 50, 100, 500, 750 µg/L)

for quantitative assessments were prepared from stock solutions in 50/50 (v/v) Milli-Q water: methanol and also stored at -20^{0}C.

5.2.3. Chromatographic Analysis

Chromatography was performed with an Agilent 1200 UHPLC, with separation using a Waters Acquity HSS T3 C_{18} column (2.1 mm × 50 mm, 1.8 µm dp), coupled to a Waters Acquity HSS T3 C_{18} guard column (2.1 mm × 5 mm) at 42^{0}C at 0.4 mL/min. Injection volumes were 2 µL during optimisation and 10 µL during analysis. Mobile phase A1 was 0.05% formic acid (FA) in Milli-Q water, B1 was acetonitrile with 0.05% formic acid, A2 was 95/5 (v/v) Milli-Q water:methanol, and B2 was 100% acetonitrile. Gradient elution was performed as follows: 0-3.00 min linear ramp from 5% B1 to 70% B1, 3.01- 5.00 hold at 70% B1, then re-equilibrated from 5.01- 12.00 min at 5% B1. Upon completion of all analytical runs the columns were flushed with 20 min of 10% B2, then 25 min of 95% B2 to eliminate formic acid residues for column storage.

Qualitative assessment and quantification was performed through multiple reaction monitoring (MRM) on an Agilent 6410 triple quadrupole mass spectrometer in positive electrospray ionisation mode (ESI+), a capillary voltage of 4000 V, and a source temperature of 300°C. Nitrogen was used for desolvation and drying gas at 11 L/min, and for nebulization at 15 psi. Ultrapure nitrogen was used as collision gas at a flow of 16.8 L/min. The MS1 and MS2 heaters were set at 100°C. Compound-specific mass spectrometric parameters and ion fragments used are found in Table A3.2, and MLD, MLQ, accuracy and precision values can be found in Table A3.3.

Dynamic multiple reaction monitoring was used during quantitation to minimise noise which was especially important in quantifying in the ng/L range. Criteria for positive identification and quantification was on the most abundant $[M + H]^+$ product ion fragment (quantifier) and confirmation of analyte using the second most abundant $[M + H]^+$ ion fragment (qualifier).

5.2.4. Statistical Analysis

SAS University Edition (Toronto, Ontario) was used for all statistical analysis. Two-way analysis of variance was conducted on all data to compare the means of different treatments within the WWTP (primary, mixed liquor, secondary, final effluent). Due to the variance heterogeneity of the residual distribution, various appropriate tests and post-hoc tests were used: Satterthwaite degrees of freedom estimator, Shapiro-Wilk post-hoc test to confirm log-normality ($W > 0.9$; $P < 0.05$), Tukey-Kramer post-hoc test to compare treatment means ($\alpha = 0.05$), and restricted maximum likelihood estimation (MLE) to account for values lesser than the limits of quantification. MLE can randomly impute values between 0 and the LOQ for each respective analyte thus preventing a skewing of the data to any particular value (i.e. setting non-detects to 0 or to the LOQ, or 0.5 times the LOQ, etc.) Univariate procedures were conducted for general statistics (quantiles, means, confidence intervals, and distribution and probability plot of residuals).

5.3. Results and Discussion

5.3.1. Aqueous Phase

Given the hydrophilicity (based on lesser $\log K_{OW}$) of the sulfate and glucuronide conjugates, we expected to find them in the water fractions throughout the treatment plant. Across all four classes of pharmaceuticals, three separate trends became evident. Acetaminophen compounds showed significant differences after the primary clarifier (>99.8% change in concentration). Propranolol and thyroxine compounds showed no significant difference throughout the WWTP process. Sulfamethoxazole compounds showed a moderate significant difference (59-79%) from primary to final effluent.

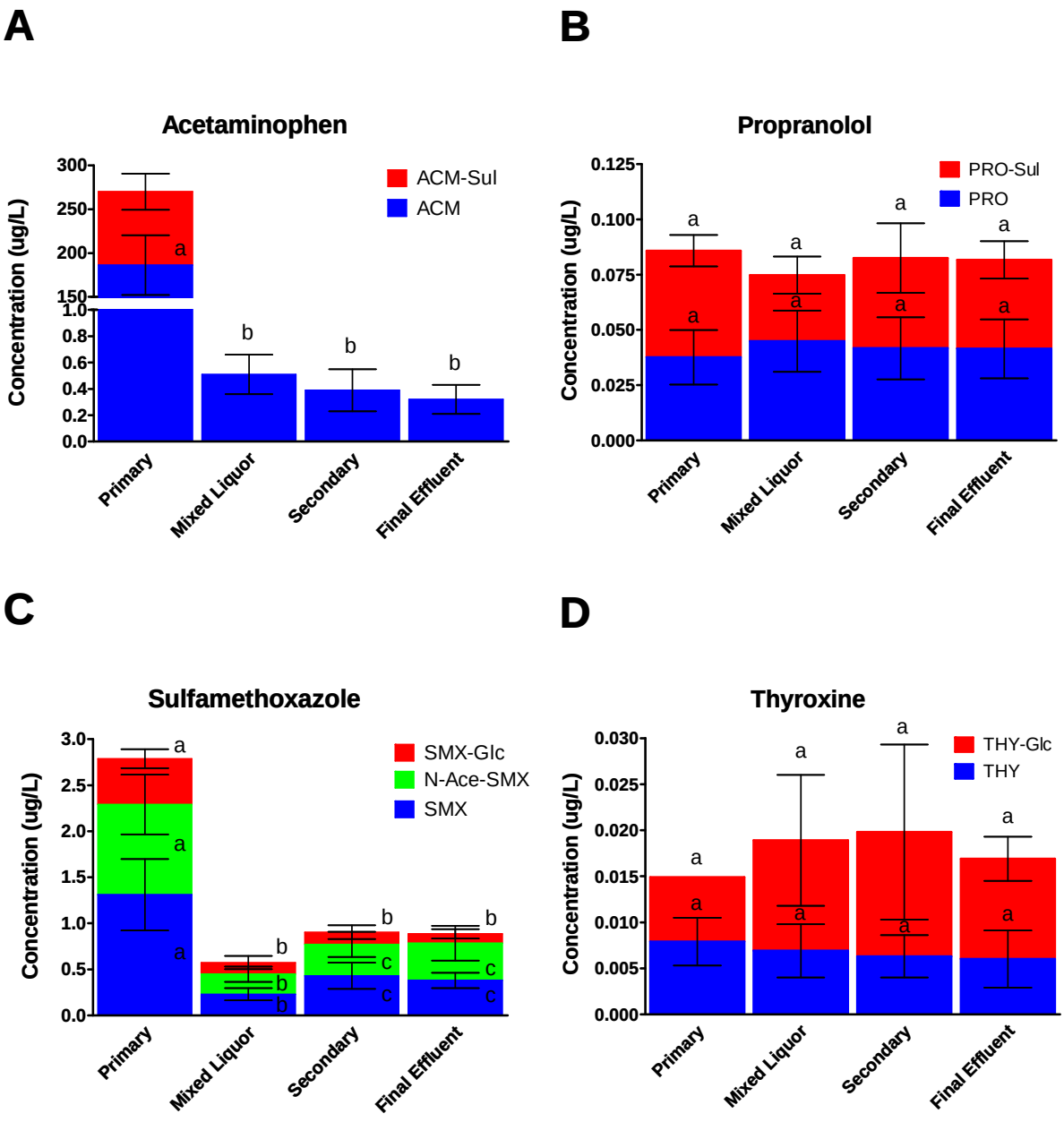

Figure 5.1: Concentrations (µg/L) for all parents and conjugates in the aqueous phase of the WWTP. Each sample is representative of a 24 h composite sample collected via autosampler. Bars represent the means, and error bars represent the standard error of the mean, and letters significantly if levels are different ($\alpha<0.05$) at each stage of treatment using Tukey-Kramer post-hoc tests.

5.3.1.1. Rapid concentration decreases of acetaminophen compounds

In the first trend, acetaminophen (Figure 5.1a) showed a dramatic significant difference from primary to mixed liquor ($P<0.0001$) from a mean of 186 to 0.51 µg/L. Subsequently there was no significant change between mixed liquor, secondary, and final effluent (means of 0.51, 0.39, and 0.32 µg/L respectively). Acetaminophen sulfate (Figure 5.1a) also showed significant difference from primary to mixed liquor ($P< 0.0001$), with means from 83.7 µg/L to <LOQ for all subsequent stages of treatment. These observations indicate both acetaminophen and acetaminophen sulfate are sufficiently labile such that aerobic biodegradation was most likely a major mechanism for removal of these chemicals [35, 36]. Overall, a 99.8% decrease in acetaminophen concentrations was observed from primary to final effluent. A similar magnitude was estimated for acetaminophen sulfate as well. acetaminophen is known to be a labile compound in a wastewater environment; [37] also reported 99% removal from crude to final effluent with concentrations of 138 and 1.5 µg/L respectively, in agreement with our findings.

5.3.1.2. No decrease in concentrations of propranolol and thyroxine compounds

In the second trend, both propranolol and propranolol sulfate (Figure 5.1b) showed no significant apparent difference throughout the treatment process. 4-Hydroxypropranolol is a major human metabolite for propranolol [19, 38], and as such it is most likely expected to yield phase II conjugates propranolol sulfate and 4-hydroxypropranolol glucuronide [5]. Levels of propranolol throughout the WWTP were 0.038, 0.045, 0.042, and 0.041 µg/L for the primary, mixed liquor, secondary, and final effluent respectively. Propranolol concentrations have been

reported within WWTPs to range from 0.001 to 0.5 µg/L [33, 39]. Our levels are also consistent within an order of magnitude and with similar resistance to removal with those of [37] who reported primary and final effluent concentrations of 0.12 µg/L in both stages of treatment. [40] reviewed 43 German WWTP occurrences of propranolol and reported inconsistency in levels found in both influent and effluent waters with concentrations varying from 0.010-0.17 µg/L, nevertheless our results are within that range. Moreover, those authors demonstrated inconsistency in removal of propranolol, ranging from 0% to 96% [40]. Levels of propranolol sulfate in our current study were 0.048, 0.030, 0.041, and 0.040 µg/L for the primary, mixed liquor, secondary, and final effluent respectively. Analysing for this single conjugate alone, the mass of total propranolol species throughout the WWTP and of that entering the receiving fluvial system is effectively doubled. This is a minimum estimate, and excludes metabolites and conjugates for which we had no authentic standards and did not quantify. Thus, conjugates can make up a significant reservoir of drugs in the wastewater environment, and by extension the aquatic environment to which wastewaters discharge. This observation is consistent with findings of estrogen conjugates at comparable proportions to their parent compounds as the ones in the present study, as well as that of lamotrigine glucuronide at levels up to 50 times that of the parent and surviving to reach ng/L levels in surface water [11, 13, 17, 18, 41, 42].

As with the propranolol compounds, thyroxine demonstrated a similar persistence throughout the WWTP in that no process seemed to result in a net augmentation or decrease in concentrations. As with propranolol, there is no evidence to suggest that deconjugation of thyroxine glucuronide [43] is occurring at a rate to increase levels of thyroxine within the residence time of the WWTP process. As such, there can be multiple transformation mechanisms simultaneously occurring on both parent and conjugate, as with propranolol above. Average

levels of thyroxine (Figure 5.1d) were 0.0079, 0.0069, 0.0063, and 0.0060 µg/L for the primary, mixed liquor, secondary, and final effluent respectively. Levels of thyroxine glucuronide (Figure 5.1d) were 0.007, 0.012, 0.014, and 0.011 µg/L for the primary, mixed liquor, secondary, and final effluent respectively. The total concentration of both thyroxine and thyroxine glucuronide together was approximately 3 times greater than that of thyroxine alone. thyroxine and thyroxine glucuronide were quantified at the same concentration range as propranolol, in the several ng/L range. Thus it appeared as though thyroxine and thyroxine glucuronide, much like propranolol and propranolol sulfate, were resistant to abiotic and biotic transformation processes.

There was no evidence to suggest that deconjugation of propranolol sulfate was occurring at a rate great enough to indicate a significant increase in propranolol throughout the treatment process. However, it is plausible that there are multiple mechanisms occurring simultaneously that may effectively cancel each other out (i.e. degradation of propranolol, while deconjugation of propranolol sulfate is occurring).

5.3.1.3. Moderate concentration decreases of sulfamethoxazole

In the third trend, there were moderate decreases in concentrations (59-79%) of sulfamethoxazole compounds from primary to final effluent. Levels of sulfamethoxazole (Figure 5.1c) were 1.31, 0.23, 0.43, and 0.38 µg/L for the primary, mixed liquor, secondary and final effluent respectively. [37] previously reported concentrations of sulfamethoxazole were 0.11 and 0.048 µg/L in the primary and final effluent respectively. These values are an order of magnitude in variation; however, of importance note is the similar removal of 58% in comparison to the 71% in this study. Levels of *N*-acetyl sulfamethoxazole (Figure 5.1c) were 0.98, 0.22, 0.34, and 0.40 µg/L for the primary, mixed liquor, secondary, and final effluent respectively. Levels of

sulfamethoxazole glucuronide (Figure 5.1c) were 0.50, 0.13, 0.13, 0.10 µg/L for the primary, mixed liquor, secondary, and final effluent respectively. sulfamethoxazole, *N*-acetyl sulfamethoxazole, and sulfamethoxazole glucuronide all reflect a significant difference from primary to mixed liquor ($P<0.0001$) indicating that aerobic biotransformation is most likely a major process. Subsequent to the mixed liquor, there is no significant change of sulfamethoxazole glucuronide. There is a slight significant increase of sulfamethoxazole ($P< 0.0001$), from 0.23 to 0.43 µg/L, and *N*-acetyl sulfamethoxazole ($P= 0.0419$) from 0.22 to 0.34 µg/L, from mixed liquor to secondary, then no further significant change. These slight increases cannot be directly correlated to the removal of sulfamethoxazole glucuronide because there was no observed decreases in concentration of that conjugate, thus this could just be a quantitative variance. Nevertheless, the total mass for these sulfamethoxazole compounds throughout the treatment process is approximately double that of sulfamethoxazole alone. [3] used high-resolution Orbitrap™ in MS/MS mode in combination with isotopically-labeled sulfamethoxazole-d_4 as a surrogate for *N*-acetyl sulfamethoxazole and sulfamethoxazole glucuronide pseudo-quantitation, and found in reclaimed water 2.85±1.37, 1.98±1.41, and 2.86±1.53 µg/L for sulfamethoxazole, *N*-acetyl sulfamethoxazole, and sulfamethoxazole glucuronide respectively. Substantial proportions of *N*-acetyl sulfamethoxazole were also found in other wastewaters along with negative attenuation [44], suggesting but not proving deconjugation may nonetheless exist in WWTPs in addition to the one we studied.

Several caveats are apparent when comparing data from the literature to our current study: concentrations of sulfamethoxazole would be population-dependent in usage; levels would be dependent on WWTP treatment type and associated hydraulic retention times (HRTs); and

using surrogates for quantitation as opposed to using identical isotopically-labelled standards to compensate for matrix effects for the individual compounds as we do here.

5.3.1.4. UV treatment had no effect on compound levels

Overall, it was apparent that regardless of compound class or conjugate type, there was no data to support removal or transformation during the tertiary UV treatment process. This is not particularly surprising given the short UV residence times (i.e. a few seconds) in combination with the ubiquity of molecules (e.g. DNA, RNA, proteins) that absorb light < 300nm, and the amount of dissolved and suspended particulate organic matter present within the wastewater system. For example, with an average flow rate of 165 ML/day, approximately 1900 L of wastewater flows by the UV treatment bulbs every second. Moreover, the sampling point for the final effluent was approximately 10 m downstream of the UV treatment; thus the opportunity to determine the effect of breaking pharmaceutical structural bonds at that rate and in that proximal location becomes problematic.

5.3.2. Solid Phase

5.3.2.1. Scope of findings and predicted levels

The scope of analysis for our set of conjugates and pharmaceuticals was limited to what was extracted *in-situ.* Sorption isotherms were not performed and so experimental determination of mechanistic possibilities cannot be concluded. However, sorptive tendencies were outlined in light of the mass of analytes associated with the suspended solids. Low levels of suspended particulate matter (Table A3.1) in the secondary clarifier and final effluent (means of 14 mg/L

and 16 mg/L respectively) resulted in concentrations below the LOQ in these stages for many compounds.

Table 5.1 Calculation of *in situ* K_D for propranolol, sulfamethoxazole, thyroxine, and thyroxine-*O-β-D*-glucuronide. C_S and C_W are the concentrations of compound associated with the suspended solids and water, respectively in the primary clarifier. $logk_{OW}$ was estimated using ECOSAR via EPISuite v 1.1. Predicted $logK_D$ was estimated using an LFER (Eqn. 9-26a) from Schwarzenbach (2003)[27]. $LogD_{OW}$ was calculated using an extension of Henderson-Hasselbalch equation, and estimated using the LogD calculation function in Chem Axon Marvin Sketch v 6.2 in parentheses. Predicted $logK_d$ was calculated using the Henderson-Hasselbalch derived values.

Compound	**C_S (µg/g solids dry weight)**	**C_W (µg/L)**	**Measured $logK_d$**	**$logk_{OW}$**	**Predicted $logK_d$**	**$logD_{OW}$**	**Predicted $logK_d$**
Propranolol	0.0518	0.0376	3.14	3.50	2.74	1.26[a] (0.36)	1.08
Sulfamethoxazole	0.153	1.31	2.07	0.89	0.81	-0.56[b] (0)	-0.26
Thyroxine	0.0567	0.00791	3.86	4.15	3.18	3.58[b] (3.44)	2.80
Thyroxine-glucuronide	0.0546	0.00697	3.89	2.65	2.11	-2.86[b] (-1.93)	-1.97

[a] Calculated using $logD_{OW} = logK_{OW} + \log (1/ 1+10^{pKa-pH})$

[b] Calculated using $logD_{OW} = logK_{OW} + \log (1/ 1+10^{pH-pKa})$

Given the moderate $logK_{OW}$ values (Figure A3.2) and $logD_{OW}$ values (pH 7.4; Table 5.1) for the classes of compounds analysed, it was predicted to see propranolol ($logK_{OW}$ = 3.5, $logD_{OW}$= 1.26), and thyroxine ($logK_{OW}$ = 4.1, $logD_{OW}$= 3.58) associated with solid matter [37, 45, 46], but less so with sulfamethoxazole ($logK_{OW}$ = 0.89, $logD_{OW}$= -0.56). [47] calculated a linear free energy relationship (LFER) based on experimental evidence of numerous pharmaceuticals

(neutral, basic, and acidic) and compared to other known LFERs. They determined that given the high degree of organic matter in WWTP sludge, that the LFER (eqn. 9-26a) reported by [26] gave sufficient approximation for $\log K_d = \log K_{iOC} = 0.74(\log K_{OW}) + 0.15$. Thus, using $\log K_{OW}$ the predicted calculated K_d values (L/kg) were 550 (propranolol), 6.5 (sulfamethoxazole), and 1500 (thyroxine). Using $\log D_{OW}$ values calculated at pH 7, the predicted calculated K_d values were 8.9 (propranolol), -0.04 (sulfamethoxazole), and 630 (thyroxine). Thus, using eqn. 9-11 reported by [26] in conjunction with the K_d values and measured mass of suspended solids per litre of primary wastewater (Table A3.1), predictions of aqueous fractions were calculated: 94.0% (propranolol), 100.0% (sulfamethoxazole), 93.4% (thyroxine). Therefore, it was predicted to see the vast majority of the parent compounds in the dissolved fraction of the primary wastewater; however, we saw marked greater amounts in the sorbed phase, as outlined in 5.3.2.2. below. Thus, these predicted aqueous fraction values can be considered a maximum for cationic species given electrostatic attractions with negatively-charged organic matter moieties.

5.3.2.2. Compound-specific sorption

For propranolol (Figure 5.2a), levels fell below the LOQ in the secondary and final effluent. Taking this caveat in mind for this and other analytes < LOQ, there were no significant differences ($P=0.6783$) among primary, mixed liquor, RAS, and WAS (means= 0.0518, 0.0447, 0.0496, and 0.0306 µg/g solids dry weight, respectively).

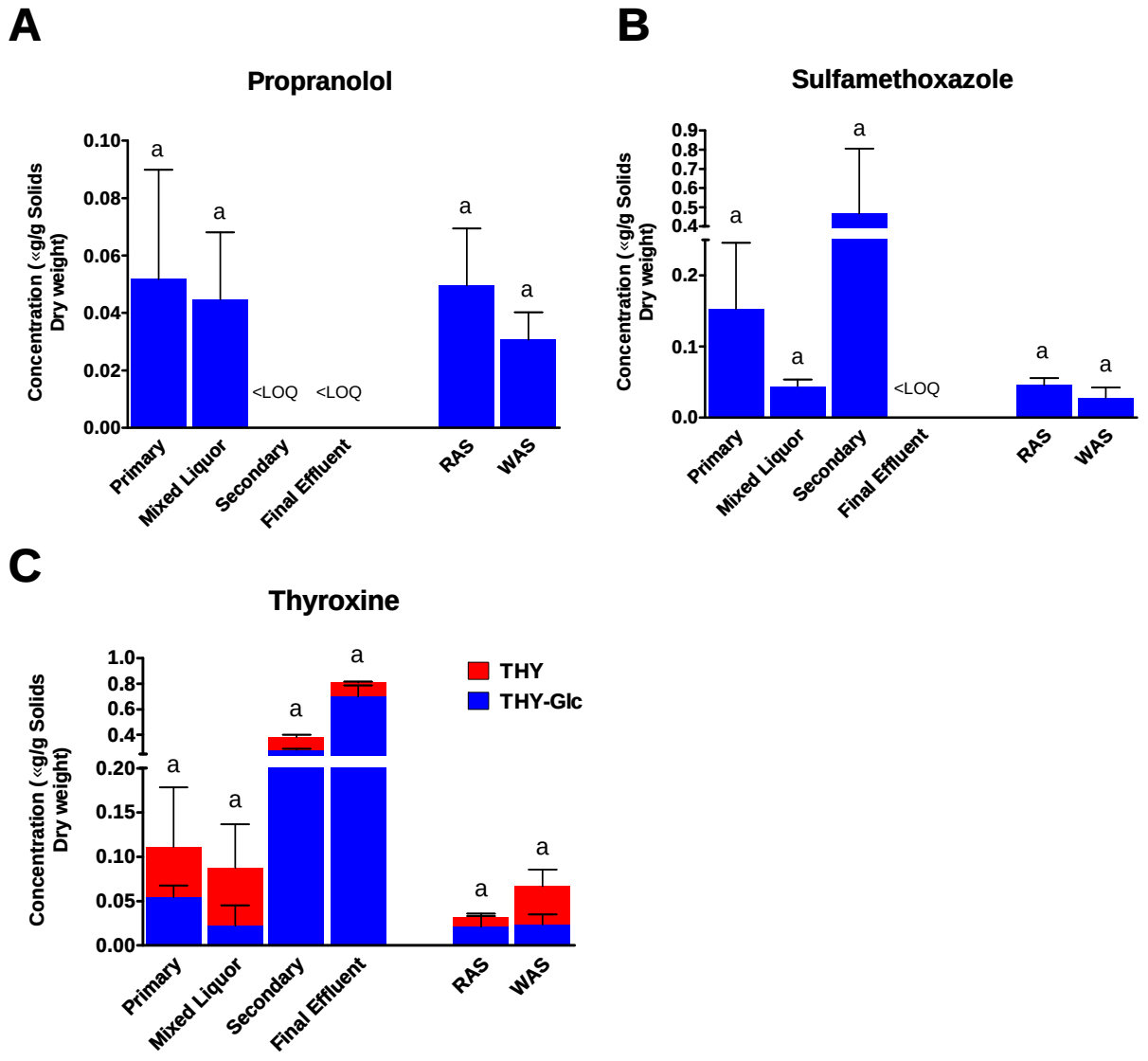

Figure 5.2: Concentrations (µg/g solids dry weight) for propranolol, sulfamethoxazole, thyroxine, and thyroxine-*O*-β-D-Glucuronide in the suspended solids of each stage of aqueous treatment. Concentrations in waste activated sludge and return activated sludge are also highlighted. Each sample is representative of a 24 h composite sample collected via autosampler. Bars represent the means, and error bars represent the standard error of the mean, and letters significantly if levels are different ($\alpha<0.05$) at each stage of treatment using Tukey-Kramer post-hoc tests.

Sulfamethoxazole demonstrated a similar distribution to propranolol, with sulfamethoxazole levels in the final effluent < LOQ (Figure 5.2b). There were no significant differences (P=0.2669) among primary, mixed liquor, secondary, RAS, and WAS (means= 0.153, 0.0445, 0.0467, 0.0460, and 0.0281 μg/g solids dry weight, respectively). The range of K_d for sulfonamides is reported to be 0.27-256 L/kg [48], and thus a major proportion is expected in the aqueous phase with some amounts associated with the solids [49]. Based on this K_d range, the predicted total sulfamethoxazole loaded on solids in wastewater would range 0.031-30 ng/L WW, 0.0038-3.6 ng/L WW, and 0.0043-4.1 ng/L WW for primary, secondary and final effluent respectively, based on the low levels of suspended solids (means= 116 mg/L (primary), 14.2 mg/L (secondary) 16.1 mg/L (final effluent). It was previously reported that the proportion of sorbed sulfonamides is likely not augmented in the presence of increased di-or tri-valent ions [46], thus indicating the fact that sulfonamides sorb primarily via hydrophobic interactions and not cation-bridging or other surface complexations in the presence of significant amounts of organic matter.

Sorbed thyroxine (Figure 5.2c) also showed no significant differences (P= 0.5161) across primary, mixed liquor, secondary, final effluent, RAS, and WAS (means= 0.0567, 0.0656, 0.100, 0.111, 0.0103, and 0.0433 μg/g solids dry weight, respectively). A similar distribution for thyroxine glucuronide (Figure 5.2c) was observed with no significant differences (P=0.9415) across primary, mixed liquor, secondary, final effluent, RAS, and WAS (means= 0.0546, 0.0221, 0.282, 0.698, 0.0210, and 0.0236 μg/g solids dry weight, respectively).

5.3.3. Temporal and Hydrodynamic Trends

When the aqueous concentrations are plotted against time, there was no obvious or statistically significant trend to indicate that acetaminophen, propranolol, sulfamethoxazole, or thyroxine compounds exhibit temporal correlations over the four-month timeframe. This intuitively makes sense given that these drugs are most likely prescribed and consumed year-round, with the potential exception of elevated sulfamethoxazole usage during cold/flu season. Given the logistical constraint of the samples being collected and analysed only during the winter months, there is no way to compare these values to another season (e.g. summer). However, there was evidence (Figure A3.3) to suggest a very mild inverse correlation of flow rate to concentration for all compounds except acetaminophen sulfate. Upon linear regression of all concentrations determined in the primary clarifier, all compounds showed poor R^2 values ranging from 0.027 (*N*-acetyl sulfamethoxazole) to 0.30 (propranolol sulfate). None of the slopes were significantly different from zero. Propranolol did show a significant deviation from 0 (R^2= 0.54; P=0.010); and all regressions displayed negative slopes, suggesting that as flow rate increased, concentrations mildly decreased. In fact, at the beginning of the study, Nov, 29, 2016 saw the flow rate dramatically increase to 361 ML/day (mean= 165 ML/day) due to the first melt of the first major autumnal snowfall in Winnipeg. The only compounds detected in the primary clarifier above the LOQ were sulfamethoxazole (0.56 µg/L) and sulfamethoxazole glucuronide (0.28 µg/L) (Figure A3.3). These were the lowest concentrations quantified during the study, suggesting that increased flow rate through the WWTP yielded lesser concentrations due to dilution.

5.3.4. In-Situ Solid-Water Distribution

Solid-water distribution coefficients (K_d) were calculated by dividing $[C]_S$ in suspended solids by $[C]_W$ in the primary clarifier (Table 5.1). This stage was chosen to maximise the concentrations of pharmaceutical and thus yield a representative distribution prior to transformation by the WWTP processes. In general, $\log K_d$ values (Table 5.1) seemed to increase as $\log K_{OW}$ increased: sulfamethoxazole (2.07)< propranolol (3.14)< thyroxine (3.86), and thyroxine glucuronide (3.89). The noted exception being thyroxine glucuronide demonstrating a similar sorptive tendency to the parent thyroxine even though the $\log K_{OW}$ (2.65 vs 4.15) is lesser. Based on the LFER noted above [26], the estimates for $\log K_d$ (Table 5.1) were sulfamethoxazole (0.81), propranolol (2.74), thyroxine (3.18), and thyroxine glucuronide (2.11). For sulfamethoxazole, this implies that there could be mechanisms occurring (e.g. inner and/or outer sphere complexation, electrostatic interactions) that aid in the retention of the glucuronide in addition to the hydrophobic interactions. The values for propranolol and thyroxine seem to be in agreement with what others found [47], although changes in speciation occur as pH changes. When $\log D_{OW}$ values were used in the LFER, the comparisons to the measured values exhibited greater variation (Table 5.1). Therefore, it appears that in a WWTP environment the sorption of these pharmaceuticals can generally be predicted by the hydrophobic interactions of the neutral species, given the significant amount of organic matter in the solid phase; thereby limiting the importance of site-specific reactivity of these compounds.

5.3.5. Final Effluent Mass Loadings and Apparent Net Removal

With these chemical distributions and the WWTP metadata in hand, it was important to estimate the final effluent mass loading of each compound into the fluvial receiving waters (Figure 5.3). What was important to note for all calculations, both aqueous and suspended solids, was the fact that defined units of water were not followed throughout the treatment plant primarily for logistical reasons. The North Main WWTP services approximately half a million people daily and extensive daily routine analytical chemistry protocols are conducted. We were not afforded the opportunity to follow a specific packet of water based on HRT in order to isolate temporospatial variables for concentrations of pharmaceuticals [50]. Several complications have been noted by [51, 52], and several solutions have been noted by [50, 53] in using a fractionated approach regarding HRTs. However, based on the good precision of the levels afforded via our LC-MS/MS quantification methods, it could be assumed that the levels we saw are representative based on the impacts of WWTP processes. i.e. these compounds have a continuous input on a daily basis regardless of seasonal or temporospatial variation, and thus, as a first estimation, can be viewed as fairly accurate representations of typical WWTP levels. However, if logistically possible, it would be of great value to follow a specific unit of water using the aforementioned techniques.

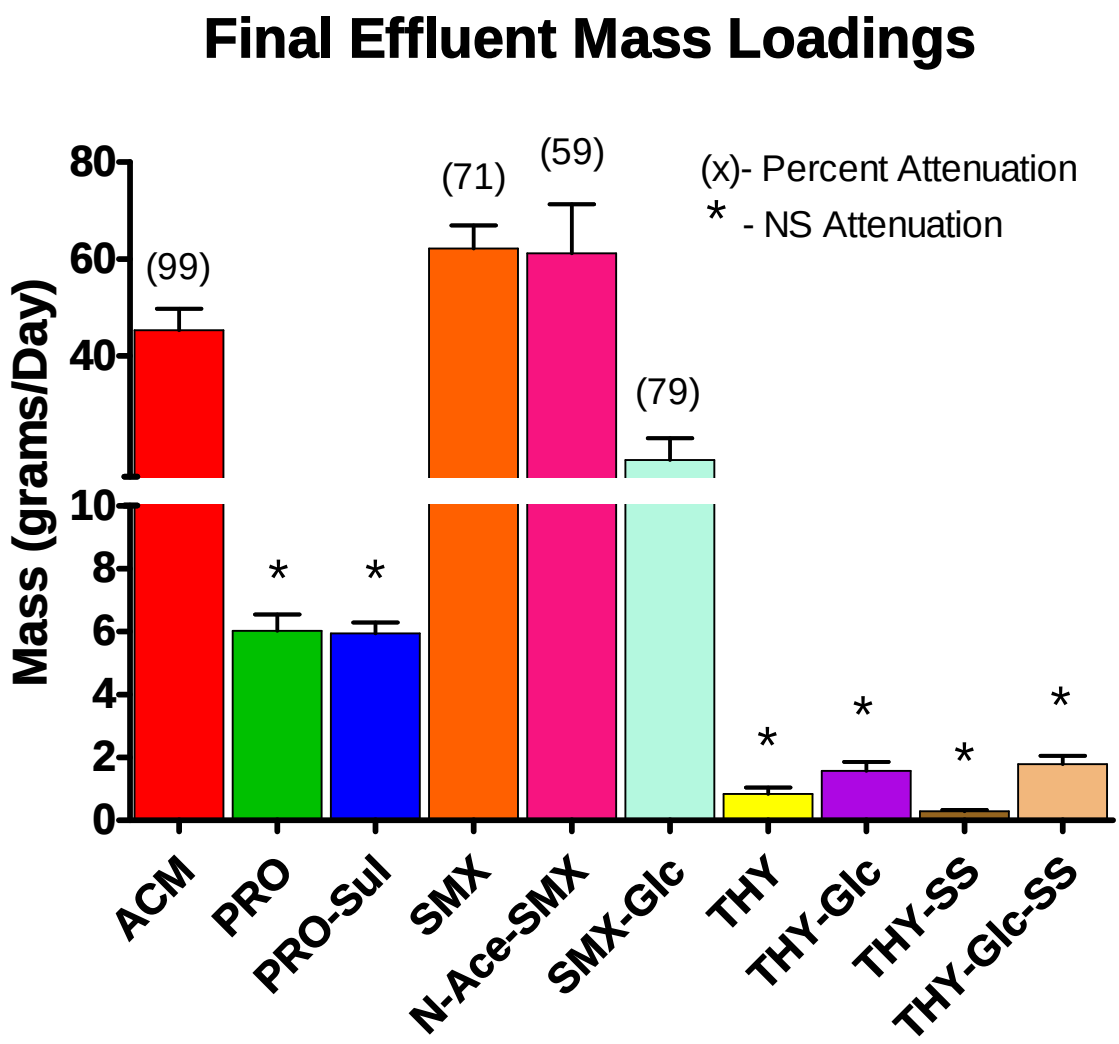

Figure 5.3: The mass loading (g/day) of all compounds present in the final effluent of the WWTP. Values in parentheses represent the significant percent decreases of concentration ($\alpha<0.05$) using Tukey-Kramer post-hoc tests from primary to final effluent for each compound; and asterisks represent no significant differences in concentration. Values above thyroxine and thyroxine glucuronide indicate the nominal concentrations in the final effluent for comparison purposes. Each sample is representative of a 24 h composite sample collected via autosampler. Bars represent the means, and error bars represent the standard error of the mean. NS = not significant.

For aqueous compounds, mass loaded (g/day) = flow rate (ML/day) × concentration of compound (µg/L); and for suspended solids, mass loaded (g/day) = flow rate (ML/day) × concentration of compound (ng/mg solids dry weight) x concentration of solids (mg/L). This allowed a direct comparison of concentrations in both aqueous to solid phases by essentially normalising them to a unit mass/day (g/day). In summary, the only compound not quantified in the final effluent was acetaminophen sulfate. Propranolol, propranolol sulfate, thyroxine, and thyroxine glucuronide all had no significant decreases in concentration throughout the wastewater treatment process (from primary to final effluent).

The mean (±SEM) of mass loadings calculated in the aqueous fraction of the final effluent for each compound were (Figure 5.3): acetaminophen: 45.3±4.2, propranolol: 6.0±0.5, propranolol sulfate: 5.9±0.3, sulfamethoxazole: 62.2±4.6, *N*-acetyl sulfamethoxazole: 61.2±9.6, sulfamethoxazole glucuronide: 18.5±4.3, thyroxine: 0.84±0.2, and thyroxine glucuronide: 1.6± 0.2 g/day. Of important note, is the mean (SEM) masses of thyroxine 0.29 (0.025) g/day and thyroxine glucuronide 1.8 (0.59) g/day. These values equate to 26% of total thyroxine and 53% of total thyroxine glucuronide reaching the Red River receiving waters being associated with suspended particulate matter. This significant proportion being particle-bound was agreement with the pharmacological literature that reports circulating T_4 (thyroxine) is bound to serum carrier proteins especially thyroxine-binding globulin and thyroxine-binding prealbumin to a great extent (>99%) [54]. Moreover, it has been reported that only 0.02% of thyroxine is considered bioavailable [55], that is the mass of thyroxine in a free soluble form. It is important to note that bacteria in the lower intestine (jejunum and ileum) possess glucuronidases, which results in very little thyroxine glucuronide being excreted via the feces [4, 56]. Thus, thyroxine glucuronide

potentially found in a wastewater environment would most likely be due to the sorption to suspended particulate matter from the aqueous phase, and this is what we expected. Hence, excreted thyroxine and thyroxine glucuronide maintain strong affinities for the solids within the WWTP.

In comparison for propranolol mass loadings, [39] reported 56.1% of total propranolol sorbed to suspended particulate matter and a total load of 12.6 mg/day/1000 inhabitants. In the current study, Winnipeg has approximately 700,000 inhabitants and the North Main WWTP services approximately 70% of the population. Therefore, based on approximately 500,000 inhabitants and similar script and usage patterns as Albany, NY, a mass loading of 6.3 g/day would be expected using the estimation by Subedi *et al*. The current study calculated a mean of 6.0 g/day loaded into the Red River, albeit all propranolol was found in the aqueous phase ostensibly due to the aforementioned lesser amounts of suspended solids present in the final effluent, resulting in levels < LOQ.

Studies have been conducted at the various stages of WWTPs to elucidate the most effective areas of removal for pharmaceuticals. [35] reported influent and effluent concentrations for acetaminophen 18.5 and 0.031 µg/L, sulfamethoxazole 0.36 and 0.18 µg/L, and *N*-acetyl sulfamethoxazole 0.19 and <LOD, respectively. This corresponded to removal rates of 99.8% (acetaminophen), 50% (sulfamethoxazole), and >99% (*N*-acetyl sulfamethoxazole). [36] reported that within a 4 hour HRT using basic lysates of extracted native enzymes from activated sludge, acetaminophen was completely attenuated (>99), *N*-acetyl sulfamethoxazole (59%), and the appearance of sulfamethoxazole of approximately 23% ostensibly due in part to deconjugation of *N*-acetyl sulfamethoxazole. The former results are virtually identical in direct comparison to our observed overall decreases in concentrations of 99.8% (acetaminophen), 71%

(sulfamethoxazole), and 59% (*N*-acetyl sulfamethoxazole). This result suggests that for these compounds, aerobic biodegradation seems to be the dominant mechanism of removal. When not in a wastewater environment, [22] found that during sorption experiments of acetaminophen and sulfamethoxazole to various soils, quantification was problematic due to the high biodegradability confirmed in their removal experiments where only 20% acetaminophen remained after 48 h when sorbed in combination with microorganisms as opposed to <10% removal with sorption alone with autoclaved soil. Moreover, < 20% removal was found for sulfamethoxazole under both sorption conditions, with or without microbial degradation. Thus, biodegradation of sulfamethoxazole within a WWTP environment is greater than that found in a terrestrial one.

