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Analysis and Characterization of Halogenated Transformation Products of Pharmaceuticals and Personal Care Products in Wastewater Effluent

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by

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Acknowledgement of Copyright

ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy, Graduate Program in Chemistry
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Dr. Cynthia K. Larive, Chairperson

Wastewater is a complex mixture of natural and anthropogenic pollutants including inorganic and organic species. The use of disinfection treatments such as chlorine is necessary to attenuate pollutants encountered in wastewater prior to its discharge into the environment. In recent years, the presence of pharmaceuticals and personal care products (PPCPs) in wastewater has received increasing attention due to the concern that these compounds may disrupt the normal endocrine function of aquatic biota. Less attention has been paid to the potential for PPCP transformation into new, chemically unique compounds via disinfection treatments. This dissertation focuses on the identification and quantification of halogenated pharmaceutical analogs in wastewater effluent generated by disinfection treatment with chlorine. The goal of this research is twofold: 1. Identify chlorination transformation products (TPs) of selected PPCPs, synthesize and purify standards (with and without deuterium labeling) for each unique TP and characterize the structure and purity of the TP standards; 2. Use these TP standards to develop targeted high performance

liquid chromatography hyphenated to mass spectrometry (HPLC-MS) methods for the analysis of the selected PPCPs and their TPs and accurately quantify each analyte in solid phase extracts (SPE) of several wastewater effluents from Southern California.

This dissertation initially focuses on the chlorination TPs of the lipid regulator gemfibrozil and their occurrence in advanced primary wastewater effluent. Standards of gemfibrozil's TPs were synthesized, purified by HPLC and characterized by nuclear magnetic resonance (NMR) spectroscopy and accurate mass MS. This research is extended to identify and quantify the chlorination TPs of the analgesic salicylic acid, the non-steroidal anti-inflammatory (NSAID) diclofenac and the plasticizer bisphenol A in secondary wastewater effluent. Standards for TPs of the additional compounds were synthesized, isolated and characterized by the same methods as used for the gemfibrozil TPs. The dissertation culminates by expanding the selected PPCPs for secondary and tertiary wastewater analysis to include the surfactants octylphenol and nonylphenol, the NSAID naproxen, in addition to the synthesis of deuterated analogs of each PPCP and TP. Several of the identified TPs were quantified for the first time at ng/L levels in several of the analyzed wastewaters.

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Concentrations (ng/L) of selected PPCPs and TPs in tertiary and secondary effluent samples.

Chapter One

Introduction

The disinfection of polluted fresh waters is one of the most important advancements in terms of sanitation in modern society. However, the disinfection of water containing a mixture of both naturally and anthropogenically derived organic and inorganic pollutants can lead to the unintentional creation of hazardous byproducts. Disinfection byproducts (DBPs) are recognized for their deleterious health effects on a wide variety of biota. Pharmaceuticals and personal care products (PPCPs) are one such class of organic anthropogenic pollutants whose presence in wastewaters has been increasingly studied in the past several decades. The potential for PPCPs to be transformed by wastewater disinfection processes, and the occurrence, fate and transport of the resultant disinfection transformation products (TPs), has received considerably less attention.

This research focuses on the identification and analysis of halogenated TPs resulting from treatment of aqueous dissolved pharmaceuticals with the commonly used oxidative disinfectant chlorine, introduced as sodium hypochlorite (NaOCl). The goals of this thesis are outlined in the objectives stated below, which are addressed in Chapters 2-4.

Objective 1: Identify halogenated TPs of selected PPCPs and create a library of purified standards for compounds not commercially available. *In situ* chlorination

reactions with parent PPCP compounds are monitored by ultra performance liquid chromatography with time of flight mass spectrometric detection (UPLC-QTOF-MS) to identify potential halogenated TPs based on retention time shifts, m/z shifts and halogen isotope signatures. Reversed phase high performance liquid chromatography with UV detection (RP-HPLC-UV) is then used to purify the major TPs from *in situ* chlorination reactions.

Objective 2: Structurally characterize halogenated TP standards. The RP-HPLC-UV purified TP standards are characterized by accurate mass QTOF-MS, with additional confirmation from comparison of the *in silico* predicted and experimentally determined mass spectral isotopic patterns. Regiochemistry of the halogenated TPs are assigned using 1- and 2-D nuclear magnetic resonance (NMR) spectroscopy, including ^{1}H survey spectra and [^{1}H,^{13}C] heteronuclear multiple bond correlation (HMBC) and [^{1}H,^{1}H] nuclear Overhauser effect spectroscopy (NOESY) experiments.

Objective 3: Quantify PPCPs and their major TPs in wastewater effluents from Southern California. Effluent samples from primary, secondary and tertiary wastewater treatment facilities are concentrated by either liquid-liquid extraction (LLE) or solid phase extraction (SPE). Concentrated extracts then undergo targeted analysis by either UPLC-QTOF-MS or HPLC with triple quadrupole mass spectrometric detection (QQQ-MS).

1.1 Wastewater and wastewater treatment

Wastewater is defined by the United States Environmental Protection Agency's (USEPA) terms of environment[1] as:

"The spent or used water from a home, community, farm or industry that contains dissolved or suspended matter."

Wastewater is produced by a combination of point source (e.g. single factory or hospital) and non-point source (e.g. runoff from fertilizer used in a neighborhood), and is a complex mixture of both natural and anthropogenic wastes.[2] The characteristics of wastewater vary greatly depending on the sources of influent pollution, often containing high amounts of both organic (i.e. carbon-containing) and inorganic (e.g. metals, salts) components. To reduce the impact of wastewater on the downstream environment upon discharge, pathogens (e.g. bacteria) and the nutrients upon which microorganisms subsist (e.g. phosphate, organic carbon) must be removed or attenuated through a wide variety of processes collectively known as wastewater treatment.[3, 4]

Wastewater treatment plants (WWTPs) are generally divided into three successive treatment sectors, consisting of primary, secondary and tertiary treatment.[5] Primary treatment mainly involves the settling of suspended solids, which can remove up to 60% of the solids content of influent municipal wastewaters. Chemical pretreatment with ionic flocculants such as ferric chloride to assist in the settling of solids is common, often garnering the name advanced primary treatment when such chemicals are used.[6] Primary effluent often

contains higher amounts of solid particulate matter compared with secondary and tertiary effluent, measured by the total suspended solids parameter with values ordinarily in the low (<100) mg/L range.[7, 8] Secondary treatment involves biological degradation of organic wastewater contaminants through the use of a bacterial soup known as activated sludge. Activated sludge is an aerated soup of saprotrophic bacteria and protozoa which help flocculate and attenuate the organic matter content of wastewater.[6] Tertiary treatment, less common than primary and secondary, involves the use of filtration techniques (e.g. reverse osmosis and nanofiltration) as well as advanced oxidation processes including chemical additives (e.g. ozone and peroxyacetic acid) as well as physiochemical processes (e.g. UV irradiation combined with a catalyst such as TiO_2).[9, 10] While less common due to the increased cost of operation, many WWTPs are currently being upgraded to tertiary treatment capabilities due to the extremely heavy input of pollution into the planet's fresh waters.[5] The organization schema of WWTPs varies greatly depending on the source of the influent pollution, and may include a combination of portions of primary, secondary and tertiary treatment. An example of a wastewater treatment plant organization scheme from Los Angeles County Sanitation District (LACSD) in Southern California is seen in Figure 1.1.[11]

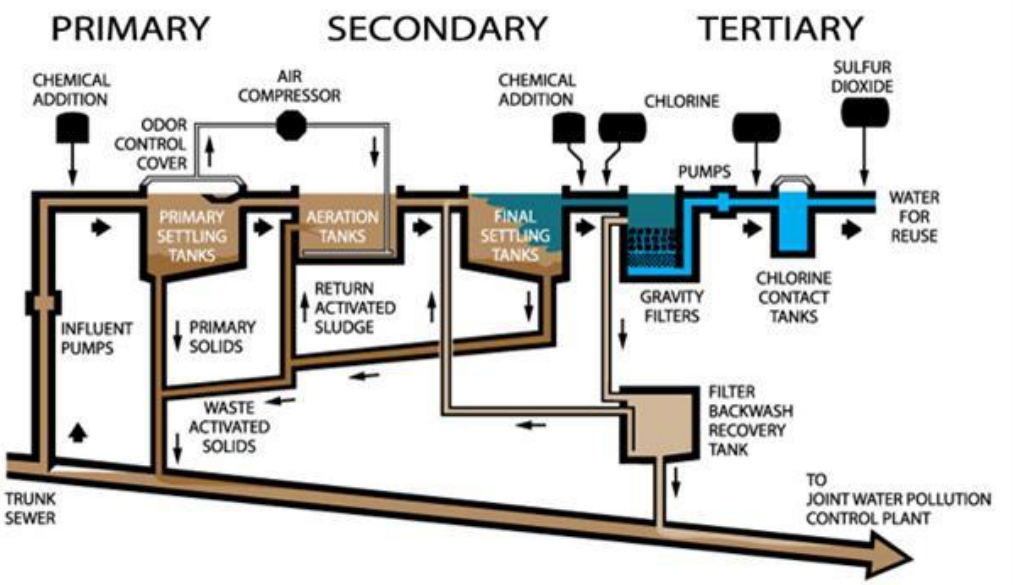


Figure 1.1. Los Angeles County Sanitation District wastewater treatment plant scheme of operation.[11]

The LACSD treatment plant in Figure 1.1 is a tertiary plant, using a combination of physical, microbial and chemical means to decontaminate the influent wastewater prior to discharge. The criteria for ensuring the cleanliness of wastewater prior to discharge varies by municipality, state and country. In the United States, discharge limits applying to regulated pollutants include parameters such as the amount of suspended solids or nitrate content which are enforced by the United States Environmental Protection Agency (USEPA) under the Clean Water Act (CWA) through programs such as the National Pollutant Discharge Elimination System (NPDES).[12] Specific values or ranges of values for the discharge limits of regulated pollutants according to the USEPA and parameters regarding their measurement are given across numerous technical documents including the Effluent Guidelines and Standards.[13]

Regardless of the arrangement of treatment schemes, nearly all WWTPs in the U.S. employ an oxidative disinfection step prior to effluent discharge. Oxidative disinfection of wastewater serves two main purposes: (1) attenuation of potentially pathogenic microbes and (2) removal of organic matter. The most common disinfectant used in WWTPs is chlorine.[14]

1.1.1 Chlorine disinfection of wastewater. Chlorine disinfection involves the addition of (in decreasing popularity of usage): NaOCl, chloramine, molecular chlorine (Cl_2 gas) or chlorine dioxide, generally applied at concentrations between 1-20 ppm.[2, 14] These chlorinating agents were compared by Lebedev

and coworkers in terms of their positive and negative characteristics as WWTP disinfectants, presented in Table 1.1.[15]

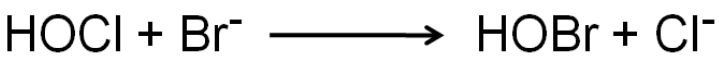
Table 1.1. Comparison of chlorinating agents.[15]

Chlorinating agent	Positive results	Negative results
Molecular chlorine	Cheap, efficient, stable	High levels of THM* and HAA5**
Sodium hypochlorite	Cheap, efficient, stable	High levels of THM* and HAA5**
Chloramine	Efficient, stable, less THM and HAA5	Formation of DMNA***, Pb removal from metal piping
Chlorine dioxide	Efficient, stable, less THM and HAA5	High levels of bromites, bromates, chlorites, chlorates

*THM = trihalomethanes
**HAA5 = halogenated acetic acids
***DMNA = dimethylnitrosamine

As shown in Table 1.1, the use of chlorine as a disinfectant can lead to the unintended generation of compounds such as trihalomethanes (THMs, regulated in the US and EU) and haloacetic acids (HAA5, regulated in the US), often referred to as classic DBPs. These compounds are recognized for their toxicity and carcinogenicity.[16, 17] Several recent reviews concerning these classic DBPs, as well as emerging related DBPs, are discussed elsewhere.[18-20] Dissolved organic matter can be either chlorinated, brominated or form a mixture of

chlorinated and brominated byproducts when treated with chlorine as bromide that is naturally present in most fresh waters is oxidized to hypobromous acid (Equation 1.1) which can then react to form brominated compounds.[21]



Equation 1.1. Halogen exchange reaction showing the oxidation of bromide to hypobromous acid by hypochlorous acid.

It is worth noting that the HOBr formed by this halogen exchange reaction is more reactive towards organic species by several orders of magnitude compared to HOCl.[22] While halogen exchange chemistry can extend to oxidation of iodide, this is extremely rare in the environment as the concentration of iodide in most wastewaters is extremely low except for hospital wastewater in which iodinated contrast agents are discharged.[23]

1.2 Pharmaceuticals in wastewater

The initial discovery of pharmaceuticals as anthropogenic environmental pollutants in fresh waters occurred in the 1970s, when clofibric acid, caffeine, salicylic acid and nicotine were identified in wastewater effluent.[24, 25] In the past several decades, the number of investigations concerning the presence, transport and fate of PPCPs in wastewaters and various other natural fresh waters (e.g. drinking water) has greatly increased. This has resulted in the

discovery and quantification of hundreds of PPCPs in various fresh waters including wastewaters around the world.[26, 27] Much of this research interest has been spurred by a growing body of evidence that some PPCPs can exert deleterious effects on non-target organisms as a result of unintentional exposure.[28] PPCPs are often present at very low, e.g. ng-µg/L, levels in aqueous matrices, and thus their analysis requires selective sample preparation and modern analytical instrumentation which afford lower limits of detection than previously achievable.[18]

1.2.1. Oxidation of organic micropollutants during wastewater treatment.

The study of the attenuation of PPCPs through wastewater treatment plants (WWTPs) is of great interest from both chemical and environmental perspectives. While WWTPs were not expressly designed for the removal of PPCPs, they are nevertheless often effective in their removal. Characterization of the amounts of PPCPs in effluent discharges compared with the influent waters can give a quantitative removal efficiency for a given compound. This is an incomplete model of the attenuation of a given compound however, since it assumes that any fraction of the compound which has been lost has been mineralized (i.e. completely degraded into carbon dioxide, water and inorganic constituents). The formation of TPs can explain this “loss” of the parent compound, where new chemical entities with potentially altered physiochemical and toxicological properties have been generated. While the formation of TPs from PPCPs in the

aqueous environment as a result of water disinfection has been known for several decades, progress in this emerging field has been slowed by a variety of factors inherent in the study of environmental micropollutants.

When PPCPs are present in source waters undergoing chlorination, halogenated TPs of these compounds can result. TPs in fresh waters can form as a result of a large variety of natural processes (e.g. bacterial metabolism and photolysis), and have in some instances been detected at higher concentrations than their parent compounds.[29, 30] Depending on the functional groups present in a given PPCP, chlorination can result in a variety of transformations including the addition of one or more halogens (especially for aromatic compounds e.g. phenolics[31, 32,]) and oxidation products (sulfur containing compounds e.g. cysteine[33]). An example of the number of halogenated TPs which can form from a single parent PPCP is given in Figure 1.2, with salicylic acid as the parent PPCP which has been shown to form multiple halogenated species upon exposure to chlorine in the presence of bromide.[34] As many PPCPs contain one or more aromatic rings, TPs incorporating additional halogens into the molecular structure can dominate observed transformation products. Chlorination TPs of PPCPs can be more toxic than the parent compound, as seen with acetaminophen which forms the toxicants 1,4-benzoquinone and *N*-acetyl-*p*-benzoquinone imine upon exposure to aqueous NaOCl.[35] This phenomenon can also be seen with the biocide triclosan which can also be transformed at environmentally relevant concentrations into the stable toxicants 2,4-

dichlorophenol and 2,4,6-trichlorophenol in the presence of low ppm doses of chlorine.[36]

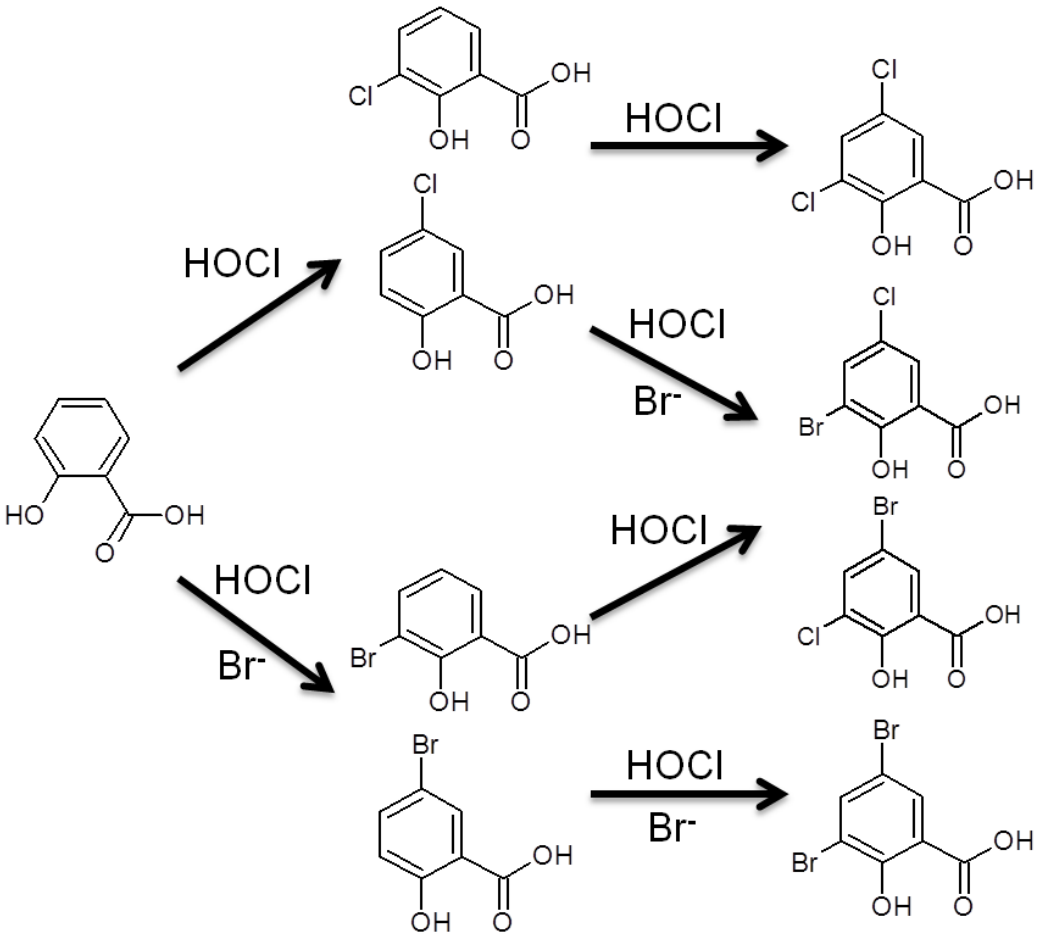


Figure 1.2. Reaction scheme for salicylic acid with chlorine in the presence of bromide. A total of 8 TPs can form from the parent PPCP.

1.3 Sample preparation and analytical platforms for the separation and detection of PPCPs in wastewater

1.3.1 Sample concentration. Wastewater sample preparation for PPCP analysis involves the extraction of dissolved organic compounds from an aqueous wastewater sample, achieving enrichment factors of up to several hundred-fold. Sample volumes for extraction are often between 0.1 and 1 L, and can be either a grab sample (single timepoint) or composite sample (average of multiple timepoints, e.g. by combining 24 discrete samples collected over 24 consecutive hours). Solid phase extraction (SPE) is the most popular sample concentration technique, owing to its ease of use, reproducibility and low solvent usage. Common SPE sorbent materials include C18 and mixed-mode sorbents, with Waters Technology Corporation's Oasis HLB (Hydrophilic-Lipophilic Balance) being an especially popular mixed mode sorbent because of its ability to trap analytes with a wide range of lipophilicities and polarities.[37] Other SPE sorbents, such as anion or cation exchange, provide selectivity to enrich compounds with ionizable functional groups which often correlate well with PPCPs of a given therapeutic class.[38] Ion exchange SPE generally involves adjusting the wastewater sample pH prior to extraction, with low pH being used for cation exchange and high pH for anion exchange. Several newer iterations of SPE are becoming popular in wastewater analysis, such as solid phase microextraction (SPME).

Liquid-liquid extraction (LLE) for wastewater sample preparation is still employed, though becoming less common due to its high solvent waste generation and laborious nature which can hinder analytical throughput.[39] Several related techniques, such as membrane assisted liquid-liquid extraction,[40] liquid-liquid microextraction (LLME),[41] ultrasound-assisted ionic liquid dispersive liquid-liquid microextraction (US-IL-DLLME),[42] and single-drop microextraction (SDME)[43] are gaining in popularity due to their increased extraction efficiencies compared with traditional LLE in addition to their use of smaller sample and solvent volumes. Liquid extraction of solid samples, such as soil, activated sludge or suspended particulate matter prevalent in primary wastewater effluent was traditionally carried out by Soxhlet extraction.[44] More recently, extractions of solids have been carried out by accelerated solvent extraction techniques such as microwave assisted extraction[45] and pressurized liquid extraction (PLE),[46] which use less solvent, have higher extraction efficiencies and take less time compared with Soxhlet extraction.[47]

1.3.2 Separation platforms. For the analysis of PPCPs in wastewater, high performance liquid chromatography (HPLC) is the most popular separation platform. Reversed-phase (RP-HPLC) is the most frequently encountered separation mode, with C18 stationary phases being the most prevalent; often the stationary phase is modified with a polar endcapping or embedded group to impart different selectivity towards certain classes of compounds.[17, 18] Columns

for analytical scale HPLC analysis often use particle sizes between 2.5-5 µm, and operate at backpressures below 400 bar. More recently, HPLC systems which can perform at pressures above 400 bar, termed ultra high pressure high performance liquid chromatography (UHPLC or UPLC), have replaced their lower-pressure HPLC counterparts. Higher pressures allow the use of sub-2 µm particle columns, which translates into higher theoretical plate heights and enhanced peak resolution and capacity.[48, 49] This is especially important in wastewater analysis as the matrix is extremely complex, necessitating high-resolution chromatographic separations for PPCP and TP analysis.[50]

Gas chromatography (GC) is a complementary separation platform to HPLC used less frequently in PPCP and TP analyses. The benefits of GC separations include narrow peakwidths and extremely reproducible retention times, however a drawback is that most PPCPs are non-volatile and thus must be derivatized prior to GC analysis.[51] Depending on the physiochemical properties of the TP, however, sample concentration and derivatization can be carried out in a single step thus eliminating such drawbacks.[52] Often GC is used for the analysis of polar PPCPs as they are difficult to retain by RP-HPLC, though hydrophilic interaction liquid chromatography (HILIC) has proven to be useful for the analysis of polar compounds such as 5-fluorouracil and cocaine.[53, 54] Capillary electrophoresis (CE) has seen infrequent implementation for the analysis of PPCPs, though it has been successfully implemented to analyze compounds with similar ionizable moieties such as phenolics and basic

antidepressants.[55, 56] Once a wastewater sample has been concentrated and its components separated, individual analytes can be measured with a variety of detection methods.

1.3.3 Analytical detection platforms. Mass spectrometry (MS) dominates as the detection platform of choice for the analysis of PPCPs in wastewater, most often coupled to HPLC or GC, and will be discussed in further detail below. Several other detectors, most often coupled with HPLC, have had some success in confidently identifying specific analytes in the complex wastewater matrix. The use of UV detection is popular for the qualitative characterization of PPCPs in wastewater effluent.[57] Indeed, the targeted results of analyses of wastewater by UV detection coupled with a separation platform such as HPLC or CE are often confirmed by MS due to the unspecific nature of UV detection in concert with the potential for co-eluting or co-migrating species.[58, 59] Surprisingly, direct UV analysis coupled with *in silico* spectral deconvolution has been used to identify distinct compounds such as azo dyes in effluent from textile manufacturing, however this approach is limited by the unique spectral properties of such compounds.[60] Other detection methods such as electrochemical detection,[35, 61] fluorescence spectroscopy,[62] and immunoassays such as enzyme linked immunosorbent assay (ELISA)[63, 64] have been used sparingly for PPCP analysis due to their inability to target a wide variety of compounds. The specificity of immunoassay-based techniques makes it more popular than the other two

detection methods for the targeted analysis of specific PPCPs in a matrix as complex as wastewater, though it requires the costly and laborious task of developing antibodies for each analyte of interest.[65]

1.3.4 Ionization sources. For mass spectrometry to be performed, the analyte must first be transferred into the gas phase and charged to create an ion. The ion can be either positively charged, negatively charged or both. Depending on the charge of the analyte, the MS is said to be working in positive mode for positively charged ions and negative mode for negatively charged ions. The most common source used in environmental mass spectrometry for the detection of PPCPs is the electrospray ionization (ESI) source because of its compatibility with HPLC and ability to ionize a wide variety of compounds without fragmenting the analytes. Modern ESI sources are able to rapidly switch between positive and negative ionization modes, termed polarity-switching, which has been increasingly employed in recent years to reduce the number of analyses for multiple analytes which preferentially ionize in one of the modes.[66, 67] For GC, the most commonly used source is electron impact (EI) which produces distinct fragmentation patterns upon ionization which can serve as a recognizable molecular fingerprint for many compounds.[68, 69]

Atmospheric pressure chemical ionization (APCI) is a less commonly used ionization method, typically hyphenated with HPLC. APCI can be useful for the analysis of neutral compounds and other compounds known to have a poor ESI-

MS response though this source often produces more fragment ions than ESI.[70, 71] While somewhat common in industrial pharmaceutical analysis, the use of hybrid sources able to switch rapidly between ionization types such as APCI/ESI, termed multimode sources, is currently not often implemented for wastewater analysis.[72, 73]

1.3.5 Mass spectrometers. Once a PPCP has been ionized, a mass spectrometer can measure its mass-to-charge (m/z) ratio which can be related back to the original mass of an analyte depending on how many charges the analyte carried. In addition, information about the abundance of isotopes in a given analyte is conveyed in the mass spectra by the presence of multiple peaks, each corresponding to the same molecule but with the presence of one or more isotopically heavier atoms. The distribution of the isotopes is related to their natural abundance, which provides an especially helpful signature for halogenated compounds such as PPCPs as the natural isotopes for chlorine and bromine have a distinct distribution of 76:24 for ^{35}Cl:^{37}Cl and 51:49 for ^{79}Br:^{81}Br. Such isotopic abundances are based on a statistical distribution, and thus they can be calculated for comparison to experimental spectra.[74] An example of mass spectra of halogenated compounds is seen in the predicted negative ionization mass spectra in Figure 1.3 for salicylate (1.3A) and its monochlorinated (1.3B) and monobrominated (1.3C) TPs, with molecular formulae of $C_7H_5O_3$, $C_7H_4O_3Cl$ and $C_7H_4O_3Br$, respectively.[35] The monoisotopic peak is referred to as "M" while

each peak one mass unit heavier is incrementally referred to as "M+1", "M+2" and so on.

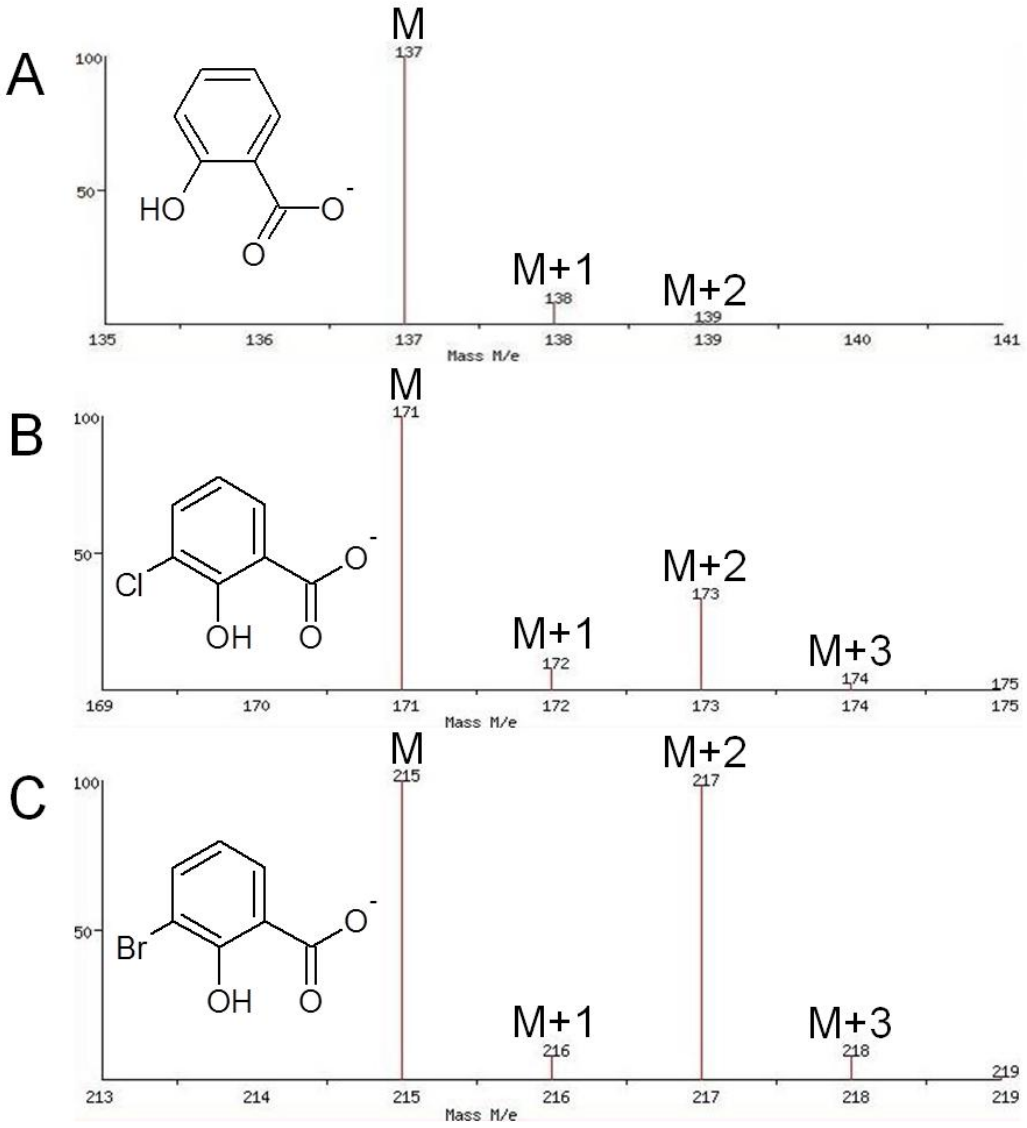


Figure 1.3. Predicted negative mode mass spectra of salicylate (A), chlorosalicylate (B) and bromosalicylate (C). Note the 3:1 ratio of M:M+2 intensities for chlorosalicylate and the 1:1 ratio of M:M+2 intensities for bromosalicylate, reflecting the natural abundance 3:1 ratio of ^{35}Cl:^{37}Cl and 1:1 ratio of ^{79}Br:^{81}Br, respectively.

Due to the complexity of wastewater, mass spectrometry is necessary to confidently identify one component from other components within a given wastewater sample. The prevalent use of low-resolution single quadrupole mass analyzers prior to ca. early 21st century for PPCP analysis potentially led to false positive identifications due to the frequently encountered problem of isobaric overlap resulting from the co-detection of two or more analytes with equivalent m/z values.[26, 75] Towards this end, the reduction of the complexity of the sample as it enters the MS through targeted sample concentration techniques coupled with high-efficiency chromatographic separations is extremely important for the analysis of PPCPs in wastewater to prevent false positive identification.[50, 76] Besides enhanced separation technologies, the problem of isobaric overlap can be solved in several ways with modern MS instruments.[77]

Recent advances in technology have led to the replacement of single quadrupole, low resolution MS instruments by increasingly more sensitive, higher resolution and faster scanning instruments.[78] New time-of-flight MS (TOF-MS) mass analyzers can provide mass accuracy (<1 ppm), fast scan times (50 Hz) and resolution ca. 40,000.[79] The mass accuracy provided by TOF-MS instruments allows the generation of empirical formulae for an observed m/z taking into account several thermodynamic rules for molecular arrangements, which can provide a nearly unambiguous assignment of a compound especially when combined with the LC or GC retention time of an authentic standard.[74, 80] In the last decade, hybrid MS instruments have become popular, such as the

quadrupole time-of-flight (QTOF-MS). The QTOF-MS employs a quadrupole prior to the TOF portion of the instrument so that a mass can be selected for tandem mass spectrometry (MS-MS). The use of MS-MS involves the collision of molecules of a certain m/z value, as selected by the quadrupole, with molecules of an inert gas such as argon or nitrogen with kinetic energies in the tens of electron volt (eV) range. Molecular collisions take place after the mass filtering quadrupole in a second quadrupole, hexapole or octapole, referred to as a collision cell. The energy from the collisions with gas molecules causes the analytes to break apart in reproducible ways, providing characteristic fragments (termed transitions) which are then analyzed by the TOF segment of the QTOF-MS.[81] Transitions are often reported as "parent mass > fragment mass", for example 137 > 93 for salicylic acid (m/z 137) as it fragments and loses its carboxyl group (m/z 44) as analyzed in the negative mode.[34] While this definition of MS-MS, termed collision induced dissociation (CID), is based on only one of many mechanisms of MS-MS fragmentation, as it is the most frequently employed in wastewater analysis this will be the only method discussed herein. It is worth noting that CID can also take place in the ionization source, termed in-source CID.[82]

Another popular instrument used for PPCP analysis is the triple quadrupole mass spectrometer (QQQ-MS), which has three quadrupoles in series. While mass accuracy is poor compared to the TOF-MS analyzer, MS-MS experiments can be extended to more fragmentation steps i.e. MS-MS-MS (MS^n).