5.4 Conclusions

Overall there were significant decreases in the concentrations of acetaminophen and sulfamethoxazole compounds, but persistence of propranolol and thyroxine compounds throughout the WWTP. The mass loadings throughout the WWTP necessitated that both aqueous and solid concentrations be taken into account. Comparable final effluent concentrations of propranolol sulfate, *N*-acetyl sulfamethoxazole, sulfamethoxazole glucuronide, and thyroxine glucuronide were released, if not more, than their respective parent. This study reflects the importance of including phase II conjugates in assessing overall compound load of pharmaceutical discharge from wastewaters, and also that substantial amounts of such contaminants are associated with wastewater solids when drugs are in the pg/L to µg/L range. However, generalisations to other classes of pharmaceuticals cannot be concluded based on the scarcity of monitoring data and the complexity of sorption mechanisms within a WWTP.

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Chapter 6

Kinetics of Human Pharmaceutical Conjugates and the Impact of Transformation, Deconjugation, and Sorption on Persistence in Wastewater Bioreactors

A version of this chapter has been previously submitted as Brown AK, Ackerman J, Cicek N, and Wong CS. Kinetics of Human Pharmaceutical Conjugates and the Impact of Transformation, Deconjugation, and Sorption on Persistence in Wastewater Bioreactors. *Water Research.* 2019. (Submitted April 7, 2019).

The author of this dissertation designed the experiment along with his supervisor, performed the experiments, interpreted the data, and wrote the manuscript. Joe Ackerman provided the bioreactor wastewater quality parameters found in the supporting information.

6. Abstract

The fate of selected pharmaceuticals and their Phase II conjugates in wastewater bioreactors was evaluated, to determine how treatment parameters such as components of primary or secondary wastewater, addition of air, and the presence of waste activated sludge (WAS) could influence the kinetics of removal. Under a realistic hydraulic residence time (HRT) (<2h), acetaminophen and its sulfate were both rapidly degraded (>99%). Propranolol was sulfated and concurrently removed. Deconjugation of *N*-acetylsulfamethoxazole and sulfamethoxazole-glucuronide contributed to increases of the parent sulfamethoxazole. Thyroxine was resistant to degradation, while thyroxine-glucuronide was rapidly deconjugated (>90% in <2h). Without WAS, sorption to suspended solids was another major removal mechanism for acetaminophen, propranolol, sulfamethoxazole, and thyroxine. However, with WAS, concentrations associated with suspended solids decreased for all analytes by 24h. These results indicate that both conjugation and back-transformation are compound-specific and dependent on parameters such as HRT, addition of WAS, and suspended solids levels. These transformation processes may strongly influence the fate and speciation of pharmaceuticals in wastewater effluents.

6.1. Introduction

There is an increasing need to determine the aquatic occurrence, fate, and toxicity (if any) of pharmaceutical metabolites[1-3]. Human metabolites and abiotic/biotic transformation products (TPs) are present in wastewater, and thus released into proximal receiving waters[4-7].

Concentrations, and thus exposure levels, can be influenced by photolysis[8], hydrolysis[9], biodegradation[10], sorption[11-13], and complexation with inorganic ions and surfaces[14].

Phase II conjugates (e.g., glucuronide, sulfate, acetyl, amino acid) of human metabolites can exist in waters at levels rivaling that of parent compounds[15-18]. Moreover, some conjugates are resistant to removal during wastewater treatment[19]. If conjugates become deconjugated, they can back-transform into the parent compound again[20], and potentially elicit toxic effects. Current knowledge gaps include the aquatic occurrence, fate, and toxicity of major human TPs such as conjugates. Thus, it is important to determine which conjugates can influence levels of their respective parent compound, and to what magnitudes, under realistic conditions.

Previously[21], we demonstrated that concentrations of acetaminophen, propranolol, sulfamethoxazole, thyroxine, and their conjugates can vary within a model wastewater treatment plant (WWTP) in a treatment-dependent manner (e.g. aerobic treatment versus removal via sorption). These pharmaceuticals are commonly used, which contributes to their pseudo-persistence[21-25]. Previously, many high mass resolution studies highlighting levels of various pharmaceutical TPs, or other low-resolution studies quantifying target metabolites, have been done in natural/engineered systems[6, 26], and for estrogen TPs in dairy wastewater[27]. Other pharmacological studies aimed to isolate previously unknown TPs[28, 29]. However, little work has been done to understand the concomitant kinetics and concentrations of multiple classes of both parent pharmaceuticals *and* TP conjugates under controlled conditions, and if these processes could corroborate conjugate levels observed in our previous field observations. For example, do conjugates back-transform (i.e. deconjugate) and contribute to parent compound concentrations[30], or is there additional *de novo* conjugation[31] (e.g. *Rhodococcus equi* conjugating sulfamethoxazole via the enzyme arylamine *N*-acetyltransferase)[32]? How would wastewater

treatment operating parameters, such as the addition of oxygen and heavily bacteria-laden waste activated sludge (WAS), influence removal or transformation kinetics? Is sorption to suspended solids important, and if so, how? Such knowledge could help explain the presence, persistence, and fate of pharmaceuticals and their TPs under full-scale scenarios (i.e. activated sludge facilities, or rural lagoon systems).

6.2. Materials and Methods

6.2.1. Materials

Details are in Appendix 4 for acetaminophen, acetaminophen sulfate, propranolol, 4-OH-propranolol sulfate (propranolol sulfate), sulfamethoxazole, *N*-acetylsulfamethoxazole, sulfamethoxazole-β-glucuronide (sulfamethoxazole-glucuronide), thyroxine, and thyroxine-*O*-β-D-glucuronide (thyroxine-glucuronide).

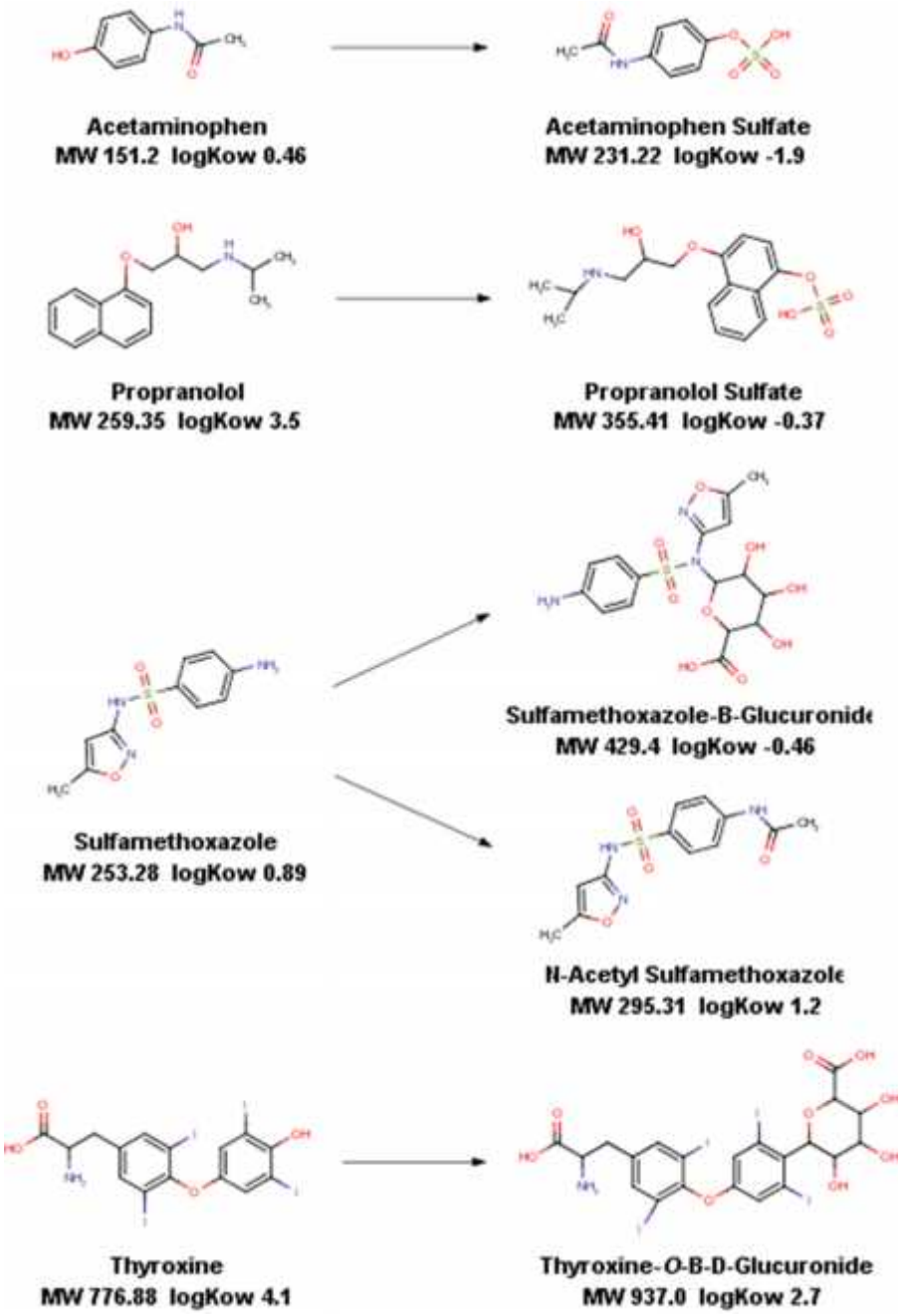

Figure 6.1 The physical structures (fully protonated), molecular masses, and logK_{OW} values for all parent pharmaceuticals and conjugates monitored in this study. Structures were drawn using Chem Axon® Marvin Sketch v. 6.2. Structures were copied as SMILES notations and entered in ECOSAR via EpiSuite v .1.1 to generate molecular masses and logK_{OW}. Parent compounds all had known values in the database; those of the conjugates are all estimates.

6.2.2. Methods

6.2.2.1. Sample Collection

Samples were collected weekly at the North End Winnipeg Pollution Control Centre WWTP in Winnipeg, Canada. Identical 24h composite batches were obtained and refrigerated in the dark (30 minutes), then transported under ice to the laboratory. Table A4.1 has details on the WWTP and chemical parameters for bioreactor wastewaters. Aerated primary and non-aerated secondary were sampled and run in triplicate, non-aerated primary, aerated secondary, and aerated activated primary were all run in duplicate.

6.2.2.2. Batch Bioreactor Set-Up

All bioreactors were 3.0 L, and consisted of either primary effluent, secondary effluent, or activated primary effluent (2.85 L with 0.15 L of waste activated sludge added). The pH values were measured prior to spiking bioreactors, and upon completion of the 24h trials. Additional 200 mL composite samples of each WWTP batch were used to calculate total phosphorus (filtered/unfiltered), phosphate, ammonia, nitrite, and nitrate (Table A4.1). Bioreactors were stirred either without aeration, or with aeration (flow rate 2.4 L/min) using a Fluval A-850 electric air pump, autoclavable tubing, and ceramic air diffuser stones. All bioreactors were wrapped in aluminum foil, covered loosely, and run in the dark to eliminate photolysis.

6.2.2.3. Extraction and Analysis

Details regarding solid phase extraction (SPE), chromatographic analysis, and quantification are published[16, 19] and in Appendix 4. In brief, 3.0 L bioreactors were spiked with a 1 mL mixture of 1000 µg/L unlabeled compounds, for a nominal concentration of 0.33 µg/L of each analyte. Aliquots (100 mL) were taken at several timepoints: 0, 1, 2, 4, 8, and 24h. These were centrifuged at 2200 g for 10 minutes, and the supernatant was filtered through 0.45 µm nitrocellulose filters, spiked with 50 µL of 1000 µg /L of internal standard mixture, and subjected to weak anion exchange (WAX) SPE. Analytes were separated by reversed-phase liquid chromatography and quantified through isotope dilution via tandem mass spectrometry. Applicable suspended solids were extracted by vortexing for 30 seconds in 5 mL of methanol, sonicating for 20 mins, vortexing again for 30 seconds, and centrifuging at 2200 g for 10 minutes. The supernatant was diluted to 200 mL with deionised water prior to filtration and WAX SPE as above.

To determine if abiotic transformations (e.g. hydrolysis) affected analyte dissipation over 24h, a kill control bioreactor (no aeration) consisting of secondary effluent was autoclaved in two consecutive 90 min liquid cycles.

6.2.3. Statistical Analysis

GraphPad Prism v.5 (GraphPad Software, La Jolla, CA, USA) was used to conduct all analyses during method development. Linear regression was performed on all kinetics plots from individual bioreactors using $\ln(A_t/A_0)$ vs. time, to determine if slopes (k) significantly deviated

from zero (α=0.05). The mean 24h rate constant is reported with the standard error of the mean in parentheses.

6.3. Results and Discussion

The wastewater from the threatment plant studied typically has flow rates of 120-370 ML/day, and a total process hydraulic residence time (HRT) of approximately 7.5-8h from raw influent to final discharge. The HRT for primary clarification is approximately 1.5-2h, aerobic reaction is approximately 1.4-2.16h, and secondary clarification is approximately 2h. Thus, pseudo-first order kinetic rates and half-lives in this study (Table A4.3) were highlighted at the 2h (applicable to activated sludge secondary WWTPs) and 24h timepoints (more applicable to a lagoon environment)[33].

The kill controls (Figures e of 6.2 to 6.5) showed no evidence of abiotic transformation or other such losses (e.g., sorption to container walls) within 24h, as no significant changes in concentration over time occurred (all slopes ns; $P > 0.05$). This observation implied that the analytes were stable in aqueous solution, and that all major fluctuations in concentrations over time were most likely from biotransformation. Thus, the varying kinetic rates were likely due to the conditions provided to bacteria present. Primary and secondary total suspended solids concentrations for this WWTP were approximately 116 mg/L and 14 mg/L, respectively. The WAS contained approximately 10000 mg/L suspended solids. All samples had appreciable levels of endogenous analytes.

As noted, each bioreactor was spiked with a nominal, environmentally-relevant (additional) concentration of 0.33 μg/L to ensure that analyte concentrations were always >LOQ.

The proportion of the total initial analyte accounted for by the spikes were: acetaminophen and acetaminophen sulfate <0.01% (primary) and 40% (secondary); propranolol and propranolol sulfate 100% (both primary and secondary); sulfamethoxazole and *N*-acetylsulfamethoxazole approximately 25% (both primary and secondary), sulfamethoxazole-glucuronide approximately 40-87% (primary), 83-100% (secondary); thyroxine and thyroxine-glucuronide 100% (both primary and secondary).

Table 6.1 Pseudo first-order half-lives of each of the 4 parent compounds: acetaminophen (ACM), propranolol (PRO), sulfamethoxazole (SMX), and thyroxine (THY), and the 5 conjugates: acetaminophen sulfate (ACM-Sul), propranolol sulfate (PRO-Sul), *N*-acetylsulfamethoxazole (N-Ace-SMX), sulfamethoxazole-glucuronide (SMX-Glc), and thyroxine-glucuronide (THY-Glc). Negative half-lives indicated an increase in concentrations for that compound by that specific timepoint.

Analyte ($logK_{OW}$)	**First Order Rate (h^{-1}) (Standard Error) Half-Life (h)**									
	Primary				**Secondary**				**Activated Primary**	
	Aerated		**Non Aerated**		**Aerated**		**Non Aerated**		**Aerated**	
	2h	**24h**	**2h**	**24h**	**2h**	**24h**	**2h**	**24h**	**2h**	**24h**
ACM		0.28		0.008		0.047		0.074		2.42
(0.46)	0.024	(0.021)	0.056	(0.002)	0.13	(0.008)	0.13	(0.006)	2.4	(0.062)
	29.4	**2.5**	**12.3**	**84**	**5.3**	**14.7**	**5.2**	**9.3**	**0.3**	**0.3**
ACM-Sul		0.23		0.023		-0.014		0.05		0.21
(-1.9)	0.18	(0.023)	0.037	(0.005)	-0.093	(0.010)	0.16	(0.005)	0.33	(0.066)
	3.9	**3**	**18.8**	**29.6**	**-7.5**	**-49.3**	**4.5**	**13.8**	**2.1**	**3.4**
PRO		0.18		-0.009		0.053	-	0.012		0.11
(3.5)	0.11	(0.009)	0.05	(0.004)	0.085	(0.003)	0.075	(0.004)	0.079	(0.010)
	6.4	**3.8**	**13.9**	**-77**	**8.2**	**13**	**-9.2**	**56.8**	**8.8**	**6.5**
PRO-Sul		-0.025		-0.009		-0.013	-	-0.006		0.051
(-0.37)	-0.17	(0.008)	-0.023	(0.004)	-0.086	(0.004)	0.055	(0.005)	-0.13	(0.010)
	-4.1	**-27.2**	**-30.4**	**-76.3**	**-8.1**	**-52**	**-12.7**	**-107**	**-5.2**	**13.5**
SMX		0.009		-0.01		-0.003		-0.016		0.010
(0.89)	-0.023	(0.003)	0.001	(0.002)	-0.038	(0.003)	-0.19	(0.005)	-0.16	(0.009)
	-29.6	**81.4**	**479**	**-66.5**	**-18.1**	**-218**	**-3.8**	**-42.9**	**-4.3**	**193**
N-Ace-		-0.004		0.001		0.005		0.005		0.13
SMX	0.002	(0.002)	0	(0.004)	0.046	(0.004)	0.063	(0.003)	0.21	(0.009)
(1.2)	**355**	**-192**	**3430**	**832**	**15.2**	**138**	**10.9**	**131**	**3.2**	**5.4**
SMX-Glc		0.033		0.055		-0.006		0.006		0.071
(-0.46)	0.08	(0.004)	0.17	(0.005)	-0.068	(0.003)	0.032	(0.004)	0.051	(0.005)
	8.7	**21.3**	**4.0**	**12.7**	**-10.2**	**-110**	**21.6**	**117**	**13.5**	**10.3**
THY		-0.006		0.007		-0.010		-0.012		0.034
(4.1)	-0.09	(0.002)	0.019	(0.004)	-0.13	(0.005)	-0.1	(0.004)	0.02	(0.006)
	-7.7	**-66.5**	**36**	**106**	**-5.5**	**-47.1**	**-7.0**	**-44**	**34.3**	**20.3**
THY-Glc		0.11		0.011		0.14		0.068		0.10
(2.7)	0.98	(0.041)	0.36	(0.046)	0.79	(0.040)	0.59	(0.063)	0.15	(0.068)
	0.7	**4.8**	**1.9**	**60.9**	**0.9**	**3.9**	**1.2**	**8.6**	**4.5**	**5.8**

6.3.1. Acetaminophen

Endogenous concentrations of acetaminophen in primary bioreactors were 115-253 µg/L (Figures 6.2a and 6.2b). In primary and secondary waters, there was a slight downward trend over time irrespective of aeration, indicating that biodegradation was occurring. However, in the aerated primary at 24h, there was a significant drop (>99%) for both acetaminophen and acetaminophen sulfate, from 201 to 0.26 µg/L and 71.9 to 0.27 µg/L, respectively. This observation indicates that in an aerobic environment, with plenty of microbial consortia and suspended solids, concentrations of both compounds decreased rapidly. Thus, overall half-lives by 24h were 2.5 and 3.0h respectively (Table 6.1). In a lagoon system, where there is much lower flow and aeration, concentrations could potentially be seen that are reflective of Figure 6.2a.

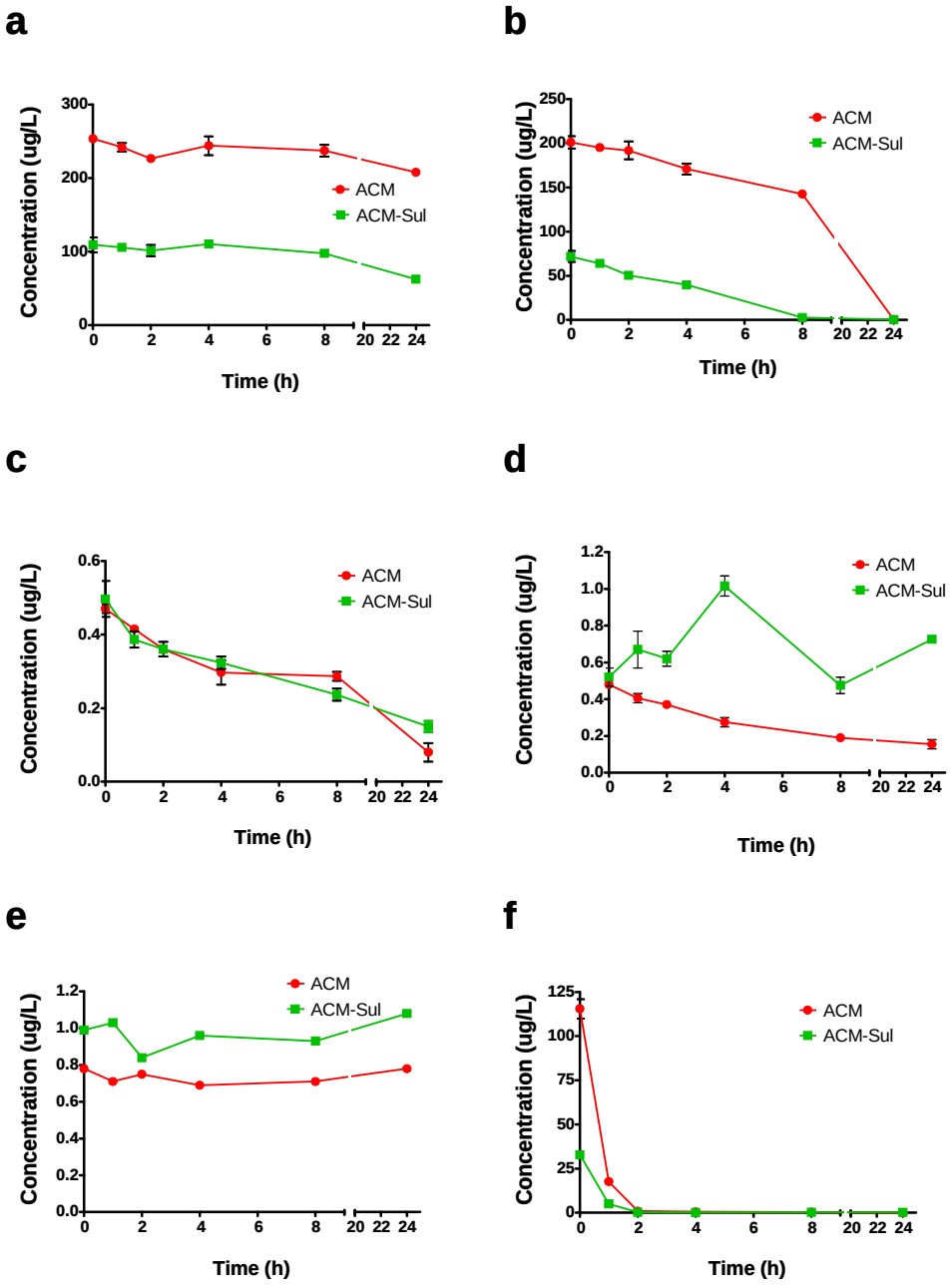

Figure 6.2 Concentrations of acetaminophen (ACM), and acetaminophen sulfate (ACM-Sul) during various treatments of wastewater in benchtop bioreactors. A) Primary effluent no air; B) Primary effluent with air; C) Secondary effluent no air; D) Secondary effluent with air; E) Autoclaved kill control; F) Primary effluent with aerated waste activated sludge. Values are means and error bars are standard error of the mean.

The presence of WAS (Figure 6.2f) rapidly reduced concentrations of acetaminophen and acetaminophen sulfate, which dropped (>99%) from 115 and 31.8 µg/L to 0.95 and 0.26 µg/L respectively after 2h, with half-lives of 0.3h each (Table 6.1). This observation agrees with our previous field observations[21] of >99% attenuation from primary to secondary effluent within the same WWTP (HRT<2h). Other studies also showed >99% reduction in acetaminophen with similar nominal concentrations[34], and 92% reduction in acetaminophen in various other WWTPs[35].

Sulfation was possible for acetaminophen given the presence of this conjugate, which has not been reported in wastewater before to our knowledge. Under aerobic conditions with much less suspended solids (Figure 6.2d), acetaminophen sulfate levels increased from 0.52 to 1.02 µg/L from 0-4h prior to the decrease to 0.72 µg/L at the 24h mark. Overall, there was no evidence for deconjugation of acetaminophen sulfate leading to increased concentrations of acetaminophen at any point. Thus, in both a WWTP environment (HRT<2h per treatment stage) without the use of WAS, and a lagoon environment (>24h), both acetaminophen and acetaminophen sulfate concentrations are unlikely to fluctuate greatly.

The addition of WAS was effective at reducing both compounds >99%. However, concentrations in the high ng/L range remained for release into receiving waters. Augmenting wastewater conditions (e.g. nitrifying or denitrifying) contributes to the proliferation of specific types of microbes that can transform pharmaceuticals[36, 37]. This is in agreement with previous literature that showed *Delftia tsuruhatensis* and *Pseudomonas aeruginosa* as major contributors for acetaminophen biodegradation[38]. In contrast, ammonia-oxidising and heterotrophic bacteria can aid in *de novo* conjugation within a WWTP[31]. While profiling the microbial consortia within

our bioreactors would have proved interesting, it is likely that similar profiles as other previous studies[39, 40] would exist (i.e. *Nitrosomonas sp.*, *Pseudomonas sp.*, *Achromobacter sp.*, *Rhodococcus sp.*)[32, 41] given the endogenous presence of all analytes within the oxic wastewater tested and the time-frame tested (24h).

6.3.2. *Propranolol*

Endogenous concentrations of propranolol and propranolol sulfate in both primary and secondary waters (Figure 6.3) were typically 0.1-0.2 µg/L. Without aeration, levels were relatively constant to 24h (Figures 6.3a and 6.3c) at 0.17-0.26 µg/L and 0.10 to 0.19 µg/L, respectively. Under aerobic conditions (Figures 6.3b and 6.3d), there was evidence for sulfation of propranolol into propranolol sulfate at 2h and clearly at 24h. Propranolol decreased from 0.21 to 0.003 µg/L and 0.19 to 0.05 µg/L for primary and secondary waters respectively, in agreement with parent levels in numerous other WWTPs[35, 42], and similar decreasing trends within realistic HRT values observed elsewhere[43]. Propranolol sulfate levels similarly increased from 0.13 to 0.24 µg/L and 0.14 to 0.20 µg/L respectively, a phenomenon also seen in our previous field study at the same WWTP[21].

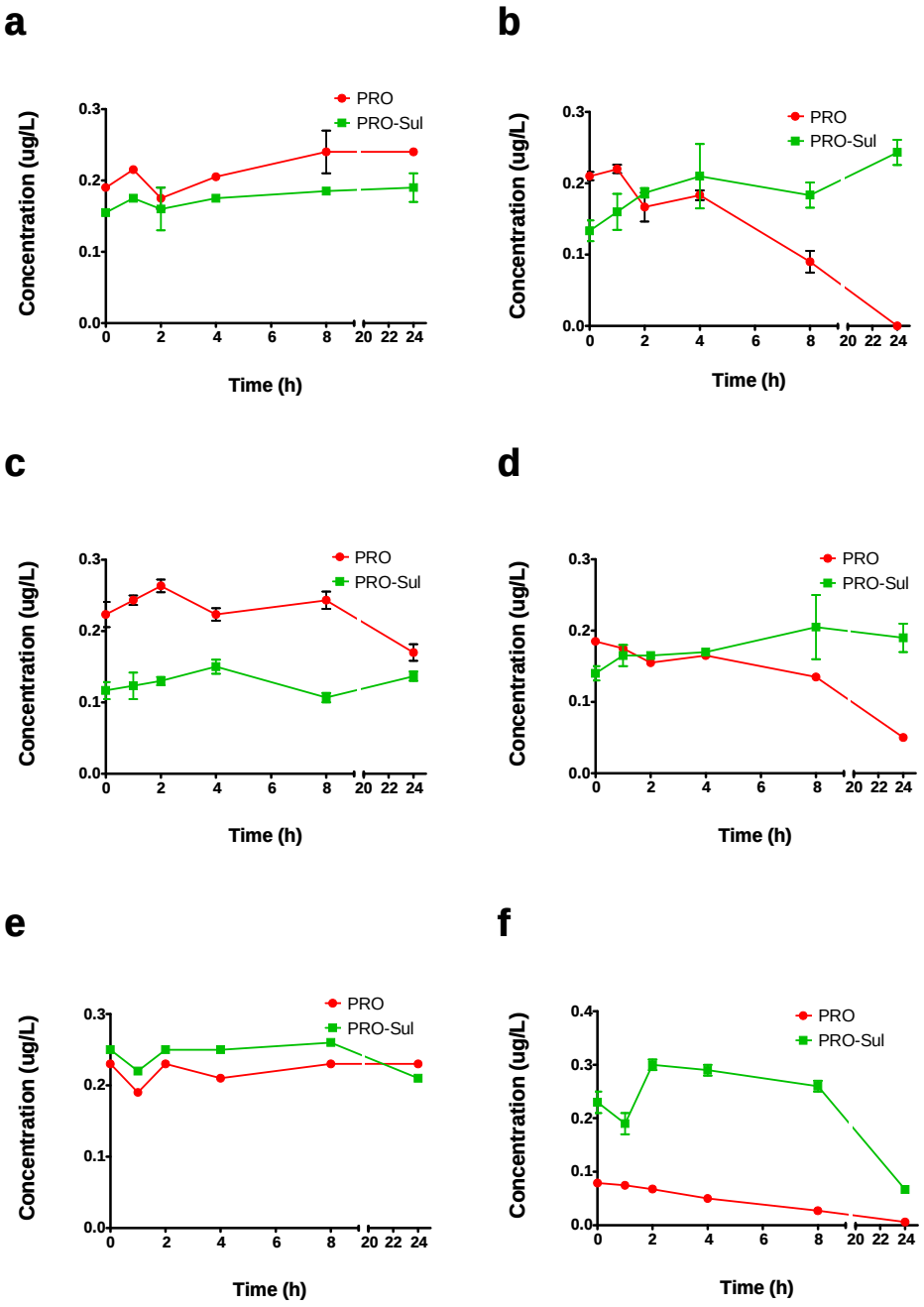

Figure 6.3 Concentrations of propranolol (PRO), and propranolol sulfate (PRO-Sul) during various treatments of wastewater in benchtop bioreactors. A) Primary effluent no air; B) Primary effluent with air; C) Secondary effluent no air; D) Secondary effluent with air; E) Autoclaved kill control; F) Primary effluent with aerated waste activated sludge. Values are means and error bars are standard error of the mean.

During activated treatment with WAS, propranolol sulfate concentrations also increased (indicating sulfation) from 0.23 to 0.30 µg/L by 2h prior to concomitant biodegradation with propranolol from 8 to 24h, with overall half-lives of 6.5 and 13.5h respectively for propranolol and propranolol sulfate (Table 6.1). These observations suggested that the addition of much more activated bacteria and suspended solids in addition to greater but unrealistic WWTP HRTs was beneficial for biodegradation of both otherwise recalcitrant compounds[37, 44]. This study corroborates the sulfotransferase potential of microbes on aryl compounds demonstrated previously via PAPS or arylsulfotransferases[45]. However, this is the first time to our knowledge that kinetics has been calculated for propranolol sulfate in wastewater. This study also demonstrates that conjugation of propranolol could potentially contribute to the environmental persistence of the drug's total inventory in receiving waters[31].

6.3.3. *Sulfamethoxazole*

The two main human conjugates of sulfamethoxazole are *N*-acetylsulfamethoxazole and sulfamethoxazole-glucuronide[15]; thus understanding relative concentrations of these compounds over time gives insights into the environmental persistence of sulfamethoxazole. Initial primary effluent levels of sulfamethoxazole, *N*-acetylsulfamethoxazole, and sulfamethoxazole-glucuronide all ranged from 0.41 to 1.24 µg/L (Figures 6.4a,b,f). Sulfamethoxazole and *N*-acetylsulfamethoxazole levels stayed constant within 24h, across all treatments of primary or secondary wastewater (Figures 6.4a-d), aerated or not. However, in all primary wastewaters (Figures 6.4a and 6.4b), sulfamethoxazole-glucuronide concentrations dropped significantly

from 0.58 to 0.27 µg/L, and 0.79 to 0.21 µg/L respectively at 2h, which corresponded to 2h HRT half-lives of 8.7 and 4h respectively (Table 6.1). Wang and Gardinali (2014)[15] also measured sulfamethoxazole, *N*-acetylsulfamethoxazole, and sulfamethoxazole-glucuronide in a Florida reclaimed water at 2.8, 2.0, and 2.9 µg/L respectively, indicating that all three compounds can survive wastewater treatment and persist environmentally.

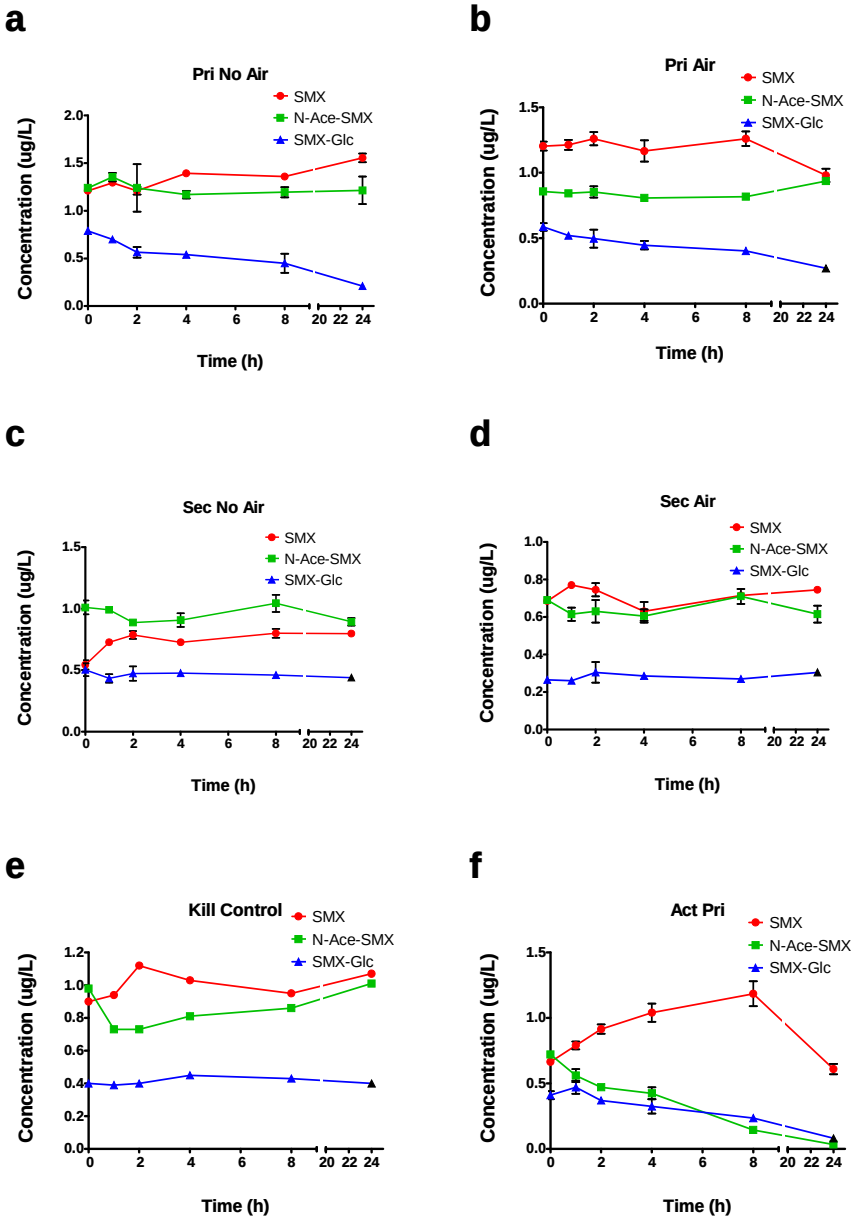

Figure 6.4 Concentrations of sulfamethoxazole (SMX), N-acetylsulfamethoxazole (N-Ace-SMX), and sulfamethoxazole glucuronide (SMX-Glc) during various treatments of wastewater in benchtop bioreactors. A) Primary effluent no air; B) Primary effluent with air; C) Secondary effluent no air; D) Secondary effluent with air; E) Autoclaved kill control; F) Primary effluent with aerated waste activated sludge. Values are means and error bars are standard error of the mean.

The decrease in both sulfamethoxazole-glucuronide and *N*-acetylsulfamethoxazole was also seen in the activated primary treatment, with similar half-lives for both at the 2h and 24h timepoints (Table 6.1) of 5.4 and 3.2h, and 10.3 and 13.5h respectively. Sulfamethoxazole increased from 0.67 µg/L to a maximum of 1.19 µg/L by 8h (Figure 6.4f), at which time subsequent biodegradation decreased concentrations back to that of the initial input (i.e. 0.61 µg/L). This observation suggested a steady deconjugation of both conjugates under greater but unrealistic HRTs using WAS (Figure 6.4f). Deconjugation to the parent of both *N*-acetylsulfamethoxazole and sulfamethoxazole-glucuronide appears to have occurred at rates great enough that any potential simultaneous degradation of sulfamethoxazole was masked. Thus, concentrations of these compounds should remain relatively constant in WWTPs under both primary and secondary treatment with 2h HRTs. Moreover, in a lagoon setting with suspended solids and endogenous microbial consortia (without WAS), levels of all three sulfamethoxazole compounds should remain relatively constant as well[15]. A study of 35 WWTPs in China[46] reported that sulfamethoxazole and *N*-acetylsulfamethoxazole had mean removal rates of approximately 45% and 90% respectively, demonstrating that sulfamethoxazole conjugates still survive wastewater treatment even though rates of removal can vary.

6.3.4. *Thyroxine*

The dominant trend across all treatments (Figures 6.5a-d, and 6.5f) was the persistence of the parent thyroxine over 24h. Primary treatments had constant thyroxine concentrations (Figure 6.5a and b) of 0.16 to 0.13 µg/L, and 0.20 µg/L to 0.26 µg/L, respectively (slopes ns; $P>0.05$).

All secondary treatments also demonstrated significant increases from 0.30 to 0.43 µg/L, and 0.35 to 0.49 µg/L respectively from 0 to 24h timepoints. In contrast, thyroxine is readily hydrolysable in non-acidic pH environments such as analytical standards or animal tissue extracts at >24h time frames[25]. Thus, aquatic persistence of thyroxine is possibly controlled by the pH of the surrounding system in addition to HRTs.

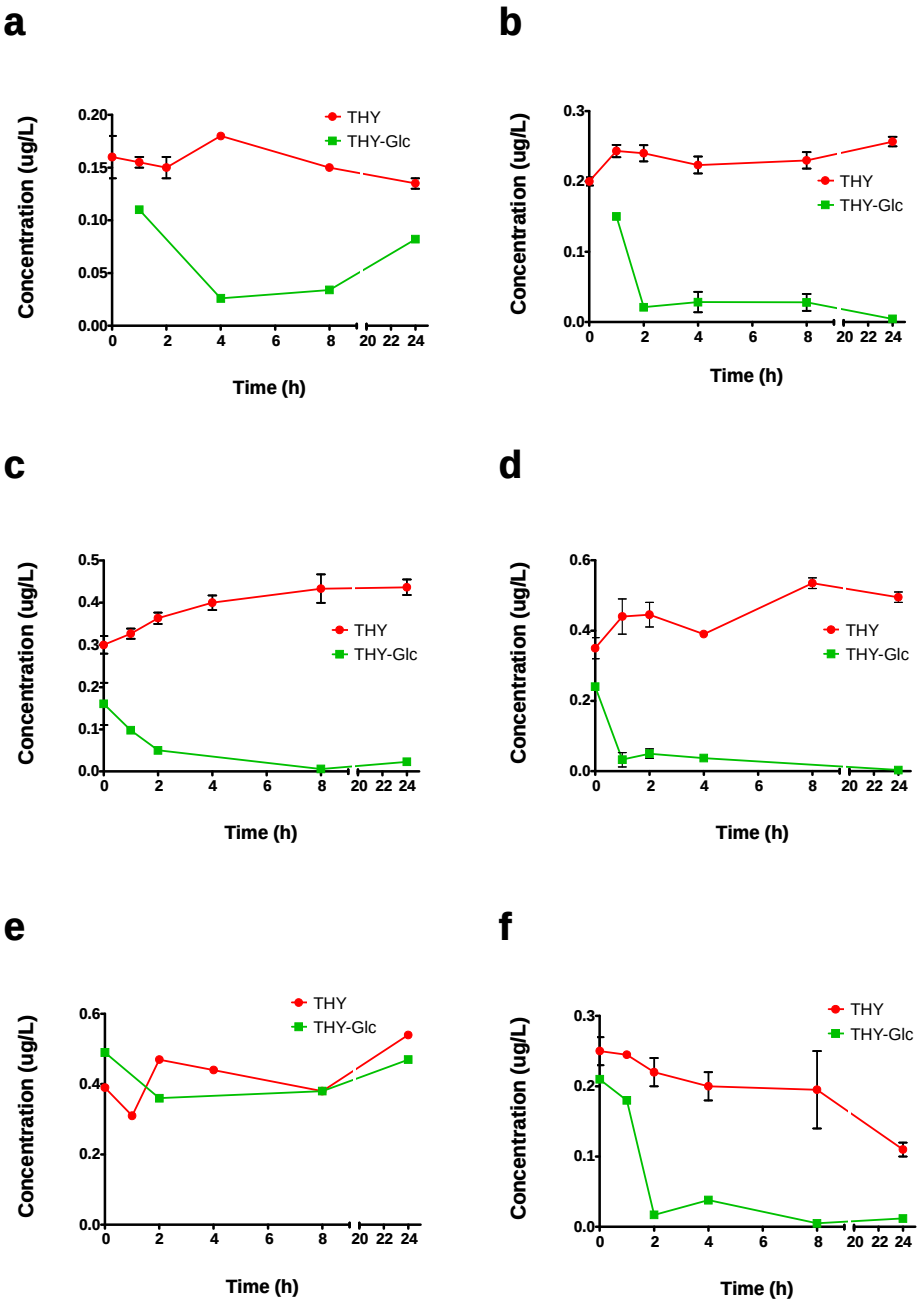

Figure 6.5 Concentrations of thyroxine (THY) and thyroxine glucuronide (THY-Glc) during various treatments of wastewater in benchtop bioreactors. A) Primary effluent no air; B) Primary effluent with air; C) Secondary effluent no air; D) Secondary effluent with air; E) Autoclaved kill control; F) Primary effluent with aerated waste activated sludge. Values are means and error bars are standard error of the mean.

Thyroxine-glucuronide deconjugated across all live treatments (Figures 6.5a-d and 6.5f), which most likely explains the increase in thyroxine concentrations during secondary treatments. Half-lives for thyroxine-glucuronide ranged from 0.7 to 1.9h by the 2h timepoint, suggesting that the deconjugation of thyroxine-glucuronide occurs at rates masking the potential concurrent biodegradation, if any, of the parent thyroxine. Given the $\log K_{OW}$ of thyroxine (4.1) and thyroxine-glucuronide (2.65)[21], appreciable amounts of both should be associated with the suspended solids (highlighted below)[21] . The greater increase in thyroxine after 24h in secondary waters (Figures 6.5c and 6.5d) is most likely explained by the 10-fold decrease in concentration of suspended solids (means=116 mg/L primary and 14 mg/L secondary). Thus, as thyroxine-glucuronide was deconjugated, the lack of sorption to solids helped drive up aqueous concentrations.

6.3.5. *Suspended Solids*

Based on our previous field study[21], we expected to measure appreciable quantities of acetaminophen, propranolol, sulfamethoxazole, thyroxine, and thyroxine-glucuronide associated with the suspended solids levels present (Table A4.4). Suspended solids at both 0h and 24h were extracted and quantified to determine whether sorption played an important role during various treatments. Sufficient quantities of suspended solids were extractable only from the two primary treatments. Nonetheless, two facts became apparent: first, concentrations associated with solids of all five compounds increased in the absence of aeration; second, concentrations associated

with solids of acetaminophen, propranolol, and sulfamethoxazole all decreased in the presence of aeration.

Particulate concentrations of acetaminophen, propranolol, and sulfamethoxazole (Table A4.4) significantly increased from 3.0 to 10.5, 0.073 to 0.27, and 0.35 to 0.46 μg/g solids (all dry weight) respectively without aeration. With aeration, acetaminophen and propranolol also significantly decreased from 6.8 to 2.0 μg/g, and 0.061 to 0.022 μg/g respectively, while sulfamethoxazole stayed constant at 0.55 μg/g. Sulfamethoxazole is known to be resistant to degradation (i.e. <5%) in WWTP solids in less than 24h[47]. Propranolol levels are in agreement with quantities sorbed to digested sludge (0.06 μg/g) elsewhere[43]. This observation strongly suggests that in the presence of WAS, the decrease in both aqueous and particulate-associated pharmaceutical concentrations are greater due to microbiological activation and degradation[38]. With lower microbial activity, sorption becomes a more important factor in removal of these compounds from the aqueous phase[38]. Overall, both sorption and biodegradation are important in lagoons or receiving waters. In a WWTP environment, though, biodegradation can dominate the removal of acetaminophen, propranolol, and sulfamethoxazole.

Thyroxine and thyroxine-glucuronide concentrations (Table A4.4) increased in both primary treatments irrespective of aeration. After 24h, thyroxine concentrations increased 20-fold from 0.04 to 0.8 μg/g solids (dry weight), and 10-fold from 0.065 to 0.64 μg/g solids (dry weight) in non-aerated and aerated treatments respectively. Thyroxine-glucuronide levels also increased from <LOQ to 0.026, and <LOQ to 0.0002 μg/g solids (dry weight) respectively during non-aerated and aerated treatments respectively. These levels are in the same range as our previous field study[21]. Thus, aqueous concentrations of thyroxine should only significantly

increase in the absence of appreciable masses of suspended solids, otherwise resultant deconjugated thyroxine-glucuronide will tend to sorb to the solid phase.

6.4 Conclusions

In summary, under oxic conditions (similar to surface waters) acetaminophen and acetaminophen sulfate were steadily removed. Moreover, both compounds were extensively (>99%) removed in the presence of activated sludge (increased bacteria). Both propranolol and propranolol sulfate appeared to persist environmentally regardless of treatment, suggesting that this sulfate conjugate could be an alternative source of propranolol. Sulfamethoxazole, sulfamethoxazole glucuronide, and *N*-acetylsulfamethoxazole were all moderately attenuated regardless of treatment. However, in the presence of activated sludge there appeared to be distinct deconjugation of both conjugates which lead to a concomitant increase of the parent sulfamethoxazole prior to subsequent removal over time. Thyroxine glucuronide most likely deconjugated and contributed to marginal increases in thyroxine concentrations over time under conditions of lesser suspended solids. However, under increased suspended solids conditions, potential simultaneous mechanisms could have been at play: both thyroxine and thyroxine glucuronide partitioned to suspended solids over time; also aqueous thyroxine concentrations appear to stay constant over time. Thus, it is plausible that as thyroxine glucuronide was deconjugated, regenerated thyroxine increased the aqueous concentration, and shifted the chemical gradient to facilitate sorption to the solids.

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Chapter 7

Selective Serotonin Reuptake Inhibitors and β-blocker Transformation Products Risk of Toxicity to Aquatic Organisms in Wastewater Effluent-Dominated Receiving Waters

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The author of this dissertation researched all topics, interpreted the peer-reviewed literature, and wrote the majority of the manuscript. Jonathan K. Challis was responsible for the exposure and effects research regarding the beta-blocker component to the manuscript. Mark L. Hanson was responsible for the risk assessment algorithm and procedure in accordance with the USEPA.

7. Abstract

A probabilistic ecological risk assessment was conducted for the transformation products (TPs) of three β-blockers (atenolol, metoprolol, and propranolol) and five selective serotonin re-uptake inhibitors (SSRIs; citalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline) to assess potential threats to aquatic organisms in effluent-dominated surface waters. To this end, the pharmacokinetic literature, the University of Minnesota's Biocatalysis/Biodegradation Database Pathway Prediction System aerobic microbial degradation software, and photolysis literature pertaining to β-blockers and SSRIs were used to determine their most likely TPs formed via human metabolism, aerobic biodegradation, and photolysis, respectively. Monitoring data from North American and European surface waters receiving human wastewater inputs were the basis of the exposure characterizations of the parent compounds and the TPs, where available. In most cases, where monitoring data for TPs did not exist, we assumed a conservative 1:1 parent to TP production ratio (i.e., 100% of parent converted). The USEPA's EPISuite and ECOSAR v1.11 software were used to estimate acute and chronic toxicities to aquatic organisms. Hazard quotients, which were calculated using the 95^{th} percentile of the exposure distributions, ranged from 10^{-11} to 10^{-3} (i.e., all significantly less than 1). Based on these results, the TPs of interest would be expected to pose little to no environmental risk in surface waters receiving wastewater inputs. Overall, we recommend developing analytical methods that can isolate and quantify human metabolites and TPs at environmentally relevant concentrations to confirm these predictions. Further, we recommend identifying the major species of TPs from classes of pharmaceuticals that could elicit toxic effects via specific modes of action (e.g., norfluoxetine via the serotonin 5-HT $_{1A}$ receptors), and conducting aquatic toxicity tests to confirm these findings.

7.1. Problem Formulation

7.1.1. Introduction

Pharmaceuticals are designed to elicit strong biological responses at low doses in their target organism. Their presence in aquatic environments globally has led to concerns regarding the potential for adverse toxicological effects on non-target organisms (fish, invertebrates, microbial communities, plants, etc.) [1]. In addition, human phase I detoxification of these compounds promotes their water solubility further for either phase II conjugation or excretion. As a result, humans excrete both parent and metabolized drug, which largely enter aquatic environments via wastewater effluent, and contribute to the pseudo-persistence of pharmaceuticals in the environment [2]. Within wastewater treatment plants (WWTPs), processes by which bacterial enzymes can either further biodegrade these parents and metabolite compounds, or deconjugate metabolites back into the parent compound can occur [3]. In addition to the biotransformation of these compounds, abiotic processes (e.g., photolysis, hydrolysis) have the potential to transform parent compounds and metabolites into either pharmacologically active, or inactive, transformation products (henceforth referred to as TPs), either of which may or may not lead to complete mineralisation [4].

7.1.2. Pharmaceutical Prevalence

This study is specifically interested in the possible ecological risks associated with the TPs of β-blockers and selective serotonin reuptake inhibitors (SSRIs- citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline). β-Blockers (atenolol, metoprolol, propranolol) are adrenergic receptor antagonists, and are typically used to prevent further cardiac arrhythmias by blocking endogenous epinephrine [5]. SSRIs are antidepressants that are used to treat anxiety and

depression by regulating levels of the neurotransmitter serotonin [6]. According to the United States National Centre for Health Statistics (NCHS), 48% of Americans used at least one prescription drug in 2007–2008, and 31% used two drugs [7]. Moreover, it was estimated that >88% of Americans over 60, regardless of sex or race, were taking at least one prescription in 2007-2008. Specifically, it is estimated that 1-in-3 Americans have hypertension (American Heart Association 2013), making β-blocking agents important and widely used therapeutic drugs [5, 8]). In fact, the β-blocker metoprolol was 77th in the top 100 drugs in 2013 by sales [9]. SSRIs were the third most prescribed class of drug in the US for adolescents aged 12-18 at 4.8%, and the number one class prescribed in adults 20-59 at 10.8% [7]. Likewise, in Canada from 2005-2009, SSRI prescriptions written by pediatricians increased by 39%, while SSRI drug prescriptions by all specialists for children and adolescents increased by 44% over the 5-year period, with fluoxetine being the most commonly recommended and dispensed SSRI [10].

SSRIs and β-blockers are commonly found in effluent-impacted surface waters, especially rivers, largely as a result of human excretion and lack of targeted removal in WWTPs [11-14]. Furthermore, the advancement in analytical technologies has facilitated the detection of both parent drugs and their biotic and abiotic transformation products (TPs), which can be more persistent and/or toxic than their parent. This is illustrated by the carcinogenic TP acridine, formed from the anticonvulsant carbamazepine and found at levels rivalling that of the parent. For example, it has been demonstrated experimentally that following UV irradiation for up to 90 minutes, levels of carbamazepine decreased and acridine increased proportionally, then both compounds stabilised in approximately equal amounts [15]. Additionally, this TP can be found environmentally in WWTP effluent, receiving waters, and finished drinking water [16]. While acute effects to aquatic biota are considered unlikely at environmentally relevant concentrations,

chronic sub-lethal effects (e.g., on feeding habits, socialisation, predation anxiety, fecundity) of SSRIs, β-blockers, and their TPs could result in ecosystem impairment, depending on trophic position and integration within the ecosystem [2, 17]. Moreover, SSRIs are agreed to elicit effects in innervated aquatic biota through the serotonin 5-HT $_{1A}$ receptors [18]. Under environmentally-relevant levels of fluoxetine (i.e. 80 µg/L) *Daphnia magna* showed increased efficiency in catabolism (reduced carbohydrate stores) and increased oxygen consumption under oxic conditions. However, under anoxic conditions females produced more, but smaller offspring, showed earlier maturation, and shortened survival times [19]. These results were representative of the administration of fluoxetine, and independent of food levels. Moreover, anoxic conditions are likely to be periodically encountered under realistic environmental conditions. Thus, it would be informative to consider TPs of compounds that could elicit adverse effects under sub-lethal conditions in ecological risk assessments.

The literature searched for in this environmental risk assessment (ERA) was typically representative of worst-case scenarios of exposure to these compounds (parent or TP). The majority of exposure data found was for WWTP or lagoon effluent, and the remainder was for proximal (within 1 km of release) receiving waters. If non-target toxic effects of TPs were to be seen in biota, it would be most likely in effluent-dominated systems where observed environmental concentrations are greatest. 'Worst-case' exposure data for parent compounds, and a 1:1 full conversion to TP was assumed. Based on pharmacological literature of SSRIs and β-blockers, this 1:1 conversion ratio is approximately a 10 fold overestimation of TP exposure, at a minimum. This is especially true considering a threshold of relevancy for human metabolites was set at 5%. This overestimation helps establish a conservative first-tier risk assessment that does not necessitate the application of an uncertainty factor.

Particular attention must be paid when assessing actual 'exposure' levels of active pharmaceutical ingredients (API) to non-target organisms. It has been shown through an extensive critical review by Daughton and Brooks (2010)[20] that API do not necessarily act like other organic pollutants (e.g. legacy contaminants such as PCBs). For instance, some API can be taken up by active transport (e.g. organic anion or cation transporters) in addition to passive diffusion. Thus, the uptake mechanisms of the TP, in addition to a variety of other factors, will contribute to the actual internal dosage. Moreover, the physiological activity of a particular API will dictate the potential toxicity to a particular non-target organism, as well as possible cumulative effects at a population level [20]. Specific modes of action of pharmaceutical TPs on physiological receptors then become more relevant than narcosis when attempting to assess the risk posed to aquatic biota. Moreover, it is of particular importance to note that particular endpoint effects are a function of the sensitivity of the non-target or test organism to the particular API. Other sub-lethal effects not routinely recognised by standard test protocols could potentially be found at lower exposure levels than the standard ecotoxicological tests (e.g. fathead minnow feeding behaviour more sensitive to fluoxetine than 7 day juvenile growth) [20]. Therefore, caution should be exercised when interpreting hazard quotients (HQs) resulting from risk assessments conducted using less sensitive toxicity assay endpoints, such as those possibly used in Ecological Structure Activity Relationships (ECOSAR).

7.1.3. Risk Assessments of β-Blockers and SSRIs

Carlsson *et al.* (2006)[21] conducted an ERA for usage patterns in Sweden that included the β-blockers atenolol and metoprolol. The resultant HQs were found to be much less than 1 (e.g., metoprolol = 0.0034). Gabet-Giraud *et al.* (2014)[22] reported that out of 10 β-blockers analysed at

15 effluent-impacted sites along the Lyon River, only propranolol had HQ values above 1 (5 sites: HQ values ranged from 1.7- 34.2). The authors calculated these values based on measured concentrations and a chronic predicted no effect concentration (PNEC) value for fish of 10 ng/L. Silva *et al.* (2014)[23] studied SSRIs and used ECOSAR to generate PNEC values for green algae and fish, applying an uncertainty factor (UF) of 1000, and used experimental data for daphnids from Henry *et al.* (2004)[24] with a UF of 10. They only detected citalopram and paroxetine in their WWTP effluent, and determined that although algae appeared to be the most sensitive, their HQ values were still under 1 (0.59 and 0.31 respectively). The variability in results seen in these ERAs is possibly due to the differences in exposure data used to construct HQ values and the application of UF values. A major benefit of constructing exposure distributions is to minimise the uncertainty of random point estimates, and allow predictions around probability of exceeding certain exposure levels. For these reasons, our ERA used this approach.

However, to our knowledge, only a very limited number of studies have assessed the risk of pharmaceutical TPs in the aquatic environment [25]. This speaks to the complexities and resources involved in identifying and measuring TPs for exposure assessment, in addition to establishing effects data. Not surprisingly, the focus has been on ERAs conducted on parent pharmaceuticals [26-29]. Still, some TP-focused work has been conducted. For example, Escher *et al.* (2006)[30] used a mode-of-action-based test and QSAR approach to assess the non-target effects and hazards posed by the same three β-blockers of interest here (and the β-blocker sotalol) and their human metabolites. The authors used experimental and literature data of the parent β-blockers to derive estimated hazards for the human metabolites, based on two underlying assumptions dealing with realistic and worst-case scenarios [30]. The

They calculated a series of toxicity ratios (toxic potential- TPmax, TPmin, and relative potency- RPmax and RPmin). RPmax and RPmin were used to estimate a worst-case scenario (using a specific mode of action) and a realistic scenario (baseline toxicity), respectively, of the human metabolites' toxicities, as compared to the parent drug. The TPmax and TPmin were then calculated as sums of the RP values along with the fractions of parent drug and metabolites excreted. Thus, TPmax values represented a specific mode of action toxicity, and TPmin represented 'baseline' toxicity, as estimated by an employed structure-activity relationship (SAR). A TP >1 indicates that the metabolite mixtures released into the environment are more toxic than the parent, and TP <1 means less toxic than the parent. The highest TPmax and TPmin for metabolites were 0.34 and 0.12 for propranolol, 0.92 and 0.18 for metoprolol, and 0.97 and 0.96 for atenolol. Escher et al. (2006)[30] concluded that while the work represents an initial low-tier hazard assessment, the findings suggest that human pharmaceutical metabolites are likely to be important factors to consider in environmental risk assessment.

This assessment by Escher *et al.* (2006)[30] notwithstanding, the toxicity ratios were calculated by several assumptions and certain experimental values. An assumption that algae are the most susceptible organism to β-blockers was made, but this may not be the most relevant adverse outcome pathway to ecosystem-level effects [31]. It may also lack conservatism as the algal toxicity values were calculated using experimental 24-h chlorophyll fluorescence EC50 values, which may not be representative of more sensitive algal responses (e.g., growth rates). Besse *et al.* (2008)[32] took a more qualitative approach to the risk assessment of a large number of pharmaceutical metabolites, including our β-blockers and SSRIs of interest. The authors used known excretion, dosage, and consumption rates, populations, and waste treatment removal, along-side known phase I and II human metabolism of parent pharmaceuticals in order to

estimate predicted environmental concentrations (PEC) of their 'important' metabolites [32]. While hazard quotients were calculated for all parent compounds, only qualitative statements regarding potential risk of certain human pharmaceutical metabolites were made, given a lack of effects data. Estimated HQ values were 0.54 for propranolol and 0.22 for sertraline, using PEC values and PNEC values for *H. azteca, and C. dubia* respectively, with an applied UF of 10.

Recently, Escher and Fenner (2011)[25] proposed a framework for dealing with potential risk from TPs in a risk assessment context for aquatic ecosystems. Their threshold of 10% TP production is adopted from the OECD Guideline 308 for testing of degradation TPs in aquatic sediment systems [33]. Escher and Fenner classified existing approaches for transformation product assessment as either exposure- or effect-driven. In the exposure-driven approach, TPs are to be identified and quantified by chemical analysis followed by effect assessment. In the effect-driven approach, a reaction mixture undergoes toxicity testing. If the decrease in toxicity parallels the decrease in parent compound concentration, the transformation products are considered to be irrelevant. Only when toxicity increases or when the decrease is not proportional to the parent compound concentration are the TPs identified.

A current review of the published experimental toxicity data is compared to those generated by ECOSAR (Table 7.5). For example, for propranolol acute 72-96h EC50 (growth) for green algae was 0.7-7.4mg/L [34, 35] when compared to ECOSAR value of 1.9mg/L. Also, chronic *C. dubia* 27d LOEC (reproduction) was 0.25mg/L [36] compared to an ECOSAR value of 0.23mg/L. For fluoxetine, an acute 48h LC50 of 0.234mg/L was seen for *C. dubia* [37], compared to the ECOSAR value of 0.18mg/L. For sertraline, an acute 96h LC50 of 0.38mg/L was seen for *O. mykiss* [38], compared to the ECOSAR acute 48h LC50 value of 0.408mg/L.

Herein we have conducted an ecological risk assessment (ERA) of both parent compounds and respective biotic/abiotic transformation products for three commonly occurring β-blockers (propranolol, metoprolol, and atenolol) and five SSRIs (citalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline). We built upon the work of others, such as Sanderson *et al.* (2003)[26] who conducted hazard quotient assessments, and Escher and Fenner (2011)[25] who outlined a TP production threshold of 10% as significant in the environment. The primary objective of our work was to establish a baseline, Tier 1 approach to estimate potential hazards posed to aquatic organisms by pharmaceutical TPs, in lieu of any substantial experimental data.

Unique to this ERA was the inclusion of aerobic biodegradation and photolysis TPs. These were seen as important processes by which toxic compounds could be formed, and potentially elicit toxic effects on aquatic biota. The photolysis literature was searched for plausible mechanisms on SSRIs and β-blockers to elucidate which TPs would be environmentally relevant. EAWAG's (formerly the University of Minnesota's) Biocatalysis/Biodegradation Database Pathway Prediction System (UM-BBD-PPS) aerobic microbial degradation software was used to estimate relevant biodegradation TPs for risk assessment. This program is unique in that it qualitatively identifies biodegradation TPs as likely or not based on existing chemical "rules" of aerobic biodegradation.

7.2. Methods

This ERA used a first-tiered approach of simplified, highly conservative probabilistic estimations of hazard quotients.

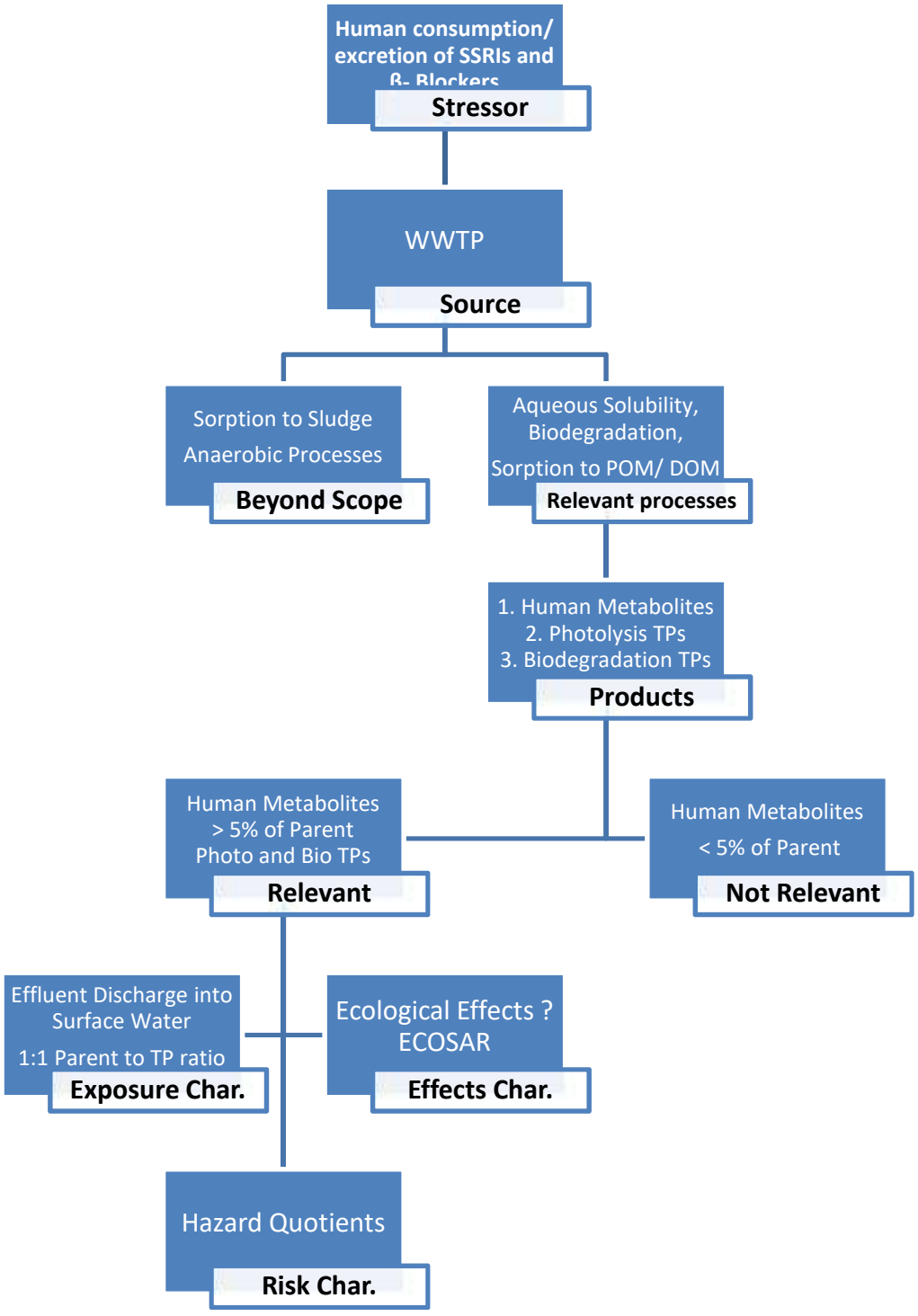


Figure 7.1 Conceptual model of probabilistic environmental risk assessment.

7.2.1. Stressor Characterisation

The major difficulty in the conductance of an ERA for TPs is the almost complete lack of data – be it occurrence, exposure, toxicity, or physicochemical. Therefore, we must rely extensively on the extrapolation of data, from the parent compound to TP, as well as modeling software, to estimate exposure and toxicity data and ultimately conduct a risk analysis for these compounds. However, actual exposure and toxicity data for the TP norfluoxetine, a metabolite of fluoxetine, was available and utilised [39, 40]. As outlined in Figure 7.1, our approach involved three steps. First, the United States Environmental Protection Agency (USEPA) EPISuite software was used to predict the physicochemical properties, namely log K_{OW} and solubility (see Table 7.1), of the TPs identified as toxicologically important. Second, exposure data for TPs was estimated from North American and European monitoring data of the parent compounds in WWTP effluent and receiving waters. TP identification was predicted using human metabolite yields and plausible transformation mechanisms (e.g., biodegradation, and photolysis). Exposures from WWTPs were used because they represented a worst-case exposure scenario for aquatic biota. Conservatism was enhanced by assuming a 1:1 parent compound to TP ratio.

Table 7.1 Physicochemical properties related to environmental fate of three β-blockers and five selective serotonin reuptake inhibitors.

Compound	Molar mass (g/mol)[a]	Solubility (mg/L)[b]	pK_a	$LogK_{ow}$	K_D (L/kg)[e]	k_{bio} (L/d g OC)[g]	Photolysis $t_{1/2}$ (d)[h]	H (atm m^3/mol)[a]
Propranolol	295.8	62.0	9.24[c]	3.48[c]	4.55-12	0.39 ± 0.07	1.5	$8.0x10^{-13}$
Metoprolol	267.3	16900	9.70[c]	1.88[c]	1.75-7.3	0.58 ± 0.05	78	$1.4x10^{-13}$
Atenolol	266.3	13300	9.55[c]	0.16[c]	1.13-3.1	0.69 ± 0.05	34	$1.37x10^{-18}$
Citalopram	324.4	31.09	9.50[d]	3.74[b]	8798-42579[f]	NA	53-462[i]	$2.69x10^{-11}$
Fluoxetine	309.3	60.28	8.70[d]	4.65[b]	785-12546[f]	NA	44-99[i]	$8.90x10^{-08}$
Fluvoxamine	318.3	22.22	9.39[d]	3.09[b]	60-649[f]	NA	0.57- 29[j]	NA
Paroxetine	329.4	35.27	9.90[d]	3.95[b]	131-5067[f]	NA	0.67[j]	$1.78x10^{-12}$
Sertraline	306.2	3.52	9.48[d]	5.29[b]	147-787[f]	NA	4-11[i]	$5.10x10^{-08}$

[a]PHYSPROP Database – www.srcinc.com. [b]EPI Suite™ via ECOSAR. [c]Escher *et al.* (2006)[41]. [d]Lajeunesse *et al.* (2008)[42]. [e]Ramil *et al.* (2010)[43]. Lower and upper range represents two different types of sediments. [f]Kwon and Armbrust (2008)[44] Lower and upper range represents two different types of sediments. [g]Maurer *et al.* (2007)[45]. k_{bio}=pseudo-first-order biodegradation rate in sludge. OC=organic carbon (in sludge). [h]Liu *et al.* (2007)[46]. Direct photolysis sunlight half-lives in June, USA, 40 °N. [i]Styrishave *et al.* (2011)[47]. [j]Black and Armbrust (2007)[48]

Effects data for TPs were acquired from the USEPA ECOSAR software v1.11. Data included acute and chronic toxicities to aquatic organisms, namely fish, invertebrates, and algae. Our assessment threshold for no significant hazard of TPs of β-blockers and SSRIs in the aquatic

environment was an HQ value under 1 without applying a UF. Chronic toxicities were anticipated to be of greater ecological relevance than acute due to the constant low-level environmental input. Therefore, the HQ values calculated for chronic toxicity estimations using ECOSAR would reflect NOEC values for green algae, daphnids, and fish.

7.2.2. Environmental Fate and Behaviour

Three β-blockers and five SSRIs were chosen as the focus for this work. While each drug class represent structurally and pharmacologically related compounds, the parent compounds both within and between classes present markedly different physicochemical properties (e.g., $LogK_{ow}$ – Table 7.1), fate data (e.g., photolysis $t_{1/2}$ – Table 7.1), and TPs (Fig. S1 – S8). These contaminants possess a wide range of hydrophobicity (Log $K_{OW} \approx$ -1.5 – 5.5, and low Henry's law constants), suggesting that these compounds could be found predominantly in the water compartment, and may accumulate in lipid tissues and/or sorb to organic carbon (OC) for the more hydrophobic chemicals. Sorption is supported by K_D values for all of the SSRIs (K_D = 60-43000; see Table 7.1), suggesting that they will sorb significantly to WWTP sludge, and natural sediments and particles containing OC. For instance, sludge can vary in OC content, depending on the influent composition and treatment process (e.g., 125 mg carbon/L influent[49]; 459 g carbon/kg sludge dry weight [50]). Additionally, these chemicals are bases and predominantly protonated at environmental pH values, thus they may also sorb to negatively-charged OC. Photolytically, propranolol, fluvoxamine, and paroxetine are known to be significantly more photo-labile than the other compounds, with an approximate direct photolysis sunlight half-life of 1.5, 0.57-29, and 0.67 days, respectively (Table 7.1).

Overall, beta blockers will have moderate water solubility, low lipid solubility, and no

bioaccumulation potential. Also, given low to moderate K_D values, they could associate mildly with sediment or activated sludge, limiting their bioavailability to organisms in the water phase. SSRIs are most likely found in the sediment of aquatic ecosystems and the activated sludge in WWTP, based on their very high K_D values [2]. They may bioaccumulate based on their moderately high K_{OW} values. Overall, direct photolysis will be more likely for propranolol, fluvoxamine, and paroxetine. Sertraline, citalopram, and paroxetine seem to have a lower propensity to biodegrade aerobically, based on UMBBD software (see Figures A5.4- A5.8).

7.2.3. Exposure Characterisation

7.2.3.1. Transformation Product Identification

The method for predicted metabolites has been employed for active pharmaceutical ingredient submission requirements from 2002 onward as per OECD Guidelines 302 and 308. Berkner and Thierbach (2014)[51] conducted a review on chemical submissions following these degradation guidelines as of 2010. They found that of 33 studies that used the guideline-recommended radio-labelled analytes, in 70% of the studies, at least one TP is formed above 10 % of the originally applied dose, but in only 26% of the studies are all TPs identified. The evaluation also revealed that some TP of pharmaceutical active ingredients show a dissipation times (DT_{50}) 410 times longer compared to the parent compound (e.g., (val)sartan acid, formed from an antihypertensive compound valsartan).

This ERA focused on TPs formed via three general pathways: 1) human metabolism products (human-TPs), 2) microbial biodegradation products (bio-TPs), and 3) photodegradation products (photo-TPs). The pharmacological literature, the UM-BBD-PPS aerobic microbial degradation software, and photolysis literature were used to determine the most likely TPs

formed via these three fate processes, respectively.

When judging the importance of specific human metabolites to the overall drug burden, only those that are known or predicted to be formed at a yield of ≥5% were considered, a slight modification of the proposed threshold of 10% by Escher and Fenner (2011)[25] and the OECD guideline 308. This lower threshold added to the conservative nature of our approach, and ultimately only leads to the inclusion of a few additional TPs as a result.

Figures A5.1 – A5.8 depict the TPs formed for each β-blocker and SSRI, separated into the three pathways; human metabolism, biodegradation, and photodegradation. These pathways encompass the majority of transformation processes possible for human pharmaceuticals [12]. All β-blocker human metabolite structures, pathways, and data (Fig. A1.1- A1.3) are from Escher *et al.* (2006)[30]. SSRI human metabolites (Fig. A1.4- A1.8) were taken from the relevant pharmacological literature [52]. For predicting relevant bio-TPs, the UM-BBD-PPS program (http://umbbd.ethz.ch) uses known enzymatic and microbial driven reactions and developed microbial degradation rules to predict transformation products potentially down to mineralisation. As outlined in SI Appendix C, the software outlines pathways as unlikely (red arrows), neutral (yellow arrows), likely (light-green arrows), or very likely (dark-green arrows). Given the extensive pathways the UM-BBD-PPS program produced, only likely and very likely products through a maximum of two arrows were considered (i.e., representing two subsequent levels of biological transformation), starting with the parent compound. Human metabolites were also run through the UM-BBD-PPS program to assess any important bio-TPs formed with the human-TP as the starting compound. This is potentially an important inclusion given that these parent drugs are, in many cases, excreted from the human body largely as metabolites (Table 7.1). The restriction to only two green arrows made this exercise feasible in terms of the total

number of TPs to be assessed, while still including a significant and representative number of TPs.

Experimentally observed photodegradation products were used to create the photochemical degradation pathways for these drugs. Given the minimal amount of experimental data available pertaining to metoprolol photo-products [4], the experimental data for atenolol (Fig. S1) was used as a model to predict other metoprolol photo-products (Fig. S2). This assumption is considered appropriate given the structural similarities that exist between these two parent β-blockers. SSRIs have been shown to be resistant to direct photolysis primarily due to their high proportion of the parent compound being associated with sediments, sludge, or other organic matter [47], most likely through a mixture of ionic and hydrophobic interactions [44](see K_D and t½ values in Table 7.1). However, pharmacokinetic data show that the majority of SSRIs are excreted as TPs not parent compounds (parent proportions: citalopram 12%, fluoxetine 11%, fluvoxamine <4%, paroxetine <2%, and sertraline 0%) [52] and would be in the aqueous phase.

Exposure distributions were constructed for TPs assuming 100% of the parent compound is converted to each TP. This 1:1 parent to TP assumption serves both as a highly conservative estimate and a significantly simplified approach. It has been shown that levels of parent pharmaceuticals can actually be greater in WWTP effluents than influents through biotic and abiotic deconjugation (e.g., venlafaxine, sulfamethoxazole[53]). These examples speak to the complexities and uncertainties in assuming even a simple conservative ratio of 1:1 parent to TP. IUPAC structure names and SMILES for the human-, bio-, and photo-TPs were determined using the chemical drawing program Marvin Sketch (ChemAxon, Cambridge, MA)[54].