In MS^n experiments, the first quadrupole (Q1) is used to select for a specific m/z, which is fragmented in the second quadrupole (Q2) and passed into the third quadrupole (Q3). The Q3 sector can then either scan ions of a specified m/z range for detection (product ion scan), filter for a specific m/z for detection (selected reaction monitoring (SRM)), or pass ions of a selected m/z back into Q2 for a second round of fragmentation (MS^n). In terms of isobaric overlap with PPCP analysis in a wastewater extract, while two compounds with identical m/z may share a single overlapping transition at one collision energy it is unlikely that they will share a second identical transition at a given collision energy. In this way more unambiguous identification of an analyte can be achieved. The combination of HPLC and QQQ-MS allows the use of SRM, frequently referred to in recent literature as multiple reaction monitoring (MRM). When employing LC-QQQ-MS in MRM mode, the retention time of the desired analytes are combined with one or more MS^n transitions for each analyte, thus allowing quantitative analysis as well as confident assignment of analyte identity (i.e. lower false positive rate). For halogenated compounds, it is possible to use a high collision energy to liberate chloride or bromide as a fragment. In this way, two transitions from the parent molecule's m/z to m/z values of 35 and 37 or 79 and 81 for chlorinated and brominated compounds, respectively. For the identification of halogenated compounds such as chlorination TPs, a precursor scan using a QQQ-MS is useful. In a precursor scan, fragment ions of a certain m/z are selected in Q3 and the masses which gave rise to the fragments are scanned by

Q1.[83] Thus, analytes which give rise to specific m/z transitions, such as 35/37 or 79/81 for identifying chlorinated or brominated compounds, can be identified even though their identity may be unknown.[84]

Several other types of MS used less frequently for the analysis of PPCPs in wastewater analysis include the ion trap (IT-MS), which can provide excellent signal-to-noise over multiple MS-MS transitions. While used less frequently in wastewater analysis, the IT-MS is quite useful for the identification of TPs formed from PPCPs as will be seen in section 1.4.[85] The Orbitrap MS is becoming increasingly popular due to its ability to provide both high resolution and high mass accuracy.[86] Several exotic hybrids of such MS instruments have become commercially available, such as the linear ion trap (LIT) Orbitrap MS or LIT-TOF.[87] Newly available hybrid MS instruments can provide the excellent MS^n capabilities of the ion trap while maintaining the high mass accuracy necessary to accurately assign compound identity during wastewater analysis. A recent review on the use of hybrid MS instruments and analysis techniques for PPCPs and TPs in drinking water and wastewater is presented by Radjenović et al.[85] This review focuses on the use of modern MS platforms such as the hybrid quadrupole linear ion trap (QqLIT-MS) and high mass accuracy instruments such as the QTOF-MS for the analysis of PPCPs and TPs. Several other recent reviews have covered the subject of emerging mass spectrometer technologies and their use in environmental analysis.[78, 88]

1.4 Halogenated TPs generated from the chlorination of PPCPs

One of the difficulties inherent in the environmental analysis of PPCP chlorination TPs is that most potential TPs have only recently been identified, meaning pure analytical standards for such compounds are rarely commercially available. The availability of authentic standards allows quantitative analysis by techniques such as LC-MS through the creation of external calibration curves. Unlike NMR where the detector response is universal relative to the nucleus being detected, ionization efficiencies in LC-MS can be very different between analytes, even those with nearly identical chemical structures. Without authentic standards, accurate and reliable quantitative analysis is difficult because the extraction efficiency (the total percentage of the analyte recovered from a given sample) during wastewater concentration steps (e.g. SPE) is unknown. The most trusted method for determining sample recovery is through the use of isotopically enriched standards spiked into the wastewater sample prior to extraction, since such compounds are not found naturally in wastewater, their chemical behavior in the extraction and separation steps is identical to the unlabeled target compound, and the MS signature is unique compared to that of the unlabeled compound. Isotopically enriched standards can also increase the accuracy of assignment of chemical identity as well as account for variations in analytical system performance such as ionization suppression.[89, 90] As noted by Annesley, the use of standards which behave chromatographically identically to the analytes in questions is necessary for the accurate characterization of ion

suppression effects especially during ESI-MS and APCI-MS analyses.[91] Interestingly, ion enhancement has also been noted in the analysis of PPCPs in wastewater, underscoring the need for isotopically labeled standards for accurate quantitation.[70, 92] As even non-isotopically labeled standards are unavailable for most chlorination TPs, the use of isotopically labeled standards often relies on using a surrogate standard which is representative of the physiochemical properties and ionizable moieties of a group of target analytes.[93]

A majority of research to date concerning *in situ* PPCP chlorination studies has focused on the degradation kinetics of a parent PPCP, where half-lives can be used to estimate PPCP removal from WWTP influent waters. In these experiments, the desired parent compound is dissolved in an aqueous matrix, often deionized or pH buffered water, and chlorine is added in various molar ratios compared to the parent. The chlorination reactions are sampled at discrete timepoints where the reaction is halted by use of a reducing agent such as thiosulfate, and the disappearance of the parent is assessed through the decreasing HPLC-UV peak area. The use of pure water as the reaction matrix may not be representative of real-world conditions such as those found in WWTPs, especially because of the absence of bromide. Acero et al. investigated the degradation kinetics of a variety of pharmaceuticals at multiple pH values in both phosphate buffered water and municipal wastewater.[94] Removal rates were higher in wastewater samples, attributed to the presence of bromide in the wastewater. There is also evidence that other common wastewater constituents,

such as tertiary amines, can enhance the degradation rates of organic compounds upon exposure to chlorine.[95] The reaction conditions necessary to generate real-world applicable chlorine degradation kinetic data is a complicated topic, addressed in a recent review by Deborde et al.[2] The next step towards the analysis of TPs in the environment is to identify potential TPs using *in situ* chlorination reactions, and to generate standards for such compounds. A general scheme for the identification of chlorination TPs and the isolation of pure standards for those TPs is given in Figure 1.4.

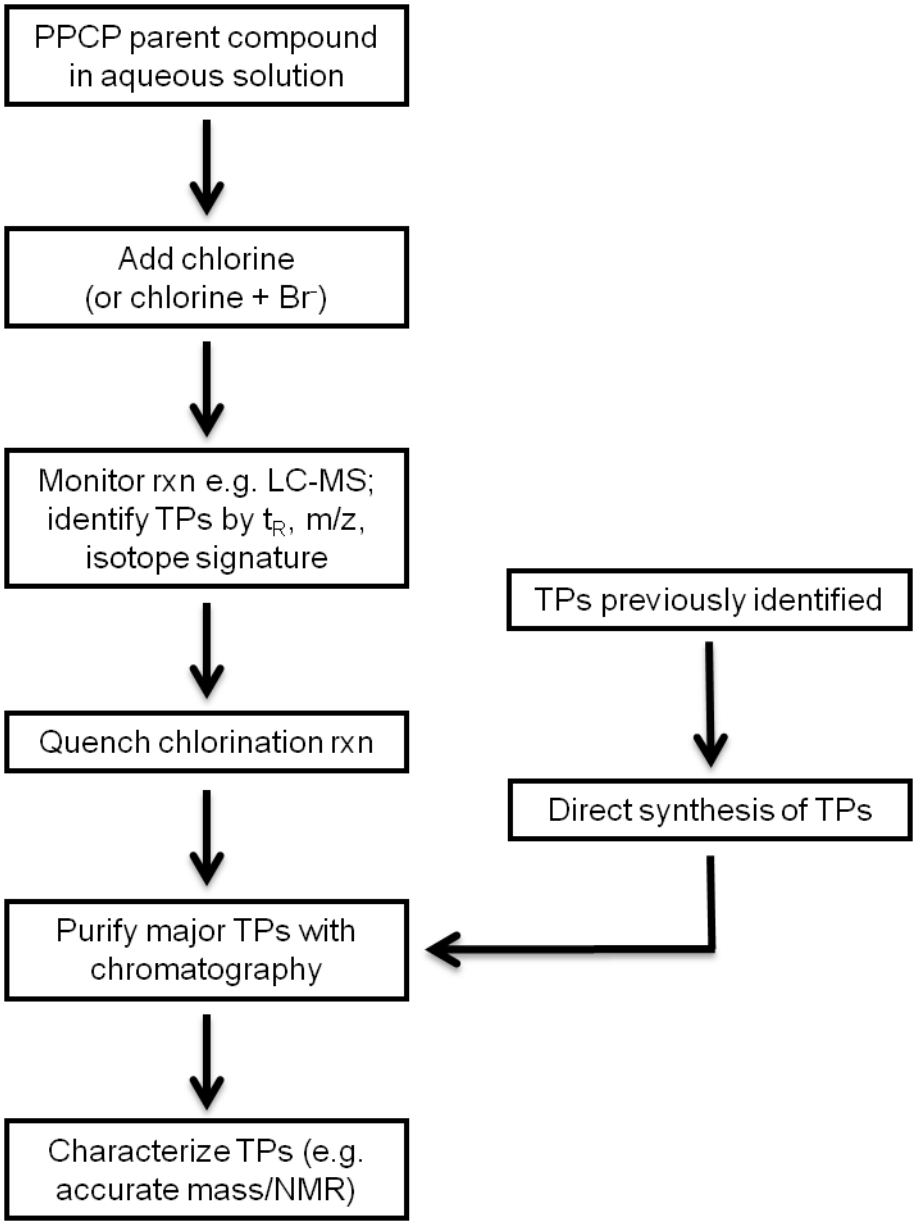


Figure 1.4. General flowchart for the generation, identification, isolation and characterization of chlorination TPs from a given parent PPCP.

Not all steps of Figure 1.4 are necessary for the characterization of TPs from a given PPCP, depending on the depth of prior research. Kuruto-Niwa et al. generated TP standards of *tert*-octylphenol, nonylphenol and bisphenol S for use in bioassays by the dropwise addition of aqueous NaOCl to each parent compound dissolved in a mixture of methanol and water.[96] In this case, previous literature reports had characterized the expected TPs from chlorination reactions, so the syntheses could move forward without the identification of the TPs formed. After reacting for 1-2 h, the chlorination reaction was quenched with sulfite, and the mixtures were acidified and purified using normal phase silica gel chromatography. Individual TP fraction identity was confirmed using single quadrupole GC-MS and ^{1}H NMR, where the MS spectrum was compared to the expected mass.

In many studies, TPs may only be putatively identified based on their mass spectra, rather than isolated for use as standards. Glassmeyer et al. reacted several pharmaceuticals at 50 mg/L in deionized water with 28 mg/L chlorine, and identified chlorinated TPs after 48 hours of reaction time by single quadrupole LC-MS.[97] The structures of the TPs formed was ambiguous given the unit mass resolution of the instrument, though the presence of one or more chlorines in observed TPs such as those arising from chlorination of acetaminophen and gemfibrozil were assigned by isotopic signature and change in retention time.

Often the TPs of a given PPCP are unknown and uncharacterized, such as in the case of the antibiotic sulfamethoxazole as investigated by Dodd et al.[98] Sulfamethoxazole in deionized water at 100 mg/L was reacted with 400 mg/L chlorine, and the reaction evolution was monitored by HPLC-UV-MS for the appearance of TP peaks. The identities of most of the TPs were assigned by post-column HPLC-UV collection of the major peaks by UV peak area, followed by concentration and reinjection for GC-MS analysis. One TP which could not be identified by LC-MS was subjected to ^{1}H NMR analysis. To verify that the TPs observed during chlorination reactions in deionized water might occur in the environment, Dodd spiked a much lower dose of 500 µg/L of sulfamethoxazole into both municipal drinking water and wastewater and added 1-2 mg/L of chlorine. These reactions were monitored by LC-MS, where the same TPs as previously observed were identified by targeted analysis. The use of lower parent compound concentrations and real-world water samples as a chlorination matrix strengthens the findings of this study, though the concentration of sulfamethoxazole found in wastewater rarely exceeds the low µg/L range.[99]

1.5 Methods for PPCP and halogenated TP analyses in wastewater

There are relatively few literature reports of halogenated PPCP TPs in the literature, owing mainly to the lack of analytical standards and suitable analytical methods. Even with standards, a great deal of method development must be accomplished before trustworthy data can be generated from wastewater

samples. A general workflow for the analysis of PPCPs in wastewater samples is given in Figure 1.5, combining the technologies and methods discussed in sections 1.4 and 1.5.

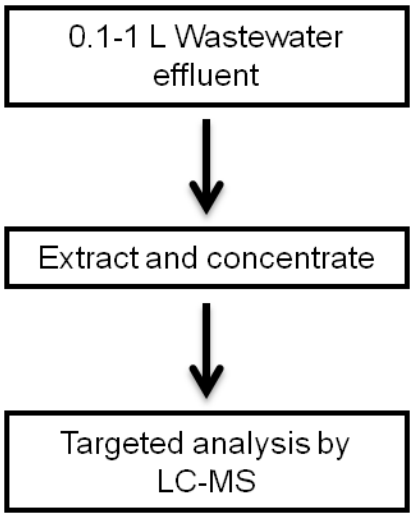


Figure 1.5. General workflow for the extraction and analysis of wastewater.

While the study of PPCP TPs is an emerging field, recently several reviews on the subject have been published. A review by Fatta-Kassinos and Kümmerer et al. highlights advances made in the study of PPCP TPs and the difficulties encountered therein, and while not solely focused on chlorine transformation the strategies for analysis of TPs are nonetheless relevant.[100] The review discusses the investigation of PPCP degradation rates, the identification and structural elucidation of TPs, and the biological potency of TPs relative to the parent compound. Importantly, the review describes the knowledge gaps which currently exist in the field and the factors which complicate progress, which include the difficulty of TP analysis in complex environmental matrices, the uncharacterized and altered physiochemical properties of TPs, the absence of a single extraction procedure for the analysis of both parent and TPs and the lack of analytical standards for most TPs. The need for coupling toxicity testing to chemical TP analyses is also emphasized, where uncertainty exists in terms of what organisms and endpoints are the most relevant for the toxicity testing of PPCPs and their TPs. A related review by Oulton et al. emphasizes similar points, identifying the dearth of research into PPCP TPs and their potential for deleterious biological effects.[101] In particular, Oulton et al. advise caution in relying too heavily upon *in vitro* toxicological data as the endpoints may not be relevant to the real-world scenario of *in vivo* toxicological effects.

An overview of prior studies involving targeted wastewater analysis of PCCP TPs produced by chlorination provides useful a backdrop for the

dissertation research presented in Chapters 2-4. Buth and coworkers investigated the occurrence of the biocide triclosan as well as several of its halogenated TPs in wastewater samples.[102] Two WWTPs employing secondary treatment, both using activated sludge followed by either UV or chlorine disinfection, were sampled at influent, pre-disinfection and final post-disinfection effluent with an appropriate time delay to ensure temporal consistency of the wastewater stream. Wastewater samples from 250-500 mL were concentrated by SPE and analyzed by UPLC-QQQ-MS, targeting the parent triclosan as well as three of its more highly chlorinated TPs. The TPs investigated were 4-Cl-triclosan, 6-Cl-triclosan and 4,6-Cl-triclosan, previously identified as potential TPs resulting from chlorination *in situ*.[103] Standards for these compounds were synthesized directly and purified by RP-HPLC. Isotope dilution using ^{13}C-labeled triclosan was used to correct extracted samples for incomplete recovery. Triclosan and its three halogenated TPs were detected in each influent sample at levels ranging from 453-4530 and 2-98 ng/L, respectively. For both WWTPs, a large decrease in the aqueous phase concentrations of each analyte was observed following activated sludge treatment, indicating effective removal by biological treatment; this result is supported by previous research demonstrating triclosan's propensity to accumulate in activated sludge.[104] After chlorination treatment the concentration of the three TPs increased by several fold to concentrations as high as 22 ng/L, concomitant with a decrease in the level of the parent triclosan. The authors attributed the formation of halogenated triclosan

TPs to the reaction of triclosan with chlorine during disinfection in the WWTP, where the total amount of halogenated TPs was approximately equal to 1/3 of the total remaining amount of the parent triclosan during the wastewater sampling period. The authors mention that the transformation of triclosan by chlorine may be a significant route for the degradation of triclosan during wastewater treatment.

One of the more robust experimental designs for *in situ* chlorination TP identification and real-world sample analysis reported to date is a study by Quintana et al.[34] A Box-Behnken experimental design methodology was used to determine the impact of the concentration of hypochlorite, bromide and pH on degradation rate for the acidic PPCPs salicylic acid, naproxen, diclofenac and indomethacine. Box-Behnken is a methodology in which greater than 2 parameters in an experiment are varied in relation to each other in a matrix, where each parameter is incremented from a minimum to maximum with at least two discrete values per parameter. In this case, the parameters were the concentration of hypochlorite (1-10 mg/L), bromide (0-100 µg/L) and the solution pH (5.7-8.3). The use of LC-QTOF-MS allowed accurate mass determination of several of the TPs of chlorination reactions with and without bromide, while the use of LC-QQQ-MS operating in MRM mode allowed the eventual analysis of real-world wastewater, tapwater and drinking water extracts for the occurrence of the observed TPs. The Box-Behnken experimental design incrementally varied chlorination reaction conditions, with the pH between 5.7-8.3, chlorine between

1-10 mg/L, bromide between 0-100 µg/L all with a 50 µg/L PPCP concentration. Aliquots of the reaction were sampled at 5 timepoints and analyzed by LC-QQQ-MS, yielding pseudo first order kinetic half-lives of 23-573 h, 13-446 min, 5-328 min and 0.4-13.4 min for salicylic acid, naproxen, diclofenac and indomethacine, respectively. The most significant factor affecting the rate of chlorination of all 4 PPCPs was the concentration of chlorine, followed secondly by the concentration of bromide (with the exception of indomethacine). The structures of the TPs were determined by accurate mass LC-QTOF-MS in addition to LC-QQQ-MS. Importantly, the MS-MS MRM transitions for the LC-QQQ-MS analyses were reported allowing rapid future method development. Salicylic acid was transformed into 4 mono- and 2 di-halogenated TPs, naproxen into chloronaproxen and bromonaproxen, diclofenac incorporated either a chlorine or a bromine as well as a decarboxylated analog of each halogenated TP while indomethacine was transformed into 4 isomeric ring oxidation products. The authors noted that the more halogenated TPs were resistant to further degradation by chlorine, degrading either negligibly once formed or resistant to degradation for several days except for one of indomethacine's TPs. A search for the identified and characterized TPs was conducted in five surface water samples, five tap water samples, a finished drinking water sample and two wastewater treatment plant samples. Between 2-500 mL of each water sample was concentrated by Oasis HLB SPE and reconstituted in 1 mL for LC-QQQ-MS-MS analysis operating in the MRM mode. Parent PPCPs were detected in

wastewater at concentrations of 300-1050 ng/L, 320-460 ng/L, 10-40 ng/L and 630-8750 ng/L for naproxen, diclofenac, indomethacine and salicylic acid, respectively. None of the parent PPCPs were detected in surface, drinking or tap water. The TPs of salicylic acid, however, were detected in all five tap water samples, the finished drinking water sample and both wastewater samples, with the dihalogenated analogs accounting for the most intense ions observed. Though the authors noted that quantitation was not possible for these TPs as authentic standards are not available, comparison to the MS response of the parent suggests they may occur in the mid-ng/L level in tap water. It was also suggested that more polluted environments be investigated for the occurrence of these TPs. A similar experimental design by the same group was used to study the degradation of the basic drugs atenolol, propranolol and salbutamol, identifying 14 TPs by accurate mass QTOF-MS.[105] The same researchers again employed the Box-Behnken methodology to examine the chlorination TPs of cocaine, where 4 non-halogenated TPs were identified by accurate mass LC-QTOF-MS to within 1 ppm mass error.[106]

1.6 Effects of halogenation on PPCPs

1.6.1 Potential impacts of PPCP halogenation: Implications for fate and transport. The addition of a chlorine or bromine atom can increase the lipophilicity of a given PPCP, which in turn can increase its ability to concentrate in biota and permeate cell membranes.[107] A study by Pothitou and coworkers

demonstrated that compounds of increasing lipophilicity preferentially adsorb to suspended particulates in wastewater.[108] Consequently, as halogenation increases a PPCP's lipophilicity it may also impart a greater tendency to sorb to particulate matter. This sorption has implications for sample preparation depending on the solids content of a wastewater, as well as impacting bioavailability for benthic species.[109] Halogenation can also affect the persistence of PPCPs in the environment.

A recent paper by González-Mariño et al. discusses the degradation of commercially available authentic halogenated PPCP standards.[110] The occurrence and biodegradability of methyl, ethyl, *n*-propyl, *n*-butyl paraben and methyl paraben's monochlorinated, dichlorinated and dibrominated analogs in wastewater and activated sludge were investigated using LC-QTOF quantitative analysis. All of the investigated compounds were detected in the wastewater samples analyzed, though >90% of each parent compound was attenuated when comparing influent and effluent concentrations prompting stability studies in activated sludge and wastewater. Though no isotopically labeled standards were available for any of the halogenated paraben analogs, deuterated analogs of methyl and *n*-propyl paraben were spiked into untreated wastewater and sludge samples as internal standards prior to SPE concentration. In activated sludge spiked at a concentration of 5 mg/L with each analyte, the non-halogenated paraben parent compounds all had half lives under 4 days, while the halogenated methyl paraben analogs had half lives of 3.3, 8.6 and 9.7 days for the

monochlorinated, dichlorinated and dibrominated species, respectively. In raw untreated wastewater at a spiked concentration of 5 µg/L, half lives ranged from 9.6-35.2 hr for the non-halogenated parabens and 28.2, 237.1 and 449.5 hr for monochlorinated, dichlorinated and dibrominated species, respectively. These results indicate that the halogenated analogs of parabens persist longer than their non-halogenated analogs in real-world scenarios. Chlorinated parabens have also been known to form in swimming pool waters as a result of chlorine disinfection in concert with the leaching of paraben compounds from human PPCP (e.g. sunscreen, cosmetics) use.[111]

The effect of halogenation on the biodegradability of nonylphenol, an xenoestrogenic and environmentally persistent alkylphenol, was studied using a bacterium which is extremely specific towards the degradation of nonylphenol. Li and coworkers investigated the metabolic transformations of halogenated nonylphenols by the bacteria *Sphingobium xenophagum* Bayram, which can survive and grow on nonylphenol as the sole carbon source.[112] Technical nonylphenol was reacted with bromine to produce reaction mixtures that contained 45% monobrominated, 40% dibrominated, and 15% unreacted nonylphenol. Reaction with and NaOCl produced 24% monochlorinated and 76% unreacted nonylphenol. The amounts of halogenated nonylphenol in each mixture were semiquantitatively determined using single quadrupole GC-MS and comparing extracted ion chromatogram peak areas to the response of chrysene-d_{12} as an internal standard. Liquid cultures of the bacteria were exposed to initial

concentrations of 10-40 mg/L of the parent nonylphenol and 1-20 mg/L of the chlorinated and brominated nonylphenol mixtures, with abiotic controls for comparison. After 6 days incubation, the 99% of the sample containing only nonylphenol had been degraded while the chlorinated mixture showed a degradation of 92% for nonylphenol and 68% for chlorinated nonylphenol. After 23 days the brominated nonylphenols in their mixture were degraded by approximately 50%, however it was noted that most of their degradation occurred in the first several days along with the degradation of the parent nonylphenol. It was thus hypothesized, and confirmed by a re-spiking of the brominated mixture into the culture, that brominated nonylphenols are cometabolized along with the parent. The degradation products were nonyl alcohols, which were identified by GC-MS. In both cases the presence of either chlorinated or brominated nonylphenols retarded the degradation of the parent nonylphenol. The test solutions of nonylphenol and halogenated nonylphenols were then exposed to nonylphenol-degrading soil cultures collected from the Mesa, AZ, USA soil aquifer treatment facility along with abiotic controls. As in the liquid cultures, the degradation of the parent nonylphenol was inhibited in the presence of chlorinated and brominated mixtures. In the case of the chlorinated mixture, degradation in the biotic and abiotic samples was equivalent indicating no bacterial degradation. For the brominated mixture, as with the liquid cultures, the degradation of brominated nonylphenols tapered off after a few days during which most of the parent in the mixture was also degraded. These results have

implications for the environmental fate and ecotoxicity of halogenated TPs, as their enhanced resistance to microbial degradation and possible refractory behavior may prolong their environmental lifetimes.

1.6.2 Biological effects of halogenated PPCP TPs. The main concern regarding the existence and persistence of PPCPs in the aquatic environment is the ability of some PPCPs to disrupt normal endocrine function at relatively low, environmentally-relevant concentrations that can have deleterious effects on natural populations of aquatic organisms.[113] A popular *in vitro* method of characterizing endocrine disruption is to measure the binding affinity of a given compound to the estrogen receptor (ER), a key nuclear hormone receptor involved in the regulation of sex hormones. High ER affinity suggests potential impacts on behavior and reproduction. One of the most studied and potent xenoestrogens (a compound that is able to bind the ER and elicit estrogenic action) *in vivo* and *in vitro* is nonylphenol.[114, 115] The commonly used nonylphenol is a technical mixture of approximately 20 para-substituted isomers (*p*-nonylphenol), which differ in branching of the alkyl chain. Cormio et al. tested four nonylphenol isomers (4*n*-, *p*353-, *p*33-, *p*363- nonylphenol) and their mono- and di-chlorinated derivatives (generated during the chlorination process in water treatment[116]) for their estrogenic and androgenic potency using yeast estrogenic and androgenic assays.[117] The estrogenic potency of the four nonylphenol isomers decreased in the following order: *p*353-nonylphenol > 4*n*-nonylphenol >

*p*33-nonylphenol > *p*363-nonylphenol. The mono-chlorination of the phenolic ring resulted in a decrease in the estrogenic potencies of three isomers; only the monochlorinated p363-nonylphenol was more estrogenic than the parent compound. All dichlorinated *p*-nonylphenol compounds displayed a decrease in the estrogenic potency. In the anti-androgenic screen, all substances exhibited a positive response; the mono- and di-chlorinated derivatives exhibited lower potency than the parent isomers. The isomer *p*363-nonylphenol and its corresponding mono- and di-chlorinated derivatives were almost inactive in the anti-androgenic assay. Furthermore, all compounds were tested for anti-estrogenic and androgenic assays, but none of them showed a positive response. These results indicate that in assessing the xenoestrogenic potency of chlorinated derivatives of nonylphenol, the use of pure compounds is essential because the mixtures may not be representative of a compound's full xenoestrogenic potency. In fact the concentrations of nonylphenol isomers differ in technical mixtures based on the producers and after chlorination different technical mixtures can generate dissimilar ratios of chlorinated derivatives. Finally, the chlorinated derivatives of nonylphenol didn't show an increase in xenoestrogenic potency compared to the parent isomers, and for this reason the many oxidized TPs generated during the chlorination process can mask the xenoestrogenic potency of the pure chlorinated isomers of nonylphenol.

Nakamura et al. investigated halogenated derivatives of estrogens, which could be produced by chlorine treatment at a sewage treatment plant.[118] The

chlorinated derivatives of estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethynylestradiol (EE2) were produced by the reaction with hypochlorous acid in organic solvents. Brominated derivatives were also formed when bromide ions were present during chlorination. The estrogenic activities of the halogenated derivatives were measured by yeast two-hybrid assays incorporating the human estrogen receptor α (hERα) or medaka fish (*Oryzias latipes*) estrogen receptor α (medERα). Although the activities of 4-chloroestrone (4-C1E1) and 10-chloro-1,4-estradiene-3,17-dione were similar to those of E1, the activities of 2-C1E1 and 2,4-dichloroestrone (2,4-diC1E1) were approximately 80% and 2% that of E1, respectively, in an agonist assay for hERα. No activity was detected for 2,4,16,16-tetrachloroestrone (2,4,16,16-tetraC1E1). The estrogenicity of chlorinated derivatives of E2, E3, and EE2 showed a similar tendency to that of E1. The brominated derivatives showed slightly weaker activity than the corresponding chlorinated derivatives. However, many estrogens halogenated at the 2 and 4 positions still had activity that was approximately 10^3-10^4 times stronger than that of bisphenol A.[118] The transformation of steroid hormones into halogenated analogs and the resultant reduction in *in vitro* estrogenic activity was recently hailed as a positive side-effect of using chlorine for water disinfection, as reported in a recent Science article by Sedlak and von Gunten, though no consideration was given to the potential for increased bioaccumulation given the higher lipophilicity of halogenated steroid TPs.[119]

The importance of selecting a relevant biological endpoint when evaluating the potential toxicity of halogenated TPs is discussed in a recent study investigating the estrogenicity as well as the peroxisome proliferator-activated receptor (PPAR) activity of the plasticizer bisphenol A (BPA) and its tetrachlorinated (TCBPA) and tetrabrominated (TBBPA) analogs [120]. The stably transfected reporter cell line HGELN was used to assess the activity of each compound against human estrogen receptors α and β (ERα and ERβ), PPARα, PPARδ and PPARγ. Results indicated that binding to both ERα and ERβ decreased along the series BPA > TCBPA > TBBPA, confirming previous *in vitro* research suggesting that the bulkier chloro- and bromo-BPA analogs decrease, but do not completely eliminate, ER binding affinity.[121, 122] Competitive binding assays against the selective PPAR agonist rosiglitazone at concentrations ranging from 10^{-5}-10^{-9} M were employed to determine the affinity of each compound for the PPARα, PPARδ and PPARγ receptors in human, *Xenopus* and zebrafish (*Danio rerio*). No activity was observed with either PPARα or PPARδ. For PPARγ, both TCBPA and TBBPA showed approximately 1% of the agonist activity of rosiglitazone, while the parent BPA showed no activity. The authors noted that while bulky halogen substitution on BPA's phenol rings decreased the *in vitro* estrogenic activity, the ability to activate PPARγ increased with the size of the substituent with IC_{50} values of 12.0 nM, 0.7 μM and 6.0 μM for rosiglitazone, TBBPA and TCBPA, respectively. The effect of 10 μM TCBPA, TBBPA and the known PPARγ agonist mono-2-ethylhexyl phthalate on the PPARγ function of

adipogenesis using NIH3T3-L1 cells (pre-adipocytes) was also investigated, where partial agonism was observed when compared to rosiglitazone. X-ray diffraction studies to determine the crystal structure of PPARγ incubated with rosiglitazone, TCBPA and TBBPA revealed nearly identical ligand density maps indicating a similar mode of action for these compounds. The enhanced PPARγ activation afforded by halogenation may have implications for the relevancy of endpoints when assessing the potential toxicity of halogenated TPs.

1.7 Summary

The presence of PPCPs in wastewater in concert with the use of chlorine as an oxidative disinfection step can create halogenated TPs. Most PPCP TPs have yet to be identified, and thus the means for their analysis and quantification in wastewater samples is precluded by their identification and the creation of analytical standards for method development. The addition of a chlorine or bromine atom to a PPCP can affect the physiochemical properties of the resultant TPs, retarding environmental degradation and affecting their potency as an endocrine disruptor. In Chapter 2, the effects of halogenation on sample preparation and analysis techniques as well as *in vivo* endocrine disruption potential will be investigated for the pharmaceutical gemfibrozil. Two halogenated TPs of gemfibrozil are targeted for analysis in primary wastewater effluent by LLE, HPLC-UV and UPLC-QTOF-MS. Chapter 3 expands the scope of work through a targeted wastewater analysis for a wider subset of PPCP TPs,

focusing on SPE and HPLC-UV purification method development, UPLC-QTOF-MS quantitation and measurement of relative lipophilicity using a chromatographic approach. Chapter 4 presents the synthesis, characterization and use of authentic standards, including isotopically labeled analogs, for an even larger number of TPs. In addition, the sensitivity of LC-QQQ-MS allows the more accurate quantification of the investigated analytes in a variety of wastewater samples collected in Southern California.

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Chapter Two

Analytical and biological characterization of halogenated gemfibrozil produced through chlorination of wastewater

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Abstract

The cholesterol-lowering pharmaceutical gemfibrozil is a relevant environmental contaminant because of its frequency of detection in US wastewaters at concentrations that have been shown to disrupt endocrine function in aquatic species. The treatment of gemfibrozil solutions with sodium hypochlorite yielded a 4'-chlorinated gemfibrozil analog (chlorogemfibrozil). In the presence of bromide ion, as is often encountered in municipal wastewater, hypobromous acid generated through a halogen exchange reaction produced an additional 4'-brominated gemfibrozil product (bromogemfibrozil). Standards of chloro- and bromogemfibrozil were synthesized, isolated and characterized using mass spectrometry and NMR spectroscopy. Mass spectrometry was used to follow the *in situ* halogenation reaction of gemfibrozil in deionized water and wastewater matrices, and to measure levels of gemfibrozil (254±20 ng/L), chlorogemfibrozil (166±121 ng/L), and bromogemfibrozil (50±11 ng/L) in advanced primary wastewater treatment effluent treated by chlorination. Chlorogemfibrozil demonstrated a significant ($p < 0.05$) reduction in the levels of 11-ketotestosterone at 55.1 µg/L and bromogemfibrozil demonstrated a significant ($p < 0.05$) reduction in the levels of testosterone at 58.8 µg/L *in vivo* in Japanese medaka in a 21 day exposure. These results indicated that aqueous exposure to halogenated degradates of gemfibrozil enhanced the anti-androgenicity of the parent compound in a model fish species, demonstrating

that chlorination may increase the toxicity of pharmaceutically active compounds in surface water.

2.1 Introduction

Gemfibrozil is a widely prescribed cholesterol-lowering drug and a member of the fibrate class of pharmaceuticals which is frequently detected in US waters at concentrations up to 63.8 μg/L.[1, 2] In the United States in 2009, gemfibrozil was prescribed over 500,000 times.[3] Gemfibrozil has been detected in the livers of wild-caught benthic-feeding fish at concentrations up to 90 ng/g in common carp (*Cyprinus carpio*) and 27.3 ng/g in White sucker (*Catostomus commersonii*),[4] and it has been shown to reduce plasma androgens in goldfish (*Carassius auratus*) following exposure to 1.5 μg/L for 4 and 14 days.[5] Given gemfibrozil's potency in aquatic organisms and the potential of enhanced bioavailability due to halogenation, the goals of this study were to characterize its halogenated products (Figure 2.1) formed under conditions mimicking wastewater treatment, determine the concentrations of post-chlorination byproducts in municipal wastewater effluent, and evaluate the endocrine disruption potential of the byproducts in relation to the parent gemfibrozil in the model aquatic organism Japanese medaka.