7.2.3.2. Occurrence Data

To characterise exposure to TPs in surface waters, the primary literature was searched for exposure data for parent compounds and human metabolites. The search was conducted from February 10 to July 17, 2014, covering the peer-reviewed literature published from 2002 to 2014, using Academic Search Premier EBSCO Host, Science Direct CRKN- Elsevier, ProQuest Research Library, Taylor and Francis Library CRKN, CRKN Wiley Online Library, and Web of Science database search engines, for all known parent compounds and human metabolites. The scope was limited initially to North American WWTP effluents, and proximal receiving waters (within 1 km downstream of release) to obtain a worst-case scenario cache. It became apparent that to increase the confidence of our exposure distributions (i.e., increase the number of exposure data points), it was necessary to expand the search to European waters. Thus, the exercise was repeated for this geographic region. Tables A1.3 and A1.4 provide these WWTP effluent and receiving water concentrations for the drugs of interest. Concentrations of TPs were determined based on measured environmental concentrations (MECs) of the parent β-blockers and SSRIs (see Tables A1.1 and A1.2), and assumed a 1:1 ratio for TP formed from the parent.

Probabilistic exposure distributions (Table 7.2) were generated using Microsoft Excel and SigmaPlot v.11. Exposure data was searched and TPs generated as above and the data was entered along with non-detects into an Excel file. Percent ranks of exposure were calculated using the Weibull equation, and the probabilities of percent ranks (y) at the 95^{th} percentile were calculated as per Solomon *et al*. (2000)[55] and Hanson and Solomon (2002)[56] (Figures 5.2-4). Data was pooled, and non- detects were included in percent rank calculation but not in the construction of the distribution.

Table 7.2 Graphical properties for the exposure distributions of β-blockers (Fig. 3.2) and SSRIs (Fig. 3.3 and 3.4). The 95th-centile was calculated as per Solomon *et al.* 2000[55]. These values are transformed into units of log and probit for the purposes of regression and back-transformations were used to calculate the intercepts. The distribution units were in mg/L. LOQ values are representative of the lowest quantifiable exposure data point from the literature values.

Compound	Slope	Intercept	LOQ (ng/L)	R^2	n	95th % (ng/L)
Atenolol	1.008	-1.861	> 1.8	0.955	66	3006
Metoprolol	0.808	-1.084	> 0.1	0.919	121	2380
Propranolol	1.152	-1.175	> 1.0	0.888	88	280
Citalopram	1.971	-3.882	> 3.4	0.911	56	637
Fluoxetine	1.389	-1.399	> 0.4	0.906	52	155
Norfluoxetine	1.961	-0.848	> 1.2	0.937	23	19
Fluvoxamine	1.806	-0.020	> 0.6	0.888	15	8
Paroxetine	1.743	-1.080	> 0.7	0.909	30	37
Sertraline	1.891	-1.513	> 0.8	0.942	29	47
Desmethylsertraline	2.190	-2.014	> 2.3	0.944	23	47

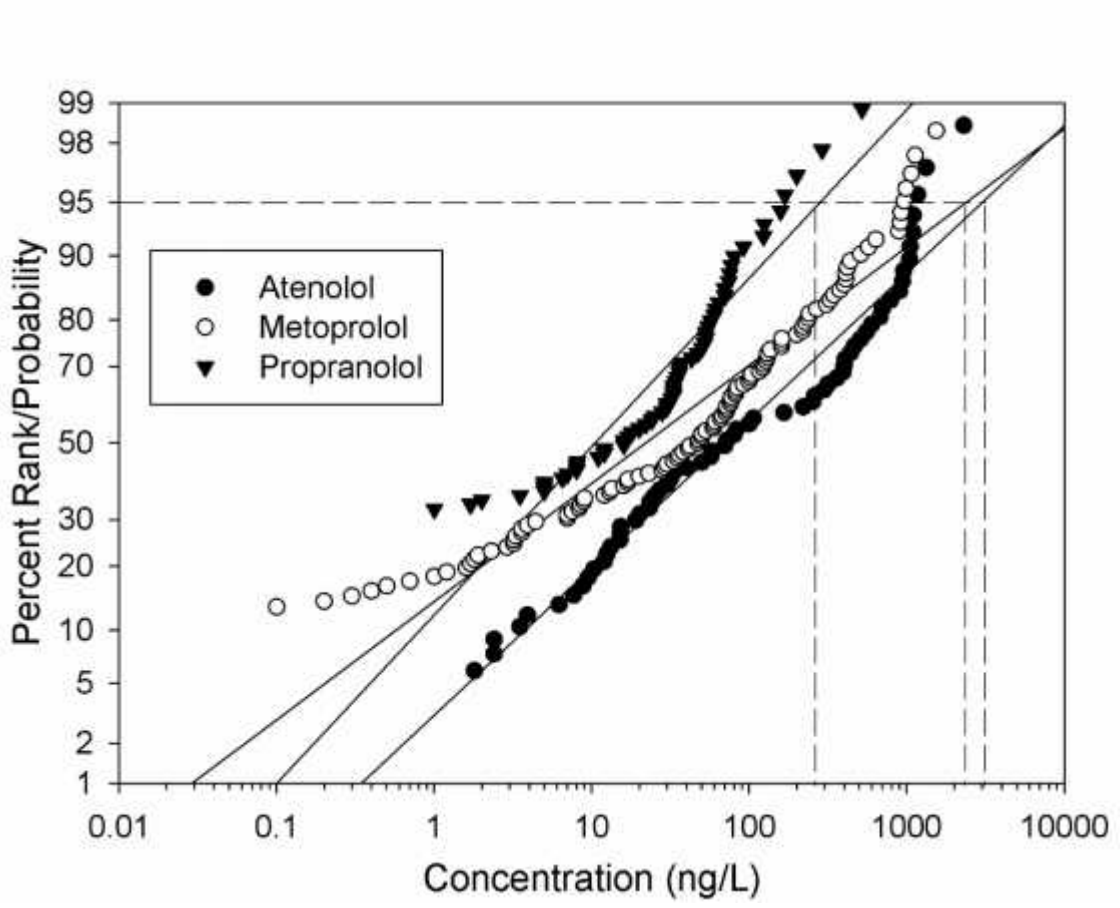


Figure 7.2 Exposure distribution of β-blockers atenolol (n=66), metoprolol (n=121), propranolol (n=88) in sewage effluent and receiving waters from North America and European countries 2005-2013. The distribution units were in ng/L, and the dashed lines represent the 95th percentile of exposure.

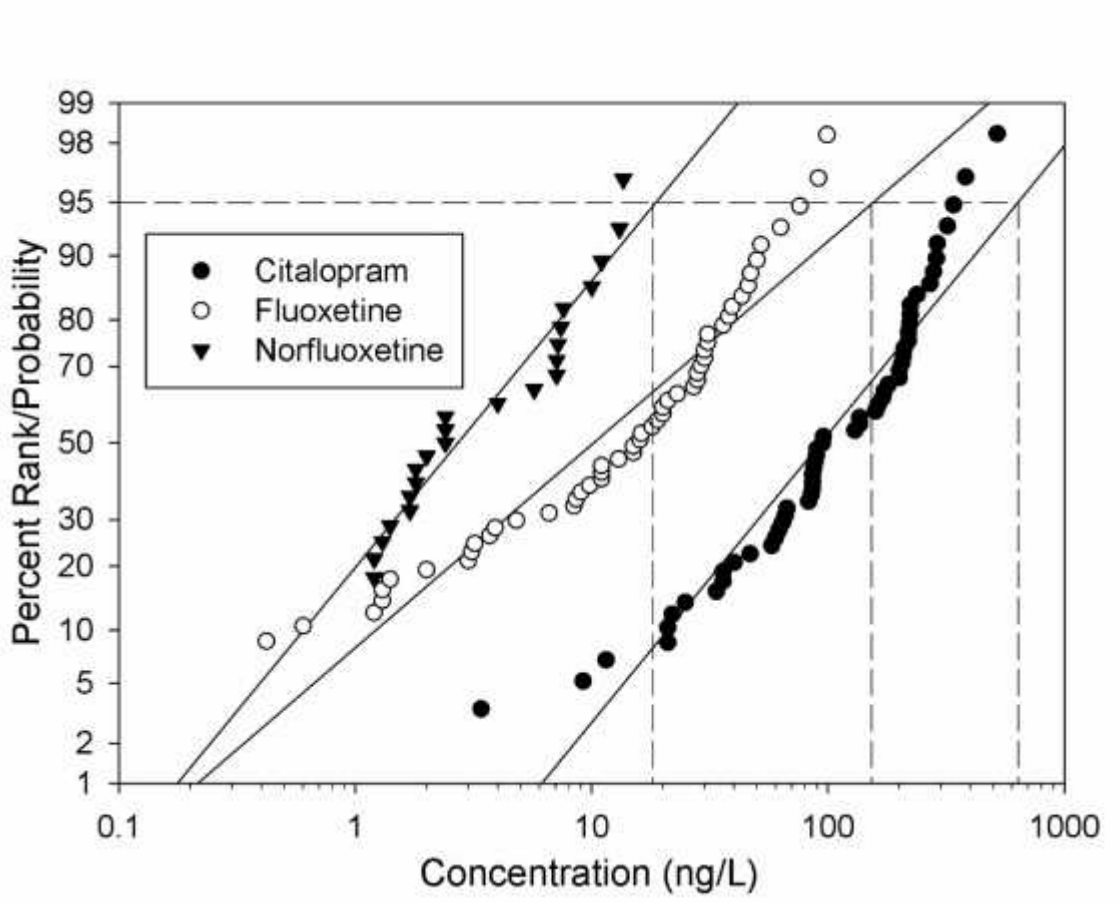


Figure 7.3 Exposure distribution of SSRIs citalopram (n=57), fluoxetine (n=56), and norfluoxetine (n=27) in sewage effluent and receiving waters from North American and European countries 2003-2012. The distribution units were in ng/L, and the dashed lines represent the 95th percentile of exposure.

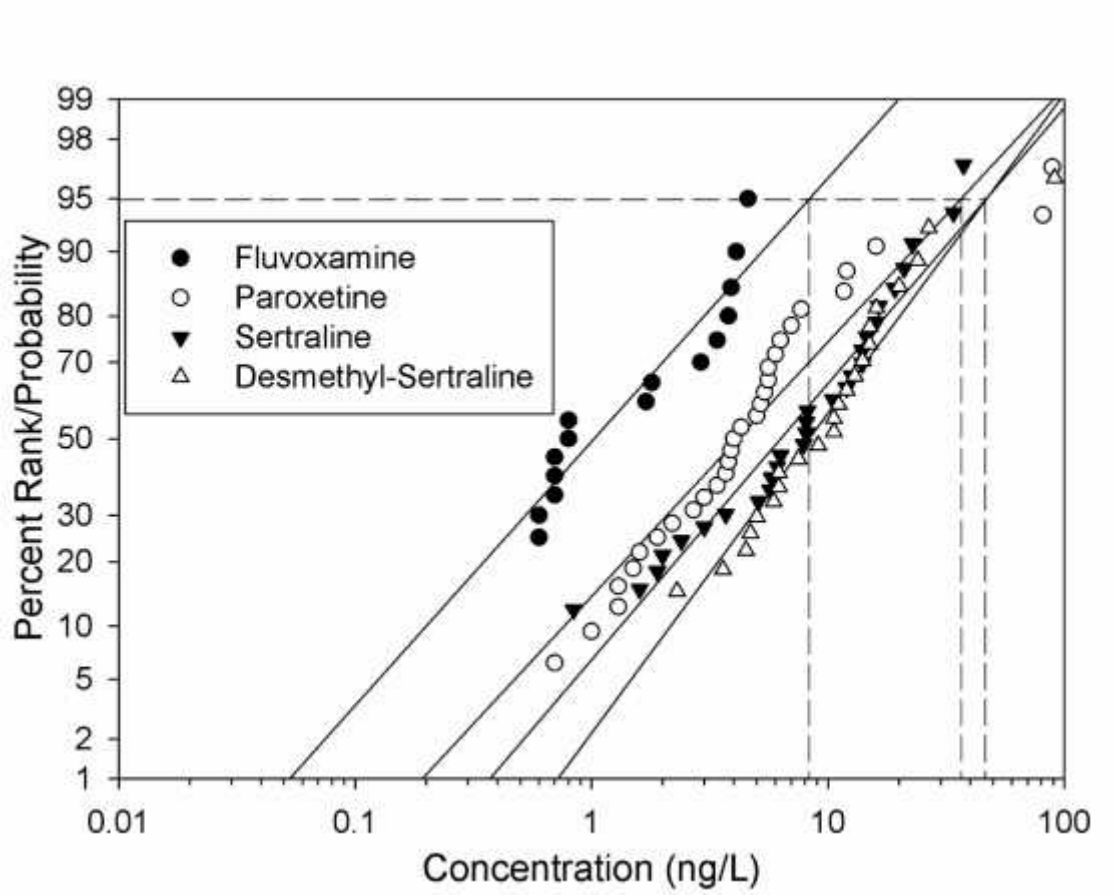


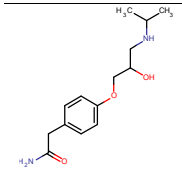
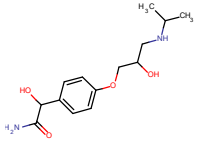
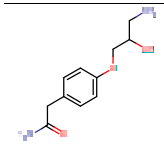
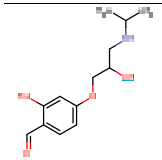
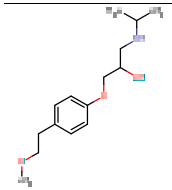
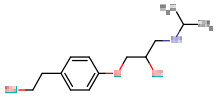
Figure 7.4 Exposure distribution of SSRIs fluvoxamine (n=19), paroxetine (n=31), sertraline (n=32), and *N*-desmethylsertraline (n=26) in sewage effluent and receiving waters from North American and European countries 2003-2012. The distribution units were in ng/L, and the dashed lines represent the 95th percentile of exposure.

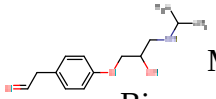
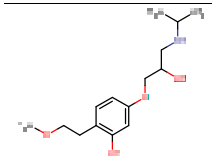
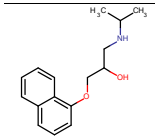
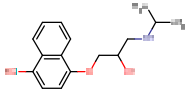
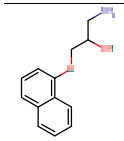
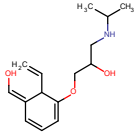
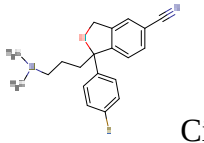
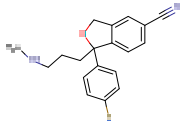
7.2.4. Effects Characterisation

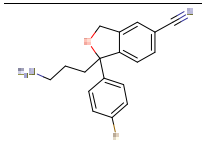
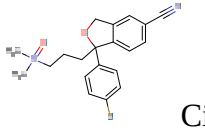
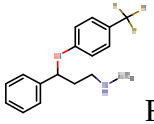
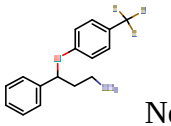
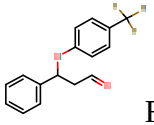
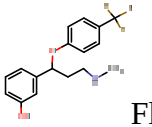
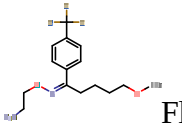
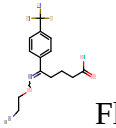
For our compounds of interest, literature on biological effects of pharmaceutical TPs is sparse and limited to the active metabolite norfluoxetine (i.e., to algae, protozoan, and crustacean) [39, 40]. Without experimentally measured effects data for these TPs, we employed modeling software to generate effects data. The ECOSAR Class Program is modelling software

designed by the USEPA for estimating organic compound toxicities based on structure [23, 26]. The program estimates a chemical's acute and chronic toxicity to aquatic organisms (e.g., fish, invertebrates, and aquatic plants) by using structure-activity relationships (SARs). ECOSAR predicts the toxicity of a chemical by identifying structural moieties that fit with a specific SAR in the ECOSAR database. Each chemical is placed in a specific ECOSAR class (e.g., aliphatic amine, amide, benzyl alcohol, vinyl/allyl aldehydes, etc.) and toxicity values are generated according to that specific SAR (Table 7.3). In cases where a given chemical is matched with multiple ECOSAR classes and it is not structurally or chemically obvious which class is most appropriate, the most sensitive ECOSAR class was chosen for our risk assessment in order to be conservative. The ECOSAR results for all 8 parent compounds were compared with the experimentally measured effects data to obtain a sense of the associated uncertainty in the ECOSAR predictions. Not all comparisons could be made due to the differences in experimental parameters as compared to those of ECOSAR (i.e test organisms, acute/chronic test, duration, bioassay endpoint).

Table 7.3 Toxicity and hazard quotient (HQ) data for a list of selected transformation products exhibiting the largest HQ values. The compound names and notation (e.g., Atenolol-U; photo) refer to Figures A5.1-A5.8.

Compound; Pathway	ECOSAR Class	Log Kow	Solubility (mg/L)	95th-centile exposure (ng/L)	ECOSAR chronic toxicity (ng/L)	95th-centile HQ	Probability of Exceedance
β-Blockers							
 Atenolol	Amides	-0.03	13300		1149000 (Fish)	0.0026	0.001
 Atenolol-B; Human	Amides	-0.41	1697	3007	1821000 (Fish)	0.0017	<0.001
 Atenolol-F; Bio	Amides	-1.40	25130		4110000 (Fish)	0.00073	<0.001
 Atenolol-U; Photo	Phenol Amines	1.44	52210		567000 (Daphnid)	0.0053	0.004
 Metoprolol	Aliphatic Amines	1.69	16900	2376	745000 (Daphnid)	0.0032	0.013
 Metoprolol-D; Human	Aliphatic Amines	1.40	14670		1027000 (Daphnid)	0.0023	0.008

 Metoprolol-D; Bio	Aliphatic Amines	0.97	35150		1773000 (Daphnid)	0.0013	0.004
 Metoprolol-Z; Photo	Phenol Amines	1.21	54520		812000 (Daphnid)	0.0029	0.011
 Propranolol	Aliphatic Amines	2.60	62		227000 (Daphnid)	0.0012	<0.001
 Propranolol-C; Human	Phenol Amines	2.12	10260	281	289000 (Daphnid)	0.00097	<0.001
 Propranolol-I; Bio	Aliphatic Amines	1.22	32730		1108000 (Daphnid)	0.00025	<0.001
 Propranolol-O; Photo	Vinyl/Allyl Alcohols	1.95	417		32000 (Daphnid)	0.0088	0.003
SSRI's							
 Citalopram	Aliphatic Amines	3.742	31.09	637	65000 (Daphnid)	0.0098	<0.001
 Desmethylcitalopram; human	Aliphatic Amines	3.531	57.01		82000 (Daphnid)	0.0078	<0.001

 Didesmethylcitalopram; Bio	Aliphatic Amines	3.065	172.1		142000 (Daphnid)	0.0045	<0.001
 Cit-*N*-Oxide;Photo	Aliphatic Amines	12.97	11.09		183000 (Daphnid)	0.00035	<0.001
 Fluoxetine	Aliphatic Amines	4.648	60.28	155	19000 (Daphnid)	0.0082	<0.001
 Norfluoxetine; human	Aliphatic Amines	4.182	35.7	18.7	34000 (Daphnid)	0.00055	<0.001
 Fluoxetine-E; bio	Aliphatic Amines	4.164	3.685	155	80000 (Fish)	0.0019	<0.001
 Fluoxetine-I; photo	Aliphatic Amines	4.168	92.97	155	38000 (Daphnid)	0.0041	<0.001
 Fluvoxamine	Aliphatic Amines	3.085	22.22	8.35	149000 (Daphnid)	5.6×10^{-5}	<0.001
 Flv Acid; human	Aliphatic Amines	-0.586	258.4		165000000 (Daphnid)	5.0×10^{-8}	<0.001

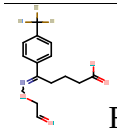
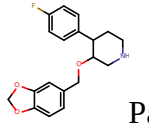
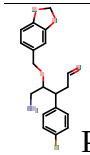
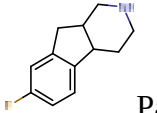
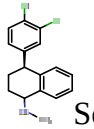
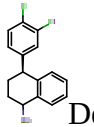
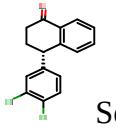
 Fluvoxamine-E; bio	Aliphatic Amines	2.595	146.7		2780000 (Daphnid)	3.0 x 10^{-6}	<0.001
 Paroxetine	Aliphatic Amines	3.954	35.27		50000 (Daphnid)	0.00073	<0.001
 Paroxetine-A; bio	Aliphatic Amines	2.602	403.7	36.6	300000 (Algae)	0.00012	<0.001
 Paroxetine-E; photo	Aliphatic Amines	3.042	1245		94000 (Daphnid)	0.00039	<0.001
 Sertraline	Aliphatic Amines	5.286	3.517	46.8	7000 (Fish)	0.0067	<0.001
 Desmethylsertraline; human	Aliphatic Amines	4.820	10.61	46.9	15000 (Daphnid)	0.0031	<0.001
 Sertraline-B; bio	Aliphatic Amines	5.007	0.732	46.8	65000 (Fish)	0.00072	<0.001

Table 7.4 Summary of calculated hazard quotient (HQ) values (range, median, mean) combined for each ECOSAR endpoint encompassing all TPs (human, bio, and photo) of each parent compound. 95^{th}-centile predicted exposure concentration (PEC) determined from exposure distributions of parent compounds. Also, herein is the comparison of each parent compound's hazard quotient value to the sum of their respective transformation products.

ECOSAR Endpoint	95th-centile PEC	Hazard Quotient				
		Range	Median	Mean	Parent	Sum of TPs
			Atenolol			
Fish 96-hr LC_{50}	3007 ng/L	$5.72x10^{-10}$ - $1.83x10^{-04}$	$8.27x10^{-08}$	$1.01x10^{-05}$	$1.35x10^{-06}$	$2.00x10^{-04}$
Daphnid 48-hr LC_{50}		$3.39x10^{-09}$ - $1.86x10^{-03}$	$5.71x10^{-08}$	$9.88x10^{-05}$	$4.80x10^{-07}$	$1.90x10^{-03}$
Green Algae 96-hr EC_{50}		$2.89x10^{-09}$ - $4.60x10^{-04}$	$5.82x10^{-06}$	$4.06x10^{-05}$	$6.62x10^{-05}$	$8.00x10^{-04}$
Fish Chronic		$1.10x10^{-09}$ - $5.07x10^{-03}$	$1.22x10^{-04}$	$8.78x10^{-04}$	$2.62x10^{-03}$	$1.67x10^{-02}$
Daphnid Chronic		$2.40x10^{-07}$ - $5.30x10^{-03}$	$2.85x10^{-06}$	$3.03x10^{-04}$	$3.26x10^{-05}$	$5.80x10^{-03}$
Green Algae Chronic		$1.45x10^{-08}$ - $2.56x10^{-03}$	$3.08x10^{-05}$	$1.83x10^{-04}$	$1.66x10^{-04}$	$3.50x10^{-03}$
			Metoprolol			
Fish 96-hr LC_{50}	2376 ng/L	$9.62x10^{-10}$ - $1.04x10^{-04}$	$2.26x10^{-06}$	$1.19x10^{-05}$	$3.00x10^{-05}$	$3.00x10^{-04}$
Daphnid 48-hr LC_{50}		$1.35x10^{-08}$ - $1.18x10^{-03}$	$7.41x10^{-06}$	$8.70x10^{-05}$	$3.00x10^{-04}$	$2.10x10^{-03}$
Green Algae 96-hr EC_{50}		$5.10x10^{-09}$ - $2.54x10^{-04}$	$5.75x10^{-06}$	$3.97x10^{-05}$	$3.00x10^{-04}$	$1.00x10^{-03}$
Fish Chronic		$2.18x10^{-09}$ - $1.62x10^{-03}$	$1.19x10^{-05}$	$1.36x10^{-04}$	$4.00x10^{-04}$	$3.30x10^{-03}$
Daphnid Chronic		$2.68x10^{-07}$ - $2.93x10^{-03}$	$1.01x10^{-04}$	$5.58x10^{-04}$	$3.19x10^{-03}$	$1.34x10^{-02}$
Green Algae Chronic		$2.48x10^{-08}$ - $1.51x10^{-03}$	$2.25x10^{-05}$	$1.56x10^{-04}$	$8.84x10^{-04}$	$3.80x10^{-03}$
			Propranolol			
Fish 96-hr LC_{50}	281 ng/L	$9.11x10^{-08}$ - $5.09x10^{-04}$	$2.07x10^{-06}$	$4.31x10^{-05}$	$1.39x10^{-05}$	$6.00x10^{-04}$
Daphnid 48-hr LC_{50}		$1.64x10^{-07}$ - $3.87x10^{-04}$	$4.37x10^{-06}$	$7.21x10^{-05}$	$1.09x10^{-04}$	$1.10x10^{-03}$
Green Algae 96-hr EC_{50}		$2.40x10^{-07}$ - $8.38x10^{-05}$	$1.72x10^{-06}$	$1.46x10^{-05}$	$1.51x10^{-04}$	$2.00x10^{-04}$
Fish Chronic		$9.56x10^{-07}$ - $8.02x10^{-03}$	$2.71x10^{-05}$	$7.82x10^{-04}$	$2.95x10^{-04}$	$1.17x10^{-02}$
Daphnid Chronic		$1.78x10^{-06}$ - $8.77x10^{-03}$	$2.29x10^{-05}$	$7.50x10^{-04}$	$1.24x10^{-03}$	$1.12x10^{-02}$

Green Algae Chronic		$9.63x10^{-07}$ - $3.83x10^{-04}$	$9.37x10^{-06}$	$7.32x10^{-05}$	$4.34x10^{-04}$	$1.10x10^{-03}$
Citalopram						
Fish 96-hr LC_{50}	637 ng/L	$2.18x10^{-06}$ – $1.43x10^{-04}$	$8.22 x10^{-05}$	$7.76 x10^{-05}$	$1.43x10^{-04}$	$4.78x10^{-04}$
Daphnid 48-hr LC_{50}		$3.49x10^{-06}$ – $9.77x10^{-04}$	$2.29 x10^{-04}$	$3.45 x10^{-04}$	$9.77x10^{-04}$	$1.78x10^{-03}$
Green Algae 96-hr EC_{50}		$3.14x10^{-06}$ – $1.77x10^{-03}$	$2.78 x10^{-04}$	$5.45 x10^{-04}$	$1.77x10^{-03}$	$2.59x10^{-03}$
Fish Chronic		$1.99x10^{-05}$ – $4.55x10^{-03}$	$1.33 x10^{-03}$	$1.61 x10^{-03}$	$4.55x10^{-03}$	$8.34x10^{-03}$
Daphnid Chronic		$2.73x10^{-05}$ – $9.80x10^{-03}$	$2.13 x10^{-03}$	$3.39 x10^{-03}$	$9.80x10^{-03}$	$1.74x10^{-02}$
Green Algae Chronic		$9.65x10^{-06}$ – $4.62x10^{-03}$	$7.50 x10^{-04}$	$1.14 x10^{-04}$	$4.62x10^{-03}$	$6.93x10^{-03}$
Fluoxetine						
Fish 96-hr LC_{50}	155 ng/L (All except) 18.7 ng/L (NorFlu)	$1.92x10^{-08}$ – $1.43x10^{-04}$	$8.95 x10^{-06}$	$3.83 x10^{-05}$	$1.43x10^{-04}$	$2.78x10^{-04}$
Daphnid 48-hr LC_{50}		$1.02x10^{-08}$ – $8.86x10^{-04}$	$3.40 x10^{-05}$	$1.60 x10^{-04}$	$8.86x10^{-04}$	$8.71x10^{-04}$
Green Algae 96-hr EC_{50}		$1.94x10^{-07}$ – $1.96x10^{-03}$	$2.34 x10^{-05}$	$2.82 x10^{-04}$	$1.96x10^{-03}$	$1.14x10^{-03}$
Fish Chronic		$3.26x10^{-07}$ – $6.20x10^{-03}$	$1.17 x10^{-04}$	$1.06 x10^{-03}$	$6.20x10^{-03}$	$5.41x10^{-03}$
Daphnid Chronic		$5.04x10^{-07}$ – $8.16x10^{-03}$	$2.16 x10^{-04}$	$1.35 x10^{-03}$	$8.16x10^{-03}$	$6.78x10^{-03}$
Green Algae Chronic		$5.86x10^{-07}$ – $4.70x10^{-03}$	$5.09 x10^{-05}$	$6.82 x10^{-04}$	$4.70x10^{-03}$	$2.80x10^{-03}$
Fluvoxamine						
Fish 96-hr LC_{50}	8.35 ng/L	$6.79x10^{-11}$ – $7.05x10^{-07}$	$2.65 x10^{-08}$	$1.94 x10^{-07}$	$7.05x10^{-07}$	$8.45x10^{-07}$
Daphnid 48-hr LC_{50}		$8.61x10^{-10}$ – $5.21x10^{-06}$	$2.11 x10^{-07}$	$1.44 x10^{-06}$	$5.21x10^{-06}$	$6.28x10^{-06}$
Green Algae 96-hr EC_{50}		$4.59x10^{-10}$ – $8.11x10^{-06}$	$2.85 x10^{-07}$	$2.22 x10^{-06}$	$8.11x10^{-06}$	$9.68x10^{-06}$
Fish Chronic		$3.29x10^{-10}$ – $1.78x10^{-05}$	$5.40 x10^{-07}$	$4.86 x10^{-06}$	$1.78x10^{-05}$	$2.11x10^{-05}$
Daphnid Chronic		$1.57x10^{-08}$ – $5.60x10^{-05}$	$2.43 x10^{-06}$	$1.55 x10^{-05}$	$5.60x10^{-05}$	$6.80x10^{-05}$

Green Algae Chronic		$1.87x10^{-09} – 2.24x10^{-05}$	$8.25\ x10^{-07}$	$6.13\ x10^{-06}$	$2.23x10^{-05}$	$2.67x10^{-05}$
Paroxetine						
Fish 96-hr LC_{50}	36.6 ng/L	$1.52x10^{-09} – 1.11x10^{-05}$	$1.37\ x10^{-06}$	$2.95\ x10^{-06}$	$1.11x10^{-05}$	$9.54x10^{-06}$
Daphnid 48-hr LC_{50}		$1.67x10^{-08} – 7.42x10^{-05}$	$1.07\ x10^{-05}$	$2.09\ x10^{-05}$	$7.42x10^{-05}$	$7.19x10^{-05}$
Green Algae 96-hr EC_{50}		$1.18x10^{-08} – 1.41x10^{-04}$	$1.49\ x10^{-05}$	$3.56\ x10^{-05}$	$1.41x10^{-04}$	$1.08x10^{-04}$
Fish Chronic		$1.14x10^{-08} – 3.81x10^{-04}$	$2.91\ x10^{-05}$	$8.69\ x10^{-05}$	$3.81x10^{-04}$	$2.27x10^{-04}$
Daphnid Chronic		$2.65x10^{-07} – 7.32x10^{-04}$	$4.28\ x10^{-05}$	$1.95\ x10^{-04}$	$7.32x10^{-04}$	$6.35x10^{-04}$
Green Algae Chronic		$4.33x10^{-08} – 3.62x10^{-04}$	$1.22\ x10^{-04}$	$1.17\ x10^{-04}$	$3.62x10^{-04}$	$4.59x10^{-04}$
Sertraline						
Fish 96-hr LC_{50}	46.8 ng/L (All except) 46.9 ng/L (DMSer)	$5.96x10^{-05} – 1.15x10^{-04}$	$9.81\ x10^{-05}$	$9.08\ x10^{-05}$	$1.15x10^{-04}$	$1.58x10^{-04}$
Daphnid 48-hr LC_{50}		$1.31x10^{-04} – 6.59x10^{-04}$	$3.61\ x10^{-04}$	$3.84\ x10^{-04}$	$6.59x10^{-04}$	$4.92x10^{-04}$
Green Algae 96-hr EC_{50}		$5.58x10^{-05} – 1.67x10^{-03}$	$8.38\ x10^{-04}$	$8.55\ x10^{-04}$	$1.67x10^{-03}$	$8.93x10^{-04}$
Fish Chronic		$7.20x10^{-04} – 6.69x10^{-03}$	$2.76\ x10^{-03}$	$3.39\ x10^{-03}$	$6.69x10^{-03}$	$3.48x10^{-03}$
Daphnid Chronic		$6.24x10^{-04} – 5.85x10^{-03}$	$3.13\ x10^{-03}$	$3.20\ x10^{-03}$	$5.85x10^{-03}$	$3.75x10^{-03}$
Green Algae Chronic		$1.15x10^{-04} – 3.90x10^{-03}$	$1.95\ x10^{-03}$	$1.99\ x10^{-03}$	$3.90x10^{-03}$	$2.07x10^{-03}$

7.3. Risk Characterisation

A probabilistic-type hazard quotient approach was used to assess potential risk posed by the target TPs. Exposure distributions were developed for each parent compound based on monitoring data. These distributions served for the respective TPs in the 1st tier assessment. The 95th-centile concentrations were used as our exposure estimates. Exposure distributions were compiled from occurrence data of the parent β-blockers (Figure 7.2) and SSRIs (Figure 7.3 and 3.4) in WWTP effluent and receiving waters. Occurrence data were compiled from North American and European sources (Tables A1.1 and A1.2). Exposure distributions were used to obtain 95th-centile exposure concentrations to be used in the risk assessment to calculate hazard quotients. Hazard quotients were derived by comparing the 95th-centile of exposures to all ECOSAR toxicity predictions relevant to our classes of compounds.

7.3.1. Ecosystems of Concern

In general, the ecosystems of interest for this ERA were river systems receiving treated sewage effluent. The SSRIs and β-blocking agents are strictly human-use pharmaceuticals, with an extremely small veterinary component, and thus, almost exclusively enter the environment via the sewage waste system. Typically, sewage effluent entering a river is quickly diluted as it travels downstream, often reducing concentrations to below levels of concern not far from the point source (e.g., fluoxetine, *N*-desmethylcitalopram) [57]. However, ecosystems of concern located in highly populated regions with dry or low-flow conditions may be largely effluent-dominated (e.g., Trinity River, Texas, Four-Mile Creek, Boulder Creek, USA) [58]. Seasonal variation of water flows in these systems can influence the degree of dilution of wastewater

inputs, and thus exposure to TPs [14]. Moreover, effluent concentrations within the discharge can vary by 1 or 2 orders of magnitude over a given 24-hour period. This contributes to the difficulty in predicting loadings to these systems and hence, to the uncertainty in exposure estimates [57]. The scope of importance in profiling persistent TPs within broader fluvial systems is becoming more apparent. For example, tributaries of the Red River in Manitoba, Canada, that are impacted by sewage effluents have the potential to channel these compounds into larger bodies of water like Lake Winnipeg [14], potentially making the biota vulnerable to toxic effects or eutrophication.

7.4. Results and Discussion

Our HQ results suggest low acute and chronic risk to aquatic organisms from these TPs. Uncertainty factors were not applied in this risk assessment due to the inherent conservative nature of assuming a 1:1 formation of TP from parent compound. Moreover, application of uncertainty factors is generally a risk management decision and can depend on jurisdictional preferences (e.g., USEPA versus EU EEA), stakeholders' interests, and political drivers. The commonly applied range of uncertainty factor of 10 to 1000 could result in many of the TPs having HQs greater than unity (Table 7.3), and thus, potentially warranting further investigation.

For both β-blockers and SSRIs, HQ values ranged from 10^{-11}-10^{-3} (Table 7.4) for all TPs, organisms, and endpoints. Mean HQ values were generally in the range of 10^{-5}-10^{-4}. Of the 29 compounds presented in Table 7.3 representing the maximum observed HQ values, 22 of those values were for chronic daphnid toxicity, suggesting that invertebrates are likely the most sensitive species to these TPs. However, it is evident from Table 7.4 that no HQ value comes close to surpassing the threshold of 1, to be considered potentially hazardous to the given aquatic organism. In fact, predicted exposure values are generally five orders of magnitude smaller than

the modeled ECOSAR effect concentrations, suggesting that no significant environmental risks are posed by these TPs.

The accuracy of ECOSAR can be assessed by comparing experimental effects data to those generated by the model (Table 7.5). This can provide a sense of the uncertainty involved in the ECOSAR estimations. In the absence of transformation product toxicity values, the parent compounds were considered. Transformation product toxicity can be inferred by structural homology to their respective parent compound. Various experimental bioassay parameters were chosen as close to the ECOSAR prediction as possible (i.e. acute/chronic, organism, duration, and endpoint). In Table 7.5 each compound was organised by acute then chronic, then ECOSAR or experimental data (green algae, then invertebrate, then fish). Any omitted ECOSAR toxicities were due to the absence of corresponding experimental data for comparative purposes. 26 out of 48 possible ECOSAR outcomes (acute and chronic; green algae, daphnid, and fish each) for the 8 parent compounds had direct experimental comparisons. Overall, the acute toxicity values between ECOSAR and experimental ones (Table 7.5) were very similar, and were within 1 order of magnitude for our compounds and test species.

Table 7.5 Comparison of ECOSAR toxicity values (this study) to experimentally determined data. In the absence of transformation product toxicity values, the parent compounds were considered. Transformation product toxicity can be inferred by structural homology to their respective parent compound. Various experimental bioassay parameters were chosen as close to the ECOSAR prediction as possible (i.e. acute or chronic, organism, duration, and endpoint). Each compound is organised by acute then chronic, then ECOSAR or experimental data (green algae, invertebrate, then fish). Any omitted ECOSAR toxicities are due to the absence of corresponding experimental data for comparison. *Desmodesmus subspicatus, Scenedesmus vacuolatus, Pseudokirchneriella subcapitata* are green algae; *Daphnia magna, Ceriodaphnia dubia are daphnids; Hyallela Azteca, Thamnocephalus platyurus, Dreissena polymorpha are invertebrates*; *Oryias latipes, Oncorhynchus mykiss, Poecilia wingei, Pimephales promelas, Perca fluviatilis are fish.* (Colour code: green algae = green, invertebrates = pink, fish = blue).

First Author, Year/ ECOSAR	Organism	Acute/ Chronic	Duration	Bioassay Endpoint	Toxicity (mg/L)
		Atenolol			
ECOSAR	Green Algae	Acute	96h EC50	Growth	136
Cleuvers, 2005	Desmodesmus subspicatus	Acute	72h EC50	Growth	620
ECOSAR	Daphnid	Acute	48h LC50	Mortality	103
Cleuvers, 2005	*Daphnia magna*	Acute	48h EC50	Immobilisation	313
Fraysse, 2005	*Ceriodaphnia dubia*	Acute	48h EC50	Immobilisation	33.4
		Metoprolol			
ECOSAR	Green Algae	Acute	96h EC50	Growth	8.3
Cleuvers, 2005	*Desmodesmus subspicatus*	Acute	72h EC50	Growth	7.9
Maszkowska, 2014	*Scenedesmus vacuolatus*	Acute	48h EC50	Reproduction	75
ECOSAR	Daphnid	Acute	48h LC50	Mortality	9.4
Cleuvers, 2005	*Daphnia magna*	Acute	48h EC50	Immobilisation	438
Fraysse, 2005	*Ceriodaphnia dubia*	Acute	48h EC50	Immobilisation	45.3
Huggett, 2002	*Daphnia magna*	Acute	48h LC50	Mortality	63.9
Huggett, 2002	*Ceriodaphnia dubia*	Acute	48h LC50	Mortality	8.8

Propranolol					
ECOSAR	Green Algae	Acute	96h EC50	Growth	1.9
Cleuvers, 2005	*Desmodesmus subspicatus*	Acute	72h EC50	Growth	0.7
Liu, 2009	*Pseudokirchneriella subcapitata*	Acute	96h EC50	Growth	7.4
Maszkowska, 2014	*Scenedesmus vacuolatus*	Acute	48h EC50	Reproduction	24
ECOSAR	Daphnid	Acute	48h LC50	Mortality	2.6
Huggett, 2002	*Hyallela Azteca*	Acute	48h LC50	Mortality	29.8
Huggett, 2002	*Daphnia magna*	Acute	48h LC50	Mortality	1.6
Huggett, 2002	*Ceriodaphnia dubia*	Acute	48h LC50	Mortality	0.85
Cleuvers, 2005	*Daphnia magna*	Acute	48h EC50	Immobilisation	7.7
Fraysse, 2005	*Ceriodaphnia dubia*	Acute	48h EC50	Immobilisation	1.4
Liu, 2009	*Daphnia magna*	Acute	48h EC50	Immobilisation	1.6
Liu, 2009	*Ceriodaphnia dubia*	Acute	48h EC50	Immobilisation	0.8
ECOSAR	Fish	Acute	48h LC50	Mortality	20.2
Huggett, 2002	*Oryias latipes*	Acute	48h LC50	Mortality	24.3
ECOSAR	Daphnid	Chronic	--	--	0.23
Huggett, 2002	*Ceriodaphnia dubia*	Chronic	27d LOEC	Reproduction	0.25
Huggett, 2002	*Hyallela Azteca*	Chronic	7d LOEC	Reproduction	0.1
Liu, 2009	*Hyallela azteca*	Chronic	27d	Reproduction	0.1
ECOSAR	Fish	Chronic	--	--	0.95
Huggett, 2002	*Oryias latipes*	Chronic	28d LOEC	# viable eggs	0.0005
Liu, 2009	*Oncorhynchus mykiss*	Chronic	10d	Growth	10
Citalopram					
ECOSAR	Daphnid	Acute	48h LC50	Mortality	0.65
Henry, 2004	*Ceriodaphnia dubia*	Acute	48h LC50	Mortality	3.9
Minguez, 2014	*Daphnia magna*	Acute	48h EC50	Immobilisation	30.14
ECOSAR	Daphnid	Chronic	--	--	0.065
Henry, 2004	*Ceriodaphnia dubia*	Chronic	7-8 d	# neonates	4.0
ECOSAR	Fish	Chronic	--	--	0.14
Olsen, 2014	*Poecilia wingei*	Chronic	21d	Freezing (predator avoid.)	0.0023
Fluoxetine					
ECOSAR	Green Algae	Acute	96h EC50	Growth	0.079
Brooks, 2003	*Pseudokirchneriella subcapitata*	Acute	48h EC50	Turbidity	0.024
ECOSAR	Daphnid	Acute	48h LC50	Mortality	0.18
Brooks, 2003	*Daphnia magna*	Acute	48h LC50	Mortality	0.82
Brooks, 2003	*Ceriodaphnia dubia*	Acute	48h LC50	Mortality	0.234
Henry, 2004	*Ceriodaphnia dubia*	Acute	48h LC50	Mortality	0.51

Minguez, 2014	*Daphnia magna*	Acute	48h EC50	Immobilisation	5.91
Nalecz-Jawecki, 2007	*Thamnocephalus platyurus*	Acute	24h LC50	Mortality	0.76
ECOSAR	Fish	Acute	48h LC50	Mortality	1.08
Brooks, 2003	*Pimephales promelas*	Acute	48h LC50	Mortality	0.705
ECOSAR	Daphnid	Chronic	--	--	0.019
Henry, 2004	*Ceriodaphnia dubia*	Chronic	7-8 d	# neonates	0.447
Lazzara, 2012	*Dreissena polymorpha*	Chronic	6d EC60	Dec. # oocytes	0.00002
Lazzara, 2012	*Dreissena polymorpha*	Chronic	6d EC 80	Dec. # spermatozoa	0.00002
Lazzara, 2012	*Dreissena polymorpha*	Chronic	6d	Spawning	0.00002
ECOSAR	Fish	Chronic	--	--	0.49
Weinberg, 2014	*Pimephales promelas*	Chronic	28d LOEC	# of eggs	0.1
Weinberg, 2014	*Pimephales promelas*	Chronic	28d LOEC	Predator Avoidance	0.001
Painter, 2009	*Pimephales promelas*	Chronic	5d LOEC	Predator Avoidance	0.000025
Fluvoxamine					
ECOSAR	Daphnid	Acute	48h LC50	Mortality	1.6
Henry, 2004	*Ceriodaphnia dubia*	Acute	48h LC50	Mortality	0.84
ECOSAR	Daphnid	Chronic	--	--	0.15
Henry, 2004	*Ceriodaphnia dubia*	Chronic	7-8 d	# neonates	1.47
Paroxetine					
ECOSAR	Daphnid	Acute	48h LC50	Mortality	0.49
Minguez, 2014	*Daphnia magna*	Acute	48h EC50	Immobilisation	6.24
Henry, 2004	*Ceriodaphnia dubia*	Acute	48h LC50	Mortality	0.58
ECOSAR	Daphnid	Chronic	--	--	0.05
Henry, 2004	*Ceriodaphnia dubia*	Chronic	7-8 d	# neonates	0.44
Sertraline					
ECOSAR	Green Algae	Acute	96h EC50	Growth	0.028
Minagh, 2009	*Pseudokirchneriella subcapitata*	Acute	72h EC50	Growth	0.14
ECOSAR	Daphnid	Acute	48h LC50	Mortality	0.071
Henry, 2004	*Ceriodaphnia dubia*	Acute	48h LC50	Mortality	0.12
Minguez, 2014	*Daphnia magna*	Acute	48h EC50	Immobilisation	1.15
Minagh, 2009	*Daphnia magna*	Acute	24h EC50	Immobilisation	3.1
Minagh, 2009	*Daphnia magna*	Acute	48h EC50	Immobilisation	1.3
Minagh, 2009	*Thamnocephalus platyurus*	Acute	24h LC50	Mortality	0.6
ECOSAR	Fish	Acute	48h LC50	Mortality	0.408
Minagh, 2009	*Oncorhynchus mykiss*	Acute	96h LC50	Mortality	0.38
ECOSAR	Daphnid	Chronic	--	--	0.008
Henry, 2004	*Ceriodaphnia dubia*	Chronic	7-8 d	# neonates	0.45
Minagh, 2009	*Daphnia magna*	Chronic	21d	Mortality	0.1

			LOEC		
Minagh, 2009	*Daphnia magna*	Chronic	21d LOEC	Reproduction	0.1
ECOSAR	Fish	Chronic	--	--	0.007
Hedgespeth, 2014	*Perca fluviatilis*	Chronic	7d LOEC	Feeding	0.89

A greater range in variation can be seen in the comparison of chronic toxicity values (see Table 7.5). The primary reason aside from different potential mechanisms of action is most likely that ECOSAR generates chronic toxicity values based on the geometric mean of the NOEC and LOEC values for a particular SAR/ compound. This difference in calculation is further augmented by the numerous different bioassay endpoints that are not particularly comparable, albeit independently viable (e.g. *P. pimephales:* # of oocytes compared to reduced feeding or predation avoidance). For example, relatively comparable values for propranolol can be seen in the chronic daphnid bioassay 0.23 (0.25). However, citalopram shows daphnid toxicities of 0.065 (4), fluoxetine 0.019 (0.447), and most notably sertraline 0.008 (0.1 death, 0.45 # of neonates). These values vary by up to 2 orders of magnitude. These variations in toxicity are most likely due to the fact that ECOSAR estimates chronic toxicity based on water solubility and K_{OW} in addition to structure-activity relationships. Active pharmaceutical ingredients are known to act through specific modes of action including physiological activation (e.g. neurological receptor) at levels lower than those necessary for typical narcosis [18]. Therefore, it is reasonable to assume, in the light of no other evidence, that the various TPs of the aforementioned parent compounds could possess comparable similarities and deviations in toxicities. Overall, ECOSAR acute toxicity values seem to be fairly robust. ECOSAR chronic toxicity values, however, exemplify the need to develop models that can account for physiological activation, or more to the point, experimental evidence. Thus, more accurate HQs can be calculated and risk inferred.

A number of assumptions were made in this ERA, contributing to the uncertainty in our conclusions that these compounds do not pose a significant ecological risk to aquatic organisms. Assumptions were made in the creation of hypothetical exposure data, in the consideration of

which data would be considered relevant, and in the use of toxicity data using modelling software. Below we discuss major points of uncertainty in our risk assessment.

7.4.1. Exposure Uncertainty

Toxicity of these chemicals in mixtures can vary from that of the individual chemicals [59] i.e., exhibit significant interactions. This scenario is especially relevant for effluent-dominated surface waters, where dilution of these mixtures is minimal, and can remain constant for significant temporal and spatial durations. Our current understanding of complex mixture toxicities, as may be exhibited in natural waters, makes it difficult and uncertain for appropriate incorporation into any ERA at this time.

Still, some research to this end has been attempted. When considering whole effluents containing β-blockers and SSRIs, various mixture effects have been shown in test organisms. Franzellitti *et al.* (2013)[60] showed antagonistic effects of SSRIs and β-blockers mixed together on 5-HT_1 receptor binding and gene transcription (i.e., reduced each other's toxic potential). Likewise, Barber *et al.* (2007)[61] showed that a mixture of alkyl phenols representing estrogens, pesticides, biocides, and antibiotics had a positive correlation with normal development of male fathead minnow secondary sexual characteristics. However, vitellogenin induction and an increase in hepatosomatic index were associated with increasing concentrations of whole effluent. Similarly, vitellogenin induction in female mussels (*Mytilus galloprovincialis*) was observed by Gonzalez-Rey *et al.* (2014)[62], reinforcing the estrogenicity of effluent mixtures. Using concentration addition (CA) theory when accounting for total toxicity, Backhaus and Karlsson (2014)[63] determined that the risk of randomly selecting a single pharmaceutical in a mixture represented 1000-fold less risk than the mixture. This suggested the necessity for

prioritisation of certain chemicals' toxicity. However, of the 26 compounds analysed, the top 10 highest concentrations could account for approximately 95% of the toxicity [63]. Interestingly, Petersen *et al.* (2014)[64] determined that although β-blockers are considered endocrine disrupting compounds, fluoxetine inhibited the growth of the marine algae *Skeletonema pseudocostatum* more than propranolol. In addition, they determined that at low concentrations of the mixture of pharmaceuticals, personal care products, organic contaminants, and biocides, independent action (IA) dominated the toxicity. At higher concentrations (i.e., > 10^{-7}M for fluoxetine, and > 10^{-6}M for propranolol), the correlation shifted to CA, possibly due to the activation of similar toxicity pathways in the marine algae; however, the mechanistic pathway still needs to be studied [64]. Intuitively, this suggests that the toxic contaminants present at the greatest concentrations will account for a greater proportion of the risk overall.

Table 7.4, in this ERA, outlines the HQs for all parent compounds and TPs. The potential for mixture toxicity can be assessed by the percentage difference between the sum of the TPs for any given compound and the parent. For example, HQs for both acute and chronic toxicities for fish, daphnid, and green algae were calculated for each TP. The sum of all HQs across all TPs for each ECOSAR category was compared to the parent compound HQ. The order of increasing potential for mixture toxicity of the sum of the TPs was sertraline (-48 to 37%) < paroxetine (-40 to 27%) < fluoxetine (-42 to 94%) < fluvoxamine (19 to 21%) < citalopram (46 to 240%) < metoprolol (230 to 880%) < propranolol (45 to 4600%) < atenolol (540 to 390000%). In fact, when all parent compounds and all TPs were summed together, the resulting HQ was still only 0.22. Although, it has been documented that generally the top 10 greatest concentrations in any mixture should account for approximately 95% of all toxicity [63], this calculation was done to stress in the complete lack of risk associated with these TPs aquatically, according to ECOSAR.

Temporal and spatial variation can affect the magnitude of exposures. Within effluent-dominated systems (i.e., those in close proximity to discharge from a WWTP or sewage lagoon), there can exist significant temporal variability in the concentrations of parent pharmaceuticals and their TPs. For instance, Writer *et al.* (2013)[57] documented that over the sampling period of one day (09:00 to 17:00) in Boulder Creek, Colorado, the ratio of total metabolites to parent of citalopram fluctuated by up to one order of magnitude. Therefore, it is logical to assume that grab samples for any of the published exposure data have their own inherent variability and uncertainty. In the future, by obtaining representative exposure data for the construction of the exposure distributions, some of this variability should be accounted for.

The UM-BBD-PPS software is limited to aerobic transformation, excluding any anaerobic processes. It has been documented that microbial consortia can biodegrade and deconjugate pharmaceutical compounds under nitrifying conditions [3, 53]. Although the software has the capability of showing plausible anaerobic transformation processes and products, the rules governing the validation of anaerobic processes are less corroborated. We limited the scope of our BBD to two light green or dark green arrows in order to limit the number of TPs to a feasible number. However, with this limitation, various potentially important biodegradation scenarios are not considered. For example, perhaps a single "neutral" step (yellow arrow) occurs, followed by a series of subsequent likely reactions. Also, it has been documented that pharmaceuticals are biodegraded not only under aerobic conditions but anoxic as well. Suarez *et al.* (2010)[65] showed that fluoxetine and natural estrogens are transformed under both aerobic and anoxic conditions (>75% combined). Moreover, citalopram was biotransformed ~60% aerobically and ~40% anaerobically [65]. Therefore, for this compound it is plausible that a very large portion of biotransformation is not being accounted for using the BBD software.

Photolysis can greatly fluctuate under a variety of conditions. If the surface water in question was a shallow, well-mixed water body, then photolysis is ostensibly more important. This notwithstanding, effluent-dominant eutrophic fluvial systems tend to be quite turbid and murky, so aside from very near surface processes, direct photolysis would be a less significant fate process. Indirect photolysis will vary given the characteristic make-up of the surface water (e.g., type and amount of dissolved organic matter, nitrate levels, pH, etc.) [4]. Given that photolysis can be a major degradation process for pharmaceuticals, it is a reasonable assumption that photo-TPs are likely an important piece in this complex puzzle. Thus, the specific photo-TPs formed in a given system are highly variable and will often change from system to system depending on water chemistry. Moreover, the inclusion of photo-TPs in this ERA is strictly limited to what has been observed in the literature for β-blockers and SSRIs. To our knowledge this is the first ERA on pharmaceuticals to include a combination of aerobic biodegradation and photolysis TPs together with human metabolites.

7.4.2. Effects Uncertainty

Relevant assessment endpoints would be ideal in determining the toxic effects of pharmaceutical TPs. Toxicity could vary from the parent compounds to TPs given the numerous different pathways and chemical structure moieties potentially transformed. A major consideration for relating the exposure of TPs to potential effects is partitioning to the internal component of the target organism. Internal dose involves a variety of partitioning processes that have been empirically correlated to a compound's physicochemical parameters. The biotransformation rate k_{BIO} is a combination of the affinities of metabolites and TPs for specific tissue components of the biota in question, taking into consideration the fraction of specific

tissue that is found within the biota (e.g., lipid, protein, carbohydrate, nucleic acid, cutin, lignin, etc.) [66]. For example, it has been shown that although typical aquatic exposure levels to norfluoxetine are very low (several ng/L), high levels of the SSRI can be found in brains (8.86 ng/g ± 5.9 ng/g) and livers (10.27 ng/g ± 5.73 ng/g) of fish found in tributaries of an effluent-dominated surface water [2].

A major limitation of ECOSAR is that it strictly estimates toxicity based on K_{OW} and not other parameters, such as specific mechanism of action. For example, SSRIs are agreed to elicit effects in innervated aquatic biota through the serotonin 5-HT $_{1A}$ receptors [18]. The β-blocking class of drugs have the common property of blocking the binding of catecholamines (epinephrine) to β-adrenergic receptor sites [5]. While inclusion of a mode-of-action type model would reduce uncertainty in the effect estimations, by no means would it eliminate it. However, identifying whether or not certain TPs exerted toxicity via the same mode-of-action as the parent compound would, for various species classes, reduce uncertainty.

7.5 Conclusions and Recommendations

A probabilistic ecological risk assessment was conducted on 104 compounds (67 β-blocker TPs and 37 SSRI TPs) using a conservative, Tier 1 hazard quotient approach. We determined that no HQ was above our threshold of 1; and furthermore, the highest values for either class were at least 2-3 orders of magnitude below 1. The majority of maximum HQ values were for chronic daphnid toxicity, suggesting that invertebrates are most sensitive to these particular compounds. Based on this assessment, it can be reasonably inferred that these compounds do not pose any immediate risk, acute or chronic, to aquatic biota. However, this type of exercise establishes the limitations of using a combination of models to predict true risk.

This is especially non-trivial with respect to applying baseline toxicity structure-activity relationships to compounds that operate via specific modes of action.

ECOSAR has typically been useful in elucidating effects and risk for well-studied contaminants (e.g., pesticides). Pharmaceuticals (like β- blockers and SSRIs) likely require a different approach for calculating risk due to the specific modes of action they employ. Parameters such as solubility and K_{OW} are generic chemical characteristics that account for their availability in water and partitioning into lipid tissues of biota via hydrophobic moieties. The moderately high K_D values of β- blockers and the higher values of SSRIs indicate that these chemicals will likely be found predominantly in WWTP sludge or natural sediments. Microbial biodegradation or abiotic transformation could result in desorption of these chemicals. Thus, the sludge and/or natural sediments could act as a reservoir for these chemicals to persist in the water through constant anthropogenic input.

Although this study shows no immediate necessity for higher tier assessments to be conducted on SSRIs and β- blockers based on the hazard quotients calculated, this study used programs that do not take into account specific modes of action or bioaccumulation potential. These are purposeful and essential parameters for compounds specifically designed to have sustained biological effects at low therapeutic doses. However, given the lack of complete data available for pharmaceutical metabolites, conservative exercises like this are beneficial for gauging the preliminary scope of environmental impact. QSAR-type toxicity models, e.g., ECOSAR, can be effective tools for conducting predictive risk assessments of pharmaceutical TPs in aquatic systems, given a significant lack of ecotoxicological data.

We recommend developing analytical methods that can isolate and quantify human metabolites and TPs at environmentally relevant concentrations, likely some type of liquid

chromatography-high resolution mass spectrometry. Also, we recommend identifying the major species of TPs from classes of pharmaceuticals that could elicit toxic effects via specific modes of action, and conduct aquatic toxicity tests to help inform realistic risk assessments. A spectrum of laboratory assessment endpoints using environmentally relevant concentrations should be conducted (e.g., feeding habits, socialization, predation anxiety, and fecundity). A focus on chronic exposure, given the probable levels in the low to mid-µg/kg range, using a benthic invertebrate (e.g. mollusk), an aquatic invertebrate (e.g. daphnid), and a vertebrate (e.g. fathead minnow) would be necessary. This would provide a more complete picture regarding how different classes of pharmaceutical TPs can act on different components of the aquatic food web, especially given the propensity of some chemicals to associate with phases other than water.

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Chapter 8

Conclusions and Future Directions

8.

8.1. Conclusions

Through metabolism and excretion by the human body, conjugates have the potential to persist based on their stereochemical stability, and applicable biotic and abiotic processes during wastewater treatment, and in the environment. The major considerations for environmental analysis are that the conjugates likely exist at the pg/L to µg/L level, *and* that a significant proportion of TPs of either the conjugate or parent drug are not identified. This thesis demonstrated that previously unquantified metabolite conjugates have the potential to make up a non-trivial proportion of the total pharmaceutical inventory in a WWTP, and thus the same may prove true in an aquatic ecosystem.

In the pursuit of the quantification of conjugates, extraction, pre-concentration, non-destructive separation, and mass analysis are all interconnected in their efficacy. However, technologies that take advantage of the more polar nature of the human conjugate, whether it be through SPE or liquid chromatography, have the greatest chance of succeeding at being coupled to a sensitive and selective mass analyser like the Q-LIT, Q-TOF, Orbitrap, or QQQ.

Initially, propranolol, sulfamethoxazole, and their respective major conjugates 4-OH-propranolol sulfate and sulfamethoxazole-β-glucuronide were simultaneously extracted through HLB, MAX, and WAX cartridges from various waters. WAX was determined as the sorbent of choice for extracting primary and secondary clarification wastewaters. Primary and secondary filtrates from the North End WWTP of Winnipeg respectively showed a significant increase of propranolol, decrease of propranolol sulfate, increase of sulfamethoxazole, and decrease of sulfamethoxazole-glucuronide concentrations. This preliminarily indicated that there was some

sort of removal from the aqueous phase occurring at some point between these stages of treatment that affected sulfamethoxazole and both conjugates, but that propranolol demonstrated persistence.

The innovative potential of this study is that multiple classes of pharmaceutical (as represented by propranolol and sulfamethoxazole) and multiple types of conjugate (sulfate and glucuronide) can be simultaneously quantified using a single chromatographic column, with one set of binary solvents on a low resolution mass spectrometer. The ubiquity of these instruments notwithstanding, the convenience of a low resolution instrument being able to quantify these compounds simultaneously allows high resolution instruments to be available for metabolite/ unknown compound investigation. This study was used as a framework to subsequently extract and quantify glucuronide, sulfate, and acetyl conjugates of both acidic and basic pharmaceuticals in the aquatic environment

As an extension to this quantitative method, additional classes of pharmaceuticals and their respective conjugates were added to the protocol. An effective solids extraction method using sonication in combination with weak anion exchange solid phase extraction was created to quantify thyroxine and thyroxine-glucuronide simultaneously from suspended solids and sludge, in addition to wastewater from a major Winnipeg WWTP. This method demonstrated the need to accurately assess the total inventory of particle-reactive anthropogenic contaminants within a wastewater system. Not only is there a necessity to quantify both the parent and metabolite conjugate of thyroxine, but also to quantify the masses of these compounds entering the ecosystem in both the aqueous and particulate phases. Once in the environment it is plausible that previously sorbed compounds could partition back into the aqueous phase due to the shifts in chemical gradients of these contaminants (i.e. surface waters diluting the wastewater).

In addition to thyroxine, acetaminophen was added to the quantitative methods to now establish this thesis' full suite of diverse pharmaceuticals and their associated conjugates: acetaminophen (analgesic- organic acid), propranolol (β-blocker- organic base), sulfamethoxazole (antibiotic- organic acid), and thyroxine (vertebrate hormone- zwitterion). Thus, this method was applied to a pilot scale monitoring study for approximately 3 months at the North Main WWTP in Winnipeg; and both aqueous and solid phases were examined. Concentrations were analysed throughout the treatment process from primary treatment, secondary treatment, aerobic treatment, and final effluent.

Overall there were significant decreases in the concentrations of acetaminophen and sulfamethoxazole compounds, but persistence of propranolol and thyroxine compounds throughout the WWTP. These concentrations were consistent with what was found in the previous two method development studies of this thesis (chapters 3 and 4). The mass loadings throughout the WWTP reinforced that both aqueous and solid concentrations be taken into account. Comparable levels of certain conjugates were released, if not more, than the parent (propranolol sulfate, *N*-acetylsulfamethoxazole, and thyroxine glucuronide) (Figure 5.3).

This study reflects the importance of including phase II conjugates in assessing overall compound load of pharmaceutical discharge from wastewaters, and also that substantial amounts of such contaminants are associated with wastewater solids when drugs are in the pg/L to μg/L aqueous range. However, generalisations to other classes of pharmaceuticals cannot be concluded based on the scarcity of monitoring data and the variability and complexity of class-dependent sorption mechanisms within a WWTP.

In order to back stop what was seen the pilot study, the fate of these same pharmaceuticals and their conjugates was evaluated in lab-scale wastewater bioreactors. It was

important to determine how treatment parameters, and by extension environmental factors, such as physical components of primary or secondary wastewater, the addition of air, and the presence of WAS could influence the kinetics of removal. Under a realistic HRT (<2h) with the addition of WAS, acetaminophen and its sulfate were both rapidly degraded (>99%). Propranolol was potentially sulfated and concurrently removed. Deconjugation of *N*-acetylsulfamethoxazole and sulfamethoxazole-glucuronide contributed to increases of the parent sulfamethoxazole. Thyroxine was resistant to degradation, while thyroxine-glucuronide was rapidly deconjugated (>90% in <2h).

Without WAS, sorption to suspended solids was another major removal mechanism for acetaminophen, propranolol, sulfamethoxazole, and thyroxine. However, with WAS, concentrations associated with suspended solids decreased for all analytes by 24h. These results indicate that both conjugation and back-transformation are most likely compound-specific and dependent on parameters such as HRT, addition of WAS, and suspended solids levels. These processes may strongly influence the fate and speciation of pharmaceuticals in wastewater effluents, and thus natural surface waters.

Environmental and human health policy must be established even in the absence of quantitative data of emerging contaminants and their metabolites. Essential to forging policy is the importance of following established protocols and frameworks to allow for calculating risk to these contaminants across various jurisdictions, whether global, regional, or institutional. In following with the USEPA risk characterisation protocols, a probabilistic ecological risk assessment was conducted on 104 compounds (67 β- blocker TPs and 37 SSRI TPs) using a conservative, first-tier hazard quotient (HQ) approach. This was done to highlight the necessary modeling processes and their associated uncertainties involved in conducting risk assessment in

the absence of concrete data. We determined that no HQ was above our threshold of 1 (which merits further investigation); and furthermore, the highest values for either class were at least 2-3 orders of magnitude below 1. The majority of maximum HQ values were for chronic daphnid toxicity, suggesting that invertebrates are most sensitive to these particular compounds. Based on this assessment, it can be reasonably inferred that these compounds do not pose any immediate risk, acute or chronic, to aquatic biota.

This type of exercise highlights the serious limitations of using a combination of models to predict true risk. This is especially non-trivial with respect to applying baseline (e.g. necrotic membrane disruption) toxicity using chemical structure-activity relationships (e.g. ECOSAR) to pharmaceuticals that elicit toxic effects in combination with specific modes of action (i.e. biological receptor). This fact was especially reflected in Table 7.5 in the comparison of acute and chronic experimental values to those generated by ECOSAR, where many pharmaceuticals were found to be more toxic than those predicted by models.

However, given the lack of complete data available for pharmaceutical metabolites, conservative exercises like this are beneficial for gauging the preliminary scope of environmental impact. QSAR-type toxicity models (e.g. ECOSAR) can be effective tools for conducting predictive risk assessments of pharmaceutical TPs in aquatic systems, given a significant lack of ecotoxicological data.

8.2. Future Directions

8.2.1. Variation of Microbial Community Structure

It has been previously demonstrated through many studies involving engineering advances in removal of emerging contaminants, and environmental chemistry that specific microbial consortia (and individual strains) can flourish in aquatic environments in the presence of emerging contaminants. Moreover, it has been shown that specific strains of bacteria are resistant to various forms of antibiotics, and have the potential to confer resistance to others through horizontal transfer. Functional redundancy displayed by microbes under various environmental stressors implies that certain microbes will prove opportunistic in the presence of pharmaceutical conjugates, much like they do in the presence of parent compounds.

It would be of value to use the current state of knowledge regarding the conjugate concentrations found throughout WWTPs, and the associated kinetics of the classes of compounds studied within this thesis to monitor for microbial persistence in wastewater/ surface waters and the resultant variation in community structure by analysing specific DNA parameters. The results of these studies could provide additional insight into modifications of wastewater treatment in engineered environments for the removal of these contaminants.

8.2.2. Toxicity Tests

Once a sufficient database of concentrations of specific conjugates is attained, toxicity tests can be conducted on various non-target organisms. Two goals could be achieved: first, to monitor for *in vivo* biotransformation of the compounds; second, to conduct specific acute and chronic toxicity tests using both worst-case scenario concentrations and more realistic exposure

concentrations. Monitoring for transformation products using high resolution mass spectrometry would give some insight into the resiliency of non-target organisms in surviving similar stressors. Acute toxicity tests (i.e. <96 h LC_{50}) at worst case scenario concentrations would provide context to compare similar toxicity tests using the parent compounds. This would be especially interesting for thyroxine compounds given that it has been shown that these conjugate forms are used by certain vertebrates as endogenous thyroid reservoirs during times of lesser circulating levels for homeostasis. Chronic tests (e.g. 21-28 day exposures) at environmentally relevant concentrations would provide whole organismal morbidity values (e.g. reduced fecundity, decreased appetite, reduced predator avoidance, reduced brood size/ # oocytes) to provide context surrounding aquatic organism health. Biomarkers are increasingly popular for non-destructive monitoring the augmentation of organismal gene expression during exposure to conjugates. Given the constant anthropogenic input of these compounds into receiving waters, these results would provide the most realistic health indicators of these stressors to non-target organisms.

8.2.3. Risk Assessment

As seen in Chapter 7 of this thesis, risk assessment can be conducted on these specific metabolite conjugates given sufficient exposure and relevant toxicity levels. Previously, risk assessment on these chemicals must be done using a suite of various modeling software to compensate for the lack of concrete data. Subsequent to the aforementioned collected data, a similar first tier HQ approach can be conducted to preliminarily infer as to whether or not further in-depth assessment must be made. In general, HQ > 1 imply the potential for further

investigation (i.e. that environmental concentrations and concentrations eliciting toxic effects are similar in magnitude).

Appendix 1

Simultaneous Quantification of Propranolol and Sulfamethoxazole and Major Human Metabolite Conjugates 4-Hydroxy-Propranolol Sulfate and Sulfamethoxazole-β-Glucuronide in Municipal Wastewater Using Liquid Chromatography-Tandem Mass Spectrometry

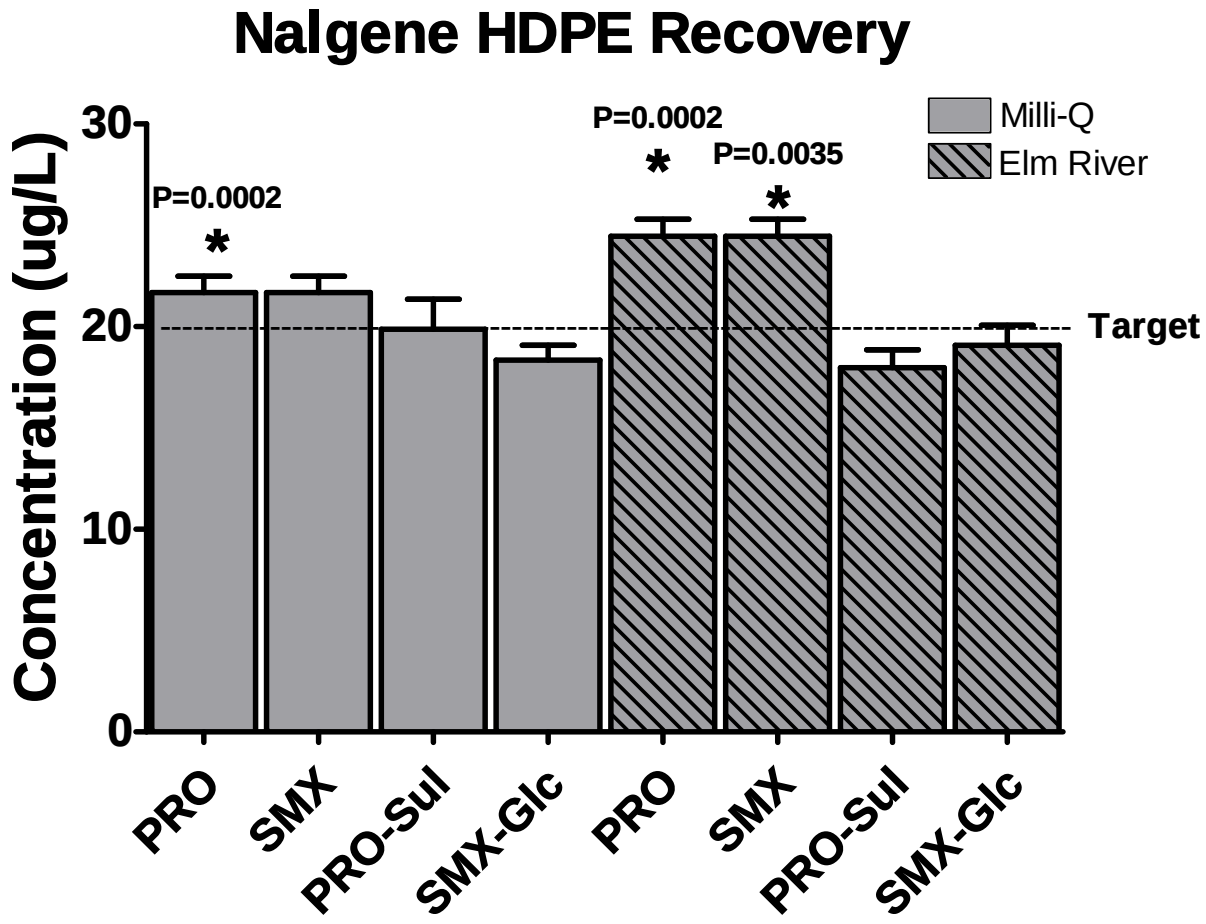

Figure A1.1 Recoveries of analytes spiked at 20 µg/L in 250 mL Nalgene® HDPE bottles (N=4), and left undisturbed for 24 hours in complete darkness. Dual elutions were performed to determine if analytes would sorb to the HDPE bottles.

Table A1.1: Precursor and product ion fragments, collision energy, abundance, and fragmentation energy for both parent and conjugate compounds. All results derived from positive mode ESI using the Agilent Mass Hunter Optimizer software. Note qualifiers could be used but for the purposes of this manuscript they were not.

Compound	Precursor Ion	Product Ion	Collision Energy	Fragmentation (V)	Quantifier-Q Qualifier-q
Propranolol	260.2	56.1	32	110	
		58.1	24		
		116.1	16		q
		183	16		Q
Propranolol-d7	267.2	56.1	32	110	
		58.1	24		
		116.1	16		Q
		72.1	20		q
Sulfamethoxazole	254.1	65.1	48	107	
		92	28		
		108	24		q
		156	12		Q
Sulfamethoxazole-d4	258.1	96.1	28	101	Q
		112	24		Q
		68.5	48		
		69	52		
rac 4'-Hydroxy Propranolol Sulfate	356.1	276.1	16	138	Q
		58.1	36		
		116.1	20		q
		56.1	44		
rac 4'-Hydroxy Propranolol-d7 Sulfate	362.2	282.2	16	122	Q
		208.6	8		
		213.3	20		
Sulfamethoxazole β-D-Glucuronide	430.1	254	0	100	Q
		92.1	48		q
		156	28		
		108	48		
Sulfamethoxazole-d4 β-D glucuronide	434.12	258.1	0	104	
		96.1	52		
		160	28		q
		112.1	44		Q

Table A1.2: Method limits of detection (MLD), method limits of quantification (MLQ), intraday precision, and interday precision were calculated by calculating the mean, standard deviation, and coefficient of variation for seven injections of each parents and conjugates standards mixtures in 50/50 (v/v) Milli-Q: methanol at the three lowest concentrations (0.1, 0.5, and 1 µg/L). MLD was determined when the mean *S/N* ratio was greater than 3, MLQ was determined when the mean *S/N* ratio was greater than 10.

	Analyte	***S/N***	**Analyte**	***S/N***	**Analyte**	***S/N***	**Analyte**	***S/N***
	PRO	**0.1ppb**	**PRO-Sul**	**0.1ppb**	**SMX-Glc**	**0.1ppb**	**SMX**	**0.1ppb**
	0.141	4.06	0.163	6.11	0.134	2.27	0.172	3.78
	0.129	5.29	0.125	3.61	0.0676	2.83	0.129	4.08
	0.119	4.46	0.166	4.04	0.202	11.32	0.0628	2.81
	0.115	3.25	0.127	6.83	0.0725	1.07	0.107	4.46
	0.112	4.34	0.122	4.45	0.141	3.0	0.0996	3.77
	0.131	5.63	0.116	4.9	0.111	3.65	0.110	4.61
	0.109	4.41	0.145	5.8			0.103	5.2
St Dev	0.0117	0.784	0.0202	1.18	0.0497	3.68	0.0331	0.761
Mean	0.122	4.49	0.137	5.11	0.121	4.02	0.112	4.10
COV	9.59	1.75	14.7	2.30	41.0	91.4	29.6	18.6
		0.5ppb		**0.5ppb**		**0.5ppb**		**0.5ppb**
	0.392	17.1	0.656	22.8	0.542	17.3	0.396	2.12
	0.422	15.2	0.513	5.19	0.548	14.8	0.448	15.0
	0.376	14.1	0.639	16.7	0.412	3.16	0.419	15.3
	0.368	16.0	0.575	17.1	0.495	9.51	0.444	14.5
	0.419	24.6	0.471	4.48	0.599	19.5	0.431	19.7
	0.346	19.0	0.579	23.2	0.572	6.12	0.400	15.1
	0.365	21.1	0.673	52.7	0.518	12.7	0.439	7.12
St Dev	0.0284	3.71	0.0752	16.1	0.0607	5.94	0.0211	5.96
Mean	0.384	18.1	0.586	20.3	0.526	11.9	0.425	12.7
COV	7.39	20.4	12.8	79.5	11.5	50.1	4.95	47.0
		1ppb		**1ppb**		**1ppb**		**1ppb**
	0.733	27.0	1.07	16.6	0.923	4.44	0.765	18.0
	0.739	37.3	0.985	118.6	1.03	78.1	0.952	41.1

	0.683	27.3	1.19	32.4	1.13	20.2	0.889	8.15
	0.697	21.5	0.971	20.9	0.991	26.2	0.886	34.0
	0.729	18.7	1.11	48.8	0.953	26.3	0.870	28.8
	0.780	31.0	1.10	26.5	0.934	42.3	0.849	40.5
	0.725	32.8	1.08	11.1	0.988	4.97	0.865	20.1
St Dev	0.0311	6.43	0.0739	37.1	0.0714	25.4	0.0558	12.4
Mean	0.727	28.0	1.07	39.3	0.993	28.9	0.868	27.2
COV	4.28	23.0	6.90	94.3	7.19	87.7	6.43	45.4

Table A1.3: Calibration curve range and accuracy was calculated by quantifying the mass of 20 µg/L native parent and conjugate standard mixture spiked in 50/50 (v/v) Milli-Q water: methanol compared to the internal standard mixture response ratio over 4 separate days.