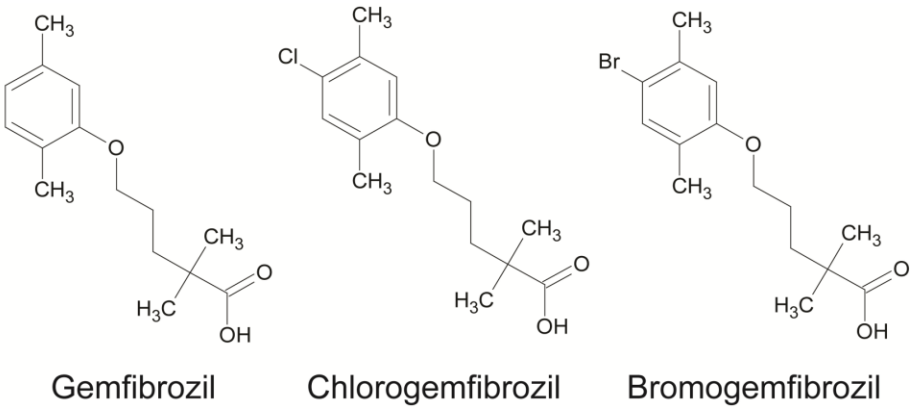

Figure 2.1. Structures of gemfibrozil, chlorogemfibrozil and bromogemfibrozil.

2.2 Materials and methods

2.2.1 Chemicals and suppliers. Sodium azide, sodium formate, nitric acid, potassium bromide, leucine enkephalin, formic acid, acetic acid, diethyl ether, bisphenol A-d_{16} and sodium thiosulfate were obtained from Sigma Aldrich (St. Louis, MO). Gemfibrozil (USP grade) was obtained from Spectrum Chemical Manufacturing Corporation (New Brunswick, NJ). Methanol, hexane, dimethyl sulfoxide, tricaine methanesulfonate, and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA), all of LC-MS purity. HPLC-grade Burdick and Jackson water was used for HPLC and LC-MS analyses (Morristown, NJ). Deionized water was obtained from a Millipore water purification system (Billerica, MA). A sodium hypochlorite solution from Acros (Geel, Belgium) containing 13% active chlorine was used for chlorination reactions. Diclofenac was obtained from Cayman Chemical Company (Ann Arbor, MI).

2.2.2. Wastewater samples. Two separate wastewater grab samples were collected locally in Southern California for this study. Both samples were collected in glass carboys which had been thoroughly cleaned with 5% nitric acid in deionized water. Samples were immediately transported to a 4 °C cold room for storage. The first sample, Wastewater A, was a blend of primary and secondary final effluents collected post-chlorination. The second sample, Wastewater B, was collected at an advanced primary wastewater treatment plant as final effluent post-chlorination. Approximately 1 g sodium azide was added per

liter of wastewater to inhibit bacterial growth. The first sample, Wastewater A, was a blend of primary and secondary final effluents collected post-chlorination. Several physical parameters were measured for this sample. The initial pH of 7 was found to be invariant over several months of storage. Residual ammonia levels several days after sampling were ca. 4 mg/L as measured by a colorimetric test kit from Aquatic Ecosystems (Apopka, FL). Bromide concentration was measured at 41.6 ± 4.2 µg/L by anion chromatography using a method previously described.[6] The second sample, Wastewater B, was collected at an advanced primary wastewater treatment plant as final effluent post-chlorination. The initial pH of the wastewater was measured at 7.5. Total suspended solids content was measured at 22.5 ± 1.7 mg/L by the Environmental Sciences Section (ESS) method 340.2.[7]

2.2.3. Chlorination reactions. *In situ* chlorination reactions were performed using either Millipore deionized water or blended primary/secondary final effluent from Wastewater A. A 13% active chlorine solution was used for the introduction of sodium hypochlorite. The activity of the hypochlorite solution was standardized weekly by iodometric titration.[8] Gemfibrozil was added to stirred deionized water or wastewater effluent solutions from a stock solution in methanol to effect a 100 µg/L concentration, with methanol accounting for no more than 0.1% of the overall reaction volume. At various time points, 500 µL of the reaction medium was sampled to determine product formation using a Waters (Milford, MA) Acquity ultra performance liquid chromatograph with a quadrupole time-of-flight mass spectrometric (UPLC-QTOF-MS) detection. To

quench the chlorination reaction, the 500 µL aliquot of the reaction medium was sampled into borosilicate Type I glass HPLC sample vials (MicroSolv, NJ) containing a molar excess (relative to the initial free chlorine concentration) of a ~1 M solution of sodium thiosulfate. An aliquot of 500 µL of methanol was added to each vial with mixing accomplished by a vortex mixer.

2.2.4 Preparation, isolation and structural characterization of halogenated gemfibrozil standards. Standards of chloro- and bromogemfibrozil were used for the extraction recovery experiments, confirmation of the chromatographic retention times and mass spectra measured in wastewater, and for the evaluation of *in vivo* biological activity. The standards were prepared by chlorination of gemfibrozil in deionized water and methanol (4:1 v/v) with and without bromide, and isolated in milligram quantities using an Agilent 1100 HPLC with UV detection at 254 nm. Separations were performed on a 4.6x150 mm Inertsil ODS-2 column from MetaChem (Torrance, CA) with mobile phase A: water + 0.1% formic acid and B: acetonitrile. A 15 min isocratic separation at 65% B and a flow rate of 2.3 mL/min was used for the isolation of chlorinated and brominated gemfibrozil products. Fractions containing the desired product were collected manually, pooled and evaporated to dryness with a Savant SC110 SpeedVac from Thermo Scientific (Asheville, NC).

The identities of the chloro- and bromogemfibrozil analogs were determined from the accurate mass m/z and isotopic ratios as shown in Table 3.1, and confirmed by elemental analysis (Table 2.2). Mass spectrometry (MS)

measurements were made by QTOF-MS as described below. Elemental analysis of the chlorine and bromine content of the isolated analogs was performed by Midwest Microlab, LLC (Indianapolis, IN), with the results summarized in Table 2.2. Purities of the chloro- and bromogemfibrozil standards were determined to be > 99% by ^{1}H NMR spectroscopy.

Table 2.1. Comparison of MS results and isotopic patterns with the expected values.

Compound	Isotopic Peak	Exp. Mass (Da)	Calc. Mass (Da)	Error (ppm)	Rel. Intensity (%)	Calc. Rel. Intensity (%)[a]
Gemfibrozil	M	249.1489	249.1490	0.40	100	100
	M+1	250.1524	250.1524	0	17	16
	M+2	251.1532	251.1533	0.40	3	2
Chlorogemfibrozil	M	283.1103	283.1101	-0.71	100	100
	M+1	284.1143	284.1134	-3.17	17	16
	M+2	285.1081	285.1071	-3.51	36	34
	M+3	286.1105	286.1105	-1.75	6	5
Bromogemfibrozil	M	327.0594	327.0596	0.61	100	100
	M+1	328.0614	328.0629	-3.66	17	16
	M+2	329.0584	329.0575	-2.74	101	99
	M+3	330.0633	330.0609	-7.27	17	16

a. Calculated using http://www.sisweb.com/mstools/isotope.htm

Table 2.2. Elemental analysis results for the halogenated gemfibrozil standards.

Compound	Expected % Halogen (w/w)	Actual % Halogen (w/w)	% Error
Chlorogemfibrozil	12.45	13.13	0.68
Bromogemfibrozil	24.39	23.95	0.44

2.2.5 Structure confirmation by NMR. The structures of the isolated chloro- and bromogemfibrozil standards were confirmed through one- and two-dimensional NMR experiments using a Bruker Avance NMR spectrometer operating at 599.84 MHz for ^{1}H. Samples were prepared in 600 µL $CDCl_3$ at a concentration of approximately 1 mM. Figure 2.2 shows the ^{1}H survey spectra of gemfibrozil (A), chloro- (B) and bromogemfibrozil (C). Resonance assignments for gemfibrozil and chlorogemfibrozil are in agreement those published by Krkošek et al.[4] The ^{1}H nuclear Overhauser effect spectroscopy (NOESY) spectrum (Figure 2.3) was used to confirm *para* halogenation for chlorogemfibrozil through the observation of a single cross peak between each of the two aromatic proton resonances and the protons on the aromatic methyl groups. The NOESY spectrum was acquired using Bruker's *noesyph* pulse program using States-TPPI, with 4096 data points acquired into 320 t_1 increments with 8 scans per increment. The spectral window in both dimensions was 6,614 Hz. A 90° pulse length of 7.50 µs at -5.00 dB attenuation was used, with a mixing time of 500 ms and a relaxation delay of 2.0 s. Comparison of the coupling patterns for the aromatic resonances of chloro- and bromogemfibrozil confirmed *para* bromination (Figure 2.2).

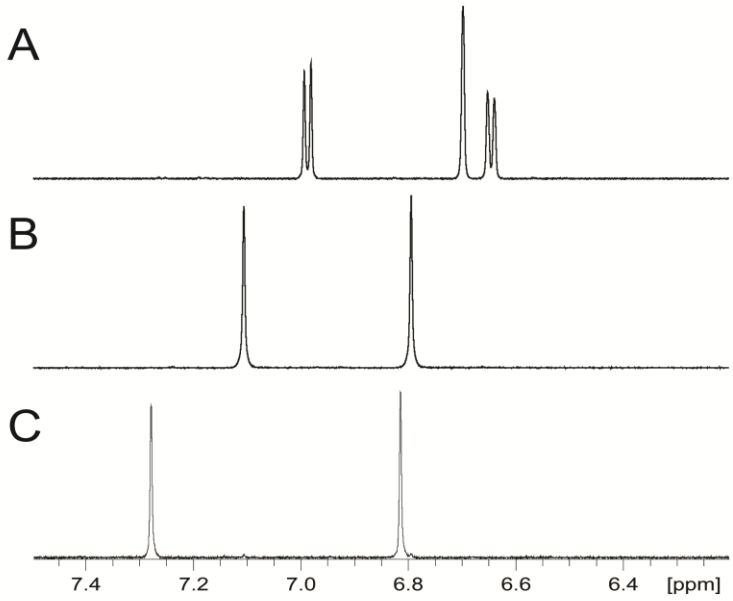

Figure 2.2. The aromatic region of the ^{1}H NMR spectra measured for $CDCl_3$ solutions of (A) gemfibrozil, (B) chlorogemfibrozil and (C) bromogemfibrozil.

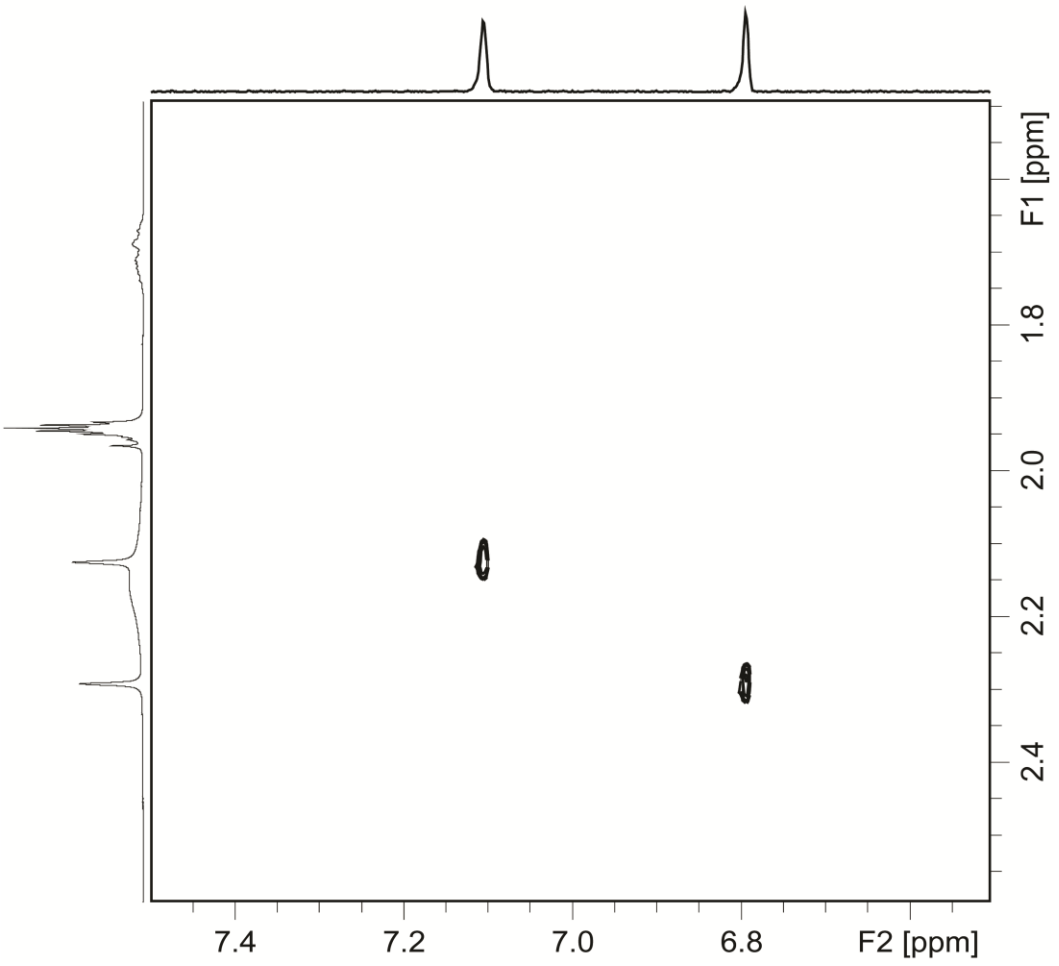

Figure 2.3. NOESY spectrum of chlorogemfibrozil. This expansion shows the NOE cross peaks observed between the aromatic resonances (F2) and the methyl resonances (F1) confirming *para*-chloro substitution.

2.2.6 Extraction of wastewater samples. Extraction recoveries were evaluated for solid phase extraction (SPE) and liquid-liquid extraction (LLE) sample treatments. Each analytical replicate was spiked with 10 µg of gemfibrozil, chlorogemfibrozil, and bromogemfibrozil standards and stirred with a Teflon stir bar on a magnetic stir plate for approximately one hr.[9] The spiked amount was chosen so that any compound already present in the wastewater samples would be negligible by comparison. The samples were extracted by either SPE or LLE as described below. After elution, samples were dried under a gentle stream of air, subject to offline HPLC fractionation, and reconstituted for UPLC-QTOF-MS analysis as described in the following section.

2.2.6.1 Solid phase extraction. Our initial attempts at wastewater analysis used SPE with 200 mg Waters Oasis HLB cartridges with minor modification of the method of Gómez et al.[10] Prior to SPE, 6 samples of 500 mL of Wastewater B were acidified to pH 3 with 5 M HCl and filtered through a 0.22 µm nylon membrane filter (Millipore, MA) to remove suspended particles. After loading the sample and rinsing with 5% methanol in deionized water, the cartridges were dried for 15 min using air flow generated by the house vacuum and eluted with 6 mL methanol. Eluates were blown to dryness by a gentle steam of air and reconstituted in 50% acetonitrile/water for offline HPLC enrichment and UPLC-QTOF-MS analysis as described below. The recoveries measured using the SPE method were: gemfibrozil (46 ± 14%), chlorogemfibrozil (7.6 ± 5%) and

bromogemfibrozil (1 ± 7%). The recoveries for the halogenated analogs were too low to be analytically useful prompting us to explore liquid-liquid extraction as an alternative sample preparation strategy.

2.2.6.2 Liquid-liquid extraction. We hypothesized that the poor SPE recoveries of chloro- and bromogemfibrozil from our spiked samples were due to binding to particulate matter which was removed by filtration prior to SPE. Therefore, we tested LLE as an alternative sample preparation method. Samples of 6 x 500 mL of Wastewater B were adjusted to pH 3 with 5 M HCl, placed in a separatory funnel without filtration and extracted twice with 100 mL aliquots of hexane with at least 5 min of vigorous shaking for each hexane aliquot. The hexane extracts were pooled along with the emulsion formed during the extraction and centrifuged at 300 g for 10 min to break the emulsion in media bottles using a Beckman Coulter J2-21M centrifuge (Brea, CA). Solids present in the samples were centrifuged to the bottom of the bottle, and remained trapped under the water layer. The hexane layer was carefully removed with a serological pipette, and rotovapped to dryness. Samples were reconstituted in 50% acetonitrile/water for offline HPLC enrichment and UPLC-QTOF-MS analysis as described below. The recoveries determined using the LLE method were: gemfibrozil (76 ± 7%), chlorogemfibrozil (38 ± 3%) and bromogemfibrozil (25 ± 4%). Although the recoveries of the more hydrophobic chloro- and bromogemfibrozil analogs are still low, they are sufficient to allow an assessment

of their levels in wastewater effluent. Method detection limits (MDL) were determined by linear regression of the signal-to-noise ratio (S/N) from a standard curve used for quantitation of gemfibrozil and its halogenated analogs. A S/N of 3 was chosen for the MDL, yielding recovery-corrected values of 0.4 ng/L, 1.1 ng/L, and 2.2 ng/L for gemfibrozil, chlorogemfibrozil, and bromogemfibrozil, respectively. The method quantification limits were taken as a S/N of 10, yielding values of 3.3 ng/L, 10.9 ng/L, and 21.8 ng/L for gemfibrozil, chlorogemfibrozil, and bromogemfibrozil, respectively.

2.2.7 UPLC-QTOF-MS experimental parameters. All UPLC separations were performed using an Acquity UPLC with an Acquity 2.1x150 mm BEH C18 column and a VanGuard 2.1x5 mm guard column (Waters, Milford, MA) at 50 °C. For *in situ* chlorination reaction monitoring (Figure 2.5), a mobile phase of A: water + 0.01% formic acid and B: acetonitrile with a flow rate of 0.5 mL/min was used. For the analysis of wastewater samples (Figure 2.3), acetic acid was substituted for formic acid after ionization optimization experiments indicated that it improved analyte ionization. The gradient for these separations was: 35% B 0-0.5 min, 35-50% B 0.5-2 min, 50-90% B 2-6 min, 100% B 6-8 min, 100-35% B 8-8.5 min, and 35% B 8.5-10 min.

A Waters Micromass QTOF mass analyzer equipped with an electrospray ionization (ESI) source operated in negative ion mode was used for the acquisition of all mass spectrometric data. Mass spectrometer operating

parameters were as follows: capillary voltage 3000 V; source temperature 120 °C, desolvation temperature 200 °C; desolvation gas flow rate 650 L/h; scan time 1 s; m/z scan range of 5-800 Da. For MS-MS experiments, a collision voltage of 12 eV was used. Accurate mass experiments used the lock-spray mode, with 5 ng/µL diclofenac as the lock mass ($[M-H]^-$ = 294.0089 Da) fed into the reference source by syringe pump at 50 µL/min and sampled at a frequency of 0.1 Hz. Accurate mass spectra were acquired with a 5 ng/µL solution of gemfibrozil, chloro- and bromogemfibrozil in 50% acetonitrile in water, analyzed by direct infusion fed by a syringe pump at 10 µL/min. Spectra were collected for 20 min and 420 scans were averaged. The mass spectrometer was calibrated prior to measurements at the start of each day using sodium formate. Calibrations of both the quadrupole and time-of-flight sectors of the spectrometer were under 1 ppm error.

2.2.8 Quantification of gemfibrozil, chlorogemfibrozil and bromogemfibrozil in spiked recovery samples and in advanced primary effluent.

Offline HPLC fractionation of wastewater samples, including the spiked samples used to determine method recovery values was carried out using an Agilent 1100 HPLC with UV detection at 254 nm. Separations were performed on a 4.6x150 mm Inertsil ODS-2 column from MetaChem (Torrance, CA) using a flow rate of 2.1 mL/min with the following gradient timetable: 0-3 min 55% B; 3-5 min 55-65% B; 5-10 min 65-100% B; 10-14 min 100% B; 14-15 min 100-55% B; 15-20 min

55% B. Standards of gemfibrozil and its halogenated analogs were analyzed by this method to establish retention times for each analyte. Fraction collection windows of approximately 45 s were set up around the elution time of each standard. Fractions from 2-5 injections were pooled and dried under a gentle stream of air, then reconstituted in hexane and transferred to a small centrifuge tube. The enriched samples were dried in the centrifuge tubes by speed vacuum, and reconstituted in 21 µL of 50% acetonitrile in water assisted by sonication. Bisphenol A-d_{16} was added as an internal standard prior to UPLC-QTOF-MS analysis.[11]

For UPLC-QTOF-MS analyses, separate calibration curves were created for gemfibrozil, chloro- and bromogemfibrozil over the range 0-200 ng injected analyte. All standards and samples were analyzed using a 20 µL injection volume. The calibration plots were forced through zero as no signal was detected in measurements for a reagent blank consisting of 500 mL of deionized water subjected to LLE and offline enrichment in parallel with wastewater samples. Extracted ion chromatogram peak areas were used to construct calibration curves. For analysis of gemfibrozil and chlorogemfibrozil a mass window of 0.1 Da was used, while extracted ion chromatograms of bromogemfibrozil used a mass window of 0.2 Da to account for its greater peak width. Chloro- and bromogemfibrozil samples were analyzed using linear calibration plots constructed for each compound spanning the range 0-75 ng injected (9 points). The equations for each calibration plot were determined by linear regression and

are reported with their respective correlation coefficient, chlorogemfibrozil: y = 0.8208x, R^2 = 0.998; bromogemfibrozil: y = 1.0777x, R^2 = 0.9956. The gemfibrozil levels exceeded the linear range and were determined using a calibration plot constructed from the standards in the range 50 – 200 ng (5 points) and fit using a second order polynomial function to account for detector saturation effects at higher concentrations. The equation and correlation coefficient obtained for the second order gemfibrozil calibration plot are y = -0.0012x^2 + 1.5861x + 1.5861, R^2 = 0.991.

MS^2 transitions were used along with retention time windows to confirm compound identification in Wastewater B as shown in Figure 2.4. Ten s retention time windows were used for MS^2 measurements bracketing the retention times of the standards of each compound. A collision energy of 12 eV was used, with the most abundant ions of gemfibrozil (249.2 m/z), chlorogemfibrozil (284.2 m/z) and bromogemfibrozil (329.2 m/z) selected as MS^1. The m/z 127 peak observed in each spectrum is the result of a non-qualifying transition corresponding to the loss of the aromatic moetiety of the parent, generating 4-carboxy-4-methylpentan-1-olate.

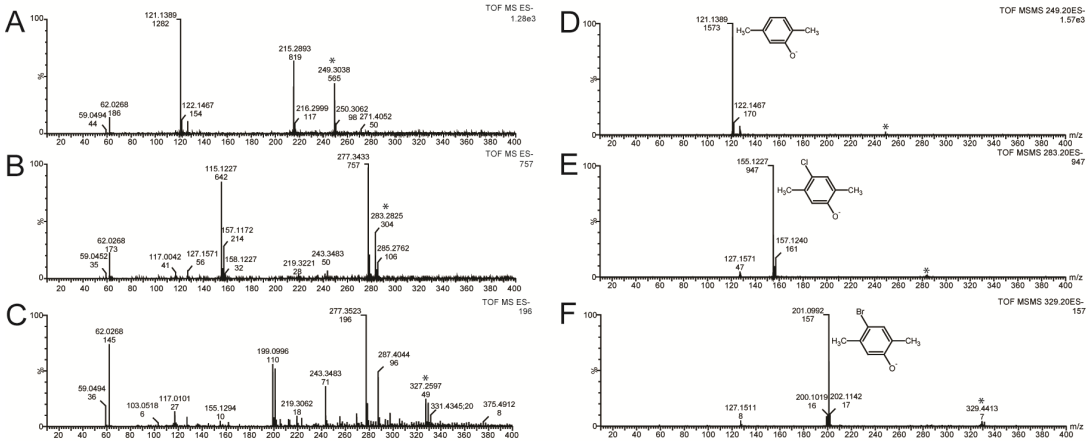

Figure 2.4. Full spectra of the XIC peaks (A-C) and MS^2 qualifying transitions and structures (D-F) corresponding to the base peak of gemfibrozil (A and D), chlorogemfibrozil (B and E) and bromogemfibrozil (C and F) as measured in Wastewater B. The parent ions are marked by *.

2.2.9. Exposure to Japanese medaka (*Oryzias latipes*) and steroid quantification. Sexually mature male Japanese medaka, approximately 6 months of age, were exposed in four replicates of two fish each. Pre-aliquotted compound that had been dried by SpeedVac in microcentrifuge tubes was reconstituted in ethanol after each water change and immediately exposed to the treatment vessels, where the solvent vehicle accounted for less than 0.005% v/v. The nominal concentrations for the waterborne exposures were: gemfibrozil at 0.5 (low), 5 (med), and 50 (high) µg/L, chlorogemfibrozil at 0.57, 5.7, and 57 µg/L, and bromogemfibrozil at 0.65, 6.5, and 65 µg/L. The concentrations of chloro- and bromogemfibrozil were selected to be equimolar with gemfibrozil's respective concentrations. A full water change was completed every other day for the duration of the exposure. Concentrations measured in selected samples (n=8) following water exchange were: gemfibrozil at 0.4±0.05, 3.7±0.4, and 43.7±4.4 µg/L, chlorogemfibrozil at 0.5±0.09, 4.5±0.4, and 55.1±5.6 µg/L, and bromogemfibrozil at 0.5±0.05, 4.4±0.5, and 58.8±3.9 µg/L. Ethanol was used as the negative control and solvent vehicle.

The 21 d exposure took place in an incubator held at 28 °C with a 14/10 day/night light cycle. Fish were fed brine shrimp once daily during the exposure. At 21 d, fish were euthanized with tricaine methanesulfonate, the wet weight recorded, and the fish were immediately placed on dry ice for several seconds. Steroid hormones were extracted from whole fish homogenate as previously described with modifications.[12] Steroid hormone levels were corrected for wet

weight and normalized to the response of the negative control. Due to temporal availability of sexually mature males of the same age, the exposures for bromogemfibrozil were performed separately from the other two compounds. Statistical significance ($p < 0.05$) was determined by the Kruskal-Wallis test with Dunn's Multiple Comparison between each treatment and the negative control using GraphPad Prism (La Jolla, CA).

2.2.10. Steroid quantification for 21 d *in vivo* exposure. At 21 d, fish were killed 2 fish (one beaker) at a time with tricaine methanesulfonate and the wet weight recorded, then immediately placed on dry ice for several seconds. Steroid hormones were extracted from whole fish homogenate as in Brion et al., with the following changes: After homogenization in phosphate buffer, homogenate was immediately transferred to a centrifuge tube and 3 mL of diethyl ether was added for the extraction of free steroids.[13] The centrifuge tube was immediately vortexed for 1 min and centrifuged for 10 min at 10,000 rpm using a Beckman JA-17 rotor at 4 °C. The organic layer was removed into a scintillation vial and the extraction repeated. The combined organic layers were blown to near dryness with a gentle stream of nitrogen and frozen at ˉ20 °C until steroid analysis. The steroid hormones testosterone and 11-ketotestosterone were measured by enzyme immunosorbent assay (EIA) from Cayman Chemical (Ann Arbor, MI). Samples were reconstituted in 500 μL of EIA buffer and sonicated for 30 minutes prior to analysis. All samples were analyzed in triplicate, and

quantified using the EIA kit's included steroid standards yielding linear logit-transformed standard curves with R^2 values of 0.9926 and 0.9828 for testosterone and 11-ketotestosterone, respectively.

2.2.11. Experimental parameters for concentration determinations of gemfibrozil, chlorogemfibrozil and bromogemfibrozil for the *in vivo* exposures.

Aliquots of 50 mL of the exposure water were set aside during water changes to evaluate the actual analyte concentrations during the 21 d *in vivo* exposure experiments. Water samples were collected once between days 2 and 6 and once between days 16 and 20, with a total n of 8 samples extracted for each exposure concentration. Samples were acidified to pH< 3 by the addition of 200 μL of 5 M HCl and subjected to SPE using Waters (Milford, MA) Sep-Pak Plus C18 cartridges. SPE could be used for these analyses due to the lower particulate matter content compared to wastewater. Cartridges were conditioned with 10 mL methanol then equilibrated with 10 mL deionized water. Samples were loaded at 5 mL/min, followed by a wash with 10 mL of 5% methanol in deionized water. Cartridges were dried for 15 min prior to elution with 2 x 5 mL methanol. The eluent was blown to dryness with a gentle stream of air and the dried samples reconstituted for analysis by UPLC-QTOF-MS as described in section 2.2.7. Recovery for each analyte was determined in quadruplicate at a spiked level of 5, 5.7, and 6.5 μg/L for gemfibrozil, chlorogemfibrozil, and bromogemfibrozil, respectively (the nominal medium exposure concentrations)

into the water used for the *in vivo* exposures (dechlorinated tap water filtered through granular activated carbon). Recovery for this method was determined to be 73±2, 70±5, and 76±3 % for gemfibrozil, chlorogemfibrozil, and bromogemfibrozil, respectively.

2.3 Results and Discussion

2.3.1. Characterization of the reaction products of *in situ* chlorination in deionized water and wastewater effluent. This study focused on gemfibrozil and its main halogenated transformation byproducts. While previous research has focused on the ability of gemfibrozil to be transformed by chlorine,[9, 14] herein this chemistry is extended to the analysis of environmental samples for the detection and quantification of levels of halogenated gemfibrozil in wastewater effluent. Selected total ion chromatograms (TICs) measured for the time-course of the reaction of gemfibrozil with hypochlorite in deionized water over a period of 60 min are presented in Figure 2.5A. A monochlorinated gemfibrozil byproduct was observed one minute after hypochlorite addition; both the m/z and the isotopic ratios indicated the addition of a single chlorine atom. This identification was confirmed by QTOF-MS and elemental analysis.[15] The absolute structure was characterized using NMR spectroscopy as described in section 2.2.5 and assigned as 4'-chlorogemfibrozil. A small amount of *o*-chlorogemfibrozil was

detected after 60 min, but this compound was not observed in reactions conducted at environmentally relevant gemfibrozil concentrations (5 μg/L).

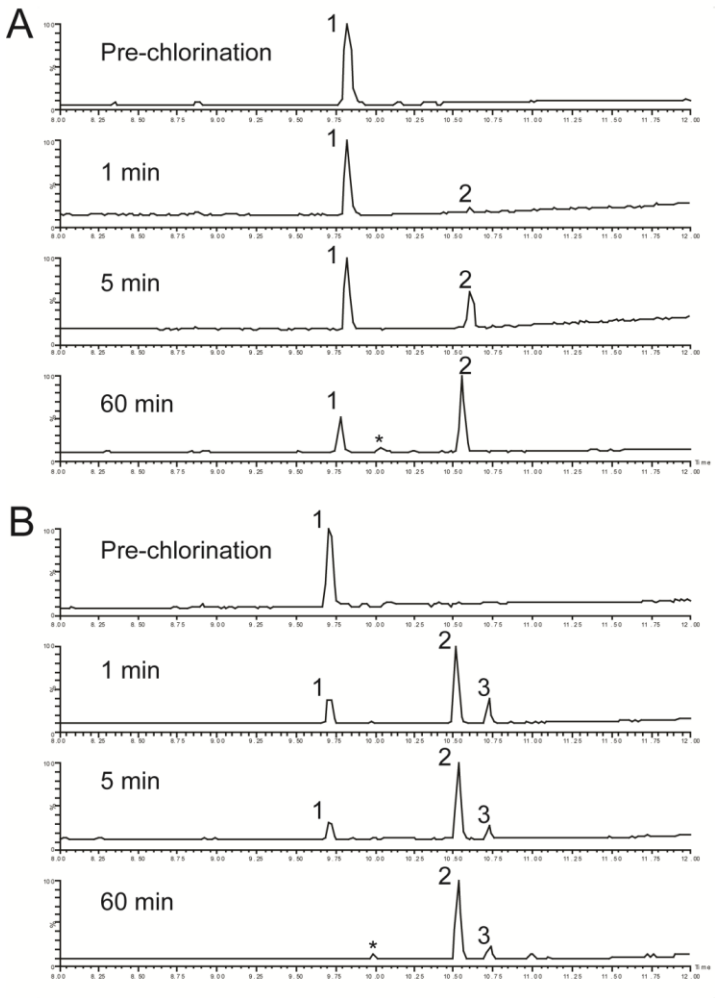

Figure 2.5. TICs of the *in situ* chlorination reaction of 10 mg/L gemfibrozil at selected time points in (A) deionized water and (B) Wastewater A. The labeled peaks correspond to gemfibrozil (1), chlorogemfibrozil (2) and bromogemfibrozil (3). The peak labeled with an asterisk corresponds to *o*-chlorogemfibrozil. Each TIC is displayed normalized to the base peak's intensity, with ion counts of 10^3-10^4.

To examine the chlorination of gemfibrozil under conditions that more accurately represent those of a wastewater treatment plant, the chlorination reactions were replicated in Wastewater A. As seen in Figure 2.5B, chlorination in Wastewater A yields the same *p*-chlorinated species as observed in Figure 2.5A as well as a new peak with a slightly longer retention time. This new transformation product has an m/z and isotopic pattern consistent with addition of a single bromine to form a *p*-bromogemfibrozil analog. The structure was confirmed by accurate mass QTOF-MS (Table 2.1), NMR (Figure 2.2), and elemental analysis (Table 2.2).

The bromine source in the wastewater matrix is naturally occurring bromide, which ranges in concentration from µg/L to mg/L in environmental waters.[14] Upon addition of hypochlorite to the effluent, hypochlorous acid is formed which quickly oxidizes bromide to hypobromous acid. Hypobromous acid has been shown to be a more reactive halogenating agent towards aromatic species compared with hypochlorous acid.[15] The greater reactivity of hypobromous acid is reflected in Figure 2.5B by the rapid formation of bromogemfibrozil (peak 3) 1 min after the addition of hypochlorite to the effluent. One may note that the TIC peak corresponding to the brominated gemfibrozil does not increase much in intensity after the first minute of the reaction. This is likely because all the available bromide was quickly converted to hypobromous acid, which reacted with gemfibrozil and the dissolved organic matter in the effluent until it was depleted. An experiment was designed to verify this hypothesis in which gemfibrozil was

treated with hypochlorite in deionized water containing a 1.2:1 molar ratio of bromide to free chlorine. The reaction proceeded rapidly to yield solely bromogemfibrozil, with no chlorogemfibrozil detected by HPLC-UV analysis. In addition, peak 2 in Figure 2.5B corresponding to chlorogemfibrozil increases more quickly in wastewater than in deionized water (Figure 2.5A). This may be attributed to the higher pH buffering capacity of the wastewater, reducing the extent of the pH increase upon hypochlorite addition. This in turn would favor the presence of hypochlorous acid over hypochlorite, which has been suggested by Pinkston et al. to be the active oxidant in aqueous chlorination reactions with gemfibrozil.[16]

The results presented in Figure 2.5 are the first report of the *in situ* formation of bromogemfibrozil. Bromination of environmental pollutants has been described previously, first in the case of nonionic surfactant alkylphenols.[17] Brominated analogs of alkylphenols have been detected in the environment in wastewater and sludge.[18] Bromination via chlorination in the presence of bromide ions has also been shown to occur in acidic pharmaceuticals *in situ* by Quintana, et al.[19] Parabens and triclosan formed chloro, bromo, and mixed chlorobromo analogs upon exposure to hypochlorite in the presence of bromide.[20, 21] It follows then that wastewaters with high concentrations of bromide undergoing disinfection treatment with chlorine may produce brominated byproducts of molecules containing aromatic moieties.

To assess which products are likely to form during disinfection, an *in situ* chlorination reaction was carried out in Wastewater A spiked with 5 µg/L gemfibrozil and exposed to 5 mg/L initial free chlorine. The reaction was monitored by UPLC-QTOF-MS as shown in Figure 2.6, where extracted ion chromatograms (EICs) after 90 min of chlorination verify the formation of chloro- and bromogemfibrozil.

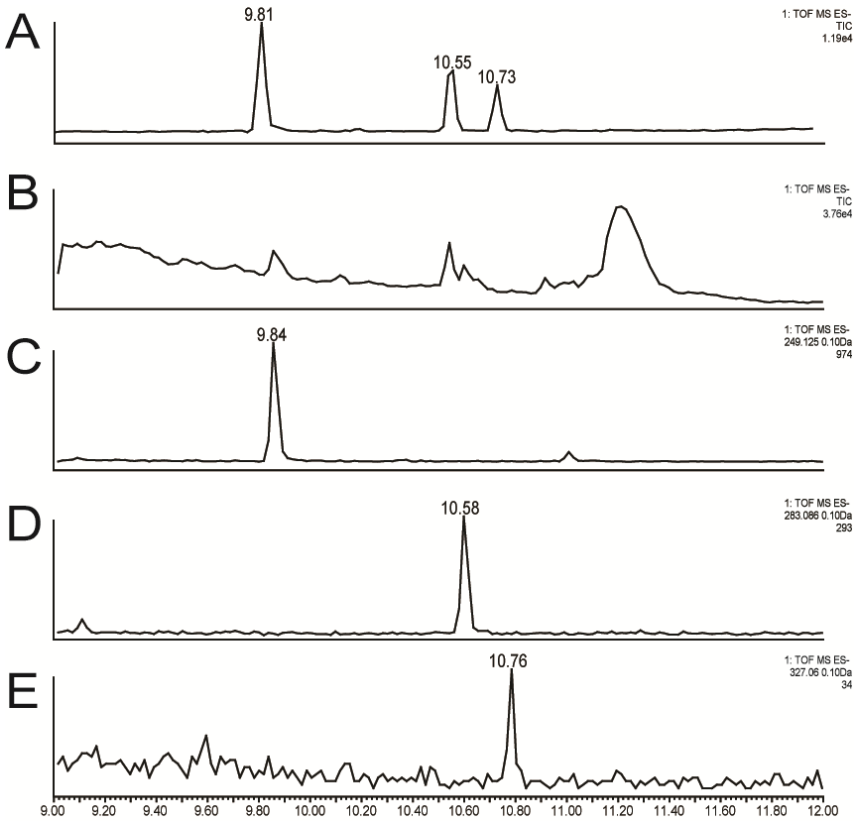

Figure 2.6. Results of the *in situ* chlorination of gemfibrozil at 5 µg/L in Wastewater A after 90 min. (A)TIC showing the retention times of the gemfibrozil (9.81 min), chlorogemfibrozil (10.55 min), and bromogemfibrozil (10.73 min) standards. (B) TIC of the *in situ* chlorination reaction products, with extracted ion chromatograms for (C) gemfibrozil, (D) chlorogemfibrozil and (E) bromogemfibrozil. A window of 0.1 Da was used for the extracted ion chromatograms. Each chromatogram is displayed with the base peak's intensity set to 100%. Note that chromatographic conditions are different than those used in Figure 2.5 therefore, retention times are not comparable.

2.3.2. Implication of particle adsorption in sample preparation. The sorption of analytes to particles complicated the analysis of the halogenated gemfibrozil analogs and provided the impetus to use LLE in place of the more commonly used SPE for sample preparation. Recoveries determined for spiked LLE samples, gemfibrozil (76 ± 7%), chlorogemfibrozil (38 ± 3%) and bromogemfibrozil (25 ± 4%), were significantly higher than those measured using SPE, gemfibrozil (46 ± 14%), chlorogemfibrozil (7.6 ± 5%) and bromogemfibrozil (1 ± 7%). The lower recoveries obtained for SPE were attributed to sorption to suspended solids which were removed by filtration through a 0.22 µm nylon filter membrane prior to SPE.[11, 22] The relative lipophilicities and water solubilities of gemfibrozil and its halogenated analogs are presented below in Table 2.3. The log P values were calculated by the Environmental Protection Agency's Estimation Program Interface suite.[23] Distribution coefficients (D) were calculated at pH 7.5 using the pK_a of gemfibrozil, 4.77, and are reported in Table 2.3.[24] These log D values may better represent the true behavior in aqueous solution, as the charge of the carboxylate implies a greater propensity for the aqueous phase than the log P indicates. Fang et al. reported a preference for aqueous gemfibrozil to sorb to silty loam soil over sandy loam soil and sand, where silty loam soil had the highest organic carbon content.[2] Considering that the organic carbon content of biosolids in wastewater can approach 40% of the dry weight, a significant fraction of gemfibrozil and its halogenated analogs may be particle-bound.[13]

Table 2.3. Selected calculated physiochemical parameters.

Compound	Log P[a]	Log D (pH 7.5)[b]	Water Solubility (mg/L @25 °C)[c]	Log BAF (L/kg wet weight)[d]
Gemfibrozil	4.77	2.04	4.96	2.97
Chlorogemfibrozil	5.41	3.32	0.89	3.28
Bromogemfibrozil	5.66	3.81	0.30	3.30

a Octanol/water partition coefficient for neutral species as calculated by US EPA's EPI Suite.

b Octanol/water partition coefficient for ionized species at a pH of 7.5.

c Milligrams of compound that can be dissolved in one liter of water as calculated by US EPA's EPI Suite.

d Log of the bioaccumulation factor (includes accumulation through trophic transfer in addition to absorption from water) as calculated by US EPA's EPI Suite.

Also presented in Table 2.3 are the calculated bioaccumulation factors (BAFs) using the same software suite, which give an estimate of the potential for a given molecule to accumulate in a high trophic species via absorption from water as well as ingestion of food/particles. Again, a trend towards higher bioaccumulation potential with increasing lipophilicity is seen across the series gemfibrozil < chlorogemfibrozil < bromogemfibrozil. Considering the potential for the more lipophilic molecules in this study to bind to particulate matter, halogenated compounds generated via wastewater treatment processes may have implications for aquatic species such as benthic organisms, which have greater contact with particles and sediment.

2.3.3. Analysis of gemfibrozil and its halogenated analogs in wastewater effluent. The concentrations of gemfibrozil, chloro- and bromogemfibrozil in Wastewater B were determined to be 249 ± 24 ng/L, 164 ± 120 ng/L and 50 ± 11 ng/L, respectively. The offline HPLC fractionation and enrichment strategy employed in this study greatly reduced sample complexity while concentrating the analytes of interest. Matrix interferents, especially problematic in complex wastewater samples, can significantly reduce the observed MS signal of a specific, low-abundance ion.[25] HPLC fractionation prior to UPLC-QTOF-MS analysis also reduced ion suppression by avoiding column overload, which can produce a continuous bleed of analytes from the column into the MS, contributing to ion suppression.[26] The extensive sample clean-up employed in this study also

reduced overloading effects such as retention time shifts, permitting the use of retention times for confirmation of analyte identity along with the qualifying MS^2 transitions (Figure 2.4).

While this analytical method was able to detect gemfibrozil and its halogenated analogs in advanced primary effluent samples, the recovery of the halogenated analogs still remains quite low. Problems in recovery from suspended solids may be specific to these analytes. An efficiency of just 17% for the supercritical fluid extraction of gemfibrozil from suspended particles was previously reported.[27] Quantitative measurements for the transformation products of gemfibrozil must be carried out with other wastewater samples to strengthen the findings reported herein.

2.3.4. Biological effects of transformation products. The extent of attenuation of PPCPs through wastewater treatment is often gauged by comparing the influent and effluent concentrations of a compound.[28] Although this is an accepted method for assessing the attenuation of a contaminant, it neglects the possible conversion of the compound to transformation products that may themselves be environmentally important.[29] As transformation products of most PPCPs have yet to be identified, pure standards are not often commercially available and therefore must be synthesized to allow a quantitative assessment of their levels in effluent, as described in this work.

An alternative approach recently described by Escher et al. uses bioassays and effects-directed analysis to prioritize the study of the transformation byproducts that are of the greatest significance.[30] If a mixture of PPCP byproducts elicits little or no response in a battery of biological assays used to assess multiple modes of toxicity, then it is of little environmental relevance to further characterize transformation byproducts, even though they may still be of analytical interest. Because gemfibrozil has been shown to disrupt endocrine function at environmentally relevant concentrations, it stands to reason that its halogenated analogs may act by a similar mode of action and thus their characterization warrants further study.[5]

The balance of steroid hormones crucial to normal function in an organism can be affected via many endogenous biochemical pathways, where perturbations in the normal levels of androgen and estrogen can be indicative of endocrine disruption. The results of *in vivo* experiments to assess androgen concentrations in Japanese medaka following a 21 d exposure to gemfibrozil and its halogenated analogs are shown below in Figure 2.7. The levels of testosterone were significantly ($P < 0.05$) reduced in the high bromogemfibrozil exposure, with a trend of reduction ($P < 0.1$) for the high dose of chlorogemfibrozil and no reduction for gemfibrozil at any concentration. For 11-ketotestosterone, a significant ($p < 0.05$) reduction can be seen for the high dose of chlorogemfibrozil along with a slight trend ($p < 0.2$) for reduction for the highest

concentration of bromogemfibrozil, but again no reduction for the parent gemfibrozil at any concentration.

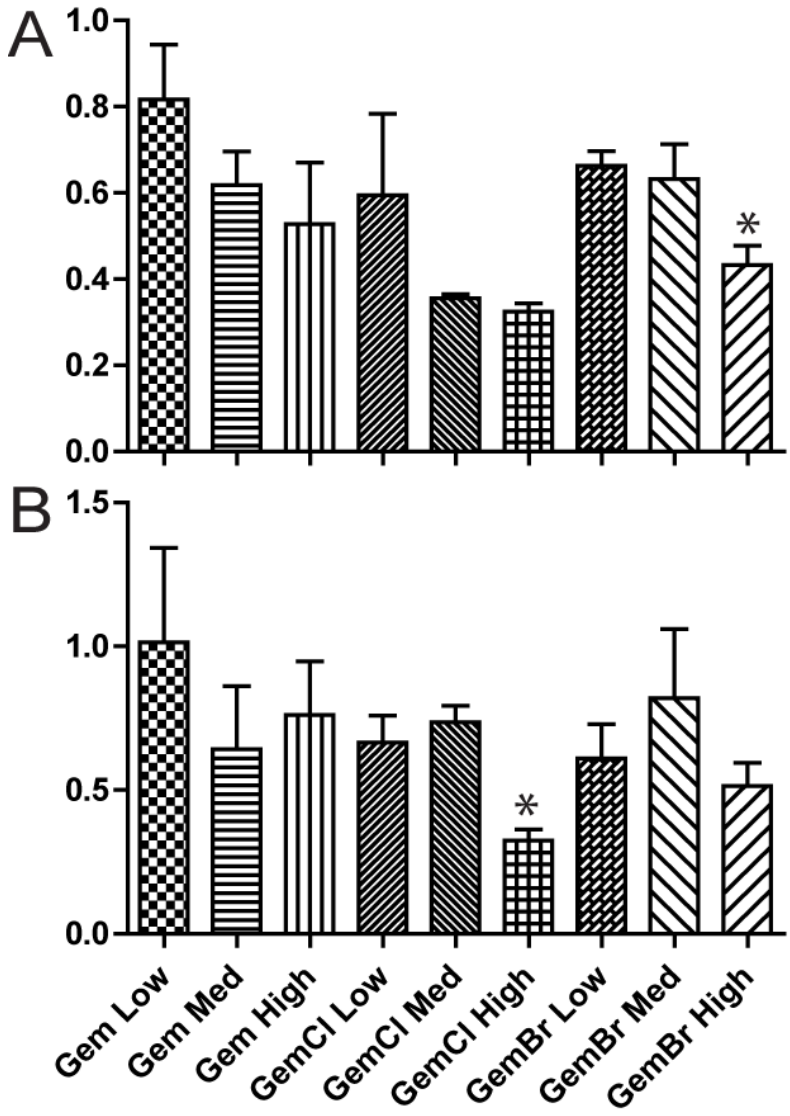

Figure 2.7. Testosterone (A) and 11-ketotestosterone (B) levels from whole Japanese medaka with standard error. Steroid hormone levels were corrected for wet weight and normalized to the response of the negative control. Statistical significance as determined by comparison of each treatment to the negative control ($p < 0.05$) is indicated by *.

Halogenation increased the anti-androgen activity of the gemfibrozil analogs, possibly due to enhanced accumulation because of their higher log P values. Ramirez et al. reported the detection of gemfibrozil in livers of wild-caught common carp and White sucker, two benthic-feeding fish.[4] Mimeault et al. reported a bioconcentration factor of 113 for gemfibrozil at the end of a 14 d exposure;[5] considering this alongside the findings of Ramirez et al. and the higher BAFs calculated in Table 2.3 for chloro- and bromogemfibrozil compared with gemfibrozil, the potential for increased biological impact for these halogenated analogs exists.

Gemfibrozil has been shown to act as an anti-androgen in goldfish.[5] In teleosts, 11-ketotestosterone rather than testosterone is the more potent androgen.[31] The results in Japanese medaka contrast those observed in goldfish since gemfibrozil was unable to reduce androgen concentrations at similar concentrations. The mechanism of impairment is unclear; it may be that goldfish are more sensitive than Japanese medaka to impairment of steroid biosynthesis. Given the effects of fibrates on lipid metabolism, previous studies evaluated the contribution of steroid acute regulatory (STAR) protein to the observed reduction of androgens but were inconclusive.[5] Gemfibrozil is a cytochrome P450 inhibitor which has been shown to inhibit the human chorionic gonadotropin-stimulated release of testosterone *in vitro* in rat Leydig cells.[32, 33] In humans, gemfibrozil is a suspected agent of erectile dysfunction with a proposed mechanism of steroidogenic impairment leading to decreased testosterone levels.[34] The specific

mechanism by which gemfibrozil and its halogenated analogs elicit anti-androgenic effects in fish requires further research to characterize the mechanism of androgen impairment.

2.4 Conclusions

Chlorination of wastewater effluent generated the formation of chloro- and bromogemfibrozil, which were found to be more potent anti-androgens than gemfibrozil in Japanese medaka. Although the concentrations of the gemfibrozil analogs in Wastewater B were below the threshold needed to elicit a response, through life-time exposure the enhanced bioaccumulation of the degradates could lead to tissue concentrations that may alter androgen levels in longer-lived species. This study supports the hypothesis that degradation of anthropogenic pollutants by oxidative disinfection processes may generate transformation products that are more toxic than the parent compounds however, additional studies are needed to characterize these unknown products and evaluate their environmental risk to aquatic organisms.

In the following chapter, the analysis of PPCPs and their halogenated transformation products in wastewater effluent will be extended to a larger number of compounds. This in turn will require the synthesis, purification and characterization of multiple halogenated analogs for each parent PPCP selected for study. In addition, secondary wastewater effluent will be used for analysis rather than primary effluent as in this chapter, enhancing the relevance of any

findings due to the increased ubiquity of secondary wastewater treatment. A modified sample preparation scheme will also be investigated, employing 2-dimensional offline chromatographic enrichment in an attempt to reduce matrix effects seen during final UPLC-QTOF-MS analysis of wastewater extracts. A chromatographic system for the characterization of the log P of the selected PPCPs and their halogenated transformation products will also be presented and compared to an *in silico* log P prediction program.

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Chapter Three

Survey of selected halogenated pharmaceutical and personal care product chlorination transformation products in secondary wastewater effluent

Based on research presented in a platform presentation given at the Society for Environmental Toxicology and Chemistry

SETAC Long Beach, November 13, 2012

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Abstract: The use of chlorination as an oxidative disinfection step prior to wastewater discharge has the potential to transform dissolved organic matter into halogenated species. Pharmaceuticals and personal care products (PPCPs), which often contain an activated aromatic moiety that increases the chances of a halogen substitution during treatment processes, constitute class of molecules especially susceptible to transformation by chlorination. This chapter focuses on the method development and sample preparation undertaken to analyze secondary wastewater effluent extracts from the Orange County Sanitation District by UPLC-QTOF-MS for the presence of the analgesic salicylic acid, non-

steroidal anti-inflammatory diclofenac, plasticizer BPA (BPA) and antilipidemic gemfibrozil in addition to their halogenated transformation products (TPs) as determined by in situ chlorination reactions. Authentic standards of the halogenated analogs were synthesized and purified for use in recovery experiments as well as in the construction of external calibration curves for quantitation of the selected analytes in secondary effluent extracts. Samples were prepared by solid phase extraction using EPA Method 1694 for PPCP analysis in wastewater, where 500 mL effluent samples were first concentrated with Waters Oasis Hydrophilic/Lipophilic balance (HLB) cartridges. To further enrich samples and reduce ion suppression from matrix components, an additional offline fraction enrichment step using reversed phase high performance liquid chromatography was used. For salicylic acid and its analogs, concentrations of 67±1, 10±2, 10±2, 25±4 and 15±4 ng/L were determined for salicylic acid, 3-chlorosalicylic acid, 5-chlorosalicylic acid, 3,5-dichlorosalicylic acid and 5-bromosalicylic acid, respectively. For diclofenac, only the parent compound was detected at a concentration of 43±14 ng/L. For BPA, neither the parent nor its tetrachlorinated analog were detected. For gemfibrozil, concentrations of 100±11, 9±2 and 16±9 ng/L were found for gemfibrozil, chlorogemfibrozil and bromogemfibrozil. Concentrations were corrected for both recovery and ion suppression effects by spiking 500 ng/L of each analyte prior to analysis. Poor sample recovery, ion suppression effects and the importance of

authentic standards in the context of method development and results obtained are discussed.

3.1 Introduction

Building on the results presented in Chapter 2 for the transformation products of the antilipidemic gemfibrozil and their anti-androgenic activity *in vivo*, the analysis of PPCP transformation products (TPs) is herein extended to a wider subset of PPCPs that have been found in US wastewater discharges.[1-3] The parent PPCP compounds chosen for this study: salicylic acid, diclofenac, BPA and gemfibrozil, were selected for a variety of reasons related to their environmental occurrence, toxicological properties and ability to be chlorinated in conditions similar to those encountered during chlorine disinfection in a wastewater treatment plant. Structures of the selected parent compounds are shown in Figure 3.1, where each R group can be replaced with either a chlorine or bromine during chlorination treatment. Bromine substitution occurs when bromide, naturally present in most waters and wastewater, is oxidized by chlorine to hypobromous acid, an effective brominating agent.

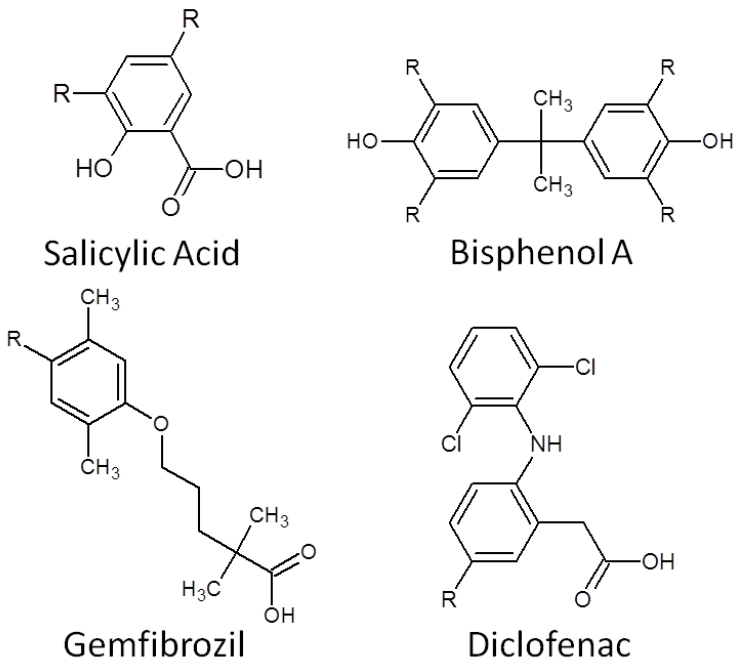

Figure 3.1. Structures of selected parent PPCP compounds.

Salicylic acid was chosen because of its omnipresence as a contaminant in the aqueous environment, and its established high reactivity with aqueous chlorination treatment.[4] In addition, the molecular signature for several halogenated TPs of salicylic acid have been previously described by Quintana et al. in extracts of Spanish wastewater, tap water and drinking water, although quantitative analysis was precluded by the lack of available analytical standards.[5] Diclofenac was chosen due to its well-known toxicity in certain bird species in addition to its potential estrogenic effects in Japanese medaka (*Oryzias latipes*) at waterborne concentrations as little as 1 µg/L.[6, 7] In addition, the *in situ* chlorination of diclofenac has been described previously, indicating a high likelihood that halogenated TPs may form during real-world chlorination treatment.[8] The plasticizer BPA was chosen because of its environmental ubiquity, persistence and suspected *in vitro* endocrine disrupting capabilities which can potentially be enhanced by the addition of halogens.[9-11] The transformation of BPA with chlorine has been described in situ, and its chlorinated TPs have been found in the discharge waters from paper bleaching treatment facilities in Japan at concentrations up to µg/L.[12, 13] In addition, several of BPAs chlorinated TPs have been found in breast adipose tissue of women in Spain, which is especially unsettling given BPAs suspected endocrine disrupting ability.[14] Finally, the antilipidemic gemfibrozil was chosen because of its potential anti-androgenic endocrine disrupting potential in vivo as described in Chapter 2, in addition to the confirmed presence of its chlorinated and brominated

derivatives in primary wastewater effluent from San Diego's Point Loma wastewater treatment facility.[15] This chapter expands on the results presented in Chapter 2 to examine a wider subset of PPCPs, and to extend this study from primary wastewater effluent to secondary wastewater effluent.

Most wastewater treatment plants around the globe, especially in densely-populated areas, employ a secondary treatment step. Orange County Sanitation District, the source of wastewater used in this chapter, employs secondary treatment and discharges a blend of primary and secondary effluent.[16] Tertiary treatment is less common, and involves advanced oxidative treatment methods such as ozonation, peroxide combined with UV, and filtration techniques such as reverse osmosis and/or nanofiltration. While tertiary treatment is the best at the removal of micropollutants such as PPCPs, it is also the most expensive and least widely used.

In addition, removal of a given parent compound through wastewater treatment does not necessarily mean that the compound has been mineralized (i.e. transformed into carbon dioxide, water, and other basic molecules). The use of chemical oxidants such as chlorine have the potential to transform a given parent PPCP into one or more TPs, thus reducing the concentration of the parent compound but not impacting the total PPCP content of the wastewater. TPs that have been halogenated generally exhibit increased lipophilicity, however, as most PPCP TPs have yet to be described their physiochemical properties can only be calculated using predictive software. Towards this end, a

chromatographic assay for the determination of lipophilicity was constructed for the comparison of software predicted lipophilicities to those determined by physical measurement. Such measurements are based on the principle that the retention of an analyte on a C18 column in isocratic reversed phase chromatography is related to its octanol-water partition coefficient (P), a common parameter for the description of a un-ionized molecule's lipophilicity given by equation 3.1.[17]

$$P = \frac{[\text{un-ionized analyte}]_{\text{octanol}}}{[\text{un-ionized analyte}]_{\text{water}}}$$

Equation 3.1. Definition of the octanol-water partition coefficient P, which describes the lipophilicity of an un-ionized molecule.

This chapter focuses on the analysis of PCCP TPs in secondary effluent due to the prevalence of secondary wastewater treatment around the world. An additional benefit for this study is relative ease of sample preparation relative to primary effluent. Secondary effluent generally has much lower suspended solids content than primary effluent, which means that sample preparation can rely on SPE rather than the more laborious and solvent-intensive liquid-liquid extraction used for gemfibrozil analyses in Chapter 2. The SPE protocol used was based on an EPA validated method, EPA 1694 which targets a wide variety of pharmaceuticals spanning multiple orders of lipophilicity.[18] As SPE treatment of

secondary effluent, with concentration factors of 100 (500 mL secondary effluent extracted and reconstituted to 500 µL), yielded no detectable UPLC-QTOF-MS signal for any of the 4 molecules and their transformation products (data not shown) this strategy was appended with a separate sample enrichment step. Enrichment of secondary effluent SPE extracts was employed using offline HPLC to further concentrate the analytes of interest and reduce matrix effects for the UPLC-QTOF-MS measurements. To generate the HPLC offline enrichment scheme, pure standards of the potential chlorination TPs for each of the 4 parent molecules needed to be synthesized in milligram quantities for use in method development. The synthesis, purification and characterization of TP standards is described and discussed, along with their use for the identification and quantification of these compounds in secondary wastewater effluent samples.

3.2 Materials and methods

3.2.1 Chemicals and suppliers. Bisphenol A, salicylic acid, phenol, 2-chlorophenol, 3-chlorophenol, 4-nitrophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, ibuprofen, triclosan, naproxen, phenolphthalein, trans-cinnamic acid, 4-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, nitric acid, potassium bromide, formic acid, acetic acid and sodium thiosulfate, all of the highest available purity, were obtained from Sigma Aldrich (St. Louis, MO). Gemfibrozil (USP grade) was obtained from Spectrum Chemical Manufacturing Corporation (New Brunswick, NJ). Methanol and acetonitrile were obtained from Fisher

Scientific (Pittsburgh, PA), both Optima™ grade. HPLC-grade Burdick and Jackson water was used for HPLC and LC-MS analyses (Morristown, NJ). Deionized water was obtained from a Millipore water purification system (Billerica, MA). A 13% active chlorine sodium hypochlorite solution from Acros (Geel, Belgium) was used for chlorination reactions. Diclofenac was obtained from Cayman Chemical Company (Ann Arbor, MI).

3.2.2. Wastewater samples. Final post-chlorination secondary effluent wastewater grab samples were collected from the Orange County Sanitation District's Fountain Valley facility. Samples were collected in 1 L amber glass bottles that had been thoroughly cleaned with methanol followed by 5% nitric acid in deionized water. Samples were immediately transported in the dark to a 4 °C cold room for storage. Approximately 80 mg of sodium thiosulfate was added to each bottle to quench residual chlorine per EPA Method 1694.[18] Several physical parameters were measured for this sample. Residual ammonia levels immediately after sampling were ca. 2 mg/L as measured by a colorimetric test kit from Aquatic Ecosystems (Apopka, FL). The initial pH of the wastewater was measured at 7.50. Dissolved organic carbon content was 365 mg/L determined using a Shimadzu TOC-VWP analyzer in the laboratory of Dr. Gan in the Environmental Sciences Department at UC Riverside. Total suspended solids and total dissolved solids content were measured at 1.3 and 2,500 mg/L, respectively, by the Environmental Sciences Section (ESS) Method 340.2.[19]

3.2.3 *In situ* TP standard synthesis. Halogenated analog standards were prepared by chlorination of the parent compound in deionized water and methanol (1:1 v/v) with and without bromide, and isolated in milligram quantities using an Agilent 1100 HPLC with UV detection at 254 nm. General reaction procedures were as follows. Between 10 and 20 mg of parent compound was dissolved in 200 mL 50% v/v H_2O/methanol. The initial solution was stirred with a Teflon coated stir bar, sampled into a glass LC-MS autosampler vial containing methanol to effect a dilution to 5 ng/µL, and analyzed by UPLC-QTOF-MS to obtain a pre-chlorination chromatogram. An aliquot of a 13% active Cl_2 NaOCl solution, standardized by iodometric titration, [20] was added to the stirred solution of parent compound to effect a 5-10 fold molar excess of chlorine. For bromination reactions, the NaOCl solution was added to 5 mL water containing bromide to give 2:1 molar excess of bromide:chlorine. This solution was briefly mixed to convert HOCl to HOBr and then introduced to the stirred reaction mixture. Due to the enhanced reactivity of HOBr over HOCl, initial doses of HOBr solutions were chosen to provide a 2-4 fold molar excess of bromine relative to the parent compound. The reaction mixture was periodically sampled and analyzed by LC-MS as described above for the pre-chlorination sample. Attenuation of the parent compound was monitored by comparing peak areas of extracted ion chromatograms (EICs) between successive sampling points. The major transformation products of each halogenation reaction were identified from the total ion chromatogram (TIC) by increasing product peak areas, shifts to

longer reversed phase retention times (indicative of halogenation), upward shifts in m/z and incorporation of halogen isotopic signatures. Additional doses of chlorine solution were added as needed based on the progression of the reaction, with the end goal being total conversion of the parent compound into its transformation products. When the EIC of a reaction sample indicated complete attenuation of the parent compound, the halogenation reaction was halted by addition of a 2:1 molar excess of thiosulfate:total chlorine dose. The reaction mixture was sampled one final time and analyzed by LC-MS to ensure that thiosulfate quenching had no effect on product distribution. The reaction products were concentrated by C18 SPE as described below following the method of Jeannot et al. with slight modification of the final elution solvent being solely methanol.[21]

For the SPE of halogenation reaction mixtures, the mixtures were first diluted with DI water to reduce the total organic solvent content to below 30% (10% for all salicylic acid reactions). A solution of 5 M HCl was used to lower the reaction mixtures to pH 3 prior to extraction. Waters Sep-Pak 500 mg C18 SPE cartridges on a Supelco Visiprep vacuum SPE manifold were employed for concentration with the following protocol. The SPE cartridges were first activated with 10 mL methanol and equilibrated with 10 mL DI water. The reaction mixture was then passed through the cartridge at a flowrate of 5 mL/min. The cartridge was dried under air flow for 30 minutes, eluted with 10 mL of methanol and the

eluate evaporated to dryness in a 50 °C water bath using a gentle stream of compressed air.

3.2.4 TP standard HPLC purification. Analytical scale separations for purification of the halogenated TPs were performed on either a 4.6x150 mm 5 µm Inertsil ODS-2 column from MetaChem (Torrance, CA) or a 4.6x150 mm 5 µm Luna Phenylhexyl column from Phenomenex (Torrance, CA) with a mobile phase of A: 0.1% formic acid (0.5% formic acid for salicylic acid), and B: ACN. The columns were held at 30 °C in a Bruker Stop Flow Oven Unit (Billerica, MA). Separations were performed using an Agilent 1100 HPLC with UV detection at 254 nm. Gradients for were optimized for chromatographic resolution for the analytical scale purification of halogenation reaction products, given below. Purification was not necessary for bisphenol A as both the parent and its tetrachlorinated analog were commercially available as pure standards.

Salicylic acid (Luna Phenylhexyl column; 2.5 mL/min): 25% B 0-8 min, 25-90% B 8-8.1 min, 90% B 8.1-10 min, 90-100% B 10-10.1 min, 100% B 10.1-11 min, 100-25% B 11-11.1 min, 25% B 11.1-12.5 min.

Gemfibrozil (Inertsil ODS-2 column; 2.1 mL/min): 54% B 0-2 min, 54-60% B 2-6 min, 60-63% B 6-10 min, 63-100% B 10-11 min, 100% B 11-12 min, 100-54% B 12-12.1 min, 54% B 12.1-13 min.

Diclofenac (Luna Phenylhexyl column; 1.5 mL/min): 50% B 0-1 min, 50-75% B 1-9 min, 75-100% B 9-9.1 min, 100% B 9.1-10.5 min, 100-50% B 10.5-10.6 min, 50% B 10.6-11.5 min.

3.2.5 TP standard accurate mass MS and NMR characterization. Accurate mass experiments used the lock-spray mode, with 10 ng/µL tetrachlorobisphenol A ($[M-H]^-$ = 362.9519 Da) in 50:50 1 mM aqueous ammonium acetate:acetonitrile as the lock mass solution; a 10 ng/µL naproxen ($[M-H]^-$ = 229.0870 Da) solution in the same mobile phase was used as a lock mass for accurate mass determination of tetrachlorobisphenol A. The lock mass solution was directly infused into the reference source by syringe pump at 25 µL/min and sampled at a frequency of 0.2 Hz. Accurate mass spectra were acquired with 1-5 ng/µL solutions of each analyte in methanol, analyzed by direct infusion fed by a syringe pump at 10-20 µL/min to generate ion counts of 300-600 cps. Spectra were collected for 2 min in the m/z scan range 130-600 Da and 20-30 scans were averaged, then processed for accurate mass measurement using the built-in accurate mass TOF correction in the MassLynx software. The mass spectrometer was calibrated after every 5 accurate mass measurements using a sodium formate solution per the manufacturer's recommendation. Calibration for the time-of-flight sector of the spectrometer was under 3 ppm RMS error for the m/z region 100-1000 Da. Accurate mass results are given below in Table 3.1, where the mass difference in ppm for the m/z of the measured value for the

monoisotopic peak for each analyte is compared to the theoretical m/z as calculated using ACD Labs ChemSketch version 12.0. All accurate mass values have mass errors <5 ppm.