	Accuracy (%)						
Concentration (µg/L)	**0.1**	**0.5**	**1**	**5**	**10**	**50**	**100**
	76.2	68.3	68.1	88.5	89.9	74	98.3
	172.5	128.5	142.4	111.2	116.5	102.6	101.4

Table A1.4: Recoveries of native spike mixtures of parents and conjugates in Milli-Q water and Elm River water. Solid phase extraction was done using the given 3 cc cartridge of 60 mg of sorbent. The values listed are the mean recoveries in % (n=3) and relative standard deviation in % in parentheses.

Compound	Target (µg/L)	Cartridge Type	Mass of Native Recovery (µg/L)							
			I.S. Spike at Given Stage of Solid Phase Extraction (SPE)							
			In Original Jar		After SPE		At Reconstitution		In LC Vial	
			Milli-Q	Elm River	Milli-Q	Elm River	Milli-Q	Elm River	Milli-Q	Elm River
PRO	20	HLB	97 (2.3)	98 (2.7)	88 (8.4)	95 (1.8)	82 (8.7)	85 (9.5)	65 (9.3)	61 (34)
		WAX	101 (10)	94 (7.2)	27 (83)	88 (6.0)	24 (93)	90 (10)	70 (83)	82 (9.7)
		MAX	101 (6.7)	95 (1.3)	88 (9.6)	91 (6.9)	86 (15)	92 (5.0)	87 (50)	86 (35)
SMX	20	HLB	98 (3.6)	104 (4.6)	54 (77)	84 (7.8)	75 (41)	76 (15)	70 (41)	51 (42)
		WAX	95 (4.9)	98 (2.6)	95 (3.2)	86 (2.7)	84 (11)	67 (3.3)	86 (38)	61 (13)
		MAX	101 (2.0)	97 (4.3)	95 (9.4)	92 (7.4)	60 (12)	77 (4.8)	53 (8.9)	72 (5.7)
PRO-Sul	20	HLB	84 (32)	64 (13)	95 (17)	54 (20)	97 (2.1)	58 (17)	94 (10)	51 (30)
		WAX	92 (13)	81 (13)	101 (7.9)	77 (13)	111 (16)	78 (8.0)	112 (47)	76 (12)
		MAX	98 (14)	7 (173)	61 (84)	6 (111)	73 (31)	4 (93)	75 (62)	4 (172)
SMX-Glc	20	HLB	82 (6.6)	87 (16)	6 (27)	27 (9.5)	6 (37)	28 (4.1)	7 (9.4)	16 (35)
		WAX	97 (8.1)	112 (22)	96 (1.8)	55 (26)	96 (4.3)	49 (16)	96 (30)	41 (8.8)
		MAX	87 (24)	48 (88)	50 (70)	17 (89)	30 (88)	13 (119)	27 (85)	13 (114)

Appendix 2

Measurement of Thyroxine and Its Glucuronide in Municipal Wastewater and Solids Using Weak Anion Exchange Solid Phase Extraction and Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry

Table A2.1: Physicochemical parameters of thyroxine and thyroxine-*O*-β-D-glucuronide.

Compound	Molar mass (g/mol)[a]	Aqueous Solubility (mg/L)[b]	pK_a[c]	$LogK_{ow}$[b]
Thyroxine	777.7	0.162	0.27, 7.43, 9.43	4.12
Thyroxine-*O*-B-D-glucuronide	953.7	N/A	0.26, 2.07, 9.43, 12.58, 13.51, 14.85	2.65

[a]PHYSPROP Database – www.srcinc.com. [b]Solubility and $LogK_{OW}$ predicted by EPISuite via ECOSAR. [c]Marvin Sketch pKa prediction.

Table A2.2: North Main wastewater treatment plant metadata including flow rate and various solids data (all dry weight, except for primary sludge). Data provided by City of Winnipeg analytical chemistry department with permission from Dave Maxwell, Senior Manager of operations. (ML/Day: megalitres/day; Mix Liq: mixed liquor; Comp: composite of three channels; %TS: % total solids; RAS: return activated sludge; WAS: waste activated sludge).

Date (YY/MM/DD)	Flow Rate (ML/Day)	Suspended Solids (24 h Composites) (mg/L)				Primary Sludge (%TS)	RAS (mg/L)	WAS Comp (mg/L)
		Primary	Secondary	Mix Liq Comp	Final Effluent			
16/11/22	189	82	11	2227	11	4.09	11533	7422
16/11/29	361	163	25	2480	44	8.08	11200	8400
16/12/08	159	97	12	2313	10	3.47	10533	8044
16/12/20	151	113	18	2007	19	4.33	13233	7644
17/01/04	124	113	17	2414	17	5.05	13567	6956
17/01/17	119	130	12	2100	11	3.51	14167	8744
17/01/24	147	110	10	3167	8	3.47	13367	13878
17/01/31	124	110	10	1967	12	3.35	10900	8600
17/02/08	128	117	9	2414	10	2.78	14100	8311
17/02/23	156	120	15	3307	19	3.96	14067	13989
17/02/28	155	123	11	2160	13	3.73	14167	8156
17/03/08	184	98	15	2414	17	5.11	13300	16653
17/03/13	158	114	12	2414	16	3.31	14200	10083
17/03/15	159	136	22	2414	19	3.55	17400	12050

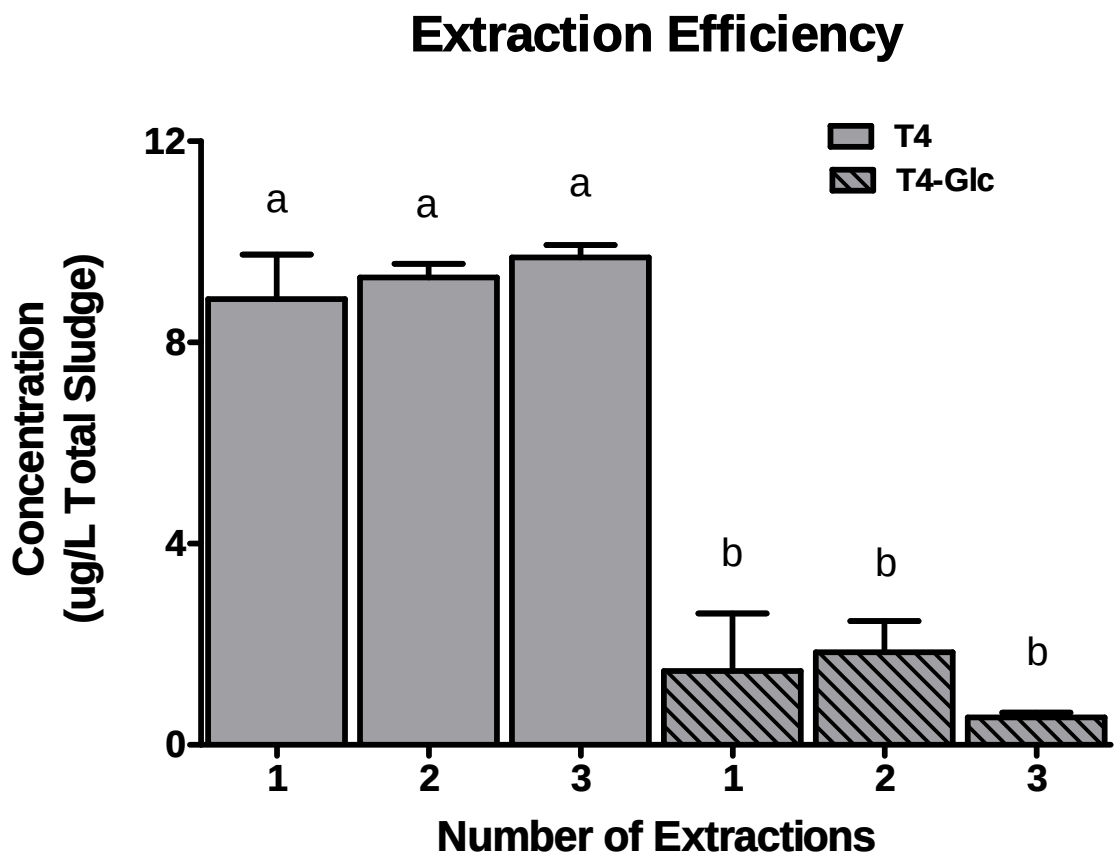

Figure A2.1: Endogenous T4 and T4-Glc extracted from nine equal 10 mL aliquots of primary sludge. The effect of extracting these compounds using 1, 2, or 3- 3mL aliquots of methanol was determined.

Appendix 3

Distribution and Fate of Pharmaceuticals and their Metabolite Conjugates in a Municipal Wastewater Treatment Plant

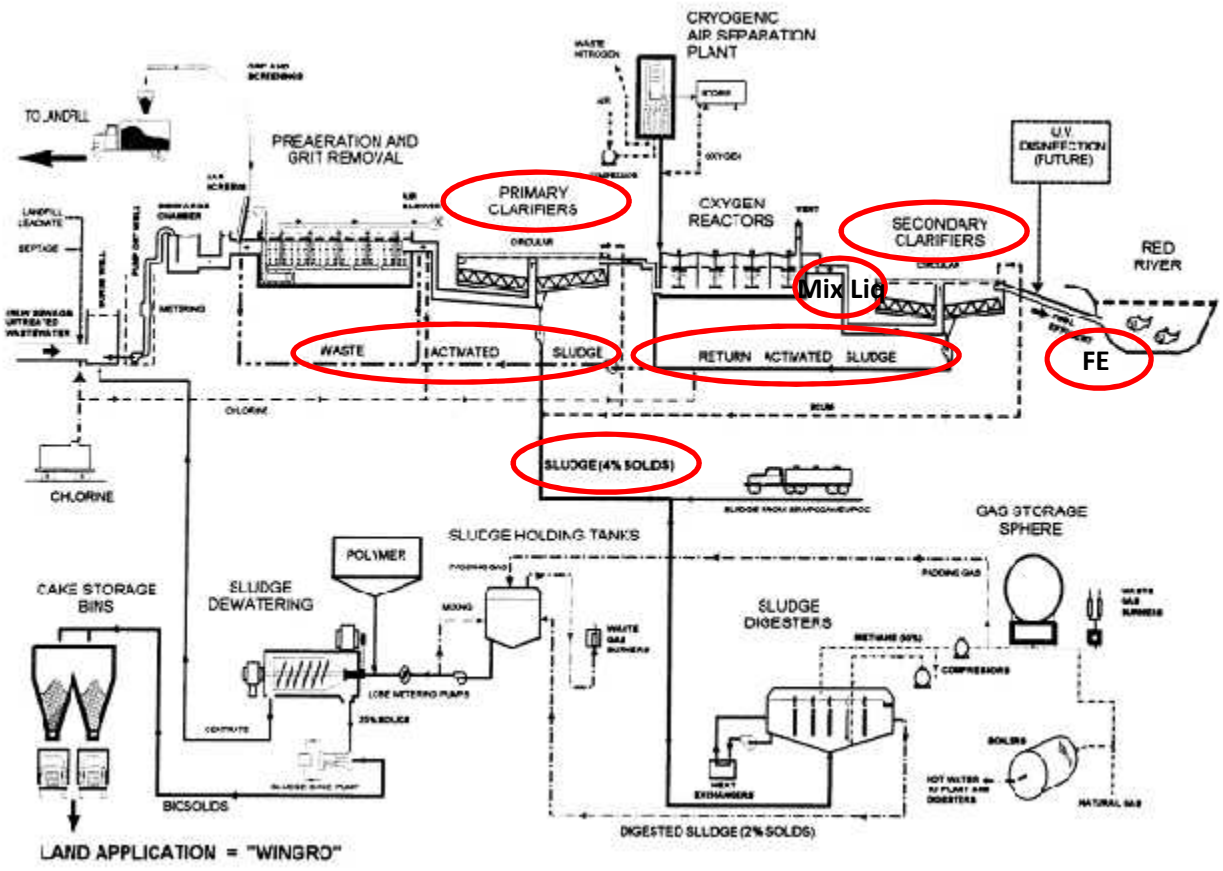

Figure A3.1: Schematic of the North Main Pollution Control WWTP in Winnipeg, Manitoba, Canada obtained from public document on operations. This schematic shows the current state of the facilities with the exception of the UV treatment upgrades introduced in 1999. However, the figure still notes the current location of the UV unit. In circles are the sampling locations in this study: primary, secondary, mixed liquor, final effluent (FE), waste activated sludge, return activated sludge, and primary sludge. Additionally, the sludge treatment process is outlined in the bottom half of the schematic. Permission to use from David Maxwell, chief manager of North Main WWTP operations.

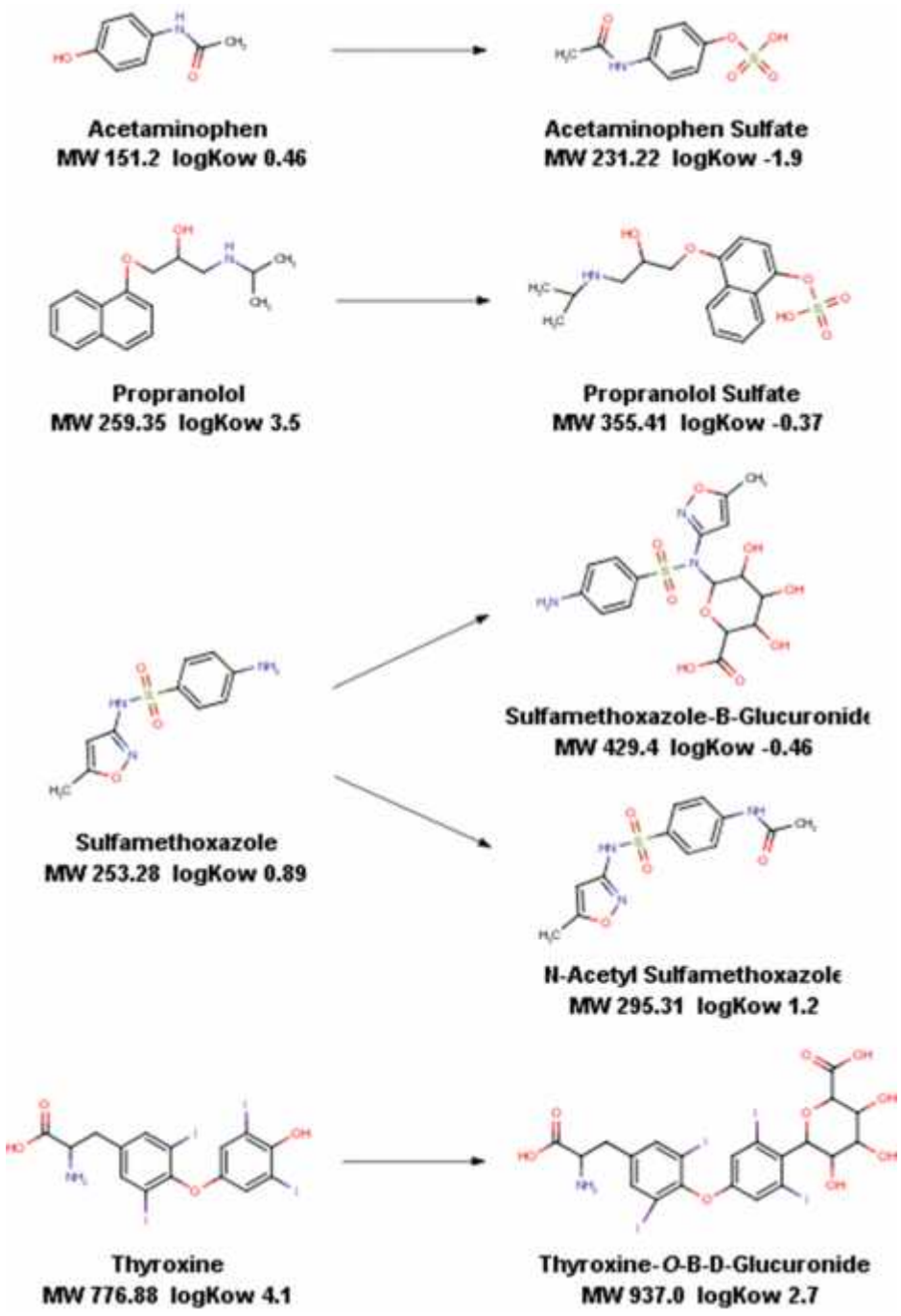

Figure A3.2: The physical structures (fully protonated), molecular masses, and logKOW values for all parent pharmaceuticals and conjugates monitored in this study. Structures were drawn using Chem Axon® Marvin Sketch v. 6.2. Structures were copied as SMILES notations and entered in ECOSAR via EpiSuite v .1.1 to generate molecular masses and logKOW. Parent compounds all had known values in the database, the conjugates are all estimates.

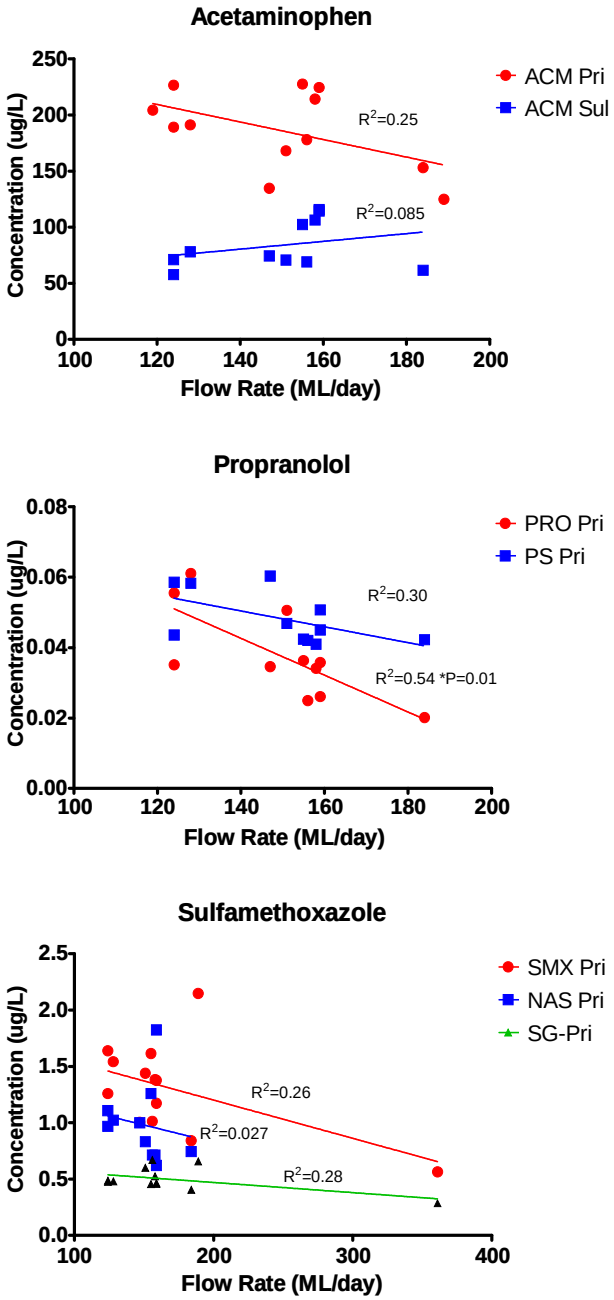

Figure A3.3: Hydrodynamic correlations of flow rate to concentrations seen in the primary clarifier from Nov.22, 2016 to March 15, 2017. Graphs are organised by class of compound, and linear regression performed with trendlines overlaid. Each plot represents a single aliquot taken from a 24 hour composite sample of the primary clarifier. ML/day represents megalitres/day. Only propranolol shows a significant deviation from 0 with respect to the R2 value.

Table A3.1: Metadata corresponding to the flow rates, and solids data collected as part of the daily operating protocols of the North Main Water Pollution Control in Winnipeg, Manitoba, Canada. All solids concentrations are listed in mg dry solids /L wastewater, except for primary sludge which was measured gravimetrically in % solids. Each data point was calculated from 24 h composite samples for each stage of treatment. Mixed liquor and waste activated sludge were additionally equal composites (1/3 each) of 3 channels (each 24 h composite) contributing to the wastewater process. WAS: waste activated sludge, RAS: return activated sludge, comp: 24h composite samples, Mix Liq: mixed liquor.

Date	**Flow Rate**	**Suspended Solids (24 h Composites) (mg/L)**				**%TS**	**mg/L**	**mg/L**
(YY/MM/DD)	(ML/Day)	Primary	Secondary	Mix Liq Comp	Final Effluent	Primary Sludge	RAS	WAS Comp
16/11/22	189	82	11	2227	11	4.09	11533	7422
16/11/29	361	163	25	2480	44	8.08	11200	8400
16/12/08	159	97	12	2313	10	3.47	10533	8044
16/12/20	151	113	18	2007	19	4.33	13233	7644
17/01/04	124	113	17	2414	17	5.05	13567	6956
17/01/17	119	130	12	2100	11	3.51	14167	8744
17/01/24	147	110	10	3167	8	3.47	13367	13878
17/01/31	124	110	10	1967	12	3.35	10900	8600
17/02/08	128	117	9	2414	10	2.78	14100	8311
17/02/23	156	120	15	3307	19	3.96	14067	13989
17/02/28	155	123	11	2160	13	3.73	14167	8156
17/03/08	184	98	15	2414	17	5.11	13300	16653
17/03/13	158	114	12	2414	16	3.31	14200	10083
17/03/15	159	136	22	2414	19	3.55	17400	12050
N	14	14	14	14	14	14	14	14
Mean	165	116	14	2414	16	4	13267	9924
Std Dev	58.0	18.5	4.6	372.5	8.6	1.3	1718.9	2890.6

Flow rates of the WWTP from Nov. 22, 2016- March 15, 2017 were 119- 361 ML/day (mean 165 ML/day). The concentrations of total suspended solids for all stages (in mg/L) were: primary 82-163 (mean 116), mixed liquor 1967-3307 (mean 2414), secondary 9-25 (mean 14), final effluent 8-44 (mean 16), RAS 10533-17400 (mean 13267), WAS 6956-16653 (mean 9924), and primary sludge (in % solids) 2.78-8.08 (mean 4.1).

Table A3.2: Optimisation parameters for the parents and conjugates of the four classes of pharmaceuticals analysed in positive ESI mode on a UHPLC-MS/MS instrument. Also listed are the corresponding labelled isotopes. Q and q are quantifier and qualifier respectively.

Compound	Frag (V)	CE (eV)	Precursor	Product	Q or q
Propranolol	110	16	260.2	116.1	q
		16		183	Q
Sulfamethoxazole	107	24	254.1	108	q
		12		156	Q
Thyroxine	147	24	777.7	731.7	Q
		44		604.8	q
Acetaminophen	94	12	152.1	110	Q
		32		65.1	q
Labeled Isoptopes					
Propranolol-d_7	110	16	267.2	116.1	Q
		20		72.1	q
Sulfamethoxazole-d_4	101	28	258.1	96.1	Q
		24		112	q
Thyroxine-^{13}C	138	28	783.7	737.7	Q
Acetaminophen-d_4	104	12	156.1	114.1	Q
		32		69.1	q
Conjugates					
***rac*-4'-Hydroxy Propranolol Sulfate**	138	16	356.1	276.1	Q
		20		116.1	q
Sulfamethoxazole β-D-Glucuronide	100	0	430.1	254	Q
		48		92.1	q
Thyroxine-4-*O*- -D-Glucuronide	141	16	953.7	777.7	Q
		40		731.7	q
4-Acetaminophen Sulfate	97	12	232	152.1	Q
		28		110	q
Labeled Isotopes					
***rac*-4'-Hydroxy Propranolol-d_7 Sulfate**	122	16	362.2	282.2	Q
Sulfamethoxazole-d_4-β-D glucuronide	104	0	434.12	258.1	Q
		52		96.1	q
Thyroxine-$^{13}C_6$-4-*O*-β-D-Glucuronide	144	16	959.8	783.7	Q

		60		85	q
4-Acetaminophen-d_3 Sulfate	85	12	235	155.1	Q
		28		111	q

Table A3.3: Method limits of quantification (MLQ), method limits of detection (MLD) for primary wastewater filtrate (WW) (ng/L), primary suspended solids (SS) (ng/g solids dry weight). Intraday precision and interday precision (% relative standard deviation- RSD) were calculated using primary wastewater.

Compound	MLQ/ MLD		Intraday Precision (%RSD)	Interday Precision (%RSD)
	WW	SS		
Acetaminophen	150/ 40	86/ 60	4.1	0.6
Acetaminophen Sulfate	160/ 120	n.q.	5.1	4.3
Propranolol	16/ 3.0	5.6/ 4.9	2.5	3.2
Propranolol Sulfate	15/ 6.5	n.q.	1.8	5.5
Sulfamethoxazole	111/ 3.9		7.6	4.1
***N*-Acetyl Sulfamethoxazole**	113/ 31	n.q.	8.4	5.6
Sulfamethoxazole Glucuronide	36/ 32	n.q.	9.2	5.8
Thyroxine	2.13/ 0.64	4.3/ 1.3	2.6	9.6
Thyroxine-Glucuronide	2.63/ 0.79	28.3/ 8.5	6.5	5.7

n.q. None quantified

Table A3.4: Raw data of the concentrations for each corresponding sampling point to the metadata found in Table S1. None quantified= n.q.

a) ACM

Date	Aqueous Concentration (µg/L)				Suspended Solids Concentration (µg/g solids)						
	Primary	Secondary	Mixed Liquor	Final Effluent	Primary	Secondary	Mixed Liquor	Final Effluent	Pri Sludge (ug/L)	RAS	WAS
16/11/22	124.9	0.35	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/11/29	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/08	n.q.	n.q.	n.q.	n.q.	2.86	9.94	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/20	168.1	0.64	n.q.	0.36	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/04	226.4	0.72	0.72	0.57	n.q.	0.86	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/17	204.2	n.q.	n.q.	0.40	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/24	134.6	0.28	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	3300	n.q.	n.q.
17/01/31	189.1	0.43	n.q.	0.38	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/08	191.1	0.20	0.28	0.15	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/23	178.0	n.q.	0.38	0.25	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/28	227.5	0.35	0.56	0.30	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/03/08	153.1	n.q.	n.q.	0.23	n.q.	17.7	n.q.	n.q.	n.q.	n.q.	n.q.
17/03/13	214.1	0.30	0.65	n.q.	1.34	n.q.	n.q.	30.5	n.q.	n.q.	n.q.
17/03/15	224.5	0.28	0.44	0.26	2.05	n.q.	n.q.	28.4	4200	n.q.	n.q.

b) ACM-Sul

Date	Aqueous Concentration (µg/L)				Suspended Solids Concentration (µg/g solids)						
	Primary	Secondary	Mixed Liquor	Final Effluent	Primary	Secondary	Mixed Liquor	Final Effluent	Pri Sludge (ug/L)	RAS	WAS
16/11/22	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/11/29	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/08	115.6	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/20	70.6	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/04	71.0	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/17	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/24	74.4	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/31	57.7	n.q.	n.q.	n.q.	n.q.	1.77	1.06	28.7	n.q.	n.q.	n.q.
17/02/08	78.1	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/23	69.1	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/28	102.3	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/03/08	61.5	n.q.	n.q.	n.q.	n.q.	n.q.	0.16	n.q.	n.q.	0.018	n.q.
17/03/13	106.2	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	17.4	n.q.	n.q.	n.q.
17/03/15	114.4	n.q.	n.q.	n.q.	0.15	n.q.	n.q.	n.q.	6.77	n.q.	n.q.

c) PRO

Date	Aqueous Concentration (µg/L)				Suspended Solids Concentration (µg/g solids)						
	Primary	Secondary	Mixed Liquor	Final Effluent	Primary	Secondary	Mixed Liquor	Final Effluent	Pri Sludge (ug/L)	RAS	WAS
16/11/22	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/11/29	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/08	0.026	0.022	0.022	0.023	n.q.	n.q.	0.071	n.q.	n.q.	n.q.	n.q.
16/12/20	0.051	0.062	0.066	0.057	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/04	0.035	0.052	0.056	0.052	n.q.	n.q.	n.q.	0.16	n.q.	n.q.	n.q.
17/01/17	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/24	0.035	0.044	0.047	0.043	0.051	n.q.	0.034	n.q.	0.93	0.055	0.027
17/01/31	0.055	0.051	0.047	0.057	0.037	n.q.	0.080	n.q.	1.04	0.064	0.034
17/02/08	0.061	0.061	0.060	0.050	0.050	n.q.	0.031	n.q.	0.62	0.093	0.035
17/02/23	0.025	0.029	0.030	0.027	n.q.	n.q.	n.q.	n.q.	0.95	0.037	0.027
17/02/28	0.036	0.043	0.042	0.045	0.13	n.q.	0.0056	n.q.	1.14	0.030	0.027
17/03/08	0.020	0.017	0.024	0.016	0.019	n.q.	0.053	n.q.	1.61	0.035	0.020
17/03/13	0.034	0.038	0.056	0.049	0.026	n.q.	n.q.	n.q.	1.05	0.034	0.053
17/03/15	0.036	0.038	0.044	0.037	n.q.	n.q.	0.039	n.q.	0.94	0.049	0.023

d) PRO-Sul

Date	Aqueous Concentration (µg/L)				Suspended Solids Concentration (µg/g solids)						
	Primary	Secondary	Mixed Liquor	Final Effluent	Primary	Secondary	Mixed Liquor	Final Effluent	Pri Sludge (ug/L)	RAS	WAS
16/11/22	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/11/29	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/08	0.051	0.030	0.022	0.051	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/20	0.047	0.029	0.016	0.036	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/04	0.058	0.030	0.032	0.042	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/17	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/24	0.060	0.041	0.036	0.031	n.q.	n.q.	0.00099	n.q.	0.043	0.0066	0.0024
17/01/31	0.044	0.047	0.037	0.051	n.q.	n.q.	n.q.	n.q.	n.q.	0.0078	n.q.
17/02/08	0.058	0.057	0.044	0.050	0.022	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/23	0.042	0.033	0.030	0.030	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/28	0.042	0.081	0.039	0.044	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/03/08	0.042	0.022	0.025	0.028	n.q.	0.032	n.q.	n.q.	n.q.	0.0012	n.q.
17/03/13	0.041	0.039	0.020	0.032	n.q.	n.q.	n.q.	n.q.	0.013	0.0044	0.00046
17/03/15	0.045	0.039	0.029	0.047	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	0.00015

e) SMX

Date	Aqueous Concentration (µg/L)				Suspended Solids Concentration (µg/g solids)						
	Primary	Secondary	Mixed Liquor	Final Effluent	Primary	Secondary	Mixed Liquor	Final Effluent	Pri Sludge (ug/L)	RAS	WAS
16/11/22	2.15	0.37	0.11	0.33	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/11/29	0.56	n.q.	0.22	0.27	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/08	1.17	0.35	0.15	0.32	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/20	1.44	0.46	0.16	0.41	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/04	1.26	0.46	0.32	0.41	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/17	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/24	1.00	0.27	0.17	0.25	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/31	1.64	0.42	0.21	0.39	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/08	1.54	0.40	0.29	0.39	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/23	1.01	0.46	0.26	0.35	n.q.	n.q.	0.033	n.q.	0.77	0.052	0.019
17/02/28	1.61	0.61	0.31	0.53	0.26	n.q.	0.051	n.q.	0.67	0.051	0.049
17/03/08	0.84	0.36	0.24	0.37	0.10	0.13	0.060	n.q.	0.63	0.035	0.014
17/03/13	1.38	0.57	0.30	0.55	0.097	0.81	0.049	0.79	0.96	0.058	0.042
17/03/15	1.37	0.43	0.24	0.37	n.q.	n.q.	0.030	n.q.	n.q.	0.034	0.017

f) N-Ace-SMX

Date	Aqueous Concentration (µg/L)				Suspended Solids Concentration (µg/g solids)						
	Primary	Secondary	Mixed Liquor	Final Effluent	Primary	Secondary	Mixed Liquor	Final Effluent	Pri Sludge (ug/L)	RAS	WAS
16/11/22	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/11/29	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/08	1.82	0.70	0.40	0.93	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/20	0.83	0.27	0.13	0.26	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/04	1.11	0.48	0.34	0.42	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/17	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/24	1.00	0.38	0.22	0.42	0.10	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/31	0.97	0.25	0.19	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/08	1.02	0.27	0.11	0.35	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/23	0.71	0.38	0.23	0.35	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/28	1.26	0.26	0.24	0.46	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/03/08	0.74	0.23	0.17	0.26	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/03/13	0.71	0.26	0.14	0.31	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/03/15	0.62	0.25	0.22	0.26	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.

g) SMX-Glc

Date	Aqueous Concentration (µg/L)				Suspended Solids Concentration (µg/g solids)						
	Primary	Secondary	Mixed Liquor	Final Effluent	Primary	Secondary	Mixed Liquor	Final Effluent	Pri Sludge (ug/L)	RAS	WAS
16/11/22	0.66	0.29	0.29	0.20	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/11/29	0.28	0.11	0.18	0.17	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/08	0.46	0.13	0.069	0.084	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/20	0.60	0.23	0.13	0.13	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/04	0.49	0	0.18	0.17	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/17	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/24	n.q.	0.072	0.076	0.042	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/31	0.47	n.q.	0.087	0.091	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/08	0.48	0.15	n.q.	0.10	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/23	0.67	0.12	n.q.	0.064	0.055	n.q.	n.q.	n.q.	n.q.	0.019	n.q.
17/02/28	0.46	0.030	n.q.	0.074	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/03/08	0.40	0.059	n.q.	0.036	0.0079	0.14	0.021	n.q.	n.q.	0.018	n.q.
17/03/13	0.52	0.12	0.073	0.068	n.q.	n.q.	n.q.	0.12	n.q.	n.q.	n.q.
17/03/15	0.47	n.q.	0.049	n.q.	n.q.	0.043	0.0033	n.q.	n.q.	n.q.	n.q.

h) THY

Date	Aqueous Concentration (µg/L)				Suspended Solids Concentration (µg/g solids)						
	Primary	Secondary	Mixed Liquor	Final Effluent	Primary	Secondary	Mixed Liquor	Final Effluent	Pri Sludge (ug/L)	RAS	WAS
16/11/22	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/11/29	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/08	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/20	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/04	0.013	0.010	0.013	0.011	0.049	0.13	0.048	0.10	n.q.	n.q.	n.q.
17/01/17	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/24	0.0060	n.q.	0.0048	0.0025	0.012	n.q.	0.053	n.q.	6.37	0.011	0.045
17/01/31	n.q.	n.q.	0.011	n.q.	0.027	n.q.	0.19	n.q.	5.73	0.013	0.085
17/02/08	n.q.	n.q.	0.0056	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/23	0.0085	0.0075	0.0072	0.0056	n.q.	n.q.	0.049	n.q.	5.25	0.011	0.028
17/02/28	n.q.	0.0065	0.0057	n.q.	0.22	n.q.	0.019	n.q.	3.39	0.0095	0.042
17/03/08	0.0075	0.0056	n.q.	n.q.	0.033	0.11	0.052	n.q.	5.48	0.010	0.022
17/03/13	0.0051	0.0050	0.0038	0.0050	0.021	0.076	0.055	0.11	5.31	0.011	0.047
17/03/15	n.q.	0.0030	0.0050	n.q.	0.037	0.087	0.057	0.12	4.27	0.0060	0.035

i) THY-Glc

Date	Aqueous Concentration (µg/L)				Suspended Solids Concentration (µg/g solids)						
	Primary	Secondary	Mixed Liquor	Final Effluent	Primary	Secondary	Mixed Liquor	Final Effluent	Pri Sludge (ug/L)	RAS	WAS
16/11/22	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/11/29	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
16/12/08	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	0.065	n.q.	n.q.	n.q.	n.q.
16/12/20	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/04	n.q.	0.0026	0.0037	0.010	n.q.	n.q.	0.0082	0.59	n.q.	n.q.	n.q.
17/01/17	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/01/24	n.q.	0.0099	n.q.	n.q.	0.039	n.q.	0.0096	n.q.	n.q.	0.046	0.020
17/01/31	0.0070	0.0065	0.011	0.0075	0.071	n.q.	n.q.	n.q.	n.q.	n.q.	0.039
17/02/08	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
17/02/23	n.q.	0.011	n.q.	0.013	n.q.	n.q.	0.0019	0.69	0.42	0.014	0.012
17/02/28	n.q.	0.034	0.021	n.q.	n.q.	0.27	0.058	n.q.	n.q.	0.017	n.q.
17/03/08	n.q.	0.0057	n.q.	n.q.	n.q.	n.q.	0.013	n.q.	n.q.	n.q.	n.q.
17/03/13	n.q.	0.021	n.q.	0.013	n.q.	n.q.	0.0071	0.81	n.q.	0.0076	n.q.
17/03/15	n.q.	0.017	n.q.	n.q.	0.054	0.29	0.014	n.q.	n.q.	n.q.	n.q.

Appendix 4

Kinetics of Human Pharmaceutical Conjugates and the Impact of Transformation, Deconjugation, and Sorption on Persistence in Wastewater Bioreactors

Details on Experimental Methods

Materials

Methanol, formic acid, ammonium hydroxide (28.9% v/v), and isopropanol (for sterilization) were obtained from Fisher Scientific (CITY, NJ, USA), while acetonitrile was purchased from Fisher and EMD Millipore). All organic solvents were HPLC-grade. Ultrapure Milli-Q (18 MΩ-cm) water was produced from a Synergy™ Milli-Q purification system from Millipore (Billerica, MA). Nitrocellulose filter paper (0.45 µm) was obtained from Merck (Ireland), and 13 mm, 0.22 µm white PTFE luer lock inlet syringe filters was purchased from Restek (Bellefonte, PA, USA). Syringe filters were attached to an Agilent 1.0 mL glass syringe (Australia). All WAX solid phase extraction cartridges were Oasis 3 cc, 60 mg from Waters Corporation (Milford, MA), Nalgene® 250 mL white HDPE bottles were purchased from Thermo Fisher, Rockwood, Tennessee, USA. Centrifuge bottles (50 mL) were purchased from VWR, Mississauga, Ontario, Canada. Glassware was pre-cleaned by ashing at 450°C for 1 hr to destroy organic materials unless otherwise indicated. PEEK tubing (Fisher Scientific, Toronto, ON) was used in the syphoning of environmental matrices through SPE cartridges.