Table 3.1. Accurate mass results for selected parent compounds and TPs.

Compound	Theoretical $[M-H]^-$ m/z (Da)	Experimental $[M-H]^-$ m/z (Da)	Mass Error (ppm)
Salicylic acid	137.0244	137.0242	-1.46
3-Chlorosalicylic acid	170.9854	170.9849	-2.92
5-Chlorosalicylic acid	170.9854	170.9859	2.92
3,5-Dichlorosalicylic acid	204.9435	204.9433	-0.98
3-Bromosalicylic acid	214.9349	214.9351	0.93
5-Bromosalicylic acid	214.9349	214.9346	-1.40
3,5-Dibromosalicylic acid	292.8454	292.8452	-0.68
Diclofenac	294.0094	294.0089	-1.70
Chlorodiclofenac	327.9704	327.9691	-3.96
Bromodiclofenac	371.9199	371.9188	-2.96
Bisphenol A	227.1078	227.1077	-0.44
Tetrachlorobisphenol A	362.9519	362.9524	1.38
Gemfibrozil	249.1496	249.1497	0.40
Chlorogemfibrozil	283.1106	283.1105	-0.35
Bromogemfibrozil	327.0601	327.0604	0.92

Assignment of the TP halogenation regiochemistry was accomplished by comparing ^{1}H NMR survey spectra of the TPs and their parent compounds. Experiments were performed using a Bruker Avance NMR spectrometer operating at 599.84 MHz for ^{1}H using the standard zgpr pulse program. Samples were prepared in 600 µL methanol-d_4 at approximately 1 mM for the parent compounds and 0.5-5 mM for the TPs, depending amount of TP available.

3.2.6.Wastewater SPE extraction. The SPE analyte concentration method from EPA Method 1694 was used for the targeted extraction of acidic/neutral analytes.[18] Briefly, 500 mL of effluent was acidified to pH 2.50 with 5 M HCl. Waters Oasis HLB 200 mg cartridges were conditioned with 6 mL methanol and equilibrated with 6 mL pH 2.50 deionized water, both at flowrates of ~6 mL/min. The effluent sample was passed through the cartridge under vacuum at ~5 mL/min followed by a wash step using 6 mL deionized water. After drying for 30 min under vacuum, the cartridge was eluted into a 15 mL centrifuge tube with 2x6 mL methanol. Eluates were blown to near dryness by a gentle steam of air and reconstituted in 300 µL methanol for offline HPLC enrichment.

3.2.7.Offline HPLC enrichment of wastewater SPE extracts. Analytical scale enrichment separations were performed on an Agilent 1100 HPLC with UV detection at 254 nm using a 4.6x150 mm 5 µm Luna phenylhexyl column from Phenomenex (Torrance, CA) with a mobile phase of A: 0.5% formic acid and B:

ACN at a flowrate of 2.0 mL/min. Detection was performed by a variable wavelength detector set to 254 nm. The gradient, optimized for baseline chromatographic resolution between each analyte, was as follows: 35% B 0-10 min, 35-60% B 10-15 min, 60% B 15-20 min, 60-70% B 20-30 min, 70-100% B 30-30.5 min, 100% B 30.5-35 min, 100-35% B 35-35.5 min. 35% B 35.5-45 min. The column was held at 60 °C in a Bruker Stop Flow Oven Unit (Billerica, MA) with a 5 inch stainless steel tubing solvent preheater on the front end of the column. Effluent SPE extracts, reconstituted in 300 µL methanol, were injected in 100 µL injections. A chromatogram of parent and TP standards is shown in Figure 3.2. Fraction collection was started 10 s before the front of each peak, and continued until 10 s after the end of each peak according to the peak times in Figure 3.2. Fractions were combined for each set of parent and TPs, e.g. salicylic acid and its TPs were all collected into the same fraction collection vessel. Fractions were blown to near dryness with a gentle steam of air in a water bath held at 40 °C, recovered in methanol and transferred to microcentrifuge tubes. The samples were evaporated with an SC110 SpeedVac from Thermo Scientific (Asheville, NC) and stored in a -20 °C freezer until UPLC-QTOF-MS analysis.

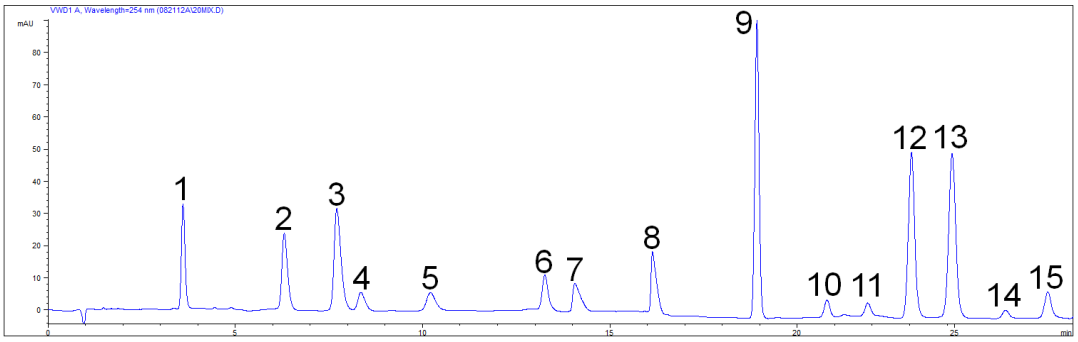

Figure 3.2. UV-detected chromatogram of parent PPCPs and their halogenated TPs. Compound identities are as follows: 1) salicylic acid, 2) 3-chlorosalicylic acid, 3) 3-bromosalicylic acid, 4) 5-chlorosalicylic acid, 5) 5-bromosalicylic acid, 6) 3,5-dichlorosalicylic acid, 7) 3,5-dibromosalicylic acid, 8) bisphenol A, 9) diclofenac, 10) gemfibrozil, 11) tetrachlorobisphenol A, 12) chlorodiclofenac, 13) bromodiclofenac, 14) chlorogemfibrozil, and 15) bromogemfibrozil.

3.2.8 UPLC-QTOF-MS experimental parameters. All UPLC separations were performed at 50 °C using an Acquity UPLC with an Acquity 2.1x150 mm BEH C18 column and a VanGuard 2.1x5 mm guard column (Waters, Milford, MA). The injection volume was 20 µL, with the injector operating in full loop mode to reduce inter-analysis systematic error. The gradients and mobile phase for each parent compound and its TP analogs were as follows:

Salicylic acid (A: 0.01% formic acid, B: ACN, 0.45 mL/min): 15% B 0-1 min, 15-45% B 1-5 min, 45% B 5.1-6 min, 100% B 6.1-8min, 100-15% B 8-8.1 min, 15% B 8.1-10 min.

Gemfibrozil (A: 0.01% acetic acid, B: ACN, 0.45 mL/min): 35% B 0-1 min, 35-80% B 1.1-5 min, 80% B 5.1-6 min, 100% B 6.1-8min, 100-35% B 8-8.1 min, 35% B 8.1-10 min.

Bisphenol A (A: 1 mM ammonium acetate, B: ACN, 0.45 mL/min): 30% B 0-1 min, 35-90% B 1.1-5 min, 90% B 5.1-6 min, 100% B 6.1-8min, 100-30% B 8-8.1 min, 30% B 8.1-10 min.

Diclofenac (A: 0.01% formic acid, B: ACN, 0.45 mL/min): 40% B 0-1 min, 40-80% B 1.1-5 min, 80% B 5.1-6 min, 100% B 6.1-8min, 100-40% B 8-8.1 min, 40% B 8.1-10 min.

A Waters Micromass QTOF Micro mass analyzer equipped with an electrospray ionization (ESI) source operated in the negative ion mode was used for the acquisition of all mass spectrometric data. Mass spectrometer operating parameters were as follows: capillary voltage 2800 V; source temperature 120

°C, desolvation temperature 250 °C; desolvation gas flow rate 650 L/h; scan time 1 s. A cone voltage of 15 V was used for salicylic acid, gemfibrozil, diclofenac and their TPs, while a value of 35 V was used for bisphenol A and tetrachlorobisphenol A. The m/z scan range was adjusted for each set of parent/TPs to account for MS-MS transitions as well as the highest m/z value from each set of TPs: Salicylic acid 50-300 Da, gemfibrozil 120-335 Da, bisphenol A 50-400 Da, diclofenac 90-380 Da. For MS-MS experiments, a collision voltage of 12 eV was used for salicylic acid, gemfibrozil, diclofenac and their TPs, while a collision voltage of 30 eV was used for bisphenol A and tetrachlorobisphenol A. The mass spectrometer was calibrated at the beginning of each day with a sodium formate solution per the manufacturer's recommendation. Calibration for the time-of-flight sector of the spectrometer was under 3 ppm RMS error for the m/z region 100-1000 Da.

3.2.9 UPLC-QTOF-MS analysis of HPLC-enriched wastewater extracts.

Extracts containing the parent compounds and TPs (described in section 3.2.7) were removed from the freezer and allowed to equilibrate to room temperature for 30 min prior to reconstitution in 50 µL of the initial mobile phase. Reconstituted extracts were sonicated for 1 min, centrifuged at 8,000 rpm for 30 s to remove particulates, and carefully transferred to glass sample vials for UPLC-QTOF-MS analysis as described in section 3.2.8. Due to the limited sample volumes, each extract was analyzed as a single injection. External

calibration curves consisting of 7 concentrations injected in triplicate were constructed over the range 1-100 ng material injected, except for bisphenol A and tetrachlorobisphenol A where the range was 5-150 ng due to lower MS response. The calibration curves were fit with 2nd order polynomials to generate R^2 values of >0.998.

All UPLC-QTOF-MS chromatograms were processed identically by the following methodology. First, the measured m/z of each compound was manually determined to the nearest 0.001 Da according to the measured values of the standards from the calibration curves; quantifying m/z values can be seen in Table 3.2. Second, the peak area of the monoisotopic molecular ion peak for each compound was measured using the embedded MassLynx integration function to ensure consistency of data treatment. The EIC was determined using a 0.1 Da extraction window centered around the exact m/z for each compound. For compounds containing one or more halogens, the peak area of the extracted ion chromatogram for both the M (monoisotopic) and M+2 was recorded and summed for additional gains in signal as seen in chapter 2 with gemfibrozil analyses.[15] For unspiked effluent extracts, qualifying MS-MS transitions were manually confirmed for samples yielding a positive hit for the analyte of interest; qualifying m/z transition ions are given in Table 3.2.

For recovery calculations, 3x500 mL wastewater effluent samples were spiked with 500, 1,000 or 5,000 ng/L of each parent compound /TP (9 total wastewater samples) from a stock solution (<100 µL) in methanol. Spiked

effluent samples were sonicated for 10 minutes prior to extraction to ensure even dissolution of the spiked analytes. Spiking levels were chosen based on previous literature values for concentration ranges for the selected parent analytes in wastewater discharges, where a spiking concentration found to be >10 fold the concentration found in unspiked samples was used for calculating recovery upon data analysis.[22] Samples were then SPE extracted and HPLC-enriched as described above for unspiked wastewater samples. An additional 3x500 mL wastewater samples were extracted and HPLC-enriched as described above, then spiked with 500 ng/L each analyte just prior to UPLC-QTOF-MS analysis for the calculation of % ion suppression by extracted ion peak area comparison. Both recovery and ion suppression samples were reconstituted prior to analysis in parallel volumes matching the initial mobile phase conditions for each set of parent compounds and their associated TPs and analyzed in triplicate. The reconstitution volumes were chosen such that a 20 µL UPLC-QTOF-MS injection volume would contain 50 ng each analyte when assuming 100% sample recovery, a mass which fell near the center of the linear range of external calibration curves constructed for each set of analytes. Limits of detection (LOD) were determined by linear regression of the signal-to-noise ratio from a standard curve used for quantitation of each analyte. A signal-to-noise ratio of 3 was chosen for the LOD, while the limit of quantitation (LOQ) was determined at a signal-to-noise ratio of 10.[15] Data on the LOD, LOQ, % recovery, % ion suppression and analyte concentration in unspiked wastewater extracts is

presented in Table 3.2. A calibration curve standard corresponding to 50 ng injected material was run after every set of unspiked and spiked recovery/ion suppression samples to ensure sensitivity remained consistent during analyses.

3.2.10 Chromatographic log P assay. An Agilent 1100 HPLC with UV detection at 254 nm was used for these measurements. A 2.1x30 mm 2.5 µm Waters Xterra MS C18 column was used at flowrate of 1.5 mL/min and isocratic elution at 30% B with A: 10 mM phosphate buffer adjusted to pH 2.1 and B: ACN. The time for each separation depended on the retention time of each analyte, and varied from 0.82 min for 4-hydroxybenzoic acid to 154.82 min for bromogemfibrozil. A library of small molecule compounds consisting of 17 carboxylic acids and phenolic compounds with known literature log P values spanning from 1.46-4.77 (all molecules in their non-ionic form) was used as a training set, where literature values were taken from the National Institute of Health's Chem ID database.[23] All samples were analyzed in triplicate, and their capacity factor (k') was calculated from the average retention time (t_R) according to Equation 3.2 using a dead volume time (t_0) of 0.40 min as determined by the UV signal from a blank air injection. The plot of the log transformed capacity factors vs. the literature log P values for the training set compounds was treated by linear regression yielding a linear equation of $y = 0.5841x - 0.7118$ ($R^2 = 0.9518$), shown in Figure 3.7. Experimentally determined capacity factors were then used to solve for the log P of the selected TPs and compared to the

predicted values as calculated by the US EPA's EPI Suite KOWWIN software version 4.10 (Table 3.4).[24]

$$k' = \frac{t_R - t_0}{t_0}$$

Equation 3.2. Formula for the calculation of the capacity factor (k') as it relates to the retention time (t_R)and dead volume time (t_0) for each analyte.

3.3.1 Results and discussion

3.3.1 *In situ* chlorination reactions and the importance of authentic standards. The analysis of TPs of environmental contaminants such as PPCPs is an emerging field, where many hurdles to rapid progress exist. One such hurdle is the lack of authentic standards for TPs of most PPCPs, which is often precluded by the fact that not all potential TPs have been identified.[25] While this dissertation focuses on TPs generated by chlorine disinfection, there are a host of other processes employed at wastewater treatment plants which can generate TPs such as bacterial metabolism during activated sludge treatment, other disinfection schemes such as ozonation and photolysis and degradation from UV/peroxide treatment.[26, 27] In wastewater (and any other environmental matrix) analysis, authentic standards allow for method development for targeted analysis of unknown samples with the generation of reference spectra for MS and MS-MS

experiments in addition to establishing LC retention times for an additional metric of confirmation.[28, 29] Standards also allow accurate quantitation in wastewater samples through the construction of external calibration curves, spiked recovery and matrix effect samples. While the use of *in situ* reactions is not necessarily the most direct method for the synthesis of TP standards, the procedure is simpler and more relevant to the aqueous chlorination undertaken at wastewater treatment plants than the use of targeted organic syntheses. As observed in Chapter 2 and again in this study, *in situ* chlorination reactions followed by analytical scale HPLC purification generate pure analytical standards in milligram quantities suitable for method development and targeted wastewater analysis.

3.3.2 Concentrations of selected analytes in secondary wastewater effluent. Reported in Table 3.2 are the concentrations (ng/L), quantifying ions and qualifying MSMS transition ions of the selected parent PPCPs and their chlorination TPs in 5 replicate 500 mL effluent samples from a secondary wastewater effluent grab sample from the Orange County Sanitation District. This is the first report concerning the quantitative analysis of halogenated derivatives of salicylic acid in wastewater effluent, though previous research by Quintana et al. qualitatively estimated the presence of the three chlorinated TPs of salicylic acid in Spanish wastewater at tens of ng/L.[30] The study by Quintana et al., however, did not employ authentic standards, and only compared the MS response of the chlorinated TPs against the response of the parent salicylic acid.

Our identification of chloro- and bromogemfibrozil in secondary effluent also confirms that the chlorination chemistry observed for primary effluent in Chapter 2 occurs in another wastewater treatment plant employing a different treatment scheme.[15] The molecular signature for tetrachlorobisphenol A was present in several of the wastewater extracts analyzed, however the average signal-to-noise was below the limit of detection. Tetrachlorobisphenol A has previously been found at low µg/L levels in the effluent discharged from a paper bleaching mill in Japan.[31] Low recoveries and high ion suppression, likely due to inadequate sample preparation in concert with the relatively low sensitivity of the QTOF-MS used in these studies, potentially led to the high number of non-detects. This will be discussed in the following section in light of the reported recoveries and % ion suppression observed. It is also possible that the targeted TPs did not form during wastewater treatment, though additional effluent samples would be required to verify this hypothesis.

Table 3.2. Analytical results for wastewater extraction and quantitation method				
Compound	Concentration in OCSD Effluent (ng/L)	Quantifying M Ion m/z (Da)	Quantifying M+2 Ion m/z (Da)	Qualifying MS-MS Transition Ion m/z (Da)
Salicylic acid	67±1	137.013	N/A	93.032
3-Chlorosalicylic acid	10±2	170.992	172.992	127.031
5-chlorosalicylic acid	10±2	170.992	172.992	127.031
3,5-Dichlorosalicylic acid	25±4	241.141	243.141	161.002
3-Bromosalicylic acid	ND	214.949	216.949	170.957
5-Bromosalicylic acid	15±4	214.949	216.949	170.957
3,5-Dibromosalicylic acid	ND	292.834	281.834	248.846
Diclofenac	43±14	294.075	296.075	250.091
Chlorodiclofenac	ND	330.036	332.036	286.093
Bromodiclofenac	ND	375.407	377.407	330.026
Bisphenol A	ND	227.246	N/A	133.145
Tetrachlorobisphenol A	<LOD	363.168	365.168	286.128
Gemfibrozil	100±11	249.023	N/A	121.125
Chlorogemfibrozil	9±2	283.263	285.263	155.106
Bromogemfibrozil	16±9	327.229	329.229	199.067

3.3.3 Recovery and ion suppression in secondary wastewater effluent.

Reported in Table 3.3 are the % recovery, % ion suppression, LOD and LOQ with standard deviations from triplicate sample analyses of spiked secondary effluent extracts. The spiking level chosen for the comparison of analyte QTOF-MS response in the recovery and ion suppression samples was 500 ng/L, as this was the closest spiked concentration to ambient analyte concentrations determined from unspiked wastewater extract analyses. This is, however, more of an estimation of the true recovery and ion suppression effects, which could be accurately determined using isotopically labeled standards.[32, 33] Isotopically labeled standards, much the same as their non-isotopically labeled counterparts synthesized for this study, were not commercially available at the time this work was performed. This limitation will be addressed in Chapter 4, which describes the preparation and use of isotopically labeled standards with a more sensitive analysis platform.

Table 3.3. Recovery, ion suppression and method information for wastewater analyses.

Compound	% Ion Suppression	% Recovery	LOD (ng/L)	LOQ (ng/L)
Salicylic acid	80 ± 4	27 ± 9	0.9	2.4
3-Chlorosalicylic acid	67 ± 5	19 ± 5	0.8	1.8
5-chlorosalicylic acid	56 ± 2	21 ± 1	0.8	1.7
3,5-Dichlorosalicylic acid	49 ± 3	17 ± 3	0.5	1.4
3-Bromosalicylic acid	57 ± 6	19 ± 8	0.8	2.3
5-Bromosalicylic acid	43 ± 2	18 ± 2	0.9	2.4
3,5-Dibromosalicylic acid	57 ± 3	5 ± 18	1.2	3.4
Diclofenac	56 ± 3	16 ± 5	2.3	7.5
Chlorodiclofenac	10 ± 0	16 ± 2	2.2	6.2
Bromodiclofenac	9 ± 1	25 ± 7	2.4	7.6
Bisphenol A	75 ± 2	0 ± 0	8.0	25.1
Tetrachlorobisphenol A	10 ± 1	32 ± 7	7.5	23.2
Gemfibrozil	24 ± 1	46 ± 4	1.1	3.8
Chlorogemfibrozil	40 ± 2	24 ± 2	1.8	4.5
Bromogemfibrozil	20 ± 1	37 ± 2	1.7	4.4

The method information presented in Table 3.3 reveals low but consistent recoveries, in addition to a large amount of ion suppression. The low recoveries could result from a variety of factors, including matrix effects in the initial SPE extraction, peak retention time shifts from column overloading in the HPLC offline enrichment step, and column overloading leading to ion suppression and retention time shifts in UPLC-QTOF-MS analyses. The use of a second HPLC separation platform is commonly referred to as offline two-dimensional chromatography, which is a commonly used technique in proteomics to increase dynamic range and proteome coverage.[34] The benefits of an added HPLC separation are highly dependent on the nature of the sample and the retention mechanisms of each LC stage, where the greater orthogonality of the retention mechanisms the greater the net peak capacity.[35, 36] This in turn means that theoretically more sample components can be resolved, with an added benefit specifically for ESI-MS of reduced matrix complexity which ideally translates into reduced ion suppression. The HPLC offline enrichment column used in this study had a hexylphenyl stationary phase, used methanol as the organic mobile phase component and a column temperature of 60 °C, while the UPLC-QTOF-MS analyses used a column with a modified C18 stationary phase, ACN as the organic mobile phase component, a column temperature of 50 °C and optimized solvent modifiers and gradients for the analysis of each set of PPCPs and their TPs. This approach is similar to the sample enrichment method used by Teng et al., where offline two-dimensional RP-HPLC systems, a cyano column separation

followed by a C18 column separation, were used to achieve higher peak capacity and resolution in the analysis of a plant resin used for traditional Chinese medicines.[37]

An extracted ion chromatogram from the analysis of a standard of 3,5-dichlorosalicylic acid compared to an unspiked wastewater sample are presented in Figure 3.3., showing a retention time shift of nearly 8 seconds. Retention time shifts between standards and samples are indicative of column overloading. For additional confirmation of sample identity, Figures 3.5 and 3.6 compare the EIC mass spectra and MS-MS spectra, respectively, of the standard and the effluent sample. The relative complexity of the baseline in Figure 3.4 and the poor signal-to-noise in Figure 3.5 underscore the complexity of the wastewater matrix, which leads to ion suppression in ESI-MS analyses.[32] One method for the reduction of ion suppression described by Kloepfer et al. is to lower the LC eluent flow entering the MS detector to a flowrate under 0.1 mL/min, however the peak broadening from such a change would severely diminish the peak capacity and resolution obtained in the currently used separations with a flowrate of 0.45 mL/min.[38]

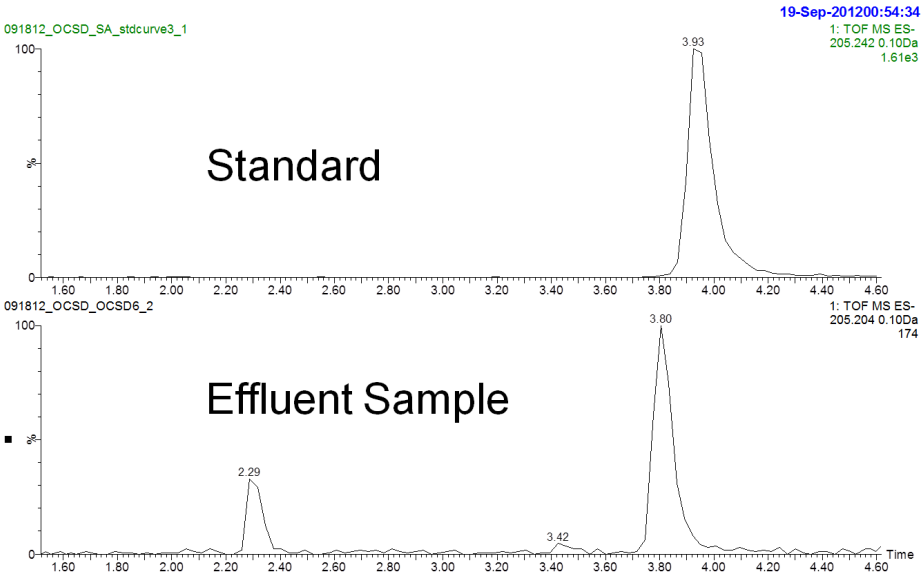

Figure 3.3. Standard and effluent sample EICs of 3,5-dichlorosalicylic acid showing differences in retention time due to column overloading. Both extracted ion chromatograms were processed with a 0.1 Da window.

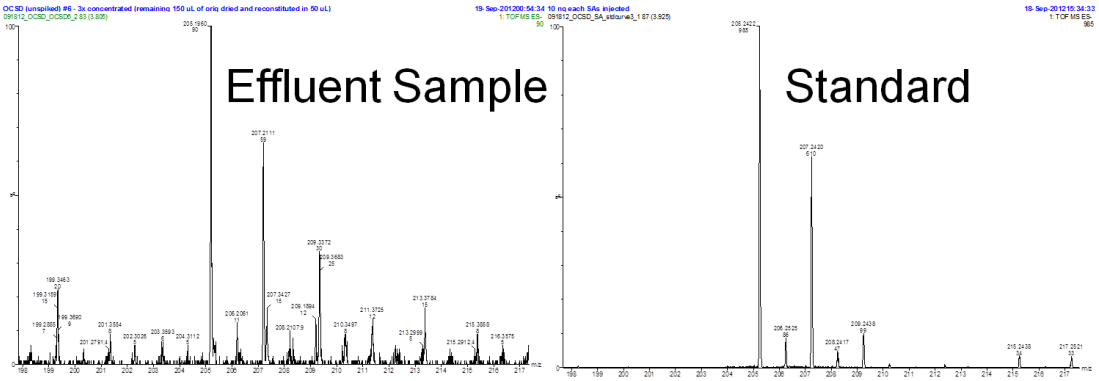

Figure 3.4. Standard and effluent sample MS spectra from EICs showing the good correspondence of intensity ratios for 3,5-dichlorosalicylic acid's M and M+2 m/z.

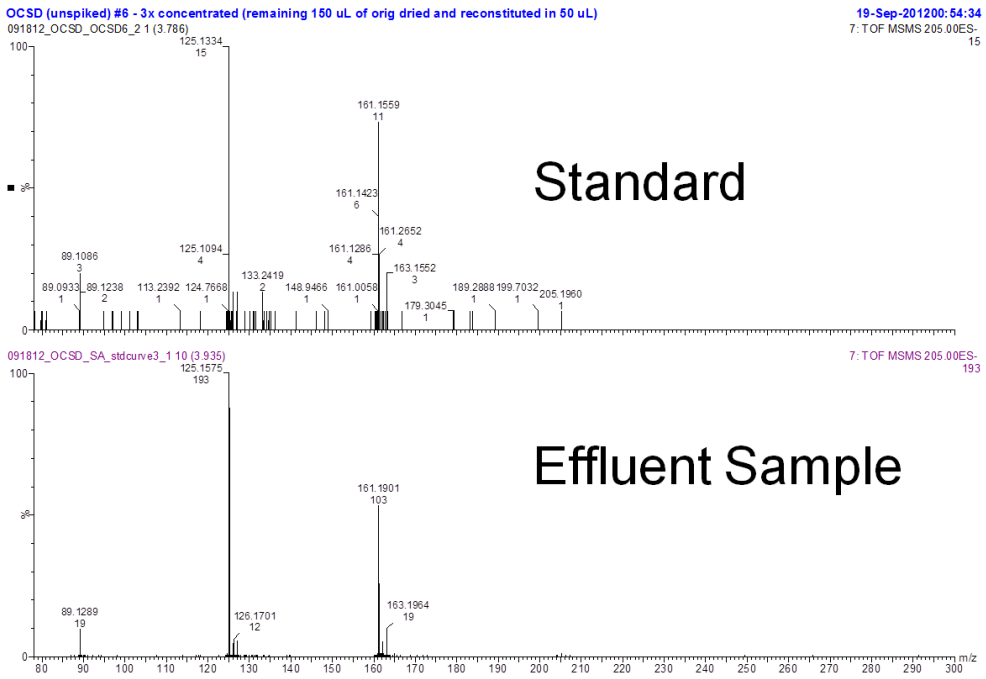

Figure 3.5. Standard and effluent sample MS-MS spectra of 3,5-dichlorosalicylic acid showing identical MS-MS transitions. Collision voltage used was 12 eV.

The chromatographic and spectral data used to determine recovery and ion suppression effects exhibit signs of sample-dependent problems that may be related to the nature of this specific wastewater grab sample and the sample enrichment and analysis methods employed. For example, spiked recovery samples for bisphenol A yielded no detectable MS signal upon UPLC-QTOF-MS analyses. Overloading of the column in the HPLC offline enrichment step could lead to retention time shifts possibly even shifting the analyte out of the fraction collection windows determined for the standard mixture shown in Figure 3.6A. Bisphenol A elutes in a crowded region of the chromatogram the complexity of effluent extract sample is considered. Figure 3.6B shows a chromatogram of a wastewater SPE extract measured using the same HPLC conditions as in Figure 3.6A . In Figure 3.6B the increased UV absorbance in the region of the chromatogram in which bisphenol A elutes is likely indicative of a large number of wastewater matrix components which are collected along with bisphenol A. The presence of these components may cause bisphenol A elute at a different retention time than in A. In addition, the UPLC-QTOF-MS method used for the analysis of the bisphenol A fraction is optimized for the ionization of phenolic compounds. Due to the complexity of wastewater matrix, it is likely that the large UV peak in the region where bisphenol A is expected to elute contains other phenolic compounds whose ionization may greatly suppress the response of the bisphenol A in spiked samples. Indeed, the ion suppression of 75% measured at a spiked level of 500 ng/L bisphenol A is one of the highest values observed for

any compound in this study. The explanations suggested for poor recovery of bisphenol A from spiked wastewater samples could be confirmed in future experiments through the use of an isotopically labeled standard, which could reveal additional reasons for the loss of this compound.

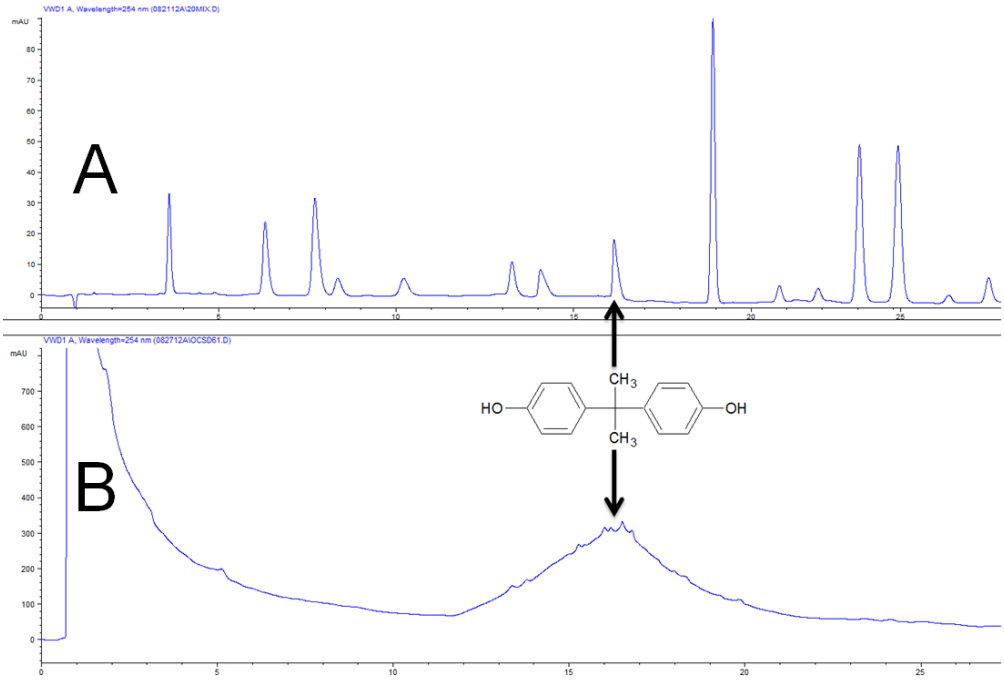

Figure 3.6. HPLC-UV chromatogram of PPCP and TP standards (A), and wastewater effluent SPE extract (B). The peak for bisphenol A standard in (A) and expected elution region in wastewater extract (B) are highlighted with arrows.

Poor analyte recovery and high ion suppression have been noted in numerous articles on wastewater PPCP analysis. In 2006, Gros et al. reported recoveries of 50% for gemfibrozil spiked at 1,000 ng/L in a Spanish wastewater effluent using sample treatment by C18 SPE followed by LC-QQQ-MS analysis.[39] Recoveries for some of the other analytes investigated were even lower, for example acetaminophen, atenolol, pravastatin, ranitidine and erythromycin at 8, 3, 0, 0 and 0%, respectively. In addition, retention time shifts between standards and samples were up to 3%, consistent with the 3% shift reported for 3,5-dichlorosalicylic acid in Figure 3.4. Measurements of gemfibrozil and diclofenac in wastewater effluent without the use of SPE sample concentration yielded detection and quantification limits similar to the values reported in Table 3.3, highlighting the relative insensitivity of the QTOF-MS platform used in this study (e.g. ng/L LODs) compared to a similar vintage QQQ-MS instrument (e.g. pg/L LODs).[39] Similar poor recoveries were noted by Ternes et al. from wastewater effluent, with values under 20% for 4-aminoantipyrene, oxylphenbutazone and phenylbutazone after correcting for ion suppression using 10,11-dihydrocarbamazepine as a surrogate standard.[40] In summary, improvements in sample cleanup and the use of a more sensitive analytical platform for analyte detection will likely lead to greater confidence in the levels of the parent contaminants and their halogenated TPs in wastewater samples. These issues will be revisited and addressed in detail in Chapter 4.