Standards of acetaminophen (ACM) CAS# 103-90-2 (chemical purity 94.16%), acetaminophen sulfate (ACM-Sul) CAS# 32113-41-0, propranolol (PRO) CAS# 525-66-6, 4-OH-propranolol sulfate (PRO-Sul) CAS# 57075-33-0, sulfamethoxazole (SMX) CAS# 723-46-6, *N*4-acetylsulfamethoxazole (N-Ace-SMX) CAS# 21312-10-7, sulfamethoxazole-β-glucuronide (SMX-Glc) CAS# 14365-52-7 , thyroxine (THY) CAS# 51-48-9, and thyroxine-*O*-β-D-glucuronide (THY-Glc) CAS# 21462-56-6 (all compounds chemical purities >98%, except ACM); and matching isotopically-labeled standards acetaminophen-d_4 CAS# 64315-36-2, acetaminophen-d_3 sulfate CAS# 1188263-45-7, propranolol-d_7 CAS# 344298-99-3, 4-OH-

propranolol-d_7 sulfate CAS# NA, sulfamethoxazole-d_4 CAS# 1020719-86-1, *N*4-acetylsulfamethoxazole-d_4 CAS# 1215530-54-3, sulfamethoxazole-d_4-β-glucuronide CAS# NA, thyroxine-$^{13}C_6$ CAS# 1217780-14-7, and thyroxine-$^{13}C_6$-*O*-β-D-glucuronide CAS# NA (all chemical purities >96%, and isotope purity >98%) (Toronto Research Chemicals, Toronto, ON) were obtained as neat powders. Methanolic stock solutions were made and stored at -20^{0}C. Calibration curve standard solutions (0.1, 0.5, 1, 5, 10, 50, 100, 500, 750 µg/L) for quantitative assessments were prepared from stock solutions in 50/50 (v/v) Milli-Q water:methanol and also stored at -20^{0}C.

Chromatography was performed with an Agilent 1200 UHPLC, with separation using a Waters Acquity HSS T3 C_{18} column (2.1 mm × 50 mm, 1.8 µm dp), coupled to a Waters Acquity HSS T3 C_{18} guard column (2.1 mm × 5 mm) at 42^{0}C at 0.4 mL/min. Injection volumes were 2 µL during optimisation and 10 µL during analysis. Mobile phase A1 was 0.05% formic acid (FA) in Milli-Q water, B1 was acetonitrile with 0.05% formic acid, A2 was 95/5 (v/v) Milli-Q water:methanol, and B2 was 100% acetonitrile. Gradient elution was performed as follows: 0-3.00 min linear ramp from 5% B1 to 70% B1, 3.01- 5.00 hold at 70% B1, then re-equilibrated from 5.01- 12.00 min at 5% B1. Upon completion of all analytical runs the columns were flushed with 20 min of 10% B2, then 25 min of 95% B2 to eliminate formic acid residues for column storage.

Qualitative assessment and quantification was performed through multiple reaction monitoring (MRM) on an Agilent 6410 triple quadrupole mass spectrometer in positive electrospray ionisation mode (ESI+), a capillary voltage of 4000 V, and a source temperature of 300°C. Nitrogen was used for desolvation and drying gas at 11 L/min, and for nebulization at 15 psi. Ultrapure nitrogen was used as collision gas at a flow of 16.8 L/min. The MS1 and MS2

heaters were set at 100°C. Compound-specific mass spectrometric parameters and ion fragments used are found in Table S2.

Dynamic multiple reaction monitoring was used during quantitation to minimise noise which was especially important in quantifying in the ng/L range. Criteria for positive identification and quantification was on the most abundant $[M + H]^+$ product ion fragment (quantifier) and confirmation of analyte using the second most abundant $[M + H]^+$ ion fragment (qualifier).

Table A4.1: Metadata corresponding to the flow rates, and solids data collected as part of the daily operating protocols of the North Main Water Pollution Control in Winnipeg, Manitoba, Canada. All solids concentrations are listed in mg dry solids /L wastewater. All concentrations of sorbed analytes were calculated from 100 mL aliquots of each 3.0 L bioreactor derived from 24 h composite sample.

Date	**Flow Rate**	**Suspended Solids (24 h Composites) (mg/L)**		**Wastewater treatment in Bioreactors**
(YY/MM/DD)	**(ML/Day)**	**Primary**	**Secondary**	
18/03/27	189	214	44	Secondary; No Aeration
18/04/03	361	117	11	Primary; No Aeration
18/04/10	159	166	17	Secondary, Aeration
18/04/18	151	106	22	Primary; Aeration
18/05/18	124	150	29	Activated Primary; Aeration

Table A4.2: Phosphorus species and nitrogen species in primary and secondary treatment samples used in the bioreactors in this study. Values are means with standard deviation in parentheses. TP was determined using Hach vial kit TNT #843, either unfiltered or filtered (0.45 µm syringe filter). PO4, NH4, NO2 and NO3 were analyzed with filtered effluent (0.45 µm) on a flow injection analyzer (QuickChem 8500 Series 2, Lachat Instruments, Loveland Co. USA).

Date	pH		TP unfiltered	TP filtered	PO4	NH4	NO2	NO3
	0h	24h						
18/03/27	6.48	NA	1.79 (0.01)	1.68 (0.01)	1.31 (0.01)	28.43 (0.06)	0.37 (0.02)	1.42 (0.04)
18/04/03	7.05	7.37	5.40 (0.21)	3.27 (0.03)	2.93 (0.02)	41.77 (0.31)	0.06 (0.00)	1.15 (0.02)
18/04/10	7.02	7.90	7.07 (0.29)	4.39 (0.02)	3.82 (0.01)	39.25 (0.50)	0.37 (0.15)	2.23 (0.02)
18/04/18	6.58	8.44	3.63 (0.03)	3.22 (0.03)	3.07 (0.04)	41.08 (0.38)	0.15 (0.00)	1.60 (0.30)
18/05/18	7.10	7.87	5.23 (0.03)	3.92 (0.02)	3.71 (0.02)	23.37 (0.12)	0.00 (0.00)	2.07 (0.13)

Table A4.3: Pseudo first-order kinetic rate constants* associated with the half-lives for each bioreactor experiment. Values in parentheses are ± standard error for the kinetics rates generated over the entire 24 h timeframe.

Compound	Primary				Secondary				Activated Primary	
	Air		No Air		Air		No Air		Air	
	2h	24h	2h	24h	2h	24h	2h	24h	2h	24h
ACM	0.024	0.28 (0.021)	0.056	0.008 (0.002)	0.13	0.047 (0.008)	0.13	0.074 (0.006)	2.4	2.42 (0.062)
ACM-Sul	0.18	0.23 (0.023)	0.037	0.023 (0.005)	-0.093	-0.014 (0.010)	0.16	0.05 (0.005)	0.33	0.21 (0.066)
PRO	0.11	0.18 (0.009)	0.05	-0.009 (0.004)	0.085	0.053 (0.003)	-0.075	0.012 (0.004)	0.079	0.11 (0.010)
PRO-Sul	-0.17	-0.025 (0.008)	-0.023	-0.009 (0.004)	-0.086	-0.013 (0.004)	-0.055	-0.006 (0.005)	-0.13	0.051 (0.010)
SMX	-0.023	0.009 (0.003)	0.001	-0.01 (0.002)	-0.038	-0.003 (0.003)	-0.19	-0.016 (0.005)	-0.16	0.010 (0.009)
N-Ace-SMX	0.002	-0.004 (0.002)	0.00001	0.001 (0.004)	0.046	0.005 (0.004)	0.063	0.005 (0.003)	0.21	0.13 (0.009)
SMX-Glc	0.08	0.033 (0.004)	0.17	0.055 (0.005)	-0.068	-0.006 (0.003)	0.032	0.006 (0.004)	0.051	0.071 (0.005)
THY	-0.09	-0.006 (0.002)	0.019	0.007 (0.004)	-0.13	-0.010 (0.005)	-0.1	-0.012 (0.004)	0.02	0.034 (0.006)
THY-Glc	0.98	0.11 (0.041)	0.36	0.011 (0.046)	0.79	0.14 (0.040)	0.59	0.068 (0.063)	0.15	0.10 (0.068)

*Note: Negative constants reflect nominal increases of analyte between 0h and the given timepoint.

Table A4.4: Concentrations of acetaminophen, sulfamethoxazole, propranolol, thyroxine, and thyroxine glucuronide in the suspended solids of primary bioreactors associated with the 0h and 24 h timepoints.

	Concentration (µg/g solids dry weight)			
	No Air		**Air**	
Compound	**0h**	**24h**	**0h**	**24h**
ACM	3.0	10.5	6.8	2.0
PRO	0.073	0.27	0.061	0.022
SMX	0.35	0.46	0.55	0.55
THY	0.04	0.8	0.065	0.64
THY-Glc	<LOQ	0.026	<LOQ	0.0002

Appendix 5

SSRI and β-blocker Transformation Products may not pose a Significant Risk of Toxicity to Aquatic Organisms in Wastewater Effluent-Dominated Receiving Waters

Supporting Data from Modeling Software

List and structures of all β- blocker transformation products.

Physicochemical properties of β- blocker metabolites and transformation products.

$LogK_{OW}$ and water solubility, were generated by EPISuite via ECOSAR v1.11. The IUPAC names and SMILES were generated via ChemAxon Marvin Sketch by drawing the chemical structure of each transformation product. Human metabolites were determined using the pharmacokinetic literature. Biodegradation products were estimated using the University of Minnesota Biocatalysis/ Biodegradation Database Pathway Prediction System aerobic microbial degradation software, and photolysis products were determined using the available experimental literature.

Atenolol (Ate) (Figure A5.1)

Ate IUPAC: 2-(4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)acetamide

SMILES: CC(C)NCC(O)COC1=CC=C(CC(N)=O)C=C1

$LogK_{OW}$ = -0.03

Wat Sol = 13300 mg/L

Human Metabolites

(Ate)A IUPAC: 6-{2-[4-(carbamoylmethyl)phenoxy]-1-{[(propan-2-yl)amino]oxy}ethoxy}-3,4,5-trihydroxyoxane-2-carboxylic acid

SMILES: CC(C)NOC(COC1=CC=C(CC(N)=O)C=C1)OC1OC(C(O)C(O)C1O)C(O)=O

$LogK_{OW}$ = -1.04

Wat Sol = 612 mg/L

(Ate)B IUPAC: 2-hydroxy-2-(4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)acetamide

SMILES: CC(C)NCC(O)COC1=CC=C(C=C1)C(O)C(N)=O

$LogK_{OW}$ = -0.41

Wat Sol = 1697 mg/L

Biodegradation Products

(Ate)C IUPAC: propan-2-one

SMILES: CC(C)=O

$LogK_{OW}$ = -0.24

Wat Sol = 1000000 mg/L

(Ate)D IUPAC: propan-2-amine

SMILES: CC(C)N

$LogK_{OW}$ = 0.27

Wat Sol = 1000000 mg/L

(Ate)E IUPAC: 2-[4-(2-hydroxy-3-oxopropoxy)phenyl]acetamide

SMILES: NC(=O)CC1=CC=C(OCC(O)C=O)C=C1

$LogK_{OW}$ = -1.42

Wat Sol = 538800 mg/L

(Ate)F IUPAC: 2-[4-(3-amino-2-hydroxypropoxy)phenyl]acetamide

SMILES: NCC(O)COC1=CC=C(CC(N)=O)C=C1

$LogK_{OW}$ = -1.40

Wat Sol = 25130 mg/L

(Ate)G IUPAC: 2-(4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)acetic acid

SMILES: CC(C)NCC(O)COC1=CC=C(CC(O)=O)C=C1

$LogK_{OW}$ = -2.34

Wat Sol = 16140 mg/L

(Ate)H IUPAC: 3-[4-(carbamoylmethyl)phenoxy]-2-hydroxypropanoic acid

SMILES: NC(=O)CC1=CC=C(OCC(O)C(O)=O)C=C1

$LogK_{OW}$ = -0.86

Wat Sol = 145600 mg/L

(Ate)I IUPAC: 2-[4-(2-hydroxy-3-oxopropoxy)phenyl]acetic acid

SMILES: OC(COC1=CC=C(CC(O)=O)C=C1)C=O

$LogK_{OW}$ = -0.54

Wat Sol = 754000 mg/L

(Ate)J IUPAC: 2-[4-(3-amino-2-hydroxypropoxy)phenyl]acetic acid

SMILES: NCC(O)COC1=CC=C(CC(O)=O)C=C1

$LogK_{OW}$ = -3.72

Wat Sol = 411000 mg/L

(AteA)K IUPAC: 6-{2-[4-(carboxymethyl)phenoxy]-1-{[(propan-2-yl)amino]oxy}ethoxy}-3,4,5-trihydroxyoxane-2-carboxylic acid

SMILES: CC(C)NOC(COC1=CC=C(CC(O)=O)C=C1)OC1OC(C(O)C(O)C1O)C(O)=O

$LogK_{OW}$ = -0.74

Wat Sol = 17080 mg/L

(AteB)L IUPAC: 2-[4-(3-amino-2-hydroxypropoxy)phenyl]-2-hydroxyacetamide

SMILES: NCC(O)COC1=CC=C(C=C1)C(O)C(N)=O

$LogK_{OW}$ = -1.78

Wat Sol = 43630 mg/L

(AteB)M IUPAC: 2-hydroxy-2-(4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)acetic acid

SMILES: CC(C)NCC(O)COC1=CC=C(C=C1)C(O)C(O)=O

$LogK_{OW}$ = -2.80

Wat Sol = 31970 mg/L

(AteB)N IUPAC: 2-hydroxy-2-[4-(2-hydroxy-3-oxopropoxy)phenyl]acetamide

SMILES: NC(=O)C(O)C1=CC=C(OCC(O)C=O)C=C1

$LogK_{OW}$ = -1.80

Wat Sol = 935800 mg/L

(AteB)O IUPAC: 2-[4-(3-amino-2-hydroxypropoxy)phenyl]-2-hydroxyacetic acid

SMILES: NCC(O)COC1=CC=C(C=C1)C(O)C(O)=O

$LogK_{OW}$ = -4.17

Wat Sol = 822600 mg/L

(AteB)P IUPAC: 3-{4-[carbamoyl(hydroxy)methyl]phenoxy}-2-hydroxypropanoic acid

SMILES: NC(=O)C(O)C1=CC=C(OCC(O)C(O)=O)C=C1

$LogK_{OW}$ = -1.24

Wat Sol = 251800 mg/L

Photodegradation Products

(Ate)Q IUPAC: 3-[(propan-2-yl)amino]propane-1,2-diol

SMILES: CC(C)NCC(O)CO

$LogK_{OW}$ = -0.88

Wat Sol = 1000000 mg/L

(Ate)R IUPAC: 2-(4-hydroxyphenyl)acetamide

SMILES: NC(=O)CC1=CC=C(O)C=C1

$LogK_{OW}$ = 0.065

Wat Sol = 340300 mg/L

(Ate)S IUPAC: 2-(3,4-dihydroxyphenyl)acetamide

SMILES: NC(=O)CC1=CC=C(O)C(O)=C1

$LogK_{OW}$ = -0.42

Wat Sol = 544900 mg/L

(Ate)T IUPAC: 2-(2-hydroxy-4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)acetamide

SMILES: CC(C)NCC(O)COC1=CC=C(CC(N)=O)C(O)=C1

$LogK_{OW}$ = -0.51

Wat Sol = 7826 mg/L

(Ate)U IUPAC: 2-hydroxy-4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}benzaldehyde

SMILES: CC(C)NCC(O)COC1=CC=C(C=O)C(O)=C1

$LogK_{OW}$ = 1.44

Wat Sol = 52210 mg/L

Metoprolol (Met) (Figure A5.2)

Met IUPAC: {2-hydroxy-3-[4-(2-methoxyethyl)phenoxy]propyl}(propan-2-yl)amine

SMILES: COCCC1=CC=C(OCC(O)CNC(C)C)C=C1

$LogK_{OW}$ = 1.69

Wat Sol = 16900 mg/L

Human Metabolites

(Met)AIUPAC: 3,4,5-trihydroxy-6-{2-[4-(2-methoxyethyl)phenoxy]-1-{[(propan-2-yl)amino]oxy}ethoxy}oxane-2-carboxylic acid

SMILES: COCCC1=CC=C(OCC(ONC(C)C)OC2OC(C(O)C(O)C2O)C(O)=O)C=C1

$LogK_{OW}$ = 0.68

Wat Sol = 1038 mg/L

(Met)BIUPAC: 1-(4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)-2-methoxyethan-1-ol

SMILES: COCC(O)C1=CC=C(OCC(O)CNC(C)C)C=C1

$LogK_{OW}$ = 0.56

Wat Sol = 51660 mg/L

(Met)CIUPAC: 2-hydroxy-3-[4-(2-methoxyethyl)phenoxy]propanoic acid

SMILES: COCCC1=CC=C(OCC(O)C(O)=O)C=C1

$LogK_{OW}$ = 0.87

Wat Sol = 39230 mg/L

(Met)DIUPAC: 2-(4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)ethan-1-ol

SMILES: CC(C)NCC(O)COC1=CC=C(CCO)C=C1

$LogK_{OW}$ = 1.40

Wat Sol = 14670 mg/L

(Met)E IUPAC: 2-(4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)acetic acid

SMILES: CC(C)NCC(O)COC1=CC=C(CC(O)=O)C=C1

$LogK_{OW}$ = -2.34

Wat Sol = 16140 mg/L

Biodegradation Products

(Met)F IUPAC: propan-2-one

SMILES: CC(C)=O

$LogK_{OW}$ = -0.24

Wat Sol = 1000000 mg/L

(Met)GIUPAC: propan-2-amine

SMILES: CC(C)N

$LogK_{OW}$ = 0.27

Wat Sol = 1000000 mg/L

(Met)HIUPAC: 2-hydroxy-3-[4-(2-methoxyethyl)phenoxy]propanal

SMILES: COCCC1=CC=C(OCC(O)C=O)C=C1

LogK_{OW} = 0.30

Wat Sol = 58470 mg/L

(Met)I IUPAC: 1-amino-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol

SMILES: COCCC1=CC=C(OCC(O)CN)C=C1

LogK_{OW} = 0.32

Wat Sol = 175200 mg/L

(MetA)None

(MetB)J IUPAC: 1-amino-3-[4-(1-hydroxy-2-methoxyethyl)phenoxy]propan-2-ol

SMILES: COCC(O)C1=CC=C(OCC(O)CN)C=C1

LogK_{OW} = -0.81

Wat Sol = 1000000 mg/L

(MetB)K IUPAC: 2-hydroxy-3-[4-(1-hydroxy-2-methoxyethyl)phenoxy]propanal

SMILES: COCC(O)C1=CC=C(OCC(O)C=O)C=C1

LogK_{OW} = -0.83

Wat Sol = 137100 mg/L

(MetB)L IUPAC: 2-hydroxy-3-[4-(1-hydroxy-2-methoxyethyl)phenoxy]propanoic acid

SMILES: COCC(O)C1=CC=C(OCC(O)C(O)=O)C=C1

$LogK_{OW}$ = -0.27

Wat Sol = 91580 mg/L

(MetC)None

(MetD)M IUPAC: 2-(4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)acetaldehyde

SMILES: CC(C)NCC(O)COC1=CC=C(CC=O)C=C1

$LogK_{OW}$ = 0.97

Wat Sol = 35150 mg/L

(MetD)N IUPAC: 1-amino-3-[4-(2-hydroxyethyl)phenoxy]propan-2-ol

SMILES: NCC(O)COC1=CC=C(CCO)C=C1

$LogK_{OW}$ = 0.027

Wat Sol = 369800 mg/L

(MetD)O IUPAC: 2-hydroxy-3-[4-(2-hydroxyethyl)phenoxy]propanal

SMILES: OCCC1=CC=C(OCC(O)C=O)C=C1

$LogK_{OW}$ = 0.008

Wat Sol = 38120 mg/L

(MetD)P IUPAC: 2-(4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)acetic acid

SMILES: CC(C)NCC(O)COC1=CC=C(CC(O)=O)C=C1

$LogK_{OW}$ = -2.34

Wat Sol = 16140 mg/L

(MetD)Q IUPAC: 2-[4-(3-amino-2-hydroxypropoxy)phenyl]acetaldehyde

SMILES: NCC(O)COC1=CC=C(CC=O)C=C1

$LogK_{OW}$ = -0.40

Wat Sol = 884300 mg/L

(MetD)R IUPAC: 2-hydroxy-3-[4-(2-hydroxyethyl)phenoxy]propanoic acid

SMILES: OCCC1=CC=C(OCC(O)C(O)=O)C=C1

$LogK_{OW}$ = 0.57

Wat Sol = 25690 mg/L

(MetE)S IUPAC: 2-[4-(3-amino-2-hydroxypropoxy)phenyl]acetic acid

SMILES: NCC(O)COC1=CC=C(CC(O)=O)C=C1

$LogK_{OW}$ = -3.72

Wat Sol = 411000 mg/L

(MetE)T IUPAC: 2-[4-(2-hydroxy-3-oxopropoxy)phenyl]acetic acid

SMILES: OC(COC1=CC=C(CC(O)=O)C=C1)C=O

$LogK_{OW}$ = -0.54

Wat Sol = 754000 mg/L

(MetE)U IUPAC: 3-[4-(carboxymethyl)phenoxy]-2-hydroxypropanoic acid

SMILES: OC(COC1=CC=C(CC(O)=O)C=C1)C(O)=O

$LogK_{OW}$ = -0.56

Wat Sol = 645600 mg/L

Photodegradation Products

(Met)VIUPAC: 3-[(propan-2-yl)amino]propane-1,2-diol

SMILES: CC(C)NCC(O)CO

$LogK_{OW}$ = -0.88

Wat Sol = 1000000 mg/L

(Met)W IUPAC: 4-(2-methoxyethyl)phenol

SMILES: COCCC1=CC=C(O)C=C1

$LogK_{OW}$ = 1.79

Wat Sol = 8422 mg/L

(Met)XIUPAC: 2-(4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}phenyl)ethan-1-ol

SMILES: CC(C)NCC(O)COC1=CC=C(CCO)C=C1

$LogK_{OW}$ = 1.40

Wat Sol = 14670 mg/L

(Met)Y IUPAC: 1-amino-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol

SMILES: COCCC1=CC=C(OCC(O)CN)C=C1

$LogK_{OW}$ = 0.32

Wat Sol = 175200 mg/L

(Met)Z IUPAC: 5-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}-2-(2-methoxyethyl)phenol

SMILES: COCCC1=C(O)C=C(OCC(O)CNC(C)C)C=C1

$LogK_{OW}$ = 1.21

Wat Sol = 54520 mg/L

Propranolol (Pro) (Figure A5.3)

Pro IUPAC: [2-hydroxy-3-(naphthalen-1-yloxy)propyl](propan-2-yl)amine

SMILES: CC(C)NCC(O)COC1=C2C=CC=CC2=CC=C1

$LogK_{OW}$ = 2.60

Wat Sol = 62 mg/L

Human Metabolites

(Pro)A IUPAC: 3,4,5-trihydroxy-6-[2-(naphthalen-1-yloxy)-1-{[(propan-2-yl)amino]oxy}ethoxy]oxane-2-carboxylic acid

SMILES: CC(C)NOC(COC1=CC=CC2=C1C=CC=C2)OC1OC(C(O)C(O)C1O)C(O)=O

$LogK_{OW}$ = 1.58

Wat Sol = 197 mg/L

(Pro)B IUPAC: 3,4,5-trihydroxy-6-{2-[(4-hydroxynaphthalen-1-yl)oxy]-1-{[(propan-2-yl)amino]oxy}ethoxy}oxane-2-carboxylic acid

SMILES: CC(C)NOC(COC1=CC=C(O)C2=C1C=CC=C2)OC1OC(C(O)C(O)C1O)C(O)=O

$LogK_{OW}$ = 1.10

Wat Sol = 1530 mg/L

(Pro)C IUPAC: 4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}naphthalen-1-ol

SMILES: CC(C)NCC(O)COC1=CC=C(O)C2=C1C=CC=C2

$LogK_{OW}$ = 2.12

Wat Sol = 10260 mg/L

(Pro)D IUPAC: 2-hydroxy-3-(naphthalen-1-yloxy)propanoic acid

SMILES: OC(COC1=CC=CC2=C1C=CC=C2)C(O)=O

$LogK_{OW}$ = 1.77

Wat Sol = 7346 mg/L

(Pro)E IUPAC: 2-(naphthalen-1-yloxy)acetic acid

SMILES: OC(=O)COC1=C2C=CC=CC2=CC=C1

$LogK_{OW}$ = 2.50

Wat Sol = 90 mg/L

Biodegradation Products

(Pro)F IUPAC: propan-2-one

SMILES: CC(C)=O

$LogK_{OW}$ = -0.24

Wat Sol = 1000000 mg/L

(Pro)G IUPAC: propan-2-amine

SMILES: CC(C)N

$LogK_{OW}$ = 0.27

Wat Sol = 1000000 mg/L

(Pro)H IUPAC: 2-hydroxy-3-(naphthalen-1-yloxy)propanal

SMILES: OC(COC1=C2C=CC=CC2=CC=C1)C=O

$LogK_{OW}$ = 1.20

Wat Sol = 10920 mg/L

(Pro)I IUPAC: 1-amino-3-(naphthalen-1-yloxy)propan-2-ol

SMILES: NCC(O)COC1=C2C=CC=CC2=CC=C1

$LogK_{OW}$ = 1.22

Wat Sol = 32730 mg/L

(Pro)J IUPAC: 2-hydroxy-3-(naphthalen-1-yloxy)propanoic acid

SMILES: OC(COC1=C2C=CC=CC2=CC=C1)C(O)=O

$LogK_{OW}$ = 1.77

Wat Sol = 7346 mg/L

(ProA) None

(ProB) None

(ProC)K IUPAC: 4-(3-amino-2-hydroxypropoxy)naphthalen-1-ol

SMILES: NCC(O)COC1=C2C=CC=CC2=C(O)C=C1

$LogK_{OW}$ = 0.74

Wat Sol = 262700 mg/L

(ProC)L IUPAC: 2-hydroxy-3-[(4-hydroxynaphthalen-1-yl)oxy]propanal

SMILES: OC(COC1=C2C=CC=CC2=C(O)C=C1)C=O

$LogK_{OW}$ = 0.72

Wat Sol = 87670 mg/L

(ProC)M IUPAC: 2-hydroxy-3-[(4-hydroxynaphthalen-1-yl)oxy]propanoic acid

SMILES: OC(COC1=C2C=CC=CC2=C(O)C=C1)C(O)=O

$LogK_{OW}$ = 1.29

Wat Sol = 58690 mg/L

(ProD) None

(ProE) None

Photodegradation Products

(Pro)N IUPAC: (2E)-3-[(6Z)-2-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}-6-(hydroxymethylidene)cyclohexa-2,4-dien-1-yl]prop-2-enal

SMILES: CC(C)NCC(O)COC1=CC=C\C(=C\O)C1\C=C\C=O

$LogK_{OW}$ = 0.88

Wat Sol = 2396 mg/L

(Pro)O IUPAC: [(1Z)-6-ethenyl-5-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}cyclohexa-2,4-dien-1-ylidene]methanol

SMILES: CC(C)NCC(O)COC1=CC=C\C(=C\O)C1C=C

$LogK_{OW}$ = 1.95

Wat Sol = 417 mg/L

(Pro)P IUPAC: 4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}naphthalene-1,2-diol

SMILES: CC(C)NCC(O)COC1=CC(O)=C(O)C2=C1C=CC=C2

$LogK_{OW}$ = 1.64

Wat Sol = 21350 mg/L

(Pro)Q IUPAC: 4-{2-hydroxy-3-[(propan-2-yl)amino]propoxy}naphthalene-1,2,3-triol

SMILES: CC(C)NCC(O)COC1=C(O)C(O)=C(O)C2=C1C=CC=C2

$LogK_{OW}$ = 1.32

Wat Sol = 8408 mg/L

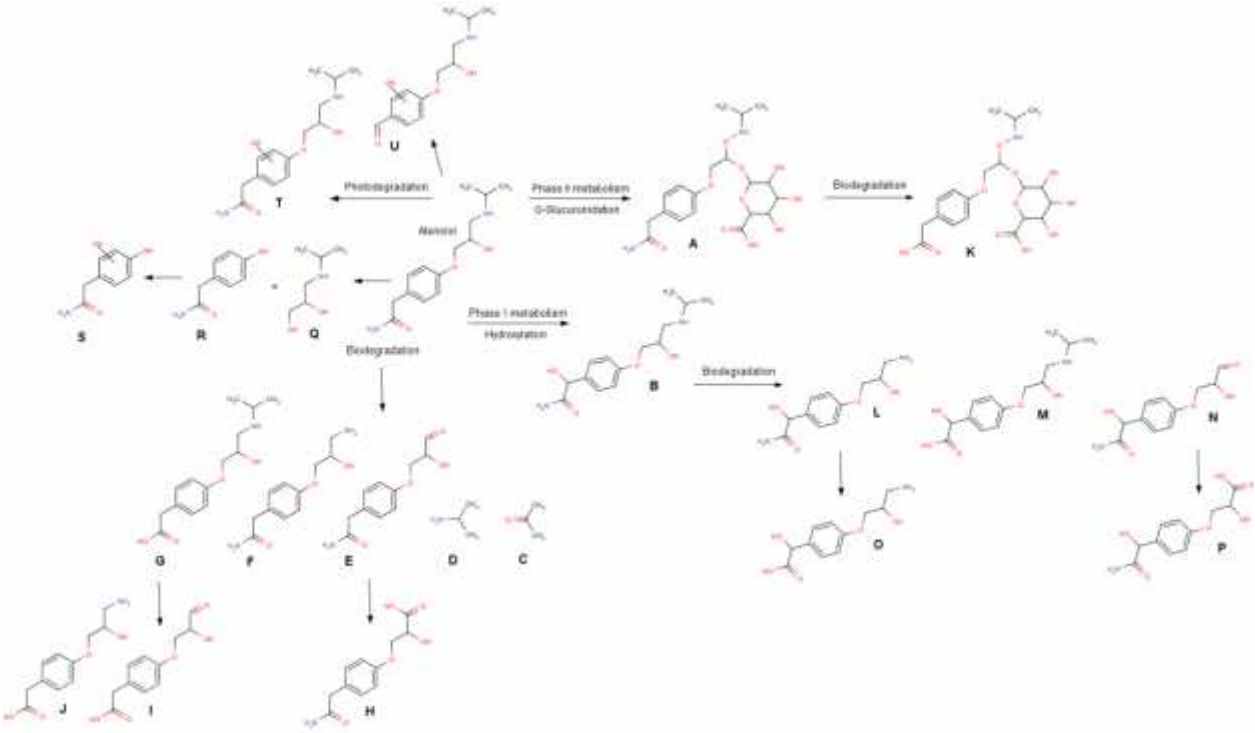

Figure A5.1: Degradation pathway of atenolol via biotic and abiotic pathways. Products A and B and K-P are human metabolites and biodegradation products of those human metabolites, respectively. Product formation and pathways of A and B borrowed from Escher *et al.* (2006). Products C – J are the most likely aerobic biodegradation products predicted using the University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD) Pathway Prediction System. Products Q – U are experimentally observed photodegradation products from Ji *et al.* (2012), Wang *et al.* (2012), and Zeng *et al.* (2012).

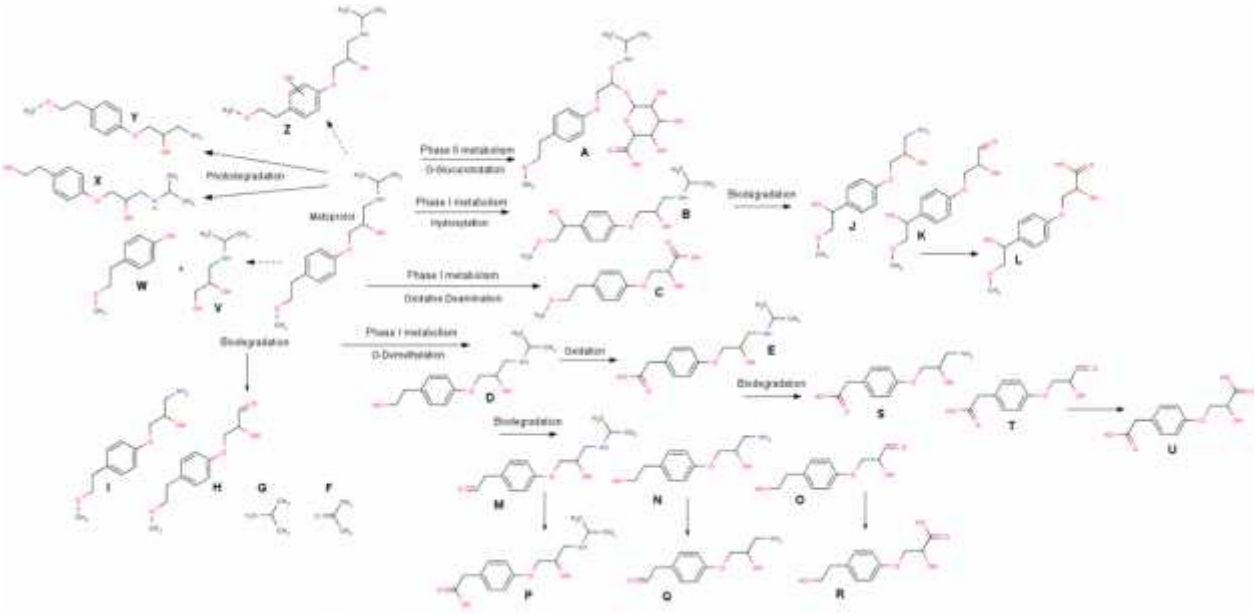

Figure A5.2: Degradation pathway of metoprolol via biotic and abiotic pathways. Products A – E are human metabolites – product formation and pathways borrowed from Escher *et al.* (2006). Products F – U are the most likely biodegradation products predicted using the University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD) Pathway Prediction System. Products V and Z are experimentally observed and predicted (dashed arrows) photodegradation products from Liu *et al.* (2009). Due to a lack of experimental photo-product data for metoprolol, the photo-products X and Y were predicted based on experimentally observed atenolol photo-products, given the similar structures of the two parent β-blockers.

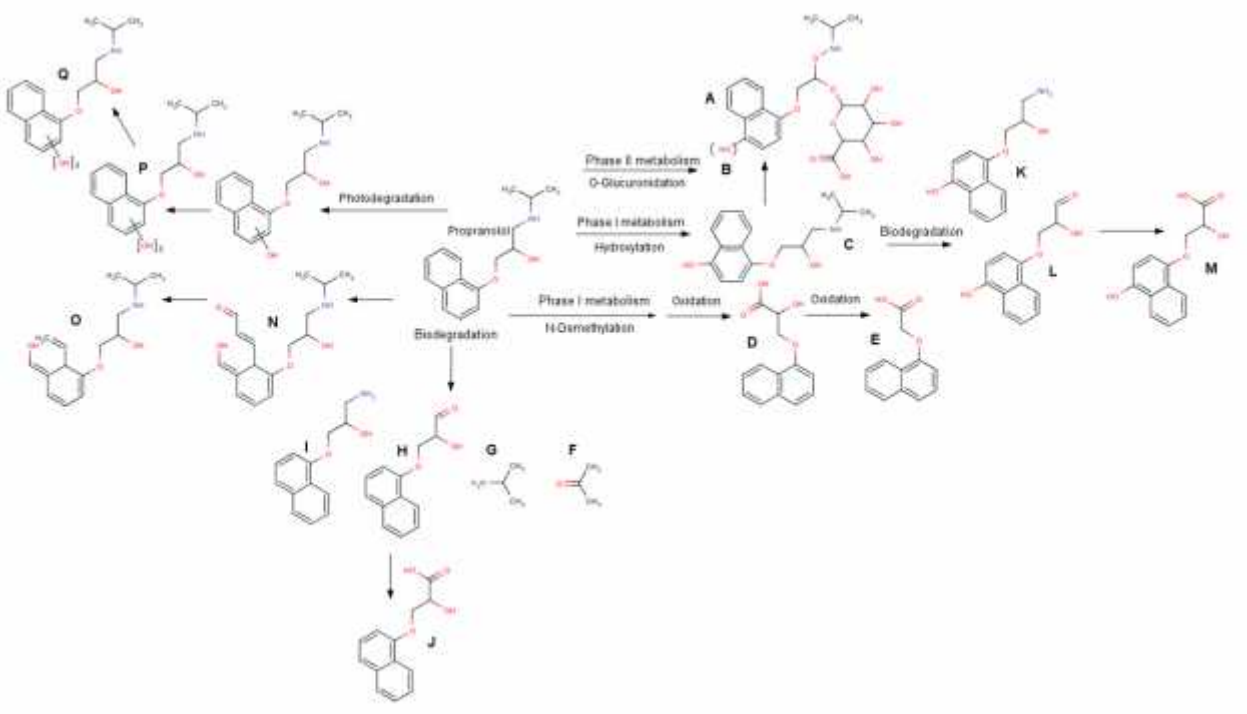

Figure A5.3: Degradation pathway of propranolol via biotic and abiotic pathways. Products A – E are human metabolites – product formation and pathways borrowed from Escher *et al.* (2006). Products F – O are the most likely biodegradation products predicted using the University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD) Pathway Prediction System. Products P and Q are experimentally observed photodegradation products from Liu and Williams (2007) and Piram *et al.* (2012).

List and structures of all SSRI transformation products.

Physicochemical properties of SSRI metabolites and transformation products.

$LogK_{OW}$, water solubility, and SMILES were generated by EPISuite via ECOSAR v1.11. The IUPAC names were generated using the SMILES via Chem Axon Marvin Sketch. Human metabolites were determined using the pharmacokinetic literature. Biodegradation products were estimated using the University of Minnesota Biocatalysis/ Biodegradation Database Pathway Prediction System aerobic microbial degradation software, and photolysis products were determined using the available experimental literature.

Citalopram (Cit) (Figure A5.4)

Cit IUPAC: 1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile

SMILES: CN(C)CCCC1(OCC2=CC(=CC=C12)C#N)C1=CC=C(F)C=C1

$LogK_{OW}$= 3.742

Wat Sol= 31.09

Human Metabolites

(Cit)A IUPAC: 1-(4-fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydro-2-benzofuran-5-carbonitrile

(DMCit) SMILES: CNCCCC1(OCC2=CC(=CC=C12)C#N)C1=CC=C(F)C=C1

$LogK_{OW}$= 3.531

Wat Sol= 57.01

(Cit)B IUPAC: 1-(3-aminopropyl)-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile

(DiDMCit) SMILES: NCCCC1(OCC2=CC(=CC=C12)C#N)C1=CC=C(F)C=C1

LogK_{OW}= 3.065

Wat Sol= 172.1

Biodegradation Products

(Cit)C IUPAC: 1-(4-fluorophenyl)-1-(3-oxopropyl)-1,3-dihydro-2-benzofuran-5-carbonitrile

SMILES: FC1=CC=C(C=C1)C1(CCC=O)OCC2=CC(=CC=C12)C#N

LogK_{OW}= 3.046

Wat Sol= 17.77

(CitB)D IUPAC: 3-[5-cyano-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-1-yl]propanoate

SMILES: [O-]C(=O)CCC1(OCC2=CC(=CC=C12)C#N)C1=CC=C(F)C=C1

LogK_{OW}= 3.049

Wat Sol= 35.38

(CitB)E IUPAC: 3-[5-cyano-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-1-yl]propanoate

SMILES: [O-]C(=O)CCC1(OCC2=CC(=CC=C12)C#N)C1=CC=C(F)C=C1

$LogK_{OW}$= 3.049

Wat Sol= 35.38

Photodegradation Products

(Cit)F IUPAC: 3-[5-cyano-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-1-yl]-N,N-dimethylpropanamine oxide

SMILES: C[N](C)(=O)CCCC1(OCC2=CC(=CC=C12)C#N)C1=CC=C(F)C=C

$LogK_{OW}$= 12.97

Wat Sol= 11.09

Fluoxetine (Flu) (Figure A5.5)

Flu IUPAC: methyl({3-phenyl-3-[4-(trifluoromethyl)phenoxy]propyl})amine

SMILES: CNCCC(OC1=CC=C(C=C1)C(F)(F)F)C1=CC=CC=C1

$LogK_{OW}$= 4.648

Wat Sol= 60.28

Human Metabolites

(Flu)A IUPAC: 1-(3-amino-1-phenylpropoxy)-4-(trifluoromethyl)benzene

(NorFlu) SMILES: NCCC(OC1=CC=C(C=C1)C(F)(F)F)C1=CC=CC=C1

$LogK_{OW}$= 4.182

Wat Sol= 35.7

(Flu)B IUPAC: 4-(trifluoromethyl)phenol

SMILES: OC1=CC=C(C=C1)C(F)(F)F

LogK_{OW}= 2.82

Wat Sol= 2847

(Flu)C IUPAC: 2-(phenylformamido)acetate

SMILES: [O-]C(=O)CNC(=O)C1=CC=CC=C1

LogK_{OW}= 0.31

Wat Sol= 3750

(FluA)D IUPAC: 3,4,5-trihydroxy-6-[({3-phenyl-3-[4-trifluoromethyl)phenoxy]propyl}amino)oxy]piperidine-2-carboxylic acid

SMILES: OC1C(O)C(NC(C1O)C(O)=O)ONCCC(OC1=CC=C(C=C1)C(F)(F)F)C1=CC=CC=C1

LogK_{OW}= 1.97

Wat Sol= 44.28

Biodegradation Products

(Flu)E IUPAC: 3-phenyl-3-[4-(trifluoromethyl)phenoxy]propanal

SMILES: FC(F)(F)C1=CC=C(OC(CC=O)C2=CC=CC=C2)C=C1

$LogK_{OW}$= 4.164

Wat Sol= 3.685

Photodegradation Products

(Flu)F IUPAC: 4-(difluoromethylidene)cyclohexa-2,5-dien-1-one

SMILES: FC(F)=C1C=CC(=O)C=C1

$LogK_{OW}$= 1.37

Wat Sol= 10.97

(Flu)G IUPAC: 3-(methylamino)-1-phenylpropan-1-ol

SMILES: CNCCC(O)C1=CC=CC=C1

$LogK_{OW}$= 0.979

Wat Sol= 9.617×10^{-4}

(Flu)H IUPAC: 4-[3-(methylamino)-1-phenylpropoxy]benzoic acid

SMILES: CNCCC(OC1=CC=C(C=C1)C(O)=O)C1=CC=CC=C1

$LogK_{OW}$= 3.567

Wat Sol= 13.43

(Flu)I IUPAC: 3-[3-(methylamino)-1-[4-(trifluoromethyl)phenoxy]propyl]phenol

SMILES: CNCCC(OC1=CC=C(C=C1)C(F)(F)F)C1=CC(O)=CC=C1

$LogK_{OW}$= 4.168

Wat Sol= 92.97

Fluvoxamine (Flv) (Figure A5.6)

Flv IUPAC: (Z)-(2-aminoethoxy)({5-methoxy-1-[4-(trifluoromethyl)phenyl]pentylidene})amine

SMILES: COCCCC\C(=N\OCCN)C1=CC=C(C=C1)C(F)(F)F

$LogK_{OW}$= 3.085

Wat Sol= 22.22

Human Metabolites

(Flv)A IUPAC: 5-[(2-aminoethoxy)imino]-5-[4-(trifluoromethyl)phenyl]pentanoate

SMILES: NCCON=C(CCCC([O-])=O)C1=CC=C(C=C1)C(F)(F)F

$LogK_{OW}$= -0.586

Wat Sol= 258.4

Biodegradation Products

(Flv)B IUPAC: 2-{[(Z)-{5-methoxy-1-[4-(trifluoromethyl)phenyl]pentylidene}amino]oxy}acetaldehyde

SMILES: COCCCC\C(=N\OCC=O)C1=CC=C(C=C1)C(F)(F)F

$LogK_{OW}$= 3.066

Wat Sol= 23.37

(Flv)C IUPAC: 2-{[(Z)-{5-methoxy-1-[4-(trifluoromethyl)phenyl]pentylidene}amino]oxy}acetate

SMILES: COCCCC\C(=N\OCC([O-])=O)C1=CC=C(C=C1)C(F)(F)F

LogK$_{OW}$= 3.319

Wat Sol= 28.66

(FlvA)D IUPAC: 5-[(2-oxoethoxy)imino]-5-[4-(trifluoromethyl)phenyl]pentanoate

SMILES: [O-]C(=O)CCCC(=NOCC=O)C1=CC=C(C=C1)C(F)(F)F

LogK$_{OW}$= 2.595

Wat Sol= 146.7

(FlvA)E IUPAC: 3-[(2-aminoethoxy)imino]-3-[4-(trifluoromethyl)phenyl]propanoate

SMILES: NCCON=C(CC([O-])=O)C1=CC=C(C=C1)C(F)(F)F

LogK$_{OW}$= -1.569

Wat Sol= 2602

(FlvA)F IUPAC: 5-[(carboxylatomethoxy)imino]-5-[4-(trifluoromethyl)phenyl]pentanoate

SMILES: [O-]C(=O)CCCC(=NOCC([O-])=O)C1=CC=C(C=C1)C(F)(F)F

LogK$_{OW}$= 2.261

Wat Sol= 227.2

(FlvA)G IUPAC: 3-[(2-oxoethoxy)imino]-3-[4-(trifluoromethyl)phenyl]propanoate

SMILES: [O-]C(=O)CC(=NOCC=O)C1=CC=C(C=C1)C(F)(F)F

$LogK_{OW}$= 1.613

Wat Sol= 1477

Photodegradation Products

None Documented

Paroxetine (Par) (Figure A5.7)

Par IUPAC: 3-(2H-1,3-benzodioxol-5-ylmethoxy)-4-(4-fluorophenyl)piperidine

SMILES: FC1=CC=C(C=C1)C1CCNCC1OCC1=CC=C2OCOC2=C1

$LogK_{OW}$= 3.954

Wat Sol= 35.27

Human Metabolites

None Documented

Biodegradation Products

(Par)A IUPAC: 5-amino-4-(2H-1,3-benzodioxol-5-ylmethoxy)-3-(4-fluorophenyl)pentanal

SMILES: NCC(OCC1=CC=C2OCOC2=C1)C(CC=O)C1=CC=C(F)C=C1

$LogK_{OW}$= 2.602

Wat Sol= 403.7

(Par)B IUPAC: 5-amino-2-(2H-1,3-benzodioxol-5-ylmethoxy)-3-(4-fluorophenyl)pentanal

SMILES: NCCC(C(OCC1=CC=C2OCOC2=C1)C=O)C1=CC=C(F)C=C1

$LogK_{OW}$= 2.602

Wat Sol= 403.7

(Par)C IUPAC: 5-amino-4-(2H-1,3-benzodioxol-5-ylmethoxy)-3-(4-fluorophenyl)pentanoate

SMILES: NCC(OCC1=CC=C2OCOC2=C1)C(CC([O-])=O)C1=CC=C(F)C=C1

$LogK_{OW}$= -0.345

Wat Sol= 88.76

(Par)D IUPAC: 5-amino-2-(2H-1,3-benzodioxol-5-ylmethoxy)-3-(4-fluorophenyl)pentanoate

SMILES: NCCC(C(OCC1=CC=C2OCOC2=C1)C([O-])=O)C1=CC=C(F)C=C1

$LogK_{OW}$= -0.345

Wat Sol= 88.76

Photodegradation Products

(Par)E IUPAC: [4-(4-fluorophenyl)piperidin-3-yl]methanol

SMILES: OCC1CNCCC1C1=CC=C(F)C=C1

$LogK_{OW}$= 1.976

Wat Sol= 8202

(Par)F IUPAC: 7-fluoro-1H,2H,3H,4H,4aH,9H,9aH-indeno[2,1-c]pyridine

SMILES: FC1=CC=C2C3CCNCC3CC2=C1

$LogK_{OW}$= 3.042

Wat Sol= 1245

Sertraline (Ser) (Figure A5.8)

Ser IUPAC: (4S)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine

SMILES: CNC1CC[C@@H](C2=CC(Cl)=C(Cl)C=C2)C2=C1C=CC=C2

$LogK_{OW}$= 5.286

Wat Sol= 3.517

Human Metabolites

(Ser)A IUPAC: (4S)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydronaphthalen-1-amine

(DMSer) SMILES: NC1CC[C@@H](C2=CC(Cl)=C(Cl)C=C2)C2=C1C=CC=C2

$LogK_{OW}$= 4.820

Wat Sol= 10.61

Biodegradation Products

(Ser)B IUPAC: (4S)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydronaphthalen-1-one

SMILES: ClC1=C(Cl)C=C(C=C1)[C@@H]1CCC(=O)C2=C1C=CC=C2

$LogK_{OW}$= 5.007

Wat Sol= 0.732

Photodegradation Products

None Documented

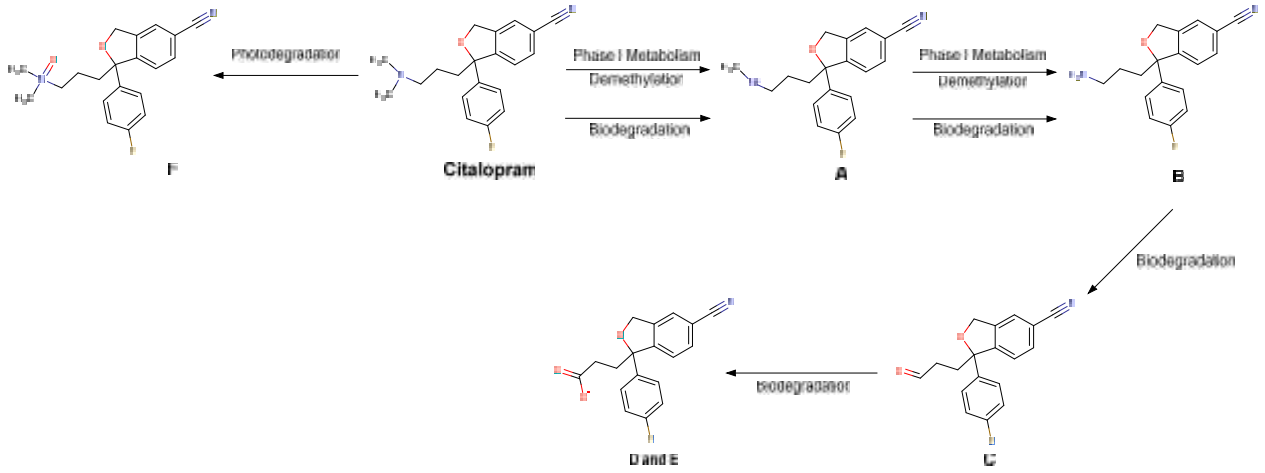

Figure A5.4: Degradation pathway of citalopram via biotic and abiotic pathways. Products A and B are human metabolites – product formation and pathways borrowed from DeVane (1999). Products A – E are also the most likely aerobic biodegradation products predicted using the University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD) Pathway Prediction System. Product F is experimentally observed photodegradation product from Kosjek and Heath (2010).

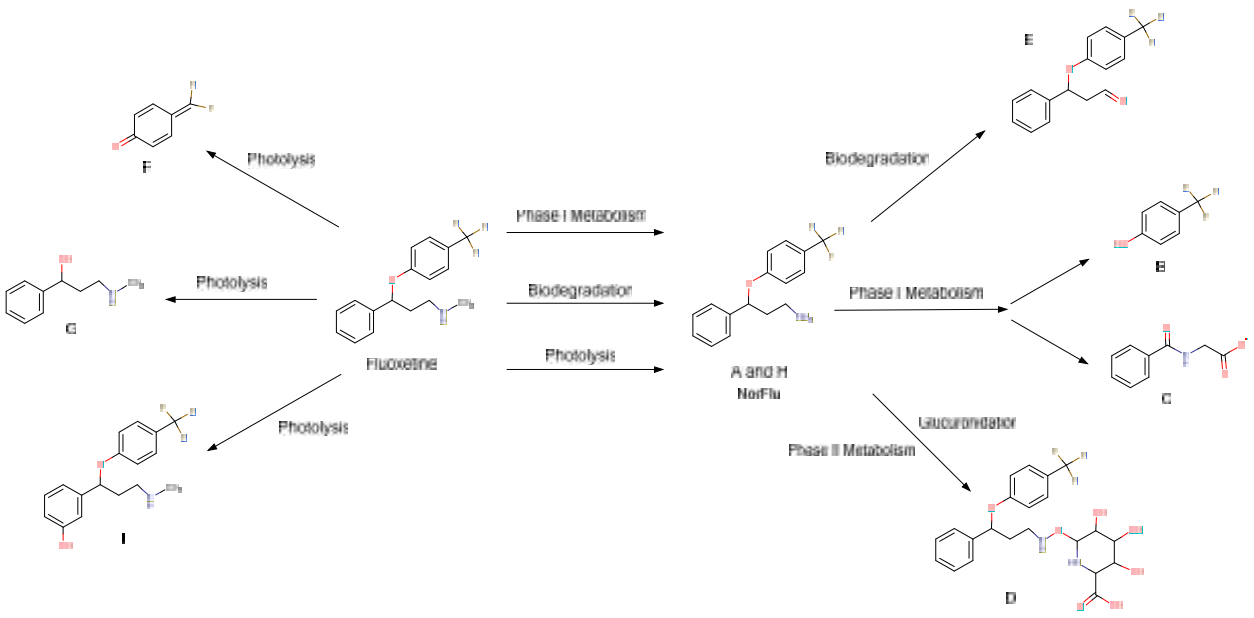

Figure A5.5: Degradation pathway of fluoxetine via biotic and abiotic pathways. Products A – D are human metabolites – product formation and pathways borrowed from DeVane (1999). Products A and E are also the most likely aerobic biodegradation products predicted using the University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD) Pathway Prediction System. Products F – I are experimentally observed photodegradation product from Kosjek and Heath (2010).

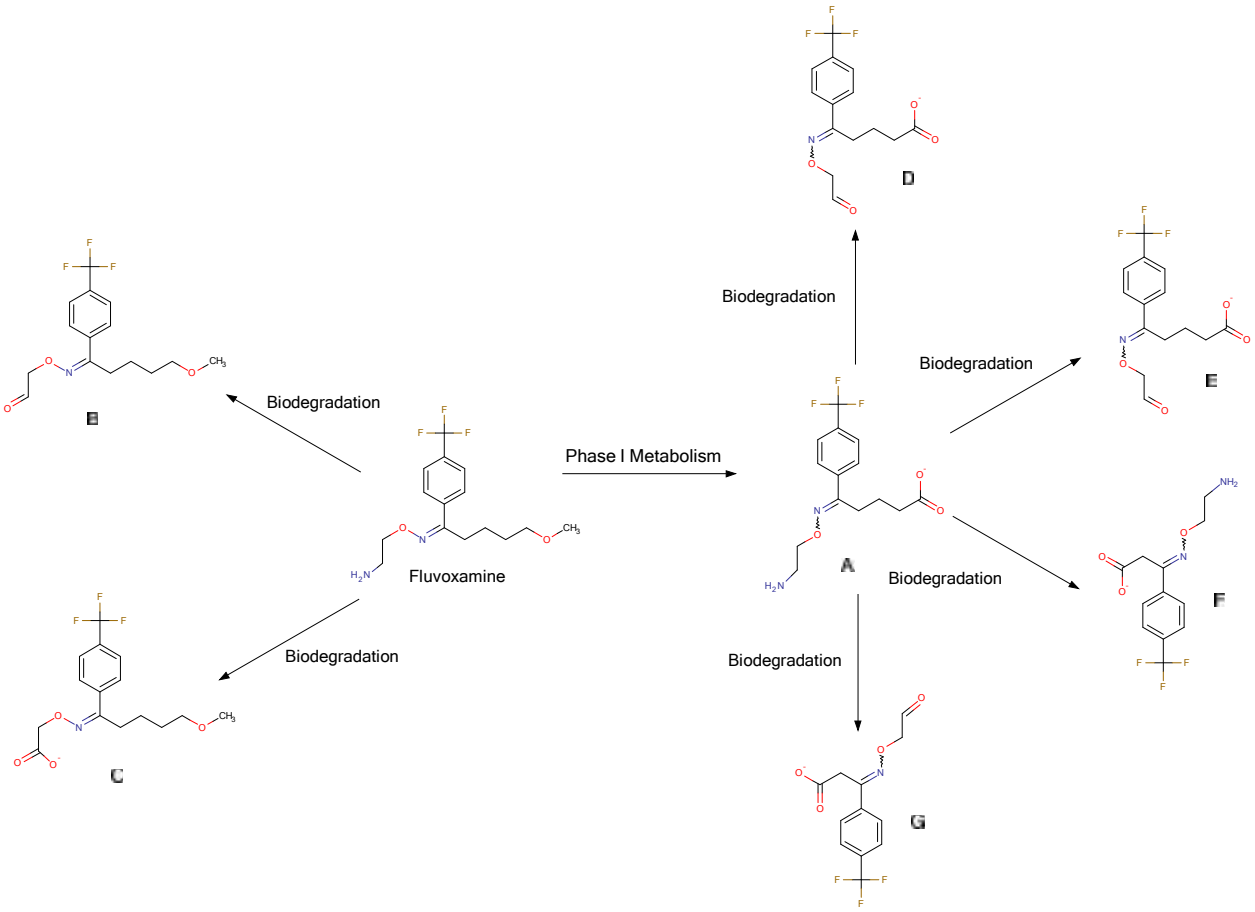

Figure A5.6: Degradation pathway of fluvoxamine via biotic and abiotic pathways. Product A is a human metabolite – product formation and pathways borrowed from DeVane (1999). Products A- G are also the most likely aerobic biodegradation products predicted using the University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD) Pathway Prediction System.

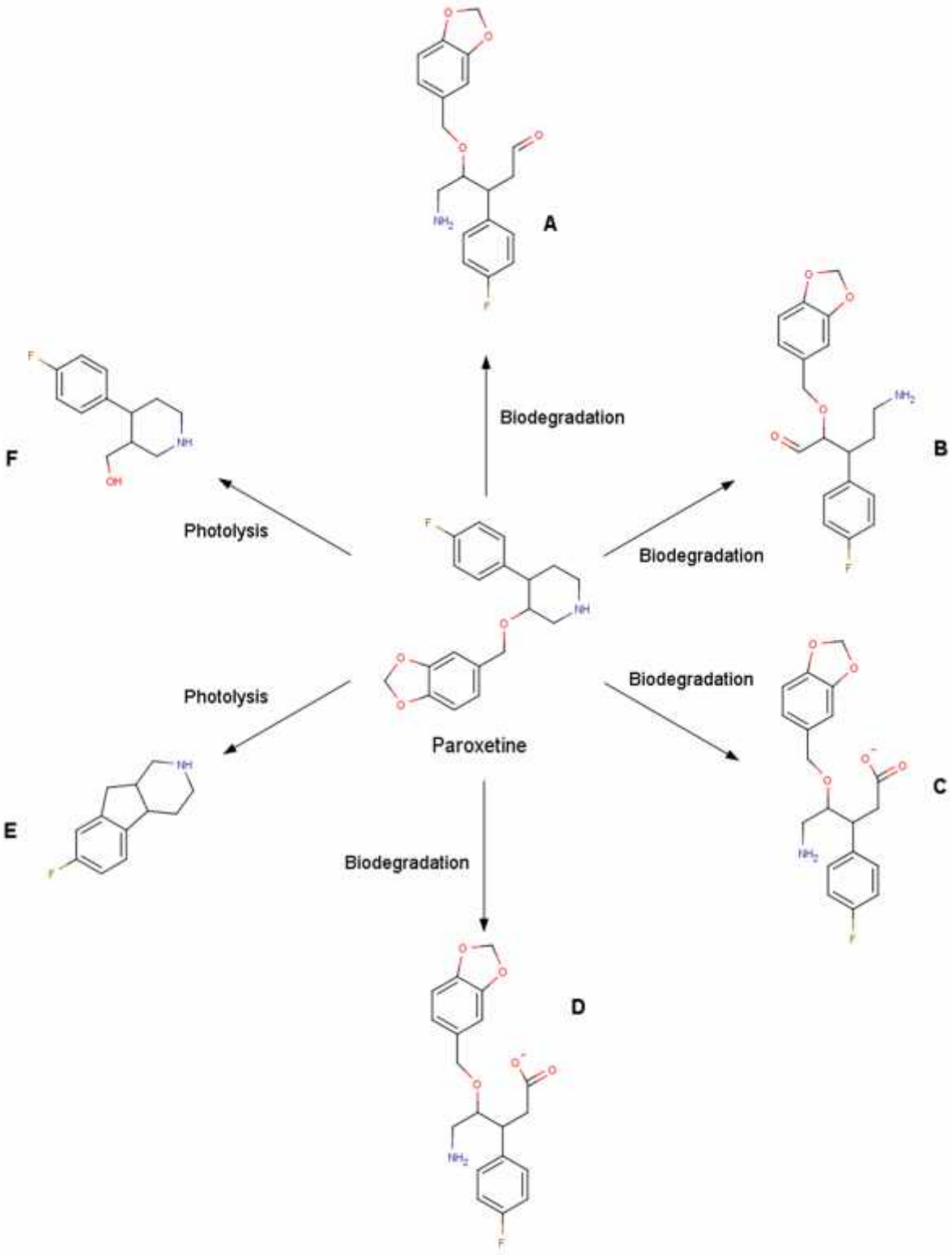
A
Biodegradation
B
Biodegradation
F
Photolysis
Paroxetine
Biodegradation
C
Photolysis
E
Biodegradation
D

Figure A5.7: Degradation pathway of paroxetine via biotic and abiotic pathways. Products A – D are the most likely aerobic biodegradation products predicted using the University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD) Pathway Prediction System. Products E and F are experimentally observed photodegradation product from Kosjek and Heath (2010).

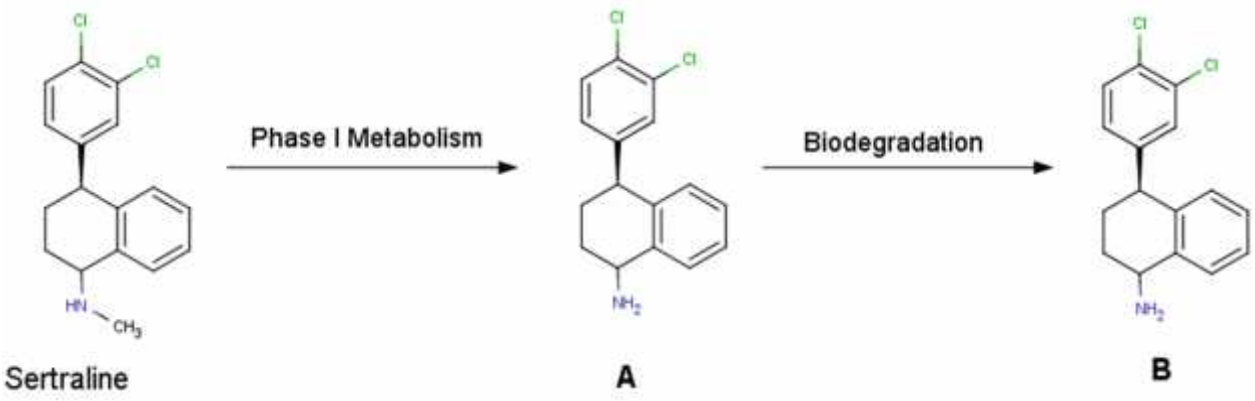

Figure A5.8: Degradation pathway of sertraline via biotic and abiotic pathways. Product A is a human metabolite – product formation and pathways borrowed from DeVane (1999). Product B is the most likely aerobic biodegradation product predicted using the University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD) Pathway Prediction System.

Figure A5.9: A snapshot of a specific parent compound (sertraline) aerobic biodegradation products predicted using the University of Minnesota Biocatalysis/ Biodegradation Database (UM-BBD) Pathway Prediction System. Green arrows indicate likely, yellow arrows indicate neutral likelihood. We chose a limit of two subsequent green arrows as the limit for inclusion in this study.

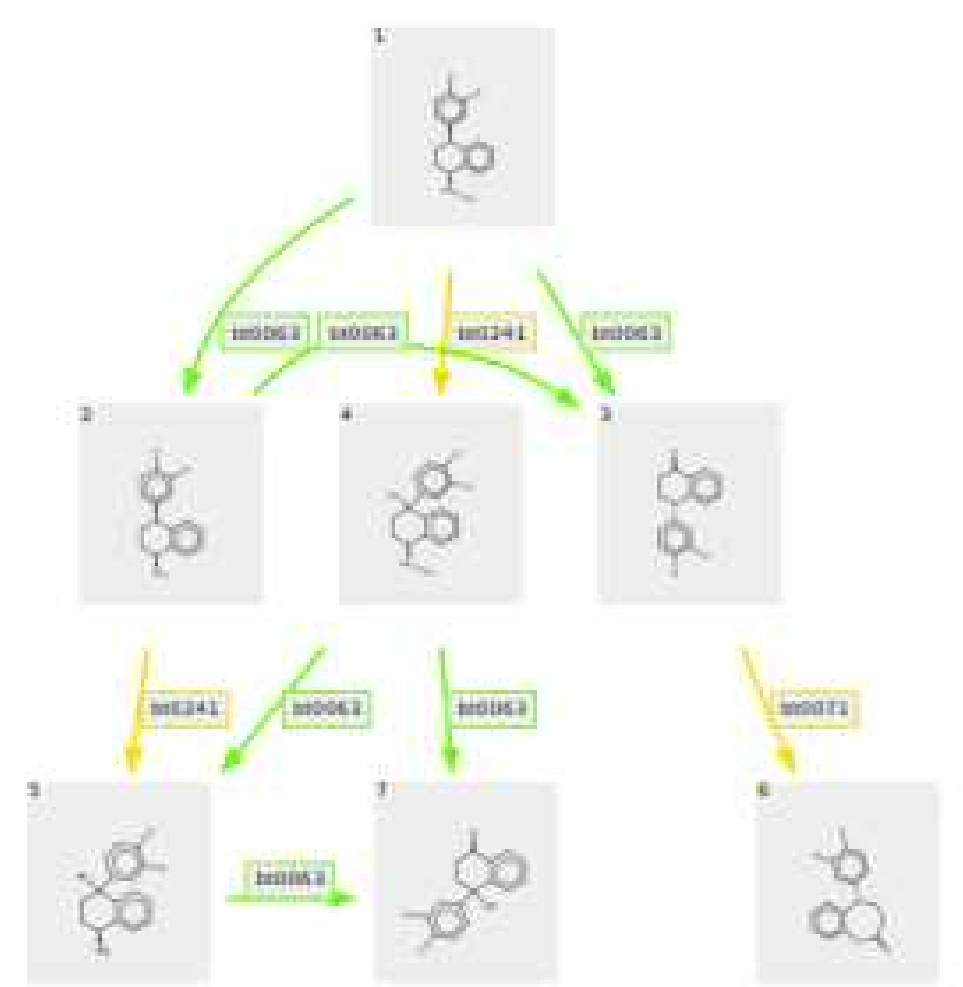

Table A5.1: Measured concentrations from North American and European waste water treatment plants effluent or proximal receiving waters, compiled from the literature, for the three β-blockers.

Compound	Exposure Concentrations (ng/L)									
Atenolol	nq [a]	58 [a]	83 [a]	360 [a]	510 [a]	1330 [a]	30 [b]	1091 [b]	27 [c]	70 [c]
	254 [c]	260 [c]	466 [c]	554 [c]	955 [c]	1168 [c]	2.4 [d]	2.4 [d]	3.9 [d]	
	6.2 [d]	7.7 [d]	9.5 [d]	10.3 [d]	13.2 [d]	15.1 [d]	15.2 [d]	15.3 [d]	20.2 [d]	
	23.2 [d]	23.4 [d]	32.8 [d]	50 [e]	1043 [e]	72 [f]	395 [f]	400 [f]	3.5 [g]	
	12.5 [g]	31.5 [g]	57 [g]	940 [h]	317 [i]	404 [i]	678 [i]	2300 [j]	220 [k]	
	600 [l]	700 [l]	790 [l]	1100 [l]	8.8 [m]	100 [n]	300 [n]	900 [n]	1050 [n]	
	1.8 [o]	106.3 [p]	166 [q]	nq [r]	nq [r]	12 [r]	19 [r]	25 [r]	40 [r]	
	80 [r]	440 [r]								
Metoprolol	nq [a]	29 [a]	36 [a]	160 [a]	200 [a]	240 [a]	nq [b]	3.6 [b]	0.1 [d]	0.2 [d]
	0.3 [d]	0.4 [d]	0.5 [d]	0.7 [d]	1.2 [d]	1.6 [d]	1.7 [d]	1.8 [d]	1.9 [d]	
	2.3 [d]	2.9 [d]	3.2 [d]	3.2 [d]	3.3 [d]	3.6 [d]	3.9 [d]	8.3 [d]	8.5 [d]	
	8.9 [d]	13.4 [d]	16.9 [d]	nq [s]	nq [s]	41 [s]	50 [s]	77 [s]	82 [s]	
	82 [s]	99 [s]	122 [s]	124 [s]	131 [s]	136 [s]	219 [s]	220 [s]	228 [s]	
	268 [s]	326 [s]	344 [s]	376 [s]	439 [s]	571 [s]	636 [s]	921 [s]	964 [s]	
	1132 [s]	1539 [s]	2269 [s]	4.4 [e]	77 [e]	nq [f]	nq [f]	nq [f]	7 [g]	
	7 [g]	7.5 [g]	59 [g]	128 [g]	410 [h]	84 [t]	310 [t]	72 [i]	103 [i]	
	161 [i]	890 [j]	50 [k]	400 [l]	410 [l]	420 [l]	510	102 [m]	1 [o]	
	47.4 [p]	nq [u]	nq [u]	nq [u]	nq [u]	nq [u]	nq [u]	nq [u]	9 [u]	

		12 [u]	13 [u]	16 [u]	17 [u]	23 [u]	28 [u]	30 [u]	33 [u]	42 [u]
		48 [u]	53 [u]	60 [u]	61 [u]	66 [u]	67 [u]	71 [u]	72 [u]	76 [u]
		92 [u]	130 [u]	160 [u]	237 [q]	nq [r]	2nq [r]	35 [r]	38 [r]	116 [r]
		910 [r]	990 [r]	1070 [r]						
Propanolol	nq [a]	7 [a]	8 [a]	30 [a]	60 [a]	70 [a]	1.7 [b]	77 [b]	nq [s]	nq [s]
		nq [s]	nq [s]	nq [s]	nq [s]	nq [s]	nq [s]	nq [s]	nq [s]	nq [s]
		nq [s]	nq [s]	nq [s]	16 [s]	17 [s]	24 [s]	25 [s]	35 [s]	37 [s]
		47 [s]	50 [s]	52 [s]	57 [s]	74 [s]	92 [s]	124 [s]	200 [s]	8.1 [e]
		158 [e]	nq [f]	168 [f]	290 [f]	5 [g]	5 [g]	6.5 [g]	33 [h]	1 [t]
		3.5 [t]	32 [i]	43 [i]	123 [i]	520 [j]	30 [k]	55 [l]	70 [l]	75 [l]
		80 [l]	2 [o]	8 [p]	nq [u]	nq [u]	nq [u]	nq [u]	nq [u]	nq [u]
		nq [u]	nq [u]	nq [u]	nq [u]	nq [u]	nq [u]	5 [u]	11 [u]	12 [u]
		12 [u]	16 [u]	18 [u]	20 [u]	22 [u]	28 [u]	33 [u]	33 [u]	34 [u]
		36 [u]	36 [u]	53 [u]	61 [u]	64 [u]	53 [q]			

[a]Alder (2010)- Switzerland; [b]Bagnall* (2012)- UK; [c]Castiglioni (2005)- Italy; [d]Carlson (2013)* - Canada; [e]Giraud* (2014)- France; [f]Gros* (2006)- Spain; [g]Kasprzyk- Hordern* (2007)- UK and Poland; [h]Kostich* (2014)- USA; [I]Maurer* (2007)- Switzerland; [j]Morasch* (2010)- Switzerland; [k]Munoz* (2009)- Spain; [l]Nikolai* (2006)- Canada; [m]Nodler* (2013)- Germany; [n]Palmer* (2008)- USA; [o]Piram (2008)- France; [p]Roldan* (2010)- Spain; [q]Thurman* (2012)- USA; [r]Vieno* (2006)- Finland; [s]Fono (2006)- USA; [t]Kunkel (2012)- Germany; [u]Sedlak* (2005)- USA. (*) Indicates the mean or median of a minimum of triplicates, with a reported standard deviation or a relative standard deviation.

Table A5.2: Measured concentrations from North American and European waste water treatment plants effluent or proximal receiving waters, compiled from the literature, for the five SSRIs and the two SSRI metabolites.

Compound	Exposure Concentration (ng/L)									
Citalopram	21 [a]	288 [a]	46.8 [b]	57.8 [b]	86 [c]	136 [c]	163 [c]	173 [c]	208 [c]	219 [c]
		223 [c]	3.4 [d]	11.5 [d]	136 [e]	223 [e]	60 [f]	85 [f]	159 [f]	205 [f]
		219 [f]	82.8 [g]	87.1 [g]	89.7 [g]	94.9 [g]	95.6 [g]	9.2 [h]	6.2 [h]	382 [h]
		21.9 [i]	24.9 [i]	33.7 [i]	40.1 [i]	64.1 [i]	85.9 [i]	89.3 [i]	238.4 [i]	21 [j]
		36 [j]	36 [j]	66 [j]	67 [j]	86 [j]	88 [j]	130 [j]	170 [j]	180 [j]
		200 [j]	200 [j]	210 [j]	222 [j]	270 [j]	280 [j]	290 [j]	320 [j]	340 [j]
		520 [j]	nq [j]							
Fluoxetine	nq [a]	28 [a]	0.42 [b]	1.3 [b]	2 [b]	3.7 [c]	6.6 [c]	8.6 [c]	9.8 [c]	11 [c]
		11 [c]	13 [c]	20 [c]	38 [d]	50 [d]	99 [d]	20 [e]	91 [e]	9 [f]
		16.2 [f]	29 [f]	39.2 [f]	43.2 [f]	nq [g]	1.2 [h]	1.3 [h]	0.6 [i]	1.4 [i]
		3 [i]	3.1 [i]	3.2 [i]	3.9 [i]	4.8 [i]	nq [h]	8.4 [i]	nq [j]	11 [j]
		15 [j]	15 [j]	16 [j]	18 [j]	19 [j]	21 [j]	23 [j]	27 [j]	28 [j]
		30 [j]	30 [j]	31 [j]	31 [j]	36 [j]	46 [j]	47 [j]	52 [j]	63 [j]
		76 [j]								
Norfluoxetine	nq [a]	nq [a]	1.2 [b]	1.3 [b]	1.7 [b]	1.8 [b]	5.7 [c]	7.1 [c]	7.1 [c]	7.2 [c]
		7.4 [c]	7.6 [c]	10 [c]	11 [e]	1.8 [f]	2.4 [f]	4 [f]	13.1 [f]	13.6 [f]
		nq [i]	nq [i]	1.2 [i]	1.4 [i]	1.7 [i]	2 [i]	2.4 [i]	2.4 [i]	

Fluvoxamine	2.9 [c]	3.4 [c]	3.9 [c]	0.7 [f]	0.8 [f]	1.7 [f]	4.1 [f]	4.6 [f]	nq [i]	1.8 [i]
		nq [i]	nq [j]	0.6 [j]	0.6 [j]	0.7 [j]	0.7 [j]	0.8 [j]	3.8 [j]	nq [j]
Paroxetine	nq [a]	89 [a]	1.3 [b]	3 [b]	4.3 [b]	5.2 [b]	1.3 [c]	3.7 [c]	5 [c]	5.6 [c]
		6 [c]	6.3 [c]	12 [c]	7 [e]	16 [e]	2.2 [f]	2.7 [f]	3.9 [f]	4 [f]
		5.4 [f]	81.1 [g]	0.7 [h]	1.6 [h]	0.5 [h]	1 [i]	1.9 [i]	3.4 [i]	3.8 [i]
		5.6 [i]	7.7 [i]	11.7 [i]	3.4 [i]					
Sertraline	nq [a]	nq [a]	0.84 [b]	2.4 [b]	5.1 [b]	5.8 [b]	5.7 [c]	8.1 [c]	8.1 [c]	12 [c]
		14 [c]	16 [c]	21 [c]	14 [e]	34 [e]	3 [f]	16.4 [f]	19.2 [f]	22.8 [f]
		37.5 [f]	nq [g]	1.6 [h]	1.9 [h]	2 [h]	3.7 [i]	6.1 [i]	6.3 [i]	7.9 [i]
		8.2 [i]	10.5 [i]	12.6 [i]	14.6 [i]					
Desmethyl sertraline	2.3 [b]	3.6 [b]	4.5 [b]	4.7 [b]	12 [c]	13 [c]	14 [c]	15 [c]	15 [c]	16 [c]
		24 [c]	20 [e]	91 [e]	5 [f]	5.9 [f]	6.2 [f]	11.1 [f]	26.7 [f]	nq [i]
		nq [i]	6.2 [i]	7.5 [i]	9.1 [i]	10.6 [i]	10.6 [i]	nq [i]		

[a]Gros* (2012)- Spain; [b]Lajeunesse* (2008)- Canada; [c]Lajeunesse* (2012)- Canada; [d]Metcalfe* (2003)- Canada; [e]Metcalfe (2009)- Canada; [f]Schultz (2010)- USA; [g]Silva (2014)- Spain; [h]Vasskog* (2006)- Norway; [i]Vasskog* (2008)- Norway; [j]Writer (2013)- USA. (*) Indicates the mean of a minimum of triplicates, with a reported standard deviation or a relative standard deviation.