3.3.4 Chromatographic log P assay: Comparison of experimental results to *in silico* prediction. Reported in Table 3.4 are the results from the chromatographic log P assay for the compounds selected for analysis in this chapter, calculated using the linear regression reported in Figure 3.7. Since the parent compounds salicylic acid, diclofenac, bisphenol A and gemfibrozil were used in the training set for calibration of the assay, their log Ps were not determined using the linear regression reported in section 3.2.10. In addition, the data for tetrachlorobisphenol A was omitted from this data set as its log P of 6.22 translates into a retention time of well over 300 min; at the flowrate used in this assay, the observed peak was broad and non-Gaussian rendering retention times determined by integration unreliable. This compound and others of a similar log P value could be accommodated in a chromatographic log P assay by raising the % B used during HPLC analysis, which would reduce its retention time. Such an alteration would require a recalibration of the training compound set data, and would likely require the addition of more training compounds with similar and higher log P values which were not available at the time of these analyses.

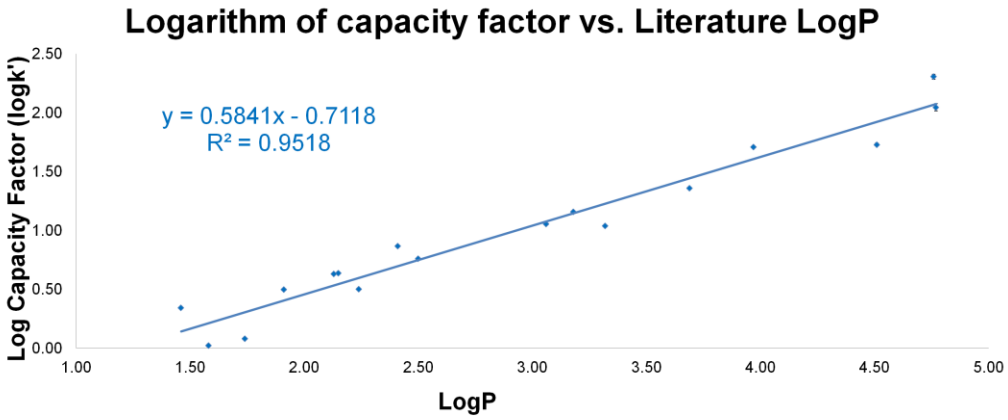

Figure 3.7. Linear regression showing the relationship between the log transformed capacity factor (log k') and the literature log P value for the 17 training set compounds. The small size of the vertical error bars, reflective of the error in analyte retention time, indicates the excellent reproducibility of the method.

Table 3.4. Log P values for selected compounds from experimental chromatographic results and US EPA EPI Suite 4.0 *in silico* predictor.

Compound	Literature Log P	Chromatographic Log P	*In Silico* Log P
Salicylic acid	2.24	N/A	2.24
3-Chlorosalicylic acid	N/A	2.61	2.89
5-Chlorosalicylic acid	N/A	2.70	2.89
3,5-Dichlorosalicylic acid	N/A	3.24	3.53
3-Bromosalicylic acid	N/A	2.75	3.13
5-Bromosalicylic acid	N/A	2.83	3.13
3,5-Dibromosalicylic acid	N/A	3.51	4.02
Diclofenac	4.51	N/A	4.02
Chlorodiclofenac	N/A	4.68	4.66
Bromodiclofenac	N/A	4.78	4.91
Bisphenol A	3.32	N/A	3.64
Gemfibrozil	4.77	N/A	4.77
Chlorogemfibrozil	N/A	5.28	5.41
Bromogemfibrozil	N/A	5.46	5.66

For the most part there is good agreement (<0.5 log P) between the chromatographically determined log P and the software predicted log P values. In addition, the error on the measured log P values is <0.01, indicating excellent reproducibility. The R^2 of 0.95 for the linear relationship between measured and literature log P values for the training set is acceptable, however this is lower than literature reported correlation coefficients which can be >0.99 though most literature studies focus only on non-ionizable analytes probably due to their relative ease of analysis.[41] The results in Table 3.4 concern ionizable analytes, and though the pH of the mobile phase was adjusted to 1 pH unit lower the lowest analyte pKa (salicylic acid pKa = 3) to ensure all analytes were predominantly in their non-ionized form, equilibrium dictates that some population of the analyte injected on the HPLC will exist in the ionized form on the timescale of the measurement.[23] The column choice is extremely important in chromatographic log P assays, as the theoretical basis for the experiment assumes that analytes are only partitioning between the mobile phase and C18 stationary phase.[42] This model does not account for effects such as ionization of analytes, peak deformation due to dead volume or unwanted interactions with impurities in the column stationary phase such as residual silanol groups. Towards this end, the Waters Xterra MS C18 column used in this study was chosen for its exceedingly low silanol content, reported to be ~0% by Mendéz et al.[43] In addition, the training compounds were chosen due to their similar functional groups and physiochemical properties (i.e. molecular weight, pKa, log

P) as the selected analytes and thus can be expected to have similar chromatographic behavior. As performing physical log P measurements using the traditional shake-flask experiment is solvent, sample and labor intensive, the use of chromatographic log P assays can provide automation, increased throughput and reduced sample usage.[44] Such measurements still require the use of authentic pure standards. Since most PPCP TPs are not commercially available, log P measurements first require the synthesis and purification of the TPs as performed in this chapter.

The use of software prediction for log P data is an alternative to physical measurements. The use of *in silico* prediction for calculating molecule lipophilicities has become increasingly popular, and there are many free software packages available which can accomplish such calculations (e.g. EPI Suite, ChemMine, VCCL, ChemAxon, Molinspiration). The estimation of log P with software is useful because of its ease and speed, which are unmatched when compared with physical measurements. Log P is an important metric when considering environmental contaminants such as PPCPs and their TPs, as it correlates well with toxicity and can be used to predict parameters relating to the fate and transport of such molecules as long as their structure is known.[45-48] Predicting physiochemical properties is extremely useful when considering environmental risk assessment of a large set of chemicals, a large number of which have not been adequately characterized.[49, 50] Much like the training set of compounds used in the chromatographic log P assay described in this chapter,

predictive software packages must be trained with a large enough set of known physiochemical quantitative structure-activity relationships to be able to yield accurate predictions. As the knowledge base for physiochemical parameters for PPCPs and their TPs widens, *in silico* predictions will likely improve.[51] However, for now there is some disagreement between software prediction and physical measurements as evidenced by the data in Table 3.4, namely that the predicted value is greater than the measured log P value in almost every case.

3.4 Conclusions

The methods developed and implemented in this chapter allowed for the successful generation of pure standards of PPCP TPs, the targeted analysis of secondary wastewater effluent extracts for a set of 15 compounds resulting in the quantification of 3 parent PPCPs and 5 of their halogenated TPs. Difficulties encountered in the analysis of wastewater extracts led to poor recoveries and problems due to ion suppression. The relatively poor sensitivity of the QTOF-MS instrument utilized in this study compared with that provided modern QQQ-MS instruments further hampered these experiments. In addition, the lack of isotopically labeled standards for the selected PPCPs and their TPs prevented truly unambiguous identification and quantification. These limitations are addressed in Chapter 4.

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Chapter Four

Occurrence of Halogenated Transformation Products of Selected Pharmaceuticals and Personal Care Products in Secondary and Tertiary Wastewaters from Southern California

Acknowledgements: I would like to acknowledge my collaborators at Los Angeles County Sanitation District, Eric Nelson and Steve Carr, for providing several deuterated standards and allowing the use of their facilities, instrumentation and manpower for the extraction and analysis of wastewater samples. I am also grateful to Dr. Kemal Solakyildirim for his assistance in the structure elucidation of several halogenated compounds using NMR.

Abstract: The study of halogenated TPs of PPCPs is hampered by the availability of authentic standards. This study combines the use of isotopically labeled and unlabeled authentic standards, synthesized, isolated and characterized herein, to accurately identify and quantify selected compounds in several secondary and tertiary wastewater samples. This is the first literature report of the detection of halogenated TPs of diclofenac, naproxen, and *tert*-octylphenol at ng/L levels in real-world wastewater effluents.

4.1 Introduction

The research presented in this chapter focuses on the occurrence of seven PPCPs and their halogenated TPs in secondary and tertiary chlorinated wastewaters from several WWTPs in Southern California. Halogenated TPs of the plasticizer bisphenol A, surfactants technical nonylphenol and *tert*-octylphenol, the antilipidemic gemfibrozil, the non-steroidal anti-inflammatories naproxen and diclofenac and the analgesic metabolite salicylic acid were synthesized, purified and characterized. The structures of the selected compounds and their TPs are presented in Figures 4.1.1 and 4.1.2. The use of authentic standards for the identification of TPs in wastewater extracts allows accurate determination of the concentrations of the compounds as reported in this study. In addition, deuterium-enriched analogs of halogenated TPs were synthesized and purified, allowing for accurate determination of analyte recovery and ion suppression due to matrix effects during LC-QQQ-MS analyses.[1] The use of isotopically labeled standards provides for more confident quantification and identification of the selected analytes as their chromatographic behavior is identical to the unlabeled compound.[2] Implementation of multiple reaction monitoring (MRM) in conjunction with authentic standards allows the additional use of retention time for identification of PPCPs and their TPs in wastewater effluent samples.

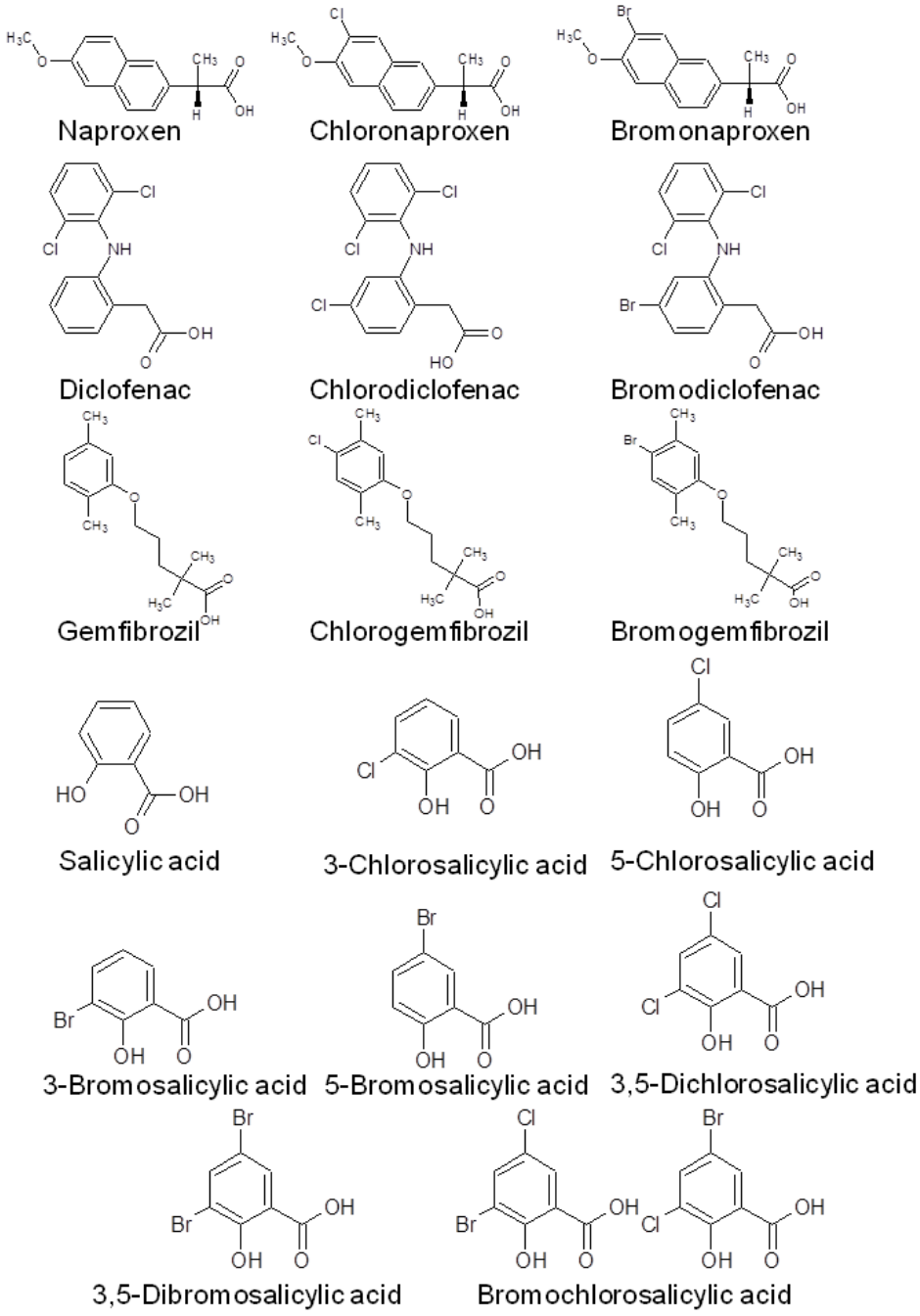

Figure 4.1.1. Selected PPCPs and their TPs which were synthesized, isolated and characterized for use as authentic standards. Structures were created using Advanced Chemistry Development Labs (Toronto, CA) 12.0

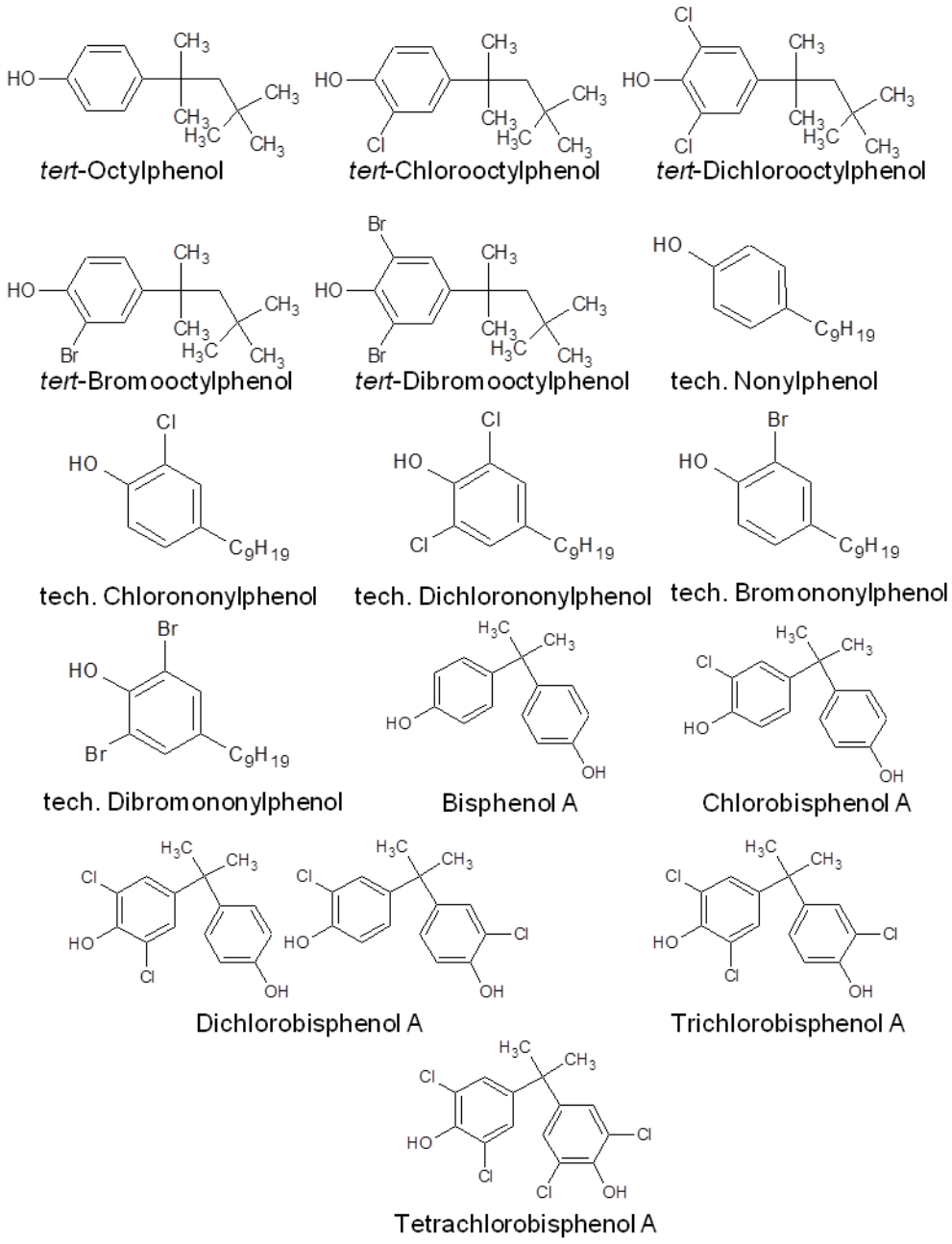

Figure 4.1.2. Selected PPCPs and their TPs which were synthesized, isolated and characterized for use as authentic standards. Structures were created using Advanced Chemistry Development Labs (Toronto, CA) 12.0

4.2 Materials and methods

4.2.1 Chemicals and suppliers. A 13% active Cl_2 solution of NaOCl was obtained from Acros (Geel, Belgium). Gemfibrozil was obtained from Spectrum Chemical Manufacturing Company (New Brunswick, NJ). Sodium formate, potassium bromide, formic acid, acetic acid, hydrochloric acid, methanol, technical nonylphenol, naproxen, bisphenol A, *tert*-octylphenol, bisphenol A-d_{16} and sodium thiosulfate were obtained from Sigma Aldrich (St. Louis, MO). LC-MS grade acetonitrile and salicylic acid were obtained from Fisher Scientific (Pittsburgh, PA). HPLC-grade water was obtained from Burdick and Jackson (Morristown, NJ). Diclofenac was obtained from Cayman Chemical Company (Ann Arbor, MI). Tetrachlorobisphenol A was obtained from TCI America (Portland, OR). Salicylic acid-d_4, diclofenac-d_4, gemfibrozil-d_6, naproxen-d_3, bisphenol A-d_6 and 4-n-octylphenol-d_{17} were obtained from C/D/N Isotopes (Quebec, Canada).

4.2.2 Wastewater samples. Final post-chlorination secondary and tertiary effluent wastewater samples were collected from several WWTPs in Southern California. Three tertiary WWTPs chlorinate effluent using Cl_2 gas, designated effluents A (n=4), B (n=2) and C (n=1), while five tertiary WWTPs use NaOCl, designated effluents D (n=3), E (n=2), F (n=2), G (n=1) and H (n=1). Both secondary effluents, designated I (n=9) and J (n=4), use NaOCl as their chlorinating agent. Samples were collected in 1 L amber glass bottles which had

been thoroughly cleaned with methanol and rinsed with deionized water. Approximately 2 g of sodium thiosulfate was added to each liter of wastewater to quench residual chlorine. Samples were immediately transported in the dark to a 4 °C refrigerator for storage and extracted within 7 days of collection.

4.2.3 Synthesis and isolation of halogenated TP standards. Halogenated TP standards of the selected PPCPs in this study were generated by the addition of NaOCl solution to a stirred mixture of the parent PPC dissolved in methanol:water 1:1 (*p*-dioxane:water 1:1 for the alkylphenols to prevent the formation of methoxylated TPs [3]), analogous to the methods presented in sections 2.2.4 and 3.2.3-3.2.4. Evolution of halogenation reactions was monitored by LC-QTOF-MS, and reactions were quenched once the parent compound had fully attenuated into its TPs. Reactions were then quenched with sodium thiosulfate, acidified to pH ~3, concentrated by C18 SPE and evaporated to near-dryness in a 40 °C water bath under a gentle stream of compressed air. Concentrated reaction mixtures were purified by analytical scale RP-HPLC-UV.

4.2.4 TP standard accurate mass MS and NMR characterization. Accurate mass experiments used the lock-spray mode, with 10 ng/µL tetrachlorobisphenol A ($[M-H]^-$ = 362.9519 Da) in 50:50 1 mM ammonium acetate:acetonitrile as the lock mass solution; a 10 ng/µL naproxen ($[M-H]^-$ = 229.0870 Da) solution in the same mobile phase was used as a lock mass for

accurate mass determination of tetrachlorobisphenol A. The lock mass solution was directly infused into the reference source by syringe pump at 25 µL/min and sampled at a frequency of 0.2 Hz. Accurate mass spectra were acquired with 1-5 ng/µL solutions of each analyte in methanol, analyzed by direct infusion fed by a syringe pump at 10-20 µL/min to generate ion counts of 300-600 cps. Spectra were collected for 2 min in the m/z scan range 130-600 Da and 20-30 scans were averaged, then processed for accurate mass measurement using the built-in accurate mass TOF correction in the MassLynx software. The mass spectrometer was calibrated after every 5 accurate mass measurements using a sodium formate solution per the manufacturer's recommendation. Calibration for the time-of-flight sector of the spectrometer was under 3 ppm RMS error for the m/z region 100-1000 Da. Accurate mass results are reported in Table 4.1, where the mass difference in ppm for the m/z of the measured value for the monoisotopic peak for each analyte is compared to the theoretical m/z as calculated using ACD Labs ChemSketch version 12.0. All accurate mass values have mass errors <5 ppm. The normalized intensities of the predicted vs. measured isotopic abundances for the M (monoisotopic peak) and M+2 (M+1 for non-halogenated parent PPCPs) are reported, all <2% difference, to provide further confirmation of halogenation. The M+4 isotopic abundance is calculated and reported for compounds where the M+4 peak is greater than 2% of the normalized intensity of the base peak.

Table 4.1. Calculated vs. measured accurate mass m/z and normalized isotopic intensity QTOF-MS data for labeled and unlabeled PPCPs and TPs

	Accurate mass m/z (Da)			Calculated/measured isotopic intensities (normalized counts)		
Compound	Calc. $[M-H]^-$	Meas. $[M-H]^-$	Error (ppm)	M	M+2*	M+4**
Salicylic acid	137.0244	137.0242	-1.46	100/100	7.8/9.2	-
3-Chlorosalicylic acid	170.9854	170.9849	-2.92	100/100	33.2/34.8	-
5-Chlorosalicylic acid	170.9854	170.9859	2.92	100/100	33.2/34.7	-
3,5-Dichlorosalicylic acid	204.9435	204.9433	-0.98	100/100	65.6/67.2	11/12.3
3-Bromosalicylic acid	214.9349	214.9351	0.93	100/100	98.4/98.3	-
5-Bromosalicylic acid	214.9349	214.9346	-1.40	100/100	98.4/98.4	-
3,5-Dibromosalicylic acid	292.8454	292.8452	-0.68	51.1/52.8	100/100	49.4/50.8
Bromochlorosalicylic acid	248.8960	248.8960	0	76.5/78.4	100/100	25/27.4
Salicylic acid-d_4	141.0495	141.0498	2.13	100/100	7.7/9.0	-
3-Chlorosalicylic acid-d_3	174.0043	174.0050	4.02	100/100	33.2/34.6	-
5-Chlorosalicylic acid-d_3	174.0043	174.0037	-3.45	100/100	33.2/34.5	-
3,5-Dichlorosalicylic acid-d_2	206.959	206.9586	-1.93	100/100	65.6/66.9	11/11.6
3-Bromosalicylic acid-d_3	217.9538	217.9540	0.92	100/100	98.4/98.1	-
5-Bromosalicylic acid-d_3	217.9538	217.9529	-4.13	100/100	98.4/97.1	-
3,5-Dibromosalicylic acid-d_2	294.8580	294.8590	3.39	51.1/50.9	100/100	49.4/49.6
Naproxen	229.0870	229.0866	-1.75	100/100	15.3/17.1	-
Chloronaproxen	263.0480	253.0475	-1.90	100/100	34.1/35.1	-
Bromonaproxen	306.9975	306.9966	-2.93	100/100	99.2/99.2	-
Naproxen-d_3	232.1058	232.1051	-3.02	100/100	15.3/16.6	-
Chloronaproxen-d_3	266.0669	266.0677	3.01	100/100	33.5/35.3	-
Bromonaproxen-d_3	310.0164	310.0159	-1.61	100/100	98.6/96.6	-
Diclofenac	294.0094	294.0089	-1.70	100/100	66.3/66.5	11.4/12.4
Chlorodiclofenac	327.9704	327.9691	-3.96	100/100	98.7/99.2	33/34.2
Bromodiclofenac	371.9199	371.9188	-2.96	61/61.6	100/100	46.5/46.6
Diclofenac-d_4	298.0345	298.0341	-1.34	100/100	66.3/67.3	11.4/12
Chlorodiclofenac-d_3	330.9893	330.9878	-4.53	100/100	98.7/100	33/33.4
Bromodiclofenac-d_3	374.9387	374.9380	-1.87	61/62.1	100/100	46.5/48
Gemfibrozil	249.1496	249.1497	0.40	100/100	16.7/18.6	-
Chlorogemfibrozil	283.1106	283.1105	-0.35	100/100	34.3/35.7	-
Bromogemfibrozil	327.0601	327.0604	0.92	100/100	99.4/99.2	-
Gemfibrozil-d_6	255.1873	255.1875	0.78	100/100	16.6/18.2	-
Chlorogemfibrozil-d_6	289.1483	289.1491	2.77	100/100	34.2/34.8	-
Bromogemfibrozil-d_6	333.0978	333.0983	1.50	100/100	99.3/99.9	-
Bisphenol A	227.1078	227.1077	-0.44	100/100	16.5/15.7	-
Chlorobisphenol A	261.0688	261.0701	4.98	100/100	34/35.6	-
Dichlorobisphenol A	295.0298	295.0291	-2.37	100/100	66.4/68.2	11.6/13.1
Trichlorobisphenol A	328.9908	328.9903	-1.52	100/100	98.8/98.6	33.1/34.3
Tetrachlorobisphenol A	362.9519	362.9524	1.38	76.2/77.6	100/100	49.6/51.5
Bisphenol A-d_6	233.1454	233.1443	-4.72	100/100	16.5/18.1	-
Chlorobisphenol A-d_{13}	274.1504	274.1501	-1.09	100/100	34/35.6	-
Dichlorobisphenol A-d_{12}	307.1051	307.1049	-0.65	100/100	66.4/68	11.6/12.3
Trichlorobisphenol A-d_{11}	340.0599	340.0614	4.41	100/100	98.8/99.5	33.1/33.5
Tetrachlorobisphenol A-d_{10}	373.0146	373.0151	1.34	76.2/77.6	100/100	49.6/50.9

*M+1 for non-halogenated compounds
**Only reported if M+4 > 2% base ion normalized intensity

Table 4.1. Calculated vs. measured accurate mass m/z and normalized isotopic intensity QTOF-MS data for labeled and unlabeled PPCPs and TPs

	Accurate mass m/z (Da)			Calculated/measured isotopic intensities (normalized counts)		
Compound	Calc. $[M-H]^-$	Meas. $[M-H]^-$	Error (ppm)	M	M+2*	M+4**
n-Octylphenol-d_{17}	222.2665	222.2668	1.35	100/100	15.2/15.4	-
n-Chlorooctylphenol-d_{17}	256.2275	256.2277	0.78	100/100	33.7/34	-
n-Dichlorooctylphenol-d_{17}	290.1885	290.1888	1.03	100/100	66.4/66.8	11.3/11.8
n-Bromooctylphenol-d_{17}	300.1770	300.1768	-0.67	100/100	98.8/99.8	
n-Dibromooctylphenol-d_{17}	378.0875	378.0868	-1.85	50.9/51.7	100/100	49.7/50.8
t-Octylphenol	205.1598	205.1597	-0.49	100/100	15.5/16.8	-
t-Chlorooctylphenol	239.1208	239.1202	-2.51	100/100	33.7/34.5	-
t-Dichlorooctylphenol	273.0818	273.0831	4.76	100/100	66.1/67.7	11.3/12.2
t-Bromooctylphenol	283.0703	283.0711	2.83	100/100	98.8/97.7	-
t-Dibromooctylphenol	360.9808	360.9823	4.16	50.9/49.5	100/100	49.7/51
tech. Nonylphenol	219.1754	219.1753	-0.46	100/100	16.6/18.2	-
tech. Chlorononylphenol	253.1365	253.1354	-4.35	100/100	33.9/34	-
tech. Dichlorononylphenol	287.0975	287.0963	-4.18	100/100	66.3/66.1	11.4/12.8
tech. Bromononylphenol	297.0859	297.0870	3.70	100/100	99/99.5	-
tech. Dibromononylphenol	374.9964	374.9963	-0.27	50.9/52.2	100/100	49.9/50.6

*M+1 for non-halogenated compounds
**Only reported if M+4 > 2% base ion normalized intensity

Confirmation of the regiochemistry of halogenation presented in Figures 4.1.1 and 4.1.2 for natural abundance TPs was carried out by one-dimensional ^{1}H and two-dimensional [^{1}H,^{13}C] heteronuclear multiple bond correlation (HMBC) NMR spectroscopy using a 600 MHz Bruker Avance spectrometer (Billerica, MA). The aromatic region of the ^{1}H NMR spectra of salicylic acid, bisphenol A, gemfibrozil, diclofenac, naproxen, technical nonylphenol and *tert*-octylphenol and their halogenated TPs are presented in Figures 4.2, 4.3, 4.4, 4.5, 4.6, 4.7 and 4.8, respectively. Samples were prepared by dissolving 0.5-10 mg of compound in methanol-d_4 with the residual ^{1}H resonance of the solvent serving as the chemical shift reference. Spectra were measured using a 3 mm sample tube and standard Bruker pulse programs. Integration of the ^{1}H NMR resonances between 0 and 8 ppm, ignoring the methanol-d_4 signals, was used to calculate purity. All TP standards were >97% pure except for three of the monohalogenated salicylic acid TPs which were difficult to completely resolve during RP-HPLC-UV isolation of the TP standards due to their similar chromatographic behavior. The sample of 3-chlorosalicylic acid contains 5% of 5-chlorosalicylic acid, 3-bromosalicylic acid contains 10% of 5-bromosalicylic acid, and 5-bromosalicylic acid contains 5% of 3-bromosalicylic acid. These purity values were taken into account during solution preparation for mixtures of standards.

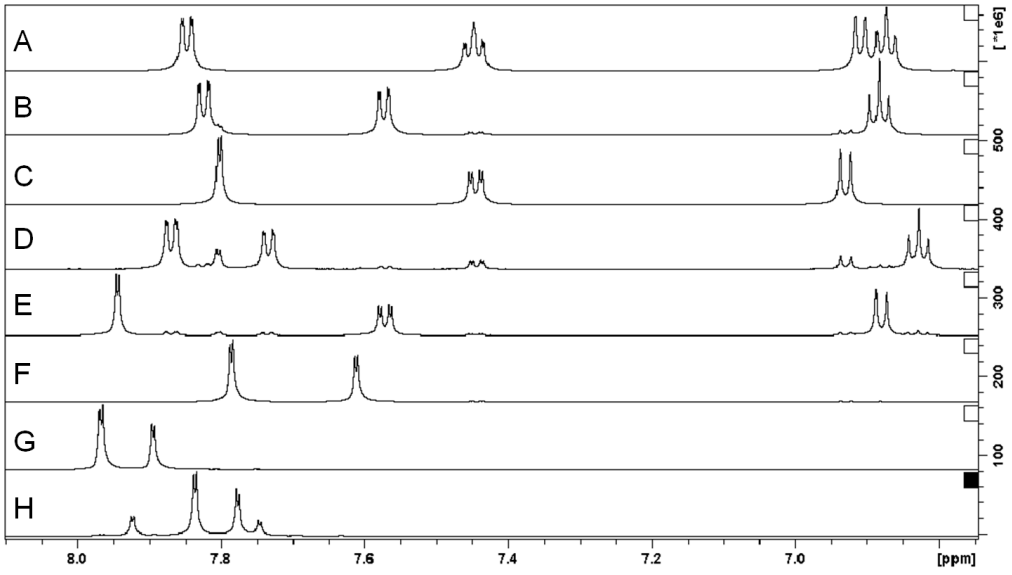

Figure 4.2. ^{1}H NMR spectra of (A) salicylic acid, (B) 3-chlorosalicylic acid, (C) 5-chlorosalicylic acid, (D) 3-bromosalicylic acid, (E) 5-bromosalicylic acid, (F) 3,5-dichlorosalicylic acid, (G) 3,5-dibromosalicylic acid, and (H) bromochlorosalicylic acid. Bromochlorosalicylic acid (H) can be seen to be a mixture of 3-chloro-5-bromosalicylic acid and 3-bromo-5-chlorosalicylic acid in a 1:6 ratio, though it is not possible to distinguish the two compounds by NMR.

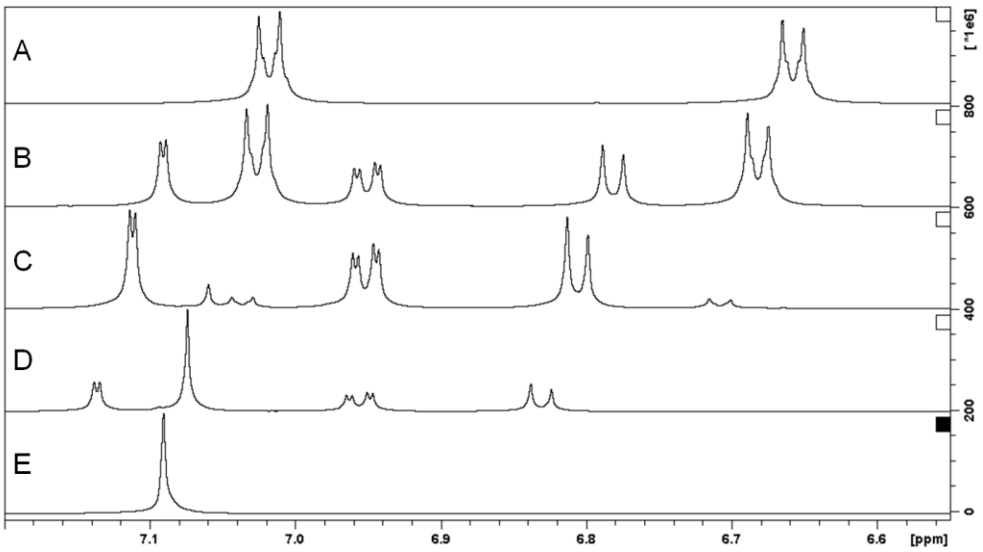

Figure 4.3. ^{1}H NMR spectra of (A) bisphenol A, (B) monochlorobisphenol A, (C) dichlorobisphenol A, (D) trichlorobisphenol A, and (E) tetrachlorobisphenol A. Dichlorobisphenol A (C) is a mixture of 2,6-dichlorobipshenol A and 2,2'-dichlorobisphenol A in a 1:10 ratio.

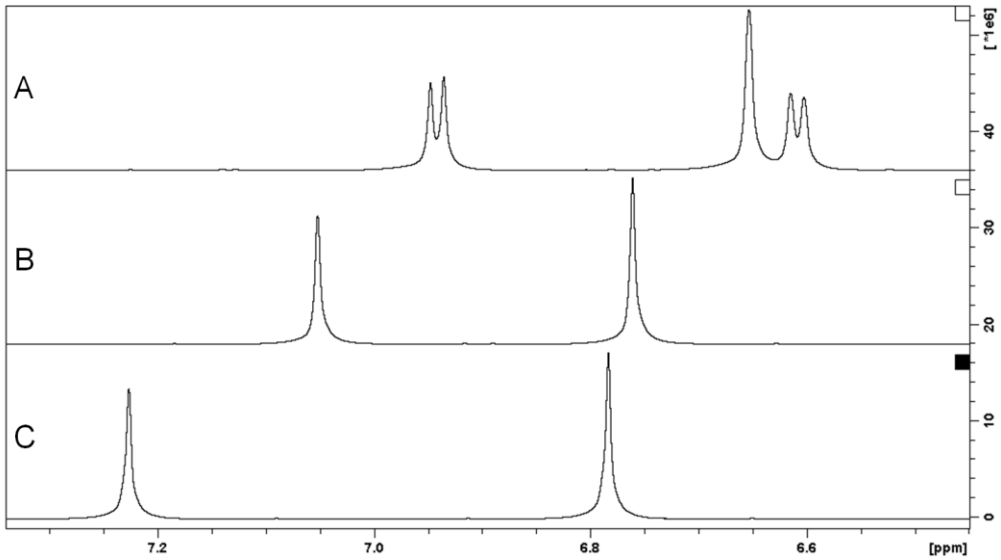

Figure 4.4. ^{1}H NMR spectra of (A) gemfibrozil, (B) chlorogemfibrozil, and (C) bromogemfibrozil.

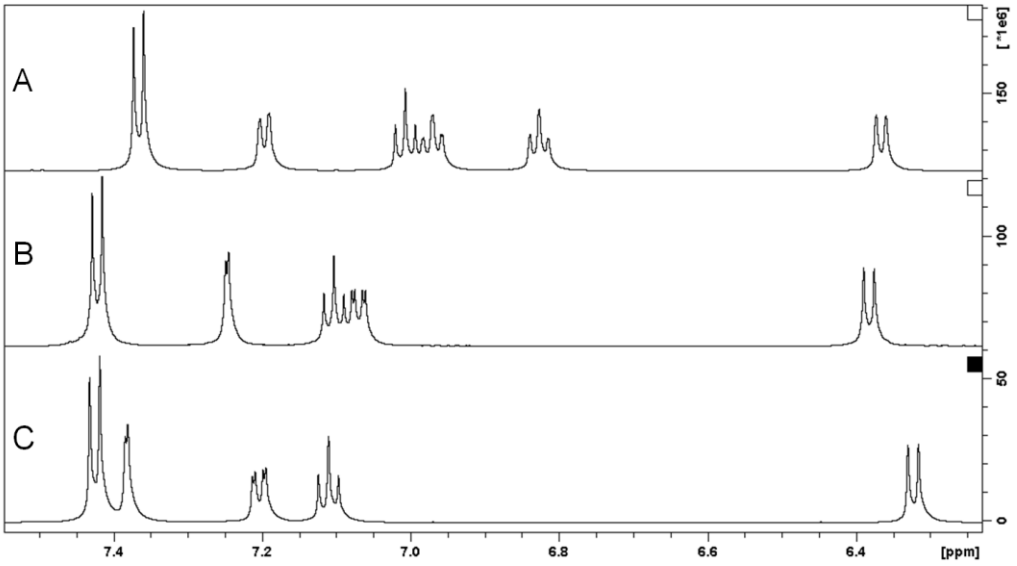

Figure 4.5. ^{1}H NMR spectra of (A) diclofenac, (B) chlorodiclofenac, and (C) bromodiclofenac.

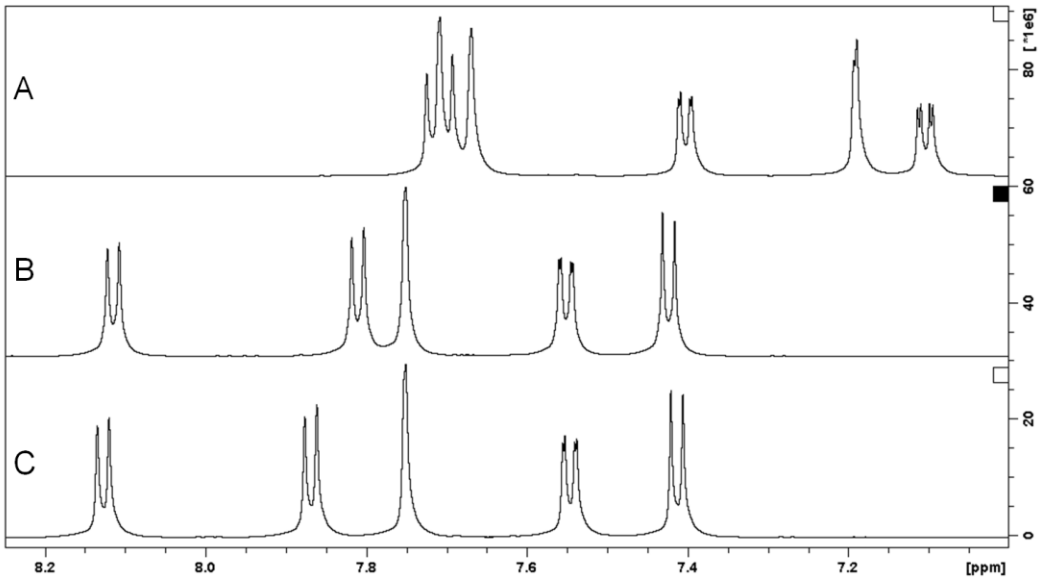

Figure 4.6. ^{1}H NMR spectra of (A) naproxen, (B) chloronaproxen, and (C) bromonaproxen.

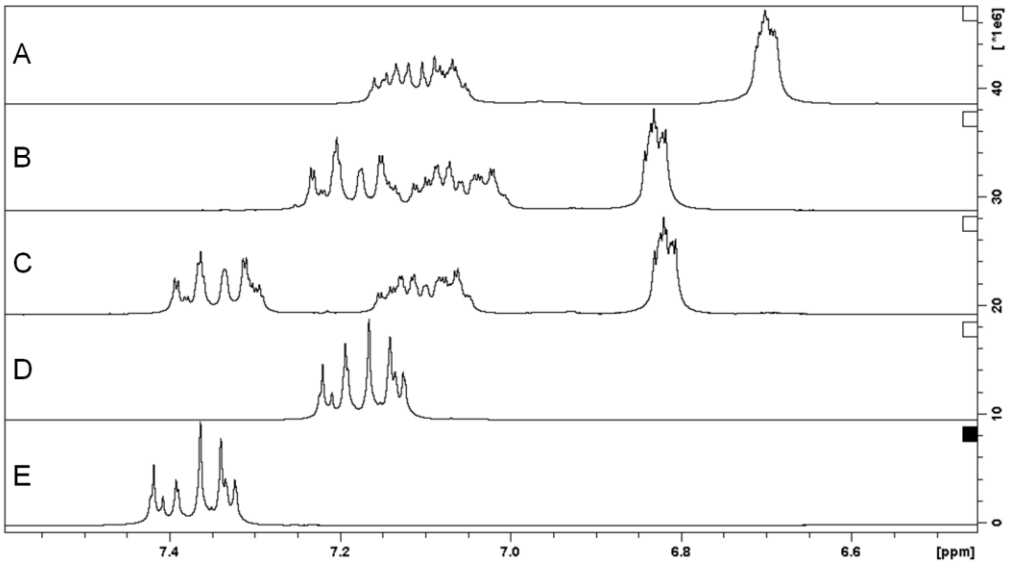

Figure 4.7. 1D 1H NMR spectra of (A) technical nonylphenol, (B) technical monochlorononylphenol, (C) technical dichlorononylphenol, (D) technical monobromononylphenol and (E) technical dibromononylphenol.

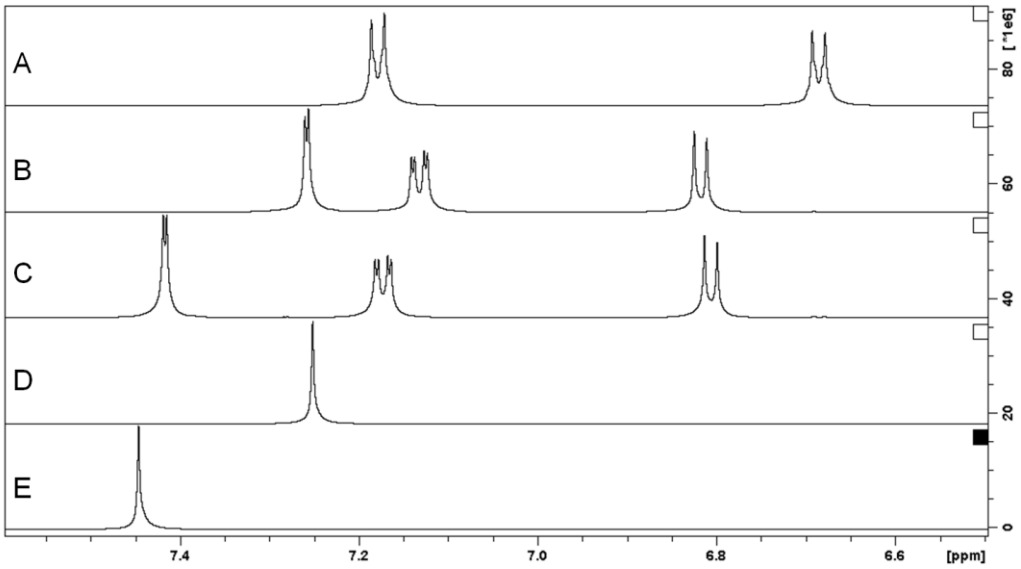

Figure 4.8. 1D 1H NMR spectra of (A) *tert*-octylphenol, (B) *tert*-monochlorooctylphenol, (C) *tert*-dichlorooctylphenol, (D) *tert*-monobromooctylphenol and (E) *tert*-dibromooctylphenol.

4.2.5 Automated SPE extraction of wastewater samples. All wastewater samples were extracted using 200 mg Waters Oasis HLB SPE cartridges (Milford, MA). Extractions were carried out using an Autotrace automated SPE extractor (Caliper Life Sciences, Hopkinton, MA) using the following procedure. Internal standards for each compound were added at concentrations ranging from 100-250 ng/L to 200 mL wastewater samples with no pH adjustment. Samples were shaken briefly to allow equilibration of the internal standards. The SPE cartridges were sequentially conditioned with 5 mL dichloromethane, 5 mL methanol, then 8 mL water all at 20 mL/min. The 200 mL wastewater sample was then passed through the cartridge at 10 mL/min. The cartridge was washed with 3 mL water followed by 4.5 mL of 5% methanol in water both at 20 mL/min. The extracts were blown to near-dryness with a gentle stream of nitrogen, and reconstituted in 1 mL methanol for LC-QQQ-MS analysis. All samples and standards were analyzed in duplicate.

Laboratory control duplicate samples consisting of 200 mL deionized water spiked with unlabeled standards at concentrations between 100-250 ng/L were extracted and analyzed after every 10 wastewater extractions. Recoveries were 100±20% for all compounds except technical nonylphenol (100-130%) and *tert*-octylphenol (90-170%) and their TPs. The results obtained for technical nonylphenol and *tert*-octylphenol are consistent with the poor recovery of their internal standards as previously reported by Nelson et al.[4] Recoveries between duplicate laboratory control spikes agreed within 20% in all cases. Triplicate

method blanks consisting of deionized water were extracted and analyzed to ensure no contamination was introduced during the extraction and analysis process. No contamination was observed except for a small amount of technical nonylphenol, attributed to trace surfactant residues present in the extraction and analysis laboratories.[4] A composite sample of tertiary effluent B was used to determine recovery for all tertiary effluent samples, while a composite sample of secondary effluent I was used to determine recovery for all secondary effluent samples. Both effluent samples were spiked with deuterated internal standards in the low ng/L range, SPE extracted and the amounts quantified. Recoveries (Table 4.2) were calculated by comparing the MS peak areas measured for the deuterated standard mixture and the spiked and SPE extracted effluent samples analyzed by LC-QQQ-MS. The recoveries were corrected for ion suppression effects by adding the deuterated standards mixture into effluent extracts post SPE concentration, and comparing the peak areas for the quantifying MRM transitions (Table 4.3) of the extract and the pure standards samples.

Table 4.2. Recovery from spiked tertiary and secondary effluent samples (n=4) and laboratory control spike average recoveries (n=6).			
Compound	Recovery from wastewater B (%)	Recovery from wastewater I (%)	Laboratory control spike recovery (%)
Salicylic acid	98±1	100±4	-
3-Chlorosalicylic acid	96±6	101±5	-
5-Chlorosalicylic acid	100±11	109±11	-
3,5-Dichlorosalicylic acid	99±1	103±8	-
3-Bromosalicylic acid	100±1	103±0	-
5-Bromosalicylic acid	101±8	103±5	-
3,5-Dibromosalicylic acid	103±0	113±5	-
Bromochlorosalicylic acid	105±6	100±7	-
Naproxen	94±0	110±16	93±6
Chloronaproxen	99±1	101±16	94±8
Bromonaproxen	102±5	108±15	98±9
Diclofenac	106±4	107±5	98±8
Chlorodiclofenac	102±3	115±24	98±11
Bromodiclofenac	100±0	97±10	104±12
Gemfibrozil	98±3	106±16	93±8
Chlorogemfibrozil	101±3	118±11	99±7
Bromogemfibrozil	99±10	110±16	100±10
Bisphenol A	103±14	100±3	101±9
Chlorobisphenol A	91±7	100±13	92±7
Dichlorobisphenol A	98±2	110±14	98±9
Trichlorobisphenol A	99±3	115±13	94±7
Tetrachlorobisphenol A	102±11	114±17	98±8
tech. Nonylphenol	130±4	97±5	132±11
tech. Monochlorononylphenol	112±4	124±20	122±8
tech. Dichlorononylphenol	108±11	119±23	109±7
tech. Monobromononylphenol	122±12	135±26	119±10
tech. Dibromononylphenol	91±5	105±23	102±10
t-Octylphenol	100±9	98±3	91±9
t-Monochlorooctylphenol	125±3	153±19	158±8
t-Dichlorooctylphenol	101±3	124±34	145±10
t-Monobromooctylphenol	110±5	143±12	95±14
t-Dibromooctylphenol	131±11	134±22	141±8

4.2.6 LC-QQQ-MS parameters. All separations were performed using a Shimadzu HPLC system (Columbia, MD) using a 10ADvp binary pump running at 0.4 mL/min and SIL-HTc autosampler unit held at 4 °C. The mobile phases used were A: 0.5 mM ammonium acetate; B: methanol except for salicylic acid and its TPs, which use A: 0.1% formic acid.

Salicylic acid and its halogenated TPs were analyzed on a 2.1x50 mm, 3 µm Agilent (Santa Clara, CA) Zorbax C18 column with a 2 µL sample injection. The gradient used was 3% B 0-1 min, 3-25% B 1-5 min, 25-99% B 5-6 min, 99% B 6-11 min, 99-3% B 11-13 min, 3% B 13-20 min. A standard chromatogram showing the quantifying MRM transitions is presented in Figure 4.9.

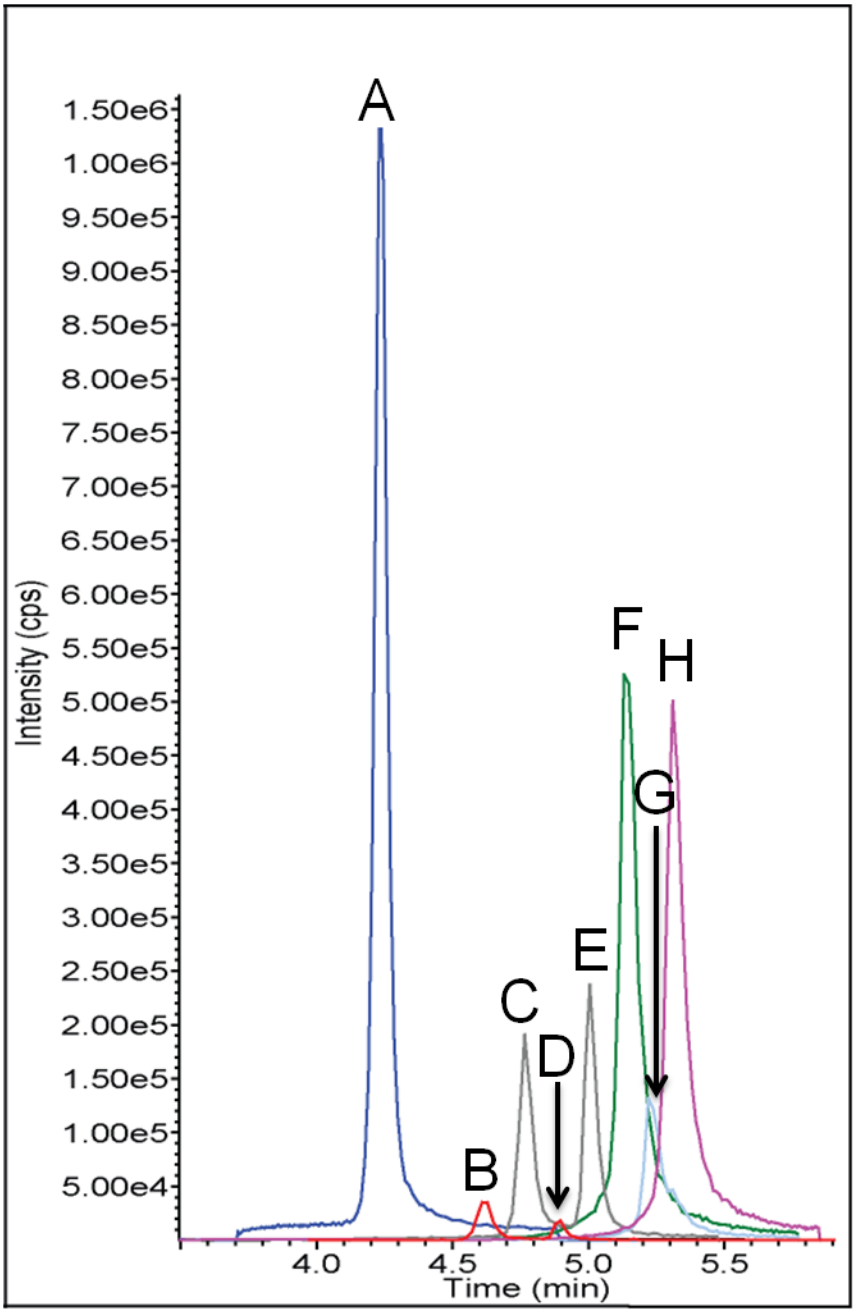

Figure 4.9. Standard LC-QQQ-MS chromatogram of the quantifying MRM transitions for (A) salicylic acid, (B) 3-chlorosalicylic acid, (C) 5-chlorosalicylic acid, (D) 3-bromosalicylic acid, (E) 5-bromosalicylic acid, (F) 3,5-dichlorosalicylic acid, (G) bromochlorosalicylic acid and (H) dibromosalicylic acid.

Naproxen, diclofenac, bisphenol A, gemfibrozil and their TPs were analyzed on a 2.1x50 mm, 3 µm Zorbax C18 column with a 5 µL sample injection. The gradient used was 3-25% B 0-1 min, 25-99% B 1-6 min, 99% B 6-11 min, 99-3% B 11-13 min, 3% B 13-20 min. A standard chromatogram of the quantifying MRM transitions is presented in Figure 4.10.

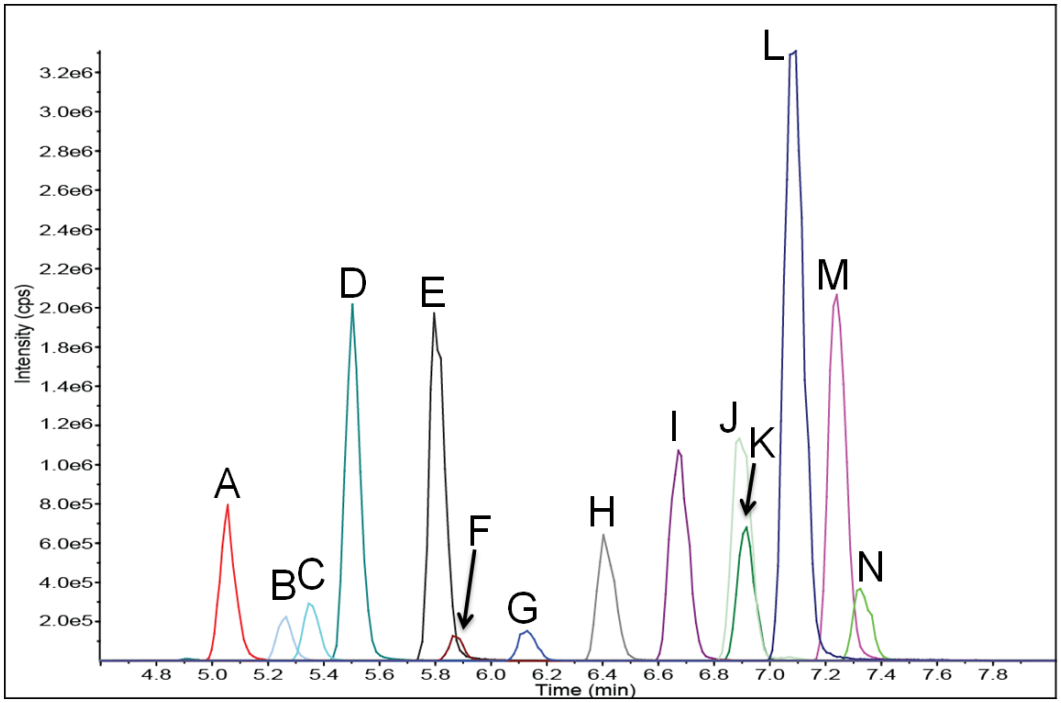

Figure 4.10. Standard LC-QQQ-MS chromatogram of the quantifying MRM transitions for (A) naproxen, (B) chloronaproxen, (C) bromonaproxen, (D) diclofenac, (E) chlorodiclofenac, (F) bromodiclofenac, (G) bisphenol A, (H) chlorobisphenol A, (I) dichlorobisphenol A, (J) trichlorobisphenol A, (K) gemfibrozil, (L) tetrachlorobisphenol A, (M) chlorogemfibrozil, and (N) bromogemfibrozil.

Technical nonylphenol, *tert*-octylphenol and their TPs were analyzed on a 2.1x50 mm, 3 µm Thermo (Waltham, MA) Aquasil column with a 5 µL sample injection. The gradient used was 50% B 0-1 min, 50-95% B 1-4.5 min, 95% B 4.5-8 min, 95-50% B 8-8.1 min, 50% B 8.1-13 min.

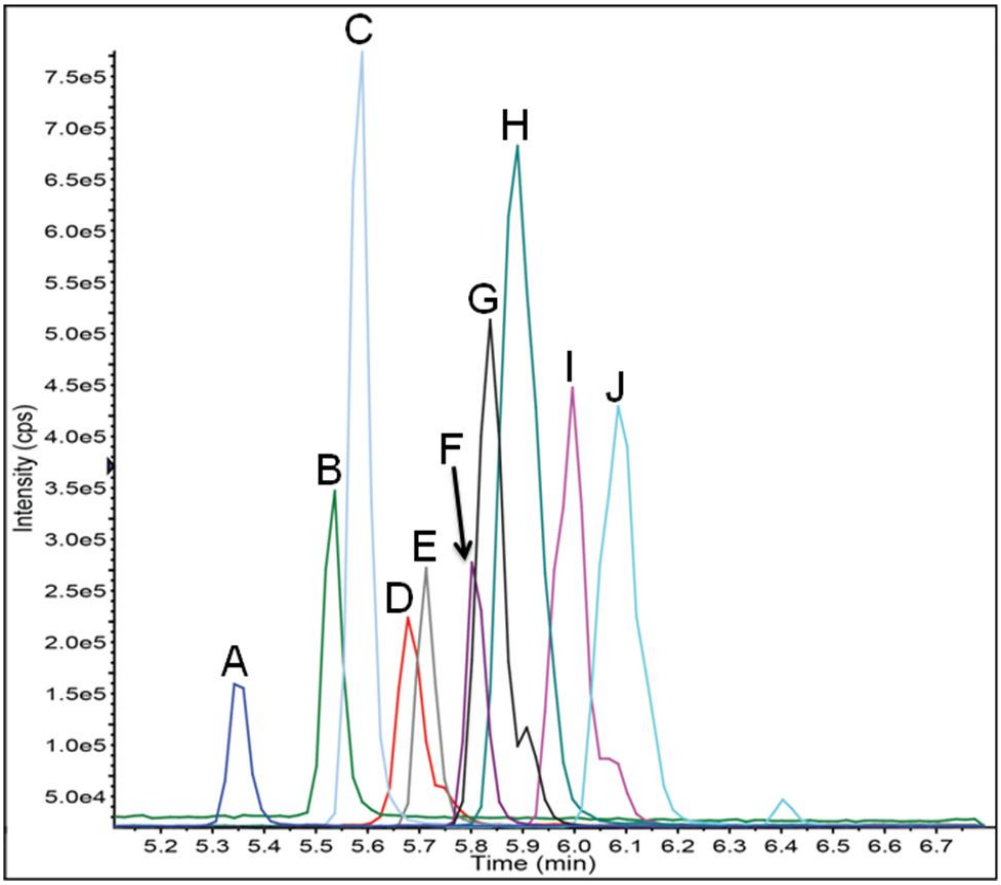

Figure 4.11. Standard LC-QQQ-MS chromatogram of the quantifying MRM transitions for (A) *tert*-octylphenol, (B) *tert*-chlorooctylphenol, (C) *tert*-bromooctylphenol, (D) technical nonylphenol, (E) *tert*-dichlorooctylphenol, (F) *tert*-dibromooctylphenol, (G) technical chlorononylphenol, (H) technical bromononylphenol, (I) technical dichlorononylphenol, and (J) technical dibromononylphenol.

An AB SCIEX (Framingham, MA) API 5000 QQQ-MS was used for detection, operating in the multiple reaction monitoring (MRM) mode. An electrospray ionization source was used operating in the negative mode with a capillary voltage of 4.5 kV. The source temperature was 400 ºC with a probe height of 5 mm. Gas 1 was set to 50, gas 2 to 60 and the curtain gas to 30. The dwell time for all ions was 40 ms. The specific MRM transitions, collision energies, collision cell exit potentials, entrance potentials and declustering potentials for each compound are given in Table 4.3.

Table 4.3. QQQ-MS MRM transitions and MS parameters for PPCPs and TPs.

Compound	Q1→ Q3 m/z (Da)	Collision energy (V)	Collision cell exit potential (V)	Entrance potential (V)	Declustering potential (V)
Salicylic acid	137→93	-35	-10	-10	-60
3-Chlorosalicylic acid	170.8→91 170.8→34.9	-30 -65	-15 -12	-10 -10	-55 -50
5-Chlorosalicylic acid	170.8→91 170.8→34.9	-30 -65	-15 -12	-10 -10	-55 -50
3,5-Dichlorosalicylic acid	204.8→125 204.8→89.2	-31 -50	-15 -15	-10 -10	-55 -100
3-Bromosalicylic acid	214.7→78.9 214.7→171	-50 -21	-9 -15	-10 -10	-70 -65
5-Bromosalicylic acid	214.7→78.9 214.7→171	-50 -21	-9 -15	-10 -10	-70 -65
3,5-Dibromosalicylic acid	294.8→80.8 292.8→78.8	-55 -55	-9 -9	-10 -10	-40 -40
Bromochlorosalicylic acid	249→78.9 250.9→80.9	-46 -46	-9 -9	-10 -10	-40 -40
Salicylic acid-d_4	141→97	-35	-10	-10	-60
3-Chlorosalicylic acid-d_3	173.9→129.9 173.9→34.9	-20 -42	-15 -12	-10 -10	-40 -40
5-Chlorosalicylic acid-d_3	173.9→93.9 173.9→34.9	-35 -40	-11 -11	-10 -10	-50 -50
3,5-Dichlorosalicylic acid-d_2	210.8→129 210.8→36.9	-31 -53	-15 -13	-10 -10	-35 -50
3-Bromosalicylic acid-d_3	219.8→80.9	-50	-9	-10	-45
5-Bromosalicylic acid-d_3	219.8→80.9	-50	-9	-10	-45

Table 4.3. QQQ-MS MRM transitions and MS parameters for PPCPs and TPs.

Compound	Q1→ Q3 m/z (Da)	Collision energy (V)	Collision cell exit potential (V)	Entrance potential (V)	Declustering potential (V)
3,5-Dibromosalicylic acid-d_2	298.8→80.9	-55	-9	-10	-40
	296.8→78.8	-55	-9	-10	-40
Naproxen	229→170	-20	-10	-10	-40
Chloronaproxen	263→204	-20	-10	-10	-55
	265→206	-19	-10	-10	-50
Bromonaproxen	306.9→79	-30	-8	-10	-35
	306.9→262.9	-9	-15	-10	-40
Naproxen-d_3	232→171	-42	-10	-10	-40
Chloronaproxen-d_3	266→207	-20	-13	-10	-55
Bromonaproxen-d_3	310→251	-24	-8	-10	-60
Diclofenac	294→249.8	-20	-15	-10	-50
	294→35	-68	-12	-10	-40
Chlorodiclofenac	328→284	-17	-25	-10	-60
	330→286	-17	-25	-10	-60
Bromodiclofenac	371.9→79	-65	-8	-10	-40
	373.8→81	-60	-9	-10	-40
Diclofenac-d_4	298→217	-30	-15	-10	-50
Chlorodiclofenac-d_3	332.8→288.9	-24	-15	-10	-90
Bromodiclofenac-d_3	376.8→80.9	-60	-9	-10	-40
Gemfibrozil	249.2→120.8	-50	-17	-10	-145

Table 4.3. QQQ-MS MRM transitions and MS parameters for PPCPs and TPs.

Compound	Q1→ Q3 m/z (Da)	Collision energy (V)	Collision cell exit potential (V)	Entrance potential (V)	Declustering potential (V)
Chlorogemfibrozil	283.2→154.9	-22	-20	-10	-150
	285.2→156.9	-17	-23	-10	-150
Bromogemfibrozil	329→81	-50	-8	-10	-150
	327→79	-50	-8	-10	-150
Gemfibrozil-d_6	255→120.9	-58	-17	-10	-135
Chlorogemfibrozil-d_6	288.9→154.9	-45	-15	-10	-150
Bromogemfibrozil-d_6	332.9→198.9	-28	-10	-10	-150
Bisphenol A	227→132.8	-35	-15	-10	-145
Chlorobisphenol A	260.9→210.1	-33	-13	-10	-130
	260.9→181.9	-39	-10	-10	-130
Dichlorobisphenol A	294.9→244	-33	-12	-10	-140
	294.9→34.9	-68	-12	-10	-140
Trichlorobisphenol A	328.7→249.8	-43	-15	-10	-170
	328.7→34.9	-85	-12	-10	-160
Tetrachlorobisphenol A	362.8→312	-35	-25	-10	-120
	362.8→34.9	-80	-12	-10	-130
Bisphenol A-d_6	233→215	-27	-20	-12	-50
Chlorobisphenol A-d_{13}	274→220.4	-33	-13	-10	-100
Dichlorobisphenol A-d_{12}	306.9→34.9	-68	-12	-10	-140
Trichlorobisphenol A-d_{11}	339.7→34.9	-85	-12	-10	-160

Table 4.3. QQQ-MS MRM transitions and MS parameters for PPCPs and TPs.

Compound	Q1→ Q3 m/z (Da)	Collision energy (V)	Collision cell exit potential (V)	Entrance potential (V)	Declustering potential (V)
Tetrachlorobisphenol A-d_{10}	374.8→34.9	-100	-12	-10	-165
n-Chlorooctylphenol-d_{17}	255→34.9	-70	-11	-10	-140
n-Dichloro octylphenol-d_{17}	290.1→34.9	-80	-12	-10	-180
n-Bromooctylphenol-d_{17}	300→78.9	-26	-8	-10	-130
n-Dibromo octylphenol-d_{17}	379.8→80.8	-45	-8	-10	-170
t-Octylphenol	205.1→132.7	-30	-15	-2	-100
t-Chlorooctylphenol	238.8→34.9 238.8→167.8	-65 -30	-12 -15	-10 -10	-80 -70
t-Dichlorooctylphenol	272.9→34.9 275→35	-70 -70	-12 -12	-10 -10	-130 -130
t-Bromooctylphenol	283→78.9 283.1→239	-50 -24	-9 -11	-10 -10	-100 -100
t-Dibromo octylphenol	362.7→78.9 362.7→291.9	-50 -37	-15 -19	-10 -10	-100 -180
tech. Nonylphenol	219.2→132.8 219.2→146.9	-40 -30	-15 -15	-10 -10	-200 -200
tech. Chlorononylphenol	253→167	-35	-15	-10	-90
tech. Dichlorononylphenol	287.1→200.8 287.1→34.9	-38 -85	-12 -12	-10 -10	-130 -120
tech. Bromononylphenol	296.9→80.9 296.9→78.9	-34 -34	-9 -9	-10 -10	-80 -80

Table 4.3. QQQ-MS MRM transitions and MS parameters for PPCPs and TPs.

Compound	Q1→ Q3 m/z (Da)	Collision energy (V)	Collision cell exit potential (V)	Entrance potential (V)	Declustering potential (V)
tech. Dibromononylphenol	377→79	-55	-8	-10	-110
	379→81	-45	-8	-10	-110
nonylphenol-$^{13}C_6$	225.2→153.2	-35	-15	-14	-105
t-octylphenol-$^{13}C_6$	211.1→138.7	-30	-15	-2	-100

Quantitation of the selected analytes was performed using six-point calibration curves ranging from 2-1,000 ng/L constructed for each analyte, with second-order polynomial fits and r^2 values >0.995 in all cases. Quantitation was performed in AB SCIEX Analyst 1.5.1 software by the ratio of the peak area to internal standard for the first MRM transition listed in Table 4.2. Concentrations above the calibration curve range analytes required the addition of a 2,000 ng/L calibration curve point. A second MRM transition for halogenated TPs provided qualitative identification for wastewater analyses. Chromatographic retention time differences of >0.1 min between standards and PPCPs and TPs in wastewater samples resulted in a null identification. Concentrated mixtures of standards were stored in methanol in a -20 °C freezer at 10x the concentration of the upper limit of the calibration curve range for each analyte, and diluted into working solutions as needed for analysis.

4.3 Results and Discussion

Concentrations of the selected PPCPs and TPs in chlorinated WWTP from the 10 WWTPs sampled are presented in Table 4.4, corrected for recovery and ion suppression. Several compounds for which targeted analyses were carried out were not detected in any effluent sample, and thus were omitted from Table 4.4. After Table 4.4, the results for each parent PPCP and its halogenated TPs will be discussed.

Table 4.4. Concentrations (ng/L) of selected PPCPs and TPs in tertiary and secondary effluent samples.

	Wastewater ID				
Compound	A	B*	C*	D	E*
Salicylic acid	52±28	70	168	79±35	78
3-Chlorosalicylic acid	10±6	14	23	14±3	11
5-Chlorosalicylic acid	5±4	7	6	9±1	9
3,5-Dichlorosalicylic acid	38±19	70	71	55±8	56
3-Bromosalicylic acid	4±2	3	7	5±1	3
5-Bromosalicylic acid	1±0	1	ND	1±1	ND
3,5-Dibromosalicylic acid	9±5	12	28	14±5	11
Bromochlorosalicylic acid	5±2	11	8	11±4	6
Naproxen	138±103	22	20	57±44	86
Chloronaproxen	1±2	ND	ND	8±14	ND
Bromonaproxen	9±1	4	29	7±3	7
Diclofenac	215±50	127	303	75±65	189
Chlorodiclofenac	18±11	23	15	11±5	12
Gemfibrozil	813±284	226	889	223±182	185
Chlorogemfibrozil	35±23	24	42	18±7	5
Bromogemfibrozil	4±3	1	3	5±1	0
Bisphenol A	7±6	4	20	2±4	4
tech. Nonylphenol	232±100	151	**	187±150	36
tech. Monochlorononylphenol	17±8	16	17	7±3	13
tech. Dichlorononylphenol	3±3	2	3	1±1	2
tech. Monobromononylphenol	8±4	7	7	5±4	2
tech. Dibromononylphenol	ND	ND	**	1	ND
4-*t*-Octylphenol	64±30	29	ND	15±8	51
4-*t*-Monochlorooctylphenol	3±4	3	7	1±1	3

*n<3, no standard deviation reported
**Unreliable internal standard peak area
ND- non-detect

Table 4.4. Concentrations (ng/L) of selected PPCPs and TPs in tertiary and secondary effluent samples.

	Wastewater ID				
Compound	F*	G*	H*	I	J
Salicylic acid	73	127	124	385±508	239±46
3-Chlorosalicylic acid	26	24	8	23±5	8±1
5-Chlorosalicylic acid	25	17	6	17±6	25±5
3,5-Dichlorosalicylic acid	78	66	39	150±26	162±32
3-Bromosalicylic acid	10	8	3	36±9	3±1
5-Bromosalicylic acid	3	3	0	13±18	4±2
3,5-Dibromosalicylic acid	16	3	15	11	129
Bromochlorosalicylic acid	26	21	7	11±2	27±7
Naproxen	25	29	146	2,602±855	725±268
Chloronaproxen	1	8	4	ND	ND
Bromonaproxen	7	3	10	26±3	109±19
Diclofenac	57	10	57	395±50	315±34
Chlorodiclofenac	6	5	2	2±2	0±0
Gemfibrozil	175	142	562	3,393±299	1,373±247
Chlorogemfibrozil	26	35	12	3±1	8±2
Bromogemfibrozil	6	12	2	ND	4±3
Bisphenol A	ND	ND	2	568	14
tech. Nonylphenol	25	265	**	2,844±426	991±487
tech. Monochlorononylphenol	5	4	17	75±31	25±13
tech. Dichlorononylphenol	1	ND	7	27±9	12±9
tech. Monobromononylphenol	2	3	11	12±5	3±4
tech. Dibromononylphenol	ND	ND	ND	141	ND
4-*t*-Octylphenol	30	15	**	1,407±256	340±169
4-*t*-Monochlorooctylphenol	ND	ND	8	13±4	ND

*n<3, no standard deviation reported
**Unreliable internal standard peak area
ND- non-detect

4.3.1 Salicylic Acid. Salicylic acid is a metabolite of acetylsalicylic acid (aspirin), an over-the-counter and widely available analgesic. The parent compound was found in tertiary and secondary wastewater effluent at concentrations as high as 174 ng/L and 1,680 ng/L, respectively. Nearly all of salicylic acid's mono- and dihalogenated TPs were quantified in each wastewater sample analyzed, indicating the ubiquitous nature of the parent compound and the efficiency of its transformation during WWTP chlorination. The potential for formation of halogenated TPs of salicylic acid during water chlorination processes was previously investigated by Quintana et al.[5] A targeted screening of several SPE extracts of wastewater, tap water and drinking water samples for the halogenated TPs of salicylic acid yielded the positive identification of several TPs, though no quantitative results were obtained due to the lack of available standards. The authors noted that the most intense TP peaks by MS response were the dihalogenated TPs, consistent with the results reported in Table 4.4. It has also be demonstrated that dihalogenated salicylic acid TPs can be formed upon chlorination of Suwanee river humic acid dissolved in simulated drinking water.[6] While the parent salicylic acid is non-toxic at environmentally relevant concentrations, 3,5-dichlorosalicylic acid has been identified as a potent inhibitor of the enzyme 20α-hydroxysteroid dehydrogenase (5.9 nM K_i) indicating the potential for disruption of the steroidogenic pathway.[7]

4.3.2 Bisphenol A. Bisphenol A is a widely-used plasticizer often recognized for its potentially deleterious health effects, notably as an endocrine disrupting xenoestrogen capable of binding to the estrogen receptor.[8, 9] Only the chlorinated TPs of bisphenol A were selected for investigation in this study as tetrabromobisphenol A is a widely used flame retardant (>150,000 tons/year), which can debrominate in the environment by bacterial metabolism into its lesser brominated analogs.[10] While the parent bisphenol A was found at levels up to 20 and 697 ng/L in tertiary and secondary effluent, respectively, none of its multiply chlorinated analogs were identified. Given the reactivity of bisphenol A with aqueous hypochlorite this result is unexpected. As this is a survey study of a limited number of wastewater samples, additional experiments would need to be performed to adequately describe the formation, fate and transport of chlorinated bisphenol A TPs during real-world WWTP chlorination processes.[11] Fukazawa et al. have reported the presence of bisphenol A and its mono, di, tri and tetrachlorinated analogs at concentrations ranging from 0.2-370, 0.2-2.0, 0.4-1.0, 0.9-1.2, and 1.3-1.4 µg/L, respectively, in final effluents from paper manufacturing plants in Japan.[12] The levels of bisphenol A and doses of chlorine encountered in effluents from paper manufacturing plants are much higher than those encountered in WWTPs, potentially explaining the positive identifications made by Fukazawa et al. Bisphenol A's halogenated TPs have also been reported in human breast adipose tissue at concentrations of 5.83±3.48, 3.05±0.28, 9.21±9.26, 0.74±0.15 ng/g and <LOD for the parent, mono, di, tri and

tetrachlorinated analogs, respectively, indicating that bioconcentration can occur in humans.[13] While chlorinated bisphenol A's are also used as flame retardants, their production (<10,000 tons/year) is low enough that chlorinated bisphenol A detected in the environment is attributed to the aqueous chlorination of the parent compound, as noted by Riu et al.[14] Bisphenol A's chlorinated TPs generally exhibit enhanced affinity for the estrogen receptor compared to the parent molecule depending on the number of chlorine substituents, with the doubly halogenated TP exhibiting a 38-fold greater response in a yeast two-hybrid expression system for the human estrogen receptor α.[12] Mixtures of chlorinated bisphenol A TPs, which may be more environmentally relevant than single TPs, also show enhanced estrogen receptor binding.[11, 15] Bisphenol A has been implicated as a thyroid hormone interfering obesogen due to its peroxisome proliferator-activated receptor γ binding affinity, where its tetrachlorinated analog is a more potent ligand than the non-halogenated parent.[14]

4.3.3 Gemfibrozil. Gemfibrozil is a widely-prescribed peroxisome proliferator antilipidemic drug in the fibrate class frequently found in wastewater effluents. The parent compound was detected in this study at concentrations up to 1,110 mg/L and 3,830 ng/L in tertiary and second effluent, respectively. The maximum concentrations in tertiary and secondary effluent, respectively, for the chlorinated TP were 37 and 56 ng/L and for the brominated TP were 8 and 13 ng/L. Gemfibrozil's chlorinated and brominated TP analogs have previously been

detected in primary effluent at concentrations of 166 and 50 ng/L, respectively, a fair amount higher than reported in the current chapter.[16] The parent compound has also been found in seawater, indicative of its ubiquity in the aqueous environment.[17] Considering the mg/L bromide content of seawater in combination with the residual chlorine content of wastewater discharged into the ocean, bromination may be a substantial transformation pathway for gemfibrozil.[17]

The parent gemfibrozil is an anti-androgenic endocrine disruptor in goldfish (*Carassius auratus*) with a bioconcentration factor of 113 at 1.5 µg/L, where both 4 and 14 day exposures to 1.5 µg/L waterborne gemfibrozil decreased plasma testosterone levels by over 50%.[18] Gemfibrozil's halogenated TPs have also been shown to be more potent anti-androgenic endocrine disruptors in terms of whole-animal steroid levels in Japanese medaka (*Oryzias latipes*), where a 21 day exposure to 55.1 µg/L chlorogemfibrozil and 58.8 µg/L bromogemfibrozil significantly ($p<0.05$) reduced the levels of 11-ketotestosterone and testosterone, respectively.[16]

4.3.4 Diclofenac. Diclofenac is a non-steroidal anti-inflammatory drug which is known for its toxic effects on vulture populations,[19] and has also been shown to cause estrogenic effects in the model aquatic species Japanese medaka at concentrations as low as 1 µg/L.[20] The parent diclofenac was found at levels up to 287 and 440 ng/L in tertiary and secondary effluents, respectively. Diclofenac's chlorinated TP was found at maximum concentrations of 26 and 6

ng/L in tertiary and secondary effluents, respectively, while its brominated TP was not detected in any effluent sample. This is the only TP which was detected at higher concentrations in tertiary rather than secondary effluent. The non-detection of the brominated analog may be due to the low response of its MRM quantifying transition as shown in Figure 4.10. We believe that this is the first reported detection of chlorodiclofenac in wastewater effluent. The aqueous chlorination TPs of diclofenac have been previously described by Quintana et al., identifying an additionally monochlorinated and monobrominated analog as TPs by LC-QQQ-MS. The absolute structure of diclofenac's TPs have not been previously characterized in terms of halogen substitution position; these results are indicated in Figure 4.1 as assigned by [^{1}H, ^{13}C] HMBC NMR spectroscopy.

4.3.5 Naproxen. Naproxen is a non-steroidal anti-inflammatory drug, unique in the set of PPCPs chosen for this study as it contains a fused bicyclic conjugated ring system. The parent naproxen was found at levels up to 285 and 3,680 ng/L in tertiary and secondary effluents, respectively. Chloronaproxen was detected in a few wastewater samples at concentrations up to 23 ng/L in tertiary effluent, while bromonaproxen was detected at concentrations up to 132 ng/L in secondary effluent. This suggests a possible preference for bromination chemistry over chlorination, perhaps explained by the higher reactivity of HOBr compared to HOCl towards activated aromatic species.[21] The main chlorination TPs of naproxen have not been previously identified, though laboratory-based

kinetic studies suggest that naproxen degradation is favored at lower pH values (<6) than are commonly encountered in chlorinated wastewaters (~7-8.5).[22] This observation may in part explain why bromonaproxen was detected more frequently than chloronaproxen despite the near omnipresence of the parent compound in all effluent samples.

4.3.6 Technical nonylphenol and *tert*-octylphenol. Because of their chemical similarity, the results obtained for the alkylphenol surfactants technical nonylphenol (a mixture of branched-chain isomers, referred to as nonylphenol hereon) and *tert*-octylphenol will be discussed together. Both nonylphenol and *tert*-octylphenol are metabolites of polyethoxylate alkylphenols, used in industrial surfactant formulations.[23] The internal standard used for quantification of all surfactant TPs was *n*-octylphenol-d_{17} due to the cost-prohibitive nature of synthesizing standards from isotopically labeled *tert*-octylphenol or technical nonylphenol. The parent technical nonylphenol was found at levels up to 287 and 440 ng/L in tertiary and secondary effluents, respectively. Technical nonylphenol's mono- and dichlorinated TPs were found at concentrations up to 115 and 39 ng/L, respectively, in secondary effluent, potentially indicating a preference for the formation of the monochlorinated analog rather than chlorination to the dichlorinated analog. Technical nonylphenol's mono- and dibrominated TPs were found in secondary effluent at maximum concentrations of 19 and 372 ng/L, respectively, an opposite trend to that observed for the

chlorinated analogs. Brominated TPs of nonylphenol and its ethoxylates rather than chlorinated TPs have been previously reported in waters and sludge from a drinking water treatment facility by Petrovic et al.[24] Concentrations of the parent nonylphenol and its ethoxylates in influent water were 0.45 and 12.9 µg/L, respectively, while concentrations of 0.21 and 4.0 µg/L were reported for brominated nonylphenol and its brominated ethoxylates, respectively. Sludge weights of 220 µg/kg and 430-1600 µg/kg were also reported for brominated nonylphenol and its brominated ethoxylates, respectively, demonstrating the preference of these TPs to accumulate in sludge rather than remain dissolved in the aqueous fraction. Another study by the same group evaluated the formation and removal of halogenated TPs of alkylphenols during various stages of drinking water treatment.[25] Prechlorination treatment transformed the parent alkylphenols into their corresponding halogenated TPs (<2-105 ng/L aqueous concentrations), where most of the TPs concentrated in flocculation sludge (<2-280 ng/g dry weight). The parent *tert*-octylphenol was found at levels up to 1,860 ng/L in secondary effluent, while only its monochlorinated TP was found at concentrations up to 19 ng/L in secondary effluent. This is interesting because its chlorination chemistry has been shown to be similar to that of nonylphenol, however it does not appear that this bench-scale chemistry necessarily translates into real-world WWTP chemistry.[26]

The parent surfactant compounds have been recognized as endocrine disruptors due to their *in vitro* estrogen receptor affinities.[27] *In vivo*, *tert*-

octylphenol has been shown to be a more potent endocrine disruptor than nonylphenol in rainbow trout, though both exhibit similar *in vivo* endocrine disruption potential in several other aquatic species.[28] Though most toxicology studies focus on the linear form of nonylphenol (4-*n*-nonylphenol), this compound is not present in technical mixtures of nonylphenol and thus may not be as environmentally relevant.[29]

4.4 Conclusions

This chapter combined the use of authentic isotopically labeled and unlabeled standards in conjunction with sensitive and specific LC-QQQ-MS MRM analysis to provide accurate quantification of the selected PPCPs and TPs. Compared to prior reports, this study represents one of the most exhaustive surveys of halogenated TPs of PPCPs in terms of use of standards and adherence to strict quality assurance and quality criteria put forth by our collaborators at LACSD. Multiple halogenated TPs were detected for the first time at low ng/L levels, further bolstering the case for transformation of PPCPs by chlorine during WWTP disinfection processes.

The use of analytical instrumentation such as LC-QQQ-MS is indispensable in targeted wastewater analyses for the identification and quantification of PPCPs and TPs. Despite such advanced technologies, the field of TP analysis is hindered by problems related to sample preparation and the lack of available authentic standards. Untargeted screening methods and the use

of orthogonal detection platforms may solve some of these issues, as will be discussed in chapter 5.

4.5 References

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Chapter Five

Conclusions and future directions

5.1 Conclusions

The presence of PPCPs in wastewater effluents is potentially worrisome as some compounds are able to exert endocrine disrupting effects on non-target aquatic organisms. The use of chlorine as a disinfectant prior to wastewater discharge is extremely common due to its ease of use and effectiveness at attenuating a wide variety of microbes. Chlorination of wastewater can transform PPCPs into their corresponding halogenated TPs, that have unknown physiochemical and toxicological properties that can be significantly different than those of the parent PPCP. This dissertation focuses on the use of LC-MS and NMR to characterize halogenated PPCP TPs and quantify their levels in various wastewater effluents from Southern California.

In chapter 2, the targeted analysis of advanced primary wastewater effluent extracts for the presence of the antilipidemic gemfibrozil and its monochlorinated and monobrominated TPs was carried out by sequential LLE/HPLC-UV/UPLC-QTOF-MS. Standards of chlorogemfibrozil and bromogemfibrozil were synthesized via *in situ* chlorination reactions, and purified to greater than 99% purity by analytical scale RP-HPLC-UV. These standards allowed the quantification of recovery for the LLE extraction of primary effluent, as well as the creation of external calibration curves for the quantification of all three analytes in advanced primary wastewater effluent samples from a WWTP

in San Diego, CA. Initial recovery determinations employing the widely used concentration technique of SPE demonstrated poor recovery compared with LLE. The necessary filtering of suspended solids prior to SPE was proposed to explain this difference, where gemfibrozil and its monohalogenated TPs exhibited a preference for particle binding that increased across the series gemfibrozil < chlorogemfibrozil < bromogemfibrozil according to increasing lipophilicity. This demonstrated the first report of the detection of chlorogemfibrozil and bromogemfibrozil in wastewater effluent.

In chapter 3, the targeted wastewater analyses presented in chapter 2 were expanded to include three more PPCPs and their halogenated TPs: the non-steroidal anti-inflammatory diclofenac, the analgesic salicylic acid and the plasticizer bisphenol A. Secondary wastewater effluent from the Orange County Sanitation District (OCSD) was used for analysis, which allowed the use of SPE for extraction due to the low suspended solids content. Wastewater extraction by SPE was followed by HPLC-UV offline sample enrichment which grouped each parent PPCP and its associated TPs into a separate fraction. These fractions were analyzed using a UPLC-QTOF method optimized for the quantification of each analyte. Poor recoveries and a high degree of ion suppression limited the quantification of the selected analytes, as most were under the limit of quantification. The presence of halogenated TPs of salicylic acid and gemfibrozil at low ng/L levels was described, mirroring previous results found in the literature for targeted wastewater analysis for these analytes.[1, 2]

Chapter 4 focused on addressing the issues of poor recovery and the variability of the results presented in the previous two chapters via a collaboration with Los Angeles County Sanitation District (LACSD). The LACSD facility provided the use of automated SPE extractors which reduced extraction variability to under 5% sample-to-sample based on the quantification of spiked recovery samples. Importantly the use of an LC-QQQ instrument afforded several orders of magnitude greater sensitivity than could be obtained by UPLC-QTOF analyses. In addition, the use of dual MRM transitions for each analyte afforded enhanced specificity. The analytes for targeted analysis were expanded from chapter 3 to include the non-steroidal anti-inflammatory naproxen and the surfactants nonylphenol and *tert*-octylphenol. Both non-isotopically labeled standards as well as deuterated standards were synthesized, isolated and characterized for all halogenated TPs. The use of isotopically labeled standards allowed the accurate determination of analyte recovery during wastewater extraction as well as the quantification of ion suppression effects during LC-QQQ analysis. The selected PPCPs and TPs were analyzed in secondary wastewater from OCSD as well as tertiary wastewater from LACSD. This work represents the first report of the occurrence of halogenated naproxen, diclofenac and *tert*-octylphenol in wastewater effluent.

5.2 Future directions

This dissertation focused on the use of authentic analytical standards for PPCP TPs for purposes of method development for the accurate quantification of the selected analytes in wastewater effluent. While no novel techniques were developed, chapter 4 presents one of the most comprehensive targeted wastewater studies to date, combining authentic isotopically and non-isotopically labeled standards, automated SPE for wastewater extraction and high-sensitivity and high-specific LC-QQQ MRM analyses. Each chapter presented difficulties and uncertainties which future experiments could address. In addition, newer analytical platforms and combinations of existing technologies with *in silico* methods can enhance the future of PPCP and TP analysis in wastewater.

In chapter 2, it appeared that an appreciable portion of gemfibrozil, chlorogemfibrozil and bromogemfibrozil was particle bound rather than in the dissolved phase as their log D values would predict. This was assumed since filtering the particles from spiked advanced primary wastewater samples with a 0.45 um filter removed nearly all of the chlorogemfibrozil and bromogemfibrozil. To confirm the hypothesis that these analytes were particle bound, the particles themselves would have to be extracted. Two methods to accomplish this are pressurized liquid extraction (PLE) and sonication assisted extraction (SAE). Following the method of Stasinakis et al., suspended solid particles can be separated from the aqueous phase and extracted for quantification of selected analytes.[3] First, a sample of wastewater with suspended solids is filtered with a

0.45 µm glass fiber filter. Then, the aqueous fraction is extracted by SPE while the particles and filter are extracted by SAE into organic solvent and concentrated. Both fractions can then be quantified, and by mass balance the fraction of the compound dissolved in the aqueous phase vs. particle bound can be determined. Recovery from the aqueous and particle fractions can be measured by spiking each sample separately with isotopically labeled standards, though there is no established method describing exactly how to spike the particle fraction.

Isotopically labeled standards are used with increasing frequency in wastewater analysis due to their absence from the environment and their identical physiochemical properties to their non-labeled analogs. Authentic standards for most PPCP chlorination TPs are not commercially available, mainly because the major TPs of PPCPs have yet to be identified. The most used method for the identification of TPs is to add the parent PPCP to an aqueous matrix, ideally one simulating wastewater, adding hypochlorite and monitoring the formation of TPs as a function of time by LC-MS. This method generally involves using concentrations of the parent PPCP that are orders of magnitude greater than the concentrations typically encountered in wastewater. In addition, due to the highly variable nature of the distribution and identity of the organic and inorganic constituents of wastewater, it is difficult to know for sure whether the observed TPs are the same as one would encounter in the environment. The identification of TPs is a targeted analysis, which relies on the identification of

new chromatographic peaks compared to a standard of the parent compound by itself. Tentative structures can be proposed based on the new peak's m/z and the ratios of the various isotopic peaks (M vs M+2 etc.), which can be narrowed further by the use of MS instruments such as the TOF that can determine an m/z to within 1 ppm mass error. For a small molecule such as a PPCP, measurements with less than 1 ppm mass error restrict the potential empirical formulae for a given m/z to only a handful of arrangements of atoms which can be further narrowed by the isotopic peak ratios. Still, such analyses are targeted, for which assumptions have to be made. For instance, reaction monitoring for phenolic compounds such as bisphenol A is often carried out using negative mode ESI-MS as the ionization source and detector. Detection is based on the removal of a phenolic proton; thus, if one or more TPs had modified structures in which there was no phenol (or other ionizable group amenable for negative ESI-MS detection) no signal for this TP would be detected. This highlights the needs for multiple modes of detection, and especially for MS analysis, multiple modes of ionization to account for all possible TPs. From an environmental perspective, however, if a chlorination TP is extremely minor e.g. <1% of the total TPs formed, the question must be asked - is this TP important?

To better address the question of the environmental significance a method complementary to targeted TP identification can be employed. Gervais and coworkers borrowed a method commonly employed in the field of metabolomics for use in identifying chlorination TPs of the synthetic estrogen ethinylestradiol.[4]

Differential global profiling uses a semi-targeted or non-targeted approach to compare the metabolite profiles of any number of experimental conditions to the profile of a control sample. This is analogous to experiments performed by other members of our research group to compare the metabolite profile of rice plants stressed by submergence vs. the profile of plants in the control group.[5] This method relies on the use of *in silico* databases and processing commonly used to probe LC-MS datasets for metabolites, or in this case TPs. Gervais et al. used differential global profiling of LC-MS datasets to monitor the chlorination of ethinylestradiol spiked into samples from a drinking water treatment plant.[4] Chlorination reactions were performed in drinking water spiked with 0.5 µg/L of ethinylestradiol and chlorinated with 0.8 mg/L NaOCl for 10, 30 and 120 min, with non-spiked control reactions run in parallel for comparison. This spiking level is about 1.5 orders of magnitude higher than levels of ethinylestradiol measured in wastewater, but several of orders of magnitude lower than spiking levels used in other recent literature reports of *in situ* chlorination reaction TP identification.[2, 6] Aliquots of 500 mL of each reaction mixture were then extracted by Oasis HLB SPE and analyzed by RP-HPLC-ESI-Orbitrap-MS for the collection of high resolution (30,000) mass spectra. Precursor ion scans were performed to identify fragments of TPs generated by chlorination, with a range of ±3 Da to preserve the isotopic spectral information afforded by halogenation (i.e. M and M+2 intensities). The LC-MS total ion chromatograms and extracted ion chromatograms were first analyzed and compared manually, which the authors

noted would not be a suitable approach for a larger dataset. A third approach employed XCMS data processing software, which compared the unspiked or non-chlorinated (control) vs. spiked (treated) chlorination and non-chlorinated reaction extracts. This process assumed that much of the skeleton of the structure of ethinylestradiol was preserved during transformation, thus assisting in the generation of potential TP molecular formulae and associated accurate mass values. A set of 708 ions of potential TPs was manually narrowed to 80 ions by visual comparison of ion and fragment m/z, isotopic pattern, adduct presence and retention time. Structures were proposed for 8 potential ethinylestradiol TPs assuming a mass accuracy of 0.001 Da, which were then compared with known TPs. Two of the potential TPs were in good agreement with previously reported TPs and 6 were not previously described, however, the authors noted the uncertainty of the absolute structures of these compounds. Though these results were not entirely conclusive, this semi-targeted data analysis approach could be useful for the analysis of TPs formed by chlorination of wastewater. It is important to note that this method requires the use of sensitive and high-resolution MS instruments, as high mass accuracy is required to match theoretical empirical formulae for TP identification. This method may yield incomplete results, however, if TPs are poorly ionized by the ESI source used with the Orbitrap-MS detector.

The use of ESI-MS is nearly ubiquitous in PPCP and TP analysis due to its sensitivity, specificity when operating in MS^n modes and ability to ionize a

wide range of small molecules. This detection platform has a drawback in the need for the ionization of a given analyte, without which there is no MS signal. NMR has seen less frequent use due to its inherent lack of sensitivity, however it has an advantage over MS in that it can universally and quantitatively detect a given nucleus (e.g. ^{1}H or ^{13}C) without sample manipulation. In addition, the use of 2D-NMR can provide additional spectral separation without additional purification or isolation in a given sample. One excellent example of the use of NMR was recently reported by Godejohann et al., describing the use of LC-ESI-TOF-MS with time-slice SPE-NMR to analyze pesticides in WWTP effluent samples collected following pesticide application to the surrounding area.[7] After an initial concentration step of 1 L of wastewater effluent by SPE, the SPE extracts were measured. The extracts were further fractionated by HPLC into one min wide segments and mixed with an aqueous make-up flow prior to post-column trapping onto SPE cartridges. Four injections of the 1 L effluent extract subjected to this procedure with replicate injections trapped post-column on the same SPE cartridges to increase the sample loading. A small fraction of the HPLC flow was split off for ESI-TOF-MS analysis using a T-splitter. The MS was calibrated prior to each chromatographic run to ensure mass accuracy. Fractions were eluted from the post-column SPE cartridges with deuterated methanol into a cryofit NMR flow cell in a cryogenic probe in a 500 MHz Bruker spectrometer, where 128 scan ^{1}H NMR spectra were acquired. Standards of several pesticides, including linuron, metazachlor and isoproturon, spiked into deionized water

samples were also analyzed by this method to build an MS and NMR signal database. A total of 152 NMR spectra were analyzed by principal component analysis (PCA), with 250 equidistant bins between 0 and 10 ppm in the ^{1}H dimension, excluding the methanol signal region. The identification of contaminants relied on statistically high levels of signals from the ^{1}H NMR PCA, which led to manual discrimination of the MS data from the corresponding time-slice. Empirical formulae were generated for the observed ions, which were correlated to an in-house database of molecular formulae for the selected pesticides. Comparison of the ^{1}H NMR spectra for the first, whole SPE extract, and the time-sliced one min wide HPLC-SPE fractions allowed the identification of "matrix" peaks which were present in the spectra of the whole extract but not in the post-column SPE fractions. Finally, tentatively identified pesticides were confirmed by comparison the MS and NMR spectra of the standards investigated, positively identifying 8 pesticides and one well-known TP of the pesticide metamitron. In addition, four other PPCPs including one drug metabolite, 3-carboxymefanamic acid, were tentatively identified based on their MS and NMR spectra. Interestingly, a number of the time-slice fractions exhibited high intensity ^{1}H resonances with almost no corresponding MS signal for the same fraction. This implies that unknown compounds present in the wastewater effluent were not ionized by the ESI source, illustrating the need for orthogonal ionization and detection platforms for the identification of such compounds. A recent review on non-targeted wastewater analysis for identification of PPCPs

and their TPs by Zedda and coworkers echoes the need for multiple ionization sources in addition to the construction of larger and more robust *in silico* MS processing tools and databases, which when combined with high-resolution MS platforms can increase the confidence in mass-based compound identification.[8]

Finally, when identifying chlorination TPs the question arises - is this compound important? From an analytical chemistry perspective, all potential TPs can be considered relevant due to interest in elucidating the identity of even trace TPs. From an environmental impact or aquatic toxicology standpoint, however, not all TPs may be important. A method for the culling of "irrelevant" TPs would direct fields such as wastewater TP analysis towards the pollutants of greatest potential environmental impact. This in turn would be beneficial in terms of risk assessment and prioritization of TPs for environmental monitoring programs. Escher and Fenner recently published a review proposing a scheme for the prioritization of TPs in terms of their potential impact on the environment involving a two-prong approach of exposure-driven and effect-driven assessments.[9] In the exposure-driven approach, TPs must first be identified, though if the conversion of parent compound to a given TP is less than 10% then it is considered not relevant for further study. If the TP forms in a relative amount greater than 10%, then it should be synthesized and then subjected to a battery of toxicity tests to determine its relevance. The identification and synthesis of TPs can be carried out by monitoring *in situ* chlorination reactions with LC-MS, while relative TP amount can be determined by HPLC-UV as reported in chapters 2-4.

The exposure-driven assessment approach ignores TPs which form in trace amounts, and thus the analytical platforms for the analysis of TP formation need not be state-of-the-art instrumentation as sensitivity is less of a concern. In the effect-driven assessment approach, a mixture toxicity test is the first step. An example of a mixture relevant to this thesis would be an *in situ* chlorination reaction of a PPCP, quenched after the evolution of several halogenated TPs without any further purification or separation step. If the toxicity of the mixture is proportional to the toxicity of the parent PPCP, then any TPs in the mixture are not considered relevant for further study. If the mixture toxicity is greater than the toxicity of the parent PPCP, then the TPs must be identified and synthesized for individual toxicity testing. Both of these approaches would require facilities for toxicity testing, such as the collaboration with Dr. Daniel Schlenk's lab in the Environmental Sciences department at UC Riverside which produced the endocrine disruption data on gemfibrozil and its TPs presented in chapter 2.[2] Toxicity testing, including endocrine disruption, should be carried out using *in vitro* as well as *in vivo* assays as evidenced by gemfibrozil's *in vivo* but lack of *in vitro* toxicity. Using these two approaches, the identification and study of TPs relevant for environmental risk can be streamlined, thus potentially reducing the number of compounds which must be studied to ensure and safeguard public and environmental health.

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Appendix A

List of Acronyms

APCI	Atmospheric pressure chemical ionization
CE	Capillary electrophoresis
CID	Collision induced dissociation
CWA	Clean water act
DBP	Disinfection byproduct
E1	Estrone
E2	17β-estradiol
E3	Estriol
EE2	17α-ethynylestradiol
EI	Electron impact
ESI	Electrospray ionization
ELISA	Enzyme-linked immunosorbent assay
EIC	Extracted ion chromatogram
eV	Electron volt
GC	Gas chromatography
HAA5	Haloacetic acids
hERα	Human estrogen receptor alpha
HILIC	Hydrophilic interaction liquid chromatography
HLB	Hydrophilic lipophilic balance

HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
IT	Ion trap
LACSD	Los Angeles County Sanitation District
LC	Liquid chromatography
LIT	Linear ion trap
LLE	Liquid-liquid extraction
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
M/z	Mass-to-charge
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser spectroscopy
NPDES	National pollutant discharge elimination system
NSAID	Non-steroidal anti -inflammatory
OCSD	Orange County Sanitation District
PPAR	Peroxisome proliferator-activated receptor
PLE	Pressurized liquid extraction
PPCP	Pharmaceutical and personal care product
QQQ	Triple quadrupole
QTOF	Quadrupole time-of-flight
RP	Reversed phase

SDME	Single-drop microextraction
SPE	Solid phase extraction
SPME	Solid phase microextraction
THM	Trihalomethane
TIC	Total ion chromatogram
TP	Transformation product
UPLC	Ultra performance liquid chromatography
USEPA	United States Environmental Protection Agency
UV	Ultra violet
WWTP	Wastewater treatment plant